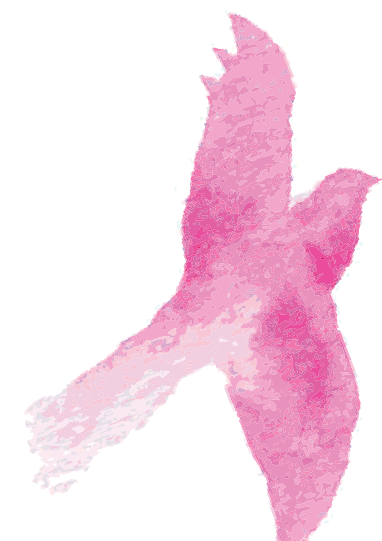
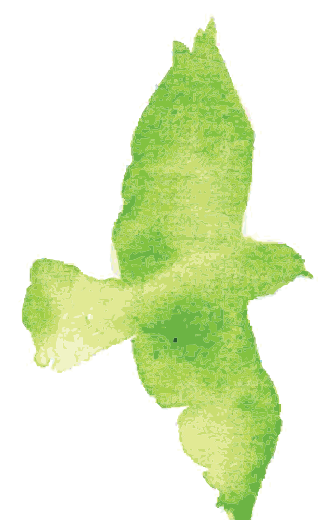




EVO-DEVO OF COLOR PATTERN FORMATION

EDITED BY: Ricardo Mallarino, Marcus Kronforst and Marie Manceau
PUBLISHED IN: *Frontiers in Ecology and Evolution* and
Frontiers in Cell and Developmental Biology





frontiers

Frontiers eBook Copyright Statement

The copyright in the text of individual articles in this eBook is the property of their respective authors or their respective institutions or funders. The copyright in graphics and images within each article may be subject to copyright of other parties. In both cases this is subject to a license granted to Frontiers.

The compilation of articles constituting this eBook is the property of Frontiers.

Each article within this eBook, and the eBook itself, are published under the most recent version of the Creative Commons CC-BY licence.

The version current at the date of publication of this eBook is CC-BY 4.0. If the CC-BY licence is updated, the licence granted by Frontiers is automatically updated to the new version.

When exercising any right under the CC-BY licence, Frontiers must be attributed as the original publisher of the article or eBook, as applicable.

Authors have the responsibility of ensuring that any graphics or other materials which are the property of others may be included in the CC-BY licence, but this should be checked before relying on the CC-BY licence to reproduce those materials. Any copyright notices relating to those materials must be complied with.

Copyright and source acknowledgement notices may not be removed and must be displayed in any copy, derivative work or partial copy which includes the elements in question.

All copyright, and all rights therein, are protected by national and international copyright laws. The above represents a summary only. For further information please read Frontiers' Conditions for Website Use and Copyright Statement, and the applicable CC-BY licence.

ISSN 1664-8714

ISBN 978-2-88971-416-2

DOI 10.3389/978-2-88971-416-2

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.

Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: frontiersin.org/about/contact

EVO-DEVO OF COLOR PATTERN FORMATION

Topic Editors:

Ricardo Mallarino, Princeton University, United States
Marcus Kronforst, University of Chicago, United States
Marie Manceau, Collège de France, France

Citation: Mallarino, R., Kronforst, M., Manceau, M., eds. (2021). Evo-Devo of Color Pattern Formation. Lausanne: Frontiers Media SA.
doi: 10.3389/978-2-88971-416-2

Table of Contents

05	<i>Editorial: Evo-Devo of Color Pattern Formation</i> Ricardo Mallarino, Marie Manceau and Marcus Kronforst
08	<i>Developmental and Cellular Basis of Vertical Bar Color Patterns in the East African Cichlid Fish <i>Haplochromis latifasciatus</i></i> Yipeng Liang, Jan Gerwin, Axel Meyer and Claudius F. Kratochwil
23	<i>Molecular Mechanisms Underlying Pupal Protective Color Switch in <i>Papilio polytes</i> Butterflies</i> Shinichi Yoda, Emi Otaguro, Mayumi Nobuta and Haruhiko Fujiwara
37	<i>Gene Regulatory Network Homoplasy Underlies Recurrent Sexually Dimorphic Fruit Fly Pigmentation</i> Jesse T. Hughes, Melissa E. Williams, Rachel Johnson, Sumant Grover, Mark Rebeiz and Thomas M. Williams
54	<i>Mutations in Domestic Animals Disrupting or Creating Pigmentation Patterns</i> Leif Andersson
62	<i>What Drives the Diversification of Eyespots in Morpho Butterflies? Disentangling Developmental and Selective Constraints From Neutral Evolution</i> Vincent Debat, Nicolas Chazot, Silvère Jarosson, Patrick Blandin and Violaine Llaurens
74	<i>Anterior–Posterior Patterning in Lepidopteran Wings</i> Kenneth Z. McKenna, Anna M. Kudla and H. Frederik Nijhout
88	<i>The Arctiid Archetype: A New Lepidopteran Groundplan</i> Richard Gawne and H. Frederik Nijhout
100	<i>Convergent Evolution of Broadband Reflectors Underlies Metallic Coloration in Butterflies</i> Anna Ren, Christopher R. Day, Joseph J. Hanly, Brian A. Counterman, Nathan I. Morehouse and Arnaud Martin
116	<i>Wnt Genes in Wing Pattern Development of Coliadinae Butterflies</i> Jennifer Fenner, Caleb Benson, Luis Rodriguez-Caro, Anna Ren, Riccardo Papa, Arnaud Martin, Federico Hoffmann, Ryan Range and Brian A. Counterman
128	<i>ebony Affects Pigmentation Divergence and Cuticular Hydrocarbons in <i>Drosophila americana</i> and <i>D. novamexicana</i></i> Abigail M. Lamb, Zinan Wang, Patricia Simmer, Henry Chung and Patricia J. Wittkopp
142	<i>Diversity of UV Reflection Patterns in Odonata</i> Ryo Futahashi
150	<i>Avian Pigment Pattern Formation: Developmental Control of Macro- (Across the Body) and Micro- (Within a Feather) Level of Pigment Patterns</i> Masafumi Inaba and Cheng-Ming Chuong

- 158** *Molecular Evolution and Developmental Expression of Melanin Pathway Genes in Lepidoptera*
Muktai Kuwalekar, Riddhi Deshmukh, Ajay Padvi and Krushnamegh Kunte
- 168** *Expression of Multiple engrailed Family Genes in Eyespots of Bicyclus anynana Butterflies Does Not Implicate the Duplication Events in the Evolution of This Morphological Novelty*
Tirtha Das Banerjee, Diane Ramos and Antónia Monteiro
- 180** *From Patterning Genes to Process: Unraveling the Gene Regulatory Networks That Pattern Heliconius Wings*
W. Owen McMillan, Luca Livraghi, Carolina Concha and Joseph J. Hanly
- 195** *Mechanisms of Change: A Population-Based Perspective on the Roles of Modularity and Pleiotropy in Diversification*
James J. Lewis and Steven M. Van Belleghem
- 203** *Blue, Black, and Stripes: Evolution and Development of Color Production and Pattern Formation in Lizards and Snakes*
Takeo Kuriyama, Arata Murakami, Matt Brandley and Masami Hasegawa
- 217** *On the Potential Role of the Neural Crest Cells in Integrating Pigmentation Into Behavioral and Physiological Syndromes*
Luis M. San-Jose and Alexandre Roulin
- 226** *Characterization of the Leucistic Texas Rat Snake Pantherophis obsoletus*
Asier Ullate-Agote and Athanasia C. Tzika



Editorial: Evo-Devo of Color Pattern Formation

Ricardo Mallarino^{1*}, Marie Manceau² and Marcus Kronforst³

¹ Department of Molecular Biology, Princeton University, Princeton, NJ, United States, ² Center for Interdisciplinary Research in Biology, Collège de France, Paris, France, ³ Department of Ecology and Evolution, University of Chicago, Chicago, IL, United States

Keywords: evolution, development, pigmentation, Evo-Devo, color pattern formation

Editorial on the Research Topic

Evo-Devo of Color Pattern Formation

Because of their diversity, visual accessibility, and profound impact on the fitness of organisms in the wild, animal pigment patterns are one of the most fascinating traits found in nature. Although biologists have long studied the ultimate causes underlying pigment patterns (i.e., why they evolve) the molecular, genetic, and developmental mechanisms underlying their formation and evolution (i.e., how they are established) are less well-understood. In recent years, advances in genomic, molecular, and imaging approaches have opened an exciting opportunity to explore the mechanistic basis of color pattern formation in unprecedented detail.

Do the same genetic/developmental mechanisms underlie similar traits in different animals? Are the same/homologous genes repeatedly used by evolution to achieve the same purpose? What are the mechanisms by which gene regulator complexity is translated into phenotypic outputs? Unlike many other fields, in which the bulk of the research is predominantly carried out in a single species, researchers studying the mechanistic basis of color pattern formation capitalize on the strengths of a variety of model organisms, representing a wide taxonomic range. This approach provides an excellent framework for addressing long-standing questions in evolutionary developmental biology and gaining general insights into the molecular processes by which phenotypic diversity is generated.

This Research Topic on the evo-devo of color pattern formation brings together original research papers, perspectives, and reviews showcasing the wide variety of model systems (e.g., insects, reptiles, birds, amphibians, and mammals) and multidisciplinary approaches (e.g., experimental embryology, genomics, modeling, transcriptomics, imaging, etc.) that researchers are taking to study the mechanistic basis of color pattern formation. Below, we highlight the main findings of the contributions that form part of this fascinating Research Topic.

INSECTS

Butterfly wing pattern features known as “eyespot” have been well-characterized from the perspective of developmental genetics but we still lack a clear understanding of the function of eyespots or the cause of their divergence among species. Debat et al. study the evolution of eyespot number, size, and patterning across the neotropical butterfly genus *Morpho* (the big, flashy-blue butterflies). Interestingly, they identify traits, like eyespot number, that appear to evolve neutrally, and others, like position and size, that suggest selection.

Concealing mimicry is one of the most fascinating survival strategies: prey species evolve a color or shape resembling that of other species or their habitat, thereby deceiving predators. In this study, Yoda et al. describe a molecular interaction between products of pigment-producing genes in the control of butterfly pupae color dimorphism due to green or brown background matching.

OPEN ACCESS

Edited and reviewed by:

Mark A. Elgar,
The University of Melbourne, Australia

*Correspondence:

Ricardo Mallarino
rmallarino@princeton.edu

Specialty section:

This article was submitted to
Evolutionary Developmental Biology,
a section of the journal
Frontiers in Ecology and Evolution

Received: 18 June 2021

Accepted: 14 July 2021

Published: 06 August 2021

Citation:

Mallarino R, Manceau M and
Kronforst M (2021) Editorial: Evo-Devo
of Color Pattern Formation.
Front. Ecol. Evol. 9:727516.
doi: 10.3389/fevo.2021.727516

Hughes et al. investigate the gene regulatory networks (GRN) underlying dimorphic tergite pigmentation in *Drosophila*. By surveying an extensive number of fly species, they find that male-specific melanism is phylogenetically widespread and is established by the use of a common metabolic pathway. Interestingly, however, they find that the mechanisms underlying the regulation of this metabolic pathway differs among species, thereby indicating that there are different degrees of flexibility and constraint on GRNs.

Because of their marked differences in pigmentation patterns and powerful molecular tools, species in the genus *Drosophila* have provided fascinating insights into the genetic basis of phenotypic traits. Using state of the art genome editing tools, Lamb et al. demonstrate that the gene *ebony*, which had been previously found in a QTL mapping experiment, plays a major role in establishing pigmentation differences between two closely related fly species. In addition, they find that *ebony* affects cuticular hydrocarbon abundance in both species but does not control between species variation in this trait, demonstrating that cis-regulatory changes are able to cause divergence in some, but not all, traits affected by the same gene.

Lepidopteran color pattern diversity may seem boundless, yet through a detailed phenotypic survey of color distribution in 200 genera of butterflies, McKenna et al. identify common trends in four typical regions. By correlating these common themes with genetic marker distribution in imaginal disks, the authors show that positional information along the antero-posterior axis serves an important role in butterfly wing color pattern evolution.

The Odonota (Dragonflies and damselflies) display a considerable diversity of color patterns and have a visual system that is capable of recognizing such patterns, suggesting that this trait plays an important role in their ecology. In this review, Futahashi surveys the wide diversity of color patterns seen in this group and suggests a way of classifying them. In addition, he describes some of the physical and chemical properties that the bodies of members of this insect Order have evolved to produce such diversity of patterns.

The nymphalid ground plan (NGP), a theoretical archetype that describes characters in butterfly wings, has been widely adopted in comparative studies because it allows for the identification of homologous elements across species. Here, Gawne and Nijhout present a thorough analysis of the diverse wing patterns found in arctiid tiger moths and conclude that, while some of the wing elements of moths are present in the NGP, others are not. Based on these findings, the authors present a new theoretical groundplan that will provide a more comprehensive framework for comparative studies of Lepidopteran wing pattern evolution.

Wing patterns in *Heliconius* butterflies serve as emblematic study systems in evolutionary genetics and developmental biology. McMillan et al. provide here a thorough review of decades of work in this system to illustrate how recent progress opened new opportunities to answer long-standing questions on the spatio-temporal hierarchy of patterning signal propagation, the evolution of new regulatory variation, or the mechanisms constraining pattern diversification.

Developmental genes *engrailed* and *invected*, which trace back to an ancient gene duplication event, have both been implicated in various aspects of butterfly wing patterning. By studying a classic evo-devo model system, the butterfly *Bicyclus anynana*, Das Banerjee et al. find evidence for a third member of this gene family, which they name *invected-like*. They discover that these genes have diverged in their expression patterns, with *engrailed* being expressed in the posterior portion of the pupal wing as well as the eyespot center and a ring around the eyespot. *Invected* and *invected-like*, in contrast, are expressed only in the center of eyespots.

Wnt is a signaling pathway that participates in a myriad of developmental processes. Specific members of this pathway, like *wingless* and *WntA*, have been shown to play a role in butterfly wing patterning. Fenner et al. explore the potential role of Wnt signaling on wing patterning in the butterfly family Pieridae, where little work has been done. They show that while *WntA* does not appear to be expressed during wing development, pharmacological treatment with heparin enhances melanization and alters UV reflectance, indicating a role for one or more Wnt genes during wing pattern development.

Shiny, metallic wing scales occur in a number of butterfly species but the exact structural features responsible for this trait remain a mystery. The unique appearance of these scales is known to be a product of their morphology, as opposed to pigments, but how do different butterfly clades produce these metallic scales and do they do it the same way? Ren et al. image metallic scales across five butterfly families and find some recurring themes as well as instances of unique scale morphologies in certain lineages. Their results suggest both similarities and differences in the origin of an apparently convergent phenotype.

Melanin is a central pigment across the animal kingdom and a core constituent of virtually all butterfly wing patterns. Furthermore, the biosynthetic pathway that produces melanin in insects is well-described. Kuwalekar et al. study the molecular evolution of six critical melanin pathway enzymes across Lepidoptera and show that these genes are largely shaped by purifying selection. Analysis of developmental expression patterns in one butterfly species, *Papilio polytes*, reveals marked instances of sexually dimorphic expression. Together, these results show how a core set of conserved genes can produce the diversity of lepidopteran wing patterns by altering the time and place of their expression.

VERTEBRATES

Domesticated animals, such as pigs, horses, and chickens, display a rich variety of coat and plumage patterns because these traits have been directly selected by breeders throughout many generations. In his review article, Andersson provides a detailed account of some of the mutations that disrupt existing color patterns or generate new ones. The examples illustrate the utility of using domesticated animals to map the genetic basis of traits.

While reviews of color pattern formation and evolution abound, none has thus far been dedicated to reptilian sauropsids

(snakes, lizards) despite the extreme diversity in color, geometry, and ecological function of motifs adorning their skin. Kuriyama et al. fill this gap, linking current knowledge of pigmentation genetics and cell-based pigment production to theoretical and empirical work taking advantage of reptile color pattern attributes such as stripes and blue tails.

Cichlid fishes represent one of the classic examples of adaptive radiation and are widely known for their marked diversity in pigment patterns. Vertical bar patterns have independently evolved in multiple instances across the cichlid phylogeny but their developmental basis remains a mystery. By examining the ontogeny of vertical bar pattern formation in a cichlid species, Liang et al. provide a thorough account of the cellular basis underlying the formation of this trait and discover that it is primarily established by spatially localized changes in melanophore density, melanin synthesis, and melanophore dispersal.

Melanin-based coloration is associated with various behaviors and physiological traits. In this review, San Jose and Roulin discuss a breadth of recent work suggest that the link between these traits is their embryonic origin, namely the neural crest. Studying genes controlling the specification and differentiation of neural crest cell lineages for associated traits can thus provide a unique and powerful framework to integrate developmental biology and ecology.

Bird species have evolved a wide variety of pigment patterns, both across the body (macro) and within each feather (micro). In a review paper, Inaba and Chuong provide a thorough account of the cellular and developmental mechanisms by which macro- and micro-scale color patterns in birds are established and conclude that the marked differences seen in this group results from a complex interplay between tissues, cell types of different developmental origins, and interacting environmental factors.

Light skin color mutants are frequent in vertebrates: albino and leucistic phenotypes have been linked to genetic mutations in pigment production enzymes or pigment-cell differentiation. In this study, Ullate-Agote and Tzika show that leucistic Texas rat snakes lack melanophores and xanthophores, but not iridophores. They pinpoint single nucleotide deletions in the coding sequence of MITF or decreases in MITF expression in leucisitic individuals, suggesting this gene controls the specification of neural-crest derived pigment-cells but not that of iridophores.

EVO-DEVO THEORY

Developmental genes are frequently thought to be expressed in a modular fashion, temporally and/or spatially, due to the action of modular cis-regulatory elements (CREs). This is a core concept in the field of evolutionary developmental biology (evo-devo). However, Lewis and Van Belleghem point out a

number of examples in which CREs have been found to function in a pleiotropic, rather than modular, fashion. They propose “evolutionary modularity” as a potential explanation for how seemingly modular phenotypes might be controlled by non-modular developmental genes.

CONCLUDING REMARKS

As evidenced by the contributions to this special issue, the evo-devo of color pattern formation is a thriving field, where researchers are employing a large variety of species and using multidisciplinary approaches to tackle a broad range of questions. Thanks to recent technical advances, the approaches and types of analyses that were once restricted to traditional model species are now widely applicable to a myriad of species with fascinating phenotypes. We anticipate that this trend will grow exponentially over the next few years, as researchers from more disciplines and backgrounds become engaged with the rich set of questions that remain wide open. By integrating the studies that are being done at multiple levels of biological organization—from molecules, to gene regulatory networks, to tissues and whole organisms—we will be able to obtain unprecedented mechanistic and conceptual insights into the processes that control color pattern formation and evolution.

AUTHOR CONTRIBUTIONS

RM, MM, and MK co-edited the Research Topic. All authors contributed to the article and approved the submitted version.

FUNDING

RM was supported by NIH grant: 5R35GM133758. MK was supported by: NIH grant GM131828 and NSF grant IOS-1922624.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2021 Mallarino, Manceau and Kronforst. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Developmental and Cellular Basis of Vertical Bar Color Patterns in the East African Cichlid Fish *Haplochromis latifasciatus*

Yipeng Liang, Jan Gerwin, Axel Meyer and Claudius F. Kratochwil*

Zoology and Evolutionary Biology, Department of Biology, University of Konstanz, Konstanz, Germany

OPEN ACCESS

Edited by:

Ricardo Mallarino,
Princeton University, United States

Reviewed by:

Pedro Martinez,
University of Barcelona, Spain
Jose Maria Martin-Duran,
Queen Mary University of London,
United Kingdom

*Correspondence:

Claudius F. Kratochwil
Claudius.Kratochwil@uni-konstanz.de

Specialty section:

This article was submitted to
Evolutionary Developmental Biology,
a section of the journal
Frontiers in Cell and Developmental
Biology

Received: 15 December 2019

Accepted: 22 January 2020

Published: 11 February 2020

Citation:

Liang Y, Gerwin J, Meyer A and
Kratochwil CF (2020) Developmental
and Cellular Basis of Vertical Bar Color
Patterns in the East African Cichlid
Fish *Haplochromis latifasciatus*.
Front. Cell Dev. Biol. 8:62.
doi: 10.3389/fcell.2020.00062

The East African adaptive radiations of cichlid fishes are renowned for their diversity in coloration. Yet, the developmental basis of pigment pattern formation remains largely unknown. One of the most common melanic patterns in cichlid fishes are vertical bar patterns. Here we describe the ontogeny of this conspicuous pattern in the Lake Kyoga species *Haplochromis latifasciatus*. Beginning with the larval stages we tracked the formation of this stereotypic color pattern and discovered that its macroscopic appearance is largely explained by an increase in melanophore density and accumulation of melanin during the first 3 weeks post-fertilization. The embryonal analysis is complemented with cytological quantifications of pigment cells in adult scales and the dermis beneath the scales. In adults, melanic bars are characterized by a two to threefold higher density of melanophores than in the intervening yellow interbars. We found no strong support for differences in other pigment cell types such as xanthophores. Quantitative PCRs for twelve known pigmentation genes showed that expression of melanin synthesis genes *tyr* and *tyrp1a* is increased five to sixfold in melanic bars, while xanthophore and iridophore marker genes are not differentially expressed. In summary, we provide novel insights on how vertical bars, one of the most widespread vertebrate color patterns, are formed through dynamic control of melanophore density, melanin synthesis and melanosome dispersal.

Keywords: vertical bars, pigment development, gene expression, chromatophores, Cichlidae

INTRODUCTION

Pigment patterns play important roles in many aspects of animal biology. Yet, until now, only in a few “model” organisms we do have insights into the molecular and developmental underpinnings of color pattern formation and evolutionary diversification. Among teleosts, the zebrafish *Danio rerio* and the Medaka *Oryzias latipes* are the main model organisms for investigation of pigmentation (Meyer et al., 1993, 1995; Kelsh et al., 1996; Nagao et al., 2014; Irion and Nüsslein-Volhard, 2019; Patterson and Parichy, 2019). More recently, African cichlid fishes with their richness in color patterns are increasingly studied to understand the molecular mechanisms of color pattern formation including but not limited to egg spot patterns (Henning and Meyer, 2012; Santos et al., 2014, 2016), blotch patterns (Streelman et al., 2003; Roberts et al., 2009), amelanism (Kratochwil et al., 2019b), horizontal stripe patterns (Ahi and Sefc, 2017; Kratochwil et al., 2018;

Hendrick et al., 2019) and pigment distribution more generally (Albertson et al., 2014). And although progress has been made identifying target genes and loci that drive evolutionary diversification in cichlids (Roberts et al., 2009; Kratochwil et al., 2018) and play key roles in adaptation and speciation (Seehausen et al., 1999; Elmer et al., 2009; Maan and Sefc, 2013), the developmental and cellular mechanisms of pigmentation phenotypes have been barely studied.

Pigment patterns are ultimately caused by spatial variation in pigmentary and/or structural tissue properties. Those can be generated by different distribution, density and aggregation state of pigment cells (chromatophores) and their multi-layered arrangement, as well as variation in the synthesis and arrangement of light-absorbing pigments or molecules causing structural coloration (Irion and Nüsslein-Volhard, 2019; Patterson and Parichy, 2019). In teleosts several types of chromatophores, including melanophores, xanthophores, iridophores, erythrophores, leucophores, and cyanophores have been described (Burton and Burton, 2017). Melanophores (containing the brown to black pigment melanin), xanthophores/erythrophores (containing yellow to red pigments) and iridophores (containing reflective guanine platelets causing structural coloration) have been also found in cichlids (Maan and Sefc, 2013).

For the mechanisms of color pattern formation, mainly (horizontal) stripe patterns have received attention because the most commonly studied “model” teleost, the zebrafish, carries this characteristic pattern. Vertical bars are less studied, with the exception of recent detailed description of the convict cichlid *Amatitlania nigrofasciata* (Prazdnikov and Shkil, 2019), studies in Amphiprioninae, the anemone fishes (Salis et al., 2018; Roux et al., 2019) and Lake Malawi cichlids (Hendrick et al., 2019). Bar patterns are presumably adaptive as such disruptive coloration breaks the outline of the individual and thereby constitutes a form of camouflage, in particular in visually complex habitats (Seehausen et al., 1999; Maan and Sefc, 2013). Additionally color patterns are often involved in species recognition (Hemingson et al., 2019).

Here, we focus on the vertical bar pattern of *Haplochromis latifasciatus* (Figure 1E) from Lake Kyoga, a lake north of Lake Victoria. *H. latifasciatus* is a species of the haplochromine cichlids, the most species-rich cichlid lineage that forms the adaptive radiations of Lake Victoria and Malawi with 500 and 800 species respectively. We describe the formation of the pattern during development and compare it to other teleosts, characterize which cell types and properties underlie this conspicuous pattern, and use a candidate gene approach to obtain insights into the underlying molecular mechanisms.

RESULTS

Developmental Progression of Vertical Bar Formation in *H. latifasciatus*

Both male and female *H. latifasciatus* in adult stage are characterized by four (in some strains five) vertical melanin bars. Individuals of our breeding stock had consistently four bars

($n > 30$; Figure 1E): one anterior bar above the operculum (vertical bar 1; vb1), two vertical bars in the trunk area that cover the whole dorso-ventral axis (vb2 and vb3) and a more posterior vertical bar (vb4) at the anterior caudal peduncle that only covers the dorsal part up to the horizontal myoseptum. The regions between the bars (referred to as interbars, ib) are yellow to beige with dominant males often having nuptial colors with stronger yellow but anteriorly also red to orange hues (Figures 1Q,R). In contrast to the closely related Lake Victoria cichlids bars are thicker and more pronounced in *H. latifasciatus* with a clearer demarcation, lower number and without as pronounced physiological color change (Greenwood, 1974; Seehausen et al., 1999).

To investigate the formation of vertical bars (Figure 1E), we described the development of *H. latifasciatus* larvae between 7 and 21 days post-fertilization (dpf; Figures 1A–D,F–P and Supplementary Figure S1). The developmental progression of vertical bar formation is fully consistent in all three individuals examined (Supplementary Figures S1, S2). At 7 dpf, melanophores are present in the dorsal head region as well as on the dorsal part of the yolk sac. In the trunk area only a few melanophores have formed without any obvious indication of a bar-like pattern (Figure 1A). Starting at 8 dpf vertical bars form in an antero-posterior sequence (Figures 1B–D,F–P and Supplementary Figure S1). Trunk melanophore number has increased considerably within an anterior dorsal patch (dc1) forming at 8 dpf (data not shown), followed by a more posterior one at 9 dpf (dc2; Figure 1B). The melanophore patches anticipate the position of vb1 and vb2, respectively (Figures 1B,C). At 10 dpf a third and fourth dorsal patch (dc3 and dc4) are appearing at the positions where vb3 and vb4 will later form, respectively (Figures 1C,G and Supplementary Figures S1, S2). After the appearance of the dorsal patches they expand dorsally into the dorsal fin and ventrally forming the four bars. One exception is the posterior bar vb4, where the bar only extends to the horizontal myoseptum. The formation of vb3 is furthermore contributed by a second melanophore cluster (lateral cluster; lc) that forms in a more posterior-ventral position and merges with the developing bar between 12 and 13 dpf (Figures 1C,I,J and Supplementary Figures S1, S2). After this time, at around 2 weeks post-fertilization, the complete adult vertical pattern is already fully formed, but the contrast of the bars further increases until 21 dpf (Figures 1D,P and Supplementary Figure S1) and beyond.

Cellular Correlates of Vertical Bar Formation

In order to understand the formation of the characteristic bar pattern of *H. latifasciatus*, we analyzed the progression of the bar pattern formation over time. Specifically, we analyzed how the darkening of the bar regions is generated at the cellular level. We hypothesized that three processes might contribute to the darker appearance of the bar regions: (a) melanosome dispersal (the aggregation and dispersion of melanosomes, the melanin-containing organelles, within melanophores) as it increases the fraction of the tissue covered by melanin, (b) the density of

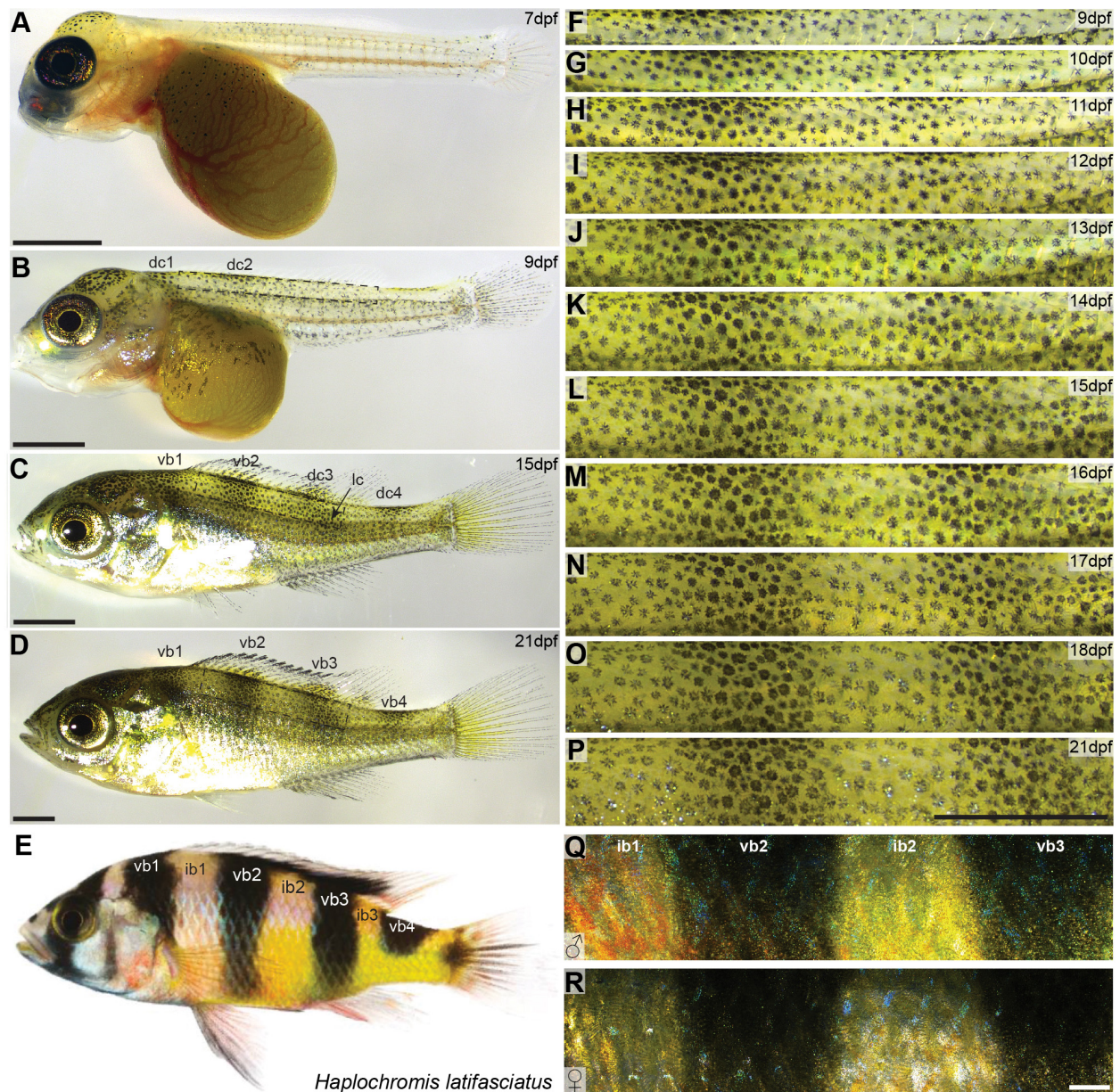


FIGURE 1 | Vertical bar patterns in *Haplochromis latifasciatus*. **(A–E)** Developmental stages of *H. latifasciatus* at 7 dpf **(A)**, 9 dpf **(B)**, 15 dpf **(C)**, 21 dpf **(D)** and an adult individual **(E)**. At 7 dpf Melanophores are mainly located on the head and yolk sac **(A)**. At 9 dpf, two melanophore dorsal clusters (dc1 and dc2) are appearing indicating the position of vb1 and vb2, respectively **(B)**. The adult vertical pattern is already fully formed at 15 dpf **(C)** and further increases in contrast until 21 dpf **(D)** and beyond. Adult *H. latifasciatus* are characterized by four dark vertical bars **(E)**. The region between the bars (interbars) have yellow to orange-red hues. **(F–R)** High magnification microscopy images showing the development of ib1, vb2, vb3 from 9 dpf to 21 dpf **(F–P)** and adult male **(Q)** and female **(R)**. Interbar melanophores are often lighter, with parts of the chromatophore or the center being almost pigment-free **(F–P)**. Vertical bar pattern does not differ between sexes in adults **(Q,R)**. Abbreviations: dc: dorsal cluster, lc: lateral cluster, vb: vertical bar, ib: interbar. Scale bars are 1 mm in **(A–D,F–P)**, 2 mm in **(Q,R)**.

melanophores, and (c) the darkness of the melanophores, i.e., the concentration of melanin.

To investigate the development and the importance of these factors we followed the development of three individuals over twelve days of development between 9 and 21 dpf focusing on the dorsal portion of the two bars in the trunk region, vb2 and vb3 and the yellow interbar region in between

(ib2). To assess melanosome dispersal, we calculated the diameter of the melanin-covered part of all melanophores (see section Materials and Methods). We found no strong spatial difference in melanosome aggregation (**Figure 2A** and **Supplementary Figure S1**), yet dispersal diameters increased with age, likely because cells are still growing during these early developmental stages (**Supplementary Figure S3**). Cell

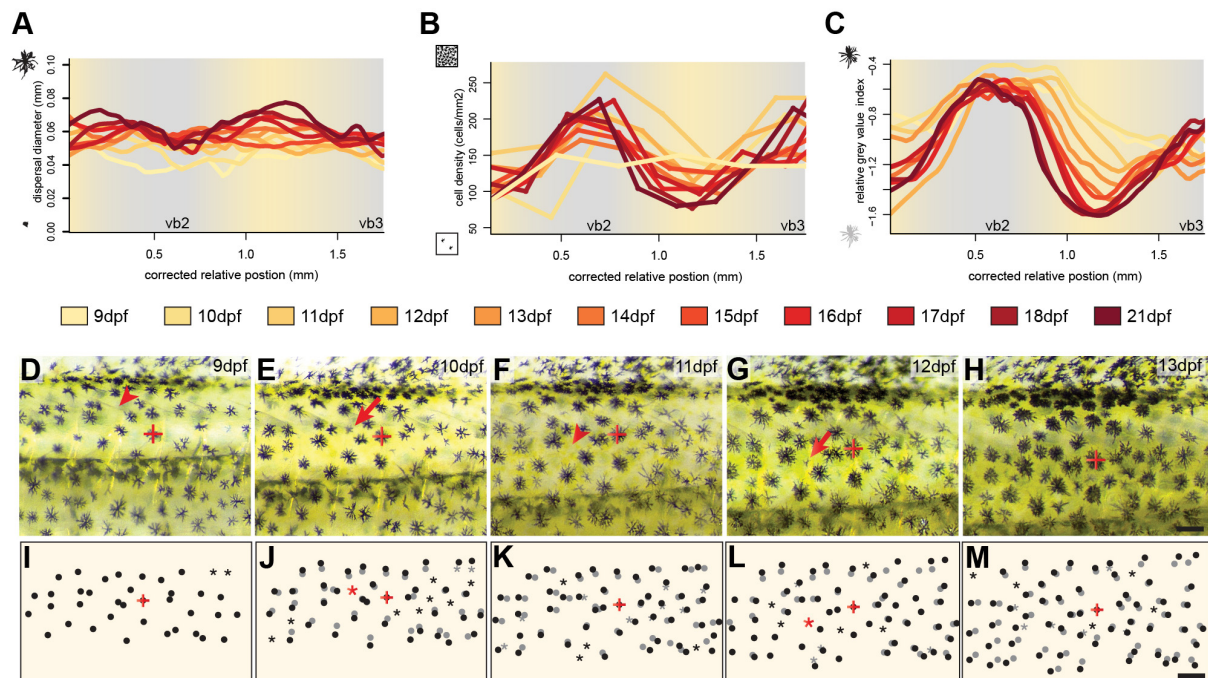


FIGURE 2 | Cellular changes during vertical bar formation. (A–C) Measurement of melanosome dispersal diameter, melanophore density and the darkness of the melanophores focusing on the dorsal portion of vb2, ib2, and vb3 illustrate changes in chromatophore number and characteristics during bar pattern formation between 9 and 21 dpf. No obvious difference could be found in melanophore dispersal diameter between bars and interbars (A). The melanophore density increases in bars compared to interbars (B). Relative gray values of melanophores increase as well (C). (D–M) Time-lapse vertical bar development in the same individual from 9 to 13 dpf as photographs (D–H) and schematics (I–M). The melanophore with the red cross in (D–M) was used to align the images. Dots show the position of melanophores in (I–M). Position on the previous days (based on image overlay) are labeled in gray. Red arrowhead in (D–H) and asterisks in (I–M) indicate where the formation of a new melanophore will occur, red arrows in (D–H) and black dots next to a gray asterisk in (I–M) the forming melanophore on the consecutive day. Scale bars are 0.5 mm in (D–M).

density was evenly distributed at 9 dpf, but during the formation of the bars, cells became more densely packed in the bar regions, while cell density decreased in the interbar regions (Figure 2B and Supplementary Figure S1). To measure the darkness of individual melanophores we measured relative gray values (see section Materials and Methods). Here the difference between bar and interbar regions continuously increased suggesting stronger accumulation of melanin in bar melanophores (Figure 2C and Supplementary Figure S1). Indeed, closer observation of melanophores shows that interbar melanophores are often not fully filled with melanin, with parts of the chromatophore, often the center of the cell being poorly pigmented (Figures 1N–P and Supplementary Figure S1).

Next, we investigated the cellular behavior of single melanophores between 9 and 13 dpf. To do so, we photographed the vb2 region of the same individual on five consecutive days and tracked cellular migration and the formation of new melanophores (Figures 2D–M). The data suggests that melanophores mainly move indirectly through the general expansion of the skin. Newly differentiating melanophores could be found across the whole examined area, but at an increased rate in the forming bars. They grow to the full size (diameter: ~0.06 mm) within 1–2 days. We found no evidence of proliferating melanin-containing cells.

In summary, these results suggest that the bars of *H. latifasciatus* form through spatial variation in melanophore properties (i.e., melanin content) and melanophore cell density that mainly arise in the second week after hatching (standard length 6–8 mm). The increase in melanophore density is most likely caused by an increased differentiation of melanoblasts within the bar regions.

Adult Patterns in *H. latifasciatus*

To investigate the distribution of chromatophores in different integument regions, we estimated cell density and size of both, melanophores and xanthophores in the two interbars (ib1, ib2) and the two bars (vb2, vb3) of the trunk region (Figure 1E). Here, we only quantified pigment cells in female individuals as the vertical bar pattern does not differ between sexes, but substantial interindividual variation in red coloration of males would have complicated quantifications (Figures 1Q,R). Scales and dermis without scales (from hereon called “scales” and “skin”, respectively) were analyzed separately. Three types of chromatophores could be found in both the dark bars and the light interbars: melanophores with black/dark brown pigments, xanthophores with yellow to orange and reddish pigments (we did not differentiate between xanthophores and erythrophores; see discussion in section Materials and Methods), and iridophores with

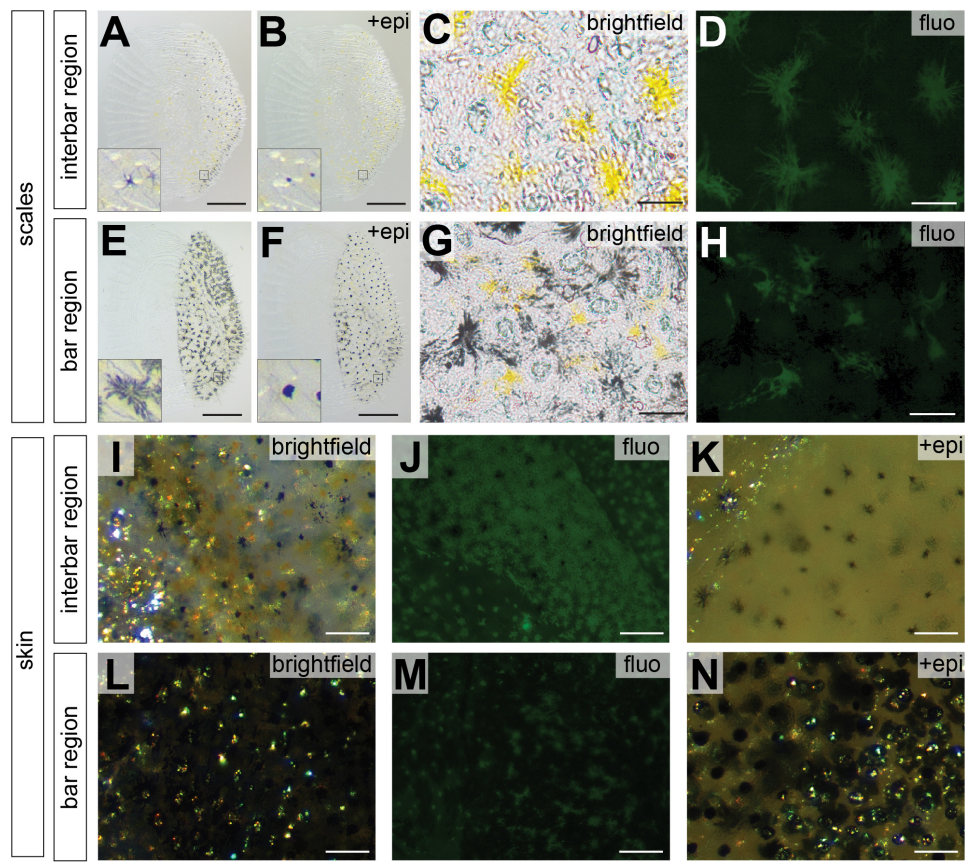


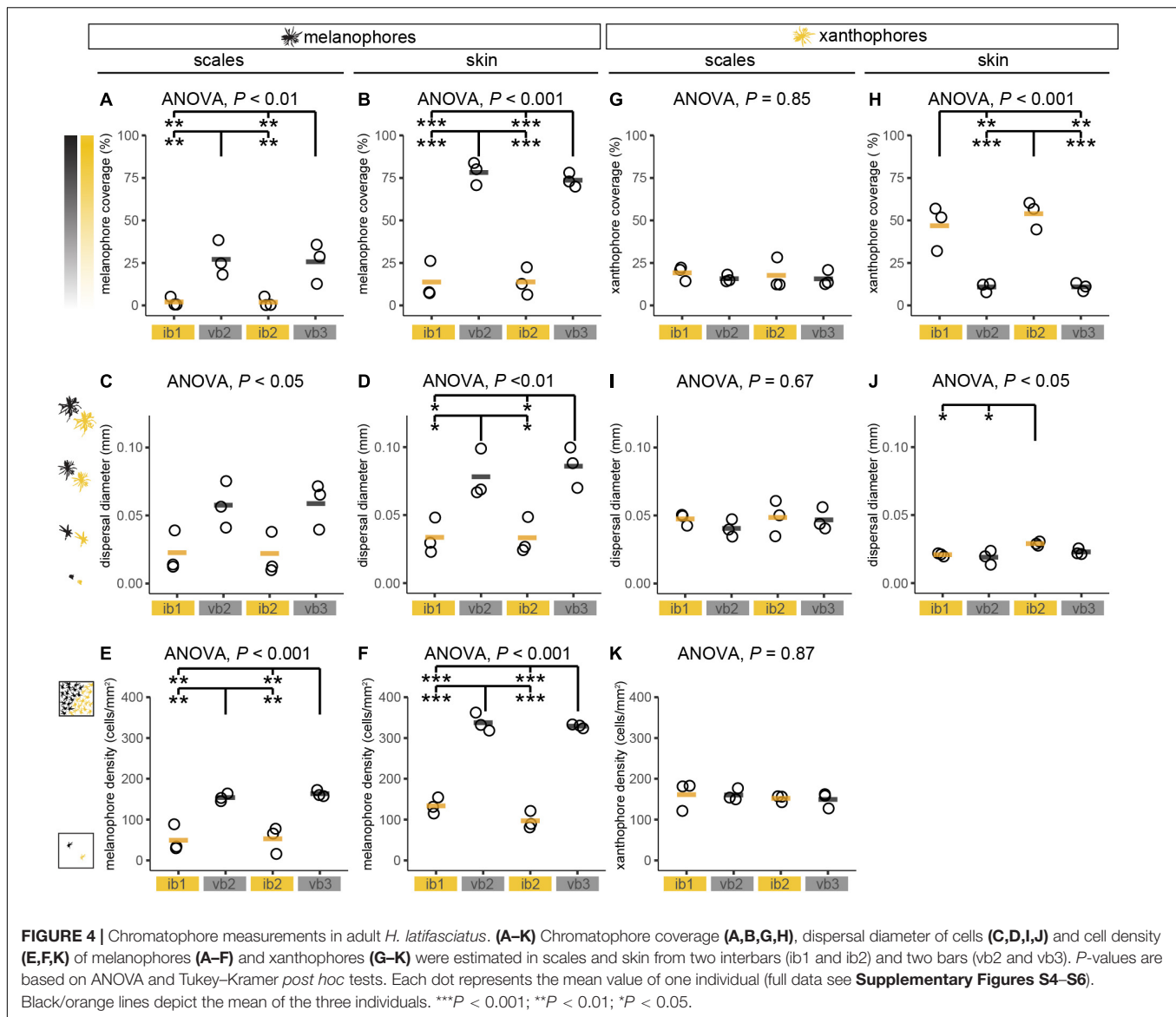
FIGURE 3 | Photographs of scale and skin dissections in bar and interbar region. (A–H) Photographs of scales from interbar region (A–D) and bar region (E–H). Melanophores from scales of interbars (A) and bars (E) aggregate after epinephrine treatment (B,F) allowing accurate quantification. Insets of (A,B,E,F) show the single melanophores before and after epinephrine treatment. Xanthophores (yellow) from scales of interbars (C) and bars (G) were detected via their autofluorescence (D,H). (I–N) High magnification photographs skin, where scales have been removed. Brightfield images of interbar (I) and bar (L) fluorescent images of interbar (J) and bar (M); brightfield images after epinephrine treatment for interbar (K) and bar region (N). Scale bars are 500 μm in (A,B,E,F); 50 μm in (C,D,G,H); 100 μm in (I–N).

iridescent/reflective properties (Figures 3I,L). To quantify pigment cell quantity and properties we used three measurements: (a) pigment cell coverage, (b) pigment cell dispersion, and (c) pigment cell density.

Pigment cell coverage is influenced by both cell number and size (or intracellular dispersal of pigments in the cell) and was measured by estimating the percentual coverage of the tissue with pigment using light microscopy for melanophores and fluorescence microscopy to detect the autofluorescence of xanthophores. Consistent with the visual impression, melanophore coverage in bars was significantly higher than in the yellowish interbars in both scales (26.4% in bars, 2.0% in interbars) and skin (75.9% in bars, 13.7% in interbars) (Figures 3, 4A,B, Supplementary Figure S4, and Supplementary Table S1). Both melanophore density and melanosome dispersal contributed to the difference in melanophore coverage. The average melanosome dispersal diameter is larger in dark bars ($\bar{O}$ 0.058 mm in scale, $\bar{O}$ 0.082 mm in skin) than light interbars ($\bar{O}$ 0.022 mm in scale, $\bar{O}$ 0.033 mm in skin) (Figures 4C,D, Supplementary Figure

S5, and Supplementary Table S1). However, variation within the same skin region was quite high suggesting that both dispersed and aggregated melanophores are widely distributed (Supplementary Figure S5 and Supplementary Table S1). The density of melanophores in both scale and skin were significantly higher in the bars (158 cells/ mm^2 in scale, 333 cells/ mm^2 in skin) than in interbars (52 cells/ mm^2 in scale, 115 cells/ mm^2 in skin) (Figures 4E,F, Supplementary Figure S6, and Supplementary Table S1).

Xanthophore coverage in bars and interbars is similar on scales (15.7% in bars, 18.4% in interbars), but significantly different in the skin (10.9% in bars, 50.4% in interbars) (Figures 3D,H,J,M, 4G,H, Supplementary Figure S4, and Supplementary Table S1). However, xanthophore coverage might be underestimated in the skin of the bar regions due to the high density of melanophores that could aggravate detection of the xanthophore autofluorescence. The dispersal diameter of xanthophores did not differ between interbars ($\bar{O}$ 0.048 mm in scale, $\bar{O}$ 0.025 mm in skin) and bars ($\bar{O}$ 0.044 mm in scale, $\bar{O}$ 0.021 mm in skin), neither in



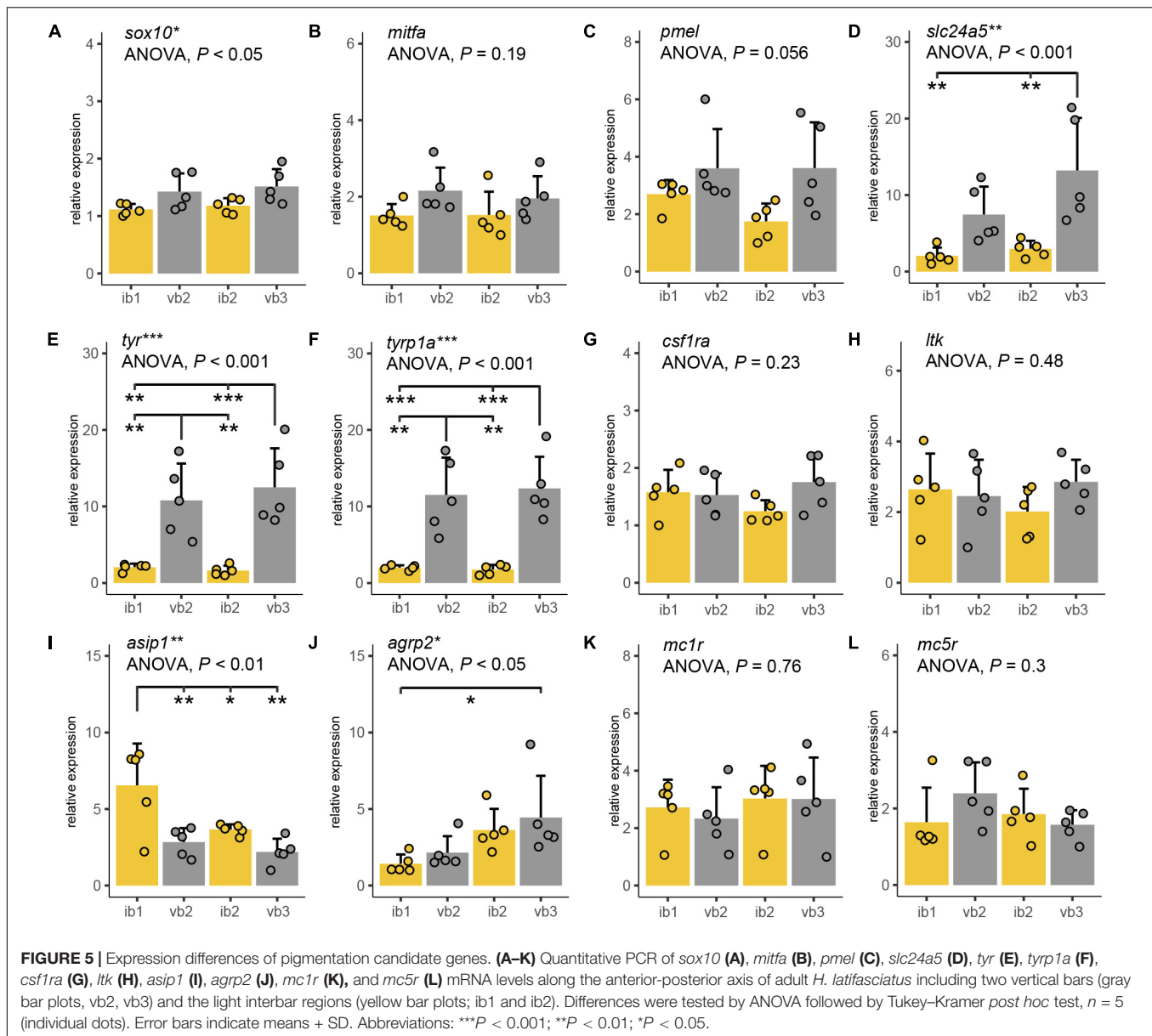
scales nor in skin preparations (Figures 4I,J, **Supplementary Figure S5**, and **Supplementary Table S1**). Cell densities were comparable in scales of interbars (157 cell/mm²) and bars (155 cell/mm²) (Figure 4K, **Supplementary Figure S6**, and **Supplementary Table S1**).

Gene Expression Associated With the Bar Patterns in *H. latifasciatus*

Next, we analyzed the molecular correlates of the observed differences in pigment cell density and pigment synthesis. Molecular markers for iridophore and xanthophore were also used as we could not analyze differences in these cells' number due to the high melanophore density in the bar regions. Therefore, we compared expression levels of twelve candidate genes across the same two bar and interbar regions for the chromatophore measurement (ib1, vb2, ib2, vb3) using

quantitative real-time PCR (qPCR; Figure 5 and Table 1). The selected genes are including marker genes for chromatophores, melanin synthesis genes and genes involved in the melanocortin signaling pathway. The latter was a particular focus as they have been previously implicated in color pattern formation of cichlids, teleosts and vertebrates more generally (Table 1).

Sox10 is a marker gene for chromatophore progenitors (Dutton et al., 2001). Expression was slightly higher in bar than in interbar regions (Figure 5A) and significantly differed between regions (ANOVA: *P* < 0.05). However, we found no significant *sox10* expression variation between bars and interbars (Tukey HSD: *P* = 0.067–0.37; **Supplementary Table S2**). Expression of the melanophores marker, *mitfa* (Lister et al., 1999; Béjar et al., 2003) was higher within the dark vertical bars than in the adjacent light interbars (Figure 5B and **Supplementary Table S2**), yet differences were not significant (ANOVA: *P* = 0.19). Similar expression profiles can be also found in *pmel*, a melanophore



specific gene important for melanin deposition in melanosomes (Schonthaler et al., 2005). Also here, we find that *pmel* is expressed at a higher yet not significantly higher (ANOVA: $P = 0.056$) level in dark bars compared to interbars (Figure 5C and Supplementary Table S2). The gene *slc24a5*, a melanosome-specific cation exchanger (Lamason et al., 2005), showed differential expression in some pair-wise comparisons of bar and interbar regions (ANOVA: $P < 0.01$; Tukey HSD: $P = 0.002$ – 0.18 ; Figure 5D and Supplementary Table S2). The two melanophore-specific genes that express melanogenic enzymes and are essential for the production of melanin, *tyr* (Hidehito et al., 1994; Camp and Lardelli, 2001) and *tyrp1a* (Braasch et al., 2009; Krauss et al., 2014) showed significantly higher expression levels in bars (ANOVA: both $P < 0.001$; Tukey HSD: all $P < 0.01$; Figures 5E,F and Supplementary Table S2).

In order to compare the distribution of iridophores and xanthophores, we used the iridophore lineage-specific marker *ltk* (Lopes et al., 2008) and the xanthophore marker *csf1ra* (Parichy and Turner, 2003). Notably, both *ltk* and *csf1ra* were expressed at similar levels across the differently pigmented bar and interbar regions (Figures 5G,H and Supplementary Table S2). This is in support of a rather homogenous distribution of iridophores and xanthophores across the trunk.

We also examined the gene expression of Agouti family genes (namely *Asip/asip1* across all vertebrates and *agrp2* in cichlid fishes) and melanocortin receptors, as they have been previously implicated in pigmentation. Two proteins of the Agouti family, *Asip1* and *Agrip2*, likely act as the antagonists for the melanocortin receptors *Mc1r* and/or *Mc5r* in teleost skin (Cal et al., 2017). As previous studies suggest,

asip1 is a key regulator of dorso-ventral countershading, presumably by regulating melanophore number (Ceinos et al., 2015; Cal et al., 2017, 2019). Interestingly we also found significant variation along the anterior-posterior axis (ANOVA: $P < 0.01$) with *asip1* being expressed significantly higher in some pair-wise *post hoc* comparisons between bars and interbars (Tukey HSD: $P = 0.0016$ – 0.8222 ; **Figure 5I** and **Supplementary Table S2**).

Previous work in cichlids showed that *agrp2* regulates presence/absence of stripe patterns while not contributing to shaping the pigment pattern itself through expression variation across the skin (Kratochwil et al., 2018). In contrast to results from the Lake Victoria species *Pundamilia nyererei*, we found significant differences between skin regions (ANOVA: $P = 0.041$; **Figure 5J** and **Supplementary Table S2**). However, expression mainly differed between anterior and posterior regions (Tukey HSD between *ib1* and *vb2*: $P < 0.05$) and not consistently between bars and interbars (Tukey HSD: $P = 0.046$ – 0.895) (**Supplementary Table S2**).

Mcl1r and *Mc5r* are two receptors antagonized by *Agrp2* and/or *Asip1* signaling and *mcl1r* and *mc5r* have been shown to be expressed in chromatophores. The expression of *mcl1r* has been reported in skin melanophores of zebrafish, barfin, and Japanese flounder (Kobayashi et al., 2010, 2012b; Higdon et al., 2013), xanthophores of goldfish (Kobayashi et al., 2011) and iridophores of zebrafish (Higdon et al., 2013). The expression of *mc5r* was detected in melanophores and xanthophores of flatfish (Kobayashi et al., 2010, 2012b). Here we found no significant expression differences for *mcl1r* (ANOVA: $P = 0.296$) and *mc5r* (ANOVA: $P = 0.764$) which suggest that the melanocortin receptors may not contribute to shaping the vertical bar pattern (**Figures 5K,L** and **Supplementary Table S2**).

DISCUSSION

Melanic Pattern Development: *H. latifasciatus* vs. Other Teleosts

Most previous investigations of teleost pigment pattern formation focused on the horizontal stripe patterns of the model teleost *Danio rerio*, the zebrafish. In zebrafish, melanoblasts, the progenitors of melanophores, migrate from the dorsal neuroectodermal margin along nerve fibers between the myotomes (Dooley et al., 2013). After they settled at their final position, melanoblasts differentiate into melanophores and accumulate melanin to form the dark horizontal stripes that gave the zebrafish its name.

In the haplochromine cichlid *H. latifasciatus* [divergence time with zebrafish approximately 220 million years (Hughes et al., 2018)] color pattern formation starts with the development of four melanophore clusters that arise in the dorsal rim of the trunk (**Figures 1B–D**). The dorsal clusters initiate the formation of bar patterns later in development, by spreading dorsally into the dorsal fin and ventrally forming bars on the trunk. The third vertical bar (*vb3*) fuses with the lateral melanophore cluster (**Figures 1C,D,I–P** and **Supplementary Figure S1**). In contrast to the zebrafish where adult color pattern develops indirectly

during a post-embryonic metamorphic phase (Parichy et al., 2009), the vertical bars in *H. latifasciatus* develop directly and are already visible at a time when the larvae are still feeding from their yolk. This is in line with previous reports on the direct development of other morphological traits of African cichlid fishes (Woltering et al., 2018).

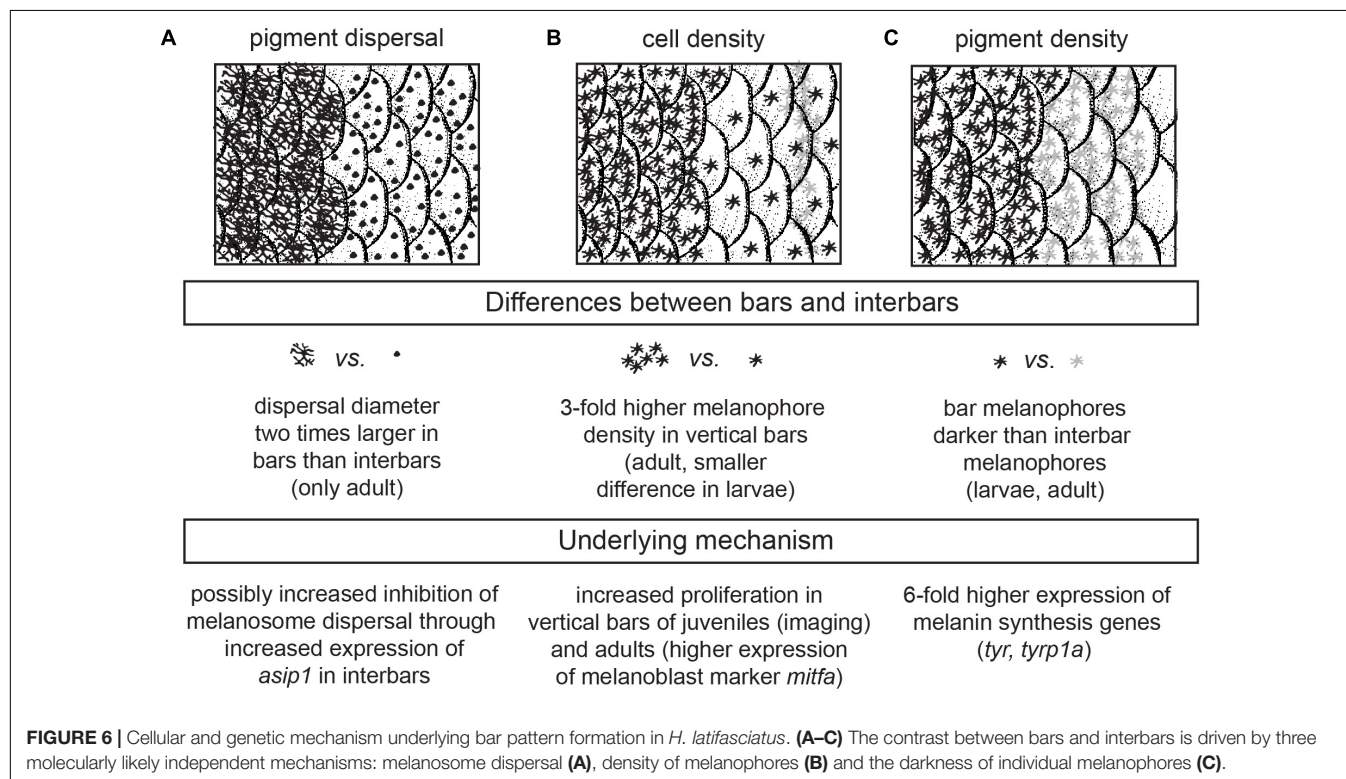
By tracking the behavior of individual melanophores we could observe how new melanophores form over a period of 1–2 days (**Figure 2** and **Supplementary Figure S1**). It is unclear how the melanoblasts reach this position. However, it is likely that they migrate dorsally from the neuroectodermal margin and then ventrally within the skin, possibly explaining the gradual expansion of bars from dorsal to ventral. This is interesting, because in zebrafish clones of single pigment cell progenitor cells have been shown to mainly spread dorso-ventrally (Singh and Nusslein-Volhard, 2015; Nüsslein-Volhard and Singh, 2017). It is possible that vertical bars are prepatterned at the progenitor level, potentially already at the level of the dorsal neuroectodermal ridge. Position and size of bars could be driven by anterior-posterior differences in progenitor number or transcriptional identity that later influence proliferation and differentiation within the dermis. It is also possible that migratory routes, environmental factors along the migratory pathways and in the skin as well as cell-cell interactions including reaction-diffusion systems contribute to or constitute the basis of this process. The anterior-posterior sequence of bar formation could be a consequence of the anterior-posterior sequence of somitogenesis. Anterior melanoblast would hereby first receive guidance cues from dermamyotome, sclerotome, and emerging dermis. Anterior neural-crest cells including pigment cell precursors would therefore migrate first as previously described (Bronner and LeDouarin, 2012).

As soon as melanophores are visible in the skin they do not migrate anymore and grow to the full size within 1–2 days. Hereby the cell-size increases, new dendritic arbors form and melanin levels visibly increase (**Figure 2** and **Supplementary Figure S1**). Therefore, the appearance of the bar pattern is mainly driven by an increase in cell density in the developing bar region as well as changing cellular characteristics including increased melanosome dispersal and melanin production (**Figures 2, 6** and **Supplementary Figure S1**).

As one of the most common pigment patterns in haplochromine cichlids, vertical melanic bars can vary in contrast and number, both between as well as within species (Witte et al., 1976; Kocher et al., 1993; Seehausen et al., 1999). However, compared to most other banded haplochromine cichlids, the vertical bars in *H. latifasciatus* are fixed in number (mostly four and in some strains five) and are relatively thick. Our study shows that vertical bar number remains constant over the course of development in *H. latifasciatus* (**Figures 1F–R, 2D–M** and **Supplementary Figure S1**), which contrasts with a recent study on the Lake Malawi cichlid *Copadichromis azureus*, a species with unfixed bar number and thinner bars (Hendrick et al., 2019). In *C. azureus*, vertical bars split during development resulting in an increase of bar number (Hendrick et al., 2019). However, both processes are not driven by rearrangement of existing

TABLE 1 | Selected candidate genes involved in coloration and pigment patterns in fish.

Gene	Full name and described function	References
<i>agrp2</i>	<i>agouti related peptide 2</i> : A member of the agouti gene family that has been shown to repress (horizontal) stripe formation in cichlids. It is also involved in background matching in zebrafish.	Zhang et al., 2010; Shainer et al., 2017; Kratochwil et al., 2018
<i>asip1</i>	<i>agouti signal peptide 1</i> : Another member of the agouti gene family that regulates dorsal-ventral pigmentation in the fish skin.	Cerda-Reverter et al., 2005; Kurokawa et al., 2006; Guillot et al., 2012; Cal et al., 2017, 2019
<i>csf1ra</i>	<i>colony stimulating factor 1 receptor a</i> : A <i>kit</i> -related tyrosine receptor kinase, which is essential for migration and survival of xanthophores.	Parichy et al., 2000; Parichy and Turner, 2003
<i>ltk</i>	<i>leukocyte receptor tyrosine kinase</i> : A tyrosine kinase receptor that is crucial for fate specification of iridophores from neural crest cells.	Lopes et al., 2008
<i>mc1r</i>	<i>melanocortin 1 receptor</i> : A G-protein-coupled seven transmembrane helix receptor that regulate several pigment cell specific processes. Loss-of-function mutation are associated with loss of melanin pigmentation.	Selz et al., 2007; Richardson et al., 2008
<i>mc5r</i>	<i>melanocortin 5 receptor</i> : Another melanocortin receptors expressed in both melanophore and xanthophores.	Kobayashi et al., 2010, 2012a
<i>mitfa</i>	<i>microphthalmia-associated transcription factor a</i> : A master regulator of melanophore/melanocyte differentiation across vertebrates.	Lister et al., 1999; Béjar et al., 2003
<i>pmel</i>	<i>premelanosome protein a</i> : A melanosome protein that plays an important role in the structural organization of premelanosomes and the formation of intraluminal fibrils during melanosome biogenesis.	Chakraborty et al., 1996; Béjar et al., 2003; Du et al., 2003; Schonhaler et al., 2005; Theos et al., 2005
<i>slc24a5</i>	<i>solute carrier family 24 member 5</i> : A transporter protein localized in the melanosomal membrane that is essential for melanin synthesis.	Lamason et al., 2005
<i>sox10</i>	<i>sex determining region Y box 10</i> : A transcription factor that is essential for the specification of chromatophore progenitors.	Dutton et al., 2001; Elworthy et al., 2003; Hou et al., 2006
<i>tyr</i>	<i>tyrosinase</i> : An oxidase that can controls the melanogenesis as the rate-limiting enzyme by catalyzing tyrosine into dopaquinone via L-dopa.	Korner and Pawelek, 1982; Hidehito et al., 1994; Camp and Lardelli, 2001
<i>tyrp1a</i>	<i>tyrosinase-related protein 1a</i> : Another enzyme of the tyrosinase family that catalyzes the melanin biosynthesis.	Braasch et al., 2009; Krauss et al., 2014



pigment cells, but both broadening of bars in *H. latifasciatus* and increase in bar number in *C. azureus* is driven by the formation of new melanophores.

Cellular Correlates of Bar Patterns and Underlying Mechanism

Comparisons of juvenile and adult patterns largely demonstrate that the juvenile patterns bear already most cellular and morphological characteristics of adult patterns. Vertical bars have a higher density of melanophores and melanophores are also evidently darker in the melanic regions (Figures 4E,F, 6). In contrast to juveniles, pigments are more dispersed in adult bar melanophores. Xanthophores show no clear differences, yet reliable quantification was only partially possible due to the high density of melanophores.

Gene expression analyses of known pigmentation genes gave complementary information as well as mechanistic insights on how the spatial variation of chromatophore properties and densities are achieved (Figures 5, 6). Clearly, melanophore-specific genes such as *tyr*, *tyrp1a*, but also *slc24a5* are differentially expressed between bars and interbars (Figures 5D–F and Supplementary Table S2). All three genes are essential for melanin production. The two tyrosinases are directly involved in the synthesis of melanin, *slc24a5*, a potassium-dependent sodium/calcium exchanger is thought to modulate melanosomal pH, which is a crucial parameter for melanin synthesis (Ginger et al., 2008). Also *pmel*, the premelanosome protein, another melanophore-specific gene is slightly, yet not significantly, upregulated in bar regions compared to interbar regions (Figure 5C and Supplementary Table S2). Overall, expression data show a sixfold higher expression of tyrosinases in bars providing an explanation for the darker appearance of bar melanophores (Figure 6 and Supplementary Table S2). Even, if we standardize the differential expression by the increased cell-number or the melanophore-lineage marker *mitfa* we still find a 2–4.5-fold (Supplementary Figure S7 and Supplementary Tables S3, S4) increase of tyrosinase expression and therefore likely melanin synthesis.

Although we did not find significant differential expression of the transcription factors *sox10* and *mitfa* (Figures 5A,B), both crucial factors for melanophore differentiation, expression is slightly elevated in bar regions. As *mitfa* expression has been reported to be only weak in differentiated melanophores (Johnson et al., 2011), this result might indicate an elevated number of melanoblasts. Although this would have to be analyzed in detail, it would explain the increased number of newly forming melanophores in the bars of juveniles (Figure 2 and Supplementary Figure S1) and consequently an increase in melanophore density.

Additionally, we used a marker gene for the xanthophore lineage (*csflra*) and the iridophore lineage (*ltk*). None of them showed any expression differences (Figures 5G,H), suggesting that there is no obvious difference in xanthophore and iridophore number. Therefore, melanophores seem to be the main cell type that clearly differs both in number and expression of marker genes between bar and interbar regions. Although

there is some indication of a decreased number and coverage of xanthophores in the bar regions, this analysis was greatly hindered by the dense melanophore coverage. Use of mutant lines as for example of the *oca2* gene that will likely not affect the pattering of xanthophores and iridophores (Kratochwil et al., 2019b) would be a possibility to better assess this question. Still, this result is somewhat surprising as cell-cell interactions between xanthophores, melanophores and iridophores seem to have a completely different dynamic in cichlids compared to zebrafish, where these cell types are spatially segregated (Mahalwar et al., 2014; Patterson et al., 2014; Singh et al., 2014; Eom and Parichy, 2017). In zebrafish, especially xanthophores and melanophores are almost mutually exclusive, with a decreased cell number of xanthophores in the melanic stripes. No evidence of such a strong antagonistic interaction could be found in *H. latifasciatus*. In xanthophore morphology though, zebrafish and *H. latifasciatus* have some shared features: xanthophores in melanic regions are more irregular and seem smaller and have fewer and not as evenly distributed dendritic arbors (Figures 3, 4, Supplementary Figure S5, and Supplementary Table S1), although we did not find significant differences in xanthophore diameter (Figures 4I,J and Supplementary Table S1).

Our result on the expression of *mc1r* and *mc5r* shows that the melanocortin receptors likely play no role in shaping the vertical bar patterns in *H. latifasciatus* (Figures 5K,L). The antagonists *asip1* and *agrp2* show significant differences in expression (Figures 5I,J and Supplementary Table S2). The gene *agrp2* has been shown to inhibit the formation of horizontal stripe patterns: species with high *agrp2* expression lack any horizontal stripe patterns, while species with horizontal stripes have generally low expression of *agrp2* in skin. Yet, spatial variation in *agrp2* expression does not seem to be necessary for the formation of stripes (Kratochwil et al., 2018). On the other hand, a gradient in *agrp2* expression along antero-posterior axis could be found in both *H. latifasciatus* with significant difference from this study (Figure 5J and Supplementary Table S2) and *P. nyererei* without significant difference from our previous study (Kratochwil et al., 2018). However, based on our previous work that showed no changes in bar patterns in a knockout of *agrp2* in *P. nyererei* (Kratochwil et al., 2018) or association with bar presence and absence (Kratochwil et al., 2019a), a role in vertical bar formation seems rather unlikely.

The variation in *asip1* expression is intriguing. The melanocortin signaling antagonist *asip1* has higher expression in interbars (twofold difference; Supplementary Table S2) with a particularly high expression in the first interbar. This is surprising as *asip1* (and also the tetrapod homolog *Asip*) was previously known to vary along the dorso-ventral axis where it is involved in generating the dorso-ventral countershading that can be seen in many vertebrates (Manceau et al., 2011; Cal et al., 2017, 2019; Haupaix et al., 2018; Kratochwil, 2019). Here we observed variation along the antero-posterior axis (Figure 5I and Supplementary Table S2), suggesting that gene expression differences of *asip1* might contribute to variation in pigmentation on both axes. As *Asip1* acts as *Mc1r*/*Mc5r* antagonist, one of the responses of decreased melanocortin signaling would be an increased aggregation of melanosomes. This would be in

line with the significant difference in dispersion diameter we observe between bars and interbars. In interbars the diameter is on average 2.8 times smaller (**Figures 4C,D** and **Supplementary Table S1**), contributing to the lighter appearance of the interbar region (**Figure 6**). Yet the variation of *asip1* expression serves only as a partial explanation as (a) the expression level in int2 is similar to the adjacent bars and (b) the dispersion/aggregation states of melanophores vary greatly within bars and interbars as well as between individuals. Still, based on these findings, it might be an important take-home-message to consider functions of *asip1* beyond dorso-ventral patterning (i.e., countershading).

CONCLUSION

In summary, we investigated the formation of the vertical bar color pattern of *H. latifasciatus* – a member of the phenotypically diverse East African haplochromine cichlid fish radiations – during embryonic and larval development, how the macroscopic patterns is formed through variation in pigment cell distribution and properties, and how this variation links to known coloration gene expression. Our work provides novel insights into the molecular and cellular properties that contribute to the formation of color patterns in this famously diverse family of fish. More specifically we demonstrate that bar pattern formation is facilitated by three molecularly likely independent mechanisms: increased melanosome dispersal (controlled by melanosome migration along the cytoskeleton), density of melanophores (controlled by proliferation in progenitors) and melanin synthesis (controlled by melanin synthesis pathways and melanosome micro-environment) (**Figure 6**). *H. latifasciatus* with its – as we describe here – morphologically and transcriptomically well defined bar and interbar regions that can be tracked throughout development at a cellular level provides a unique system to further understand the molecular and cellular underpinnings of color patterns. However, further investigations on the development of vertical bars by assaying gene expression using RNA-seq, *in situ* hybridization and immunohistochemistry as well as comparative analyses with closely related species will be particularly informative for further understanding the molecular mechanisms underlying vertical bar formation. Cichlids with a rich and expanding repertoire of experimental approaches including hybrid crosses, transgenesis and CRISPR-Cas9 genome editing (Kratochwil and Meyer, 2015; Juntti, 2019) will make this species an excellent choice for further investigating the causal genetic variants, genes and molecular mechanisms that influenced evolution of and variation in vertical bar pattern formation.

MATERIALS AND METHODS

Fish Husbandry and Embryo Culture

H. latifasciatus were kept in groups of 10–25 individuals. Fertilized eggs were removed from the gravid females as early as possible. The larvae and juvenile fish were then raised in egg incubators (ZET-E55, Ziss aqua) at room temperature. At 5 days

post-fertilization (dpf) three larvae were separated from their siblings and each of them was raised in a single incubator to be able to keep track of a single individual in the following weeks. The hatching fish larvae were provided with nutrients by their yolk sac which lasts for ~14 days. After 14 dpf the larvae were fed on *Artemia nauplii* twice a day. Experiments were performed in accordance with the rules of the animal research facility of the University of Konstanz, Germany and with the permission of the animal care committee (Regierungspräsidium) Freiburg, Germany (G18/60 and T16/13).

Fish Larvae Photography and Analysis

Photographs of *H. latifasciatus* embryos and larvae were captured with a stereomicroscope (Leica MZ10F) with a Leica DMC2900 color camera. Fish were first anesthetized with 0.04% tricaine (MS-222, Sigma-Aldrich). Images were taken as previously described (Kratochwil et al., 2015; Kratochwil et al., 2017). In order to capture the color pattern development of *H. latifasciatus*, photographs were taken from 7 to 21 dpf of three individuals.

For quantitative analyses, a comparable area including vb2 and vb3 of photographs of three individuals from stages between 9 and 21 dpf were put together, aligned (using the anterior dorsal fin as landmark), transformed into a black-and-white image and quantified in three different ways using Fiji (Schindelin et al., 2012). Melanosome dispersal was estimated by measuring the diameter of the minimally sized circle that encloses all melanic parts of a melanophore (minimal enclosing circle). It is therefore affected both by the melanophore size and the dispersal/aggregation state of melanosomes within the melanophores. The relative gray value was measured by taking the mean gray value of the same circle and dividing it by the mean of all melanophore gray value measurements of the same individual and stage. This relative value was used to account for differences between images and stages. Melanophore density was calculated from manual melanophore counts. As the position of the bars would shift as the fish is growing, we corrected the position values by the growth of the individual (size of individual at X dph/size of individual at 9 dph). The values were plotted using non-parametric regression (locally weighted scatterplot smoothing; LOWESS) in R (f parameter: 0.1). For the density calculation the area was split into ten equally-sized zones. To track individual melanophores we took images of the same individual on five consecutive days (from 9 to 13 dpf; **Figures 2D–H**). The identity of single melanophores across stages was estimated using overlays of the images from multiple days.

Image Acquisition in Adult Fish

Three female individuals (standard length ~12 cm) were examined for pigment quantification. We only quantified the chromatophores in females as the vertical bar pattern does not differ between sexes, we found more variation in red coloration in males (**Figures 1Q,R**). For each fish, in total 156 photos were obtained from scales and skin with scales removed (referred to as skin). Firstly, we separated the two flanks with scales still attached to skin. Scales from study regions were then carefully

removed from the left flank and kept separately in Ringer's solution (6.5 g/L NaCl, 0.25 g/L KCl, 0.3 g/L CaCl₂ and 0.2 g/L NaHCO₃) at 4°C before imaging. Five light microscope photographs were taken on each melanistic and non-melanistic region (the two melanistic bar regions vb2 and vb3, and the interbar ib1 which anterior of vb2 and ib3 which between vb2 and vb3) from dorsal to ventral with a Leica MZ10F stereomicroscope equipped with a Leica DMC2900 color camera. Four photos were taken also along the dorsal-ventral axis on both region using Leica MZ10F equipped with a Leica DFC 3000G black-and-white camera with GFP filter to image the auto-fluorescence of adult xanthophores (Kelsh et al., 1996; Guyader and Jesuthasan, 2002). For each pigmented region, ten scales were imaged using a Leica DMC2900 camera on Leica DM6B upright microscope. Leica Application Suite X software was used to capture the photos using the same setting. To count the number of melanophores, the right flank including both scales and skin tissue was treated with 10 mg/ml epinephrine (SIGMA-ALDRICH) for 20 min at room temperature to aggregate the melanosomes and thereby permit robust cell number quantifications (Figures 3B,F,K,N). After epinephrine treatment, tissue was washed by Phosphate Buffered Saline (PBS, pH7.4) three times and then kept in 4% Paraformaldehyde (PFA) in PBS. Epinephrine-treated scales were removed from skin and photos were taken for both skin and scales as described above. Fluorescence images were not taken as this treatment caused a high autofluorescence background.

Image and Data Analysis of Adult Patterns

All photos were analyzed with the image analysis software Fiji (Schindelin et al., 2012). At the beginning of the analysis we manually adjusted the color threshold to obtain reliable quantification of the bright field photos from non-treated skin and scales. Using this setting we executed the "Analyze Particles" function to obtain the melanophore coverage. For 20 randomly selected melanophores from each skin image and 10 melanophores from each scale image we measured the dispersal diameter of melanin covered parts of the melanophores (as described above). For xanthophore coverage we used the same approach. The dispersal diameter of the pigment filled part of 20 xanthophores of each skin image and 10 xanthophores of each scale image was measured from each photograph. The number of melanophore and xanthophore was counted from epinephrine-treated skin and scale specimens. Single xanthophores on scales could be observed easily by fluorescence microscopy, while not all boundaries of xanthophores in the skin could be easily identified as the cells often overlapped. Therefore, we were able to count the xanthophore number in scales but not in the skin dissections, while dispersal diameter measurements for xanthophores was possible on both scales and skin. Xanthophores and (what likely are erythrophores) was treated as the same cell type. Several studies show that erythrophores also exist in cichlids (Chen et al., 2014, 2015). However, vesicles containing pteridine and carotenoids could be found in the same cells, in which case the overall color depends on the ratio of red and yellow pigments

(Matsumoto, 1965; Bagnara, 1966). Hence, the distinction between xanthophores and erythrophores is not always clear. Therefore, we classified yellow/orange/red colored cells all as xanthophores. Although we could identify iridophores in both dark bars and light interbars before and after epinephrine treatment (Figures 3I,K,L,N), iridophores were mostly below or above melanophores hindering reliable measurements. We, therefore, used gene expression of the iridophore lineage marker gene *ltk* (Lopes et al., 2008).

RNA Extraction

To compare the expression of coloration and pigment genes between melanistic bars and interbars regions, we sampled the whole melanistic and non-melanistic skin region. Skin tissue was dissected and kept in RNAlater (Invitrogen) at 4°C overnight and then transferred to -20°C for long-term storage. RNAlater was removed prior to homogenization. Skin samples and the appropriate amount of TRIzol (Invitrogen) (1 ml TRIzol per 100 mg sample) were homogenized in 2 ml Lysing Matrix A tube (MP Biomedicals) using FastPrep-24 Classic Instrument (MP Biomedicals). RNA was extracted according to the manufacturer's recommendations (Invitrogen) with an additional wash step by 75% Ethanol. Subsequent purification and on-column DNase treatment were performed with the RNeasy Mini Kit (Qiagen) and RNeasy-Free DNase Set (Qiagen). Following extraction and purification, RNA was quantified using Qubit RNA BR Assay Kit (Invitrogen) with Qubit Fluorometer (Life Technologies).

Quantitative Real-Time PCR (RT-qPCR)

Gene expression analyses were performed on two melanistic (vb2 and vb3) and two non-melanistic (ib1 and ib2) skin regions. First strand cDNA was synthesized by using 1 µg of total RNA with a GoScript Reverse Transcription System (Promega). qPCRs were performed with 2 µl of 100 µl synthesized first strand cDNA that was diluted ten times from 20 µl of initial reaction volume as a template, 10 pmol of each forward primer and reverse primer, and GoTaq qPCR Master Mix (Promega) with nuclease-free water to make the final volume of 20 µl in a 96-well plate. Twelve genes were processed to examine the expression level including *sox10*, *mitfa*, *csf1ra*, *ltk*, *pmel*, *slc24a5*, *tyr*, *tyrp1a*, *asip1*, *agrp2*, *mc1r*, *mc5r* (Table 1). Primers are listed in Supplementary Table S5. We used 40 cycles of amplification on a CFX96 Real-Time PCR Detection System (Bio-Rad). The amplification program was: initial denaturation at 95°C for 10 min, 40 cycles of 95°C for 20 s, 60°C for 60 s. At the end of the cycles, melting curve of the products was verified for the specificity of PCR products. Only samples with one peak in the melting curves were processed to analyses. We assayed gene expression in triplicate for each sample and normalized the data using the reference genes *β-actin* and *gapdh*. Ct values were defined as the point at which fluorescence crossed a threshold (R_{CT}) adjusted manually to be the point at which fluorescence rose above the background level. Next, we compared the relative expression between samples using the $2^{-\Delta\Delta CT}$ method (Nolan et al., 2006). For group comparisons, we used ANOVA followed by Tukey's HSD. All statistical tests were performed in R (R Development Core Team, 2019).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation, to any qualified researcher.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Care Committee (Regierungspräsidium) Freiburg, Germany.

AUTHOR CONTRIBUTIONS

YL: investigation, visualization, methodology, formal analysis, funding acquisition, validation, writing – original draft, and writing – review and editing. JG: investigation. AM: supervision, funding acquisition, and writing – review and editing. CK: conceptualization, resources, formal analysis, supervision, funding acquisition, visualization, methodology, writing – original draft, project administration, and writing – review and editing.

REFERENCES

- Ahi, E. P., and Sefc, K. M. (2017). Anterior-posterior gene expression differences in three Lake Malawi cichlid fishes with variation in body stripe orientation. *PeerJ* 5:e4080. doi: 10.7717/peerj.4080
- Albertson, R. C., Powder, K. E., Hu, Y., Coyle, K. P., Roberts, R. B., and Parsons, K. J. (2014). Genetic basis of continuous variation in the levels and modular inheritance of pigmentation in cichlid fishes. *Mol. Ecol.* 23, 5135–5150. doi: 10.1111/mec.12900
- Bagnara, J. T. (1966). "Cytology and cytophysiology of non-melanophore pigment cells," in *International Review of Cytology*, Ed. K. W. Jeon, (Amsterdam: Elsevier), 173–205. doi: 10.1016/s0074-7696(08)60801-3
- Béjar, J., Hong, Y., and Scharf, M. (2003). Mitf expression is sufficient to direct differentiation of medaka blastula derived stem cells to melanocytes. *Development* 130, 6545–6553. doi: 10.1242/dev.00872
- Braasch, I., Liedtke, D., Volff, J. N., and Scharf, M. (2009). Pigmentary function and evolution of tyrp1 gene duplicates in fish. *Pigment Cell Melanoma Res.* 22, 839–850. doi: 10.1111/j.1755-148x.2009.00614.x
- Bronner, M. E., and LeDouarin, N. M. (2012). Development and evolution of the neural crest: an overview. *Dev. Biol.* 366, 2–9. doi: 10.1016/j.ydbio.2011.12.042
- Burton, D., and Burton, M. (2017). *Essential Fish Biology: Diversity, Structure, and Function*. Oxford: Oxford University Press.
- Cal, L., Megias, M., Cerda-Reverter, J. M., Postlethwait, J. H., Braasch, I., and Rotllant, J. (2017). BAC Recombineering of the Agouti Loci from Spotted Gar and Zebrafish Reveals the Evolutionary Ancestry of Dorsal-Ventral Pigment Asymmetry in Fish. *J. Exp. Zool. B Mol. Dev. Evol.* 328, 697–708. doi: 10.1002/jezb.22748
- Cal, L., Suarez-Bregua, P., Comesana, P., Owen, J., Braasch, I., Kelsh, R., et al. (2019). Countershading in zebrafish results from an Asip1 controlled dorsoventral gradient of pigment cell differentiation. *Sci. Rep.* 9:3449. doi: 10.1038/s41598-019-40251-z
- Camp, E., and Lardelli, M. (2001). Tyrosinase gene expression in zebrafish embryos. *Dev. Genes Evol.* 211, 150–153. doi: 10.1007/s004270000125
- Ceinos, R. M., Guillot, R., Kelsh, R. N., Cerda-Reverter, J. M., and Rotllant, J. (2015). Pigment patterns in adult fish result from superimposition of two largely independent pigmentation mechanisms. *Pigment Cell Melanoma Res.* 28, 196–209. doi: 10.1111/pcmr.12335

FUNDING

This work was supported by a stipend from the China Scholarship Council (CSC, to YL), the Baden-Württemberg Foundation (to CK) grants by the Deutsche Forschungsgemeinschaft (DFG, to AM and KR 4670/2-1 and KR 4670/4-1 to CK), an ERC Advanced grant GenAdap number 293700 by the European Research Council (to AM), and the University of Konstanz (YL, JG, AM, and CK).

ACKNOWLEDGMENTS

We thank the staff of the animal facility of the University of Konstanz for their excellent help.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcell.2020.00062/full#supplementary-material>

- Cerda-Reverter, J. M., Haitina, T., Schioth, H. B., and Peter, R. E. (2005). Gene structure of the goldfish agouti-signaling protein: a putative role in the dorsal-ventral pigment pattern of fish. *Endocrinology* 146, 1597–1610. doi: 10.1210/en.2004-1346
- Chakraborty, A. K., Platt, J. T., Kim, K. K., Kwon, B. S., Bennett, D. C., and Pawelek, J. M. (1996). Polymerization of 5,6-dihydroxyindole-2-carboxylic acid to melanin by the pmel 17/silver locus protein. *Eur. J. Biochem.* 236, 180–188. doi: 10.1111/j.1432-1033.1996.t01-1-00180.x
- Chen, S. C., Hornsby, M. A., Robertson, R. M., and Hawryshyn, C. W. (2014). The influence of chromatic background on the photosensitivity of tilapia erythrophores. *Biol. Open* 3, 117–120. doi: 10.1242/bio.2014.6742
- Chen, S. C., Xiao, C., Troje, N. F., Robertson, R. M., and Hawryshyn, C. W. (2015). Functional characterisation of the chromatically antagonistic photosensitive mechanism of erythrophores in the tilapia *Oreochromis niloticus*. *J. Exp. Biol.* 218, 748–756. doi: 10.1242/jeb.106831
- Dooley, C. M., Mongera, A., Walderich, B., and Nusslein-Volhard, C. (2013). On the embryonic origin of adult melanophores: the role of ErbB and Kit signalling in establishing melanophore stem cells in zebrafish. *Development* 140, 1003–1013. doi: 10.1242/dev.087007
- Du, J., Miller, A. J., Widlund, H. R., Horstmann, M. A., Ramaswamy, S., and Fisher, D. E. (2003). MLANA/MART1 and SILV/PMEL17/GP100 are transcriptionally regulated by MITF in melanocytes and melanoma. *Am. J. Pathol.* 163, 333–343. doi: 10.1016/s0002-9440(10)63657-7
- Dutton, K. A., Pauliny, A., Lopes, S. S., Elworthy, S., Carney, T. J., Rauch, J., et al. (2001). Zebrafish colourless encodes sox10 and specifies non-ectomesenchymal neural crest fates. *Development* 128, 4113–4125.
- Elmer, K. R., Lehtonen, T. K., and Meyer, A. (2009). Color assortative mating contributes to sympatric divergence of neotropical cichlid fish. *Evolution* 63, 2750–2757. doi: 10.1111/j.1558-5646.2009.00736.x
- Elworthy, S., Lister, J. A., Carney, T. J., Raible, D. W., and Kelsh, R. N. (2003). Transcriptional regulation of mitfa accounts for the sox10 requirement in zebrafish melanophore development. *Development* 130, 2809–2818. doi: 10.1242/dev.00461
- Eom, D. S., and Parichy, D. M. (2017). A macrophage relay for long-distance signaling during postembryonic tissue remodeling. *Science* 355, 1317–1320. doi: 10.1126/science.aal2745

- Ginger, R. S., Askew, S. E., Ogborne, R. M., Wilson, S., Ferdinando, D., Dadd, T., et al. (2008). SLC24A5 encodes a trans-Golgi network protein with potassium-dependent sodium-calcium exchange activity that regulates human epidermal melanogenesis. *J. Biol. Chem.* 283, 5486–5495. doi: 10.1074/jbc.M707521200
- Greenwood, P. H. (1974). Cichlid fishes of Lake Victoria, East Africa: the biology and evolution of a species flock. *Bull. Br. Mus. Nat. Hist. Zool. Suppl.* 6, 1–134.
- Guillot, R., Ceinos, R. M., Cal, R., Rotllant, J., and Cerda-Reverter, J. M. (2012). Transient ectopic overexpression of agouti-signalling protein 1 (asip1) induces pigment anomalies in flatfish. *PLoS One* 7:e48526. doi: 10.1371/journal.pone.0048526
- Guyader, S. L., and Jesuthasan, S. (2002). Analysis of xanthophore and pterinosome biogenesis in zebrafish using methylene blue and pteridine autofluorescence. *Pigment Cell Res.* 15, 27–31. doi: 10.1034/j.1600-0749.2002.00045.x
- Haupaix, N., Curant, C., Baillieu, R., Beck, S., Robic, A., and Manceau, M. (2018). The periodic coloration in birds forms through a prepattern of somite origin. *Science* 361:eaar4777. doi: 10.1126/science.aar4777
- Hemingson, C. R., Cowman, P. F., Hodge, J. R., and Bellwood, D. R. (2019). Colour pattern divergence in reef fish species is rapid and driven by both range overlap and symmetry. *Ecol. Lett.* 22, 190–199. doi: 10.1111/ele.13180
- Hendrick, L. A., Carter, G. A., Hilbrands, E. H., Heubel, B. P., Schilling, T. F., and Le Pabic, P. (2019). Bar, stripe and spot development in sand-dwelling cichlids from Lake Malawi. *EvoDevo* 10:18. doi: 10.1186/s13227-019-0132-7
- Henning, F., and Meyer, A. (2012). Eggspot number and sexual selection in the cichlid fish *Astatotilapia burtoni*. *PLoS One* 7:e43695. doi: 10.1371/journal.pone.0043695
- Hidehito, I., Yoshitaka, B., Akihiko, K., and Hiroshi, H. (1994). Expression of the tyrosinase-encoding gene in a colorless melanophore mutant of the medaka fish *Oryzias latipes*. *Gene* 150, 319–324. doi: 10.1016/0378-1119(94)90445-6
- Higdon, C. W., Mitra, R. D., and Johnson, S. L. (2013). Gene expression analysis of zebrafish melanocytes, iridophores, and retinal pigmented epithelium reveals indicators of biological function and developmental origin. *PLoS One* 8:e67801. doi: 10.1371/journal.pone.0067801
- Hou, L., Arnheiter, H., and Pavan, W. J. (2006). Interspecies difference in the regulation of melanocyte development by SOX10 and MITF. *Proc. Natl. Acad. Sci. U.S.A.* 103, 9081–9085. doi: 10.1073/pnas.0603114103
- Hughes, L. C., Orti, G., Huang, Y., Sun, Y., Baldwin, C. C., Thompson, A. W., et al. (2018). Comprehensive phylogeny of ray-finned fishes (*Actinopterygii*) based on transcriptomic and genomic data. *Proc. Natl. Acad. Sci. U.S.A.* 115, 6249–6254. doi: 10.1073/pnas.1719358115
- Irion, U., and Nüsslein-Volhard, C. (2019). The identification of genes involved in the evolution of color patterns in fish. *Curr. Opin. Genet. Dev.* 57, 31–38. doi: 10.1016/j.gde.2019.07.002
- Johnson, S. L., Nguyen, A. N., and Lister, J. A. (2011). Mitfa is required at multiple stages of melanocyte differentiation but not to establish the melanocyte stem cell. *Dev. Biol.* 350, 405–413. doi: 10.1016/j.ydbio.2010.12.004
- Juntti, S. (2019). The future of gene-guided neuroscience research in non-traditional model organisms. *Brain Behav. Evol.* 93, 108–121. doi: 10.1159/000500072
- Kelsh, R. N., Brand, M., Jiang, Y. J., Heisenberg, C. P., Lin, S., Haffter, P., et al. (1996). Zebrafish pigmentation mutations and the processes of neural crest development. *Development* 123, 369–389.
- Kobayashi, Y., Chiba, H., Mizusawa, K., Suzuki, N., Cerda-Reverter, J. M., and Takahashi, A. (2011). Pigment-dispersing activities and cortisol-releasing activities of melanocortins and their receptors in xanthophores and head kidneys of the goldfish *Carassius auratus*. *Gen. Comp. Endocrinol.* 173, 438–446. doi: 10.1016/j.ygcen.2011.06.019
- Kobayashi, Y., Mizusawa, K., Chiba, H., Tagawa, M., and Takahashi, A. (2012a). Further evidence on acetylation-induced inhibition of the pigment-dispersing activity of α -melanocyte-stimulating hormone. *Gen. Comp. Endocrinol.* 176, 9–17. doi: 10.1016/j.ygcen.2011.12.001
- Kobayashi, Y., Mizusawa, K., Saito, Y., and Takahashi, A. (2012b). Melanocortin systems on pigment dispersion in fish chromatophores. *Front. Endocrinol.* 3:9. doi: 10.3389/fendo.2012.00009
- Kobayashi, Y., Tsuchiya, K., Yamanome, T., Schiöth, H. B., and Takahashi, A. (2010). Differential expressions of melanocortin receptor subtypes in melanophores and xanthophores of barfin flounder. *Gen. Comp. Endocrinol.* 168, 133–142. doi: 10.1016/j.ygcen.2010.04.017
- Kocher, T. D., Conroy, J. A., Mckaye, K. R., and Stauffer, J. R. (1993). Similar morphologies of cichlid fish in Lakes Tanganyika and Malawi are due to convergence. *Mol. Phylogenet. Evol.* 2, 158–165. doi: 10.1006/mpev.1993.1016
- Korner, A., and Pawelek, J. (1982). Mammalian tyrosinase catalyzes three reactions in the biosynthesis of melanin. *Science* 217, 1163–1165. doi: 10.1126/science.6810464
- Kratochwil, C. F. (2019). Molecular mechanisms of convergent color pattern evolution. *Zoology* 134, 66–68. doi: 10.1016/j.zool.2019.04.004
- Kratochwil, C. F., Liang, Y., Gerwin, J., Woltering, J. M., Urban, S., Henning, F., et al. (2018). Agouti-related peptide 2 facilitates convergent evolution of stripe patterns across cichlid fish radiations. *Science* 362, 457–460. doi: 10.1126/science.aao6809
- Kratochwil, C. F., Liang, Y., Urban, S., Torres-Dowdall, J., and Meyer, A. (2019a). Evolutionary dynamics of structural variation at a key locus for color pattern diversification in cichlid fishes. *Genome Biol. Evol.* 11, 3452–3465. doi: 10.1093/gbe/evz261
- Kratochwil, C. F., Urban, S., and Meyer, A. (2019b). Genome of the Malawi golden cichlid fish (*Melanochromis auratus*) reveals exon loss of *oca2* in an amelanistic morph. *Pigment Cell Melanoma Res.* 32, 719–723. doi: 10.1111/pcmr.12799
- Kratochwil, C. F., and Meyer, A. (2015). Closing the genotype-phenotype gap: emerging technologies for evolutionary genetics in ecological model vertebrate systems. *Bioessays* 37, 213–226. doi: 10.1002/bies.201400142
- Kratochwil, C. F., Sefton, M. M., Liang, Y., and Meyer, A. (2017). Tol2 transposon-mediated transgenesis in the Midas cichlid (*Amphilophus citrinellus*) - towards understanding gene function and regulatory evolution in an ecological model system for rapid phenotypic diversification. *BMC Dev. Biol.* 17:15. doi: 10.1186/s12861-017-0157-x
- Kratochwil, C. F., Sefton, M. M., and Meyer, A. (2015). Embryonic and larval development in the Midas cichlid fish species flock (*Amphilophus* spp.): a new evo-devo model for the investigation of adaptive novelties and species differences. *BMC Dev. Biol.* 15:12. doi: 10.1186/s12861-015-0061-1
- Krauss, J., Geiger-Rudolph, S., Koch, I., Nüsslein-Volhard, C., and Irion, U. (2014). A dominant mutation in *tyrp1* A leads to melanophore death in zebrafish. *Pigment Cell Melanoma Res.* 27, 827–830. doi: 10.1111/pcmr.12272
- Kurokawa, T., Murashita, K., and Uji, S. (2006). Characterization and tissue distribution of multiple agouti-family genes in pufferfish *Takifugu rubripes*. *Peptides* 27, 3165–3175. doi: 10.1016/j.peptides.2006.09.013
- Lamasón, R. L., Mohideen, M. A., Mest, J. R., Wong, A. C., Norton, H. L., Aros, M. C., et al. (2005). SLC24A5, a putative cation exchanger, affects pigmentation in zebrafish and humans. *Science* 310, 1782–1786. doi: 10.1126/science.1116238
- Lister, J. A., Robertson, C. P., Lepage, T., Johnson, S. L., and Raible, D. W. (1999). nacre encodes a zebrafish micropthalmia-related protein that regulates neural-crest-derived pigment cell fate. *Development* 126, 3757–3767.
- Lopes, S. S., Yang, X., Muller, J., Carney, T. J., Mcadow, A. R., Rauch, G. J., et al. (2008). Leukocyte tyrosine kinase functions in pigment cell development. *PLoS Genet.* 4:e1000026. doi: 10.1371/journal.pgen.1000026
- Maan, M. E., and Sefc, K. M. (2013). Colour variation in cichlid fish: developmental mechanisms, selective pressures and evolutionary consequences. *Semin. Cell Dev. Biol.* 24, 516–528. doi: 10.1016/j.semcdb.2013.05.003
- Mahalwar, P., Walderich, B., Singh, A. P., and Nüsslein-Volhard, C. (2014). Local reorganization of xanthophores fine-tunes and colors the striped pattern of zebrafish. *Science* 345, 1362–1364. doi: 10.1126/science.1254837
- Manceau, M., Domingues, V. S., Mallarino, R., and Hoekstra, H. E. (2011). The developmental role of Agouti in color pattern evolution. *Science* 331, 1062–1065. doi: 10.1126/science.1200684
- Matsumoto, J. (1965). Studies on Fine structure and cytochemical properties of erythrophores in Swordtail *Xiphophorus Hellei* with special reference to their pigment granules (Pterinosomes). *J. Cell Biol.* 27, 493–504. doi: 10.1083/jcb.27.3.493
- Meyer, A., Biermann, C. H., and Orti, G. (1993). The phylogenetic position of the zebrafish (*Danio rerio*), a model system in developmental biology: an invitation to the comparative method. *Proc. Biol. Sci.* 252, 231–236. doi: 10.1098/rspb.1993.0070
- Meyer, A., Ritchie, P. A., and Witte, K. E. (1995). Predicting developmental processes from evolutionary patterns - a molecular phylogeny of the zebrafish (*Danio-rerio*) and Its Relatives. *Philos. Trans. R. Soc. B Biol. Sci.* 349, 103–111. doi: 10.1098/rstb.1995.0096

- Nagao, Y., Suzuki, T., Shimizu, A., Kimura, T., Seki, R., Adachi, T., et al. (2014). Sox5 functions as a fate switch in Medaka pigment cell development. *PLoS Genet.* 10:e1004246. doi: 10.1371/journal.pgen.1004246
- Nolan, T., Hands, R. E., and Bustin, S. A. (2006). Quantification of mRNA using real-time RT-PCR. *Nat. Protoc.* 1, 1559–1582. doi: 10.1038/nprot.2006.236
- Nüsslein-Volhard, C., and Singh, A. P. (2017). How fish color their skin: a paradigm for development and evolution of adult patterns: multipotency, plasticity, and cell competition regulate proliferation and spreading of pigment cells in zebrafish coloration. *BioEssays* 39:1600231. doi: 10.1002/bies.201600231
- Parichy, D. M., Elizondo, M. R., Mills, M. G., Gordon, T. N., and Engeszer, R. E. (2009). Normal table of postembryonic zebrafish development: staging by externally visible anatomy of the living fish. *Dev. Dyn.* 238, 2975–3015. doi: 10.1002/dvdy.22113
- Parichy, D. M., Ransom, D. G., Paw, B., Zon, L. I., and Johnson, S. L. (2000). An orthologue of the kit-related gene *fms* is required for development of neural crest-derived xanthophores and a subpopulation of adult melanocytes in the zebrafish. *Danio rerio*. *Development* 127, 3031–3044.
- Parichy, D. M., and Turner, J. M. (2003). Temporal and cellular requirements for Fms signaling during zebrafish adult pigment pattern development. *Development* 130, 817–833. doi: 10.1242/dev.00307
- Patterson, L. B., Bain, E. J., and Parichy, D. M. (2014). Pigment cell interactions and differential xanthophore recruitment underlying zebrafish stripe reiteration and *Danio* pattern evolution. *Nat. Commun.* 5:5299. doi: 10.1038/ncomms6299
- Patterson, L. B., and Parichy, D. M. (2019). Zebrafish pigment pattern formation: insights into the development and evolution of adult form. *Ann. Rev. Genet.* 53, 505–530. doi: 10.1146/annurev-genet-112618-043741
- Prazdnikov, D. V., and Shkil, F. N. (2019). Experimental evidence of the role of heterochrony in evolution of the Mesoamerican cichlids pigment patterns. *Evol. Dev.* 21, 3–15. doi: 10.1111/ede.12272
- R Development Core Team (2019). *R: A Language and Environment for Statistical Computing*. Vienna: R Foundation for Statistical Computing.
- Richardson, J., Lundegaard, P. R., Reynolds, N. L., Dorin, J. R., Porteous, D. J., Jackson, I. J., et al. (2008). *mc1r* Pathway regulation of zebrafish melanosome dispersion. *Zebrafish* 5, 289–295. doi: 10.1089/zeb.2008.0541
- Roberts, R. B., Ser, J. R., and Kocher, T. D. (2009). Sexual conflict resolved by invasion of a novel sex determiner in Lake Malawi cichlid fishes. *Science* 326, 998–1001. doi: 10.1126/science.1174705
- Roux, N., Salis, P., Lambert, A., Logeux, V., Soulat, O., Romans, P., et al. (2019). Staging and normal table of postembryonic development of the clownfish (*Amphiprion ocellaris*). *Dev. Dyn.* 248, 545–568. doi: 10.1002/dvdy.46
- Salis, P., Roux, N., Soulat, O., Lecchini, D., Laudet, V., and Frédérick, B. (2018). Ontogenetic and phylogenetic simplification during white stripe evolution in clownfishes. *BMC Biol.* 16:90. doi: 10.1186/s12915-018-0559-7
- Santos, M. E., Baldo, L., Gu, L., Boileau, N., Musilova, Z., and Salzburger, W. (2016). Comparative transcriptomics of anal fin pigmentation patterns in cichlid fishes. *BMC Genomics* 17:712. doi: 10.1186/s12864-016-3046-y
- Santos, M. E., Braasch, I., Boileau, N., Meyer, B. S., Sauter, L., Bohne, A., et al. (2014). The evolution of cichlid fish egg-spots is linked with a cis-regulatory change. *Nat. Commun.* 5:5149. doi: 10.1038/ncomms6149
- Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., et al. (2012). Fiji: an open-source platform for biological-image analysis. *Nat. Methods* 9, 676–682. doi: 10.1038/nmeth.2019
- Schonthaler, H. B., Lampert, J. M., Von Lintig, J., Schwarz, H., Geisler, R., and Neuhauss, S. C. (2005). A mutation in the silver gene leads to defects in melanosome biogenesis and alterations in the visual system in the zebrafish mutant fading vision. *Dev. Biol.* 284, 421–436. doi: 10.1016/j.ydbio.2005.06.001
- Seehausen, O., Mayhew, P. J., and Van Alphen, J. J. M. (1999). Evolution of colour patterns in East African cichlid fish. *J. Evol. Biol.* 12, 514–534. doi: 10.1046/j.1420-9101.1999.00055.x
- Selz, Y., Braasch, I., Hoffmann, C., Schmidt, C., Schultheis, C., Scharlt, M., et al. (2007). Evolution of melanocortin receptors in teleost fish: the melanocortin type 1 receptor. *Gene* 401, 114–122. doi: 10.1016/j.gene.2007.07.005
- Shainer, I., Buchshtab, A., Hawkins, T. A., Wilson, S. W., Cone, R. D., and Gothilf, Y. (2017). Novel hypophysiotropic AgRP2 neurons and pineal cells revealed by BAC transgenesis in zebrafish. *Sci. Rep.* 7:44777. doi: 10.1038/srep44777
- Singh, A. P., and Nüsslein-Volhard, C. (2015). Zebrafish stripes as a model for vertebrate colour pattern formation. *Curr. Biol.* 25, R81–R92. doi: 10.1016/j.cub.2014.11.013
- Singh, A. P., Schach, U., and Nüsslein-Volhard, C. (2014). Proliferation, dispersal and patterned aggregation of iridophores in the skin prefigure striped colouration of zebrafish. *Nat. Cell Biol.* 16, 607–614. doi: 10.1038/ncb2955
- Streelman, J. T., Albertson, R. C., and Kocher, T. D. (2003). Genome mapping of the orange blotch colour pattern in cichlid fishes. *Mol. Ecol.* 12, 2465–2471. doi: 10.1046/j.1365-294x.2003.01920.x
- Theos, A. C., Truschel, S. T., Raposo, G., and Marks, M. S. (2005). The Silver locus product *Pmel17/gp100/Silv/ME20*: controversial in name and in function. *Pigment Cell Res.* 18, 322–336. doi: 10.1111/j.1600-0749.2005.00269.x
- Witte, F., Barel, C., Witte-Maas, E. L., and Van Oijen, M. (1976). An introduction to the taxonomy and morphology of the haplochromine Cichlidae from Lake Victoria. *Netherlands J. Zool.* 27, 333–380. doi: 10.1163/002829677x00199
- Woltering, J. M., Holzem, M., Schneider, R. F., Nanos, V., and Meyer, A. (2018). The skeletal ontogeny of *Astatotilapia burtoni* - a direct-developing model system for the evolution and development of the teleost body plan. *BMC Dev. Biol.* 18:8. doi: 10.1186/s12861-018-0166-4
- Zhang, C., Song, Y., Thompson, D. A., Madonna, M. A., Millhauser, G. L., Toro, S., et al. (2010). Pineal-specific agouti protein regulates teleost background adaptation. *Proc. Natl. Acad. Sci. U.S.A.* 107, 20164–20171. doi: 10.1073/pnas.1014941107

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2020 Liang, Gerwin, Meyer and Kratochwil. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Molecular Mechanisms Underlying Pupal Protective Color Switch in *Papilio polytes* Butterflies

Shinichi Yoda, Emi Otaguro, Mayumi Nobuta and Haruhiko Fujiwara*

Department of Integrated Biosciences, Graduate School of Frontier Sciences, The University of Tokyo, Kashiwa, Japan

OPEN ACCESS

Edited by:

Mark Rebeiz,
University of Pittsburgh, United States

Reviewed by:

Arnaud Martin,
George Washington University,
United States
Thomas Williams,
University of Dayton, United States

*Correspondence:

Haruhiko Fujiwara
haruh@edu.k.u-tokyo.ac.jp

Specialty section:

This article was submitted to
Evolutionary Developmental Biology,
a section of the journal
Frontiers in Ecology and Evolution

Received: 17 December 2019

Accepted: 19 February 2020

Published: 06 March 2020

Citation:

Yoda S, Otaguro E, Nobuta M and
Fujiwara H (2020) Molecular
Mechanisms Underlying Pupal
Protective Color Switch in *Papilio*
polytes Butterflies.
Front. Ecol. Evol. 8:51.
doi: 10.3389/fevo.2020.00051

Mimicry is a survival strategy in which organisms deceive their predators by imitating other organisms or the surrounding environment. One example of this involves pupal color polymorphism, which is widely observed in butterflies and moths. It has been suggested that the pupal colors of *Papilio* butterflies are selected according to the tactile stimulation experienced before pupation (P). Specifically, larvae crawling on smooth leaves become green pupae, but those crawling on rough stems become brown pupae. These protective colors fit with the surrounding environment. However, the detailed molecular mechanisms underlying pupal polymorphism have generally remained unknown. To reveal these mechanisms, we first established control over the green and brown pupal coloration in *Papilio polytes* under laboratory conditions and clarified temporal and spatial changes of pupal pigments under both conditions. We also analyzed the expression of coloration genes during the pre-pupal stage and at P in the epidermis under the green and brown conditions, by RNA sequencing and quantitative RT-PCR. These analyses revealed that the brown pupal color is regulated mainly by melanin synthesis genes, *tyrosine hydroxylase* (*TH*) and *laccase 2*. In contrast, the green pupal color was suggested to be formed mainly through the expression of both multiple *bilin binding protein* (*BBP*)-related genes responsible for blue pigmentation and multiple *juvenile hormone binding protein* (*JHBP*)-related genes responsible for yellow pigmentation. Electroporation-mediated RNAi showed that the knockdown of *TH* or *laccase 2* blocked the brown pupal coloration, and that the knockdown of *BBP*- or *JHBP*-related genes caused yellow or blue coloration in the green-conditioned pupae, respectively, supporting the above hypothesis. We here report how genes involved in the pupal coloration of *P. polytes* are regulated, which sheds light on the evolutionary process of pupal protective colors among Lepidoptera.

Keywords: pupal polymorphic color switch, *Papilio polytes*, swallowtail butterfly, electroporation-mediated gene knockdown, mimicry, melanin synthesis genes, bilin binding protein, juvenile hormone binding protein

INTRODUCTION

More than 150 years ago, pioneering evolutionary biologists identified the phenomenon of mimicry, in which prey deceives its predator by adopting a color, shape, or other characteristic to mimic other objects (Bates, 1862; Wallace, 1865). Among several types of mimicry, concealing mimicry, also called camouflage or crypsis, is one of the most interesting traits involved in prey-predator interactions and has long attracted the interest of many researchers (Prudic et al., 2007;

Futahashi and Fujiwara, 2008; Li et al., 2015; Fujiwara and Nishikawa, 2016; Jin et al., 2019). Pupae of many butterfly species belonging to the families Papilionidae, Pieridae, Nymphalid, and Lycaenid exhibit pupal cryptic colors, which fit with the background color to enable the avoidance of predators (Maisch and Bückmann, 1987; Jones et al., 2007). Pupae of most species in the *Papilio* family show green or brown colors in response to the environment (Figure 1). Hidaka et al. (1959) reported that brown and green pupae of *Papilio xuthus* were preyed upon less in lawns with the same background color, suggesting that pupal color dimorphism is effective for protection against predators. It has also been suggested that some environmental factors such as temperature, relative humidity, wavelength of light, and photoperiod perceived during the larval growth period influence the protective pupal coloration in some Papilionid and Lycaenid species (Ishizaki and Kato, 1956; Bückmann, 1960; Smith, 1978; Honda, 1981; Yamamoto et al., 2011). However, in *P. xuthus* and *P. protenor*, it was shown that the pupal color was not determined by the background color (Ohnishi and Hidaka, 1956).

In *P. xuthus*, determination of the alternative pupal coloration is mainly dependent on the tactile stimuli received in a critical period both before and after girdling (G) (Hiraga, 2006). Under conditions of sufficient light, larvae on smooth surfaces such as leaves or stalks in this period become green pupae (Figure 1). On the other hand, larvae on rough surfaces such as branches or trunks become brown pupae (Figure 1). It is suggested that the stimulus from a rough surface is transmitted to the brain and some unknown neuropeptide for brown color (termed pupal cuticle melanizing hormone: PCMH) is produced and secreted to the larval hemolymph (Awiti and Hidaka, 1982).

Most body colors in the larvae and pupae of butterflies are made by various chemical pigments, such as melanin, bilin, carotenoid, and their related pigments. Our previous studies on *Papilio* larval color revealed the association of the specific expression of several genes with each coloration: black, red, blue, and yellow. The expression of melanin-related genes, *tyrosine hydroxylase* (*TH*), *dopa decarboxylase* (*DDC*), *yellow*, *ebony*, *tan*, and *laccase 2*, is involved in black pigmentation (Futahashi and Fujiwara, 2005, 2006, 2007, 2008; Futahashi et al., 2010, 2012). Expression of the *ebony* gene, which converts dopamine to *N*-beta alanyl dopamine (NBAD), is associated with the eyespot-specific red pigmentation (Shirataki et al., 2010). In addition, from the comparison of the larval patterns of three *Papilio* species (*xuthus*, *machaon*, and *polytes*), it was suggested that the green color is produced by the co-expression of *bilin-binding protein* (*BBP*) (blue) and *yellow-related gene* (*YRG*) or *carotenoid binding protein 1* (*CBP1*) (Shirataki et al., 2010; Futahashi et al., 2012). This information on larval pigmentation suggests that the same or a similar set of genes involved in larval pigmentation is used for producing green or brown pupae in *Papilio* species, although the detailed mechanism of pupal coloration and regulation of pigmentation genes has not been clarified.

To clarify how green and brown pupal coloration occurs via the expression of pigmentation-specific genes, we here used *P. polytes* as an experimental model. Using this species, we studied the molecular mechanisms of larval marking formation (Shirataki et al., 2010) and female-specific Batesian mimicry

(Iijima et al., 2018, 2019); we also recently clarified the whole-genome sequence of this species (Nishikawa et al., 2015), which facilitated analysis of the gene regulation involved in pupal coloration. Furthermore, we established a method of electroporation-mediated gene analysis (Ando and Fujiwara, 2013; Nishikawa et al., 2015) that can provide direct evidence of the functional role of each gene.

In this study, we first established control over green and brown pupal coloration in *P. polytes* under laboratory conditions. Using this system, we specified temporal and spatial changes of pupal pigments in brown and green pupae anatomically. We further identified pigmentation genes responsible for producing green and brown pupae, and clarified their functional roles in pigmentation by electroporation-mediated RNAi. We found that some genes exhibit expression patterns for pupal color induced specifically under green or brown conditions. When the expression of these genes is reduced by electroporation-mediated knockdown, the specific coloration is blocked. We here report how genes involved in the pupal green and brown coloration of *P. polytes* are regulated, which sheds light on the evolutionary process of pupal protective colors among Lepidoptera.

MATERIALS AND METHODS

Insect Rearing

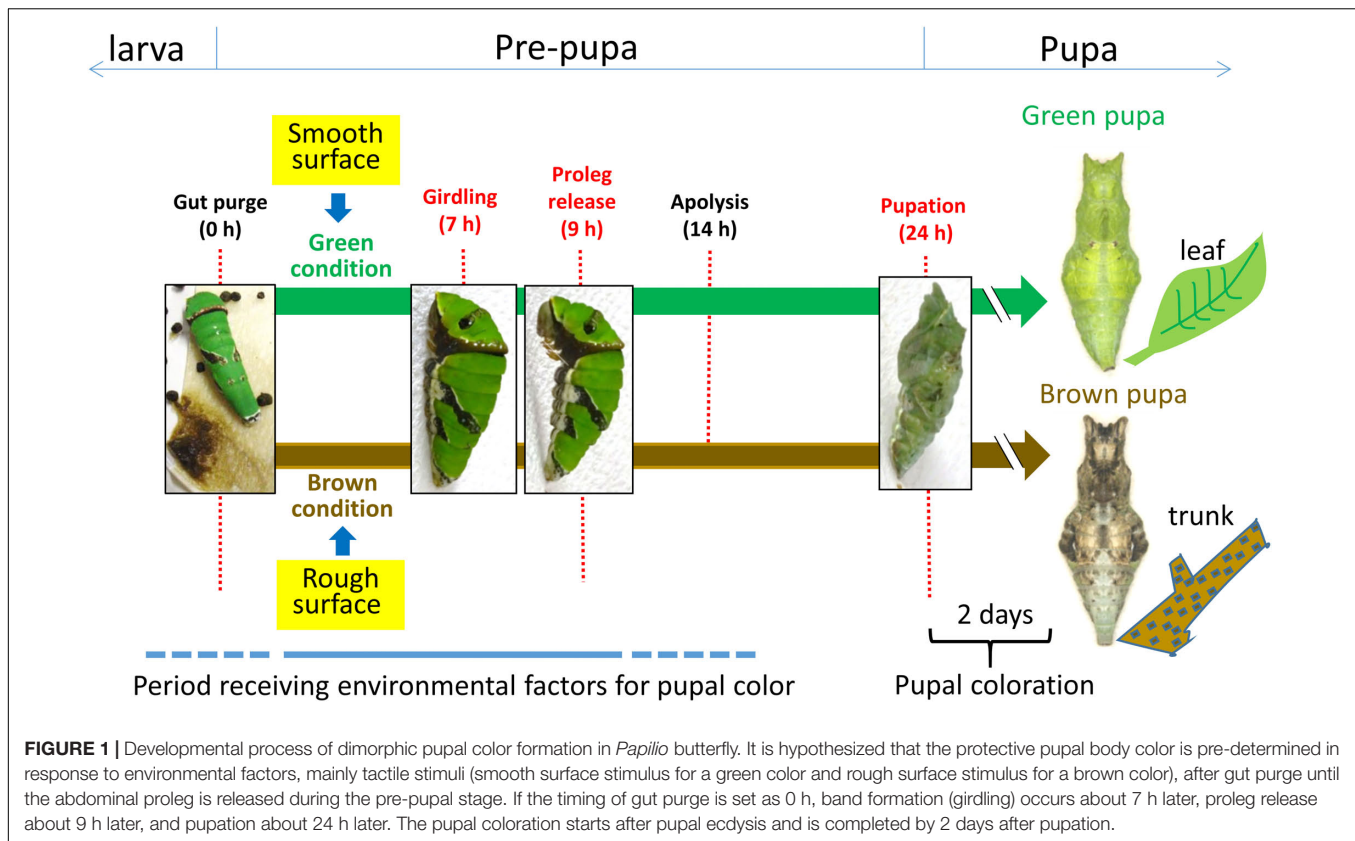
Adults of the swallowtail butterfly, *P. polytes*, were purchased from Chokan-Kabira (Okinawa, Japan). We obtained eggs from mother butterflies and reared the hatched larvae at 25°C under conditions of 16 h light and 8 h darkness (16L/8D). Larvae were fed with *Citrus natsudaoidai* (Rutaceae) leaves or an artificial diet including *Citrus* leaf powder. Young larvae (first to fourth instar) and last-instar larvae (fifth instar) were reared in Petri dishes and plastic containers, respectively (Shirataki et al., 2010). Under these conditions, fifth-instar larvae pupated 24 h after gut purge (GP) and emerged 12 days after pupation (P).

Pupal Coloration System

To obtain green and brown pupae under laboratory condition, we followed the method by Hiraga (2006). The color of pupae was controlled as follows. To obtain green pupae (green pupal conditions), fifth-instar larvae within 1 h after GP were put in a plastic container and placed on the brightest shelf (nearest to the light) in an incubator. To obtain brown pupae (brown pupal conditions), fifth-instar larvae within 1 h after GP were put in a plastic container, the interior of which was covered with the rough side of Kimtowel® (Nippon Paper Crexia Co., Tokyo, Japan) and placed on the darkest shelf in the incubator.

RNA Isolation and Quantitative Reverse-Transcription Polymerase Chain Reaction

Papilio polytes pre-pupae at G (6–7 h after GP), proleg release (PR) (8–10 h after GP), and P (24–30 h after GP) were used for RNA sampling. Epidermal samples from four to six abdominal segments were dissected and washed in cold phosphate-buffered



saline (PBS). Total RNA was isolated using precooled TRI reagent (400 μ l per sample; Sigma-Aldrich) and treated with 0.2 U of DNase I (Takara) at 37°C for 15 min, purified by phenol–chloroform extraction, and then reverse-transcribed with a random primer (N6) using Verso cDNA synthesis kit (Thermo Fisher Scientific). Reverse transcription was carried out at 42°C for 60 min, followed by inactivation by incubation at 95°C for 2 min. The StepOne™ Real-Time PCR System (ABI) was used to carry out quantitative real-time PCR. Analysis was carried out using StepOne™ Software v 2.1 by the relative standard curve method. PCR reaction was carried out using Power SYBR Green PCR Master Mix (ABI) at 95°C for 15 s and 60°C for 1 min for 40 cycles. **Supplementary Table S1** summarizes the primers used for Quantitative Reverse-Transcription Polymerase Chain Reaction (qRT-PCR).

Screening of Differentially Expressed Genes in Prepupae/Pupae Under Green and Brown Conditions

Epidermal RNA was extracted and purified by the above method. BGI Japan Corporation was contracted to carry out the RNA-seq analysis. Sequencing was carried out with 100 bp paired-end reads using the Illumina HiSeq 2500 platform. The obtained read data were mapped to the Ppolytes.v1.0.0 transcriptome [with full-length open reading frame of *BBP6* (XM_013280002.1) and *DDC* (XM_013278236.1)] (Nishikawa et al., 2015) using the analytical software Bowtie 2 (Langmead and Salzberg, 2012). We

used DESeq R package (Anders and Huber, 2010) for estimating differential gene expression. After calculating the expression level of each gene as the FPKM (fragments per kilobase of transcript per million mapped reads) value, genes with significantly different expression levels ($p_{adj} < 0.1$) in comparisons between green and brown conditions during G, PR, and P stages were identified. Heatmaps were generated in R Bioconductor using the heatmap.2 function of the gplots package¹.

Functional Analysis by RNAi Using *in vivo* Electroporation

siRNA to be injected was designed using siRNA design support software siDirect version 2.0² (Ui-Tei et al., 2004; Yamaguchi et al., 2011). After obtaining the sequence of the open-reading frame region of the gene of interest from PapilioBase (Nishikawa et al., 2015), candidate sequences were searched for, using siDirect and based on the sequence information. To reduce off-target effects, we used the BLAST search function of PapilioBase to investigate the specificity of the designed sequence to the gene of interest and selected one with high specificity from among the candidate sequences. Synthesis of siRNA was contracted to FASMAC Corporation. **Supplementary Table S2** summarizes the details of the prepared siRNAs. The siRNA was dissolved to 500 μ M in annealing buffer [100 mM CH₃COOK, 2 mM Mg (CH₃COO)₂, 30 mM HEPES-KOH, pH 7.4]. For the injection,

¹<http://cran.r-project.org/web/packages/gplots/index.html>

²<http://sidirect2.rnai.jp/>

the microinjector FemtoJet (Eppendorf) was used, and the glass needle for injection was fabricated using a puller to process a glass tube with a core. The siRNA was drawn up into the glass needle and placed in a micromanipulator M 401 with a glass needle set under a stereoscopic microscope; the siRNA (250 μ M; 2 μ l) was injected from the intersegmental membrane between the fifth and sixth abdominal segments into hemolymph at the prepupal stage within 1 h after GP. Immediately after injection, 0.1% PBS agarose gel was placed on the left dorsal side of the abdominal fourth and fifth segments and siRNA was introduced (five square pulses of 30 V, 280 ms width) by electrostimulation using an electroporator (Ando and Fujiwara, 2013).

Construction of Phylogenetic Trees

To comprehensively obtain lipocalins from eight lepidopteran species (*P. polytes*, *P. xuthus*, *P. machaon*, *P. glaucus*, *Danaus plexippus*, *Heliconius melpomene*, *Manduca sexta*, and *Bombyx mori*), we downloaded Refseq/predicted proteins with InterProScan annotation files from Lepbase³ (Challis et al., 2016) and selected genes that were annotated as being members of the lipocalin family (PF00061). Codon alignment was generated from a multiple sequence alignment of predicted amino acid sequences by MUSCLE (Edgar, 2004). The phylogenetic tree was constructed using the neighbor joining method with the MEGA6 program (JTT model) (Tamura et al., 2013). The confidence levels for various phylogenetic lineages were assessed by bootstrap analysis (1,000 replicates).

Gene Synteny of Lipocalins in Lepidopteran Species

To obtain orthologous scaffolds for the *P. polytes* *BBP* locus from seven lepidopteran species (*P. xuthus*, *P. machaon*, *P. glaucus*, *D. plexippus*, *H. melpomene*, *M. sexta*, and *B. mori*), we used the following four neighboring genes of the *BBP* locus, which were highly conserved in almost all of these species. We performed a Blastp search on Lepbase (Challis et al., 2016) based on two genes upstream of the *BBP* locus, *uncharacterized LOC106101173* (XM_013280315.1) and *hemicentin-1-like* (XM_013280340.1), and two genes downstream of it, *uncharacterized LOC106101173* (XM_013280315.1) and *hemicentin-1-like* (XM_013280340.1); we then obtained the corresponding scaffolds from each species. We obtained the transcriptional orientation and genomic location of each gene from Lepbase (Challis et al., 2016). To obtain orthologous scaffolds for *P. polytes* *JHBP* (*CBP1*) locus from the seven species, we used *toll-like* gene (XM_013282841.1) as a landmark which is conserved in most of the species. Since the scaffold length of several species is relatively short and lacks the *toll-gene*, we used a reciprocal blast strategy to look for *P. polytes* *JHBP* orthologs: *P. polytes* sequences were blasted (blastp, *E*-value < $e-10$) against protein database of the seven lepidopteran species of interest as well as the opposite strategy (blastp, *E*-value < $e-10$). Only when both pairs (*P. polytes* vs. the others) of top blast hits were the same, we considered them as true orthologs.

³<http://lepbase.org/>

Statistics and Reproducibility

Statistical analysis was performed in three biological replicates, except for analyses on the RNA-Seq data. All data are presented as mean $\pm$ standard deviation (SD). All statistical tests were two-sided. The statistical significance of differences was analyzed using Student's *t*-test; *p* < 0.05 was considered statistically significant.

RESULTS

Temporal and Spatial Change of Color Pigments in Green and Brown Pupae

To the best of our knowledge, the conditions resulting in the production of green and brown pupae in *P. polytes* have not been reported. Thus, with reference to the detailed conditions for selective pupal coloration in *P. xuthus* (Hiraga, 2006), we attempted to establish these conditions in *P. polytes* (see the section "Materials and Methods") to produce green and brown pupae. When last-instar larvae just before GP were placed in a plastic cup on the shelf exposed to the most amount of light in an incubator, 50 of 50 pre-pupae (100%) developed into green pupae. In contrast, when last-instar larvae just before GP were placed on a Kimtowel wiper in a plastic cup and positioned on the shelf exposed to the least amount of light in the incubator, 49 of 50 pre-pupae (98%) developed into brown pupae (**Supplementary Figure S1**).

Almost all of the pupae just after pupal ecdysis (P), about 24 h after GP, appeared green in terms of their external appearance in both conditions. To clarify when and where the green or brown color pigments were expressed, we observed the pigmentation process from pre-pupal to pupal stages under both conditions (**Figure 2A**). During the pre-pupal stage to P, there was little difference in the external appearance. In the pre-pupal stage, the old cuticle layer appeared green and new cuticle appeared transparent in both conditions. Even just after pupal ecdysis, new cuticle appeared transparent, except for only the outer region of cuticle, which exhibited fragmentary green or brown pigment. Since the hemolymph was green in all stages in both conditions, the green color of pre-pupae and pupae at P corresponds to the hemolymph color, which is seen through the transparent cuticle (**Figure 2**).

The green and brown pupal colors were clearly differentiable about 1.5 days after GP (**Figure 2A**, 34 h). When 2-day-old pupae were observed, both the external color and the color of the cuticle surface were completely differentiated into two colors between the green and brown conditions, while the colors of other regions and hemolymph were the same in these two conditions (**Figure 2B**). This suggests that the green or brown appearance of pupae depends mainly on the pigments localized in the cuticle surface. To further characterize the pigments, we treated the cuticles with 6N HCl at 90°C for 120 min (**Figure 2B**, right photos). We found that the green color in green pupae was lost upon this treatment, while the brown color of brown pupae was retained, suggesting that the brown color but not the green color is mainly composed of melanin-related

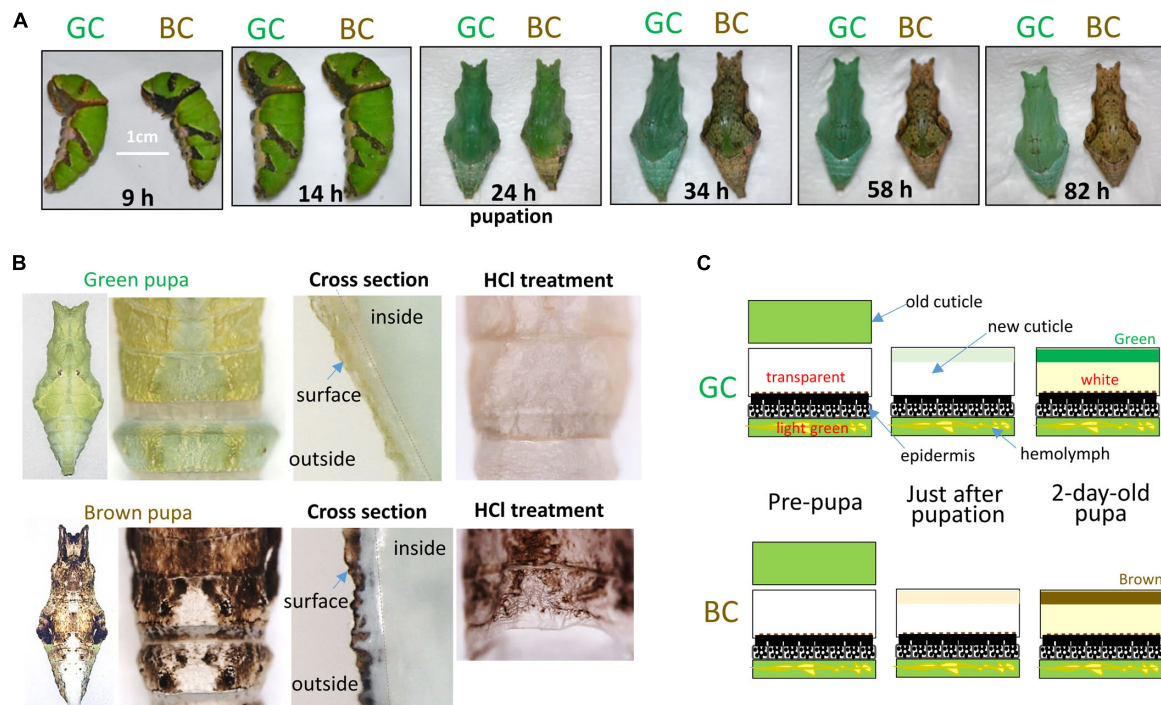


FIGURE 2 | Color change from pre-pupal to pupal stages under green (GC) and brown conditions (BC). **(A)** Surface color change from pre-pupal to pupal stages. Pupation occurs about 24 h after gut purge (0 h). **(B)** Cuticle structure and HCl treatment of green and brown pupae. Left: Whole dorsal view and enlarged view of each pupa. Middle: Cross-section of cuticle regions. Right: Dorsal view of pupal cuticle after HCl treatment (see the text). **(C)** Schematic diagram of cuticle coloration from pre-pupal to pupal stages. Respective cuticle structure and colors under GC and BC are shown with epidermis and hemolymph.

pigment (Shirataki et al., 2010). The above observation indicates that green and brown pupal coloration (pigment formation) occurs at the exterior of the cuticle (epicuticle) mainly after P (Figure 2C). We also observed the *P. xuthus* samples and found that they exhibited a coloration process similar to that in *P. polytes*.

Genes Differentially Expressed Between Green and Brown Conditions

It has been suggested that the *P. xuthus* pre-pupae sense environmental factors (mainly in the form of tactile stimulation) before and after G (about 7 h after GP) and that the pupal colors are pre-determined during the pre-pupal stage (Hiraga, 2006). It is assumed that an unidentified neuropeptide that induces the brown coloration is excreted from the brain at least before P because the brown (and green) pigment formation mainly occurred after P, as shown above. Thus, we speculate that gene regulation during the pre-pupal stage to P is essential for dimorphic pupal coloration.

Next, as an attempt to screen the genes involved in green and brown pupal coloration, we performed RNA sequencing (RNA-seq) analyses using RNAs prepared from G (7 h after GP), PR (9 h after GP), and P (24 h after GP), under green and brown conditions (Supplementary Figure S2). We mapped short read sequences on the transcriptome (Ppolytes v1.0.0; Nishikawa et al., 2015) and calculated the read count for each gene using Bowtie 2

(Langmead and Salzberg, 2012). Total read numbers in RNA-seq were 112,039,832 (G, under the green condition), 112,716,020 (G, under the brown condition), 101,964,764 (PR, GC), 137,897,900 (PR, BC), 133,278,184 (P, GC), and 108,870,344 (P, BC). Next, we screened the genes differentially expressed (DE) between green and brown conditions using DESeq (see the section “Materials and Methods”), with padj (adjusted p -value) < 0.1 . Under these conditions, we identified DE genes as follows: 48 (G, GC), 72 (G, BC), 62 (PR, GC), 169 (PR, BC), 73 (P, GC), and 53 (P, BC) (Supplementary Figure S2). RNA-seq-based screening in this study mainly has an exploratory value because each comparison consists of one biological replicate.

Notably, seven lipocalin family genes, *BBP1*, 2, 4, 5, two *insecticyanin-A-like* genes (*PpolytesGene0006744*, *PpolytesGene0006745*), and *lopap-like*, were included in the list of green condition-specific genes at P. *BBP* genes are reported to be involved in the blue coloration in the green region of final-instar larvae of *Papilio* species (Shirataki et al., 2010), suggesting that these genes are involved in the green pupal coloration.

In the list of brown-specific genes, some pigmentation-related genes were screened: *opsin-1-like* (at G) is related to red pigmentation of *Drosophila* eye; *kynurenine formamidase* (at PR) is related to brown/red pigment papiliochrome R; and *yellow-c* and *yellow-h3* (at G) are related to melanin synthesis (Supplementary Figure S2) (Futahashi et al., 2012). In addition, some Toll signaling pathway genes, namely, *trypsin-1-like*, *peptidoglycan recognition protein-like*, *serine protease easter-like*,

trypsin-7-like (all at G), and serine protease *snake-like* (at PR), were also screened (**Supplementary Figure S2**). It is possible that these genes are involved in the brown coloration because the Toll signaling pathway genes are suggested to be involved in red/black pigmentation in earlier reports on *P. polytes* wings (Nishikawa et al., 2013) and silkworm *Zebra* mutant (KonDo et al., 2017).

Comparison of Expression Profiles of Genes Putatively Involved in Brown Pupal Coloration – Melanin-Related Genes

Since the brown pupal color is composed of melanin-related pigments as shown above, we next used RNA-seq to analyze the expression profiles of six melanin-synthesis genes at three stages: G, PR, and P (**Figure 3B**). In eyespot marking of the fifth-instar larvae of *P. xuthus*, we previously found that *TH*, *yellow*, *laccase 2*, and *tan* are expressed specifically in the black region, and *ebony* specifically in the red region (Futahashi et al., 2010; Shirataki et al., 2010). Based on the RNA-seq data for the abdominal region at P, *TH*, *ebony*, and *yellow* were expressed more highly under the brown condition than under the green one (**Figure 3A**). In contrast, *tan* exhibited the opposite pattern. Since the purpose of the RNA-seq analysis was to screen notable genes using only one sample, we next performed quantitative RT-PCR for five genes (not for *DDC*) with triplicate samples to confirm the above results. The expression profiles of *tan* and *ebony* were similar between the RNA-seq and qRT-PCR, suggesting the dominant expression of *tan* at P under green conditions, but the dominant expression of *ebony* at P under the brown condition. Comparison of the data obtained by the two methods also showed similar patterns in *TH* and *laccase 2*, but a different one in *yellow*. Judged from the results of qRT-PCR, significant differences in expression were observed in *tan* at P, which was induced specifically in the green condition, and in *laccase 2* at P, which was induced specifically in the brown condition.

Comparison of Expression Profiles of Genes Putatively Involved in Green Pupal Coloration: *BBP*- and *JHBP*-Related Genes

Our earlier results on larval coloration in *Papilio* species showed that *BBP* and *CBP1* genes are related to blue and yellow color formation, respectively, and that the co-expression of both genes produces a green region (Shirataki et al., 2010; Futahashi et al., 2012). According to phylogenetic analysis, *BBP* is categorized as a kind of lipocalin; here, we found a new *BBP* gene named *BBP6* from RefSeq (XP_013135456.1) (**Supplementary Figure S3**). Based on the whole-genome sequence of *P. polytes*, we revealed that one single scaffold, NW_013525180.1, included six *BBP* genes and five lipocalin genes (I–V), suggesting that these genes are clustered at a genetic locus on chromosome (linkage group) 25 (**Figure 4**). Although this gene cluster is conserved among lepidopteran species with a sequenced genome, *P. polytes* has the largest number of *BBP* and lipocalin family genes. *CBP1* (*PpolytesGene0004815*) is categorized as

a juvenile hormone binding protein (*JHBP*), and six *JHBP* genes (*PpolytesGene0004811* to *0004816*) were included in a single scaffold (NW_013525336.1). This suggests that these genes constitute another genomic locus seems to reside in chromosome (linkage group) 23 (**Supplementary Figure S4**). We compared the *JHBP* synteny with other lepidopteran species as for *BBP* genes. The analysis of the *CBP1* locus comparisons revealed that *JHBP* genes including *CBP1* are clustered in all *Papilio* species, indicating that *JHBP* (*CBP1*) cluster is highly conserved between *Papilio* species (**Supplementary Figure S4**). Although we could not detect true *CBP1* orthologs in the rest of lepidopteran species (*D. plexippus*, *H. melpomene*, *M. sexta*, and *B. mori*), we found *JHBP* gene repeats in the corresponding locus (except for *M. sexta*) but the gene numbers are different between species, suggesting that *JHBP* genes have been gained or lost independently in the evolutionary process of Lepidoptera. The variation of *JHBP* gene copies might involve body color formation but future study is needed.

To determine the possibility that these genes are involved in the pupal green color formation, we analyzed their expression profiles between the green and brown pupal conditions (**Figure 5**). In the results of RNA-seq analysis, we found that the expression of five *BBP* genes, *BBP1*, 2, 4, 5, and 6, was induced only at P more highly under the green condition than under the brown condition. However, *BBP3* was expressed in three stages under both color conditions. By qRT-PCR, we confirmed this expression profile of four *BBP* genes (1, 2, 4, and 5) found in RNA-seq. Considering that the pupal coloration occurs mainly after pupal ecdysis, as shown above, it is suggested that the green-specific expression of these *BBP* genes just after P is involved in blue pigmentation.

We further compared the expression patterns of six *JHBP* genes, and found that *PpolytesGene0004811* (*JHBP4811*) and *PpolytesGene0004813* (*JHBP4813*) showed expression profiles similar to those of *BBP* genes, the expression of which is induced at P in the green condition but not in the brown one (**Supplementary Figure S5**). *CBP1* (*PpolytesGene0004815*) and *JHBP4812* showed similar levels of expression between green and brown conditions in all stages. The qRT-PCR analysis of *CBP1* confirmed its expression profile as identified by RNA-seq. The expression of two *JHBP*-related genes [*PpolytesGene0004814* (*JHBP4814*) and *PpolytesGene0004816* (*JHBP4816*)] was induced in the brown condition at the PR stage and at the G stage, respectively.

Functional Analyses of Genes Involved in Brown and Green Pupal Colors

To determine the functional role of each gene, we next performed electroporation-mediated knockdown experiments. The siRNA for each gene was injected into the dorsal side of the abdominal segment and incorporated into the right side of the abdominal region (the fourth and fifth segments) by touching the plus (+) electrode. We found that the knockdown of *TH* in nine brown-conditioned pupae caused decreased brown coloration in the siRNA-introduced region of eight samples, compared with that on the non-introduced side (**Figure 6**). Similarly,

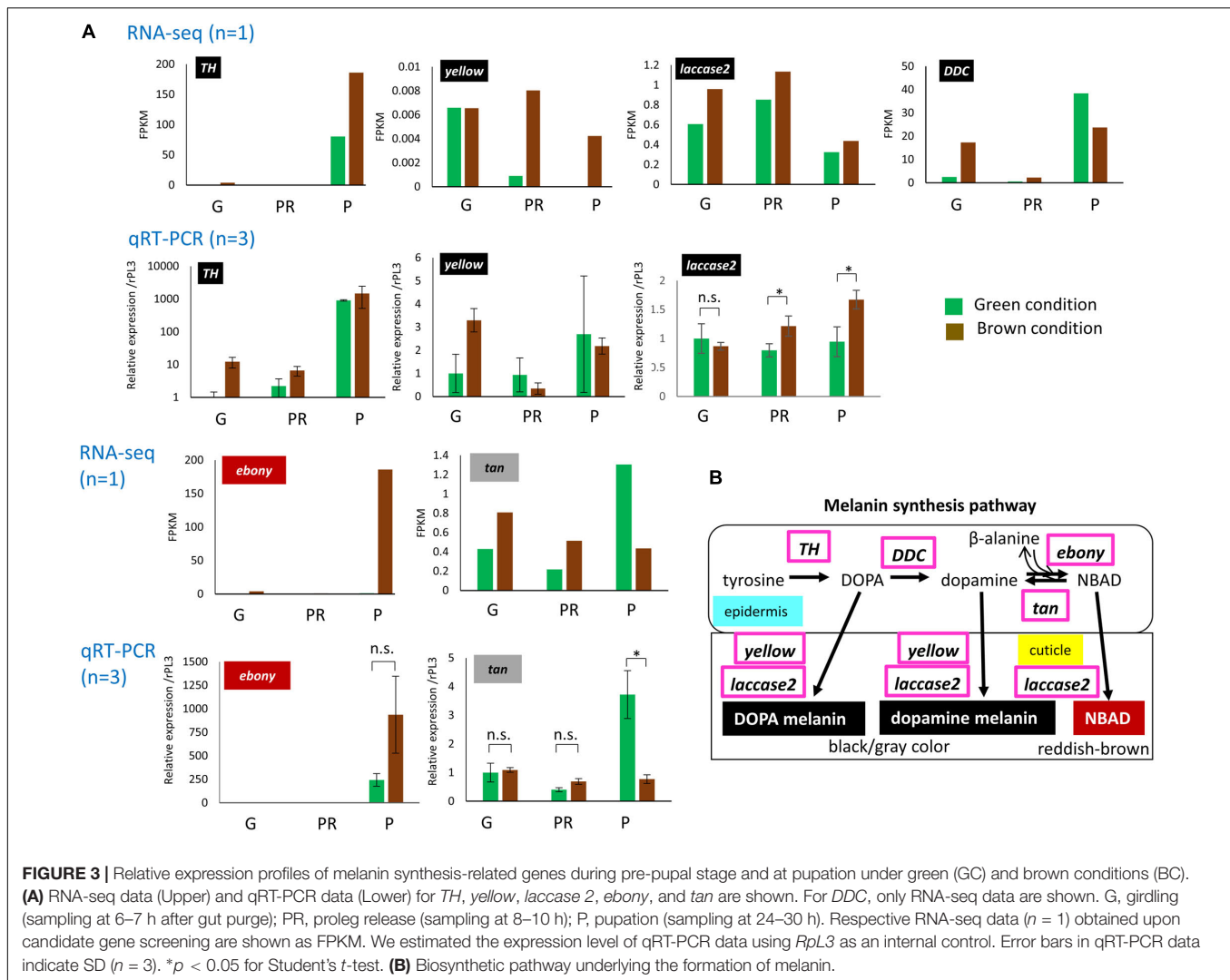


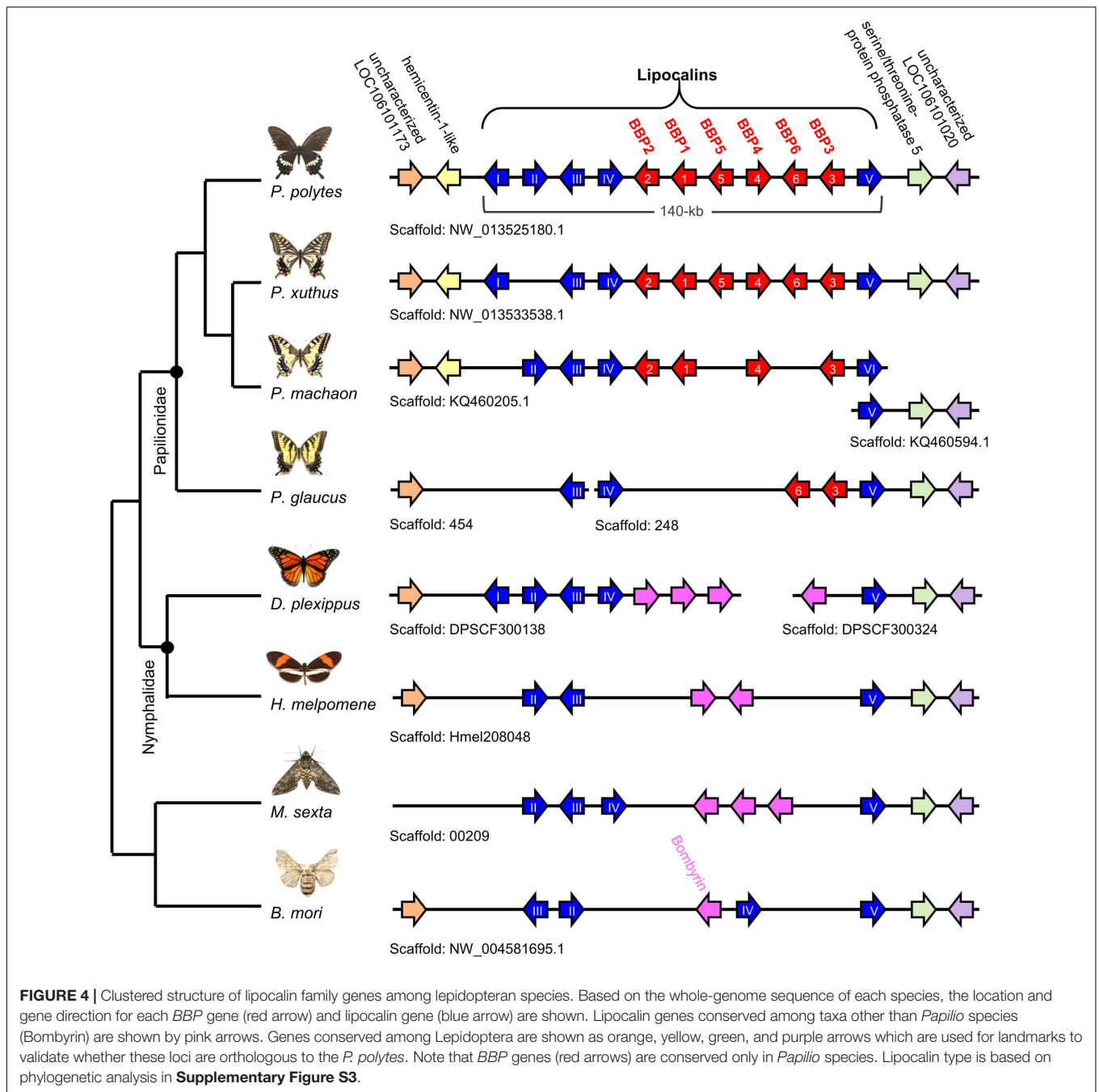
FIGURE 3 | Relative expression profiles of melanin synthesis-related genes during pre-pupal stage and at pupation under green (GC) and brown conditions (BC). **(A)** RNA-seq data (Upper) and qRT-PCR data (Lower) for *TH*, *yellow*, *laccase 2*, *ebony*, and *tan* are shown. For *DDC*, only RNA-seq data are shown. G, girdling (sampling at 6–7 h after gut purge); PR, proleg release (sampling at 8–10 h); P, pupation (sampling at 24–30 h). Respective RNA-seq data ($n = 1$) obtained upon candidate gene screening are shown as FPKM. We estimated the expression level of qRT-PCR data using *Rpl3* as an internal control. Error bars in qRT-PCR data indicate SD ($n = 3$). * $p < 0.05$ for Student's *t*-test. **(B)** Biosynthetic pathway underlying the formation of melanin.

knockdown of *laccase 2* in four brown pupae resulted in the decrease of brown coloration in all cases. Notably, the same siRNA-introduced region showed the complete loss of black pigmentation in the later adult stage (Figure 6). Thus, melanin-synthesis pathway genes, *TH* and *laccase 2*, are involved in the brown coloration. Although we attempted to knock down the expression of other melanin-related genes (*yellow*, *tan*, and *ebony*), we could not detect phenotypic changes in the brown coloration.

To check whether *BBP* and *JHBP* genes are involved in the green pupal coloration, we next knocked down genes whose expression was induced by the green conditions at P (*BBP1*, *BBP2*, *BBP4*, *BBP5*, *JHBP4811*, and *JHBP4813*). Single gene knockdown of *BBP1* or *BBP2* in green-conditioned samples showed a yellowish color change in the introduced region, indicating the loss of blue pigmentation (Figure 7). However, no phenotypic change was observed upon the single knockdown of *BBP4* or *BBP5* (Figure 7). Since the blue coloration could potentially be formed by redundant

functions of *BBP* family genes, we further knocked down several genes simultaneously. Double knockdown of *BBP1/BBP2* or *BBP4/BBP5*, and simultaneous knockdown of four *BBP* genes, made the change from green to yellow in the siRNA-introduced region clearer than the case upon single gene knockdown (Figure 7). This suggests that each of the four *BBP* genes has a redundant function and cooperatively contributes to the pupal blue color formation.

Furthermore, we knocked down two *JHBP* genes in the green pupae, *4811* and *4813*, since their expression was observed specifically under the green conditions at P. While a small change of color from green to blue was observed upon the single knockdown of each gene, double knockdown of both genes showed the blue region more clearly due to the decreased yellow pigmentation (Figure 8). Although *CBP1* (*4815*) was indicated to be involved in the larval green coloration (Shirataki et al., 2010; Futahashi et al., 2012), its knockdown did not show a phenotypic change in the green pupae, suggesting that the expression of *CBP1* is not related to the pupal green coloration.

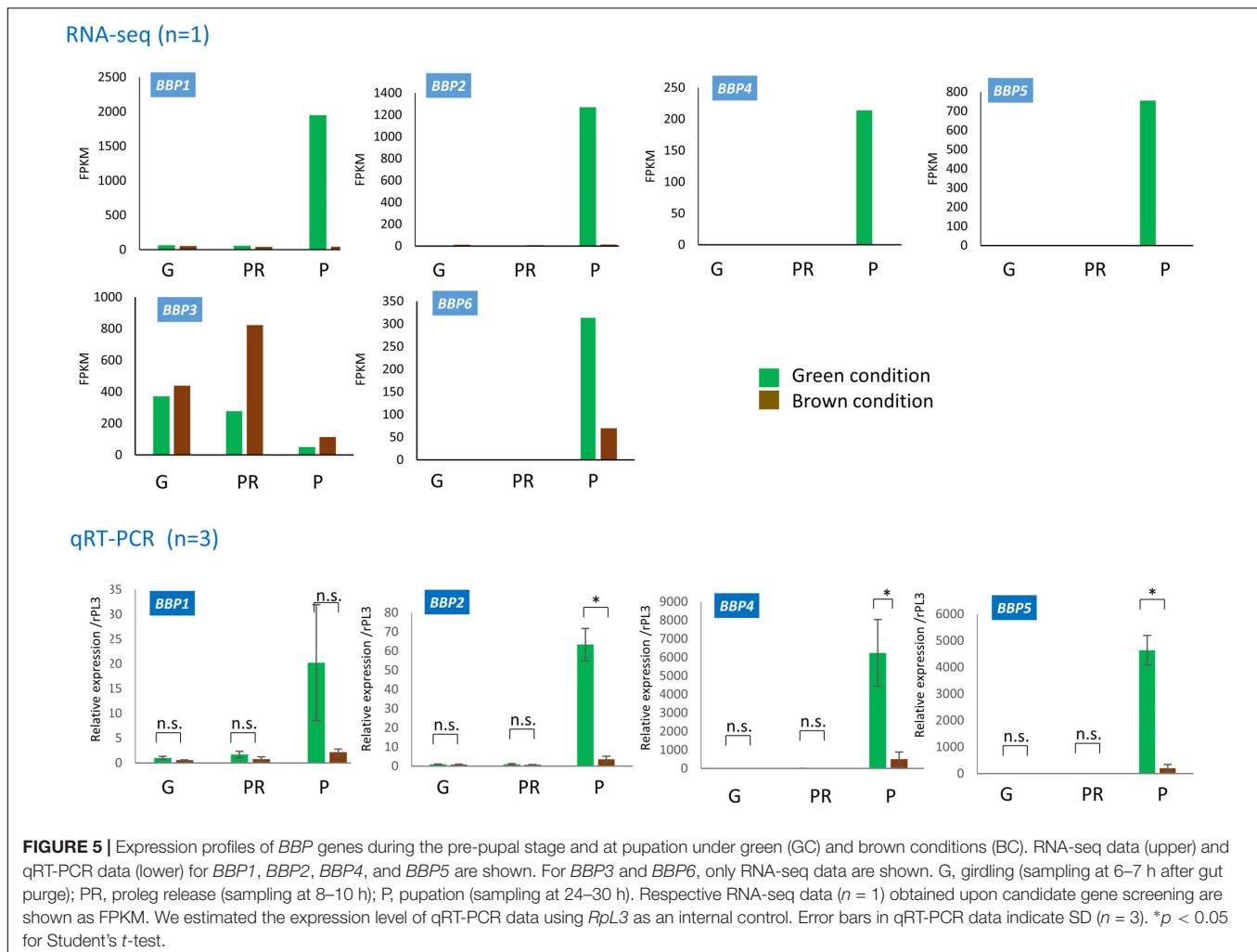


DISCUSSION

Hiraga (2006) reported that *P. xuthus* pupae become green when the surface of their pupation site is smooth, while they become brown when the surface is rough, under conditions with sufficient light. Although light suppresses the induction of the brown pupal coloration, the main environmental factors influencing pupal coloration are considered to be tactile signals in this *Papilio* species. Hiraga (2005) also found that the protective pupal coloration in another butterfly in Papilioninae, *Graphium sarpedon*, is strongly dependent on the brightness in

the pupation site. Although some environmental factors, such as light, humidity, temperature, and photoperiod, influence the pupal coloration of *Papilio* species, none of these factors appears to be the main determinant (Hazel and West, 1979; Honda, 1981). In *P. polytes*, *P. demoleus*, and *P. polyxenes*, Smith (1978) reported that brown pupae were often found on rough surfaces and green pupae on smooth ones, although the main environmental factor affecting pupal coloration was not identified.

In this study, we observed that the pupal coloration of *P. polytes* occurs via a process similar to that in *P. xuthus*, and established the laboratory conditions causing an almost



complete switch in the pupal coloration to green or brown (98–100%) (**Supplementary Figure S1**). This enables us to pursue the gene expression profiles leading to each coloration precisely after the pre-determining period before the pigmentation period. Hiraga (2006) suggested that a critical period for exposure to environmental factors pre-determining pupal coloration ranges from before G (about 7 h after GP) to after G. We found that all pre-pupae and pupae just after P (about 24 h after GP), even under brown conditions, appeared almost completely green in appearance, and brown pupae were visible at least 24 h after P (**Figure 2**). Just after P, the green color of hemolymph was seen through the transparent cuticle newly synthesized in pupae under both green and brown conditions. Notably, the green and brown pigments were located mainly in the epicuticle of newly synthesized pupal cuticle. This indicates that there is a time gap, of at least 1 day, between the pre-determination and pigmentation periods.

We thought that the genes controlling the pigmentation processes should be expressed before pupal coloration, which appear to occur mainly after P. Thus, we analyzed

the temporal expression patterns of genes by RNA-seq and qRT-PCR at three developmental stages: G, PR, and P. Among the melanin synthesis pathway genes, *TH*, *laccase 2*, *ebony*, and *tan* showed similar expression patterns between RNA-seq and qRT-PCR analyses (**Figure 3**). However, *yellow* showed different patterns between the two analyses, probably because its expression level was very low and easily fluctuated. The expression levels of *TH* and *laccase 2* were higher under the brown condition in all three stages. Combining this observation with the result that the knockdown of each gene caused the loss of melanin pigments (**Figure 6**), it is supposed that *TH* and *laccase 2* are essential factors for inducing the brown pupal coloration. Interestingly, the expression of *ebony* and *tan* was induced at P specifically under the brown and green conditions, respectively. However, *ebony* knockdown in the brown pupae or *tan* knockdown in the green pupae did not alter the color. Although we do not know the exact reason for this result, the timing of injection of siRNA before the GP may not be appropriate for effective knockdown of the expression of these genes.

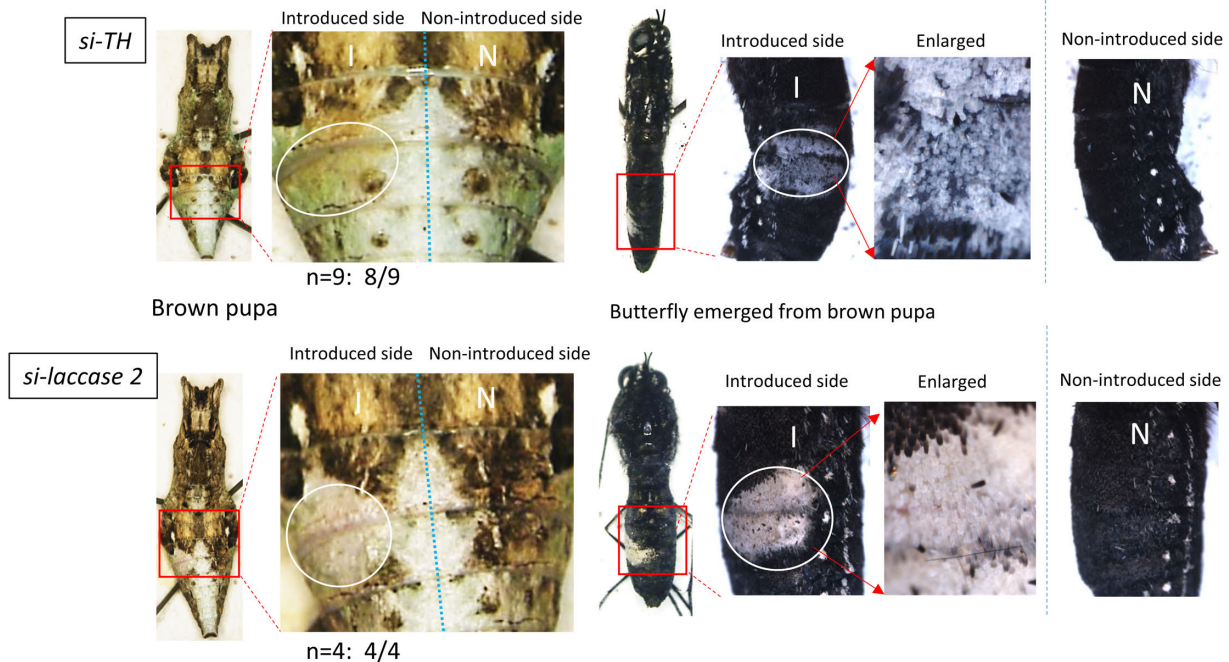
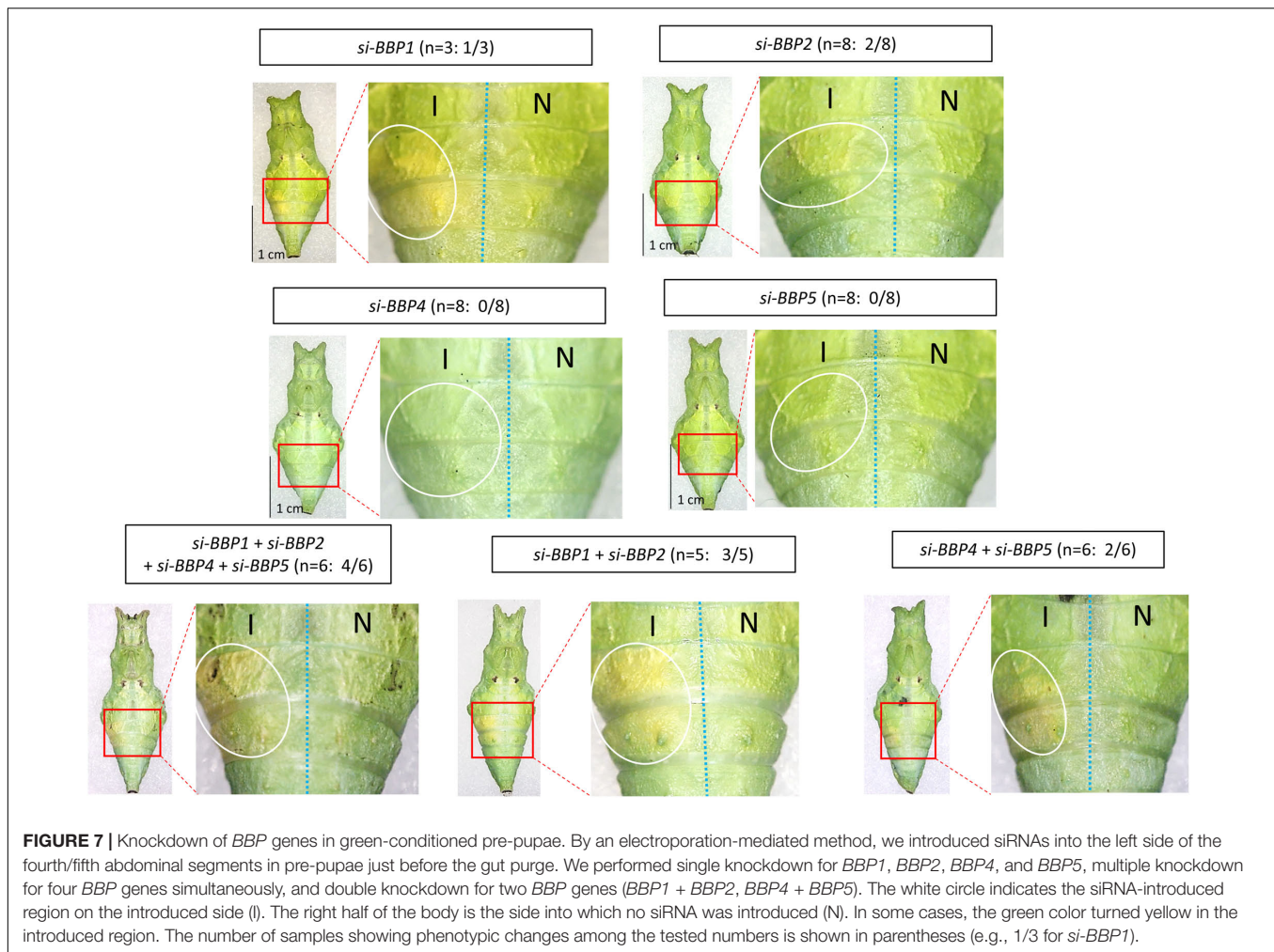


FIGURE 6 | Knockdown of *TH* and *laccase 2* in brown-conditioned pre-pupae. By an electroporation-mediated method, we introduced siRNA for *TH* or *laccase 2* into the left side of the fourth/fifth abdominal segments in pre-pupae after or just before gut purge. The white circle indicates the siRNA-introduced region. The right half of the body is the side into which siRNA was not introduced. Eight of nine pre-pupae treated with *si-TH* and four of four pre-pupae treated with *si-laccase 2* showed the repression of brown coloration in the pupal stage (Left), and the loss of black pigmentation in the adult stage (Right). To clearly show the effects in butterflies, we removed their wings.

BBP1 and *BBP2* were shown to be expressed specifically in the blue spot region of *P. polytes* larvae (Futahashi et al., 2012), suggesting that these genes are involved in the blue pigmentation. In addition, *BBP1*, *BBP2*, and *CBP1* were also co-expressed in the larval green region of *P. polytes* (Futahashi et al., 2012), suggesting that *CBP1* is involved in the yellow pigmentation, at least during larval development. Notably, five *BBP* genes (1, 2, 4, 5, and 6) were highly expressed in the green-conditioned pupae only at the P stage (Figure 5). However, these *BBP* genes were not expressed in two other pre-pupal stages, nor under the brown condition. Among six *JHBP* genes tested, *4811* and *4813* also showed similar P-specific expression only under the green condition (Supplementary Figure S5), while *CBP1* (4815) was constantly expressed under both green and brown conditions. Therefore, it is suggested that the expression of these genes is regulated to ensure that they work together at P to effectively produce a green color. Each protein encoded by these genes should be produced after P, which is consistent with the observation that green (or brown) coloration occurs mainly after P. Although single knockdown of *BBP4* or *BBP5* could not block the green coloration, double knockdown of both genes repressed it (Figure 7). In addition, simultaneous knockdown of multiple *BBP* or *JHBP* genes generally showed the green coloration more effectively than single-gene knockdown (Figures 7, 8). This indicates the redundant function of *BBP* and *JHBP* genes. It is noteworthy that *CBP1* is involved in the larval green coloration, but

seemingly not in the pupal green (Figure 8). In contrast, *BBP4* and *BBP5* could not block the larval green coloration, even upon their double knockdown, so these genes are involved only in the pupal green coloration (Supplementary Figure S6 and Figure 7). These results suggest different color regulation between larval and pupal stages, even in the same individual of *P. polytes*.

This study also clarified the existence of multiple copies of the *BBP* and *JHBP* gene families, which are each located in a single locus of the genome. In *P. polytes*, six *BBP* and five other lipocalin genes are clustered in a 140 kb genomic region, the structure of which is conserved among *Papilio* species, while the gene numbers differ in each species (Figure 4). *P. glaucus*, the pupae of which are only brown in color, has only two *BBP* genes (*BBP3* and *BBP6*), while *P. machaon*, *P. xuthus*, and *P. polytes*, the pupae of which show dimorphic green and brown colors, have four, six, and six *BBP* genes, respectively (Figure 4). This indicates that the numbers of *BBP* genes have increased (or decreased) as an adaptation to the environment experienced during each life cycle over the course of evolution. In contrast to other *BBP* genes, *BBP3* was expressed under both conditions and was not suggested to be involved in pupal green coloration, which is supported by the above finding in *P. glaucus*. The *BBP* gene cluster is conserved specifically among the *Papilio* species, while it is replaced by lipocalin family *bombyrin* genes in other lepidopteran species (Figure 4 and Supplementary Figure S3).



Bombyrin in *B. mori* is known to be localized in the central nervous system (Sakai et al., 2001), but its function has not yet been clarified. It would be interesting to determine the functional and evolutionary relationships between *BBP* and *bombyrin* family genes.

The clustered structure of *BBP* and *JHBP* multigene families may provide a clue to understanding the evolution and mechanisms underlying the green color formation. Each set of *BBP* genes located side by side in the *P. polytes* genome appears to have different functional roles: the set of *BBP1* and *BBP2* is involved in both larval and pupal green coloration, the set of *BBP4*, *BBP5*, and *BBP6* is involved only in pupal green coloration, while the set of *BBP3* appears to be a prototype because *BBP3* is the only gene that is conserved in *Papilio* species (Figure 4). The main *JHBP* genes involved in pupal green coloration, *4811* and *4813*, are located on the same locus (Supplementary Figure S4), and the distance between them is about 10 kb. These findings indicate that genes with similar functions in green coloration have evolved as sets. A regulatory *cis*-element enabling the similar expression patterns may be shared among each gene set, but this has not yet been clarified. A famous example of multigene families in

insects is chorion (eggshell protein) genes involved in the eggshell formation (choriogenesis). In *Bombyx*, it has been shown that 127 chorion genes are clustered within 750 kb on chromosome 2 (Chen et al., 2015). The vast majority of *Bombyx* chorion genes are organized as coordinately expressed pairs that are transcribed from opposite strands, under the direction of a short shared 5' flanking promoter located on the middle of the pair (Kafotos et al., 1995). Recent study showed that this bidirectional promoter contains *cis*-elements interacting with transcription factors such as HMGA, GATA, and C/EBP to achieve precise timing of chorion genes' expression (Tsatsarounos et al., 2015). In contrast to chorion genes, regulatory mechanisms for the differential expression of *BBP* and *JHBP* genes remained unknown, but it is suggested that the pupal color switch in *Papilionid* depends on the release of a brown inducing factor, pupal cuticle melanizing hormone (PCMH), which is secreted from brain subesophageal ganglion thoracic ganglion (Br-SG-TG) complexes during the pharate pupal stage (Awiti and Hidaka, 1982; Starnecker and Hazel, 1999; Yamanaka et al., 1999). Interestingly, *Papilio glaucus*, a species that only produces brown pupae, midbody ligation causes the posterior compartment to become green,

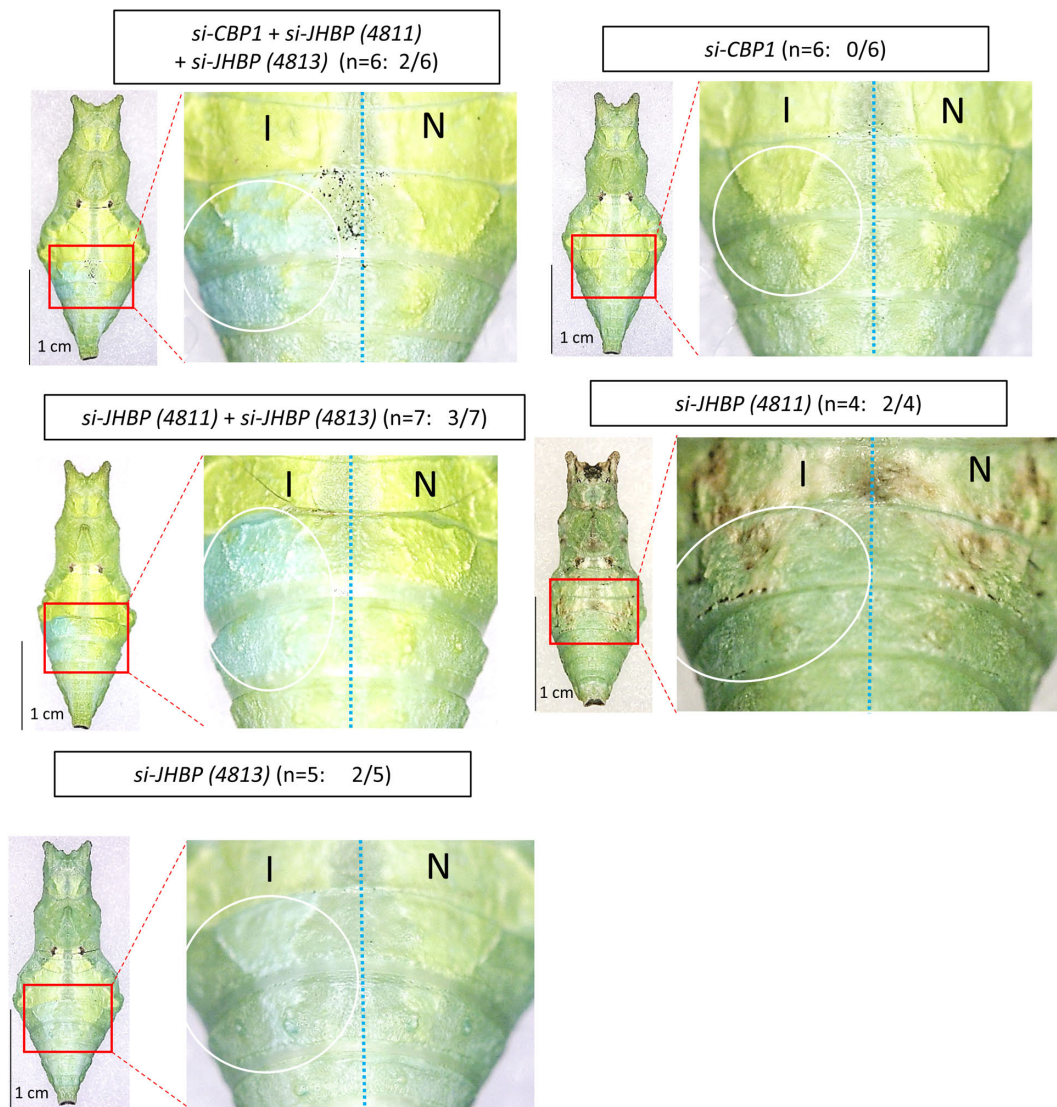


FIGURE 8 | Knockdown of *JHBP*-related genes in green conditioned pre-pupae. By an electroporation-mediated method, we introduced siRNAs into the left side of the fourth/fifth abdominal segments in pre-pupae just before the gut purge. We performed single knockdown for *JHBP4811*, *JHBP4813*, and *JHBP4815* (*CBP1*), multiple knockdown for three *JHBP* genes simultaneously, and double knockdown for two *JHBP* genes (*JHBP4811* + *JHBP4813*). The white circle indicates the siRNA introduced region on the introduced side (I). The right half of the body is the side with no siRNA introduction (N). In some cases, the green color turned blue in the introduced region. The number of samples showing phenotypic changes among the tested numbers is shown in parentheses (e.g., 2/5 for *si-JHBP4813*).

and the brown color can be rescued with an injection of PCMH. The observation indicates that environmentally cued pupal color could evolve by facultative inhibition of PCMH release (Jones et al., 2007; Nijhout, 2010). By performing gel filtration and high-performance liquid chromatography (HPLC), Yamanaka et al. (1999) estimated that the molecular weight of the *B. mori* factor showing PCMH activity was 3,000–4,000 Da, although the PCMH molecule has not yet been clarified not only in *B. mori* but also in *Papilio* species. Identifying the PCMH itself should help address the crosstalk between the PCMH-related hormonal pathway and the gene networks involved in the pupal color polymorphism in *Papilio* species.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in NCBI BioProject, NCBI Accession No. PRJDB9103.

ETHICS STATEMENT

We conducted the study in accordance with the policy of life science research ethics and safety in the University of Tokyo. The ethics committee of the University of Tokyo declares that higher invertebrates are not included in regulated animals.

AUTHOR CONTRIBUTIONS

EO and MN provided the organisms. EO and SY isolated the RNA and performed qPCR and RNAi, RNA-seq, and phylogenetic analyses. HF and SY wrote the manuscript. HF supervised the project.

FUNDING

This work was supported by the MEXT/JSPS KAKENHI (20017007, 22128005, 15H05778, 18H04880, 16H06279 to HF).

REFERENCES

- Anders, S., and Huber, W. (2010). Differential expression analysis for sequence count data. *Genome Biol.* 11:R106. doi: 10.1186/gb-2010-11-10-r106
- Ando, T., and Fujiwara, H. (2013). Electroporation mediated somatic transgenesis for rapid functional analysis in insects. *Development* 140, 454–458. doi: 10.1242/dev.085241
- Awiti, L. R. S., and Hidaka, T. (1982). Neuroendocrine mechanism involved in pupal colour dimorphism in swallowtail *Papilio xuthus* L. *Insect Sci. Appl.* 3, 181–191. doi: 10.1017/S1742758400005798
- Bates, H. R. (1862). Contributions to and insect fauna of the amazon valley (Lepidoptera, Heliconidae). *Trans. Linn. Soc.* 23, 495–556. doi: 10.1111/j.1095-8312.1981.tb01842.x
- Bückmann, D. (1960). Die determination der puppenfärbung bei *Vanessa urticae* L. *Naturwissenschaften* 47, 610–611. doi: 10.1007/BF00602233
- Challis, R. J., Kumar, S., Dasmahapatra, K. K., Jiggins, C. D., and Blaxter, M. (2016). Lepbase: the Lepidopteran genome database. *bioRxiv* [Preprint] doi: 10.1101/056994
- Chen, Z., Nohata, J., Guo, H., Li, S., Liu, J., Guo, Y., et al. (2015). A comprehensive analysis of the chorion locus in silkworm. *Sci. Rep.* 5:16424. doi: 10.1038/srep16424
- Edgar, R. C. (2004). MUSCLE: a multiple sequence alignment method with reduced time and space complexity. *BMC Bioinform.* 5:113. doi: 10.1186/1471-2105-5-113
- Fujiwara, H., and Nishikawa, H. (2016). Functional analysis of genes involved in color pattern formation in Lepidoptera. *Curr. Opin. Insect Sci.* 17, 16–23. doi: 10.1016/j.cois.2016.05.015
- Futahashi, R., Banno, Y., and Fujiwara, H. (2010). Caterpillar color patterns are determined by a two-phase melanin gene prepatterning process: new evidence from tan and laccase2. *Evol. Dev.* 12, 157–167. doi: 10.1111/j.1525-142X.2010.00401.x
- Futahashi, R., and Fujiwara, H. (2005). Melanin-synthesis enzymes coregulate stage-specific larval cuticular markings in the swallowtail butterfly, *Papilio xuthus*. *Dev. Genes Evol.* 215, 519–529. doi: 10.1007/s00427-005-0014-y
- Futahashi, R., and Fujiwara, H. (2006). Expression of one isoform of GTP cyclohydrolase I coincides with the larval black markings of the swallowtail butterfly, *Papilio xuthus*. *Insect Biochem. Mol. Biol.* 36, 63–70. doi: 10.1016/j.ibmb.2005.11.002
- Futahashi, R., and Fujiwara, H. (2007). Regulation of 20-hydroxyecdysone on the larval pigmentation and the expression of melanin synthesis enzymes and yellow gene of the swallowtail butterfly, *Papilio xuthus*. *Insect Biochem. Mol. Biol.* 37, 855–864. doi: 10.1016/j.ibmb.2007.02.014
- Futahashi, R., and Fujiwara, H. (2008). Juvenile hormone regulates butterfly larval pattern switches. *Science* 319:1061. doi: 10.1126/science.1149786
- Futahashi, R., Shirataki, H., Narita, T., Mita, K., and Fujiwara, H. (2012). Comprehensive microarray-based analysis for stage-specific larval camouflage pattern-associated genes in the swallowtail butterfly, *Papilio xuthus*. *BMC Biol.* 10:1. doi: 10.1111/j.1525-142X.2010.00401.x
- Hazel, W. N., and West, D. A. (1979). Environmental control of pupal colour in swallowtail butterflies (Lepidoptera: Papilionidae): *Battus philenor* (L.) and

ACKNOWLEDGMENTS

We thank Mr. Yousuke Muraoka and Drs. Shinji Nagata, Hideki Nishikawa, and Junichi Yamaguchi for helpful suggestions on our research. We also thank Dr. Tetsuya Kojima for helpful comments on the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fevo.2020.00051/full#supplementary-material>

- Papilio polyxenes* Fabr. *Ecol. Ent.* 4, 393–400. doi: 10.1111/j.1365-2311.1979.tb00599.x
- Hidaka, T., Kimura, T., and Onosaka, M. (1959). Experiments on the protective coloration of pupae of the swallowtail, *Papilio xuthus* L. *Dobutsu. Zasshi* 68, 222–226.
- Hiraga, S. (2005). Two different sensory mechanisms for the control of pupal protective coloration in butterflies. *J. Insect Physiol.* 51, 1033–1040. doi: 10.1016/j.jinsphys.2005.04.018
- Hiraga, S. (2006). Interactions of environmental factors influencing pupal coloration in swallowtail butterfly *Papilio xuthus*. *J. Insect Physiol.* 52, 826–838. doi: 10.1016/j.jinsphys.2006.05.002
- Honda, K. (1981). Environmental factors affecting the pupal coloration in *Papilio protenor* Demetrius Cr. (Lepidoptera: Papilionidae) II. *Effect Phys. Stimuli. Appl. Entomol. Zool.* 16, 467–471. doi: 10.1303/aez.16.467
- Iijima, T., Kajitani, R., Komata, S., Lin, C.-P., Sota, T., Itoh, T., et al. (2018). Parallel evolution of Batesian mimicry supergene in two *Papilio* butterflies, *P. polytes* and *P. memnon*. *Sci. Adv.* 4:eaao5416. doi: 10.1126/sciadv.aao5416
- Iijima, T., Yoda, S., and Fujiwara, H. (2019). The mimetic wing pattern of *Papilio polytes* butterfly is regulated by *doublesex*-orchestrated gene network. *Commun. Biol.* 2:257. doi: 10.1038/s42003-019-0510-7
- Ishizaki, H., and Kato, M. (1956). Environmental factors affecting the formation of orange pupae in *Papilio xuthus*. *Mem. Coll. Sci. Univ. Kyoto Ser. B* 23, 11–18.
- Jin, H., Seki, T., Yamaguchi, J., and Fujiwara, H. (2019). Pre-patterning of *Papilio xuthus* caterpillar camouflage is controlled by three homeobox genes: clawless, abdominal-A and Abdominal-B. *Sci. Adv.* 5:eaav7569. doi: 10.1126/sciadv.aav7569
- Jones, M., Rakes, L., Yochum, M., Dunn, G., Wurster, S., Kinney, K., et al. (2007). The proximate control of pupal color in swallowtail butterflies: implications for the evolution of environmentally cued pupal color in butterflies (Lepidoptera: Papilionidae). *J. Insect Physiol.* 53, 40–46. doi: 10.1016/j.jinsphys.2006.09.013
- Kafotos, F. C., Tzertzinis, G., Spoerel, N. A., and Nguyen, H. T. (1995). “Chorion genes: an overview of their structure, function, and transcriptional regulation,” in *Molecular Model Systems in the Lepidoptera*, eds M. R. Goldsmith, and A. S. Wilkins (New York, NY: Cambridge University Press), 181–216. doi: 10.1017/CBO9780511529931.008
- KonDo, Y., Yoda, S., Mizoguchi, T., Ando, T., Yamaguchi, J., Yamamoto, K., et al. (2017). Toll ligand Spätzle3 controls melanization in the stripe pattern formation in caterpillars. *Proc. Natl. Acad. Sci. U.S.A.* 114, 8336–8341. doi: 10.1073/pnas.1707896114
- Langmead, B., and Salzberg, S. L. (2012). Fast gapped-read alignment with Bowtie 2. *Nat. Methods* 9, 357–359. doi: 10.1038/nmeth.1923
- Li, X., Fan, D., Zhang, W., Liu, G., Zhang, L., Zhao, L., et al. (2015). Outbred genome sequencing and CRISPR/Cas9 gene editing in butterflies. *Nat. Commun.* 6:8212. doi: 10.1038/ncomms9212
- Maisch, A., and Bückmann, D. (1987). The control of cuticular melanin and lutein incorporation in the morphological colour adaptation of a nymphalid pupa, *Inachis io* L. *J. Insect Physiol.* 33, 393–402. doi: 10.1016/0022-1910(87)90017-5
- Nijhout, F. H. (2010). “Photoperiodism in insects: effects on morphology, in photoperiodism,” in *The Biological Calendar*, eds R. J. Nelson, D. L. Denlinger,

- and D. E. Somers (New York, NY: Oxford University Press), 318–341. doi: 10.1093/acprof:oso/9780195335903.003.0013
- Nishikawa, H., Iga, M., Yamaguchi, J., Saito, K., Kataoka, H., Suzuki, Y., et al. (2013). Molecular basis of wing coloration in a Batesian mimic butterfly, *Papilio polytes*. *Sci. Rep.* 3:3184. doi: 10.1038/srep03184
- Nishikawa, H., Iijima, T., Kajitani, R., Yamaguchi, J., Ando, T., Suzuki, Y., et al. (2015). A genetic mechanism for female-limited Batesian mimicry in *Papilio* butterfly. *Nat. Genet.* 47, 405–409. doi: 10.1038/ng.3241
- Ohnishi, E., and Hidaka, T. (1956). Effect of environmental factors on the determination of pupal types in some swallowtail, *Papilio xuthus* L. and *P. protenor demetrius*. *Cr. Zool. Mag.* 65, 185–187.
- Prudic, K. L., Oliver, J. C., and Sperling, F. A. (2007). The signal environment is more important than diet or chemical specialization in the evolution of warning coloration. *Proc. Natl. Acad. Sci. U.S.A.* 104, 19381–19386. doi: 10.1073/pnas.0705478104
- Sakai, M., Wu, C., and Suzuki, K. (2001). Nucleotide and deduced amino acid sequences of a cDNA encoding a lipocalin protein in the central nervous system of *Bombyx mori*. *J. Insect Biotechnol. Sericol.* 70, 105–111. doi: 10.11416/jjbs2001.70.105
- Shirataki, H., Futahashi, R., and Fujiwara, H. (2010). Species-specific coordinated gene expression and trans-regulation of larval color pattern in three swallowtail butterflies. *Evol. Dev.* 12, 305–314. doi: 10.1111/j.1525-142X.2010.00416.x
- Smith, A. G. (1978). Environmental factors influencing pupal colour determination in Lepidoptera I. Experiments with *Papilio polytes*, *Papilio demoleus* and *Papilio polyxenes*. *Proc. R. Soc. Lond. Ser. B* 200, 295–329. doi: 10.1098/rspb.1978.0021
- Starnecker, G., and Hazel, W. (1999). Convergent evolution of neuroendocrine control of phenotypic plasticity in pupal colour in butterflies. *Proc. R. Soc. Lond. B* 266, 2409–2412. doi: 10.1098/rspb.1999.0939
- Tamura, K., Stecher, G., Peterson, D., Filipski, A., and Kumar, S. (2013). MEGA6: molecular evolutionary genetics analysis version 6.0. *Mol. Biol. Evol.* 30, 2725–2729. doi: 10.1093/molbev/mst197
- Tsatsaronos, S. P., Rodakis, G. C., and Lecanidou, R. (2015). Analysis of developmentally regulated chorion gene promoter architecture via electroporation of silk moth follicles. *Insect Mol. Biol.* 24, 71–81. doi: 10.1111/imb.12136
- Ui-Tei, K., Naito, Y., Takahashi, F., Haraguchi, T., Ohki-Hamazaki, H., Juni, A., et al. (2004). Guidelines for the selection of highly effective siRNA sequences for mammalian and chick RNA interference. *Nucleic Acids Res.* 32, 936–948. doi: 10.1093/nar/gkh247
- Wallace, A. R. (1865). On the phenomena of variation and geographical distribution as illustrated by the Papilionidae of the Malayan region. *Trans. Linn. Soc.* 25, 1–71. doi: 10.1111/j.1096-3642.1865.tb00178.x
- Yamaguchi, J., Mizoguchi, T., and Fujiwara, H. (2011). siRNAs induce efficient RNAi response in *Bombyx mori* embryos. *PLoS One* 6:e25469. doi: 10.1371/journal.pone.0025469
- Yamamoto, K., Tsujimura, Y., Kometani, M., Kitazawa, C., Islam, A. T., and Yamanaka, A. (2011). Diapause pupal color diphenism induced by temperature and humidity conditions in *Byasa alcinous* (Lepidoptera: Papilionidae). *J. Insect Physiol.* 57, 930–934. doi: 10.1016/j.jinsphys.2011.04.002
- Yamanaka, A., Endo, K., Nishida, H., Kawamura, N., Hatase, Y., Kong, W., et al. (1999). Extraction and partial characterizations of pupal-cuticle-melanizing hormone (PCMH) in the swallowtail butterfly, *Papilio xuthus* L. (Lepidoptera, Papilionidae). *Zool. Sci.* 16, 261–268. doi: 10.2108/zsj.16.261

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2020 Yoda, Otaguro, Nobuta and Fujiwara. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Gene Regulatory Network Homoplasia Underlies Recurrent Sexually Dimorphic Fruit Fly Pigmentation

Jesse T. Hughes¹, Melissa E. Williams¹, Rachel Johnson¹, Sumant Grover¹, Mark Rebeiz^{2*} and Thomas M. Williams^{1,3*}

¹ Department of Biology, University of Dayton, Dayton, OH, United States, ² Department of Biological Sciences, University of Pittsburgh, Pittsburgh, PA, United States, ³ The Integrative Science and Engineering Center, University of Dayton, Dayton, OH, United States

OPEN ACCESS

Edited by:

Ricardo Mallarino,
Princeton University, United States

Reviewed by:

Élio Sucena,
Universidade de Lisboa, Portugal
Berta Verd,
University of Cambridge,
United Kingdom

*Correspondence:

Mark Rebeiz
rebeiz@pitt.edu
Thomas M. Williams
twilliams2@udayton.edu

Specialty section:

This article was submitted to
Evolutionary Developmental Biology,
a section of the journal
Frontiers in Ecology and Evolution

Received: 13 December 2019

Accepted: 12 March 2020

Published: 26 March 2020

Citation:

Hughes JT, Williams ME,
Johnson R, Grover S, Rebeiz M and
Williams TM (2020) Gene Regulatory
Network Homoplasia Underlies
Recurrent Sexually Dimorphic Fruit Fly
Pigmentation. *Front. Ecol. Evol.* 8:80.
doi: 10.3389/fevo.2020.00080

Traits that appear discontinuously along phylogenies may be explained by independent origins (homoplasia) or repeated loss (homology). While discriminating between these models is difficult, the dissection of gene regulatory networks (GRNs) which drive the development of such repeatedly occurring traits can offer a mechanistic window on this fundamental problem. The GRN responsible for the male-specific pattern of *Drosophila* (*D.*) *melanogaster* melanistic tergite pigmentation has received considerable attention. In this system, a metabolic pathway of pigmentation enzyme genes is expressed in spatial and sex-specific (i.e., dimorphic) patterns. The dimorphic expression of several genes is regulated by the Bab transcription factors, which suppress pigmentation enzyme expression in females, by virtue of their high expression in this sex. Here, we analyzed the phylogenetic distribution of species with male-specific pigmentation and show that this dimorphism is phylogenetically widespread among fruit flies. The analysis of pigmentation enzyme gene expression in distantly related dimorphic and monomorphic species shows that dimorphism is driven by the similar deployment of a conserved metabolic pathway. However, sexually dimorphic Bab expression was found only in *D. melanogaster* and its close relatives. These results suggest that dimorphism evolved by parallel deployment of differentiation genes, but was derived through distinct architectures at the level of regulatory genes. This work demonstrates the interplay of constraint and flexibility within evolving GRNs, findings that may foretell the mechanisms of homoplasia more broadly.

Keywords: *Drosophila*, pigmentation, gene regulatory network (GRN), evo-devo (evolution and development), morphological evolution, gene expression, homoplasia, homology

INTRODUCTION

Recurring traits are widespread in nature, suggesting that evolution has predictable solutions to certain ecological challenges (Conway Morris, 2003; Losos, 2017; Blount et al., 2018). This discontinuous presence of similar phenotypes on phylogenies can result from different historical processes. Notably, the trait in question could be ancestral, and repeated loss events could explain

the recurrent absence of the trait (Wiens, 2001). Alternatively, the trait could be derived and evolved independently in multiple lineages, a phenomenon known as homoplasy (Wake et al., 2011). Although methods for ancestral character reconstruction can help discriminate losses from gains, these methods are often inconclusive and are sensitive to estimated differences in the relative rates of trait gain and loss (Cunningham et al., 1998; Joy et al., 2016). Distinguishing between these two scenarios ultimately comes down to whether the genetic processes that build the trait are homologous. Thus, dissecting the individual genetic components underlying trait formation represents the most granular way to determine the elusive historical nature of recurring traits.

For morphological traits, their construction during development is recognized to depend upon precise spatial and temporal patterns of gene expression among the genes within gene regulatory networks (GRNs) (Peter and Davidson, 2011; Rebeiz et al., 2015). Each GRN utilizes numerous regulatory genes, many encoding transcription factors that govern the expression of the differentiation genes that produce the morphological feature. The patterns of expression for genes within any GRN depends upon combinations of transcription factors binding to *cis*-regulatory elements (CREs) that control the activation of target genes. For well-studied traits, dozens to more than hundreds of genes are known to comprise the GRNs (Bonn and Furlong, 2008). In addition to explaining how phenotypes develop, GRNs are the lens through which we can observe important aspects of a phenotype's evolution. Important here, GRNs provide a critical context in which we might be able to detect the presence (common GRNs) or absence (distinct GRNs) of homology relationships (Wagner, 2016). Moreover, the examination of GRNs that govern recurring traits could reveal whether and how similar traits converge at the molecular level.

One of the premiere morphological traits for the study of GRN evolution is the rapidly diverging patterns of body pigmentation in *Drosophila*. Melanic pigmentation is widespread in nature, playing important ecological roles. Pigmentation has many uses for insects, including wound healing, desiccation resistance, thermal regulation, and sexual selection (Majerus, 1998). In fruit flies of the *Drosophila* (*D.*) genus, pigmentation traits are quite diverse (Wittkopp et al., 2003; Werner et al., 2010; Arnoult et al., 2013; Pham et al., 2017). Pigmentation patterns exist on the wings, legs, thorax, and the cuticle plates (known as tergites) that cover the dorsal abdomen surface. Work on *Drosophila* pigmentation traits has advanced rapidly by virtue of the ability to study the GRNs for pigmentation in the highly genetically tractable *Drosophila melanogaster* model system.

D. melanogaster belongs to the *melanogaster* species group within the subgenus *Sophophora* (Markow and O'Grady, 2006). Tergite pigmentation in this species is sexually dimorphic (Kopp et al., 2000), with males possessing fully melanic A5 and A6 tergite pigmentation, whereas female A5 and A6 tergites are only partially pigmented (Figure 1A). Species with similar dimorphic patterns of pigmentation are common among all three clades of the *melanogaster* species group,

while members of more distantly related *Sophophora* groups such as *D. pseudoobscura* and *D. willistoni* bear monomorphic tergite pigmentation patterns (Figure 1A). Bolstered by an ancestral character reconstruction analysis (Jeong et al., 2006), it was inferred that dimorphic pigmentation is a derived trait that evolved in the lineage of the *melanogaster* species group (Rebeiz and Williams, 2017). Additionally, species outside of *Sophophora* are known to have dimorphic patterns of tergite pigmentation that resemble *D. melanogaster* (Gompel and Carroll, 2003). Hence, this system represents a tractable system to study homoplasy at the level of participating GRNs.

The *D. melanogaster* abdominal pigmentation GRN has received considerable attention, which includes a metabolic pathway of differentiation genes whose expression correlates or anti-correlates with the dimorphic phenotype. Among the first acting genes in this pathway are *pale* and *Ddc*, which are expressed monomorphically in the abdominal epidermis (Grover et al., 2018), and their enzyme activities catalyze the production of Dopamine. *ebony* is expressed in a dimorphic pattern, with prominent expression in the female A5 and A6 segments (Rebeiz et al., 2009), and its encoded enzyme converts Dopamine to NBAD that is used to make yellow-colored sclerotin. NBAD can be converted back into Dopamine by the enzyme encoded by *tan*, and converting Dopamine into black Dopamine-melanin requires the activity of the protein encoded by *yellow* (Grover et al., 2018). Both *tan* and *yellow* expression is upregulated in the male A5 and A6 segments (Camino et al., 2015).

The temporal, spatial, and sex-specific expression patterns of the pigmentation pathway genes are achieved by the regulatory genes of the GRN (Figure 1B). Two key regulators are *bab1* and *bab2*, collectively referred to as *bab*, which encode the Bab1 and Bab2 transcription factor proteins, which function as dominant repressors of black pigmentation (Kopp et al., 2000; Couderc et al., 2002; Roeske et al., 2018). In *D. melanogaster* and species of all three clades of the *melanogaster* species group, the Bab1 and Bab2 proteins are expressed in a dimorphic pattern. Expression in the abdominal epidermis of males is downregulated, while expression can be observed throughout the A2–A6 segments of females (Salomone et al., 2013). One key function of Bab is to directly bind to the body element CRE of the *yellow* gene and thereby repress *yellow* expression in the female A5 and A6 segments (Roeske et al., 2018). Bab additionally represses *tan* expression in females, though the mechanism of action remains unknown. A previous study showed that Bab2 expression is broadly downregulated in the abdomen epidermis underlying melanic tergite regions during early pupal development, including distantly related species with dimorphic pigmentation (Gompel and Carroll, 2003). These results were interpreted to indicate that Bab has a long history as a suppressor of melanic pigmentation, and thus its expression might generally evolve to shape diverse patterns of tergite coloration.

Here, we performed an expanded survey of tergite pigmentation phenotypes in the *Drosophila* genus (Figure 2). Our results draw attention to how dimorphic pigmentation

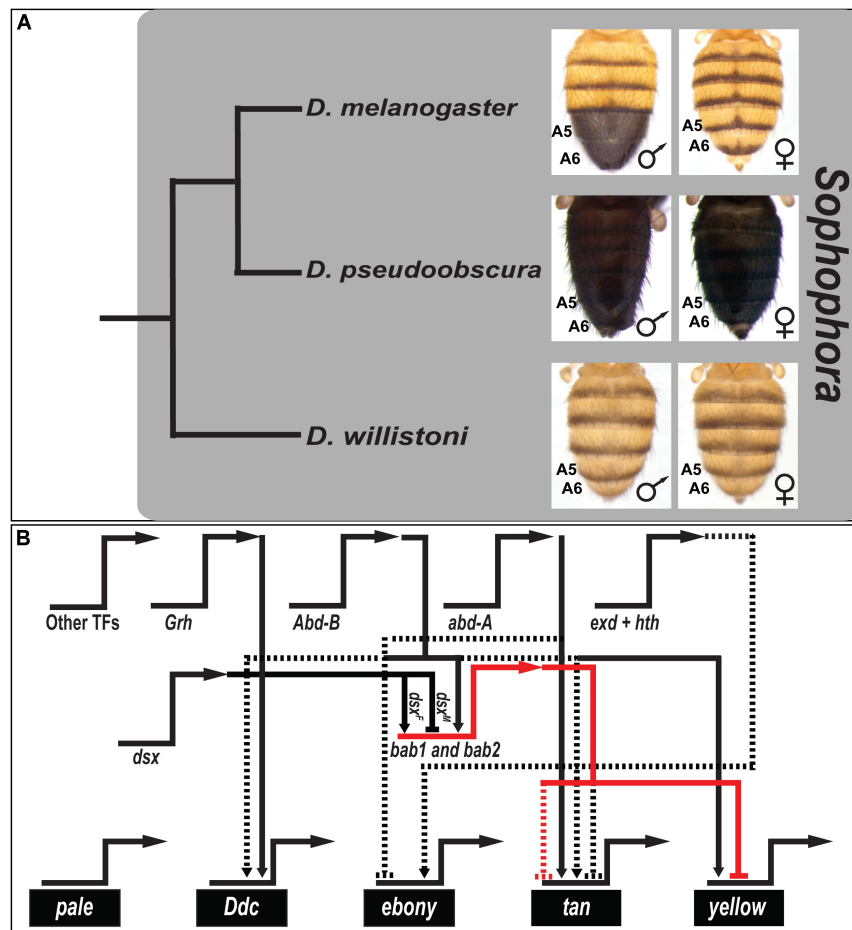


FIGURE 1 | The canonical model for the origin of sexually dimorphic abdomen pigmentation of *D. melanogaster*, and its underlying gene regulatory network. **(A)** Phylogenetic relationship of model species of the *Sophophora* subgenus. Here, monomorphism is presumed to be ancestral, and dimorphism derived. **(B)** The current understanding of the gene regulatory network for the *D. melanogaster* phenotype has key transcription factor genes (regulatory genes) driving the expression of genes that encode proteins that participate in a pigment metabolic pathway (differentiation genes). The horizontal-pointing arrows represent expression occurring from the named loci. Key nodes within this network drive the male-limited expression of the *tan* and *yellow* genes, whose expression in females is repressed by the activity of the *bab1* and *bab2* transcription factor genes. Solid connectors between genes represent known direct interactions between the encoded transcription factor and a binding site(s) in the target gene's *cis*-regulatory element. Dashed lines represent regulation not confirmed as direct. Activation is indicated by connectors terminating with an arrowhead, and repression as a nail head shape.

exists in most *Sophophora* species groups, but is also common outside this subgenus. To determine whether processes proximal to pigmentation phenotypes are generated by shared or divergent enzyme pathway uses, we characterized the expression patterns of known *D. melanogaster* pigmentation pathway genes in phylogenetically disparate cases of dimorphism. This revealed how dimorphic pigmentation evolved through the formation of GRNs that similarly deploy a conserved pathway of pigmentation genes. In contrast, analysis of *Bab1* expression revealed that dimorphism in these similarly implemented pathways is mediated by different regulatory genes. We suggest that dimorphic pigmentation is indeed a product of parallel evolution in *Drosophila*, and provide an example where constraint exists at the level of the differentiation genes in contrast to the regulatory tier of this GRN.

MATERIALS AND METHODS

Fly Stocks

Fly stocks were maintained at 25°C on a sugar food medium (Salomone et al., 2013). Species stocks used in this study were *D. melanogaster* (14021-0231.04), *D. willistoni* (14030-08 11.24), *D. affinis* (14012-0141.09), *D. algonquin* (14012-0161.04a), *D. azteca* (14012.0171.08), *D. persimilis* (14011-0111.00), *D. miranda* (14011-0101.08), *D. ambigua* (14013-1011.00), *D. bifasciata* (14012-0181.02), *D. guanche* (14011-0095.01), *D. sturtevantii* (14043-0871.07), *D. nebulosa* (14030-0761.03), *D. milleri* (14043-0861.00), *D. saltans* (14043-0871.01), *D. lusaltans* (14045-0891.00), *D. prosaltans* (14045-0901.02), *D. emarginata* (14042-0841.09), *D. neocordata* (14041-0831.00), *D. tropicalis* (14030-0801.00), *D. paulistorum*

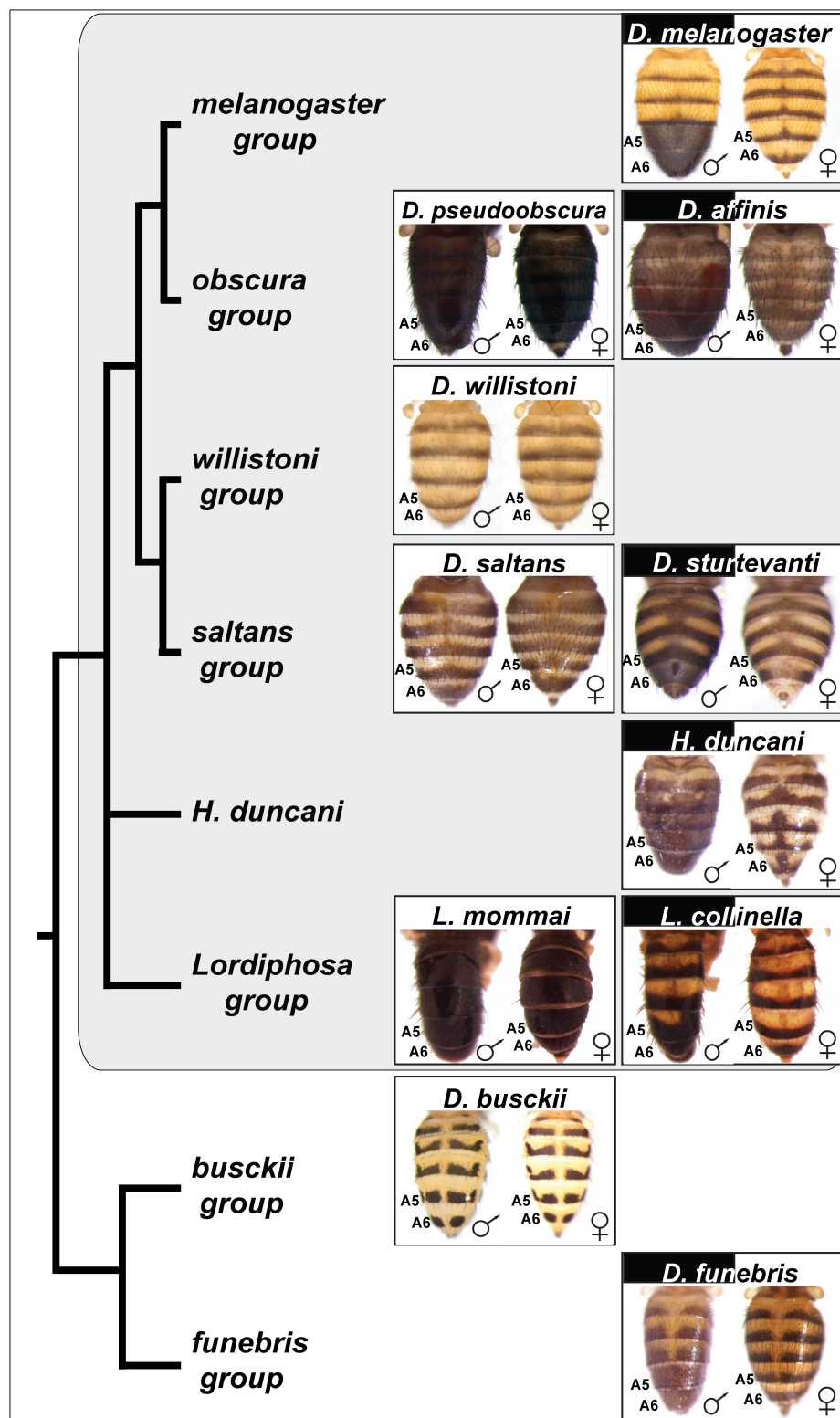


FIGURE 2 | Species with dimorphic tergite pigmentation are widespread throughout the *Drosophila* genus. *Sophophora* subgenus species groups and species are indicated by the gray background. *D. busckii* and *D. funebris* are included as non-*Sophophora* species from the *Drosophila* genus that respectively exhibit monomorphic and dimorphic patterns of tergite pigmentation. The homologous A5 and A6 segment tergites are indicated for each species, the segments bearing the dimorphic pigmentation in *D. melanogaster*. While the *obscura*, *saltans*, and *Lordiphosa* groups are predominately populated by monomorphic species (e.g., *D. pseudoobscura*, *D. saltans*, and *L. mommai*), they possess a few dimorphic species, including *D. affinis*, *D. sturtevantii*, and *L. collinella*.

(14030-0771.06), *D. equinoxialis* (14030-0741.00), *D. sucinea* (14030-0791.00), and *H. duncani* (92000-0075.00) were obtained from the National Drosophila Species Stock Center. *D. capricorni*, *D. fumipennis*, *D. obscura*, *D. pseudoobscura*, *D. funebris*, and *D. busckii* were obtained from the lab of Dr. Sean B. Carroll. *L. collinella* and *L. mommai* specimens were obtained from Dr. Masanori J. Toda.

In situ Hybridization

In situ hybridization was performed as described previously in greater detail (Jeong et al., 2008). In brief, Digoxigenin labeled RNA probes for *pale*, *Ddc*, *ebony*, *tan*, and *yellow* were prepared through *in vitro* transcription of species-specific PCR templates amplified from genomic DNA (PCR primers listed in **Supplementary Table S1**). Dorsal abdomens were dissected at various pupal developmental stages between P10 and P15ii (P15ii being newly eclosed adults). Stages were identified by the presence of various morphological markers (Ashburner et al., 2005; Grover et al., 2018) (**Supplementary Figure S1**). Male and female samples were pooled, and females were distinguished by the removal of their wings. All following steps were done with the male and female samples of the same stages together in the same tubes or plate and thereby experiencing identical conditions. Probe hybridizations were detected using an anti-digoxigenin antibody (Roche Diagnostics) and visualized by alkaline phosphatase color reaction using BCIP/NBT (Promega). Samples were allowed to stain in the dark, and once the staining reactions were stopped, the specimens were transferred to glycerol mountant (80% glycerol and 100 mM Tris pH 8) before being placed between a slide and coverslip for imaging.

Immunohistochemistry

Dorsal abdomens were dissected from pupae at the P10 and P14-15i developmental stages (Ashburner et al., 2005; Grover et al., 2018) (**Supplementary Figure S1**). Male and female samples were pooled, and females were distinguished by the removal of their wings. All following steps were done with the male and female samples of the same stages together in the same tubes or plate and thereby experiencing identical conditions. Samples were fixed for 35 min in PBST solution (phosphate buffered saline with 0.3% Triton X-100) that additionally contained 4% paraformaldehyde (Electron Microscopy Services). Following fixation, samples were washed twice with PBST and then blocked for 1 h at room temperature in blocking solution (PBST and 1% Bovine Serum Albumin). The abdomen specimens were then incubated overnight at 4°C with rabbit anti-Bab1 primary antibody (Williams et al., 2008) at a 1:200 dilution in PBST. Following four washes with PBST and then 1 h in blocking solution, specimens were incubated with goat anti-rabbit Alexa Fluor 647 (Invitrogen) secondary antibody at a dilution of 1:500 in PBST. After four washes with PBST, samples were incubated for ten minutes at room temperature in Glycerol Mount:PBST (50:50) solution. Samples were then transferred to glycerol mount before finally being placed between a glass cover slip and slide for imaging with a confocal microscope. Although we were unable to acquire a Bab2 antibody, we found in a previous study that Bab1 and Bab2 expression are indistinguishable in

the abdominal epidermis in a variety of species (Salomone et al., 2013), and these paralogs are suspected to be under the regulatory control of the same CREs (Williams et al., 2008). Therefore, the Bab1 expression shown here is anticipated to reflect Bab2 expression as well.

Microscopic Imaging of Fly Abdomens

Images for the specimens taken through the immunohistochemistry protocol, to visualize Bab1 expression in pupal dorsal abdominal tissue, were obtained using an Olympus FV3000 confocal microscope and FV31S-SW imaging software. Samples were imaged with microscope settings as follows: 10% laser power (647 laser), HV between 650 and 700, offset equal to 1, gain equal to 1, aperture set at 180 microns, Z-series step size of 5 microns, and Kalman line averaging set to 2.

Images of adult fruit fly abdomen pigmentation patterns (between two and four days old) and *in situ* hybridization specimens, were obtained using an Olympus SZX16 zoom stereoscope, running the Olympus cellSens Standard 2.2 software package, with a mounted DP72 digital camera. All samples were imaged at 63X magnification with a 1X objective lens.

Processing Images

Confocal projection images and stereoscope images were exported in TIFF, and processed with the consistent processing steps in Adobe Photoshop CS3. Figures were assembled by the use of Adobe Illustrator CS3. The Image J program (Abramoff et al., 2004) was used to measure pixel intensity from Bab1 immunohistochemistry images. Similar epidermal regions of the A5 segment were selected that lack confounding expression from muscle and oenocyte cells. For each species, the pixel intensity values were measured for three separate male and female specimens. Mean pixel intensity values were calculated, and differences between the male and female means were evaluated by a two-sided t-test.

RESULTS

Sexually Dimorphic Pigmentation Is Widespread Within the *Sophophora* Subgenus and Is Found Elsewhere in the *Drosophila* Genus

Previous phylogenetic analyses of the origin of *D. melanogaster* male-specific A5 and A6 tergite pigmentation suggested that monomorphism is the ancestral character state in the *Sophophora* subgenus, and dimorphism was derived. The origin of this trait was suspected to have occurred in the lineage of *D. melanogaster* at some point following its split from the lineage of *D. pseudoobscura* of the *obscura* species group (**Figure 1**; Jeong et al., 2006; Rebeiz and Williams, 2017). This conclusion was derived from a limited sampling of *Sophophora* species diversity (**Figure 2A**), including only a single taxon from the *saltans* and *willistoni* groups.

The *melanogaster* species group has been well characterized and includes a preponderance of species that possess a

male-specific pattern of tergite pigmentation (Kopp et al., 2000; Jeong et al., 2006). This suggests that the common ancestor for this group possessed the dimorphic trait. The *obscura* group is most closely related to the *melanogaster* group. Males and females of *D. pseudoobscura* have a similar monomorphic pattern of melanic abdominal tergites. To see whether such monomorphism is typical of this species group, we inspected the coloration phenotypes of another nine of its member species (Supplementary Figure S2). Although monomorphic melanic tergite color was the most common phenotype, we observed male-limited pigmentation phenotypes in *D. affinis* (Figure 2) and *D. algonquin* (Supplementary Figure S2).

More distantly related to the *melanogaster* species group are both the *saltans* and *willistoni* groups (Figure 2) (Markow and O'Grady, 2006). Their charter species, *D. saltans* and *D. willistoni*, are characterized by monomorphic patterns of tergite pigmentation (Supplementary Figures S3, S4). To see whether such monomorphism is typical of these groups, we inspected the coloration phenotypes of another six species from the *saltans* group (Supplementary Figure S3) and seven species from the *willistoni* group (Supplementary Figure S4). Although monomorphic non-melanic tergite color was the most common phenotype among *saltans* species (Supplementary Figure S3) (De Magalhaes, 1956), we observed male-limited pigmentation phenotypes for *D. sturtevantii* (Figure 2) and *D. emarginata* (Supplementary Figure S3). Within the *willistoni* species group, all species analyzed (Supplementary Figure S4) or reported in the literature (Zanini et al., 2015) exhibit a monomorphic non-melanic tergite color.

Phylogenetic studies have expanded the number of species and lineages within *Sophophora*. One study supported a topology that places *Hirtodrosophila* (*H.*) *duncani* as an outgroup to the clade containing *melanogaster*, *obscura*, *saltans*, and *willistoni* groups (van der Linde et al., 2010). This inclusion in the *Sophophora* subgenus is consistent with findings that *H. duncani* genital morphology is most similar to species of the *obscura* group (Nater, 1950, 1953), and has been considered close to or within *Sophophora* in other analyses (Throckmorton, 1962). *H. duncani* is a species with an extensive male-limited pattern of melanic tergite pigmentation (Gompel and Carroll, 2003), including the A5 and A6 tergites (Figure 2). Phylogenetic studies have supported a branching structure that places species of the *Lordiphosa* genus within *Sophophora* (Figure 2; Hu and Toda, 2001; Gao et al., 2011). We inspected the coloration phenotypes of seven species from the *Lordiphosa* group phylogeny (Supplementary Figure S5) (personal communication from Dr. Masanori J. Toda) (Gao et al., 2011; Katoh et al., 2018). Although monomorphic non-melanic tergite color was the prevalent common phenotype, we observed a modest male-limited pigmentation phenotype for *L. collinella* on the A6 tergite (Figure 2 and Supplementary Figure S5). Elsewhere, four additional species have been recently described as having monomorphic patterns of tergite coloration (Fartyal et al., 2017).

Outside of the *Sophophora* subgenus exist a wealth of species with monomorphic tergite phenotypes, such as *D. busckii*

(Figure 2). However, dimorphic species can be found among the diverse lineages, including *D. funebris* of the *funebris* group (Figure 2). This species possesses male-limited patterns of tergite coloration that include the A5 and A6 tergites. Thus, from this exploration of species representing diverse branches of the *Sophophora* subgenus and *Drosophila* genus, it is apparent that male-limited tergite pigmentation is widespread. This raises the question regarding whether the dimorphic trait was ancestral, or evolved on independent occasions.

Sexually Dimorphic and Monomorphic Deployments of a Pigment Metabolic Pathway

In order to discern how *D. melanogaster* develops the robust male-specific melanic pigmentation of the A5 and A6 segment tergites (Figure 2), we contrasted the expression of its core pigment metabolic pathway genes to the orthologs in the monomorphic *D. willistoni* (Figure 3). To form black melanin or yellow-colored sclerotin from this pathway, Tyrosine is first converted to Dopa by the activity of the enzyme Pale, then Dopa is converted to Dopamine by the activity of Ddc (Wright, 1987). In *D. melanogaster*, *pale* and *Ddc* are expressed robustly during pupal development, and in patterns that appear monomorphic (Figures 3A,A',B,B'). In *D. willistoni*, *pale* expression appears to be similarly robust, though *Ddc* expression was less pronounced as revealed by *in situ* hybridization (Figures 3H,H',I,I').

Dopamine can be converted to NBAD by the activity of Ebony, which provides the substrate to make the more yellow-colored sclerotin (Hovemann et al., 1998). In *D. melanogaster*, *ebony* expression occurs in a pattern that demarcates where the yellow-colored regions of the tergites will form. *ebony* expression is dimorphic, as it is absent from the male A5 and A6 segments (Figures 3C,C'). In *D. willistoni*, *ebony* expression is similarly robust, though monomorphic in a pattern that mirrors its tergite color phenotype (Figures 3J,J'). To facilitate the production of black melanin, NBAD can be converted back into Dopamine by the enzyme Tan (True et al., 2005), and then converted into Dopamine melanin through the involvement of Yellow. In *D. melanogaster*, both *tan* and *yellow* expression is upregulated in the male A5 and A6 segment epidermis to promote the final development of black tergites (Figures 3D,D',E,E'). In contrast, *tan* and *yellow* expression is modest and monomorphic in *D. willistoni* (Figures 3K,K',L,L').

Our gene expression comparisons of the pigmentation pathway genes of *D. melanogaster* and *D. willistoni* revealed apparent expression differences across this conserved pathway that we would have reasonably predicted based upon phenotype alone (Figures 3G,N). We were curious whether other distantly related species with dimorphic or monomorphic tergite color phenotypes would show predictable patterns of pathway deployment.

Pigment Metabolic Pathway Utilization Across the *Sophophora* Subgenus

The *obscura* group is the species group most closely related to that of the *melanogaster* group (Figure 2). This group is

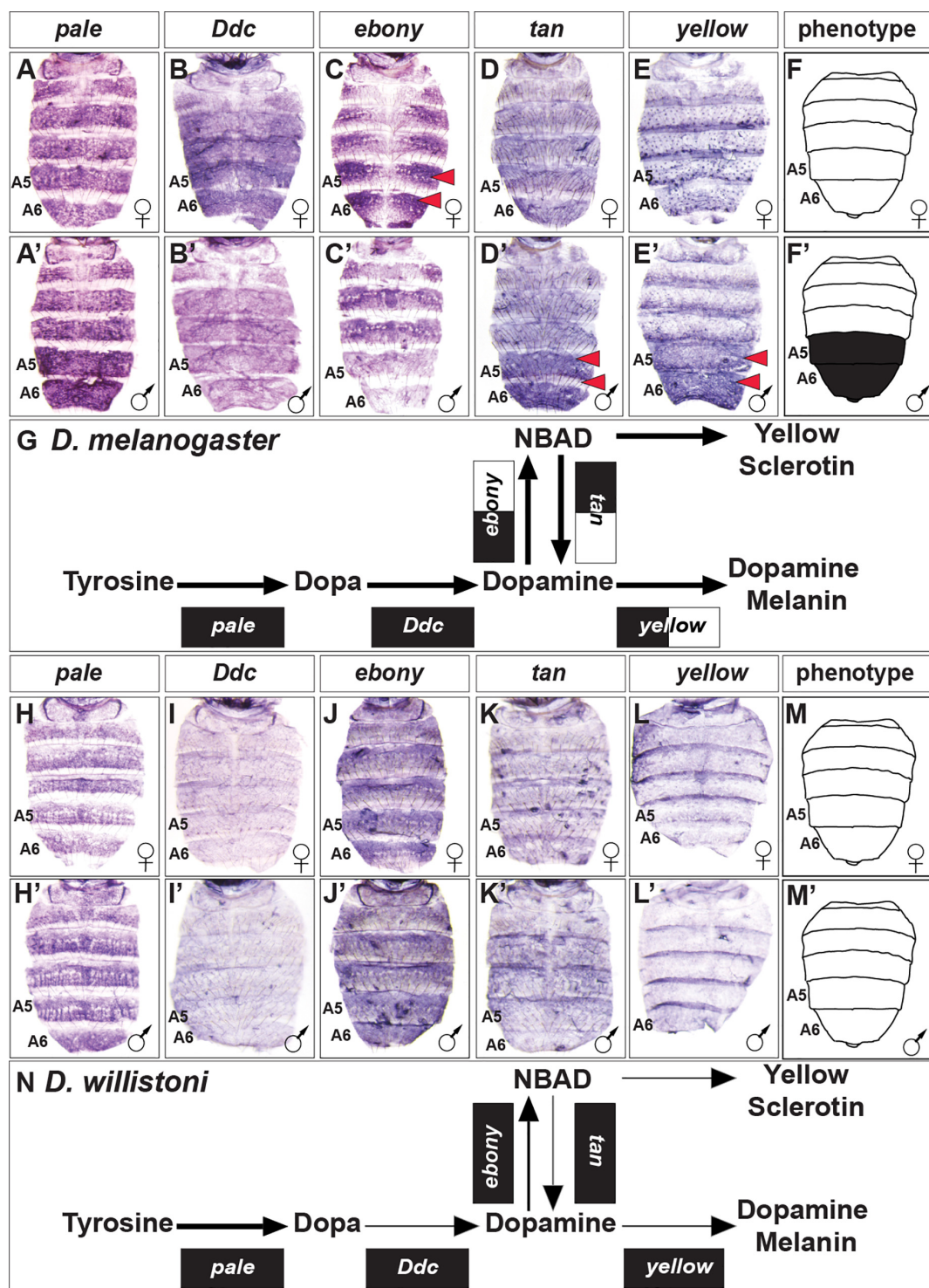


FIGURE 3 | Contrast in the pigment metabolic pathway utilization between the dimorphic *D. melanogaster* and the monomorphic *D. willistoni*. (A–E) Female and (A'–E') male expressions of *D. melanogaster* pigment metabolic pathway genes, and (F,F') cartoon representation of the pigmentation phenotype. (G) Summary of the *D. melanogaster* pathway use includes robust expression of all genes, with dimorphic expressions of *ebony*, *tan*, and *yellow*. (H–L) Female and (H'–L') male expressions of *D. willistoni* pigment metabolic pathway genes, and (M,M') cartoon of the pigmentation phenotype. (N) Summary of the *D. willistoni* pathway use includes modest and monomorphic expression of most genes. (A,A',H,H') *pale*, (B,B',I,I') *Ddc*, (C,C',J,J') *ebony*, (D,D',K,K') *tan*, and (E,E',L,L') *yellow*. Red arrowheads indicate robust patterns of dimorphic expression in the A5 and A6 segment epidermis. (G,N) Black arrow thickness represents the level of expression for the underlying gene. Monomorphic gene expression is represented by the gene names being placed upon a black rectangle. Dimorphic gene expression is represented by the gene names being placed upon a black and white colored rectangle.

predominately populated by species with broadly melanic and monomorphic pigmentation, albeit with two species for which pigmentation is sexually dimorphic (**Supplementary Figure S2**). We investigated the expression of the pigmentation pathway genes for the dimorphic species *D. affinis* and the monomorphic melanic species *D. pseudoobscura* (**Figure 4**). The dimorphic pigmentation patterns differ somewhat between *D. melanogaster* and *D. affinis*, the latter displaying broad tergite pigmentation that extends to the male A4 and A3 segment tergites (**Figure 2**). Among the pigmentation pathway genes of *D. affinis*, *pale*, and *Ddc* are expressed monomorphically in males and females (**Figures 4A,A',B,B'**). Similar to *D. melanogaster*, *D. affinis* expresses *ebony* and *yellow* in sex-specific patterns. Here, *ebony* is upregulated in the female abdomen consistent with their yellow-colored anterior tergite regions (**Figures 4C,C'**), and *yellow* is upregulated in males, prefiguring their melanic color (**Figures 4E,E'**). In contrast to *D. melanogaster*, *D. affinis tan* expression appears modest in level and monomorphic (**Figures 4D,D'**).

While *D. pseudoobscura* can be considered a monomorphic species with regards to its pigmentation, this species' tergites are melanic rather than the light yellow-brown color of *D. willistoni* (**Figure 1**). This broadly melanic phenotype is associated with monomorphic adjustments to the expression of pigmentation pathway genes. We found all genes to be similarly expressed between males and females (**Figures 4H–L,H'–L'**). The melanic coloration appears to be shaped by reduced levels of *ebony* and *tan* expression (**Figures 4J,J',K,K'**) and elevated expression of *yellow* (**Figures 4L,L'**).

H. duncani is a distant relative of *D. melanogaster* within *Sophophora* (van der Linde et al., 2010), which exhibits a striking male-specific pattern of melanic pigmentation on the A5 and A6 tergites, and this dimorphism extends to a lesser extent to the A4 and A3 tergites (**Figure 2**). Similar to *D. melanogaster* and *D. affinis*, *pale* expression is robust and monomorphic (**Figures 5A,A'**), *ebony* is upregulated in the female abdomen in the epidermis regions underlying where the yellow cuticle forms (**Figures 5C,C'**), and *tan* (**Figures 5D,D'**) and *yellow* (**Figures 5E,E'**) are upregulated in the male abdominal epidermis of segments A3–A6. One conspicuous difference with the *H. duncani* pigment metabolic pathway is the apparent upregulation of *Ddc* in the male A5 and A6 segments (**Figures 5B,B'**).

Overall, this comparison reveals how the dimorphism of *D. affinis* and *H. duncani* involves a similar deployment of the pigmentation pathway genes (**Figures 4G, 5G**) compared to *D. melanogaster* (**Figure 3G**), and how a related species develops broadly melanic and monomorphic tergite coloration through the modified use of this same metabolic pathway (**Figure 4N**). These trends in expression raise the suspicion that we can generally predict the patterns of expression for the pigmentation pathway more broadly among *Sophophora*. However, whether this predictability extends to more distantly related non-*Sophophora* species was the next question we sought to address.

Parallel Patterns of Pigment Metabolic Pathway Utilization Colors Abdomens Across the *Drosophila* Genus

To gain a perspective of pigment metabolic pathway gene expression outside of *Sophophora*, we focused our attention on the dimorphic species *D. funebris*, and the monomorphic species *D. busckii* (**Figure 2**). *D. funebris* exhibits a conspicuous male-specific pattern of melanic pigmentation on the A5 and A6 tergites, and this dimorphism extends to a lesser extent to the A4 and A3 tergites (**Figure 2**). Similar to *D. melanogaster* and *D. affinis*, *pale* and *Ddc* expression are monomorphic (**Figures 6A,A',B,B'**), and *ebony* is upregulated in the female abdomen in the epidermis regions underlying where the non-melanic cuticle forms (**Figures 6C,C'**). *tan* (**Figures 6D,D'**) and more prominently *yellow* (**Figures 6E,E'**) are upregulated in the male abdominal epidermis of segments A3–A6. The monomorphic *D. busckii* pigment metabolic pathway genes are expressed in patterns similar to orthologs from the monomorphic *D. willistoni* (Compare **Figure 6** to **Figure 3**). *D. busckii* possesses melanic interrupted stripes along the posterior region of the tergites (**Figure 2**). Interestingly, each of the five pigmentation genes were expressed in patterns that correlate (or anti-correlate in the case of *ebony*) with these stripes (**Figures 6H–L,H'–L'**).

The patterns of pigmentation pathway deployment in the abdomen epidermis of these outgroup *Drosophila* species (**Figures 6F,L**) reinforce the impression that stereotypic patterns of gene expression evolved to mediate monomorphic and dimorphic tergite color patterns. We were curious whether such similarities extend to the level of transcription factors within this gene regulatory network. Thus, we examined the Bab1 transcription factor that plays an essential role in shaping dimorphism in *D. melanogaster*.

Dimorphic Bab1 Expression Is Limited to *D. melanogaster* and Its Close Relatives

The sexually dimorphic expression of the Bab1 and Bab2 transcription factors is an essential feature of the GRN shaping the male-limited tergite pigmentation of *D. melanogaster* (Roeske et al., 2018). This dimorphism extends broadly among species of the *melanogaster* group, stimulating the interpretation that dimorphic Bab expression existed in the most recent common ancestor of this group (Kopp et al., 2000; Salomone et al., 2013). Here, we explored the expression of Bab1 in more distantly related species, including several with dimorphic tergite phenotypes, to investigate whether this is an ancestral feature of dimorphic pigmentation GRNs (**Figures 7, 8, and Supplementary Figures S6–S17**). To be consistent with a previous study, we first assessed Bab1 expression at the P14–P15i stage of pupal development (~85–88 h after puparium formation or hAPF) (Salomone et al., 2013). During this stage, Bab1 expression is highly reduced in the dorsal epidermis of *D. melanogaster* males compared to females (**Figures 7A,B'** and **Supplementary Figure S6**). This time point is concurrent with the regulation of *yellow*, which is a direct Bab target (Roeske et al., 2018), and just after *tan* expression initiated. In contrast,

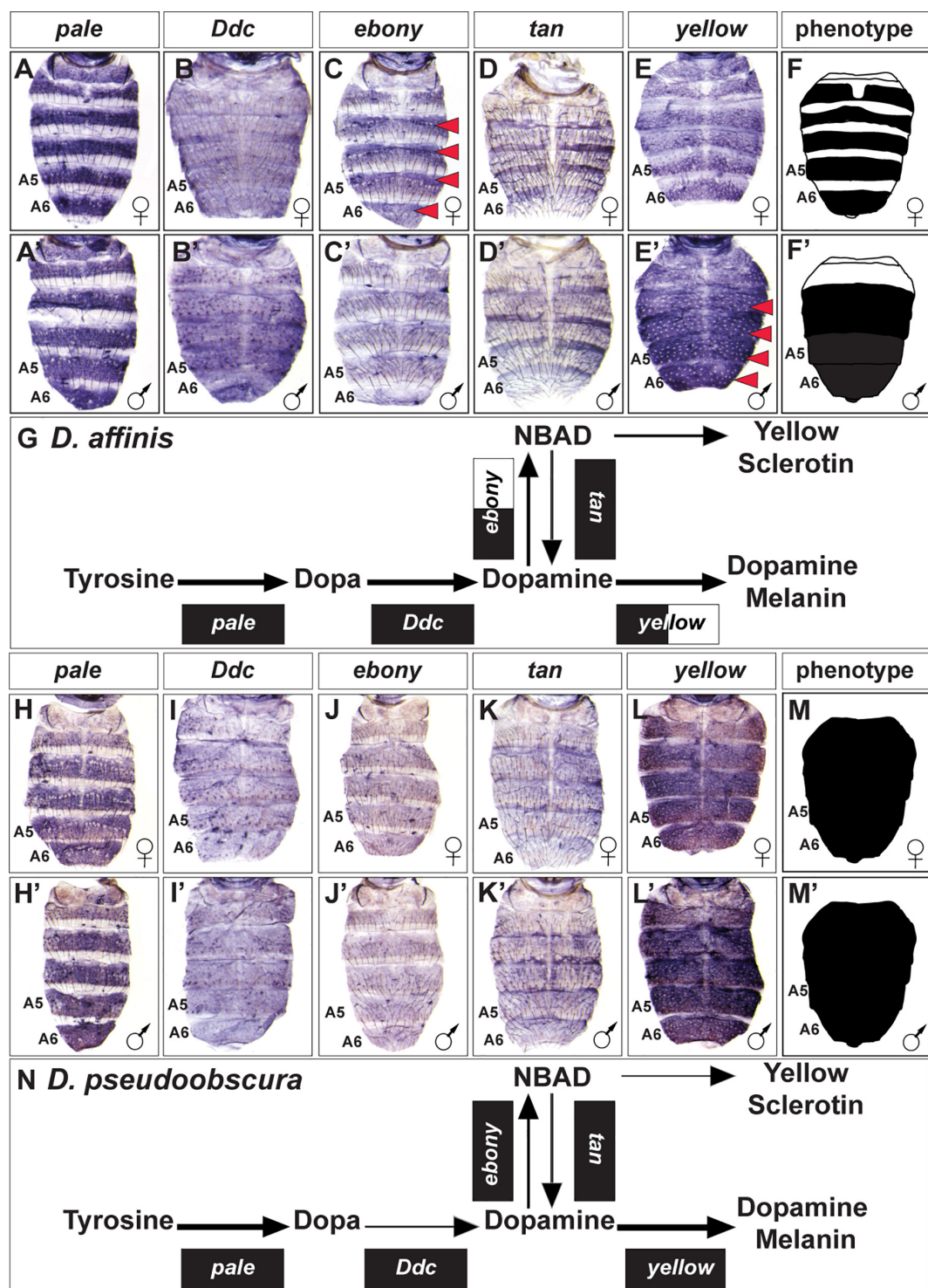


FIGURE 4 | Contrast in the pigment metabolic pathway utilization between the dimorphic *D. affinis* and the monomorphic *D. pseudoobscura*. (A–E) Female and (A'–E') male expressions of *D. affinis* pigment metabolic pathway genes, and (F,F') cartoon representation of the pigmentation phenotype. (G) Summary of the *D. affinis* pathway use includes robust expression of all genes, with dimorphic expressions of *ebony*, and *yellow*. (H–L) Female and (H'–L') male expressions of *D. pseudoobscura* pigment metabolic pathway genes, and (M,M') cartoon representation of the pigmentation phenotype. (N) Summary of the *D. pseudoobscura* pathway use includes monomorphic expression of all genes. (A,A',H,H') *pale*, (B,B',I,I') *Ddc*, (C,C',J,J') *ebony*, (D,D',K,K') *tan*, and (E,E',L,L') *yellow*. Red arrowheads indicate robust patterns of dimorphic expression in the A3–A6 segment epidermis. (G,N) Black arrow thickness represents the level of expression for the underlying gene. Monomorphic gene expression is represented by the gene names being placed upon a black rectangle. Dimorphic gene expression is represented by the gene names being placed upon a black and white colored rectangle.

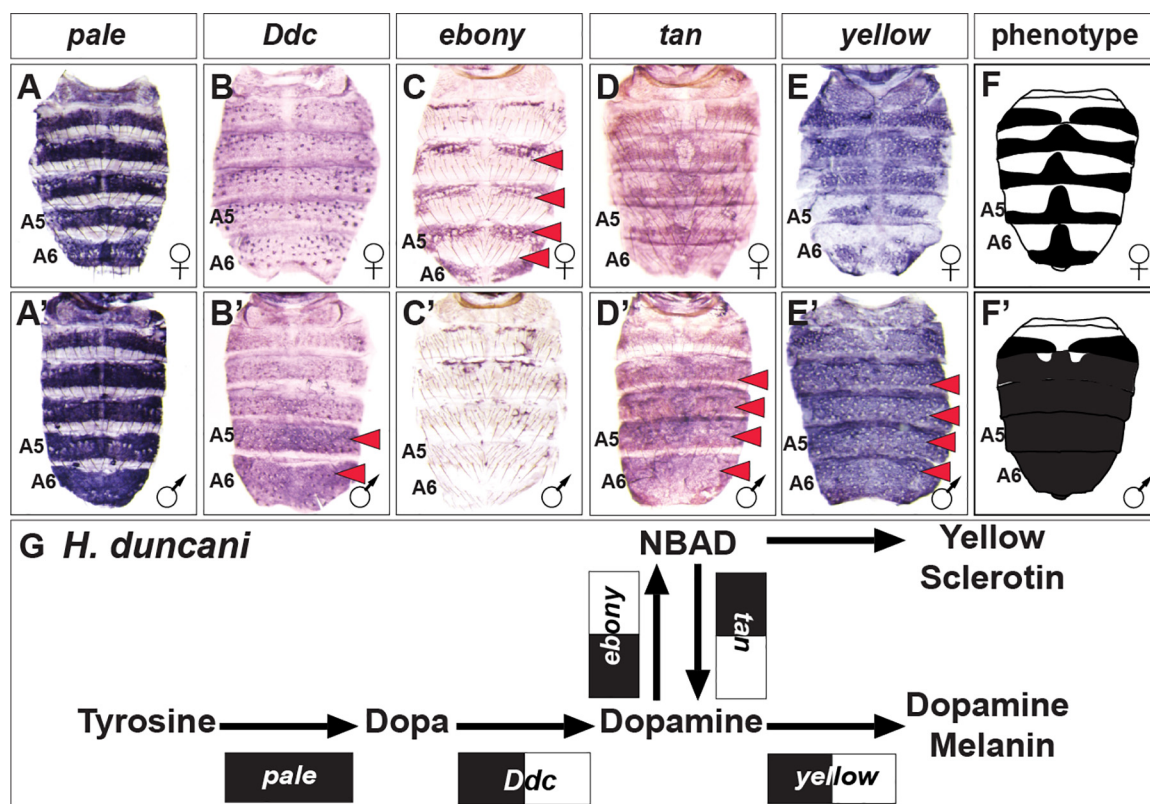


FIGURE 5 | The pigment metabolic pathway utilization for the dimorphic *H. duncani*. (A–E) Female and (A'–E') male expressions of *H. duncani* pigment metabolic pathway genes, and (F,F') cartoon representation of the pigmentation phenotype. (G) Summary of the *H. duncani* pathway use includes robust expression of all genes, with dimorphic expressions of *Ddc*, *ebony*, *tan*, and *yellow*. (A,A') *pale*, (B,B') *Ddc*, (C,C') *ebony*, (D,D') *tan*, and (E,E') *yellow*. Red arrowheads indicate robust patterns of dimorphic expression in the dorsal abdominal epidermis. (G) Black arrow thickness represents the level of expression for the underlying gene. Monomorphic gene expression is represented by the gene names being placed upon a black rectangle. Dimorphic gene expression is represented by the gene names being placed upon a black and white colored rectangle.

Bab1 expression is monomorphic in *D. affinis* (Figures 7C,D' and Supplementary Figure S7), and *D. pseudoobscura* (Figures 7E,F' and Supplementary Figure S8). This suggested that despite the dimorphic pigmentation of *D. affinis*, dimorphism in pigmentation genes is achieved through a different regulatory mechanism.

We next analyzed Bab1 expression in species with either dimorphic or monomorphic tergite pigmentation that are more distantly related to *D. melanogaster*. *D. sturtevantii* of the *saltans* group is one such dimorphic species; however, Bab1 expression was found to be monomorphic (Figures 7G,H' and Supplementary Figure S9). This monomorphic expression is comparable to that observed for the monomorphically pigmented *D. willistoni* of the *willistoni* group (Figures 7I,J' and Supplementary Figure S10). While *H. duncani* is distantly related to *D. melanogaster* within *Sophophora*, this species possesses a comparable male-specific pattern of tergite pigmentation (Figure 2). For this species, Bab1 expression is monomorphic at the P14–P15i stage (Figures 7K,L' and Supplementary Figure S11).

To see whether dimorphic Bab expression might occur outside the *Sophophora* subgenus, we investigated Bab1 expression

in the monomorphically pigmented *D. busckii*, and the dimorphically pigmented *D. funebris* (Figure 2). At the P14–P15i developmental stage, monomorphic expression was observed in both species (Figures 7M,N',O',P' and Supplementary Figures S12, S13). These results suggest that dimorphic Bab expression is limited to the *melanogaster* species group at this stage which has been shown to be critical for pigment formation (Salomone et al., 2013).

We were concerned that the widespread observation of monomorphic Bab1 expression outside of the *melanogaster* group was due to the late developmental stage that we assessed. Thus, we investigated Bab1 expression at the P10 stage (Figure 8 and Supplementary Figures S6–S17), which corresponds to ~65 hAPF in *D. melanogaster*. This is the stage that coincides with the initiation of male-specific *yellow* in *D. melanogaster* through the activity of a CRE that is directly repressed by Bab in females (Roeske et al., 2018), and when Bab expression has been shown to be relevant to the phenotype (Salomone et al., 2013). With the exception of *D. melanogaster* (Figures 8A,B' and Supplementary Figure S6) and perhaps *D. pseudoobscura* to a lesser extent (Figures 8E,F' and Supplementary Figure S8), Bab1 expression was found to be monomorphic in the

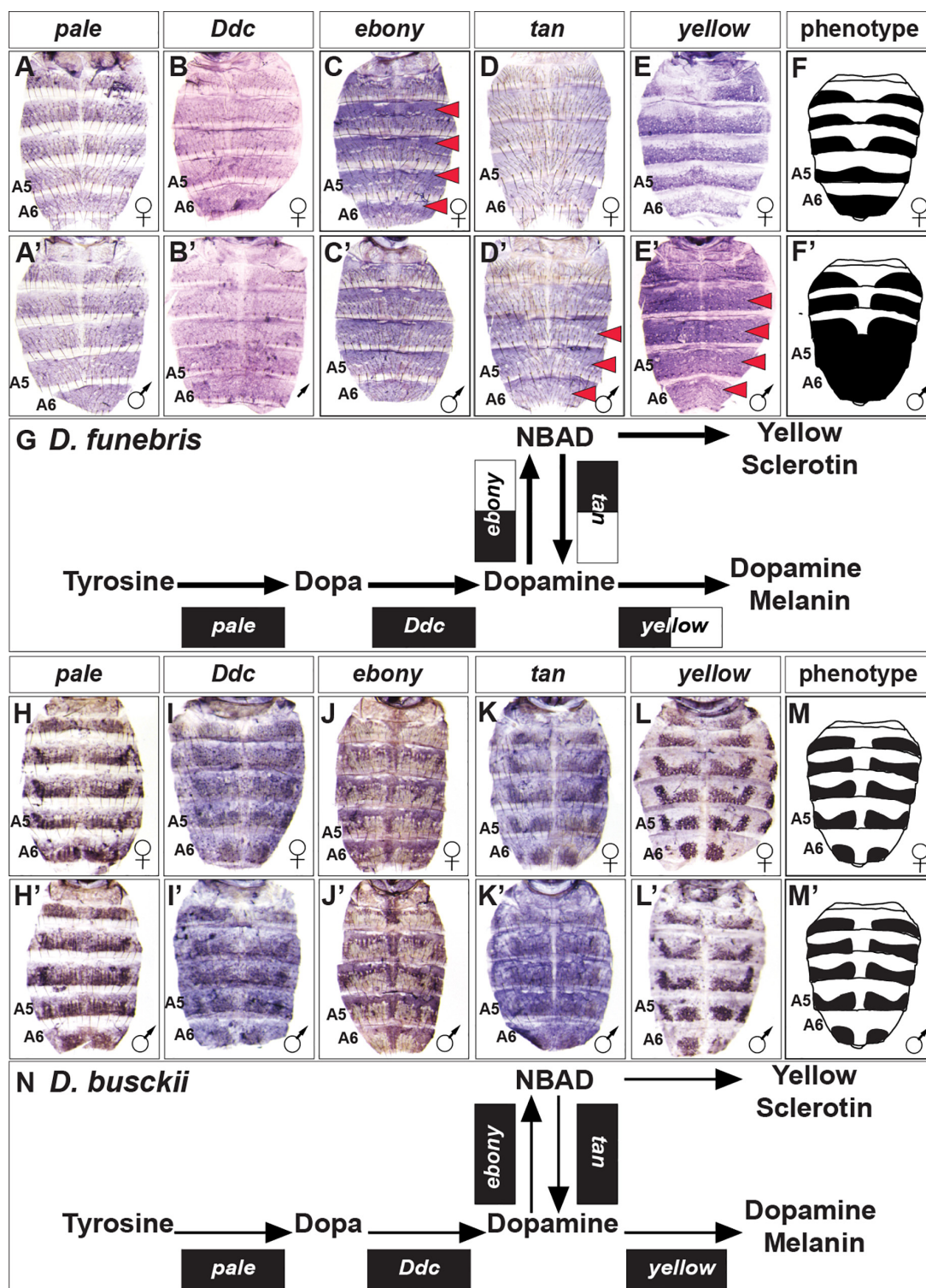
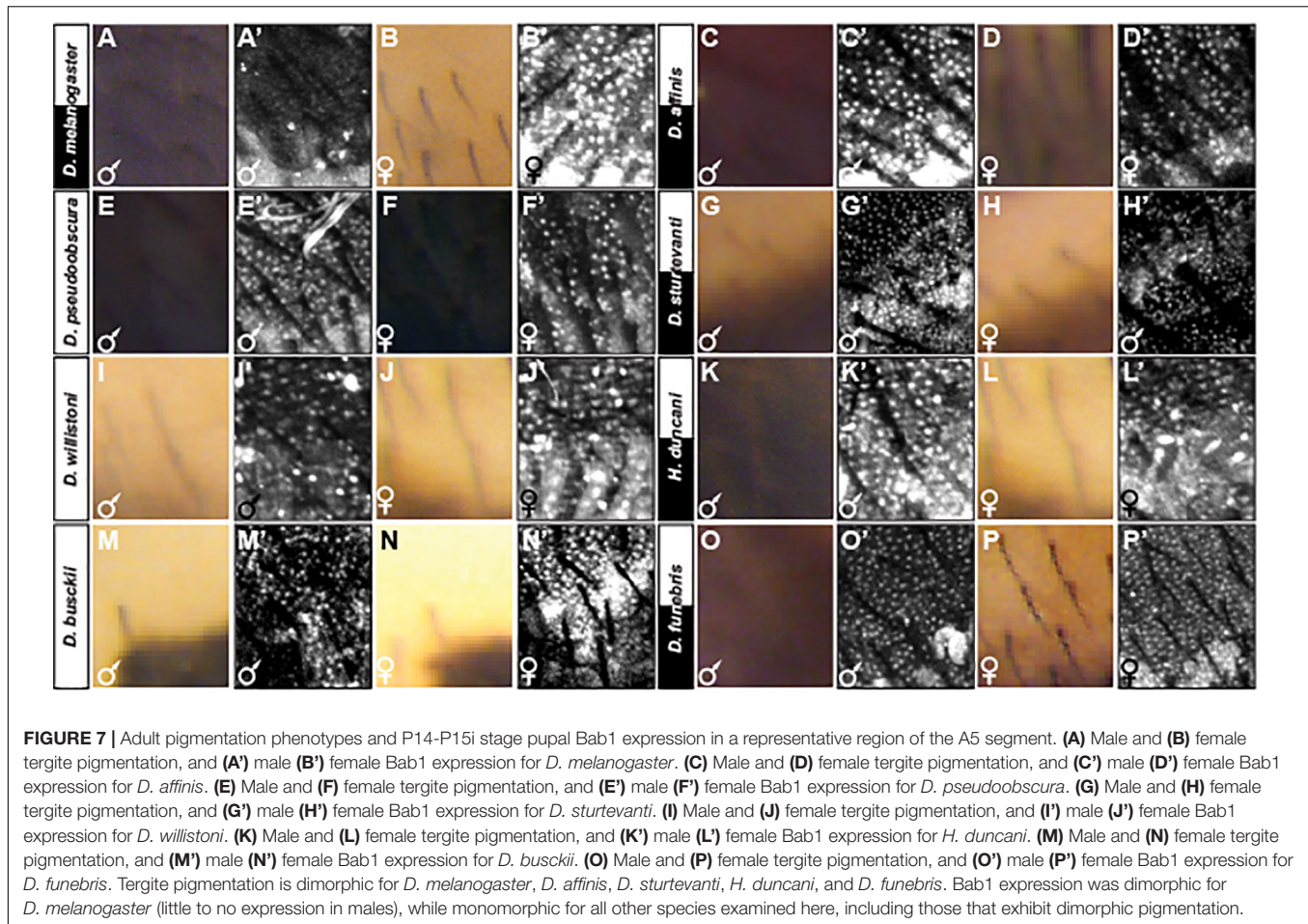


FIGURE 6 | Contrast in the pigment metabolic pathway utilization between the dimorphic *D. funebris* and the monomorphic *D. busckii*. (A–E) Female and (A'–E') male expressions of *D. funebris* pigment metabolic pathway genes, and (F,F') cartoon representation of the pigmentation phenotype. (G) Summary of the *D. funebris* pathway use includes robust expression of all genes, with dimorphic expressions of *ebony*, *tan*, and *yellow*. (H–L) Female and (H'–L') male expressions of *D. busckii* pigment metabolic pathway genes, and (M,M') cartoon representation of the pigmentation phenotype. (N) Summary of the *D. busckii* pathway use includes robust and monomorphic expression of all genes. (A,A',H,H') *pale*, (B,B',I,I') *Ddc*, (C,C',J,J') *ebony*, (D,D',K,K') *tan*, and (E,E',L,L') *yellow*. Red arrowheads indicate robust patterns of dimorphic expression in the dorsal abdominal epidermis. (G,N) Black arrow thickness represents the level of expression for the underlying gene. Monomorphic gene expression is represented by the gene names being placed upon a black rectangle. Dimorphic gene expression is represented by the gene names being placed upon a black and white colored rectangle.



other species studied here that possess dimorphic patterns of tergite pigmentation (Figures 8C,D,G,H,K,L,O,P, and Supplementary Figures S9, S11, and S13), as well for those with monomorphic patterns of tergite pigmentation (Figures 8I,J,M,N, and Supplementary Figures S10, S12). These dimorphic and monomorphic patterns of Bab1 expression were replicated in independent specimens (Supplementary Figures S14–S17). These results indicate that robust sexually dimorphic Bab expression is limited to the *melanogaster* species group and that a mild dimorphism may extend to some species of the most closely-related *obscura* species group (Figures 2, 8). However, monomorphism is broadly found across the *Sophophora* subgenus and *Drosophila* genus, indicating monomorphism as the ancestral state for Bab expression in the abdominal epidermis.

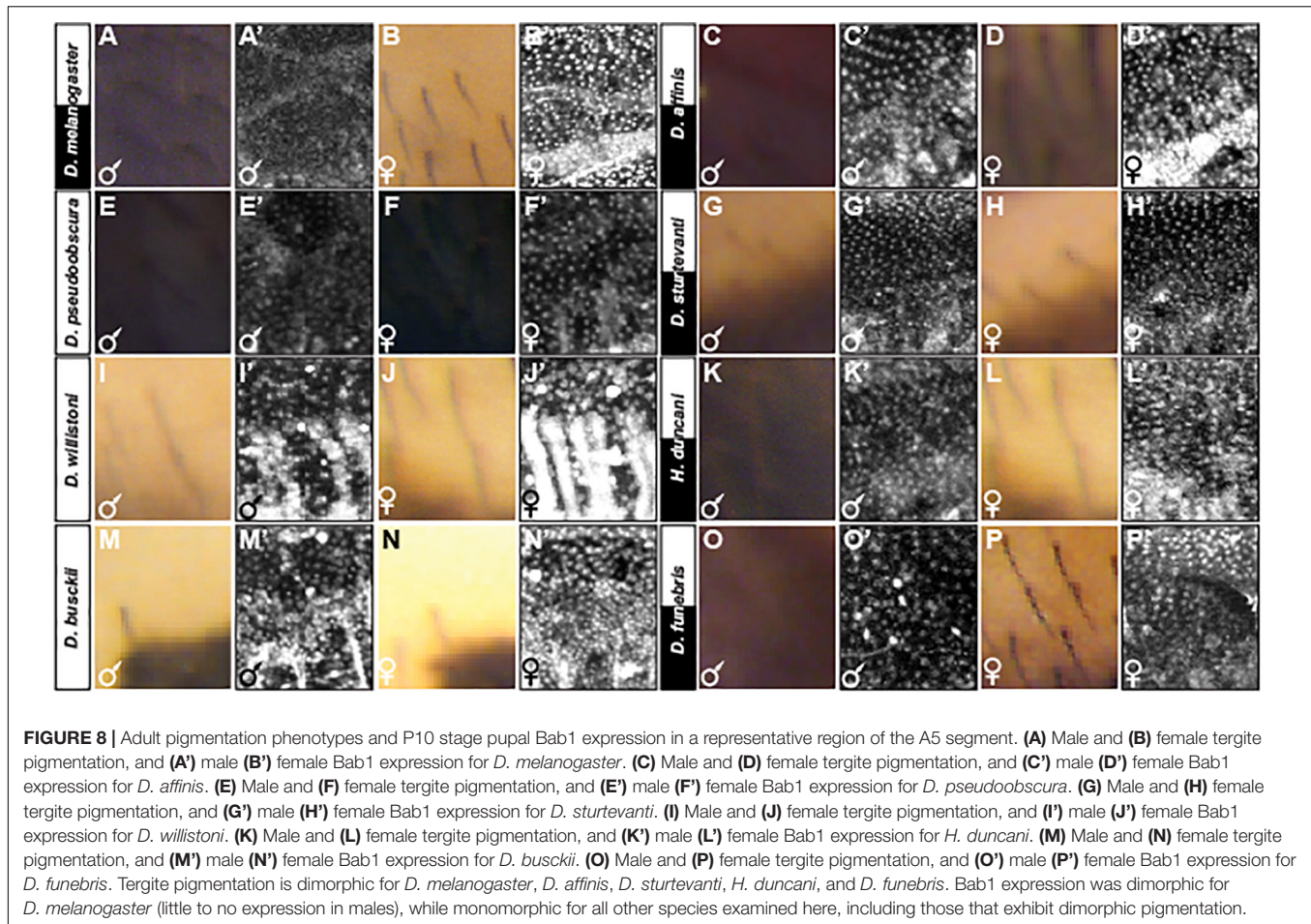
DISCUSSION

The existence of constantly recurring morphological characters within animal phylogenies raises a rarely mentioned, but important concern about our ability to infer whether such traits arose by independent gains or rampant patterns of loss from a common ancestral state. GRNs offer a unique perspective to

distinguish these possibilities at multiple levels of organization and granularity: are the same genes expressed to produce the trait? If so, are they activated by the same CREs? And are homologous binding sites used to generate similar expression patterns? Here, we explored the gene expression patterns underlying sexually monomorphic and dimorphic patterns of abdominal tergite pigmentation across a phylogeny in an extensively studied trait that has repeatedly changed states. This revealed common changes associated with dimorphism, namely dimorphic patterns of *ebony*, *tan*, and *yellow* expression. In contrast, a critical sex-specific regulator of this trait in the *D. melanogaster* species group is notably absent in other observed instances of this trait. Combining these results with previous studies in this system, we discuss how the GRN perspective reveals the developmental basis for a trait to repeatedly flicker in and out of existence.

Discriminating Homoplasy From Loss Through Analysis of Expression Patterns

For traits in which patterned gene expression is an important feature of their development, analysis of these patterns can provide critical information concerning trait gain or loss. If completely different genes were deployed to generate



dimorphic pigmentation patterns, this would support the independent convergence of these traits through separate genetic mechanisms (Stern, 2013). On the other hand, if the same genes are deployed, this could indicate ancestral homology coupled to loss or perhaps parallelism in which the same developmental mechanisms have been independently assembled multiple times. Our expression analyses of enzymes and their regulators in this system reveals a combination of these two outcomes.

Among the five enzyme-encoding genes we analyzed, we observed common themes in the deployment of this battery among dimorphic species. *yellow* and *tan* expression were upregulated in males, while *ebony* expression was reciprocally upregulated in females. *pale* and *Ddc* expression were generally monomorphic, presumably since Dopamine is a precursor for both yellow and black cuticle in males and females. The only exceptions were the dimorphic expression of *Ddc* in *H. duncani* and the underwhelming expression of *tan* in *D. affinis*. For *H. duncani*, this may reflect subtle differences in how the throughput of the pathway was arranged. In the case of *D. affinis*, this species has a dull color reminiscent of *tan* mutants (True et al., 2005), and may reflect differences relevant to generating its precise phenotype.

The patterning of the same enzymes in apparently separate instances of dimorphism is perhaps to be expected. These enzymes are certainly older than the genus *Drosophila* and encode proteins that perform the same enzymatic function in distantly related insects (Wright, 1987), including butterflies (Zhang et al., 2017) and the hemimetabolous milkweed bug *Oncopeltus fasciatus* (Liu et al., 2014, 2016). Thus, there is likely only a small number of potential paths by which a melanistic trait could evolve at the enzymatic level. However, analysis of a key regulator in this system reveals a stark contrast.

While dozens of transcription factor genes have been implicated as being a part of the *D. melanogaster* GRN (Rogers et al., 2014), two factors, Abd-B and Bab are highly patterned and play particularly important and well-understood roles. The Bab proteins play a key role in regulating the dimorphic output of the *D. melanogaster* GRN (Figure 1B). Previously, we have shown Bab1 and Bab2 expression to be indistinguishable for *D. melanogaster* and related *melanogaster* group species in the abdominal epidermis, with expression virtually absent from the male epidermis during the latter half of pupal development (Salomone et al., 2013). The reduction of either protein results in masculinized pigmentation in females (Couderc et al., 2002), while ectopic expression of either protein feminizes the

pigmentation of males (Kopp et al., 2000; Roeske et al., 2018). Previous work had suggested correlations of Bab expression with dimorphic pigmentation in instances outside of *Sophophora* (Gompel and Carroll, 2003). However, those studies were focused on very early stages that do not coincide with the physiologically relevant expression of Bab (Salomone et al., 2013). Thus, Bab was an excellent candidate regulator with which to evaluate homology in the production of this trait.

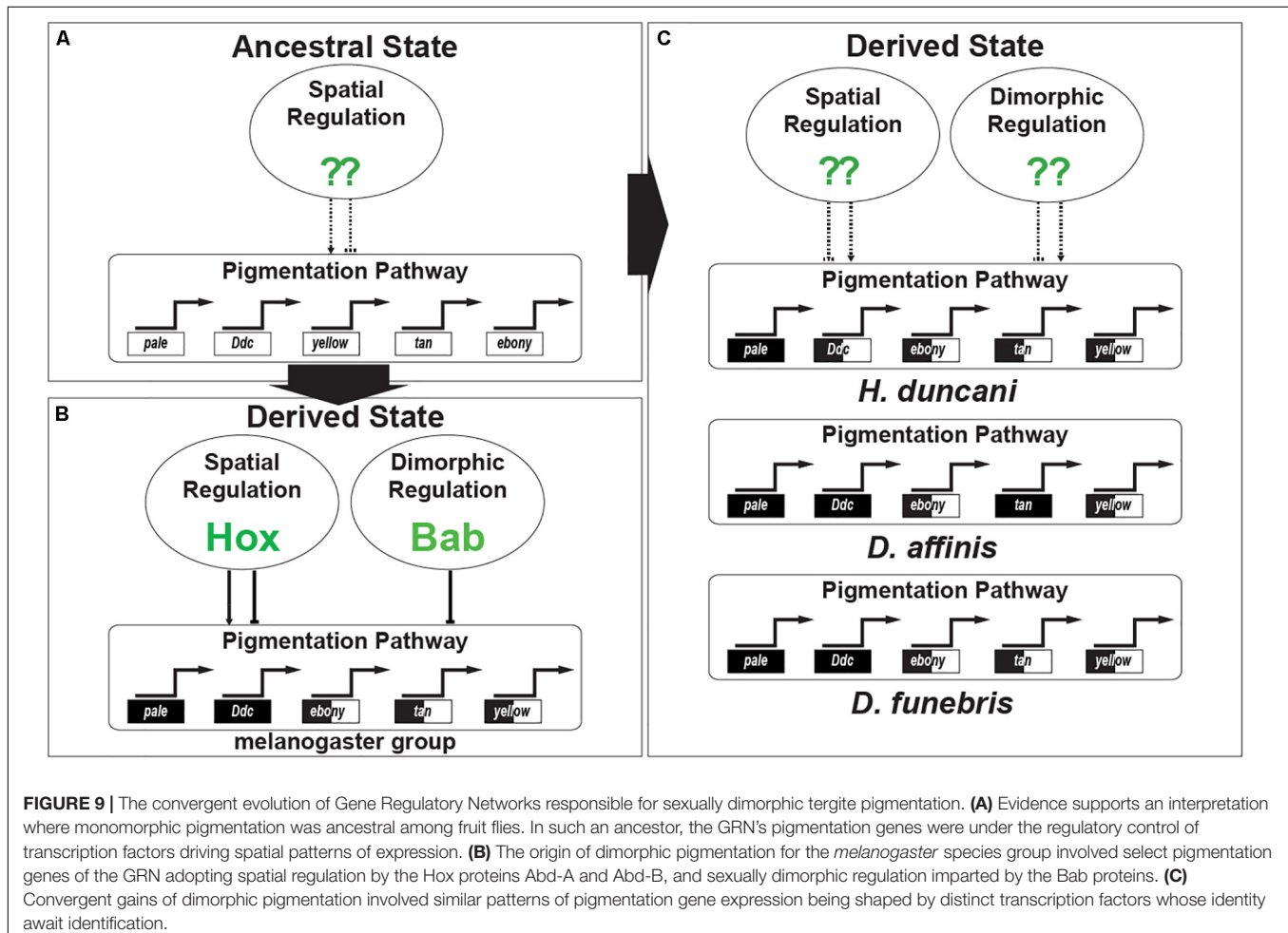
However, our results provide no such evidence for this repeated inclusion of Bab in other dimorphic GRNs. Rather our work in combination with a previous study indicates that dimorphic Bab expression evolved in the ancestry of the *melanogaster* species group, perhaps originating as early as the lineage in common between *D. melanogaster* and *D. pseudoobscura*. Thus, the dimorphic pigmentation for *H. duncani* and *D. funebris*, amongst other dimorphic species, were shaped by the origin of GRNs with another regulatory gene or genes shaping the sex-specific expression of the same pigmentation genes (Figure 9).

Comparative Assays of CRE Activity

While comparisons of gene expression can indicate whether the same genes underlie a recurring trait, analysis of the CREs which

activate these gene expression patterns can provide much higher resolution concerning homology or homoplasy. If the same CRE drives the expression in two potentially parallel instances of a trait, it would strongly support loss from an ancestor that possessed the trait. On the other hand, if distinct CREs positioned in different parts of the gene are responsible, such data would support the hypothesis of parallelism. Combining the gene expression analysis presented here with previous work suggests that the CREs underlying recurring similar patterns of enzyme expression are unique and rapidly arise, while the apparatus that could mediate dimorphic Bab expression is quite old.

CREs for the patterned pigmentation genes *yellow* and *tan* appear to have evolved uniquely with this trait in the *melanogaster* subgroup (Camino et al., 2015). This included the integration of *Hox* genes as spatial patterning factors, such as direct activation of *yellow* through the gain of Abd-B binding sites in the *yellow* body element CRE (Jeong et al., 2006), and Abd-A and Abd-B regulating dimorphic *tan* expression through its CRE with male-specific activity (Camino et al., 2015). Previous work has shown that other abdominal CREs for *yellow* can rapidly arise within the phylogeny (Kalay and Wittkopp, 2010) and that fragments of its regulatory regions may contain “pre-enhancers” that are sufficient to drive patterns when tested



in isolated reporter constructs (Kalay et al., 2019). Thus, the repeated GRN inclusion of *yellow*, *tan*, and *ebony* may indeed be due to convergence at the level of its CREs, a hypothesis that can now be tested more rigorously.

The CREs underlying the dimorphic expression of Bab are quite old, and very well could have been easily recruited to dimorphic pigmentation, but apparently were not. Dimorphic expression of Bab is mediated by the joint action of two CREs, one which drives a monomorphic pattern in anterior body segments, while a dimorphic element drives expression in female A5 and A6 segments (Williams et al., 2008). The inferred ancestral function of this element is to drive expression in the female genitalia, an activity that expanded the domain of its dimorphic activity to include the A5 and A6 segments in the *melanogaster* subgroup (Williams et al., 2008). The dimorphic element CRE and its Abd-B and DSX binding sites are conserved throughout the genus (Williams et al., 2008), and mutations in this CRE have impacts upon female pigmentation (Rogers et al., 2013). Thus, while Bab could easily have been recruited to mediate dimorphic pigmentation, its absence in these other networks is all the more surprising and suggests the existence of alternative ways to evolve the dimorphic regulation of pigmentation enzymes.

Detecting Homology in Transcription Factor Binding Sites

Ultimately, the question of trait gain or loss could be resolved at the level of individual transcription factor binding sites within CREs mediating recurrent traits. Our previous work on this topic, however, reveals how this question may nevertheless only weakly support homoplasy. We traced the binding of the Bab transcription factors to the *yellow* upstream regulatory region of *D. melanogaster* and found that only species closely related to *D. melanogaster* contain this binding site (Roeske et al., 2018). Our experiments supported an evolutionary scenario in which Bab binding evolved contemporaneously with the inferred origin of dimorphism in the lineage leading to the *melanogaster* species group. However, at the sequence level, the Bab-binding regions were not alignable outside of this group, and functional transgenic reporter assays confirmed that these distant relatives do not respond to Bab. For now, we believe that this is the best one can do to infer the origins of a transcription factor linkage within a GRN. This is because sequence divergence can erase traces of homology at the binding site level, while binding site turnover maintains ancestral functions without any trace of homology (Ludwig et al., 2000; Swanson et al., 2011). Thus, the absence of evidence supporting binding site conservation offers a poor readout for homoplasy. For this reason, we propose that analyses of expression patterns, coupled to functional assays of CRE activity or genetic tests of necessity represent the most fruitful ways to assess trait gain and loss within GRNs (Rebeiz and Williams, 2011).

Mesoevolution and the Recurrent Assembly of GRNs

It has been suggested that homoplasy and homology (loss) are two extremes on a continuum (Hall, 2007). In between

these two extremes lies parallelism in which the similar traits flicker on and off through the deployment of the same developmental programs. This process has been posited to occur most often for mesoevolutionary comparisons that represent differences between closely related species (Abouheif, 2008). The work we describe here shows how such flickering may developmentally manifest, with rapid evolution at the extremities of networks, and dramatic differences in the internal architecture of the regulators deployed. Future investigations into these parallel pigmentation patterns and their GRNs should be prioritized to identify the regulatory gene or genes driving dimorphism. The outcomes of such investigations will inform whether there are any common features to the genes that were recruited to play a pivotal role in sexually dimorphic patterning, or whether any transcription factor will suffice.

The results here may also bear upon the repeated origins of other morphological traits. Specifically, the occurrence of hotspot genes that are predictable evolutionary targets of phenotype-modifying mutations are likely to be features of GRNs for the loss or diversification of homologous traits. This was shown for the repeated loss of trichomes on *Drosophila* larvae, and flowering time for *Arabidopsis* plants, where modifications occurred to the *shavenbaby* (Sucena et al., 2003; McGregor et al., 2007; Frankel et al., 2012) and *frigida* transcription factor genes repeatedly (Johanson et al., 2000; Le Corre et al., 2002; Gazzani et al., 2003; Shindo et al., 2005; Stern, 2010). As for morphological novelties, the origin of their GRNs are likely to involve changes in the expression and function of transcription factors that are far less predictable.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

JH and TW designed the project. JH, MW, RJ, and SG performed the experiments characterized the gene expression. JH performed all further experiments and analysis. RJ and MW took care of the fruit fly stocks. JH, MR, and TW analyzed and interpreted the data, and wrote the manuscript. All authors read and approved the submitted version of the manuscript.

FUNDING

RJ received support from a Dean's Summer Fellowship from the University of Dayton, and SG and JH were supported by fellowships from the University of Dayton Graduate School. JH received support from a Graduate Research Fellowship from the National Science Foundation (DGE-1439647). MR and TW received support from a National Science Foundation grant

(IOS-1555906). The funders had no role in data collection and analysis, study design, decision to publish, or manuscript preparation.

ACKNOWLEDGMENTS

Species stocks were purchased from the San Diego Drosophila Stock Center or provided by S.B. Carroll. Specimens and

information on pigmentation phenotypes for *Lordiphosa* species were provided by Dr. Masanori J. Toda.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fevo.2020.00080/full#supplementary-material>

REFERENCES

- Abouheif, E. (2008). Parallelism as the pattern and process of mesoevolution. *Evol. Dev.* 10, 3–5. doi: 10.1111/j.1525-142X.2007.00208.x
- Abramoff, M. D., Hospitals, I., Magalhães, P. J., and Abramoff, M. (2004). Image Processing with ImageJ. *Biophotonics Int.* 11, 36–42.
- Arnoult, L., Su, K., Manoel, D., Minervino, C., Magrina, J., Gompel, N., et al. (2013). Emergence and diversification of fly pigmentation through evolution of a gene regulatory module. *Science* 339, 1423–1426. doi: 10.1126/science.1233749
- Ashburner, M., Golic, K. G., and Hawley, R. S. (2005). *Drosophila: A Laboratory Handbook*, 2nd Edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.
- Blount, Z. D., Lenski, R. E., and Losos, J. B. (2018). Contingency and determinism in evolution: replaying life's tape. *Science* 362:eaam5979. doi: 10.1126/science.aam5979
- Bonn, S., and Furlong, E. E. M. (2008). cis-Regulatory networks during development: a view of *Drosophila*. *Curr. Opin. Genet. Dev.* 18, 513–520. doi: 10.1016/j.gde.2008.09.005
- Camino, E. M., Butts, J. C., Ordway, A., Vellky, J. E., Rebeiz, M., and Williams, T. M. (2015). The evolutionary origination and diversification of a dimorphic gene regulatory network through parallel innovations in cis and trans. *PLoS Genet.* 11:e1005136. doi: 10.1371/journal.pgen.1005136
- Conway Morris, S. (2003). *Life's Solution. Inevitable Humans in a Lonely Universe*, 1st Edn. Cambridge: Cambridge University Press.
- Couderc, J.-L., Godt, D., Zollman, S., Chen, J., Li, M., Tjong, S., et al. (2002). The bric à brac locus consists of two paralogous genes encoding BTB/POZ domain proteins and acts as a homeotic and morphogenetic regulator of imaginal development in *Drosophila*. *Development* 129, 2419–2433.
- Cunningham, C. W., Omland, K. E., and Oakley, T. H. (1998). Reconstructing ancestral character states: a critical reappraisal. *Trends Ecol. Evol.* 13, 361–366. doi: 10.1016/S0169-5347(98)01382-2
- De Magalhaes, L. E. (1956). Description of four new species of the "Saltans" Group Of "*Drosophila*" (*Diptera*). *Rev. Bras. Bio.* 16, 273–280.
- Fartay, R. S., Sati, P. C., Pradhan, S., and Kandpal, M. C. (2017). A review of the genus *Lordiphosa* Basden in India, with descriptions of four new species from the Himalayan region (*Diptera: Drosophilidae*). *Zookeys* 79, 49–79. doi: 10.3897/zookeys.688.12590
- Frankel, N., Wang, S., and Stern, D. L. (2012). Conserved regulatory architecture underlies parallel genetic changes and convergent phenotypic evolution. *Proc. Natl. Acad. Sci. USA* 109, 20975–20979. doi: 10.1073/pnas.1207715109
- Gao, J., Hu, Y., Toda, M. J., Katoh, T., and Tamura, K. (2011). Phylogenetic relationships between Sophophora and Lordiphosa, with proposition of a hypothesis on the vicariant divergences of tropical lineages between the Old and New Worlds in the family *Drosophilidae*. *Mol. Phylogenet. Evol.* 60, 98–107. doi: 10.1016/j.ympev.2011.04.012
- Gazzani, S., Gendall, A. R., Lister, C., and Dean, C. (2003). Analysis of the molecular basis of flowering time variation in *Arabidopsis* accessions 1 [w]. *Plant Physiol.* 132, 1107–1114. doi: 10.1104/pp.103.021212
- Gompel, N., and Carroll, S. B. (2003). Genetic mechanisms and constraints governing the evolution of correlated traits in drosophilid flies. *Nature* 424, 931–935. doi: 10.1038/nature01863.1
- Grover, S., Williams, M. E., Kaiser, R., Hughes, J. T., Gresham, L., Rebeiz, M., et al. (2018). Augmentation of a wound response element accompanies the origin of a Hox-regulated *Drosophila* abdominal pigmentation trait. *Dev. Biol.* 441, 159–175. doi: 10.1016/j.ydbio.2018.07.001
- Hall, B. K. (2007). Homoplasy and homology: dichotomy or continuum? *J. Hum. Evol.* 52, 473–479. doi: 10.1016/j.jhevol.2006.11.010
- Hovemann, B. T., Ryseck, R. P., Walldorf, U., Stortkuhl, K. F., Dietzel, I. D., and Dessen, E. (1998). The *Drosophila* ebony gene is closely related to microbial peptide synthetases and shows specific cuticle and nervous system expression. *Gene* 221, 1–9. doi: 10.1016/S0378-1119(98)00440-5
- Hu, Y., and Toda, M. J. (2001). Polyphyly of Lordiphosa and its relationships in *Drosophilinae* (*Diptera: Drosophilidae*). *Syst. Entomol.* 26, 15–31. doi: 10.1046/j.1365-3113.2001.00135.x
- Jeong, S., Rebeiz, M., Andolfatto, P., Werner, T., True, J., and Carroll, S. B. (2008). The evolution of gene regulation underlies a morphological difference between two *Drosophila* sister species. *Cell* 132, 783–793. doi: 10.1016/j.cell.2008.01.014
- Jeong, S., Rokas, A., and Carroll, S. B. (2006). Regulation of body pigmentation by the Abdominal-B Hox protein and its gain and loss in *Drosophila* evolution. *Cell* 125, 1387–1399. doi: 10.1016/j.cell.2006.04.043
- Johanson, U., West, J., Lister, C., Michaels, S., Amasino, R., and Dean, C. (2000). Molecular analysis of frigida, a major determinant of natural variation in *Arabidopsis* flowering time. *Science* 290, 344–348.
- Joy, J. B., Liang, R. H., McCloskey, R. M., Nguyen, T., and Art, F. (2016). Ancestral reconstruction. *PLoS Comput. Biol.* 12:e1004763. doi: 10.1371/journal.pcbi.1004763
- Kalay, G., Lachowiec, J., Rosas, U., Dome, M. R., and Wittkopp, P. J. (2019). Redundant and cryptic enhancer activities of the drosophila yellow gene. *Genetics* 212, 343–360. doi: 10.1534/genetics.119.301985
- Kalay, G., and Wittkopp, P. J. (2010). Nomadic enhancers: tissue-specific cis-regulatory elements of yellow have divergent genomic positions among *Drosophila* species. *PLoS Genet.* 6:e1001222. doi: 10.1371/journal.pgen.1001222
- Katoh, T. K., Zhang, G., Toda, M. J., Zhang, W.-X., and Gau, J.-J. (2018). The Lordiphosa denticeps species group (*Diptera: Drosophilidae*) in China, with redescription of four known species and descriptions of nine new species. *Zootaxa* 4471, 37–75. doi: 10.11646/zootaxa.4471.1.2
- Kopp, A., Duncan, I., and Carroll, S. B. (2000). Genetic control and evolution of sexually dimorphic characters in *Drosophila*. *Nature* 408, 553–559. doi: 10.1038/35046017
- Le Corre, V., Roux, F., and Reboud, X. (2002). DNA Polymorphism at the FRIGIDA Gene in *Arabidopsis thaliana*: extensive nonsynonymous variation is consistent with local selection for flowering time. *Mol. Biol. Evol.* 19, 1261–1271. doi: 10.1093/oxfordjournals.molbev.a004187
- Liu, J., Lemonds, T. R., Marden, J. H., and Popadić, A. (2016). A pathway analysis of melanin patterning in a hemimetabolous insect. *Genetics* 203, 403–413. doi: 10.1534/genetics.115.186684
- Liu, J., Lemonds, T. R., and Popadić, A. (2014). The genetic control of aposematic black pigmentation in hemimetabolous insects: insights from *Oncopeltus fasciatus*. *Evol. Dev.* 27, 270–277. doi: 10.1111/ede.12090
- Losos, J. B. (2017). *Improbable Destinies: Fate, Chance, and the Future of Evolution*, 1st Edn. New York, NY: Riverhead Books.
- Ludwig, M. Z., Bergman, C., Patel, N. H., and Kreitman, M. (2000). Evidence for stabilizing selection in a eukaryotic enhancer element. *Nature* 403, 564–567. doi: 10.1038/35000615
- Majerus, M. E. N. (1998). *Melanism, Evolution in Action*. Oxford: Oxford University Press.
- Markow, T. A., and O'Grady, P. M. (2006). *Drosophila: A Guide to Species Identification and Use*. Cambridge, MA: Academic Press.
- McGregor, A. P., Orgogozo, V., Delon, I., Zanet, J., Srinivasan, D. G., Payre, F., et al. (2007). Morphological evolution through multiple cis-regulatory

- mutations at a single gene. *Nature* 448, 587–590. doi: 10.1038/nature05988
- Nater, H. (1953). Vergleichend-morphologische Untersuchung des äusseren Geschlechtsapparates innerhalb der Gattung *Drosophila*. *Zool. Jahrbucher* 81, 437–486.
- Nater, H. (1950). Der Samenpumpen-Sklerit von *Drosophila* als taxonomisches Merkmal. *Arch. der Jul. Klaus-Stiftung für Verebungsforschung. Sozialanthropologie und Rassenhygiene* 25, 623–625.
- Peter, I. S., and Davidson, E. H. (2011). Evolution of gene regulatory networks controlling body plan development. *Cell* 144, 970–985. doi: 10.1016/j.cell.2011.02.017
- Pham, T., Day, S. M., Glassford, W. J., Williams, T. M., and Rebeiz, M. (2017). The evolutionary origination of a novel expression pattern through an extreme heterochronic shift. *Evol. Dev.* 19, 43–55. doi: 10.1111/ede.12215
- Rebeiz, M., Patel, N. H., and Hinman, V. F. (2015). Unraveling the tangled skein: the evolution of transcriptional regulatory networks in development. *Annu. Rev. Genomics Hum. Genet.* 16, 103–131. doi: 10.1146/annurev-genom-091212-153423
- Rebeiz, M., Pool, J. E., Kassner, V. A., Aquadro, C. F., and Carroll, S. B. (2009). Stepwise modification of a modular enhancer underlies adaptation in a *Drosophila* population. *Science* 326, 1663–1667. doi: 10.1126/science.1178357
- Rebeiz, M., and Williams, T. M. (2011). “experimental approaches to evaluate the contributions of candidate Cis-regulatory mutations to phenotypic evolution,” in *Methods in Molecular Biology* Methods in Molecular Biology, eds V. Orgogozo and M. V. Rockman (Totowa, NJ: Humana Press), 351–375. doi: 10.1007/978-1-61779-228-1_21
- Rebeiz, M., and Williams, T. M. (2017). Using *Drosophila* pigmentation traits to study the mechanisms of cis-regulatory evolution. *Curr. Opin. Insect Sci.* 19, 1–7. doi: 10.1016/j.cois.2016.10.002
- Roeske, M. J., Camino, E. M., Grover, S., Rebeiz, M., and Williams, T. M. (2018). Cis-regulatory evolution integrated the bric-a-brac transcription factors into a novel fruit fly gene regulatory network. *Elife* 7, 1–28. doi: 10.7554/eLife.32273
- Rogers, W. A., Grover, S., Stringer, S. J., Parks, J., Rebeiz, M., and Williams, T. M. (2014). A survey of the trans-regulatory landscape for *Drosophila melanogaster* abdominal pigmentation. *Dev. Biol.* 385, 417–432. doi: 10.1016/j.ydbio.2013.11.013
- Rogers, W. A., Salomone, J. R., Tacy, D. J., Camino, E. M., Davis, K. A., Rebeiz, M., et al. (2013). Recurrent modification of a conserved Cis-Regulatory element underlies fruit fly pigmentation diversity. *PLoS Genet.* 9:e1003740. doi: 10.1371/journal.pgen.1003740
- Salomone, J. R., Rogers, W. A., Rebeiz, M., and Williams, T. M. (2013). The evolution of Bab paralog expression and abdominal pigmentation among *Sophophora* fruit fly species. *Evol. Dev.* 15, 442–457. doi: 10.1111/ede.12053
- Shindo, C., Aranzana, M. J., Lister, C., Baxter, C., Nicholls, C., Nordborg, M., et al. (2005). Role of FRIGIDA and FLOWERING LOCUS C in determining variation in flowering time of *Arabidopsis*. *Plant Physiol.* 138, 1163–1173. doi: 10.1104/pp.105.061309.1
- Stern, D. L. (2010). *Evolution, Development, & the Predictable Genome*, 1st Edn. Greenwood Village: Roberts & Company Publishers.
- Stern, D. L. (2013). The genetic causes of convergent evolution. *Nat. Rev. Genet.* 14, 751–764. doi: 10.1038/nrg3483
- Sucena, E., Delon, I., Jones, I., Payre, F., and Stern, D. L. (2003). Regulatory evolution of shavenbaby/ovo underlies multiple cases of morphological parallelism. *Nature* 424, 935–938. doi: 10.1038/nature01768
- Swanson, C. I., Schimmer, D. B., and Barolo, S. (2011). Rapid evolutionary rewiring of a structurally constrained eye enhancer. *Curr. Biol.* 21, 1186–1196. doi: 10.1016/j.cub.2011.05.056
- Throckmorton, L. H. (1962). The problem of phylogeny in the genus *Drosophila*. *Univ. Texas Publ.* 6205, 207–343. doi: 10.1016/j.jympev.2011.04.012
- True, J. R., Yeh, S.-D., Hovemann, B. T., Kemme, T., Meinertzhagen, I. A., Edwards, T. N., et al. (2005). *Drosophila tan* encodes a novel hydrolase required in pigmentation and vision. *PLoS Genet.* 1:e63. doi: 10.1371/journal.pgen.0010063
- van der Linde, K., Houle, D., Spicer, G. S., and Stepan, S. J. (2010). A supermatrix-based molecular phylogeny of the family *Drosophilidae*. *Genet. Res.* 92, 25–38. doi: 10.1017/S001667231000008X
- Wagner, G. P. (2016). What Is “Homology Thinking” and what Is it for? *J. Exp. Zool.* 326B, 3–8. doi: 10.1002/jez.b.22656
- Wake, D. B., Wake, M. H., and Specht, C. D. (2011). Homoplasy: from detecting pattern and mechanism of evolution. *Science* 331, 1032–1035. doi: 10.1126/science.1188545
- Werner, T., Koshikawa, S., Williams, T. M., and Carroll, S. B. (2010). Generation of a novel wing colour pattern by the Wingless morphogen. *Nature* 464, 1143–1148. doi: 10.1038/nature08896
- Wiens, J. J. (2001). Widespread loss of sexually selected traits: how the peacock lost its spots. *Trends Ecol. Evol.* 16, 517–523. doi: 10.1016/s0169-5347(01)02217-0
- Williams, T. M., Selegue, J. E., Werner, T., Gompel, N., Kopp, A., and Carroll, S. B. (2008). The regulation and evolution of a genetic switch controlling sexually dimorphic traits in *Drosophila*. *Cell* 134, 610–623. doi: 10.1016/j.cell.2008.06.052
- Wittkopp, P. J., Carroll, S. B., and Kopp, A. (2003). Evolution in black and white: genetic control of pigment patterns in *Drosophila*. *Trends Genet.* 19, 495–504. doi: 10.1016/S0168-9525(03)00194-X
- Wright, T. R. F. (1987). “The Genetics of Biogenic Amine Metabolism, Sclerotization, and Melanization in *Drosophila melanogaster*,” in *Advances in Genetics*, eds J. G. Scandalios and E. W. Caspari (San Diego, CA: Harcourt Brace Jovanovich), 127–222. doi: 10.1016/s0065-2660(08)60008-5
- Zanini, R., Deprá, M., and Lúcia, V. (2015). Can sibling species of the *Drosophila willistoni* subgroup be recognized through combined microscopy techniques? *Rev. Bras. Entomol.* 59, 323–331. doi: 10.1016/j.rbe.2015.09.006
- Zhang, L., Martin, A., Perry, M., van der Burg, K. R. L., Matsuoka, Y., Monteiro, A., et al. (2017). Genetic basis of melanin pigmentation in butterfly wings. *Genetics* 205, 1537–1550. doi: 10.1534/genetics.116.196451/-/DC1.1

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2020 Hughes, Williams, Johnson, Grover, Rebeiz and Williams. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Mutations in Domestic Animals Disrupting or Creating Pigmentation Patterns

Leif Andersson^{1,2,3*}

¹ Department of Medical Biochemistry and Microbiology, Uppsala University, Uppsala, Sweden, ² Department of Veterinary Integrative Biosciences, Texas A&M University, College Station, TX, United States, ³ Department of Animal Breeding and Genetics, Swedish University of Agricultural Sciences, Uppsala, Sweden

The rich phenotypic diversity in coat and plumage color in domestic animals is primarily caused by direct selection on pigmentation phenotypes. Characteristic features are selection for viable alleles with no or only minor negative pleiotropic effects on other traits, and that alleles often evolve by accumulating several consecutive mutations in the same gene. This review provides examples of mutations that disrupt or create pigmentation patterns. White spotting patterns in domestic animals are often caused by mutations in *KIT*, microphthalmia transcription factor (*MITF*), or endothelin receptor B (*EDNRB*), impairing migration or survival of melanoblasts. Wild boar piglets are camouflage-colored and show a characteristic pattern of dark and light longitudinal stripes. This pattern is disrupted by mutations in *Melanocortin 1 receptor (MC1R)*, implying that a functional MC1R receptor is required for wild-type camouflage color in pigs. The great majority of pig breeds carry *MC1R* mutations disrupting wild-type color and different mutations causing dominant black color were independently selected in European and Asian domestic pigs. The European allele evolved into a new allele creating a pigmentation pattern, black spotting, after acquiring a second mutation. This second mutation, an insertion of two C nucleotides in a stretch of 6 Cs, is somatically unstable and creates black spots after the open reading frame has been restored by somatic mutations. In the horse, mutations located in an enhancer downstream of *TBX3* disrupt the Dun pigmentation pattern present in wild equids, a camouflage color where pigmentation on the flanks is diluted. A fascinating example of the creation of a pigmentation pattern is Sex-linked barring in chicken which is caused by the combined effect of both regulatory and coding mutations affecting the function of *CDKN2A*, a tumor suppressor gene associated with familial forms of melanoma in human. These examples illustrate how evolution of pigmentation patterns in domestic animals constitutes a model for evolutionary change in natural populations.

Keywords: pigmentation, domestic animals, patterning, domestication, selection

INTRODUCTION

Pigmentation phenotypes have been under strong selection in domestic animals throughout their evolutionary history, and references to variation in pigmentation are indicated already in ancient literature and illustrations. For instance, the Greek historian Herodotus described that the Persian emperor Xerxes (in reign 485 to 465 BC) kept sacred white horses, most likely white horses caused

OPEN ACCESS

Edited by:

Ricardo Mallarino,
Princeton University, United States

Reviewed by:

Salvatore D'Aniello,
Stazione Zoologica Anton Dohrn, Italy
Claudius Frank Kratochwil,
University of Konstanz, Germany

*Correspondence:

Leif Andersson
Leif.Andersson@imbim.uu.se

Specialty section:

This article was submitted to
Evolutionary Developmental Biology,
a section of the journal
Frontiers in Ecology and Evolution

Received: 08 December 2019

Accepted: 09 April 2020

Published: 13 May 2020

Citation:

Andersson L (2020) Mutations
in Domestic Animals Disrupting or
Creating Pigmentation Patterns.
Front. Ecol. Evol. 8:116.
doi: 10.3389/fevo.2020.00116

by the Graying with age mutation (Rosengren Pielberg et al., 2008). Coat color variation is also described in old Roman literature (Forster and Heffner, 1968). Pigmentation must have been one of the first traits that were altered after domestication was initiated and extensive color diversity is a hallmark for domestic animals. The molecular characterization of mutations underlying these changes has given insight about mechanisms underlying pigmentation patterns. The evolution of pigmentation patterns in domestic animals constitutes a model for evolutionary change in natural populations. This review provides examples of mutations that disrupt pigmentation patterns and others that create pigmentation patterns. In addition to the patterns described here, it is worth noticing the Himalayan pattern that occurs in several species like cat, rabbit, mouse, and gerbil (Lyons et al., 2005), and is caused by temperature-sensitive mutations of tyrosinase producing white color but with dark pigmentation in cooler areas of the body, like the tips of the ears.

WHITE SPOTTING PATTERNS IN DOMESTIC ANIMALS

White spotting patterns occur frequently in domestic animals. The most common causes are mutations in *KIT* encoding the KIT tyrosine kinase receptor, microphthalmia transcription factor (*MITF*), or endothelin receptor B (*EDNRB*), all with a crucial role for melanoblast migration and survival. Thus, a common reason for white spotting is lack of pigment cells in skin and/or in the hair/feather follicles.

The majority of *KIT* mutations causing pigment patterns in domestic animals are structural rearrangements. There are two reasons why these are common in domestic animals. One is that structural rearrangements that do not touch the coding sequence may give a spectacular pigmentation pattern without causing negative pleiotropic effects, because *KIT* function is also essential for development of hematopoietic cells and for germ cells. The second reason is because some regulatory elements affecting *KIT* expression are located hundreds of kb both upstream and downstream of the coding sequence, disruption of these often gives spectacular spotting patterns. This is well illustrated by the domestic pig where a 450 kb duplication encompassing the entire coding sequence and more than 100 kb upstream and downstream of the coding sequence is causing the Patch phenotype characterized by large areas of the coat lacking pigmentation (Johansson Moller et al., 1996; Giuffra et al., 2002). Further, the Belt phenotype, characterized by a white belt across the foreleg, is associated with several duplications in non-coding regions of *KIT* (Rubin et al., 2012). The top dominant *KIT* allele, present in billions of pigs used for meat production worldwide, is *Dominant white* causing complete or near complete absence of skin and hair pigmentation. The *Dominant white* allele carries multiple causal mutations, including the different duplications associated with the Patch and Belt phenotypes, and in addition a splice mutation in one of the copies that leads to skipping of exon 17 encoding the tyrosine kinase domain. Thus, this results in a dominant negative receptor with normal ligand binding but inactivated tyrosine kinase signaling

(Marklund et al., 1998; Rubin et al., 2012). The *Dominant white* allele is affecting pigmentation based on the combined effect of regulatory mutations (the duplications) and a coding change (splice mutation) in one of the copies. Due to this combination, it is the most dominant *KIT* allele as regards its effect on pigmentation in any mammal and with no or only very mild pleiotropic effects on hematopoiesis and fertility. Other examples of *KIT* structural rearrangements causing striking pigmentation patterns in domestic animals are Tobiano white spotting in horses caused by a 40 Mb inversion where one of the inversion breakpoints is located about 100 kb downstream of *KIT* (Brooks et al., 2008), and color sidedness in cattle caused by two serial translocations affecting *KIT* expression (Durkin et al., 2012).

In contrast to pigs where there is an allelic series at the *KIT* locus, white spotting in dogs is largely determined by an allelic series at the *MITF* locus (Karlsson et al., 2007). This *Spotting* (*S*) locus was first described by Little (1957) and is composed of four alleles *Solid* (*S*, wild-type), *Irish spotting* (*Sⁱ*), *Piebald* (*S^P*), and *Extreme white* (*S^W*). Irish spotting occurs in breeds like Bernese mountain dogs, Collie and Basenji, and is characterized by limited white spotting on the chest and often with a white ring around the neck. The Piebald phenotype occurs in for instance Beagles and Fox terriers and is characterized by more extensive white spotting across the body. Finally, Extreme white occurs in Dalmatians, white Boxers, and white Bull terriers and presents as a near total absence of pigmentation but remaining spots of pigmentation show normal pigmentation implying no defect in pigment production *per se*. In contrast to the situation in mice where the majority of described alleles affect the coding sequence and is associated with severe negative pleiotropic effects in other tissues where *MITF* function is essential, none of the *MITF* alleles in dogs affect the coding sequence and they have no or only mild negative effects (Karlsson et al., 2007); a fraction of the Extreme white dogs show deafness. Furthermore, an interesting aspect of the dog *MITF* alleles is that the three mutant alleles do not represent three independent mutations but show haplotype sharing strongly suggesting that the three alleles have evolved by consecutive accumulation of several causal mutations in the non-coding part of *MITF*. Functional characterization indicated that a simple repeat polymorphism in the *MITF* promoter is likely one of the causal variants affecting white spotting patterns (Baranowska Körberg et al., 2014). A non-coding variant in the 5' region of *MITF* is also associated with a white spotting pattern in cattle (Hofstetter et al., 2019). In horses, mutations in both *MITF* and *PAX3* are associated with the Splashed white pigmentation pattern (Hauswirth et al., 2012).

A missense mutation in *EDNRB* Ile118Lys is causing the Overo white spotting pattern in horses and in the homozygous condition the Overo lethal white syndrome, where lethality is caused by intestinal aganglionosis (Metallinos et al., 1998; Santschi et al., 1998; Yang et al., 1998). This horse syndrome corresponds to the form of Hirschsprung disease in humans caused by mutations in the same gene. A missense mutation in *EDNRB2* is also associated with a feather pigmentation pattern in chicken (see below).



FIGURE 1 | Wild boar sow with piglets. The piglets show the characteristic camouflage pattern which requires the presence of a wild-type *MC1R* allele. Photo: Annelie Andersson.

MC1R MUTATIONS IN PIGS BOTH DISRUPT AND CREATE PIGMENTATION PATTERNING

Melanocortin 1 receptor (*MC1R*) is one of the major coat color loci in the domestic pig. The wild boar piglets show a striking camouflage color composed of longitudinal dark- and light-colored stripes (**Figure 1**). In the great majority of pig breeds of the world, this camouflage color is disrupted by *MC1R* mutations, either dominant black or recessive red mutations (Kijas et al., 1998, 2001). Fang et al. (2009) tested pigs from 68 different breeds from Europe and China and found that pigs from only one, the Hungarian Mangalica, were homozygous for the wild-type allele. Domestication of pigs occurred in parallel in Europe and Asia from two different subspecies of the wild boar, the European wild boar and the Asian wild boar that separated from each other about one million years ago (Giuffra et al., 2000; Kijas and Andersson, 2001; Groenen et al., 2012). Two different missense mutations in *MC1R* causing dominant black color were selected in European and Asian domestic pigs, D124N and L102P, respectively. A comprehensive screen of the *MC1R* coding sequences in wild boars and domestic pigs from both Europe and Asia led to the conclusion that there is purifying selection to maintain camouflage in wild boars and selection to disrupt camouflage in domestic pigs (Fang et al., 2009). A conclusion based on the observation that seven out of seven nucleotide substitutions among European and Asian wild boars were all synonymous, whereas nine out of 10 nucleotide substitutions among domestic pigs were non-synonymous changes.

The disruption of camouflage pattern in pigs carrying dominant black or recessive red alleles at *MC1R* suggests strongly that a wild-type *MC1R* receptor, whose signaling activity is controlled by the relative abundance of melanocyte-stimulating hormone (MSH) and agouti (ASIP), is required for the development of this pattern. The most likely explanation is that differential expression of agouti is causing patterning

as recently reported for periodic feather patterning in juvenile galliform birds (Haupaix et al., 2018). Furthermore, differential expression of the transcription factor *ALX3* is associated with the development of periodic dorsal stripes in the African striped mouse, which resemble the camouflage pattern in piglets (Mallarino et al., 2016).

One of the *MC1R* alleles in pigs is also creating a stochastic pigment pattern. That is the black-spotting E^P allele that evolved from the European dominant black allele (E^{D1}), carrying the D124N missense mutation, by the insertion of two C nucleotides at codon 22 creating a mononucleotide repeat of 8 C (Kijas et al., 2001). A frameshift mutation at codon 22 is expected to result in a complete loss-of-function and lack of black eumelanin in the coat. But that is not the case, the most common phenotype is red with a more or less random distribution of black spots across the body or white coat with larger black spots; whether the black spots occur on a white or red background is determined by one or more other genetic factors that have not yet been identified. The black-spotting phenotype associated with this allele ranges from almost no spots at all, in particular on the red background as in Tamworth pigs, to an entire black coat with six white points (tail, nose, and four white feet) in Berkshire. So, how is this possible? The explanation is that the 8 C mononucleotide repeat is somatically unstable and may lose two nucleotides or gain one nucleotide and thereby restore the open reading frame. When that happens, constitutive *MC1R* signaling is reactivated due to the presence of the D124N missense mutation. This somatic instability of the mononucleotide repeat was confirmed by RT-PCR analysis (Kijas et al., 2001). The phenotypic range associated with the black-spotting allele is most likely explained by sequence variants affecting the probability for somatic reversion to occur as well as loci affecting the proliferation of melanocytes after reversion has occurred.

A similar stochastic pattern of pigmented spots occurs in white horses carrying the dominant graying with age mutation. These horses are born normally colored but start to gray already during the first year of life and they are usually completely white before they are 10 years of age. Graying with age is caused by a 4.6 kb tandem duplication in an intron of *syntaxin 17* (Rosengren Pielberg et al., 2008). Many horses that are heterozygous for this mutation show large number of small pigmented spots and are called flea-bitten gray. It appears plausible that this phenotype is caused by somatic loss of one of the duplicated copies or that it is inactivated by an epigenetic mechanism. A third example of a stochastic generation of a pigmentation pattern in domestic animals is the merle patterning in dogs in which pigmentation is diluted by a retrotransposon insertion in *PMEL* (previously denoted *SILV*), somatic deletion of the retrotransposon restores normal pigmentation (Clark et al., 2006).

REGULATORY MUTATIONS IN *TBX3* DISRUPT CAMOUFLAGE COLOR IN HORSES

The majority of domestic horses have a non-dun phenotype characterized by intense pigmentation and caused by

homozygosity for a recessive allele at the *Dun* locus. The dominant Dun phenotype occurs in some horses like Icelandic horses and the Norwegian Fjord horse, but this is in fact a wild-type color present also in the Przewalski's horse, a close relative to the ancestor of domestic horses. *Dun* is causing a pattern of dilution on the flanks but leaves a dark dorsal stripe and may be associated with other dark patterns which may include facial mask, shoulder cross, and zebra-like stripes on the legs (Imsland et al., 2016). There are many mutations described in the pigmentation literature causing pigment dilution caused by various defects in the pigment machinery. It is worth noticing that Dun in horses is in fact a wild-type phenotype contributing to camouflage by reducing the intensity of pigmentation.

Histological studies revealed that the difference between hairs from Dun and non-dun horses is that the latter have a symmetric deposition of pigment whereas hair from Dun horses have an asymmetric deposition of pigments on the outward-facing side of the hair (Imsland et al., 2016). Thus, this is a pigmentation pattern affecting the individual hair. High-resolution genetic mapping combined with whole genome sequencing data from Dun and non-dun horses revealed that the non-dun phenotype is caused by cis-acting regulatory mutations affecting tissue-specific expression of the *TBX3* transcription factor gene (Imsland et al., 2016). *TBX3* had never before been associated with pigmentation, but it is important during development and loss-of-function mutations cause the ulnar-mammary syndrome in humans that involves defects in limb, apocrine gland, tooth, and genital development (Bamshad et al., 1997). Imsland et al. (2016) first showed that the majority of non-dun horses, including the reference horse used for the horse genome assembly, were homozygous for a 1609 bp deletion located about 5 kb downstream of *TBX3*, in a region showing high sequence conservation among mammals. The fact that not all non-dun horses were homozygous required further analysis which revealed the presence of two different alleles *non-dun1* (lacking the deletion) and *non-dun2* (with the deletion). The causal mutation for *non-dun1* is a single nucleotide substitution within the region deleted in *non-dun2*! Furthermore, genotyping more than 1000 horses for these two causal variants explained a phenotypic heterogeneity among non-dun horses where horses homozygous for *non-dun2* have the most intense pigmentation and non-dun1 horses show an intermediate phenotype often with a weak dorsal stripe (Imsland et al., 2016). Interestingly, *non-dun1* is in fact also a wild-type allele since it was found in two ancient horses (4400 and 42,700 years old). This implies that there existed two different color morphs of the ancestor of domestic horses, Dun and non-dun1, possibly adapted to different environmental conditions.

Imsland et al. (2016) also established a plausible molecular mechanism underlying camouflage color in Dun horses, which is disrupted in non-dun horses. In Dun horses, *TBX3* has an asymmetric expression in the hair follicle that matches the asymmetric deposition of pigment. KIT ligand (KITL) shows downregulation in the area where *TBX3* is expressed, which in turn means that pigment cells are not attracted to this part of the hair follicle explaining the lack of pigment deposition. In contrast, *TBX3* is not expressed in the hair follicle in non-dun

horses explaining the symmetric deposition of pigment. Thus, the results suggest that the deleted region contains an enhancer required for *TBX3* expression in the hair follicle.

The work on the Dun horse coat color revealed a previously unknown function for *TBX3* and a previously unknown mechanism for generation of camouflage color in mammals. This mechanism is present at least in all equids including zebras. For instance, the Somali wild ass, the wild ancestor of the donkey, shows a very clear Dun phenotype with diluted pigmentation on the flanks, a dorsal black stripe and zebra-like leg stripes. It is possible that this mechanism for camouflage pattern is also active in other mammals including different species of deer and antelopes.

MUTATIONS IN THE *CDKN2A* TUMOR SUPPRESSOR GENE CREATE PIGMENTATION PATTERN IN CHICKEN

Sex-linked barring is an iconic plumage phenotype present in breeds like Barred Plymouth Rock and Coucou de Rennes (Figure 2A). This phenotype shows sex-linked dominant inheritance and is characterized by feathers with periodic black and white bars. In the initial identification of the causal gene for this phenotype, Hellström et al. (2010) mapped this locus to a 12 kb region containing only the *CDKN2A* tumor suppressor gene encoding two transcripts INK4b and ARF. *CDKN2A* had never before been associated with a pigmentation phenotype but has a well-established link to pigment cell biology because heterozygosity for loss-of-function mutations in this gene is a major risk factor for familiar forms of malignant melanoma in humans (Hussussian et al., 1994). Sequence analysis of the 12 kb region across many breeds of chicken revealed three *CDKN2A* alleles: *wild-type* (*N*), *Sex-linked barring* (*B1*), and *Sex-linked dilution* (*B2*); the latter is a variant of Sex-linked barring but causing a more diluted pigmentation and not as sharp contrast between the black and white bars as in Sex-linked barring. Four sequence variants, all in or near the ARF transcript, were unique to the *B1* and *B2* alleles and not found in any of the sequenced wild-type chromosomes (Figure 2B). *B1* carried a V9D missense mutation while *B2* was associated with an R10C missense mutation, and both carried two SNPs in non-coding sequences. The two missense mutations were very strong candidates for being causal because they were both non-conservative and affected the MDM2-binding domain of the ARF protein. However, it was a mystery why both mutations occurred on the same very rare haplotype characterized by two non-coding changes not found among *wild-type* chromosomes.

A second study (Schwochow-Thalmann et al., 2017) characterized a fourth allele, *B0*, that carried only the two non-coding changes (Figure 2B). The *B0* allele was associated with the most extreme suppression of pigmentation with a weak barring pattern and was therefore named *Sex-linked extreme dilution*. Functional analysis revealed allelic imbalance associated with the *B0*, *B1*, and *B2* alleles, with higher expression of the mutant allele compared with the *wild-type* allele in feather follicles but not in skin or in liver. The results imply that one of the two

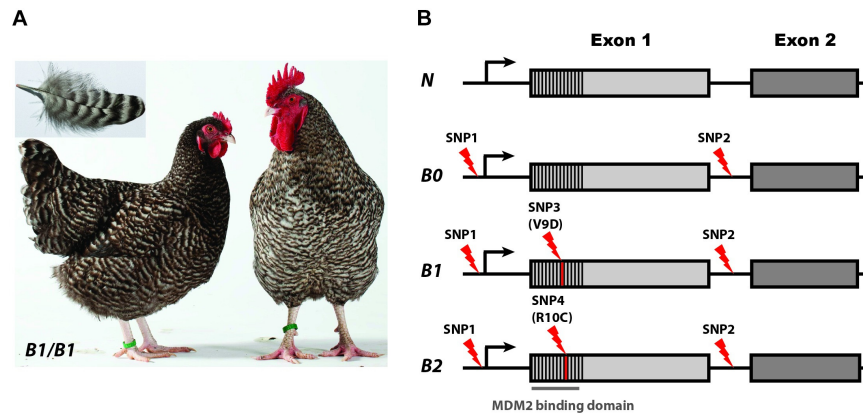


FIGURE 2 | Phenotypes and alleles at the Sex-linked barring (*CDKN2A*) locus in chicken. **(A)** Female and male Coucou de Rennes chicken illustrating the Sex-linked barring phenotype caused by the *B1* allele. **(B)** Sex-linked barring alleles and associated sequence variants. SNP1 and SNP2 are non-coding while SNP3 and SNP4 constitute non-synonymous changes in the region encoding the MDM2 binding domain. Photo credit: **(A)** Hervé Ronné, Ecomusée du pays de Rennes. From Schwochow-Thalmann et al. (2017).

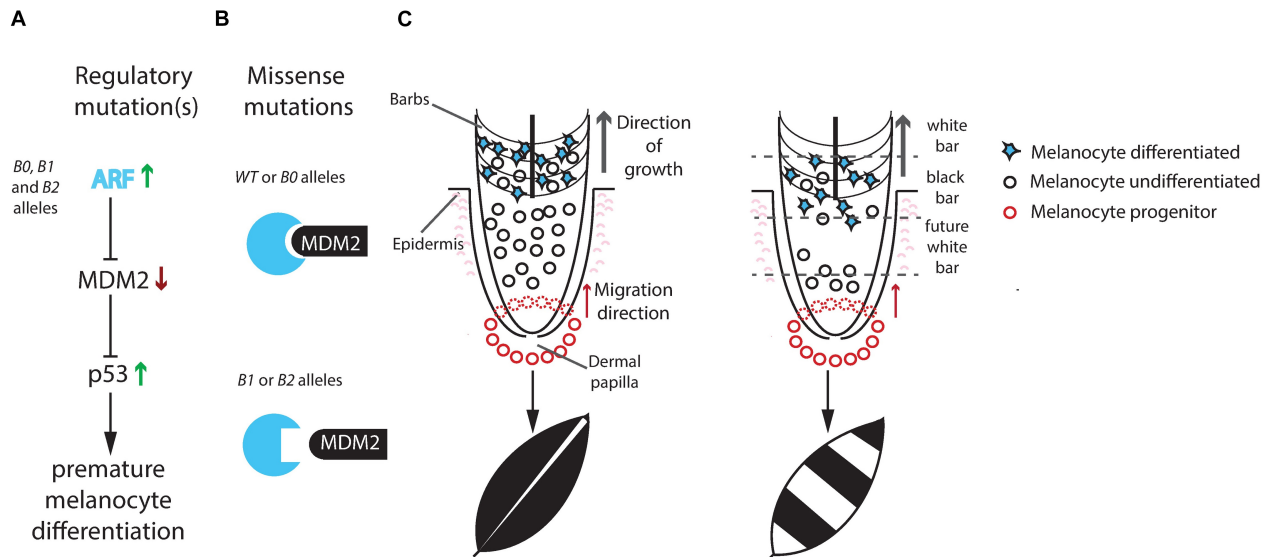


FIGURE 3 | Proposed mechanism for development of the Sex-linked barring phenotype. **(A)** The non-coding mutation(s) present in the *B0*, *B1*, and *B2* alleles cause a tissue-specific upregulation of *CDKN2A* encoding the ARF protein. ARF inhibits MDM2-mediated degradation of p53. p53 will activate downstream targets possibly initiating premature melanocyte differentiation resulting in a loss of mature pigment cells. **(B)** The amino acid substitutions associated with the *B1* and *B2* alleles impair the ARF/MDM2 interaction, which counteracts the consequences of upregulated ARF expression. **(C)** In wild-type feathers, melanocyte progenitor cells migrate up from the feather base and start expressing ARF in the barb region leading to differentiation of melanocytes and pigment production without exhausting the pool of undifferentiated melanocytes. In Sex-linked barred feathers, upregulated ARF expression may lead to premature differentiation of pigment cells and a lack of undifferentiated melanocytes that can replenish the ones producing pigment. As the feather keeps on growing, no more melanocytes are available to produce pigment resulting in the white bar. A plausible explanation for the periodic appearance of white and black bars is that new recruitment of melanocyte progenitor cells takes place after the undifferentiated melanocytes have been depleted. From Schwochow-Thalmann et al. (2017).

non-coding changes or the combined effect of the two constitutes a cis-acting regulatory mutation. Furthermore, functional characterization using far-UV circular dichroism and isothermal titration calorimetry (ITC) of the different protein variants (N, B1, and B2) showed that the missense mutations impair the interaction between ARF and MDM2 (Schwochow-Thalmann et al., 2017) and thus counteract the effect of the upregulated expression of ARF caused by the regulatory mutation. The results

are consistent with an evolutionary scenario where the regulatory mutation occurs first resulting in the *B0* allele followed by two independent missense mutations causing the *B1* and *B2* alleles.

Schwochow-Thalmann et al. (2017) proposed a plausible mechanism of action of the mutant alleles (Figure 3), partially based on the finding by Lin et al. (2013) that Sex-linked barring is associated with premature differentiation of pigment cells. First, upregulation of ARF expression blocks MDM2 and leads

to upregulation of the p53 tumor suppressor and shorter life-span of pigment cells. Thus, this is the opposite effect compared with the consequence of *CDKN2A* loss-of-function mutations predisposing for human melanoma. Second, the missense mutations in the *B1* and *B2* alleles impair the interaction between ARF and MDM2 (**Figure 3B**). The Sex-linked barring phenotype is most likely caused by a repeated process where melanocyte progenitor cells are recruited, differentiate and produce pigment resulting in a black bar, followed by exhaustion of pigment cells due to premature differentiation resulting in a white bar, and then the process start again with the recruitment of new progenitor cells (**Figure 3C**).

In addition to Sex-linked barring, there is an extensive diversity in intra-feather pigmentation patterns in the domestic chicken. Smyth (1990) described the following main types: stippling (wild-type), penciling, autosomal barring, single lacing, double lacing, spangling, and mottling. The difference between stippling and the other variants is caused by mutations at distinct loci or by the combined effect of multiple loci, some of these genes have been identified at the molecular level. Autosomal barring resembles Sex-linked barring but the difference is that the white bars in Sex-linked barring lack pigment whereas the light-colored bars in Autosomal barring usually have pigment deposition. According to Smyth (1990), autosomal barring is caused by the combined effect of mutations at the *Extension*, *Dark brown*, *Patterning*, and *Columbian* loci. *Extension* corresponds to *MC1R* in which an allelic series has been identified at the molecular level (Takeuchi et al., 1996; Kerje et al., 2003; Dávila et al., 2014). *Dark brown* is caused by an 8.3 kb deletion upstream of *SOX10* (Gunnarsson et al., 2011). The *SOX10* transcription factor plays an important role during melanocyte development, and *SOX10* mutations is causing some forms of Waardenburg syndrome, involving pigmentary disturbances of hair, skin, and eyes (Pingault et al., 1998). The *Patterning* and *Columbian* loci have not yet been identified at the molecular level. *Mottling* (white tip of the feather) has been associated with a missense mutation in *EDNRB2* encoding endothelin receptor B2 (Kinoshita et al., 2014).

DISCUSSION

An extensive phenotypic diversity of pigmentation patterns is a characteristic feature in domestic animals. It has been speculated that this, in particular white spotting patterns, is a by-product of selection for tameness (Wilkins et al., 2014). The argument is based on proposed pleiotropic effects of mutations in genes expressed in neural-crest derived cells affecting both brain function and pigmentation. However, to the best of my knowledge, there are no convincing empirical data supporting this hypothesis as a major explanation for coat color diversity in domestic animals. For instance, the white spotting locus in dogs (*S/MITF*) contradicts this hypothesis, because mutations affecting melanocyte migration with no or minor pleiotropic effects have been selected.

Furthermore, some of the friendliest dogs, like Labradors and Golden Retrievers, tend to be homozygous for the *Solid* (wild-type) allele at this locus. In fact, a recent study confirms the degree of white pigmentation do not covary with differences in behavior across dog breeds (Wheat et al., 2019). The most important reason for the extensive variation in pigmentation pattern in domestic animals is positive selection for variation (Fang et al., 2009). Selection against camouflage like the striping patterns in piglets may have facilitated animal husbandry when domestic piglets were roaming freely around early settlements. Furthermore, Columella, the Roman authority on agriculture, wrote almost 2000 years ago that shepherds prefer dogs with white color because it helps them to distinguish dogs from wolves at low light conditions (Forster and Heffner, 1968). There has also been a selection for pure beauty, exemplified by the phenotype of the white horses (Rosengren Pielberg et al., 2008) and Sex-linked barring in the domestic chicken (Schwochow-Thalmann et al., 2017). Finally, relaxed purifying selection, related to the importance of pigmentation for camouflage and mate choice in natural populations, has most certainly contributed to the observed diversity in domestic animals.

A characteristic feature of alleles affecting pigmentation in domestic animals is that alleles with strong effects on pigmentation but no or only mild negative pleiotropic effects have been preferred. This is well illustrated by the *MITF* white spotting alleles in dogs, and by *KIT* alleles in pigs where a drastic effect on pigmentation without strong negative effect on hematopoiesis and fertility became possible after the gene was duplicated. The relatively long evolutionary history of domestic animals, compared with experimental organisms, has resulted in another characteristic feature of alleles affecting pigmentation in domestic animals, namely, the common occurrence of alleles differing by more than one sequence change from the wild-type allele. This is illustrated by four examples given in this review, including *KIT* alleles in pigs and cattle, *MC1R* alleles in pigs, and *CDKN2A* alleles in chicken. In this aspect, domestic animals are better models than experimental organisms for pigmentation phenotypes in natural populations. For traits that have been under selection for many generations, the presence of multiple causal variant is expected to be the rule rather than the exception. A possible example of this is the *MC1R* allele associated with white ornamental feathers in Satellite males in the ruff that differs by four derived missense mutations compared with the wild-type allele (Lamichhaney et al., 2016).

AUTHOR CONTRIBUTIONS

LA wrote the manuscript.

FUNDING

LA was supported by The Swedish Research Council and The Knut and Alice Wallenberg Foundation.

REFERENCES

- Bamshad, M., Lin, R. C., Law, D. J., Watkins, W. S., Krakowiak, P. A., Moore, M. E., et al. (1997). Mutations in human *TBX3* alter limb, apocrine and genital development in ulnar-mammary syndrome. *Nat. Genet.* 16, 311–315. doi: 10.1038/ng0797-311
- Baranowska Körberg, I., Sundström, E., Meadows, J. R. S., Rosengren, P. G., Gustafson, U., and Hedhammar, Å (2014). A simple repeat polymorphism in the *MITF*-M promoter is a key regulator of white spotting in dogs. *PLoS One* 9:e104363. doi: 10.1371/journal.pone.0104363
- Brooks, S. A., Lear, T. L., Adelson, D. L., and Bailey, E. (2008). A chromosome inversion near the *KIT* gene and the Tobiano spotting pattern in horses. *Cytogenet. Genome Res.* 119, 225–230. doi: 10.1159/000112065
- Clark, L., Wahl, J., Rees, C., and Murphy, K. (2006). Retrotransposon insertion in *SILV* is responsible for merle patterning of the domestic dog. *Proc. Natl. Acad. Sci. U.S.A.* 103, 1376–1381. doi: 10.1073/pnas.0506940103
- Dávila, S. G., Gil, M. G., Resino-Talaván, P., and Campo, J. L. (2014). Association between polymorphism in the melanocortin 1 receptor gene and E locus plumage color phenotype. *Poult. Sci.* 93, 1089–1096. doi: 10.3382/ps.2013-03611
- Durkin, K., Coppieters, W., Drögemüller, C., Ahariz, N., Cambisano, N., Druet, T., et al. (2012). Serial translocation by means of circular intermediates underlies colour sidedness in cattle. *Nature* 482, 81–84. doi: 10.1038/nature10757
- Fang, M., Larson, G., Soares Ribeiro, H., Li, N., and Andersson, L. (2009). Contrasting mode of evolution at a coat color locus in wild and domestic pigs. *PLoS Genet.* 5:e1000341. doi: 10.1371/journal.pgen.1000341
- Forster, E. S., and Heffner, E. H. (1968). *Lucius Junius Moderatus (Columella) on Agriculture*. London: Heinemann.
- Giuffra, E., Kijas, J. M. H., Amarger, V., Carlborg, O., Jeon, J.-T., and Andersson, L. (2000). The origin of the domestic pig: independent domestication and subsequent introgression. *Genetics* 154, 1785–1791.
- Giuffra, E., Törnsten, A., Marklund, S., Bongcam-Rudloff, E., Chardon, P., Kijas, J. M. H., et al. (2002). A large duplication associated with dominant white color in pigs originated by homologous recombination between LINE elements flanking *KIT*. *Mamm. Genome* 13, 569–577. doi: 10.1007/s00335-002-2184-5
- Groenen, M. A., Archibald, A. L., Uenishi, H., Tuggle, C. K., Takeuchi, Y., Rothschild, M. F., et al. (2012). Pig genomes provide insight into porcine demography and evolution. *Nature* 491, 393–398. doi: 10.1038/nature11622
- Gunnarsson, U., Kerje, S., Bed'hom, B., Sahlgvist, A. S., Ekwall, O., Tixier-Boichard, M., et al. (2011). The Dark brown plumage color in chickens is caused by an 8.3 kb deletion upstream of *SOX10*. *Pigment Cell Melanoma Res.* 24, 268–274. doi: 10.1111/j.1755-148X.2011.00825.x
- Haupaix, N., Curantz, C., Baillieu, R., Beck, S., Robic, A., and Manceau, M. (2018). The periodic coloration in birds forms through a prepattern of somite origin. *Science* 361:eaar4777. doi: 10.1126/science.aar4777
- Hauswirth, R., Haase, B., Blatter, M., Brooks, S. A., Burger, D., Drögemüller, C., et al. (2012). Mutations in *MITF* and *PAX3* cause “Splashed White” and other white spotting phenotypes in horses. *PLoS Genet.* 8:e1002653. doi: 10.1371/journal.pgen.1002653
- Hellström, A. R., Sundström, E., Gunnarsson, U., Bed'Hom, B., Tixier-Boichard, M., Honaker, C. F., et al. (2010). Sex-linked barring in chickens is controlled by the *CDKN2A/B* tumour suppressor locus. *Pigment Cell Melanoma Res.* 23, 521–530. doi: 10.1111/j.1755-148X.2010.00700.x
- Hofstetter, S., Seefried, F., Häfliger, I. M., Jagannathan, V., Leeb, T., and Drögemüller, C. (2019). A non-coding regulatory variant in the 5'-region of the *MITF* gene is associated with white-spotted coat in Brown Swiss cattle. *Anim. Genet.* 50, 27–32. doi: 10.1111/age.12751
- Hussussian, C. J., Struwing, J. P., Goldstein, A. M., Higgins, P. A. T., Ally, D. S., Sheahan, M. D., et al. (1994). Germline p16 mutations in familial melanoma. *Nat. Genet.* 8, 15–21.
- Imsland, F., McGowan, K., Rubin, C.-J., Henegar, C., Sundstrom, E., Berglund, J., et al. (2016). Regulatory mutations in *TBX3* disrupt asymmetric hair pigmentation that underlies Dun camouflage color in horses. *Nat. Genet.* 48, 152–158. doi: 10.1038/ng.3475
- Johansson Moller, M., Chaudhary, R., Hellmen, E., Hoyheim, B., Chowdhary, B., and Andersson, L. (1996). Pigs with the dominant white coat color phenotype carry a duplication of the *KIT* gene encoding the mast/stem cell growth factor receptor. *Mamm. Genome* 7, 822–830. doi: 10.1007/s003359900244
- Karlsson, E. K., Baranowska, I., Wade, C. M., Salmon Hillbertz, N. H. C., Zody, M. C., Andersson, N., et al. (2007). Efficient mapping of mendelian traits in dogs through genome-wide association. *Nat. Genet.* 39, 1321–1328. doi: 10.1038/ng.2007.10
- Kerje, S., Lind, J., Schütz, K., Jensen, P., and Andersson, L. (2003). Melanocortin 1-receptor (*MC1R*) mutations are associated with plumage colour in chicken. *Anim. Genet.* 34, 241–248. doi: 10.1046/j.1365-2052.2003.00991.x
- Kijas, J. M. H., and Andersson, L. (2001). A phylogenetic study of the origin of the domestic pig estimated from the near complete mtDNA genome. *J. Mol. Evol.* 52, 302–308. doi: 10.1007/s002390010158
- Kijas, J. M. H., Moller, M., Plastow, G., and Andersson, L. (2001). A frameshift mutation in *MC1R* and a high frequency of somatic reversions cause black spotting in pigs. *Genetics* 158, 779–785.
- Kijas, J. M. H., Wales, R., Törnsten, A., Chardon, P., Moller, M., and Andersson, L. (1998). Melanocortin receptor 1 (*MC1R*) mutations and coat color in pigs. *Genetics* 150, 1177–1185.
- Kinoshita, K., Akiyama, T., Mizutani, M., Shinomiya, A., Ishikawa, A., Younis, H. H., et al. (2014). Endothelin receptor B2 (*EDNRB2*) is responsible for the tyrosinase-independent recessive white (mow) and mottled (mo) plumage phenotypes in the chicken. *PLoS One* 9:e86361. doi: 10.1371/journal.pone.0086361
- Lamichhaney, S., Fan, G., Widemo, F., Gunnarsson, U., and Schwochow, Thalmann, D. (2016). Structural genomic changes underlie alternative reproductive strategies in the ruff (*Philomachus pugnax*). *Nat. Gen.* 48, 84–88. doi: 10.1038/ng.3430
- Lin, S. J., Foley, J., Jiang, T. X., Yeh, C. Y., Wu, P., Foley, A., et al. (2013). Topology of feather melanocyte progenitor niche allows complex pigment patterns to emerge. *Science* 340, 1442–1445. doi: 10.1126/science.1230374
- Little, C. C. (1957). *The Inheritance Of Coat Colour In The Dogs*. Ithaca: Cornell University Press.
- Lyons, L. A., Imes, D. L., Rah, H. C., and Grahn, R. A. (2005). Tyrosinase mutations associated with siamese and burmese patterns in the domestic cat (*Felis catus*). *Anim. Genet.* 36, 119–126. doi: 10.1111/j.1365-2052.2005.01253.x
- Mallarino, R., Henegar, C., Mirasierra, M., Manceau, M., Schradin, C., Vallejo, M., et al. (2016). Developmental mechanisms of stripe patterns in rodents. *Nature* 539, 518–523. doi: 10.1038/nature20109
- Marklund, S., Kijas, J., Rodriguez-Martinez, H., Rönstrand, L., Funa, K., Moller, M., et al. (1998). Molecular basis for the dominant white phenotype in the domestic pig. *Genome Res.* 8, 826–833. doi: 10.1101/gr.8.8.826
- Metallinos, D. L., Bowling, A. T., and Rine, J. (1998). A missense mutation in the endothelin-B receptor gene is associated with lethal white foal syndrome: an equine version of hirschsprung disease. *Mamm. Genome* 9, 426–431. doi: 10.1007/s003359900790
- Pingault, V., Bondurand, N., Kuhlbrodt, K., Goerich, D. E., Préhu, M.-O., Puliti, A., et al. (1998). *SOX10* mutations in patients with Waardenburg-Hirschsprung disease. *Nat. Genet.* 18, 171–173. doi: 10.1038/ng0298-171
- Rosengren Pielberg, G., Golovko, A., Sundström, E., Curik, I., Lennartsson, J., Selténhammer, M. H., et al. (2008). A cis-acting regulatory mutation causes premature hair greying and susceptibility to melanoma in the horse. *Nat. Genet.* 40, 1004–1009. doi: 10.1038/ng.185
- Rubin, C.-J., Megens, H., Martinez Barrio, A., Maqbool, K., Sayyab, S., Schwochow, D., et al. (2012). Strong signatures of selection in the domestic pig genome. *Proc. Natl. Acad. Sci. U.S.A.* 109, 19529–19536. doi: 10.1073/pnas.1217149109
- Santschi, E. M., Purdy, A. K., Valberg, S. J., Vrotsos, P. D., Kaese, H., and Mickelson, J. R. (1998). Endothelin receptor B polymorphism associated with lethal white foal syndrome in horses. *Mamm. Genome* 9, 306–309. doi: 10.1007/s003359900754
- Schwochow-Thalmann, D., Ring, H., Sundström, E., Cao, X., Larsson, M., Kerje, S., et al. (2017). The evolution of Sex-linked barring alleles in chickens involves both regulatory and coding changes in *CDKN2A*. *PLoS Genet.* 13:e1006665. doi: 10.1371/journal.pgen.1006665
- Smyth, J. R. Jr. (1990). “Genetics of plumage, skin and pigmentation in chickens,” in *Poultry Breeding and Genetics*, ed. R. D. Crawford (New York, NY: Elsevier Science), 109–167.
- Takeuchi, S., Suzuki, H., Yabuuchi, M., and Takahashi, S. (1996). A possible involvement of melanocortin 1-receptor in regulating feather color pigmentation in the chicken. *Biochim. Biophys. Acta* 1308, 164–168. doi: 10.1016/0167-4781(96)00100-5

- Wheat, C. H., van der Bijl, W., and Wheat, C. W. (2019). Morphology does not covary with predicted behavioural correlations of the domestication syndrome in dogs. *bioRxiv* [Preprint]. doi: 10.1101/660829
- Wilkins, A. S., Wrangham, R. W., and Fitch, W. T. (2014). The “domestication syndrome” in mammals: a unified explanation based on neural crest cell behavior and genetics. *Genetics* 197, 795–808. doi: 10.1534/genetics.114.165423
- Yang, G. C., Croaker, D., Zhang, A. L., Manglick, P., Cartmill, T., and Cass, D. (1998). A dinucleotide mutation in the endothelin-B receptor gene is associated with lethal white foal syndrome (LWFS); a horse variant of Hirschsprung disease. *Hum. Mol. Genet.* 7, 1047–1052. doi: 10.1093/hmg/7.6.1047

Conflict of Interest: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2020 Andersson. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



What Drives the Diversification of Eyespots in *Morpho* Butterflies? Disentangling Developmental and Selective Constraints From Neutral Evolution

Vincent Debat^{1*}, Nicolas Chazot^{2,3}, Silvère Jarosson¹, Patrick Blandin¹ and Violaine Llaurens¹

¹ Institut de Systématique, Evolution et Biodiversité, Centre National de la Recherche Scientifique/MNHN/Sorbonne Université/EPHE, Muséum National d'Histoire Naturelle, Paris, France, ² Department of Biological and Environmental Sciences, University of Gothenburg, Gothenburg, Sweden, ³ Gothenburg Global Biodiversity Centre, University of Gothenburg, Gothenburg, Sweden

OPEN ACCESS

Edited by:

Marcus Kronforst,
The University of Chicago,
United States

Reviewed by:

Patricia Johnston Moore,
University of Georgia, United States
Jeffrey Oliver,
The University of Arizona,
United States

*Correspondence:

Vincent Debat
debat@mnhn.fr

Specialty section:

This article was submitted to
Evolutionary Developmental Biology,
a section of the journal
Frontiers in Ecology and Evolution

Received: 13 December 2019

Accepted: 06 April 2020

Published: 27 May 2020

Citation:

Debat V, Chazot N, Jarosson S,
Blandin P and Llaurens V (2020) What
Drives the Diversification of Eyespots
in *Morpho* Butterflies? Disentangling
Developmental and Selective
Constraints From Neutral Evolution.
Front. Ecol. Evol. 8:112.
doi: 10.3389/fevo.2020.00112

Eyespots are wing color pattern elements repeatedly observed in many butterflies, whose developmental genetics has been extensively documented. Nevertheless, the evolutionary forces shaping their diversification across species still remain largely unknown. Here we investigate the evolution of eyespots in the 30 species of the neotropical genus *Morpho*. *Morpho* butterflies display a series of eyespots located on the ventral side of their wings, highly variable among species, ranging from large, conspicuous structures to vestigial spots. Applying geometric morphometrics to a large sample ($N = 910$) spanning all *Morpho* species and both sexes, we assess eyespot number, position, size, and shape. We detect a divergence in eyespot position between understory and canopy species, with an L-shaped configuration in canopy species and a line pattern in understory species, where the eyespots tend to fuse and form a stripe in white and pale species. This effect is stronger than expected based on a Brownian motion model of phylogenetic divergence, suggesting an adaptation to the microhabitat and an influence of the wing color on the evolution of pattern elements. Remarkably, this shift in color pattern is strongly correlated with a shift in wing shape. However, using a thin-plate spline interpolation, we show that the shape change is insufficient to explain the evolution of eyespot position as a developmental side effect of wing shape evolution, also pointing at an adaptive effect. Finally, we find a significant negative correlation between eyespot relative size and the within-species variation in eyespot number, position, size, and shape, suggesting a relaxed or apostatic selection on small eyespots (rare phenotypes being favored as they are less likely to be remembered and thus detected by predators). We hypothesize that contrasted ecology may explain the observed differences between species: large and phenotypically stable eyespots might act as attack deflectors, small, variable faded eyespots might rather enhance crypsis, and pale species stripe pattern might disrupt the outline of the wing.

Keywords: eyespot, morphometrics, Evo-Devo, evolution, selection

INTRODUCTION

Eyespots are among the most studied wing color pattern elements in butterflies, and their developmental genetics is therefore known in great detail [e.g., Nijhout, 1991; Brakefield et al., 1996; reviewed in Monteiro (2015)]: their development has been suggested to be controlled by reaction–diffusion processes involving the focal expression of morphogens typically located in between wing veins. The evolution of eyespots may thus be influenced by variation in wing shape (e.g., Monteiro et al., 1997; Breuker et al., 2007). Direct selection on eyespots has also been demonstrated in some lepidopteran species (reviewed in Stevens, 2005; Kodandaramaiah, 2011). For instance, large conspicuous dorsal eyespots were shown to intimidate or deter predators in some butterfly and moth species (e.g., Vallin et al., 2005); marginal ventral eyespots can deflect predator attacks away from the vital body parts (e.g., Olofsson et al., 2010); in *Bicyclus anynana*, a role in mate choice was shown for both dorsal (e.g., Robertson and Monteiro, 2005) and ventral eyespots (Huq et al., 2019). Yet the relative roles of natural and sexual selection, developmental constraints linked to wing shape and neutral processes, have not been investigated in a comparative framework. In particular, a prominent role for selection is often assumed (Kodandaramaiah, 2011), and the importance of neutral factors is seldom mentioned (but see Beldade et al., 2002; Allen, 2008). This is surprising as eyespot number and morphology can be highly variable within species (e.g., *Maniola jurtina*; Brakefield, 1984), suggesting a weak selection. Many species with a cryptic ventral color pattern harbor reduced, inconspicuous, and very variable eyespots, evocative of neutral variation, or apostatic selection, a form of selection where rare phenotypes are favored as they are less likely to be remembered by predators and therefore detected (Clarke, 1969; Bond and Kamil, 2002).

In this study, we investigate the evolution of eyespots in *Morpho* butterflies, a genus that displays a particularly marked diversity of eyespot number, color, size, and shape (Blandin, 2007). This Neotropical genus is famous for its large species, some of which have bright iridescent blue wings (e.g., Debat et al., 2018). In sharp contrast with the bright colors of the dorsal wing surface, the ventral side is in most cases brownish and rather cryptic—although a few species are very pale and three even have a white background. The ventral color patterns include a series of eyespots that broadly vary among species, from large and highly conspicuous to small and barely visible (**Supplementary Figure S1**). The phylogeny of the 30 recognized *Morpho* species was recently updated (Cassildé et al., 2010, 2012; Penz et al., 2012; Chazot et al., 2016). The genus includes a basal clade of two species and two larger clades (**Figure 1A**). One clade is composed of species that mostly fly at the canopy level, often seen gliding high in the forest strata. All other species, including the two early diverging species, are mostly found flying in the understory, with a typical flapping–gliding behavior (DeVries et al., 2010). Although this binary division between canopy and understory species is likely oversimplifying, it nevertheless seems a meaningful ecological approximation. It was indeed shown that the divergence of

wing shape between species from these two microhabitats was stronger than expected from a phylogenetic effect alone (DeVries et al., 2010; Chazot et al., 2016), suggesting an adaptation to those different ecological conditions, possibly *via* selection of contrasted flight behaviors.

Here we explore the diversification of *Morpho* eyespots and, in particular, the possible roles of neutral evolution and developmental constraints in relation to wing shape evolution and ecology. We focus on eyespot number, presence and absence at each putative location, eyespot position relative to wing veins and outline, and finally on eyespot size and shape using geometric morphometrics to finely depict and quantify phenotypic variation.

We investigate whether neutral divergence is sufficient to explain the observed patterns of eyespot evolution between species. We test adaptive hypotheses against this null hypothesis of neutral evolution: (1) is there any sign of adaptive divergence among microhabitats, possibly in relation with the existence of different predator communities in the canopy and the understory habitats? (2) How much variation of eyespot position can be attributed to variation of wing shape *via* a passive structural correlation (i.e., a developmental constraint)? (3) Does variation in eyespot size and shape reflect differential selective regimes? In particular, we hypothesize that the largest eyespots might be under stabilizing selection while the smallest might be under relaxed selection. We predict accordingly that small eyespots should be more variable within species than large ones. We briefly discuss our finding in the context of our (limited) knowledge of *Morpho* ecology.

MATERIALS AND METHODS

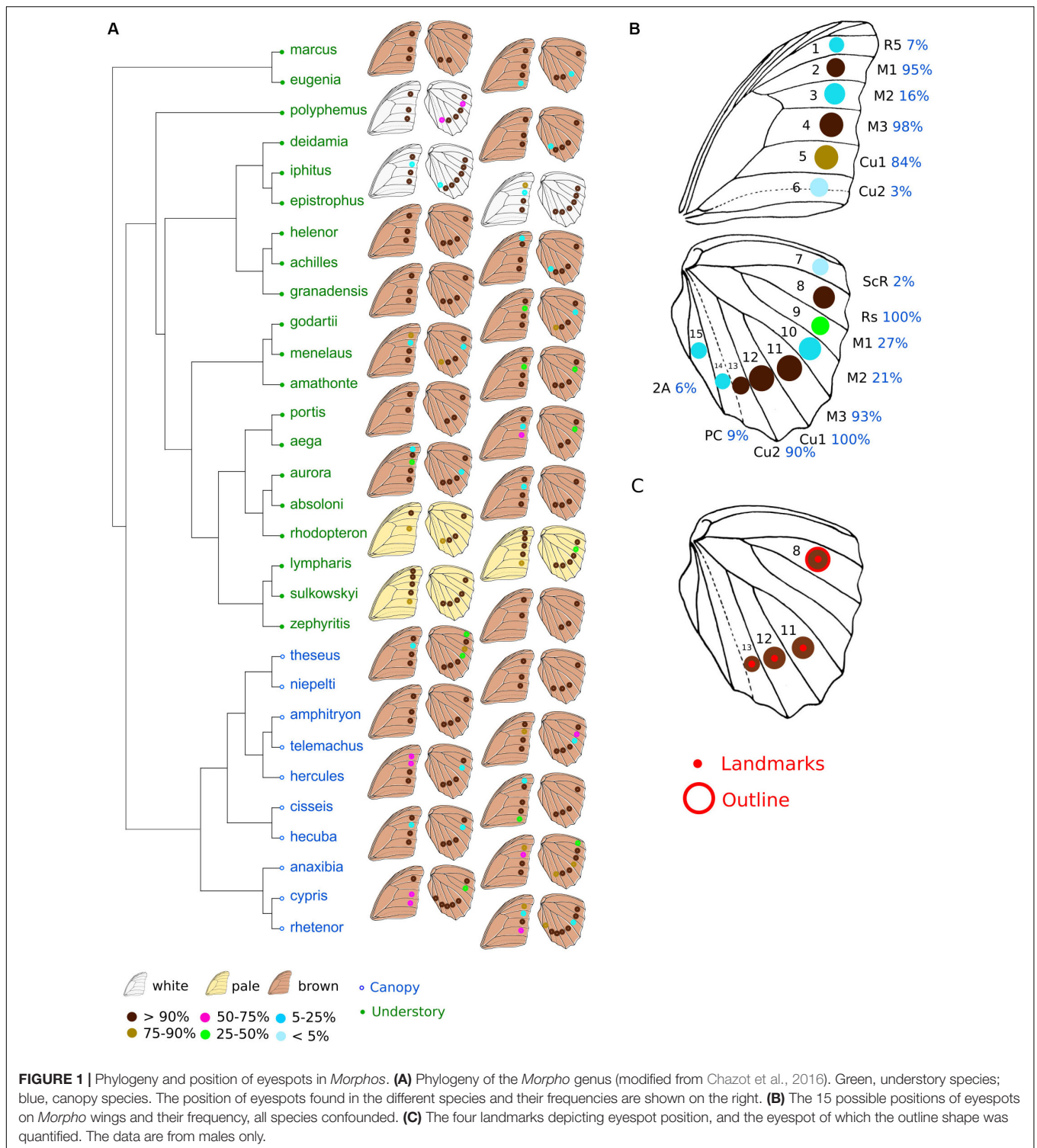
We sampled specimens from the collection of Paris Museum of Natural History to cover the 30 recognized species and include both sexes within species. We thus photographed 910 individual butterflies (642 males and 268 females) in standardized conditions using a NikonD90 camera (see Chazot et al., 2016). All measurements were performed on the ventral surface of the wings.

Eyespot Number and Configuration

The full sample was used to quantify eyespot number and position. Eyespots can occur on both hindwing and forewing at 15 different positions defined by compartments between veins (**Figure 1B**). For each individual, the presence of eyespot was assessed at every possible location, coded as 0 (absence) or 1 (presence) in a data matrix. We computed the average number of eyespots per wing and per species as well as their average positions and frequency.

Eyespot Number

We estimated the phylogenetic signal using the K statistic (Blomberg et al., 2003). We then tested the possible occurrence of a difference in eyespot number between canopy (10 species) and understory (20 species) species using a phylogenetic ANOVA as performed in the *phytools* package (Revell, 2012). Accounting



for the phylogeny is particularly important as the distribution of species across microhabitats mostly matches the phylogenetic structure in two main clades (Figure 1A). We also investigated the evolution of sexual dimorphism by computing the difference of mean eyespot number between sexes (a dimorphic species could thus be categorized as monomorphic if the two sexes have

the same number of eyespots but in different positions, but this does not occur in the genus *Morpho*).

Eyespot Configuration

Female specimens are few in the collection as males are more often collected than females in the field, so we focused our

investigation of eyespot configurations on male specimens alone. Eyespot configuration was defined as the combined presence and absence data at each of the 15 putative locations. We applied a principal component analysis (PCA) on eyespot frequencies at each of the 15 positions within species and projected the phylogenetic tree onto the first PC plane, explaining most of the variance in eyespot configurations. This allowed depicting the similarity in eyespot configuration among species together with the phylogenetic relationships between species. We then quantified the phylogenetic signal of eyespot configuration and tested its significance using the multivariate generalization of the Blomberg K [K -mult function of the R package *geomorph* (Adams, 2014; Adams et al., 2019)]. We then specifically compared the eyespot configurations of canopy and understory species using a phylogenetic MANOVA to disentangle the effects of neutral divergence from contrasted selective pressures exerted in these two habitats.

Relative Position of Four Posterior Eyespots—Association With Wing Shape

To investigate the evolution of eyespot position beyond their presence/absence within each wing compartment, we defined a landmark at the focus of the four most common eyespots of the hindwing (Figure 1C), allowing to precisely quantify their position on the wing. We then analyzed the variation of the resulting sets of landmarks using geometric morphometrics. The sample used for this analysis included 763 individuals from 25 species because some species do not exhibit these four eyespots simultaneously (*Morpho marcus*, *Morpho eugenia*, *Morpho portis*, *Morpho aega*, and *Morpho zephyritis* were discarded). The landmarks were superimposed using a generalized Procrustes superimposition (Rohlf and Slice, 1990). We applied a PCA on the species mean coordinates and projected the phylogenetic tree onto the first PC plane. The phylogenetic signal was computed and the canopy and the understory species were again compared using a phylogenetic MANOVA.

To investigate the possible link between the position of eyespot and wing shape evolution, we used another dataset previously obtained on the very same specimens (Chazot et al., 2016), describing wing size and shape using semi-landmarks placed on the wing outline and landmarks on vein intersections. We specifically quantified the covariation between hindwing shape and the position of the four eyespots by applying two-block partial least squares (PLS) regression (Rohlf and Corti, 2000) while accounting for the phylogenetic relationship among species [*phylo-integration* function of *geomorph* (Adams et al., 2019)].

We then explored whether the change in eyespot positions was a consequence of a change in wing shape—the eyespot position shifting due to a shift in vein positions. We applied an interpolation algorithm to the eyespot coordinates using the average shape of understory species as the reference (i.e., starting point) and that of the canopy species as a target (i.e., end-point) using the *tps2D* function of Claude (2008). This procedure allowed us to predict the shift in eyespot positions generated by a shift in wing shape between understory and

canopy species. Contrasting the predicted and the observed shifts allows testing whether the eyespot position observed in the canopy species results from the sole evolution of wing shape or whether specific evolutionary forces are acting on the eyespot relative position on the wing.

Eyespot Size and Shape

Finally, we quantified the variation in size and shape of one specific eyespot across the genus. We selected the posterior radial eyespot (Rs) present in all individuals (Figures 1B,C).

A subsample of ca. 15 individuals per species was selected, including the two sexes at an average of 2:1 proportion in favor of males, for a total of 403 individuals. The eyespot morphology was quantified by 149 semi-landmarks equidistantly spaced along the outline, plus two landmarks, one positioned at the center of the eyespot and the other located at the proximal intersection of the outline and the direction parallel to the adjacent veins running through the eyespot center. The landmark and the semi-landmark configurations were superimposed using a generalized Procrustes superimposition (Rohlf and Slice, 1990). The semi-landmarks were sited in *TPSrelw* (Rohlf, 2015) by minimizing the bending energy (e.g., Gunz and Mitteroecker, 2013). The resulting coordinates in the tangent space were used as shape data, and centroid size was used as a size estimate. The phylogenetic signal associated with these variables describing eyespot shape and size was assessed by the K -mult and the divergence between canopy and understory species tested using a phylogenetic MANOVA.

We then investigated the distribution of eyespot variability across the genus: in particular, we tested whether large eyespots are less variable than smaller ones as would be expected if large eyespots are under a stronger stabilizing selection than the small ones. To do so, we quantified the within-species coefficient of variation for eyespot size and the trace of the within-species covariance matrix for eyespot shape and assessed their correlation with eyespot relative size, i.e., the eyespot size standardized by wing size. This standardization was applied to account for the fact that species with large wings tend to have larger eyespots. The significance of the covariation was tested using phylogenetic regressions as performed in *phytools* (Revell, 2012).

We also explored the association between eyespot relative size and the variation of other morphological traits as eyespot number and eyespot position.

RESULTS

Eyespot Number

The most common configuration of eyespots includes three eyespots on the forewing and four eyespots on the hindwing (Figures 1A,B). These seven eyespots are observed in the same positions defined by the veins in all species in most individuals (>84% of individuals). The presence of an eyespot at each of the eight other possible locations on the wings is less frequent (2–27% of species). The total number of eyespots ranges from six (in *Morpho rhodopteron*) to 11 (in some *Morpho lympharis* individuals), involving variation of both forewing

eyespot (from two in *M. rhodopteron* and *M. portis* to five in *M. lympharis*) and hindwing eyespots (from two in some *M. aega* and *M. zephyritis* individuals to seven in *Morpho cypris* and *Morpho rhetenor*). A significant phylogenetic signal in eyespot number was detected in both sexes, with consistently stronger values in the hindwing close to the pattern expected under constant Brownian motion divergence $K = 1$ ($K_{\text{male forewing}} = 0.56$, $p = 0.014$; $K_{\text{male hindwing}} = 0.95$, $p < 0.001$; $K_{\text{female forewing}} = 0.58$, $p = 0.015$; $K_{\text{female hindwing}} = 0.89$, $p < 0.001$). No difference between canopy and understory species was detected when accounting for the phylogeny, neither for the total number of eyespots ($F = 2.14$, $p = 0.52$) and the forewing ($F = 0.49$, $p = 0.79$) nor the hindwing ($F = 4.58$, $p = 0.344$). Sexual dimorphism in eyespot number ranged from 0 (in *Morpho deidamia*, *Morpho helenor*, and *M. zephyritis*) to 1.5 in *M. cypris*, with most species presenting absolute values lower than 0.5 (Supplementary Figure S2). No difference in sexual dimorphism was detected between the hindwings and the forewings ($t = -0.08$, $df = 55.1$, $p\text{-value} = 0.9$). Canopy species were found to be more sexually dimorphic than understory species (difference between male and females mean numbers: 0.70 ± 0.17 SE in canopy vs. 0.26 ± 0.05 SE in understory), but this difference is not larger than expected given the phylogeny ($F = 7.98$, $p = 0.18$).

Eyespot Configuration

Eyespot configuration displayed a significant phylogenetic signal consistent with neutral divergence among species ($K\text{-mult} = 0.971$, $p = 0.001$). No difference between canopy and understory species was detected when accounting for the phylogeny ($Wilks = 0.43$, $F = 1.8$, $p = 0.99$). The PCA on male species mean eyespot frequencies is shown in Figure 2. Species with a stable and low number of eyespots are found on the negative PC1 values and species with a high and variable number of eyespots are found on the positive values, with a group of species displaying the “canonical” 3:4 configuration lying in the mid-PC plane. Interestingly, the three white species (*Morpho polyphemus*, *Morpho iphitus*, and *Morpho epistrophus*) cluster together with two very pale species (*Morpho sulkowskyi* and *M. lympharis*) due to a common high number of contiguous eyespots on the hindwing.

Quantitative Variation of Eyespot Position

The PCA applied on the species mean posterior eyespot position (Figure 3) shows a remarkable contrast between canopy species, where the four eyespots form an “L,” and understory species, where they tend to form a line. The phylogenetic signal was strong ($K\text{-mult} = 1.62$, $p < 0.001$), but the difference between the two microhabitats remained significant when accounting for the phylogeny ($F = 7.08$, $p < 0.001$), suggesting a stronger effect than expected under a neutral Brownian model of evolution. Interestingly, the white species *M. epistrophus* and *M. iphitus*, (and to a lesser degree *M. polyphemus*) and the pale species *M. sulkowskyi*, *M. lympharis*, and *M. rhodopteron* are all found on the positive values of PC1, harboring strikingly aligned eyespots.

The correlation between the wing shape and the position of the eyespots was strong ($r\text{-PLS} = 0.88$, $p < 0.001$) and triggered by the joint divergence for both wing shape and eyespot position between canopy and understory species (Supplementary Figure S3). The correlation was reduced when accounting for the phylogeny but remained significant ($r\text{-PLS} = 0.64$, $p = 0.049$; Supplementary Figure S3).

The interpolation of the wing shape change from understory to canopy, applied to the understory configuration of eyespots, is shown in Figure 4. The expected effect of the wing shape change is a shift from the “aligned” understory mean eyespot position to a more “L-shaped” disposition. However, the induced shift is clearly less marked than the observed difference between understory and canopy mean eyespot positions, albeit in the same direction. The observed change in eyespot position therefore cannot be fully accounted for by a correlated response of eyespot position to the evolution of wing shape, pointing at a specific selective effect on eyespots.

Quantitative Variation of Eyespot Size and Shape

The PCA on the first posterior eyespot shape (Figure 5) mainly contrasts eyespots that are elongated in the opposite direction: on the positive PC1 values, the white species *M. epistrophus* and *M. iphitus* display an eyespot markedly elongated transversally to the veins, while *M. telemachus* or *M. theseus* eyespots are slightly elongated longitudinally, on the negative values of PC1. There is a significant phylogenetic signal in eyespot shape ($K\text{-mult} = 0.77$, $p = 0.001$) and a significant difference between canopy and understory species when accounting for the phylogeny ($F = 0.42$, $p = 0.05$).

The analysis of eyespot variability suggested that relatively small eyespots tend to be more variable than larger ones: a strong negative correlation between the relative spot size and the within-species coefficient of variation of spot size was detected, which remained significant when accounting for the phylogeny ($r = -0.587$, $t = -3.39$, $p = 0.002$; Figure 6). Similarly, the shape of the small eyespots was found to be more variable (larger trace of the within-species covariance matrix) than that of the large eyespots, a trend that was higher than expected given the phylogenetic structure ($r = -0.37$; $t = -4.1$, $p < 0.001$; Figure 6). A similar pattern was found for eyespot number and position, whose variability was negatively correlated to eyespot relative size (Supplementary Figure S4). The species whose eyespots are the most variable include the white species (*M. iphitus*, *M. epistrophus*, and *M. polyphemus*) and two pigmented species whose eyespots tend to fade and almost disappear in some individuals (*M. aega* and *Morpho anaxibia*; see Supplementary Figure S1).

DISCUSSION

Overall our results suggest that the evolution of eyespots in *Morpho* butterflies results from a combination of neutral

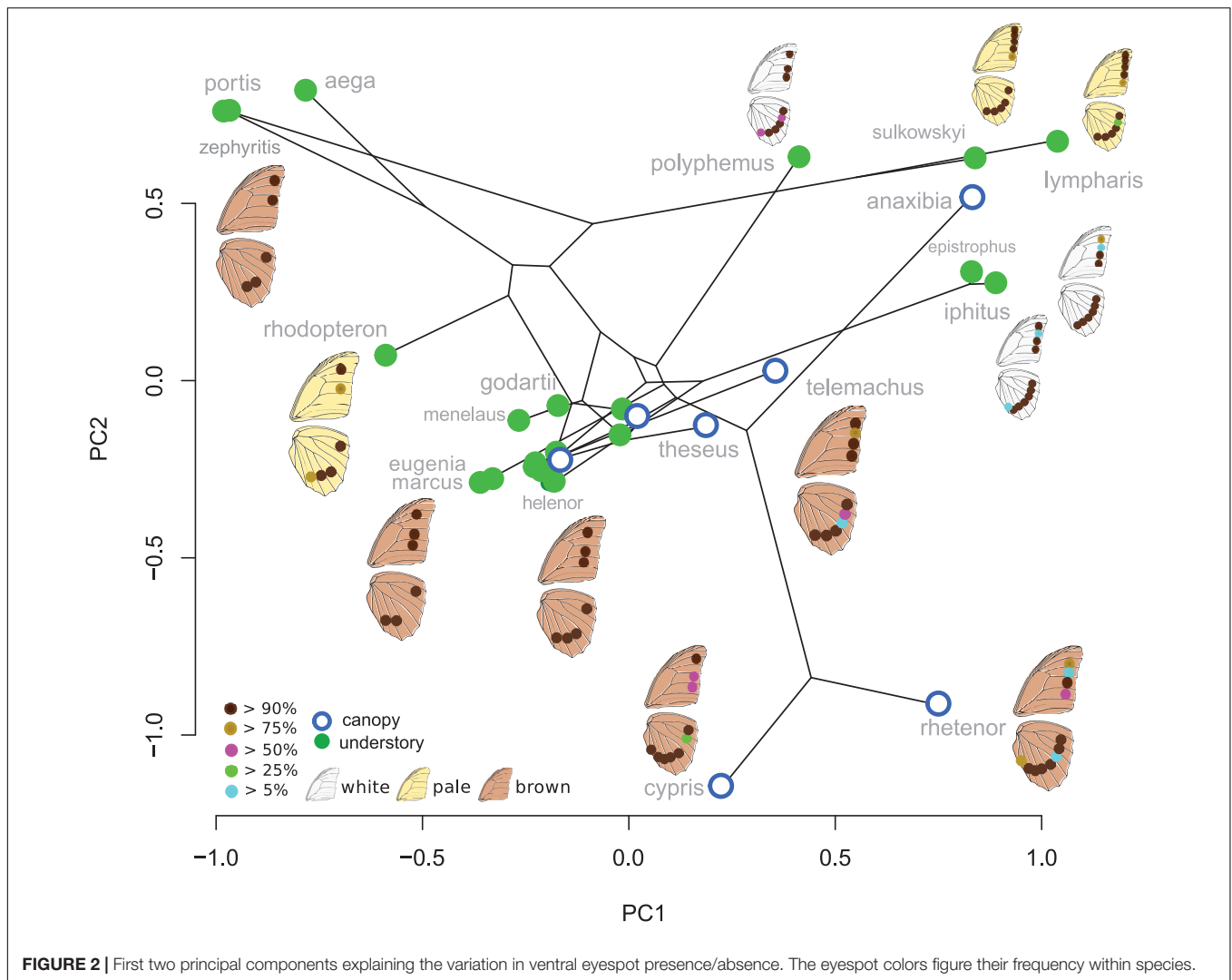


FIGURE 2 | First two principal components explaining the variation in ventral eyespot presence/absence. The eyespot colors figure their frequency within species.

divergence and selection. While the number of eyespots appears rather labile, with a strong phylogenetic signal and no divergence among microhabitats, possibly indicative of neutral evolution, their precise position and relative size are more strongly structured by ecology, which might stem from selective effects.

Diversification of Eyespots in *Morpho* Butterflies

The number and the distribution of eyespots are highly variable among species. The two early diverging species, *M. marcus* and *M. eugenia* display a 3:3 configuration on average, which is less than most other species with a typical 3:4 configuration (Figure 1). Interestingly, their two most posterior eyespots on the hindwing are located on positions Cu1 and Cu2, contrasting with all other species harboring only three posterior eyespots that are located on M3 and Cu1. This difference suggests a certain lability in the distribution of eyespots across species. The phylogenetic signal was close to the neutral $K = 1$ and no difference was detected between understory and canopy

species. These results are compatible with the hypothesis of a neutral evolution of eyespot number and distribution throughout species diversification. Such an evolutionary lability is also congruent with the known developmental basis of eyespot formation. Eyespots are indeed developmental serial homologs (e.g., Allen, 2008; Monteiro, 2015), i.e., they consist in the repeated expression at different locations of the same genetic network (Oliver et al., 2014). The presence of an eyespot at a specific position then depends on the local concentration of morphogens that triggers the expression of the eyespot genetic network [see Monteiro (2015) for a review]. Changes in size, shape, or color composition of a specific eyespot, which involve changes in the eyespot genetic network, have strong pleiotropic effects on other eyespots (e.g., Brakefield, 2001). In contrast, it is conceivable that adding or removing an eyespot, relying on a simple switch on or off of the network expression (for example, by a local change in a morphogen concentration), would be less developmentally constrained [but see Beldade and Brakefield (2003)]. The evolution of eyespot number and configuration in *Morpho* would thus be

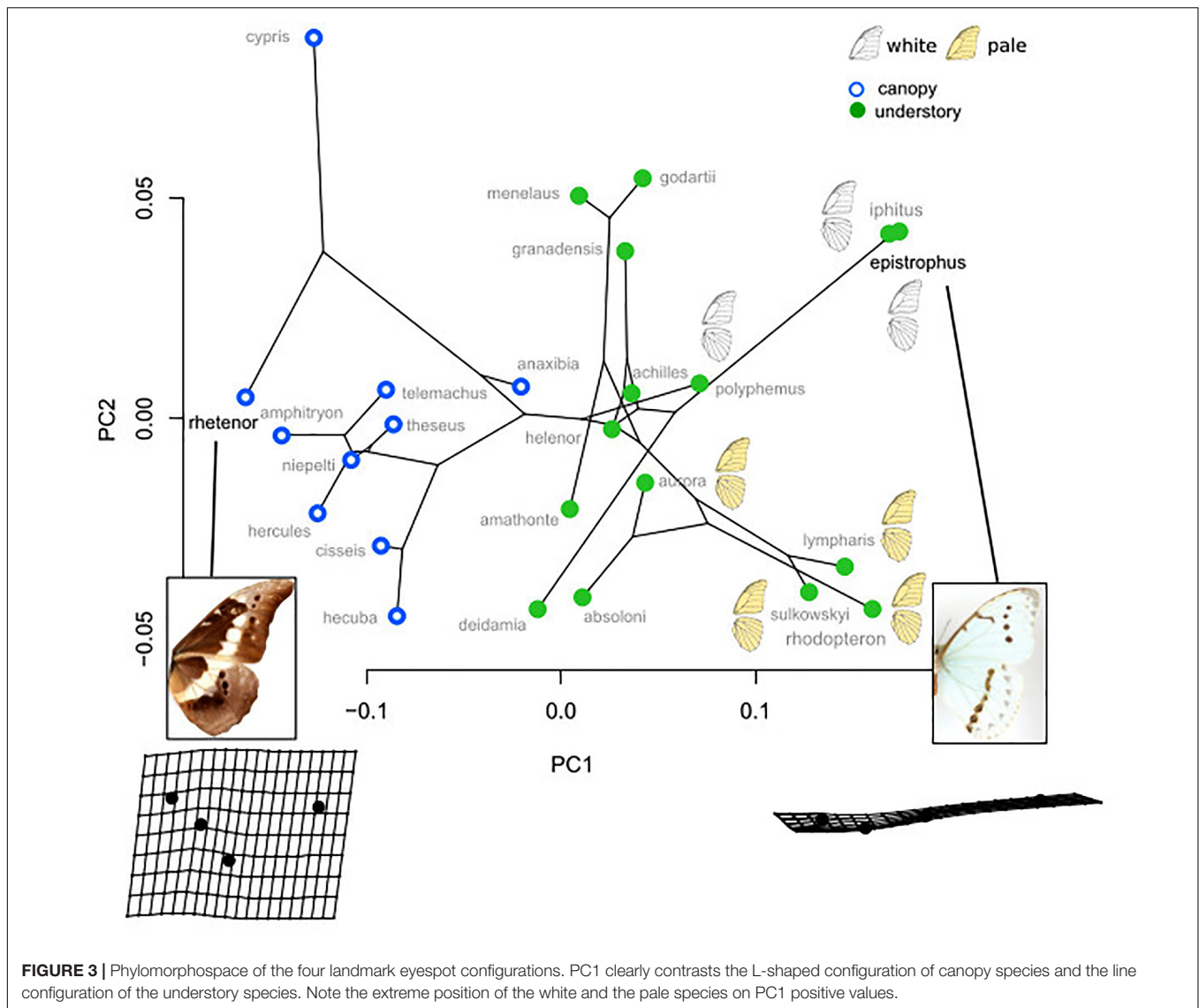


FIGURE 3 | Phylomorphospace of the four landmark eyespot configurations. PC1 clearly contrasts the L-shaped configuration of canopy species and the line configuration of the understory species. Note the extreme position of the white and the pale species on PC1 positive values.

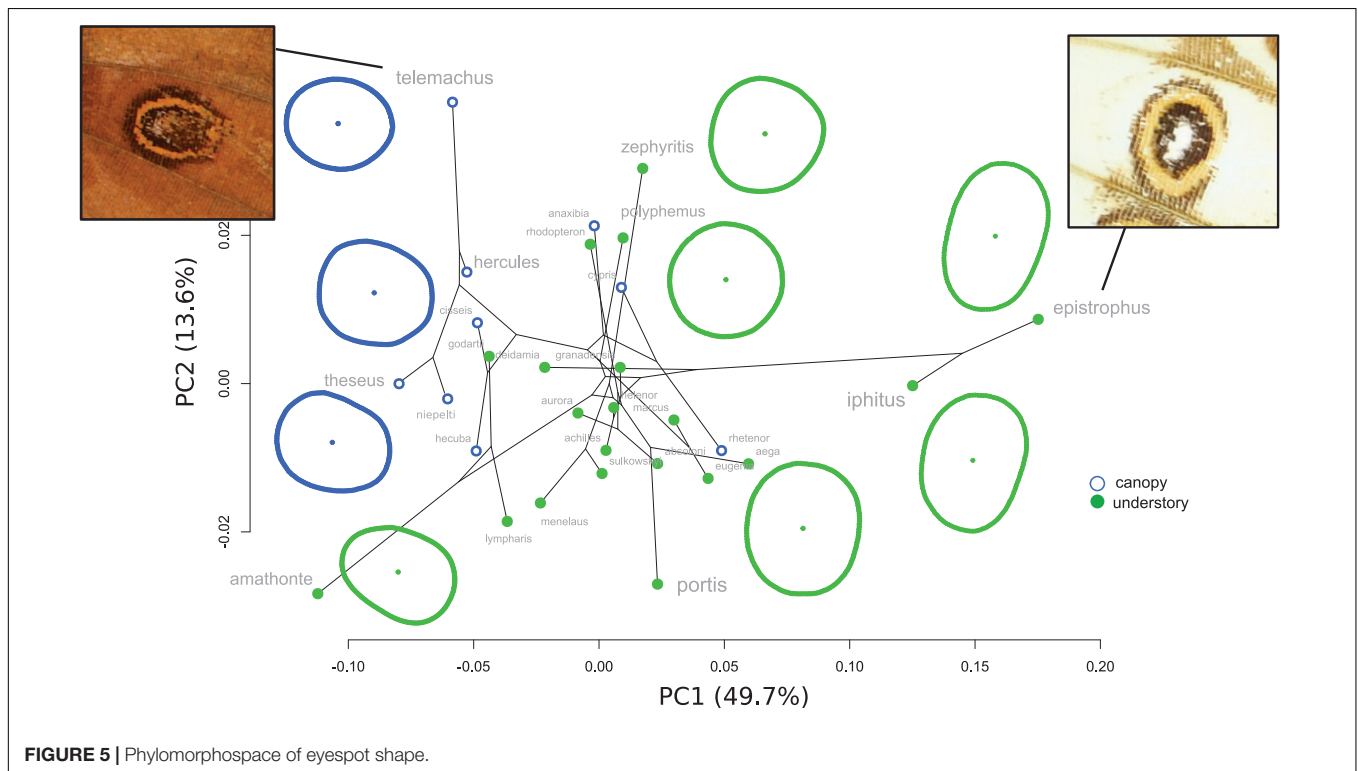
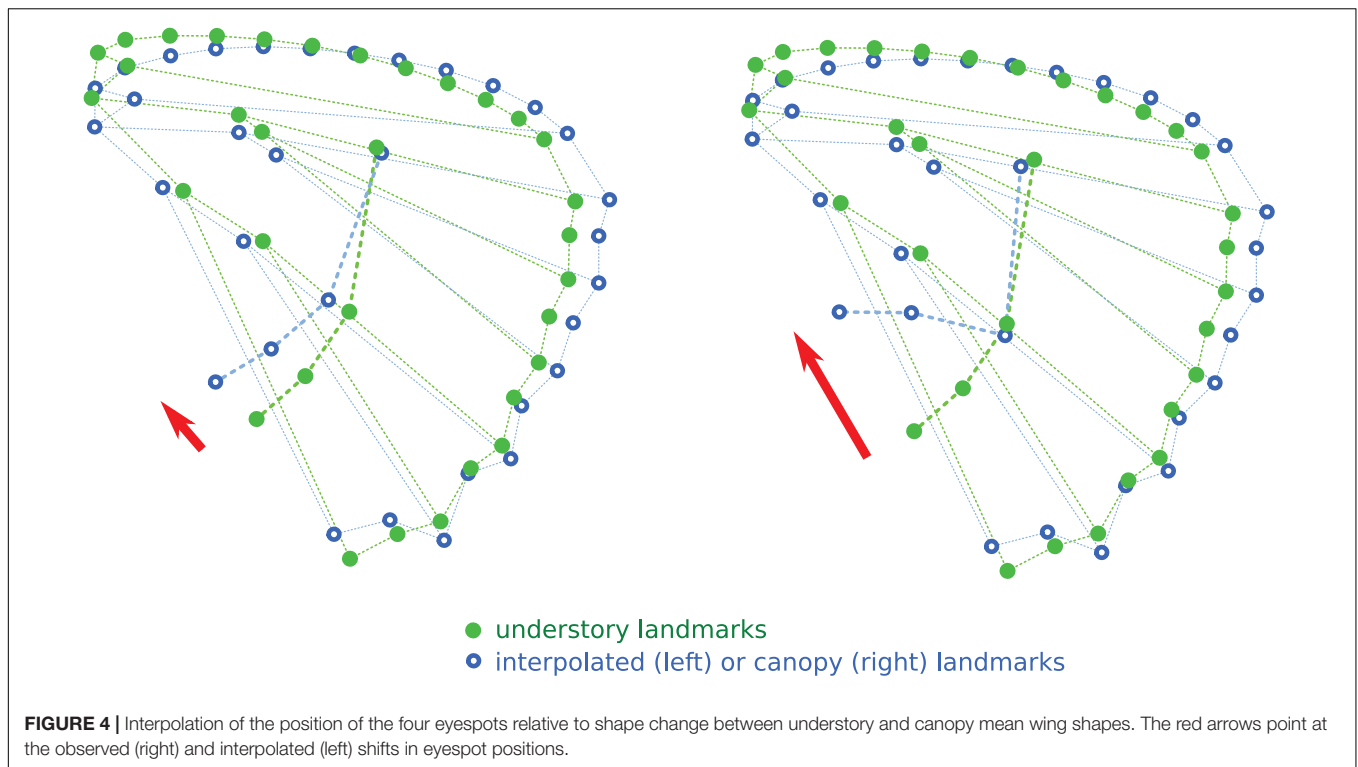
characterized by an important role of drift and a low impact of developmental constraints.

Shift in Eyespot Position: Developmental Constraint or Selection?

The absence of association between the number of eyespots and microhabitat strikingly contrasts with the evolution of the position of the four most common eyespots on the hindwing, which is driven by a strong divergence between microhabitats (Figure 3). Although partly explained by the phylogenetic signal, the divergence is stronger than expected from a neutral evolution, suggesting that selective forces are involved. The results of the interpolation analysis (Figure 4) concur with this interpretation. We had previously shown that wing shape has diverged between microhabitats (Chazot et al., 2016), a divergence interpreted as the mark of a selection on flight behavior, favoring gliding flight in the open canopy and a maneuverable flapping flight in the dense understory (DeVries et al., 2010; Chazot et al., 2016;

Le Roy et al., 2019). The interpolated shift in eyespot position induced by the wing shape change, while in the same direction as the observed difference between understory and canopy species, is markedly less pronounced (Figure 4). This demonstrates that the difference in color pattern is more than a simple by-product of a difference in wing shape that would impose a shift in eyespot position *via* a developmental correlation.

What selective pressure might thus be involved in this divergence between understory-aligned to canopy-L-shaped configurations of eyespots? The answer might partly lie in the apparent convergence between white and pale understory species, which is clear not only for the eyespot position (Figure 3) but also for their number and configuration (Figure 2). In these species, the aligned eyespots tend to form a striped pattern (Figure 7), which even involves the transversal elongation of the eyespots as shown in our shape analysis (Figure 5). This is particularly marked in the two white species *M. epistrophus* and *M. iphitus*, but remarkably a similar transversal elongation



is found on the subsequent posterior eyespots in the pale species (Figure 7). Altogether these suggest that in pale and white species a dark stripe pattern is favored over circular eyespots that tend to lose their individuality and form a stripe. Such an

alignment and elongation seem to extend to two other species (*M. portis* and *M. zephyritis*) that were not included in our position analysis due their reduced number of posterior eyespots (typically 3) and that also display marked stripes in association

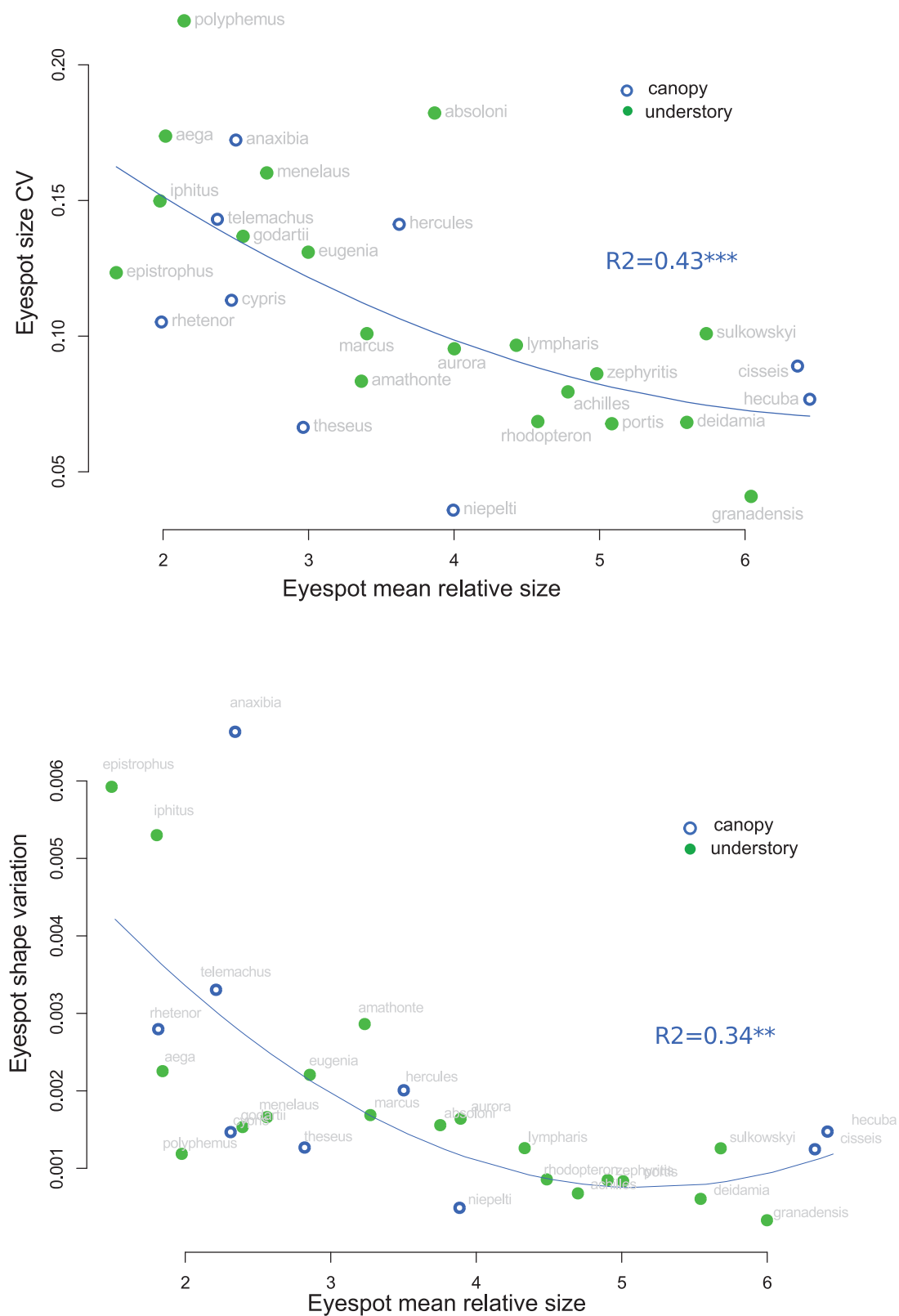


FIGURE 6 | Correlation between eyespot mean relative size and eyespot variation. **Top:** within-species coefficient of variation of size. **Bottom:** within-species trace of the shape covariance matrix (shape variation).

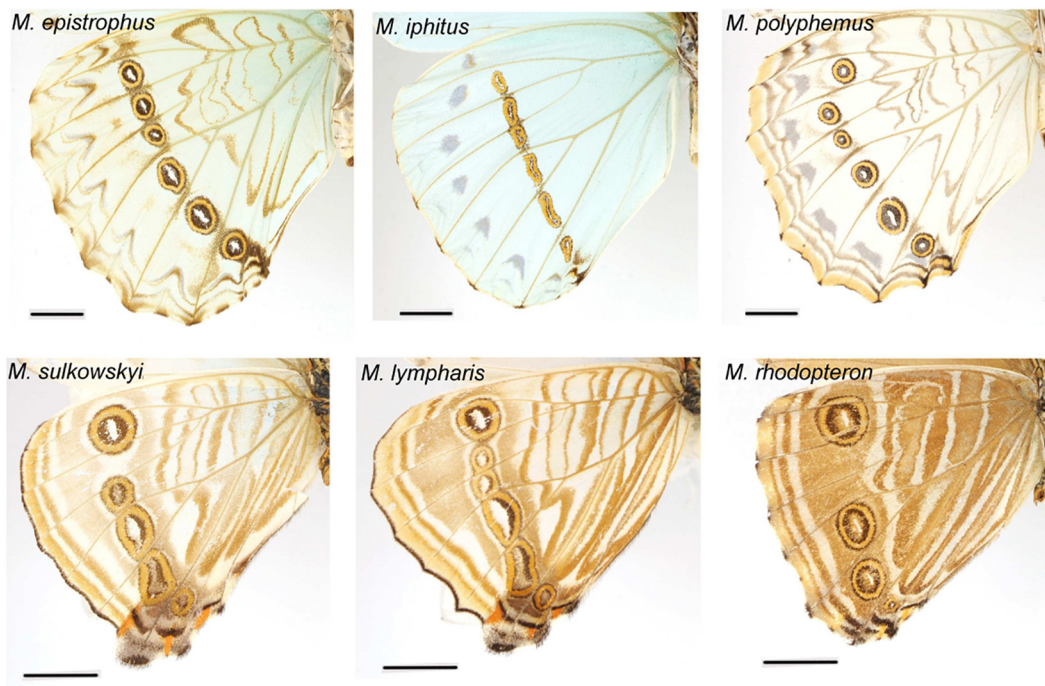


FIGURE 7 | White and pale species configurations of posterior eyespots, showing their aligned disposition. Note the strongly elongated shape of all eyespots in *M. iphitus* and in posterior eyespots in *M. sulkowskyi* and *M. lympharis*.

with their aligned eyespots. Interestingly, *M. portis* is a rather pale species, the ventral side of *M. zephyritis* males is also quite pale, with white and brown stripes, and the female *M. zephyritis* are white. The selective force promoting such a stripe pattern in the white and pale species is unknown, but stripes parallel to the wing margin have been suggested to disrupt the animal's outline and increase the difficulty of capture [e.g., Seymoure and Aiello, 2015; see Stevens and Merilaita (2009) for review]. The evolution of a pale coloration in *Morpho* may modify the way natural and sexual selection act on color pattern, possibly explaining the shift from separate eyespots to a single, fused 'stripe'. Specific ecological situations may promote the evolution of striped patterns in different *Morpho* clades: for example, all pale species are part of a clade of small-sized species that feed on bamboos (Chazot et al., in revision) and typically fly amidst very dense bushes where their striped color pattern might be cryptic. Estimating the effect of color pattern variation on predation risk in different microhabitats is now required to clarify the selective forces involved in the evolution of the striped patterns in *Morpho*.

Increased Variability of Small Eyespots: Relaxed Selection?

Stabilizing selection tends to reduce phenotypic variation both by removing genetic variants and by favoring canalized phenotypes, i.e., invariant to mutations and/or environmental influences (e.g., Waddington, 1957; Wagner et al., 1997; Meiklejohn and Hartl, 2002); relaxed selection is in turn expected to be

associated with a relatively high phenotypic variation (e.g., Crespi and Vanderkist, 1997; Billet et al., 2012). The general negative association detected in this study between eyespot mean relative size and its variation, both in size, shape, number, and location (Figure 5 and Supplementary Figure S4), might suggest that small eyespots experience reduced selective pressure relatively to large eyespots. Interspecific correlations of variability patterns are difficult to interpret as selection operates within species that may differ in ecology, demography, and genetic control of the studied phenotype. A similar selection regime might thus have contrasted effects on phenotypic variation in different species because of such differences. The observed correlation—that accounts for the phylogenetic relatedness—is nevertheless consistent with a differential selection on large and small eyespots. Large conspicuous eyespots have been suggested to have an intimidating effect upon predators, either due to their resemblance to vertebrate's eye or simply to their conspicuousness [reviewed in Stevens (2005)]. This, however, seems unlikely for ventral eyespots that are always visible when the butterfly is at rest. Alternatively, ventral eyespots might deflect predator attacks away from the body (e.g., Olofsson et al., 2010; Prudic et al., 2015). Huq et al. (2019) have recently shown that ventral eyespot color plays a role in mate choice in *B. anynana*, leaving open the possibility that eyespot size and shape might be under sexual selection. Conspicuous eyespots are thus expected to be under tight selection, which might lead to a reduced morphological variation (e.g., Nieberding et al., 2018). Notably, species exhibiting the smallest and most variable eyespots (Figure 6 and Supplementary Figure S4) are

either white species (**Figure 7**) or species wherein the eyespots are extremely faded and sometimes barely qualify as eyespots (*M. aega* and *M. anaxibia*; **Supplementary Figure S1**). In the former case, the increased variation might be related to a hypothetical modification of the selection regime that would no longer stabilize the individual eyespot size and shape but the overall stripe pattern. In the latter case, the cryptic color pattern rather than the eyespot conspicuousness might be favored by selection. The reduction in size, the fading, and the increased variation of eyespots in these species might thus reflect a relaxed selection. It has been suggested that crypsis is improved when color patterns are more variable, making it more difficult for the predators to remember and identify, a phenomenon called apostatic selection [reviewed in Bond (2007)]. Rare cryptic patterns could thus be favored by negative frequency-dependent selection, therefore promoting their variation [see Johannesson and Butlin (2017) for a discussion]. Although fully speculative, the occurrence of apostatic selection in species presenting reduced eyespots might explain their particularly strong variation. Additional research documenting the ecology of *Morpho*, and in particular the predation pressures acting on color pattern variations, are needed to investigate those hypotheses.

DATA AVAILABILITY STATEMENT

All data are available at DRYAD (accession doi: 10.5061/dryad.q573n5tfs).

AUTHOR CONTRIBUTIONS

VD, VL, and NC conceived the study. SJ acquired the data. VD and SJ analyzed the data. VD drafted

the manuscript. All authors reviewed and approved the manuscript.

FUNDING

Financial support was provided to VD by the Museum ATM Blanche 2016. Labex BCDiv (ANR-10-LABX-0003) to VD.

ACKNOWLEDGMENTS

We would like to thank Stephen Panara and Nicolas Zilbermann for their help with the picture acquisition, Marianne Elias for helpful discussions, and two reviewers for their constructive comments on previous drafts of the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fevo.2020.00112/full#supplementary-material>

FIGURE S1 | Examples of large and small eyespots. **(A)** Large conspicuous and stable eyespots in *M. hecuba* and *M. deidamia*. **(B)** Small, faded, and variable eyespots in *M. anaxibia* and *M. aega*.

FIGURE S2 | Anterior and posterior eyespot numbers in males and females.

FIGURE S3 | Covariation between wing shape and the position of the four posterior eyespots. Top: two-blocks partial least squares (PLS) on the total dataset. Bottom: two-blocks phylogenetic PLS on species means.

FIGURE S4 | Covariation between eyespot size and eyespot position variation (top) and eyespot number variation (bottom).

REFERENCES

- Adams, D. C. (2014). A generalized K statistic for estimating phylogenetic signal from shape and other high-dimensional multivariate data. *Syst. Biol.* 63, 685–697. doi: 10.1093/sysbio/syu030
- Adams, D. C., Collyer, M., and Kaliontzopoulou, A. (2019). *Geomorph: Software for Geometric Morphometric Analyses. R Package Version 3.1.0*.
- Allen, C. E. (2008). The “Eyespot Module” and eyespots as modules: development, evolution, and integration of a complex phenotype. *J. Exp. Zool. B Mol. Dev. Evol.* 310, 179–190. doi: 10.1002/jez.b.21186
- Beldade, P., and Brakefield, P. M. (2003). Concerted evolution and developmental integration in modular butterfly wing patterns. *Evol. Dev.* 5, 169–179. doi: 10.1046/j.1525-142x.2003.03025.x
- Beldade, P., Koops, K., and Brakefield, P. M. (2002). Developmental constraints versus flexibility in morphological evolution. *Nature* 416:844. doi: 10.1038/416844a
- Billet, G., Hautier, L., Asher, R. J., Schwarz, C., Crumpton, N., Martin, T., et al. (2012). High morphological variation of vestibular system accompanies slow and infrequent locomotion in three-toed sloths. *Proc. R. Soc. B Biol. Sci.* 279, 3932–3939. doi: 10.1098/rspb.2012.1212
- Blandin, P. (2007). *The Systematics of the Genus Morpho Fabricius, 1807*. Canterbury: HillsideBooks.
- Blomberg, S. P., Garland, T. Jr., and Ives, A. R. (2003). Testing for phylogenetic signal in comparative data: behavioral traits are more labile. *Evolution* 57, 717–745. doi: 10.1111/j.0014-3820.2003.tb00285.x
- Bond, A. B. (2007). The evolution of color polymorphism: crypticity, searching images, and apostatic selection. *Annu. Rev. Ecol. Syst.* 38, 489–514.
- Bond, A. B., and Kamil, A. C. (2002). Visual predators select for crypticity and polymorphism in virtual prey. *Nature* 415, 609–613. doi: 10.1038/415609a
- Brakefield, P. M. (1984). “Ecological genetics of quantitative characters of *Maniola jurtina* and other butterflies,” in *Proceedings of the Symposia of the Royal Entomological Society of London*, Rock Hill, SC.
- Brakefield, P. M. (2001). Structure of a character and the evolution of butterfly eyespot patterns. *J. Exp. Zool.* 291, 93–104. doi: 10.1002/jez.1062
- Brakefield, P. M., Gates, J., Keys, D., Kesbeke, F., Wijngaarden, P. J., Montelro, A., et al. (1996). Development, plasticity and evolution of butterfly eyespot patterns. *Nature* 384:236. doi: 10.1038/384236a0
- Breuker, C. J., Gibbs, M., Van Dyck, H., Brakefield, P. M., Klingenberg, C. P., and Van Dongen, S. (2007). Integration of wings and their eyespots in the speckled wood butterfly *Pararge aegeria*. *J. Exp. Zool. B Mol. Dev. Evol.* 308, 454–463. doi: 10.1002/jez.b.21171
- Cassildé, C., Blandin, P., Pierre, J., and Bourgoin, T. (2010). Phylogeny of the genus *Morpho fabricius*, 1807, revisited (Lepidoptera, Nymphalidae). *Bull. Soc. Entomol. France* 115, 225–250.
- Cassildé, C., Blandin, P., and Silvain, J. F. (2012). Phylogeny of the genus *Morpho fabricius* 1807: insights from two mitochondrial genes (Lepidoptera: Nymphalidae). *Ann. Soc. Entomol. France* 48, 173–188.
- Chazot, N., Panara, S., Zilbermann, N., Blandin, P., Le Poul, Y., Cornette, R., et al. (2016). *Morpho* morphometrics: shared ancestry and selection drive the evolution of wing size and shape in *Morpho* butterflies. *Evolution* 70, 181–194. doi: 10.1111/evo.12842

- Chazot, N., Blandin, P., Elias, M., Debat, V., and Condamine, F. L. (in revision). Are rates of species, and phenotypic diversification correlated in (the) *Morpho* butterflies?
- Clarke, B. (1969). The evidence for apostatic selection. *Heredity* 24:347.
- Claude, J. (2008). *Morphometrics with R*. Cham: Springer Science & Business Media.
- Crespi, B. J., and Vanderkist, B. A. (1997). Fluctuating asymmetry in vestigial and functional traits of a haplodiploid insect. *Heredity* 79:624.
- Debat, V., Berthier, S., Blandin, P., Chazot, N., Elias, M., Gomez, D., et al. (2018). Why are *Morpho Blue*? In *Biodiversity and Evolution*. Amsterdam: Elsevier, 139–174.
- DeVries, P. J., Penz, C. M., and Hill, R. I. (2010). Vertical distribution, flight behaviour and evolution of wing morphology in *Morpho* butterflies. *J. Anim. Ecol.* 79, 1077–1085. doi: 10.1111/j.1365-2656.2010.01710.x
- Gunz, P., and Mitteroecker, P. (2013). Semi-landmarks: a method for quantifying curves and surfaces. *Hystrix Ital. J. Mammal.* 24, 103–109.
- Huq, M., Bhardwaj, S., and Monteiro, A. (2019). Male *Bicyclus anynana* butterflies choose females on the basis of their ventral UV-reflective eyespot centers. *J. Insect Sci.* 19:25. doi: 10.1093/jisesa/iez014
- Johannesson, K., and Butlin, R. K. (2017). What explains rare and conspicuous colours in a snail? A test of time-series data against models of drift, migration or selection. *Heredity* 118, 21–30. doi: 10.1038/hdy.2016.77
- Kodandaramaiah, U. (2011). The evolutionary significance of butterfly eyespots. *Behav. Ecol.* 22, 1264–1271.
- Le Roy, C., Debat, V., and Llaurens, V. (2019). Adaptive evolution of butterfly wing shape: from morphology to behaviour. *Biol. Rev.* 94, 1261–1281. doi: 10.1111/brv.12500
- Meiklejohn, C. D., and Hartl, D. L. (2002). A single mode of canalization. *Trends Ecol. Evol.* 17, 468–473.
- Monteiro, A. (2015). Origin, development, and evolution of butterfly eyespots. *Annu. Rev. Entomol.* 60, 253–271. doi: 10.1146/annurev-ento-010814-020942
- Monteiro, A., Brakefield, P. M., and French, V. (1997). The relationship between eyespot shape and wing shape in the butterfly *Bicyclus anynana*: a genetic and morphometrical approach. *J. Evol. Biol.* 10, 787–802.
- Nieberding, C. M., San Martin, G., Saenko, S., Allen, C. E., Brakefield, P. M., and Visser, B. (2018). Sexual selection contributes to partial restoration of phenotypic robustness in a butterfly. *Sci. Rep.* 8:14315. doi: 10.1038/s41598-018-32132-8
- Nijhout, H. F. (1991). *The Development and Evolution Of Butterfly Wing Patterns*. Washington, DC: Smithsonian Institution Press.
- Oliver, J. C., Beaulieu, J. M., Gall, L. F., Piel, W. H., and Monteiro, A. (2014). Nymphalid eyespot serial homologues originate as a few individualized modules. *Proc. R. Soc. B Biol. Sci.* 281:20133262. doi: 10.1098/rspb.2013.3262
- Olofsson, M., Vallin, A., Jakobsson, S., and Wiklund, C. (2010). Marginal eyespots on butterfly wings deflect bird attacks under low light intensities with UV wavelengths. *PLoS One* 5:e10798. doi: 10.1371/journal.pone.0010798
- Penz, C. M., DeVries, P. J., and Wahlberg, N. (2012). Diversification of *Morpho* butterflies (Lepidoptera, Nymphalidae): a re-evaluation of morphological characters and new insight from DNA sequence data. *Syst. Entomol.* 37, 670–685.
- Prudic, K. L., Stoeck, A. M., Wasik, B. R., and Monteiro, A. (2015). Eyespots deflect predator attack increasing fitness and promoting the evolution of phenotypic plasticity. *Proc. R. Soc. B Biol. Sci.* 282:20141531. doi: 10.1098/rspb.2014.1531
- Revell, L. J. (2012). phytools: an R package for phylogenetic comparative biology (and other things). *Methods Ecol. Evol.* 3, 217–223.
- Robertson, K. A., and Monteiro, A. (2005). Female *Bicyclus anynana* butterflies choose males on the basis of their dorsal UV-reflective eyespot pupils. *Proc. R. Soc. B Biol. Sci.* 272, 1541–1546. doi: 10.1098/rspb.2005.3142
- Rohlf, F. J. (2015). The tps series of software. *Hystrix* 26, 9–12. doi: 10.4404/hystrix-26.1-11264
- Rohlf, F. J., and Corti, M. (2000). Use of two-block partial least-squares to study covariation in shape. *Syst. Biol.* 49, 740–753. doi: 10.1080/106351500750049806
- Rohlf, F. J., and Slice, D. (1990). Extensions of the Procrustes method for the optimal superimposition of landmarks. *Syst. Biol.* 39, 40–59.
- Seymour, B. M., and Aiello, A. (2015). Keeping the band together: evidence for false boundary disruptive coloration in a butterfly. *J. Evol. Biol.* 28, 1618–1624. doi: 10.1111/jeb.12681
- Stevens, M. (2005). The role of eyespots as anti-predator mechanisms, principally demonstrated in the Lepidoptera. *Biol. Rev.* 80, 573–588. doi: 10.1017/S1464793105006810
- Stevens, M., and Merilaita, S. (2009). Defining disruptive coloration and distinguishing its functions. *Philos. Trans. R. Soc. B Biol. Sci.* 364, 481–488. doi: 10.1098/rstb.2008.0216
- Vallin, A., Jakobsson, S., Lind, J., and Wiklund, C. (2005). Prey survival by predator intimidation: an experimental study of peacock butterfly defence against blue tits. *Proc. R. Soc. B Biol. Sci.* 272, 1203–1207. doi: 10.1098/rspb.2004.3034
- Waddington, C. H. (1957). *The Strategy of the Genes*. London: Allen and Unwin.
- Wagner, G. P., Booth, G., and Bagheri-Chaichian, H. (1997). A population genetic theory of canalization. *Evolution* 51, 329–347. doi: 10.1111/j.1558-5646.1997.tb02420.x

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2020 Debat, Chazot, Jarosson, Blandin and Llaurens. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Anterior–Posterior Patterning in Lepidopteran Wings

Kenneth Z. McKenna¹, Anna M. Kudla² and H. Frederik Nijhout^{2*}

¹ Division of Biological Sciences, Section of Cellular and Developmental Biology, University of California, San Diego, La Jolla, CA, United States, ² Department of Biology, Duke University, Durham, NC, United States

OPEN ACCESS

Edited by:

Marcus Kronforst,
The University of Chicago,
United States

Reviewed by:

Joseph John Hanly,
George Washington University,
United States
Arnaud Martin,
University of California, Berkeley,
United States

*Correspondence:

H. Frederik Nijhout
hfn@duke.edu

Specialty section:

This article was submitted to
Evolutionary Developmental Biology,
a section of the journal
Frontiers in Ecology and Evolution

Received: 28 February 2020

Accepted: 29 April 2020

Published: 03 June 2020

Citation:

McKenna KZ, Kudla AM and
Nijhout HF (2020) Anterior–Posterior
Patterning in Lepidopteran Wings.
Front. Ecol. Evol. 8:146.
doi: 10.3389/fevo.2020.00146

The color patterns on the wings of butterflies and moths are among the most complex manifestations of pattern formation in nature. The complexities of these patterns arise from the diversification of a conserved set of homologous elements known as the Nymphalid Ground Plan that can change color, shift position, expand, or disappear altogether. Recent work has shown that the anterior–posterior (AP) axis of the butterfly wing may also have an important role in the development and evolution of wing-pattern diversity. Here we characterize the AP axis by mapping expression domains of key regulatory genes onto the wing. We show that the butterfly wing can be subdivided into four primary regions, with the boundaries of these domains arising at the positions of the M1, M3, and Cu2 wing-veins. We find that the correlation among variation in the border ocelli is strongest for those within the same domain. We show how these domains may be used to determine phenotypic outcomes by surveying the frequency of color boundaries, tail development, and wing shape discontinuities across five major butterfly families: Lycaenidae, Nymphalidae, Papilionidae, Pieridae, and Riodinidae. Of the more than 200 genera we surveyed in this study, color pattern discontinuities emerge most often at the boundary veins M1, M3, and Cu2, and shape discontinuities and tails at veins M3 and Cu2. These findings reveal a hitherto unrecognized mode of evolution of patterning in the Lepidoptera.

Keywords: expression domains, pattern diversity, tails, color boundaries, nymphalid groundplan

INTRODUCTION

The color patterns on the wings of butterflies and moths are among the most striking and complex in nature. Although at first glance these patterns may look like random, abstract art, they are in fact composed of a series of discrete characters called pattern elements. Lepidopteran color patterns therefore differ significantly from color patterns of other organisms in that the pattern is composed of an anatomical set of homologs, or ground plan, quite analogous to that of the vertebrate skeletal system (Nijhout, 1991, 2001). This means the same stripe or spot can be found in the same location in all individuals of a species, and can also be traced from species to species and even genus to genus (Nijhout, 1991, 2001). The ground plan is therefore composed of sets of homologous characters, which makes it an ideal system to study the evolution and development of color patterns.

The ground plan for lepidopteran wing color patterns has been best studied in nymphalid butterflies, often referred to as the Nymphalid Ground Plan (NGP; Nijhout, 1991). Each color pattern element is an individuated character in the same way that a bone is an individuated character (Capdevila and Belmonte, 2001). As bones are regionally specified along the proximo-distal (PD) axis of the limb into the zeugopod, stylopod, and autopod, so too are color pattern elements. The NGP is made up of a modular series

of bands and spots along the PD axis of the wing that begins proximally with the basal symmetry system (BSS), followed distally by central symmetry system (CSS), and the border symmetry system (BoSS; **Figure 1**). The BoSS has the most complex pattern element shapes and size and is composed of border ocelli and parafoveal elements (Nijhout, 1991, 2017). Distal to this system lie the marginal bands along the distal wing margin (**Figure 1**). Changes in the size, shape, color, or position of the pattern elements belonging to this structural plan is hypothesized to have produced the enormous diversity of color patterns in butterflies (Nijhout, 1991, 2001, 2017; Otaki, 2012) and possibly even moths (Gawne and Nijhout, 2019).

Evolutionary change in any of these characteristics of symmetry systems can occur at various positions along the wing. In most cases each band or series of spots is interrupted by wing veins (Nijhout, 1991, 1994). This causes the band to look like a series of short segments that are imperfectly aligned. Developmental studies have shown that each pattern element develops independently within the regions between wing veins referred to as wing cells (Carroll et al., 1994; Panganiban et al.,

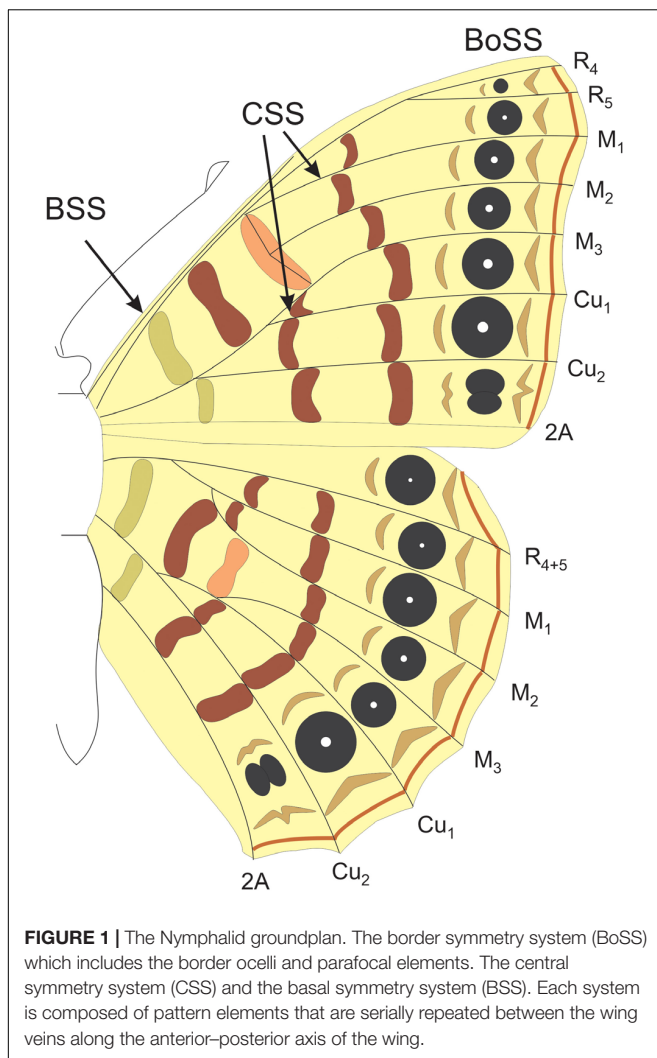
1994; Keys et al., 1999; Weatherbee et al., 1999; Brunetti et al., 2001; Saenko et al., 2011). Wing veins, and the developmental mechanisms that position them, therefore play an important role in individuating pattern elements along the anterior–posterior (AP) axis.

Thus, the size, shape, and position of a pattern element depends on which wing cell it develops in. A good example of this are the border ocelli, which in some species have been elaborated into eyespots. In *Bicyclus anayana*, eyespots develop in every wing cell on the hindwing, yet each differs dramatically in size (Brakefield et al., 1996). An artificial selection experiment in *Bicyclus* demonstrated that the size of eyespots in the anterior portion of the wing can evolve independent of those in the posterior portion of the wing (Beldade et al., 2002; Beldade and Brakefield, 2003; Allen et al., 2008). In *Junonia coenia*, pattern elements that are farther apart along the AP axis have less correlated variation than elements in the same region (Paulsen and Nijhout, 1993; Paulsen, 1994). Anterior–posterior information may play a key role in the individuation of the pattern elements within a symmetry system, allowing for independent evolution of serially homologous characters and the pattern as a whole (Nijhout, 1991, 1994; Beldade and Brakefield, 2003).

A peculiar feature of the color pattern that occurs in some species is the precise discontinuity of color and shape along the midline of the wing at or near the M3 vein. In many species, the pattern anterior to the M3 vein has a distinctly different look than the pattern posterior to the vein. This manifests in several different ways. The pigmentation of homologous pattern elements may differ on either side of M3, or background pigmentation anterior to M3 may differ from that posterior to this dividing vein. Some have hypothesized that the M3 vein may serve a special function in compartmentalizing color pattern development (Nijhout, 1991; Abbasi and Marcus, 2017, 2019).

The shape of butterfly wings also can have characteristic AP differences on either side of the M3 vein. Several species develop a tail at the M3 vein and an associated distal dislocation of the pattern elements in the M3–Cu1 wing cell (Nijhout, 1991). In others like *Hypanartia lethe*, the entire wing region posterior to the M3 veins is longer than the region anterior to M3. This size difference is associated with the distal dislocation of color pattern elements posterior to M3.

These common alterations of the ground plan suggest that AP information plays a significant role in the development and evolution of wing color patterns. In this study we aim to uncover trends in the evolution of wing color patterns in butterflies using a comparative approach, with a focus on AP differentiation. We first homologize the regions of the *Drosophila* and lepidopteran wings in order to establish AP fate maps. We show that the lepidopteran wing is divided into four unique combinatorial expression domains of key regulatory genes, which may facilitate developmental independence. To test the developmental interconnectedness of these domains, we next quantified the degree to which the positioning of border ocelli in each wing cell covaries in Nymphalidae and Satyridae. This analysis suggests that development and evolution of color pattern elements is semi-autonomous in these four domains. We then



investigated the role of the M1, M3, and Cu2 veins as pattern boundaries by analyzing color and shape heterogeneity across several butterfly families. Together, these data suggest that AP information serves an important role in butterfly wing color pattern evolution.

MATERIALS AND METHODS

Border Ocelli Analysis

Border ocelli placement in Satyridae and Nymphalidae was measured and analyzed using high-resolution digital images. Specimens were found in the collections of the Museum of Natural History in London, the Smithsonian National Museum of Natural History, the San Diego Natural History Museum, the Natural History Museum of Los Angeles, and the Nijhout lab's personal collection. Specimens were photographed with a Nikon D-5300 digital camera with a scale bar. Measurements of wings were done from scaled photographs using the Fiji distribution of ImageJ (Schindelin et al., 2012).

Wing length of forewings and hindwings was measured as the distance from the base of the Radial vein bundle to the tip of the M1 vein. To measure border ocelli placement, a straight line was drawn from the center of each ocellus to the wing margin following the ridge that is present along the midline of each wing cell (Figure 2). To generate correlation heat maps, ocelli distance was normalized to wing length and used JMP Pro to generate correlation matrices. The list of species surveyed are supplied in the **Supplementary Data File**.

Color and Tail Boundary Survey

To survey the diversity of color pattern discontinuities and tails, we used photographed specimens from the agencies listed above as well as a rich photographic dataset from books by Bernard D'Abbrera (D'Abbrera, 1980, 1981, 1982, 1984a,b, 1985, 1986, 1990). Images of *Pedaliodes* species were kindly supplied to us by Dr. Tomasz Pyrcz. We counted the number of genera in five major butterfly families (Lycaenidae, Nymphalidae, Papilionidae, Pieridae, and Riodinidae) in which a vein associated color boundary or tail occurred, focusing on the hindwing. We defined a color pattern discontinuity as a change in the background color or an element in the color pattern that appeared between intervein regions. Count data were subsequently analyzed using JMP Pro. Adobe Photoshop was used to edit images and produce the final figures. The list of the genera surveyed are supplied in the **Supplementary Data File**.

RESULTS

Defining Anterior–Posterior Organization in Lepidoptera

Anterior–posterior (AP) information serves a fundamental role in the evolution of butterfly wing color patterns. Much of what we know about AP axis differentiation of the insect wing comes from foundational work in *Drosophila melanogaster*. In this species, a cell lineage boundary occurs between veins R4 + R5 and

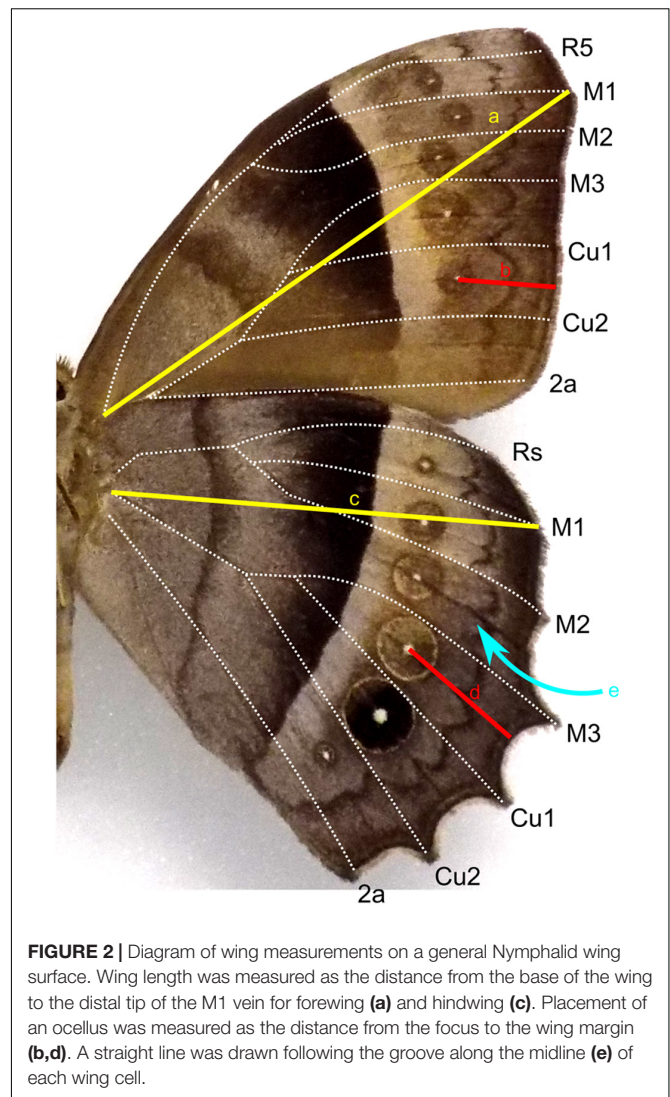
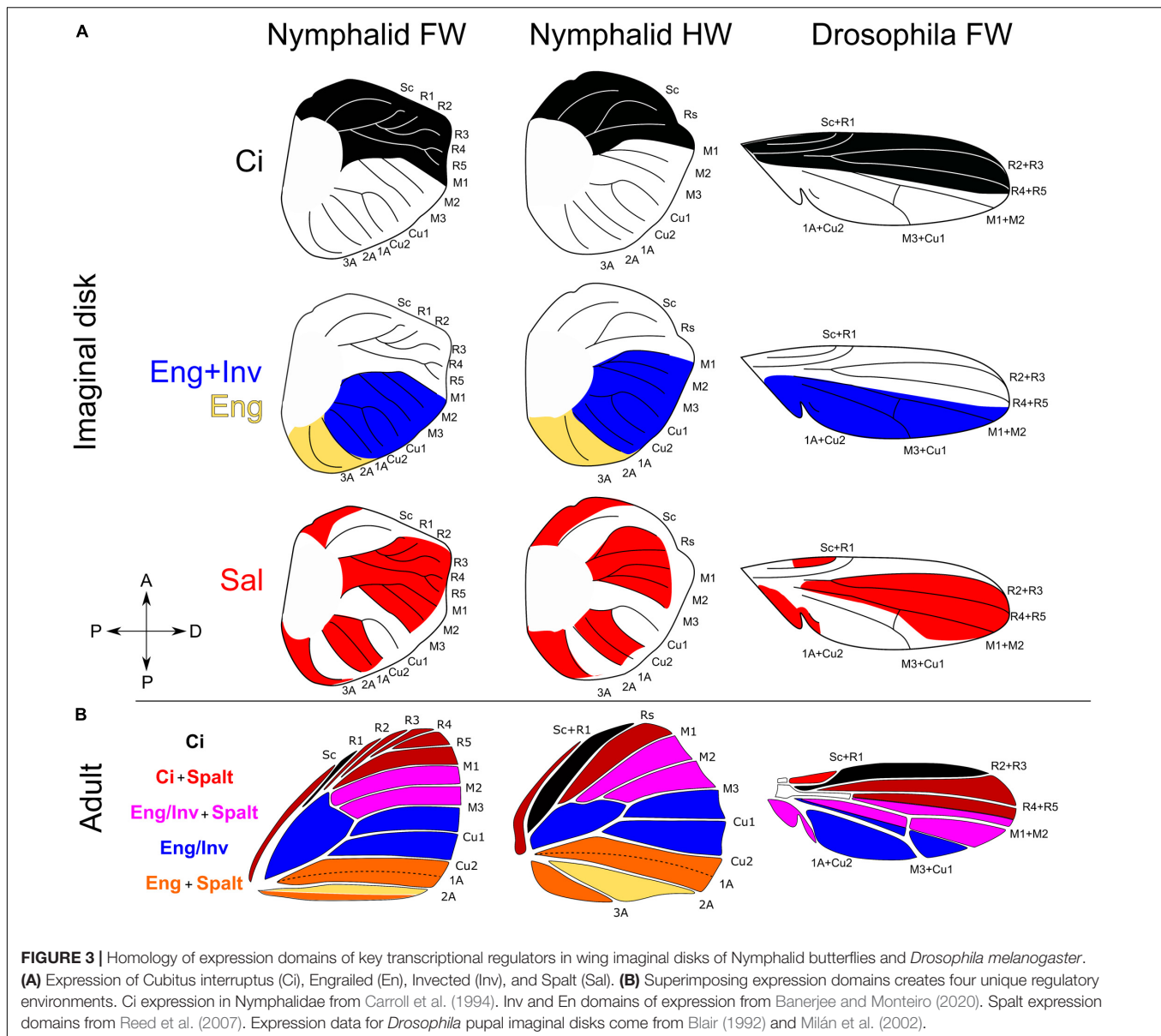


FIGURE 2 | Diagram of wing measurements on a general Nymphalid wing surface. Wing length was measured as the distance from the base of the wing to the distal tip of the M1 vein for forewing (a) and hindwing (c). Placement of an ocellus was measured as the distance from the focus to the wing margin (b,d). A straight line was drawn following the groove along the midline (e) of each wing cell.

M1 + M2 (Figure 3). Cells anterior to the boundary express *Cubitus interruptus* (Ci) and those posterior to the boundary express *Engrailed/Invected* (En/Inv) (Blair, 1992; Zecca et al., 1995; Blair and Ralston, 1997).

The cells at the AP boundary secrete the diffusible protein decapentaplegic (Dpp), stimulated by the diffusible protein Hedgehog (Hh) secreted from En/Inv cells (Zecca et al., 1995; Tanimoto et al., 2000). Decapentaplegic serves to differentiate the anterior and posterior compartments by inducing the expression of the selector genes *Spalt* (Sal) and *Optomotor blind* (Omb). Sal and Omb are induced via a threshold-diffusion mechanism: at high Dpp concentrations Sal and Omb are both expressed (Nellen et al., 1996; Sturtevant et al., 1997; Lecuit and Cohen, 1998; Cook et al., 2004). As Dpp concentrations decline anteriorly and posteriorly away from the AP boundary, Dpp fails to induce the expression of Sal but maintains the expression of Omb. Thus, at least two distinct domains are established by Dpp: a Sal + Omb domain and an Omb domain. This is important because the induction of these domains within anterior Ci and posterior



En/Inv cells, and in combination with other genes known to influence cell fate (Shen et al., 2010; Sugimori et al., 2016; Wang et al., 2016), produces combinatorial interactions that may establish semi-independent developmental microenvironments in the wing (Figure 3).

Some of these transcriptional regulators have been studied in Lepidoptera. We used published studies that described the expression of Ci, En/Inv, and Sal to homologize developmental regulatory environments in the wing of lepidoptera and *Drosophila*. In the buckeye butterfly, *J. coenia*, the AP boundary occurs between the R5 and M1 veins, where cells anterior to M1 express Ci and cells posterior express En/Inv (Keys et al., 1999). Interestingly, the fluorescence of En/Inv strains are not homogenous throughout the posterior of the wing and seem to fade posterior to M3 (Carroll et al., 1994; Reed et al., 2007; Abbasi

and Marcus, 2017). The antibody for En/Inv recognizes both En and Inv (Patel et al., 1989), and the gradual posterior dilution of the fluorescence may be due to reduced expression of one or both of these posterior selector genes. Indeed, *in situ* hybridization in *Bicyclus anyana* demonstrates that En is expressed from M1 to the posterior margin, whereas Inv expression starts at M1 and ends at 2a (Banerjee and Monteiro, 2020).

Although there have been no studies of Omb expression, Sal expression has been readily analyzed in *Junonia* and *Bicyclus* (Reed et al., 2007; Monteiro, 2015). In these species a Sal expression domain appears anterior to the Sc vein, homologous to the far anterior domain in *Drosophila* (Sturtevant et al., 1997; Milán et al., 2002). The space from the Sc vein to R2 has little to no fluorescence of Sal, but from R2 to M3 Sal expression is strong. There is weak or no expression that spans the region between

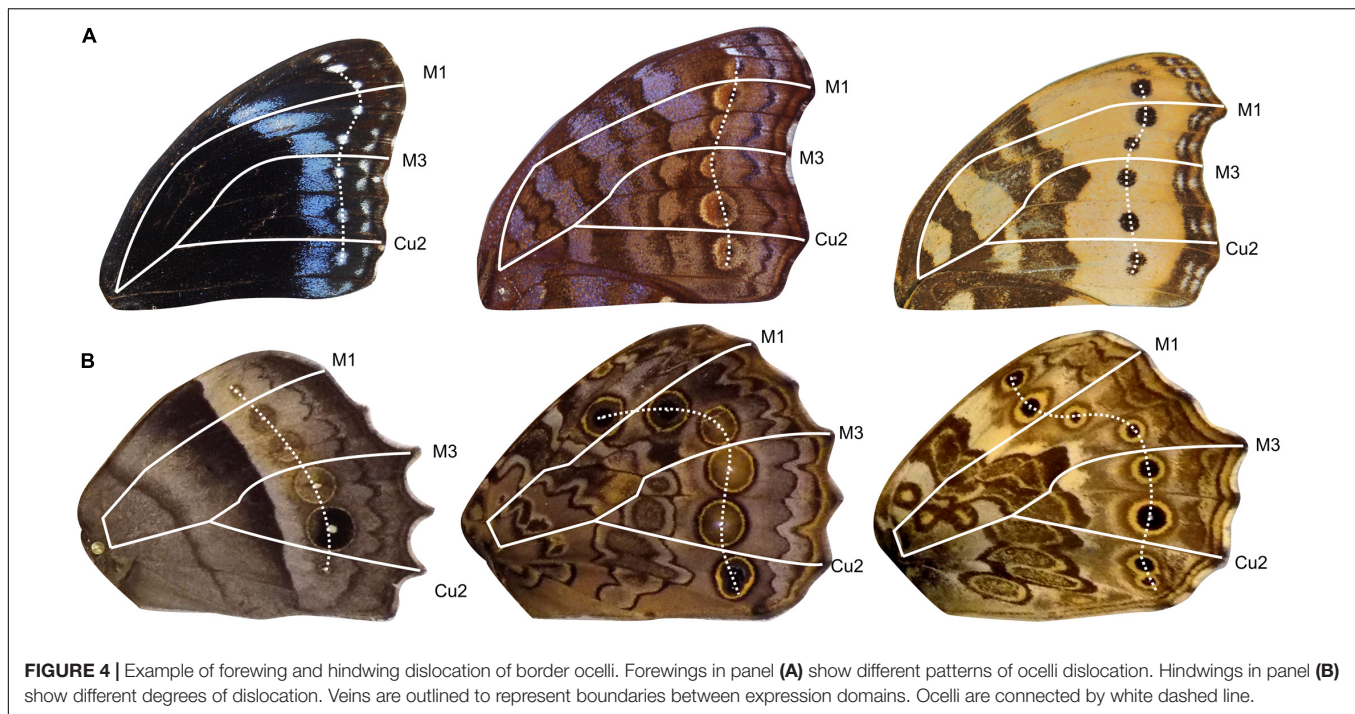


FIGURE 4 | Example of forewing and hindwing dislocation of border ocelli. Forewings in panel (A) show different patterns of ocelli dislocation. Hindwings in panel (B) show different degrees of dislocation. Veins are outlined to represent boundaries between expression domains. Ocelli are connected by white dashed line.

M3 to Cu2 (**Figure 3**). Between Cu2 and 2A another expression domain emerges that has a sharp posterior boundary along the 2A vein. No Sal expression is observed around the 2A vein, but a fourth and final expression domain occurs posterior to 2A that extends to the posterior wing margin.

When the Sal domains are superimposed onto anterior Ci and posterior En/Inv compartments, the wing is secondarily subdivided into four domains with characteristic combinations of expression of the various regulatory genes just as they are in *Drosophila* (**Figure 3**). The anterior most domain is between the R1-M1 veins (Rs-M1 on the hindwing), characterized by the expression of Ci and Sal. The next domain is between M1-M3 veins where Sal and En/Inv are co-expressed. Posterior to this domain is the M3-Cu2 domain where only En/Inv is expressed, and likely express Omb or BMP2/Dpp inhibitors. Posterior to Cu2, cells do not express Inv, but maintain En expression. The fourth domain occurs between the Cu2-2A veins where En cells also express Sal. These domains represent unique combinatorial interactions of compartment selector genes (Ci and En/Inv) and Dpp response elements. Such combinatorial interactions may allow for independent developmental regulation of the patterning mechanisms that specify serially homologous elements and background color.

Correlated Color Pattern Variation Maps to Expression Domains

Morphologically, the degree of independence of AP serial homologs can be demonstrated by measuring their correlated variation among individuals or across species. Within a species there is always some degree of individual variation in the position of pattern elements that emerges from genetic or environmental

variation (Nijhout, 2001). Similarly, within a particular genus or family and even across families, the positioning of serial homologs differs because species vary in the genetic makeup of the developmental process that specifies and positions each pattern element. If two serially homologous pattern elements covary in position from species to species, it may indicate that they share developmental regulation of the patterning mechanism. If two serially homologous pattern elements do not covary – one may vary in the opposite direction from the first or one varies more than the other – then they may not share the same developmental regulation of the mechanism that positions said pattern element.

To determine if the four AP expression domains described above influence the evolution of ocelli placement, we measured the patterns of correlated variation of ocelli in Satyridae and Nymphalidae. Both Families develop border ocelli in each of the four expression domains, thus allowing us to analyze the degree of independence of AP serial homologs (**Figure 4**).

When the measurements from Satyridae and Nymphalidae are combined, a pattern of correlated variation emerged that seems to map directly to the four domains (**Figure 5**). That is, ocelli that lie within the same domain are strongly correlated, but ocelli in different domains are not. This is true for the forewing and the hindwing. However, when the measurements for the two families are analyzed separately, the strength of these correlations are different. For Satyridae, the forewing correlations were strongest within domains but there was a stronger correlation between domains in close proximity. For the hindwing the strength of the correlations between domains was weak, strengthening the support of evolutionary independence of the domains.

For Nymphalidae, the independence between domains on the forewing was weaker than they are in Satyridae

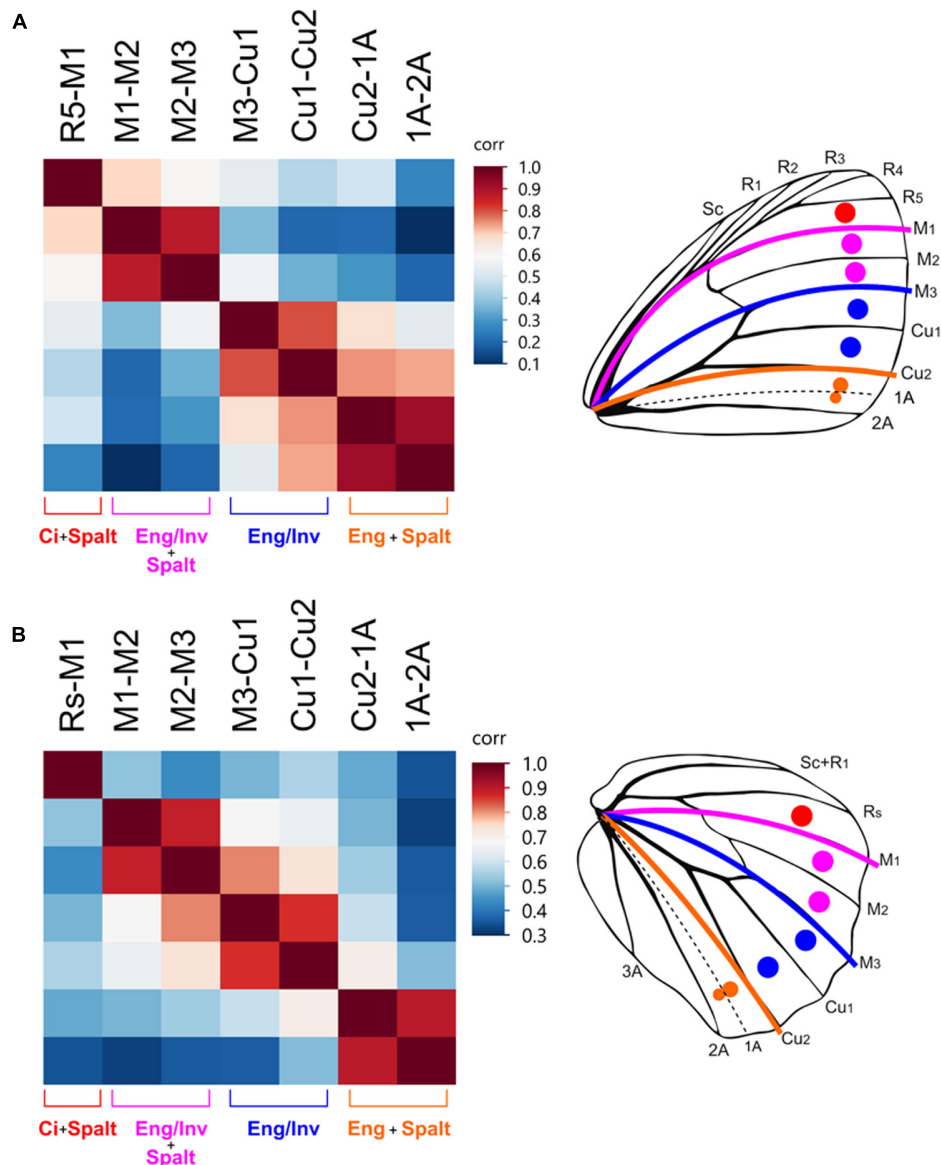


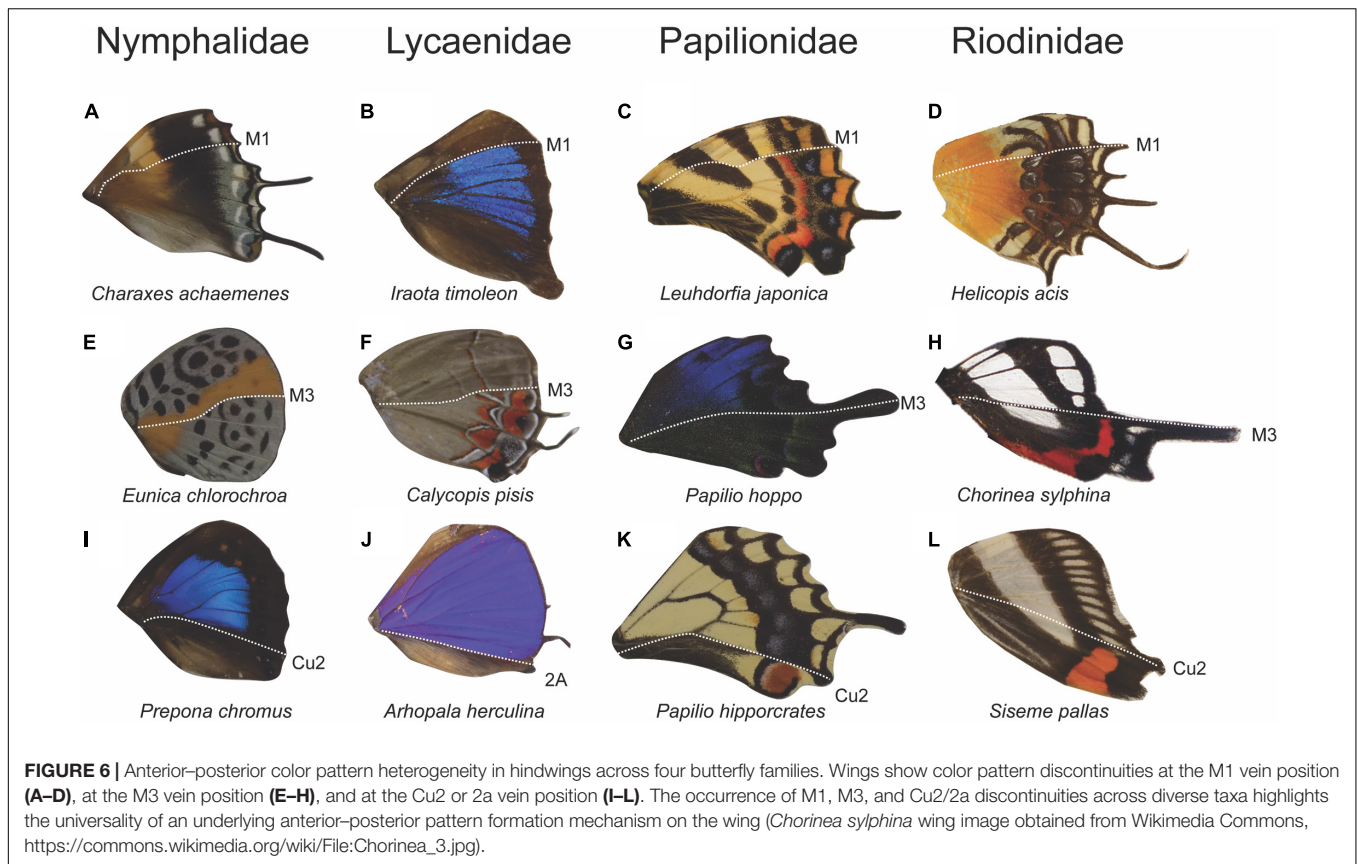
FIGURE 5 | Border ocelli placement is modular and maps to the AP expression domains. Heatmaps of correlations between the relative distance from the wing margin of border ocelli in Satyridae and Nymphalidae. Dark red colors indicate strong correlations. Light red, white, and blue represent weaker correlations. In the forewing **(A)** and hindwing **(B)**, ocelli within each expression domain are strongly correlated with each other and are weakly correlated with ocelli in other expression domains.

(**Supplementary Figure S1**). The position of ocelli in the M1-M3 domain were equally correlated with the adjacent domains. The position of the M1-M2 ocellus was equally correlated with that of the R5-M1 ocellus and the M2-M3 ocellus but was not correlated with that of the M3-Cu1 ocellus. The position of the M2-M3 ocellus was equally correlated with the M3-Cu1 ocellus but not with the Cu1-Cu2 ocellus. Thus, forewing ocelli in Nymphalidae displayed the pattern of correlations within domains but displayed stronger correlations between adjacent domains. Similarly, the pattern of correlations on the hindwing are completely different. The position of the Rs-M1 ocellus was uncorrelated with all other ocelli, but ocelli between M1-Cu2

were strongly correlated with each other and less correlated with ocelli between Cu2-2A. This suggests the positioning of ocelli in the M1-M3 and M3-Cu2 domains may share a greater degree of developmental regulation in this family despite differences in the combinatorial transcriptional regulation.

Color Boundaries

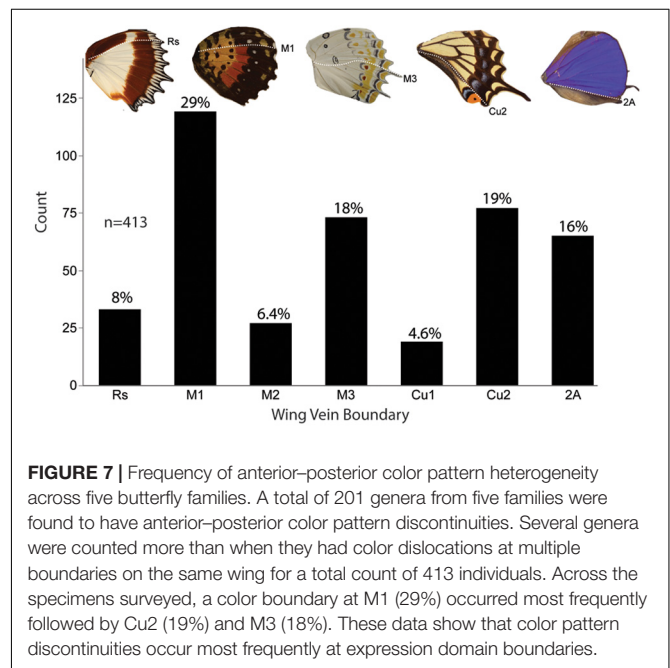
Previous studies have documented a widespread occurrence of color pattern discontinuities that form at various positions on the wing (Nijhout, 1991). The most notable discontinuity occurs around the M3 vein. In several species the pattern elements anterior to M3 are different from those posterior to the vein in



almost every aspect (size, color, and position) (Nijhout, 1991). The M3 vein, however, is not the only location on the wing where such extreme pattern differences form. Many seem to form near the M1 and Cu2 veins. Interestingly, these veins happen to occur at the boundaries between the combinatorial expression domains (Figure 3).

We sought to discover the abundance of various color pattern boundaries to see if they occur in multiple butterfly taxa and to determine whether boundaries between expression domains were more common in some taxa than others. Genera in every Family displayed some form of color discontinuities at one or more of the veins that mark a boundary between expression domains (Figure 6). Within all five Families that we surveyed, there were genera that have a color pattern boundary at M1, M3, and Cu2/2A. When the genera from each Family were combined into a single dataset, the most frequent color pattern discontinuity occurred at the M1 vein (29%), followed by the Cu2 vein (19%), M3 vein (18%), 2A vein (16%), Rs vein (8%), M2 vein (6.4%), and the Cu1 vein (4.6%). Together, the veins that represent boundaries between expression domains (M1, M3, Cu2, and 2A) represented 82% of the color pattern discontinuities observed in the 201 genera included in this study (Figure 7).

We found that the five Families we studied (Lycaenidae, Nymphalidae, Papilionidae, Pieridae, and Riodinidae) had Family-specific differences in these locations of color pattern boundaries (Figure 8). In Lycaenidae, color boundaries primarily occurred at the M1 vein (59%), followed by the M3 (21%), Cu2



(8%), 2A (4%), Cu1 (3%) and Rs (3%). In Nymphalidae, Cu2 (24%) was the most common vein boundary, followed by 2A (22%), M1 (16%), M3 (13%), Rs (11%), M2 (9%), and Cu1 (4%).

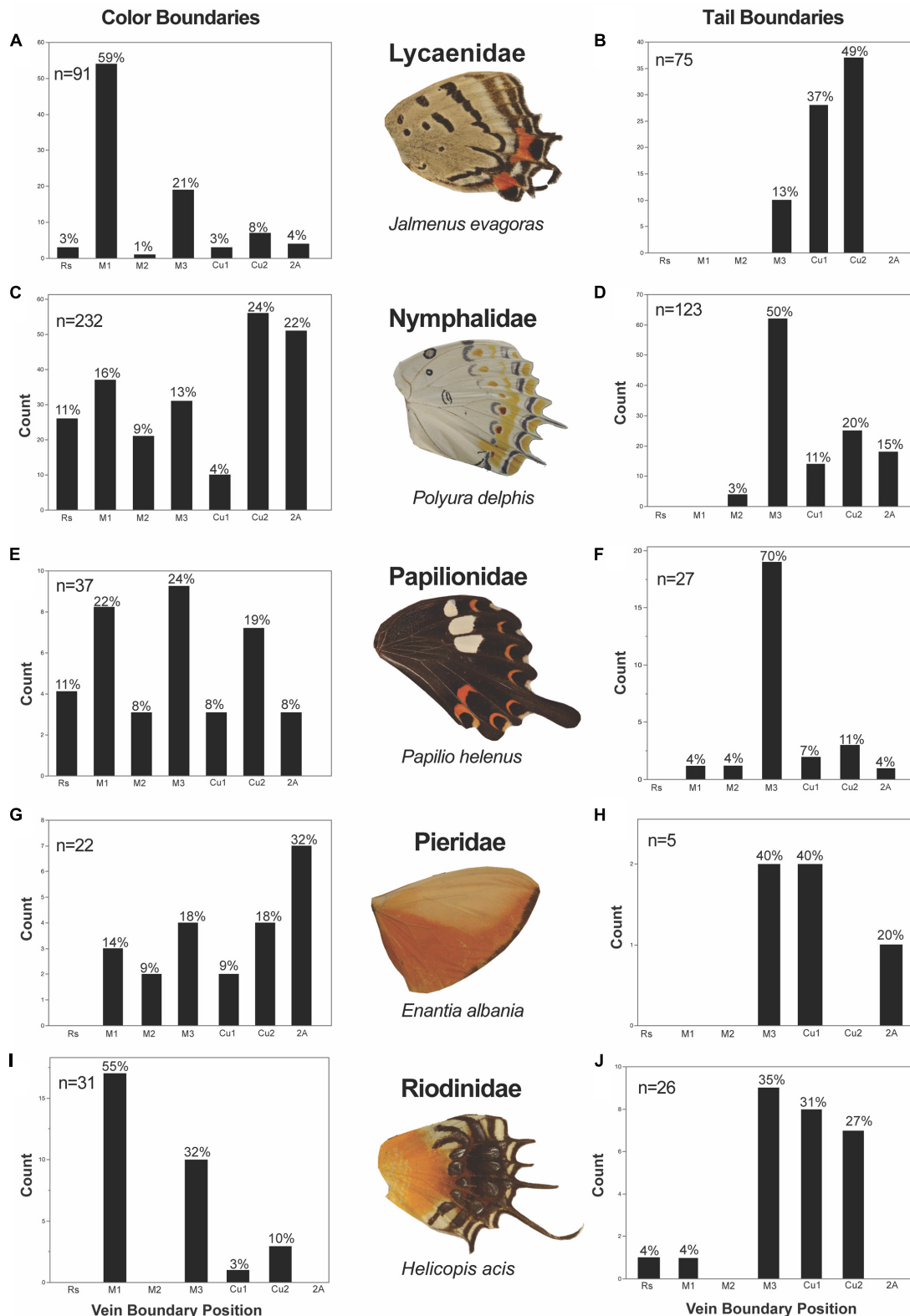
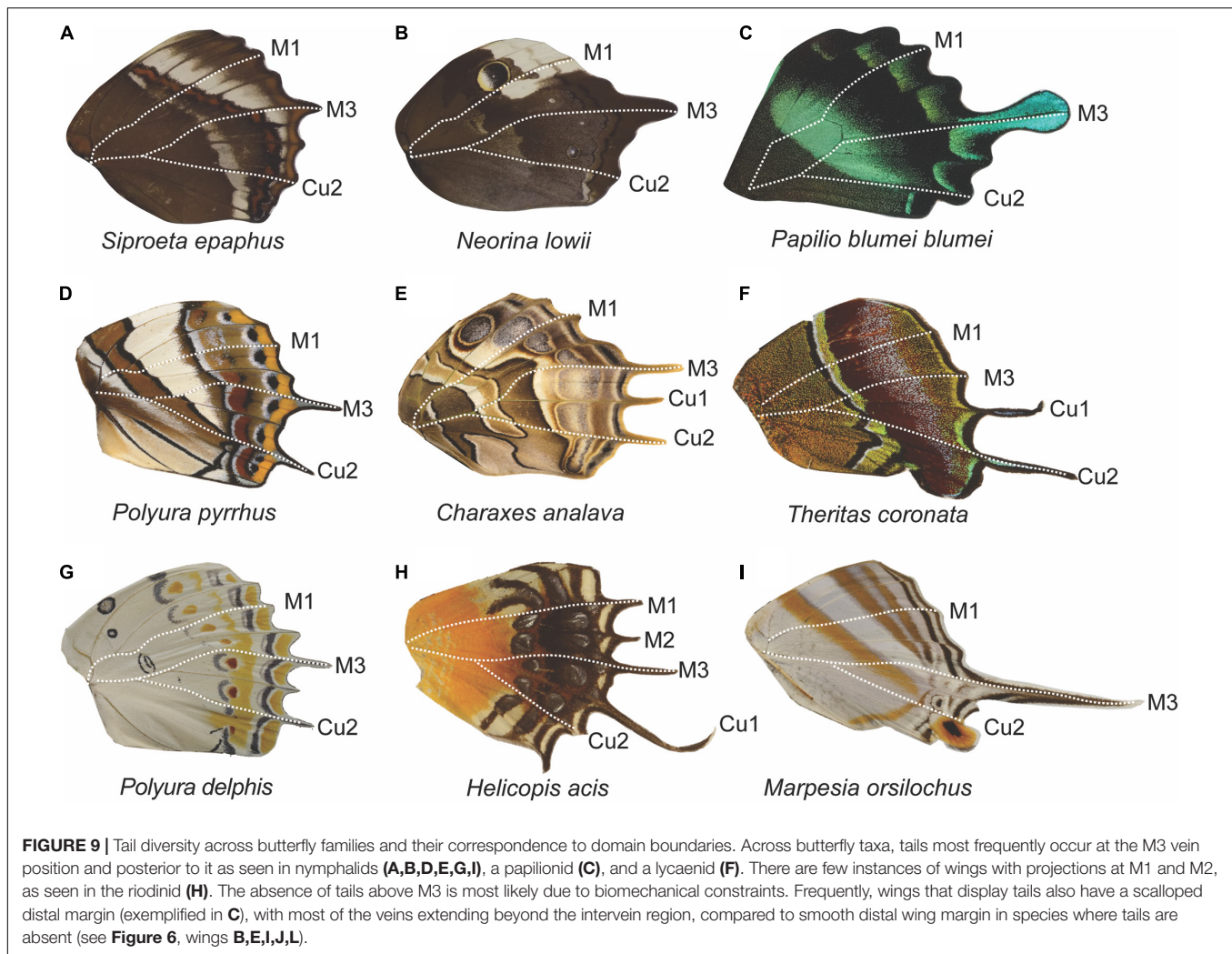


FIGURE 8 | Color pattern heterogeneity and tail occurrence frequency subdivided by Family. There is Family level variation in the position at which color boundaries and tails occur. The color boundaries in Lycaenidae (A) and Riodinidae (I) occur most frequently at M1, while color boundaries appear more frequently posterior to M3 in Nymphalidae (C) and Pieridae (G). In Papilionidae (E), color discontinuities occur most frequently at M3 and equally so at M1 and Cu2. Tails appear most frequently at M3 from all Families (D,F,H,J), but Lycaenidae (B).



In Papilionidae, most color boundaries occurred at M3 (24%) and the AP boundary at the M1 (22%) vein, followed by Cu2 (19%) and Rs (11%). Very few color boundaries occurred at M2, Cu1, and 2A (each of with approximately 8%). In Pieridae, the most common color boundary occurred at the 2A vein (32%), followed by M3 (18%), Cu2 (18%), M1 (14%), M2 (9.1%) and Cu1 (9.1%). Finally, in Riodinidae, M1 (55%) represented the most common boundary, followed by M3 (32%), Cu2 (9.7%) and Cu1 (3.2%).

Tails and Shape Boundaries

The shapes of butterfly wings can also have AP differences. For instance, a tail that develop from the outward protrusion of a vein is also frequently associated by a dislocation, exaggeration and/or reduction of pattern elements on either side of the vein. As with our analysis of color pattern discontinuities above, we sought to determine the frequency of various vein protrusions as they relate to the AP expression domains.

There is a diversity of tail shapes across the butterfly taxa we sampled as well as differences in the veins at which tails occurred (**Figure 9**). When the data were combined into a single dataset, tails largely occurred at or posterior to M3, with a 97.2%

likelihood (**Figure 10**). The most common vein to form a tail is the M3 vein (40%), followed by Cu2 (28%), Cu1 (21%), and 2A (8%). Tails anterior to M3 were very rare accounting for approximately 3% of all tails observed (M2; 2%, M1; 0.8%, Rs; 0.4%) in the 161 genera that were included in this study.

There are interesting differences in tail position among the Families we studied. In Lycaenidae, M3 tails are the least common (13%), whereas Cu2 (49%) and Cu1 (37%) tails are the most common, together making up 86% of tails in the Family (**Figure 8B**). Conversely in Nymphalidae, M3 tails are by far the most common making up 50% of tails (**Figure 8D**). The next most common tails are Cu2 (20%), 2A (15%), Cu1 (11%), and M2 (3.3%). In Papilionidae, tails almost exclusively occur at the M3 vein (89%) with the remaining 11% tails occurring at the Cu2 vein (**Figure 8F**). Tails and vein protrusions were very rare in Pieridae (**Figure 8H**). In this family, we could only identify four genera where tails were present. Of these, two had tails at M3, two at Cu1, and one at vein 2A. The Riodinidae displayed tails primarily at M3 and posteriorly, with tails at M3 most frequently followed by Cu1 and Cu2, respectively (**Figure 8J**). In some instances, the entire wing posterior to M3

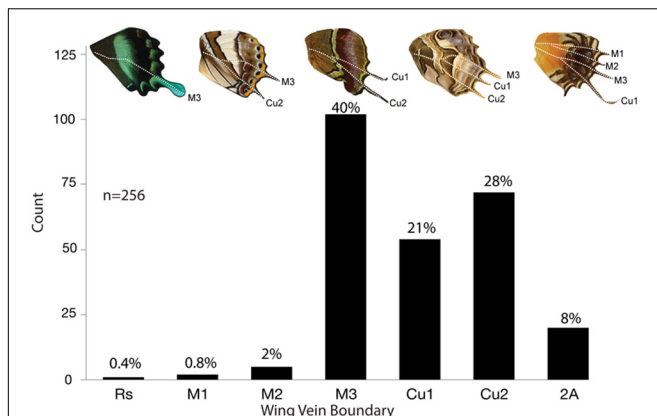


FIGURE 10 | Frequency of tails across five butterfly families. A total of 161 genera across 5 families were observed to have tails. Some genera had multiple tails and were counted more than once for a total of 256 observations. Tails emerge most frequently at M3 (40%), followed by Cu2 (28%), and Cu1 (21%).

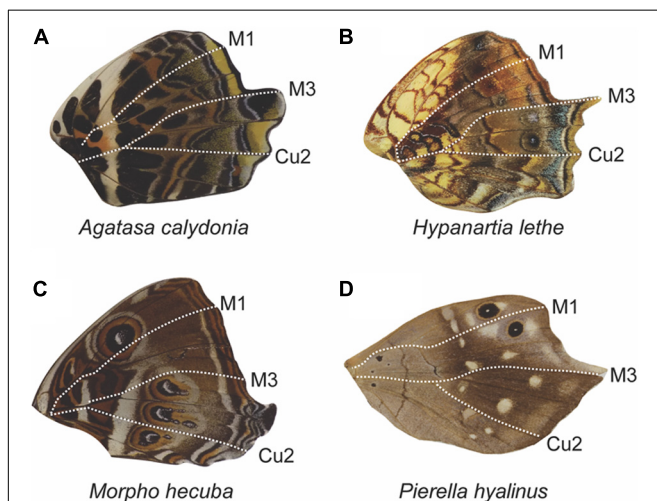


FIGURE 11 | Anterior-posterior dislocation at the M3 vein in hindwings. Butterfly wings can display elongation of the M3, Cu1, and Cu2 veins, leading to a larger posterior wing region (A–D). In some cases, the region of elongation begins at the M3 vein (A,B), in the intervein region posterior to M3 (C), or in the intervein region anterior to M3 (D), suggesting that the expression domain boundary can shift. All four wing examples are from the family Nymphalidae.

is elongated (Figure 11, and see also Figures 6G,H,I). This was most often observed in Nymphalidae, but also appears in the Riodinidae (Figure 6H). These species show a clear demarcation between the anterior and posterior regions of the wing with respect to shape, but also with regard to the pattern. In some instances, there is a color pattern dislocation (Figure 11A), a change in the predominant background color (Figures 11B,D), or the appearance of a pattern element in the elongated posterior region that was not present in the anterior region (Figure 11C).

DISCUSSION

The work presented above provides several novel insights into the evolution and development of color pattern diversity in Lepidoptera, especially the role of positional information along the AP and PD axes. We have shown that there are conserved developmental microenvironments in the wings of butterflies that divide the AP axis into 4 developmental domains, each characterized by unique combinatorial patterns of gene expression. We hypothesize that the combinatorial activity of selector genes *En*, *Inv*, *Ci*, *Sal*, and likely others, may facilitate the establishment of unique cellular states that influence color pattern formation and evolution.

Development and Evolution of Border Ocelli Placement

Lepidopteran wing color patterns are composed of three unique character systems that develop along the PD axis of the wing: the BSS, CSS, and the BoSS (Nijhout, 1991). Each system is composed of serially homologous pattern elements that are repeated along the AP axis between the veins.

The organizing principles that specify each character system are coming to be increasing well understood. Most developmental studies have targeted the BoSS, which is composed of border ocelli and parafoveal elements. Although this is perhaps the most complex symmetry system, these studies have discovered a relatively simple patterning mechanism that can explain the placement and morphology of ocelli and parafoveal elements (Nijhout, 2017).

Early studies into ocelli development discovered that several genes involved in embryonic and limb patterning are redeployed in the later stages of wing imaginal disk development (Carroll et al., 1994; Brakefield et al., 1996; Keys et al., 1999). In both *J. coenia* and *B. anyana*, the process begins with the determination of ocelli in the wing imaginal disk shortly after the vein lacuna have formed. This is characterized by an initial patterning of homeobox gene *distal-less* (*Dll*) throughout the distal region of the wing epithelia near the dorsal ventral boundary (DV; Brakefield et al., 1996; Reed and Serfas, 2004; Reed et al., 2007; Connahs et al., 2019). As the wing matures later in the instar, *Dll* begins to localize to a focus in each wing cell (Reed and Serfas, 2004; Reed et al., 2007; Nijhout, 2017).

Theoretical models such as Nijhout's grassfire model (Nijhout, 2017) and the Gray-Scott model by Connahs et al. (2019) suggest *Dll* expression is confined to a focus of cells via a diffusion-threshold mechanism. In these models, cells respond to a diffusible signal from the DV boundary to pattern *Dll* expression (Nijhout, 2017). The actual identities of the morphogenetic signal and the pathway that activates *Dll* has remained elusive, however, *Wnt1/Wg* signaling from the DV boundary is known to induce *Dll* expression in *Drosophila* (Strigini and Cohen, 2000). In Lepidoptera, several Wnts are expressed in the DV boundary cells: *Wnt1/Wg*, *Wnt6*, and *Wnt10* (Martin and Reed, 2010; Martin et al., 2012; Martin and Reed, 2014; Mazo-Vargas et al., 2017). Which of these Wnts specify border ocelli is unknown.

Other morphogenetic genes may also be involved in the patterning of eyespots. For instance, Hh and Dpp are redeployed late in butterfly wing development wherein Hh is expressed in eyespot foci (Keys et al., 1999) and Dpp is expressed throughout the wing but is noticeably absent from eyespot foci (Connahs et al., 2019). The mechanism by which they are redeployed in a novel spatial context decoupled from their role in AP organization of the wing is unknown. Their presence however may be significant for the placement of eyespots, exemplified by the Gray–Scott model (Connahs et al., 2019).

We have shown that there are homologous distinct regions of gene expression in the developing wing imaginal disks of Nymphalidae and *Drosophila*. These divide the wing into four domains with characteristic combinatorial patterns of gene expression of the transcription factors En, Inv, Ci, and Sal. The boundaries between these domains are just anterior to the M1 vein, at the M3 vein, and at the Cu2 vein. Exactly how the expression and overlap of these transcriptional regulators influences the expression of Dpp, Hh, Wnt1/Wg and the deployment of Dll remains an open question. It is entirely possible that local differences in each of these factors could lead to differential placement of ocelli. Rationale for this hypothesis comes from previous developmental studies in *J. coenia* and *B. anyana*, which clearly show differential positioning of ocelli occurs by positioning Dll in the larval wing imaginal disk (Brakefield et al., 1996; Reed and Serfas, 2004; Saenko et al., 2011; Connahs et al., 2019).

Because each expression domain spans two wing cells, regulation of the ocellus patterning process may be correlated in adjacent wing cells of the same domain. Thus, we hypothesized that patterns of correlated variation between ocelli should map to expression domains if these domains differentially regulate the ocelli patterning process. Our analysis of border ocelli positioning in Satyridae and Nymphalidae suggest that the AP expression domains do not produce the same patterns of correlated variation in these two families. In Satyridae, the positioning of ocelli displayed a pattern of correlations that supports developmental and evolutionary independence of the expression domains. In this family, the position of an ocellus was most strongly correlated with the ocellus in the same expression domain and was weakly correlated with ocelli in different domains. In Nymphalidae however, no such pattern was present. Instead, ocelli were most correlated with ocelli in wing cells directly adjacent to them. The difference in the patterns of correlation between these two Families suggest that these expression domains may not act as independent developmental and evolutionary modules by default. In fact, evolution of modularity likely requires special conditions where selection favors divergent specialization of parts (West-Eberhard, 2019). It is evident then to understand patterns of modularity in butterfly wing color patterns, we need to understand the selective pressures that favor specialization of pattern elements.

It is interesting to note the differences in forewing and hindwing patterns of correlation. These differences are most likely facilitated by local regulation of patterning events imposed by homeobox selector genes, Antennapedia (Antp) and Ultrabithorax (Ubx). Forewing development is regulated

by Antp whereas hindwing development is regulated by Ubx, and mutations affecting Ubx lead to the development of forewing color patterns on the hindwing (Weatherbee et al., 1999; Matsuoka and Monteiro, 2019). These findings suggest homeobox genes play a significant role in shaping pattern formation. The fact that forewing and hindwing correlations differ so substantially suggests that the Hox environment may influence the evolution of AP modularity, and provides a unique opportunity to understand the evolution of developmental modularity.

Color Heterogeneity

Supporting evidence for the potential of developmental autonomy of the AP expression domains stems from our analysis of color and shape discontinuities across several lepidopteran families. In many species of butterflies and moths, the color of pattern elements or of the background display sharp boundaries at various wing veins (Nijhout, 1991; Abbasi and Marcus, 2017). Across the five Families, we found that the vast majority of color discontinuities occur at veins that mark the boundary between expression domains (i.e., M1, M3, and Cu2). When examined at the individual Family level, we observed variation in the position that a color boundary most frequently occurred (**Figure 8**). This suggests that there may be Family-specific developmental patterns that influence the phenotypes that are more and less likely to occur on the wings. Lycaenids and riodinids, sister taxa, display color pattern discontinuities more frequently above the M3 vein, whereas nymphalids and pierids tend to have color discontinuities below the M3 vein. Papilionids have a more equal distribution of color discontinuities above and below M3. While the expression domains may be conserved across Lepidoptera the downstream uses of them may have become more specialized in different lineages.

Tails almost exclusively occur posterior to M3. M3, Cu1, and Cu2 tails occur in each family we surveyed and again, the frequency of each of these veins was family specific. Tails manifesting from the anal veins were far less common. Although not included in this study, butterflies in the family Hesperidae seem to break this trend and only develop tails from anal veins (Li et al., 2019). Interestingly, these shape differences along the AP axis of the wing seem to affect the color patterns non-randomly. A common feature of tail formation in each family was the alteration of pattern elements in the wing cells associated with the tails. In many cases, pattern elements were dislocated distally in the wing cells that made up the tail. If tails and other shape differences are formed via differential growth, then this may affect the placement and/or size of pattern elements because they differentiate during the growth period of the wing late in the final larval instar. In previous studies we have found that growth of lepidopteran wings is spatially patterned and changes over time (Nijhout et al., 2014). Therefore, it is possible that differential growth along the AP axis of the wing could affect the patterning and phenotypic evolution of serially homologous pattern elements.

Modes of Pattern Evolution

The results we have presented in this paper provide evidence that AP positional information influences the development and evolution of color pattern, as well as wing shape and the development of tails on the wing. In many species, color pattern and wing size show distinct discontinuities at the locations of the veins that mark boundaries between the four developmental domains we uncovered. These findings imply that both growth and color pattern formation can be influenced by factors that are different in each of the four domains. Since pattern element specification depends upon only a few variables (e.g., morphogen gradients, cellular sensitivity to morphogens, and pigment synthesis pathways), changes in the AP expression domains could have profound effects on various attributes of pattern elements.

Wing growth late in the instar is regulated by two hormones, 20-hydroxyecdysone (20E) and insulin (Kremen and Nijhout, 1998; Miner et al., 2000; Nijhout and Grunert, 2002). 20-Hydroxyecdysone primarily drives cell division while insulins seem to influence cellular growth (Nijhout et al., 2018; McKenna et al., 2019). Additionally, evidence from *Drosophila* suggests 20E influences the rate of Notch and Wnt pattern progression in the wing (Mirth et al., 2009; Oliveira et al., 2014), which therefore may play a significant role in shaping butterfly wings (Macdonald et al., 2010). In *Drosophila*, Notch induces expression of Wnt1/Wg and Cut along the peripheral margin (Panin et al., 1997). Each of these genes are expressed in butterfly wings, but there are some significant differences. First, the peripheral tissue in butterflies is relatively large and forms complex contours at the boundary between the Notch expressing cells that makeup the wing primordium and the Wg/Cut expressing peripheral cells (Macdonald et al., 2010). Second, the expression of Wnt1/Wg and Cut in peripheral region leads to programmed cell death of the periphery during the pupal stage (Macdonald et al., 2010). In Lepidoptera therefore, this mechanism works like a molecular cookie-cutter (Nijhout, 1991; Macdonald et al., 2010). In light of our results that hindwing tails and other shape differences largely occur posterior to the M3 vein, Notch/Wg patterning may be differentially regulated along the AP axis. Thus, regional differences in the sensitivity to 20E, or in the response to 20E, could control the relative rates of growth and differential patterning of the wing margin in each of the four domains, and this could account for the diversity of wing shapes found in the Lepidoptera.

Color pattern development and evolution can likewise depend on differences in the molecular and cellular properties of the four domains. For instance, Wnt signaling is involved in the development of several features of the color pattern (Martin et al., 2012). The Wnt pathway is composed of various membrane receptors and intra-cellular signaling proteins (Komiya and Habas, 2008), and the cellular context in which these components are expressed could affect their interaction kinetics or patterns of expression to alter the concentration-dependent response to the morphogens. Additionally, alteration of a pigment synthesis pathway may be the simplest way to modify the pattern. Within the expression domain of an eyespot, for instance, different pigments are synthesized at different distances from the focus.

This can happen because differences in the expression patterns of key regulatory genes modify the expression and activity of enzymes in the pigment synthesis pathways, which leads to different outputs (Nijhout, 1991; Reed and Nagy, 2005; Hughes et al., 2020). The same may be true for differences between the four AP expression domains.

In order for AP domains to modularly control pattern formation and growth, there must be some level of genetic modularity. It is entirely possible that the genes involved in Wnt, Hh, Dpp, Insulin/TOR, 20E signaling, and pigment synthesis pathways may be controlled by modular *cis*-regulatory elements (CREs) that facilitate spatial and temporal differences in gene expression. For example, pigmentation pathways (Roeske et al., 2018) and the insulin/TOR cascade (Tang et al., 2011) are differentially utilized among tissues in *Drosophila*. Finer spatial resolution of gene expression in lepidopteran wings via RNA-seq and ATAC-seq should be able to determine how various signaling pathways are used throughout the wing. It will be interesting to see if CRISPR mutagenesis of CREs and spatially restricted transcriptional regulators results in more correlated development throughout the wing. If indeed the AP expression domains within the wing differentially regulate aspects of various pathways, this could be a mechanism by which the placement of pattern elements and development of wing shape evolve.

Our findings add several new tools to the toolbox of pattern development and evolution in the Lepidoptera. Not only can color patterns be compartmentalized by wing veins (Nijhout, 1991), but the nature of the background in which patterns develop can be different in the larger regions of the wing defined by the boundaries of the expression domains we describe. As we begin to learn more about how patterns and wing shapes arise from the underlying developmental mechanisms, it will also be interesting to explore why they come about. An investigation of potential selective pressures on wing shape and color pattern may shed light on what leads certain taxa to display heterogenous color patterns and shapes, while others do not. Understanding how selection shapes the reductionist control of gene expression to produce different patterns of developmental independence represents one of the most daunting challenges in biology. Nevertheless, the study of butterfly color patterns may provide a unique opportunity to shed light on some of these fundamental principles in evolutionary developmental biology.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

KM and AK collected and analyzed the data and constructed the figures. All authors contributed equally to the conception and writing of the manuscript.

FUNDING

This work was supported by grants IOS-0744952 and IOS-1557341 from the National Science Foundation.

ACKNOWLEDGMENTS

We would like to thank Laura Grunert for help in mounting many of the butterflies used in this study.

REFERENCES

- Abbasi, R., and Marcus, J. M. (2017). A new A-P compartment boundary and organizer in holometabolous insect wings. *Sci. Rep.* 7:16337. doi: 10.1038/s41598-017-16553-5
- Abbasi, R., and Marcus, J. M. (2019). Reply to 'A refutation to 'A new A-P compartment boundary and organizer in holometabolous insect wings'. *Sci. Rep.* 9:7048. doi: 10.1038/s41598-019-42679-9
- Allen, C. E., Beldade, P., Zwaan, B. J., and Brakefield, P. M. (2008). Differences in the selection response of serially repeated color pattern characters: standing variation, development, and evolution. *BMC Evol. Biol.* 8:94. doi: 10.1186/1471-2148-8-94
- Banerjee, T. D., and Monteiro, A. (2020). Molecular mechanism underlying venation patterning in butterflies. *bioRxiv* [Preprint]. doi: 10.1101/2020.01.02.892760
- Beldade, P., and Brakefield, P. M. (2003). Concerted evolution and developmental integration in modular butterfly wing patterns. *Evol. Dev.* 5, 169–179. doi: 10.1046/j.1525-142X.2003.03025.x
- Beldade, P., Koops, K., and Brakefield, P. M. (2002). Developmental constraints versus flexibility in morphological evolution. *Nature* 416, 844–847. doi: 10.1038/416844a
- Blair, S. S. (1992). Engrailed expression in the anterior lineage compartment of the developing wing blade of *Drosophila*. *Development* 115, 21–33.
- Blair, S. S., and Ralston, A. (1997). Smoothed-mediated Hedgehog signalling is required for the maintenance of the anterior-posterior lineage restriction in the developing wing of *Drosophila*. *Development* 124, 4053–4063.
- Brakefield, P. M., Gates, J., Keys, D., Kesbeke, F., Wijngaarden, P. J., Montelro, A., et al. (1996). Development, plasticity and evolution of butterfly eyespot patterns. *Nature* 384, 236–242. doi: 10.1038/384236a0
- Brunetti, C. R., Selegue, J. E., Monteiro, A., French, V., Brakefield, P. M., and Carroll, S. B. (2001). The generation and diversification of butterfly eyespot color patterns. *Curr. Biol.* 11, 1578–1585. doi: 10.1016/S0960-9822(01)00502-4
- Capdevila, J., and Belmonte, J. C. I. (2001). Patterning mechanisms controlling vertebrate limb development. *Annu. Rev. Cell Dev. Biol.* 17, 87–132. doi: 10.1146/annurev.cellbio.17.1.87
- Carroll, S., Gates, J., Keys, D., Paddock, S., Panganiban, G., Selegue, J., et al. (1994). Pattern formation and eyespot determination in butterfly wings. *Science* 265, 109–114. doi: 10.1126/science.7912449
- Connahs, H., Tlili, S., van Creijl, J., Loo, T. Y. J., Banerjee, T. D., Saunders, T. E., et al. (2019). Activation of butterfly eyespots by Distal-less is consistent with a reaction-diffusion process. *Development* 146:dev169367. doi: 10.1242/dev.169367
- Cook, O., Biehs, B., and Bier, E. (2004). Brinker and Optomotor-blind act coordinately to initiate development of the L5 wing vein primordium in *Drosophila*. *Development* 131, 2113–2124. doi: 10.1242/dev.01100
- D'Abrera, B. (1980). *Butterflies of the Afrotropical Region*. Melbourne: Lansdowne Editions.
- D'Abrera, B. (1981). *Butterflies of the Neotropical Region. Part I. Papilionidae and Pieridae*. Melbourne: Lansdowne Editions.
- D'Abrera, B. (1982). *Butterflies of the Oriental Region. Part I. Papilionidae, Pieridae and Danaidae*. Victoria: Hill House.
- D'Abrera, B. (1984a). *Butterflies of the Neotropical Region. Part II. Danaidae, Ithomiidae, Heliconidae and Morphidae*. Victoria: Hill House.
- D'Abrera, B. (1984b). *Butterflies of South America*. Victoria: Hill House.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fevo.2020.00146/full#supplementary-material>

FIGURE S1 | Border ocelli modularity differs between Nymphalidae and Satyridae. Heatmaps of correlations between the relative distance from the wing margin of border ocelli in Satyridae and Nymphalidae. Dark red colors indicate strong correlations. Light red, white, and blue represent weaker correlations.

- D'Abrera, B. (1985). *Butterflies of the Oriental Region. Part II. Nymphalidae, Satyridae and Amathusidae*. Victoria: Hill House.
- D'Abrera, B. (1986). *Butterflies of the Oriental Region. Part III*. Victoria: Hill House.
- D'Abrera, B. (1990). *Butterflies of the Australian Region*. Victoria: Hill House.
- Gawne, R., and Nijhout, H. F. (2019). Expanding the nymphalid groundplan's domain of applicability: pattern homologies in an arctiid moth (*Utetheisa ornatrix*). *Biol. J. Linn. Soc.* 126, 912–924. doi: 10.1093/biolinnean/bly193
- Hughes, J. T., Williams, M. E., Johnson, R., Grover, S., Rebeiz, M., and Williams, T. M. (2020). Gene regulatory network homoplasy underlies recurrent sexually dimorphic fruit fly pigmentation. *Front. Ecol. Evol.* 8:80. doi: 10.3389/fevo.2020.00080
- Keys, D. N., Lewis, D. L., Selegue, J. E., Pearson, B. J., Goodrich, L. V., Johnson, R. L., et al. (1999). Recruitment of a *Hedgehog* regulatory circuit in butterfly eyespot evolution. *Science* 283, 532–534. doi: 10.1126/science.283.5401.532
- Komiya, Y., and Habas, R. (2008). Wnt signal transduction pathways. *Organogenesis* 4, 68–75. doi: 10.4161/org.4.2.5851
- Kremen, C., and Nijhout, H. F. (1998). Control of pupal commitment in the imaginal disks of *Precis coenia* (Lepidoptera: Nymphalidae). *J. Insect Physiol.* 44, 287–296. doi: 10.1016/S0022-1910(97)00121-2
- Lecuit, T., and Cohen, S. M. (1998). Dpp receptor levels contribute to shaping the Dpp morphogen gradient in the *Drosophila* wing imaginal disc. *Development* 125, 4901–4907.
- Li, W., Cong, Q., Shen, J., Zhang, J., Hallwachs, W., Janzen, D. H., et al. (2019). Genomes of skipper butterflies reveal extensive convergence of wing patterns. *Proc. Natl. Acad. Sci. U.S.A.* 116, 6232–6237. doi: 10.1073/pnas.1821304116
- Macdonald, W. P., Martin, A., and Reed, R. D. (2010). Butterfly wings shaped by a molecular cookie cutter: evolutionary radiation of lepidopteran wing shapes associated with a derived Cut/wingless wing margin boundary system. *Evol. Dev.* 12, 296–304. doi: 10.1111/j.1525-142X.2010.00415.x
- Martin, A., Papa, R., Nadeau, N. J., Hill, R. I., Counterman, B. A., Halder, G., et al. (2012). Diversification of complex butterfly wing patterns by repeated regulatory evolution of a *Wnt* ligand. *Proc. Natl. Acad. Sci. U.S.A.* 109, 12632–12637. doi: 10.1073/pnas.1204800109
- Martin, A., and Reed, R. D. (2010). *Wingless* and *Aristaless2* define a developmental ground plan for moth and butterfly wing pattern evolution. *Mol. Biol. Evol.* 27, 2864–2878. doi: 10.1093/molbev/msq173
- Martin, A., and Reed, R. D. (2014). Wnt signaling underlies evolution and development of the butterfly wing pattern symmetry systems. *Dev. Biol.* 395, 367–378. doi: 10.1016/j.ydbio.2014.08.031
- Matsuoka, Y., and Monteiro, A. (2019). Hox genes are essential for the development of novel serial homologous eyespots on the wings of *Bicyclus anynana* butterflies. *bioRxiv* [Preprint]. doi: 10.1101/814848
- Mazo-Vargas, A., Concha, C., Livraghi, L., Massardo, D., Wallbank, R. W. R., Zhang, L., et al. (2017). Macroevolutionary shifts of *WntA* function potentiate butterfly wing-pattern diversity. *Proc. Natl. Acad. Sci. U.S.A.* 114, 10701–10706. doi: 10.1073/pnas.1708149114
- McKenna, K. Z., Tao, D., and Nijhout, H. F. (2019). Exploring the role of insulin signaling in relative growth: a case study on wing-body scaling in Lepidoptera. *Integr. Comp. Biol.* 59, 1324–1337. doi: 10.1093/icb/icz080
- Milán, M., Pérez, L., and Cohen, S. M. (2002). Short-range cell interactions and cell survival in the *Drosophila* wing. *Dev. Cell* 2, 797–805. doi: 10.1016/S1534-5807(02)00169-7

- Miner, A. L., Rosenberg, A. J., and Nijhout, H. F. (2000). Control of growth and differentiation of the wing imaginal disk of *Precis coenia* (Lepidoptera: Nymphalidae). *J. Insect Physiol.* 46, 251–258. doi: 10.1016/S0022-1910(99)00177-8
- Mirth, C. K., Truman, J. W., and Riddiford, L. M. (2009). The Ecdysone receptor controls the post-critical weight switch to nutrition-independent differentiation in *Drosophila* wing imaginal discs. *Development* 136, 2345–2353. doi: 10.1242/dev.032672
- Monteiro, A. (2015). Origin, development, and evolution of butterfly eyespots. *Annu. Rev. Entomol.* 60, 253–271. doi: 10.1146/annurev-ento-010814-020942
- Nellen, D., Burke, R., Struhl, G., and Basler, K. (1996). Direct and long-range action of a Dpp morphogen gradient. *Cell* 85, 357–368. doi: 10.1016/S0092-8674(00)81114-9
- Nijhout, H. F. (1991). *The Development and Evolution of Butterfly Wing Patterns*. Washington, DC: Smithsonian Institution Press.
- Nijhout, H. F. (1994). Symmetry systems and compartments in Lepidopteran wings: the evolution of a patterning mechanism. *Development* 1994(Suppl.), 225–233.
- Nijhout, H. F. (2001). Elements of butterfly wing patterns. *J. Exp. Zool.* 291, 213–225. doi: 10.1002/jez.1099
- Nijhout, H. F. (2017). “The common developmental origin of eyespots and parafocal elements and a new model mechanism for color pattern formation,” in *Diversity and Evolution of Butterfly Wing Patterns*, eds T. Sekimura, and H. Nijhout (Singapore: Springer), 3–19.
- Nijhout, H. F., Cinderella, M., and Grunert, L. W. (2014). The development of wing shape in Lepidoptera: mitotic density, not orientation, is the primary determinant of shape. *Evol. Dev.* 16, 68–77. doi: 10.1111/ede.12065
- Nijhout, H. F., and Grunert, L. W. (2002). Bombyxin is a growth factor for wing imaginal disks in Lepidoptera. *Proc. Natl. Acad. Sci. U.S.A.* 99, 15446–15450. doi: 10.1073/pnas.242548399
- Nijhout, H. F., Laub, E., and Grunert, L. W. (2018). Hormonal control of growth in the wing imaginal disks of *Junonia coenia*: the relative contributions of insulin and ecdysone. *Development* 145:dev160101. doi: 10.1242/dev.160101
- Oliveira, M. M., Shingleton, A. W., and Mirth, C. K. (2014). Coordination of wing and whole-body development at developmental milestones ensures robustness against environmental and physiological perturbations. *PLoS Genet.* 10:e1004408. doi: 10.1371/journal.pgen.1004408
- Otaki, J. M. (2012). Color pattern analysis of nymphalid butterfly wings: revision of the nymphalid groundplan. *Zool. Sci.* 29, 568–576. doi: 10.2108/zsj.29.568
- Panganiban, G., Nagy, L., and Carroll, S. B. (1994). The role of the Distal-less gene in the development and evolution of insect limbs. *Curr. Biol.* 4, 671–675. doi: 10.1016/S0960-9822(00)00151-2
- Panin, V. M., Papayannopoulos, V., Wilson, R., and Irvine, K. D. (1997). Fringe modulates Notch–ligand interactions. *Nature* 387, 908–912. doi: 10.1038/43191
- Patel, N. H., Martin-Blanco, E., Coleman, K. G., Poole, S. J., Ellis, M. C., Kornberg, T. B., et al. (1989). Expression of engrailed proteins in arthropods, annelids, and chordates. *Cell* 58, 955–968. doi: 10.1016/0092-8674(89)90947-1
- Paulsen, S. M. (1994). Quantitative genetics of butterfly wing color patterns. *Dev. Genet.* 15, 79–91. doi: 10.1002/dvg.1020150109
- Paulsen, S. M., and Nijhout, H. F. (1993). Phenotypic correlation structure among elements of the color pattern in *Precis coenia* (Lepidoptera: Nymphalidae). *Evolution* 47, 593–618. doi: 10.1111/j.1558-5646.1993.tb02115.x
- Reed, R. D., Chen, P.-H., and Nijhout, H. F. (2007). Cryptic variation in butterfly eyespot development: the importance of sample size in gene expression studies. *Evol. Dev.* 9, 2–9. doi: 10.1111/j.1525-142X.2006.00133.x
- Reed, R. D., and Nagy, L. M. (2005). Evolutionary redeployment of a biosynthetic module: expression of eye pigment genes vermilion, cinnabar, and white in butterfly wing development. *Evol. Dev.* 7, 301–311. doi: 10.1111/j.1525-142X.2005.05036.x
- Reed, R. D., and Serfas, M. S. (2004). Butterfly wing pattern evolution is associated with changes in a Notch/Distal-less temporal pattern formation process. *Curr. Biol.* 14, 1159–1166. doi: 10.1016/j.cub.2004.06.046
- Roeske, M. J., Camino, E. M., Grover, S., Rebeiz, M., and Williams, T. M. (2018). Cis-regulatory evolution integrated the Bric-à-brac transcription factors into a novel fruit fly gene regulatory network. *eLife* 7:e32273. doi: 10.7554/eLife.32273
- Saenko, S. V., Marialva, M. S. P., and Beldade, P. (2011). Involvement of the conserved Hox gene Antennapedia in the development and evolution of a novel trait. *Evodevo* 2:9. doi: 10.1186/2041-9139-2-9
- Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., et al. (2012). Fiji: an open-source platform for biological-image analysis. *Nat. Methods* 9, 676–682. doi: 10.1038/nmeth.2019
- Shen, J., Dahmann, C., and Pflugfelder, G. O. (2010). Spatial discontinuity of Optomotor-blind expression in the *Drosophila* wing imaginal disc disrupts epithelial architecture and promotes cell sorting. *BMC Dev. Biol.* 10:23. doi: 10.1186/1471-213X-10-23
- Strigini, M., and Cohen, S. M. (2000). Wingless gradient formation in the *Drosophila* wing. *Curr. Biol.* 10, 293–300. doi: 10.1016/S0960-9822(00)00378-X
- Sturtevant, M. A., Biehs, B., Marin, E., and Bier, E. (1997). The spalt gene links the A/P compartment boundary to a linear adult structure in the *Drosophila* wing. *Development* 124, 21–32.
- Sugimori, S., Hasegawa, A., and Nakagoshi, H. (2016). Spalt-mediated *dve* repression is a critical regulatory motif and coordinates with Iroquois complex in *Drosophila* vein formation. *Mech. Dev.* 141, 25–31. doi: 10.1016/j.mod.2016.06.004
- Tang, H. Y., Smith-Caldas, M. S. B., Driscoll, M. V., Salhadar, S., and Shingleton, A. W. (2011). FOXO regulates organ-specific phenotypic plasticity in *Drosophila*. *PLoS Genet.* 7:e1002373. doi: 10.1371/journal.pgen.1002373
- Tanimoto, H., Itoh, S., ten Dijke, P., and Tabata, T. (2000). Hedgehog creates a gradient of Dpp activity in *Drosophila* wing imaginal discs. *Mol. Cell* 5, 59–71. doi: 10.1016/S1097-2765(00)80403-7
- Wang, D., Li, L., Lu, J., Liu, S., and Shen, J. (2016). Complementary expression of optomotor-blind and the Iroquois complex promotes fold formation to separate wing notum and hinge territories. *Dev. Biol.* 416, 225–234. doi: 10.1016/j.ydbio.2016.05.020
- Weatherbee, S. D., Frederik Nijhout, H., Grunert, L. W., Halder, G., Galant, R., Selegue, J., et al. (1999). Ultrabithorax function in butterfly wings and the evolution of insect wing patterns. *Curr. Biol.* 9, 109–115. doi: 10.1016/S0960-9822(99)80064-5
- West-Eberhard, M. J. (2019). Modularity as a universal emergent property of biological traits. *J. Exp. Zool. Part B Mol. Dev. Evol.* 332, 356–364. doi: 10.1002/jez.b.22913
- Zecca, M., Basler, K., and Struhl, G. (1995). Sequential organizing activities of engrailed, hedgehog and decapentaplegic in the *Drosophila* wing. *Development* 121, 2265–2278.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2020 McKenna, Kudla and Nijhout. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



The Arctiid Archetype: A New Lepidopteran Groundplan

Richard Gawne¹ and H. Frederik Nijhout^{2*}

¹ Allen Discovery Center, Tufts University, Medford, MA, United States, ² Department of Biology, Duke University, Durham, NC, United States

OPEN ACCESS

Edited by:

Marcus Kronforst,
The University of Chicago,
United States

Reviewed by:

Antonia Monteiro,
National University of Singapore,
Singapore
Joji M. Otaki,
University of the Ryukyus, Japan

*Correspondence:

H. Frederik Nijhout
hfn@duke.edu

Specialty section:

This article was submitted to
Evolutionary Developmental Biology,
a section of the journal
Frontiers in Ecology and Evolution

Received: 29 February 2020

Accepted: 18 May 2020

Published: 10 June 2020

Citation:

Gawne R and Nijhout HF (2020)
The Arctiid Archetype: A New
Lepidopteran Groundplan.
Front. Ecol. Evol. 8:175.
doi: 10.3389/fevo.2020.00175

The nymphalid groundplan (NGP) has proven to be extraordinarily useful in the study of butterfly color patterns because it allows for the identification of homologous elements across species. It has long been thought that the NGP is broadly applicable to all Lepidoptera, implying that the characters which make-up butterfly color patterns are homologous to those found in the moths. However, this conjecture remains mostly untested. We analyzed the wing patterns of the hyper-diverse arctiid tiger moths, which are represented worldwide by approximately 11,000 species, and found that the color patterns of these animals can be parsed into a limited toolkit of homologous characters. Some of the pattern elements identified, such as the basal and central symmetry system, are present on the NGP, but their morphology is often quite different from what is seen in the butterflies. The border ocelli of the NGP appear to be absent altogether in the Arctiidae, and conversely, two distal pattern element we term the “van Bemmelen” and “terminal” bands are present in the color patterns of many arctiids, but are not represented on the NGP. In light of the observed differences, we derive a new theoretical groundplan based on the original NGP that we refer to as the “arctiid archetype.” This model provides a comprehensive framework for understanding how the wing patterns of these animals develop, and yields novel insight into their evolutionary history.

Keywords: color pattern evolution and development, nymphalid groundplan, arctiid archetype, Arctiidae, arctiid moths, tiger moths

INTRODUCTION

The wing patterns of butterflies have proven to be a uniquely tractable system for research at the interface of evolution and development (Nijhout, 1991; Beldade and Brakefield, 2002; Jiggins, 2017). Unlike the stripes of zebras (Jonathan, 1977; Caro et al., 2014) or the networks of spots found on the coats of leopards (Allen et al., 2011), butterfly color patterns are composed of a limited number of homologous characters that are comparable to better known systems of homologs, such as the bones of pentadactyl limbs (Wagner, 2014). A theoretical archetype known as the nymphalid groundplan (NGP) (Nijhout, 1991) allows for the identification of these homologous characters within and across species, making it possible to precisely describe the results of experiments designed to interfere with pattern development (Monteiro et al., 2006; Martin and Reed, 2014; Mazo-Vargas et al., 2017), and identify large-scale trends in pattern evolution (Monteiro, 2008). Since its rediscovery in the 1970s (Nijhout, 1978), the NGP has directly stimulated many of the advances that have been made in our understanding of butterfly color patterns.

Although we now know a great deal about butterfly wing patterning, our knowledge of how the patterns of moths develop and evolve is comparatively limited. Researchers have often speculated that nymphalid groundplan homologs must be present in the color patterns of these animals, but attempts to test this conjecture empirically have been few and far between (Schachat and Brown, 2015, 2016; Schachat and Goldstein, 2018; Gawne and Nijhout, 2019). The aim of this paper is to broaden our understanding of lepidopteran wing patterning by determining whether the dorsal forewing patterns of the hyperdiverse arctiid “tiger moths” [Lepidoptera, Erebiidae (Arctiidae)]¹ can be decomposed into an underlying system of characters that are homologous to elements found on the nymphalid groundplan. The arctiids exhibit an enormous amount of pattern diversity both within and across species, which makes them interesting from an evolutionary and developmental point of view. A previous study has shown that it is possible to locate NGP characters such as the basal symmetry system, central symmetry system, and discal spot in the patterns of the tiger moth *Utetheisa ornatrix* (Gawne and Nijhout, 2019), but no attempt has yet been made to assess the generality of this finding through a large-scale study of the Arctiidae.

An analysis of specimens in museum collections suggested that the nymphalid groundplan homologs identified in *U. ornatrix* are widespread among the arctiids, and confirmed that tiger moth color patterns often contain two unique characters that are not depicted on modern renditions of the groundplan (Gawne and Nijhout, 2019). The fact that the arctiids deviate from the NGP in this way suggests that research on wing patterning in this subfamily will necessitate a return to earlier versions of the model, which included supernumerary characters that are primarily found in the moths (Gawne and Nijhout, 2019). To help stimulate research on arctiid color patterns, we introduce a modified version of the original groundplan (Schwanwitsch, 1924; Süffert, 1927) that we refer to as the arctiid archetype or “arctiotype.” This theoretical model has the potential to motivate novel experimental studies of the arctiids because it allows us to identify homologies, and make sense of the diversity observed their wing patterns.

We suggest that the dorsal forewing patterns of the arctiids are constructed from a maximum of five primary characters: a basal and central symmetry system, along with a discal spot, and two external characters we have termed the “van Bemmelen,” and “terminal” bands. Similar to what previous researchers have noted about NGP homologs in the butterflies (Nijhout, 1991; Otaki, 2012), there is no expectation that the entire suite of characters shown on arctiotype will be present in every species or individual. Rather, our hypothesis is that no tiger moth wing patterns contain supernumerary primary elements that are not depicted on the arctiid archetype. The combination, alteration, and interaction of the five archetypal characters, along with

the occasional inclusion of “detailed” elements such as venous stripes and stochastic melanization (Nijhout, 1991) is sufficient to generate the vast array of color patterns observed in the tiger moths. Thus, in delineating the homologies of arctiid wing patterns, we have identified the morphological toolkit these animals have employed and redeployed to move through morphospace over the course of their evolutionary history.

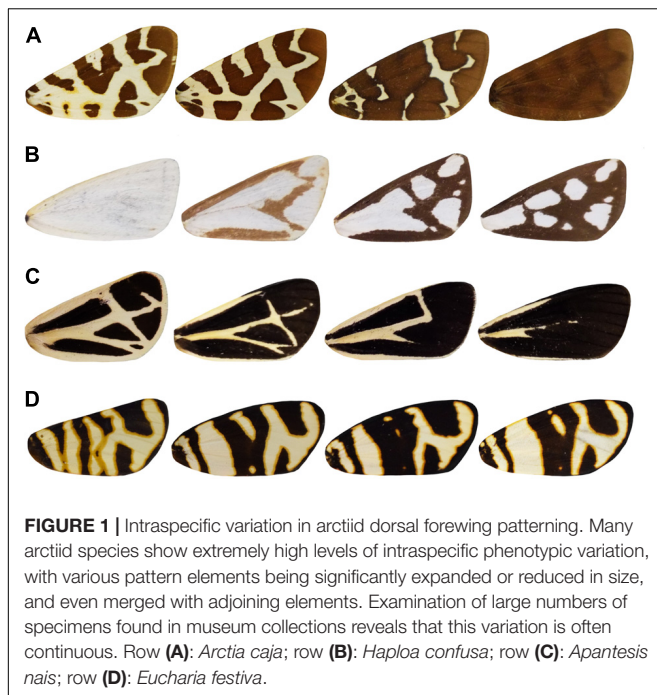
METHODS

To survey the diversity of tiger moth dorsal forewing patterns, we photographed over 1,100 mounted specimens belonging to an estimated 100 different species. These animals were found in the collections of the Smithsonian National Museum of Natural History, the Vienna Museum of Natural History, and the Hungarian Museum of Natural History. Specimens were photographed with either a Nikon Coolpix L340 (20.2 megapixels) or a Nikon D-5300 (24.1 megapixels) digital camera, using overhead florescent lighting and a scale bar. The resulting images provided the basis for the figures presented in this paper. Photoshop (Adobe Inc.) was used to edit these images, allowing for easier analysis of the macro-structure of the dorsal forewing pattern, and on-screen/in-print readability. The final, fully assembled figures were produced in Photoshop and PowerPoint (Microsoft Corp.). When relevant, left forewings were transformed via a horizontal flip to ensure a common orientation of the samples. The antennae have been drawn in on all full specimens shown (Figures 2, 3, 9).

A simple visual analysis of the photographic dataset revealed that arctiid moths show an enormous amount of variation in patterning both within (Figure 1) and across species (Figure 2). To help systematize the observed interspecific differences in phenotype, we attempted to identify recurring types of dorsal forewing patterning (Figure 2), and describe the varieties of non-homologous detailed elements (Nijhout, 1991) that occur in the photographed specimens (Figure 3). Detailed elements are common in arctiid color patterns, and similar to what has been documented in the butterflies (Nijhout, 1991), it appears that these features can sometime become the dominant component of the pattern.

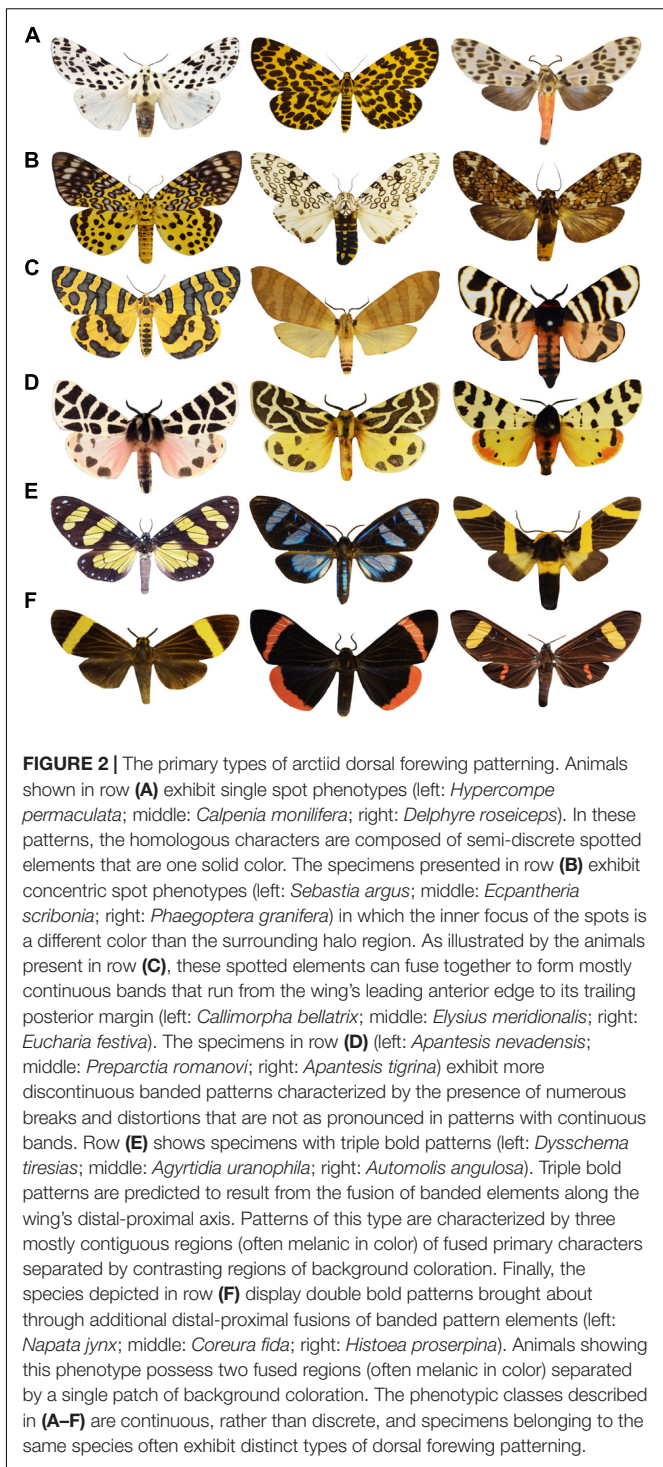
To test this hypothesis, we artificially selected for intensified venous striping in *Utetheisa ornatrix* (Figure 4). This experiment began with a large, previously established laboratory colony that contained animals with a diverse array of pattern morphologies (Gawne and Nijhout, 2019). Animals were reared under controlled day/night, temperature, and nutritional conditions that have previously been described (Gawne and Nijhout, 2019). Individuals with white stripes that outlined the contours of the veins were known to appear in the colony. To determine whether the intensity of venous striping could be increased through artificial selection, we separated a population of approximately 10–20 animals with white stripes from the main colony and allowed them to mate. Some of the animals resulting from these initial pairings displayed slightly intensified venous striping. These individuals were then put back into the experimental colony, and allowed to mate with one another, and any remaining

¹We are aware that the family Arctiidae has recently been reclassified as the sub-family Arctiinae (Zahiri et al., 2012). However, this re-classification has not yet become universally adopted and the new terminology is largely unknown to researchers in evolutionary and developmental biology. For this reason, we have chosen to retain terms such as “Arctiidae” and “arctiid,” but in doing so we are not intending to make any specific taxonomic claims.

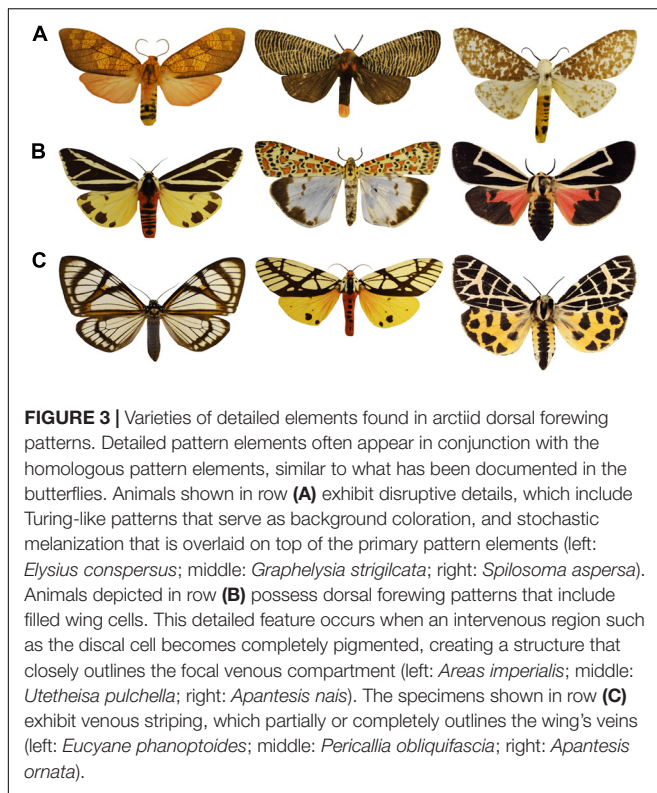


animals from the parental generation. This selection regime was continued for two additional generations. During the four generations over which the experiment was carried out, we periodically sampled animals with more pronounced venous striping to document their wing patterns. The forewings of these animals were removed, and mounted between two microscope slides. These prepared specimens were then photographed using an Olympus SZX16 stereomicroscope equipped with an Olympus DP71 camera. The resulting images were subsequently edited in Photoshop to prepare them for use in the figures, similar to our previous work on *U. ornatatrix* (Gawne and Nijhout, 2019).

The identification of morphological types provides insight into the parameters of arctiid pattern morphospace, and allows for more nuanced analyses designed to locate specific homologous characters. Based on our earlier work with *U. ornatatrix* (Gawne and Nijhout, 2019), and an examination of the positioning of elements in the specimens used for this study, we hypothesized that tiger moth color patterns are composed of a maximum of five homologous characters, three of which are found in the modern rendition of the NGP (Nijhout, 1991, 2001; Otaki, 2012). Accordingly, we developed an arctiid archetype that depicts what appear to be the primary homologous characters found in the dorsal forewing patterns of the tiger moths (Figure 5). The arctiid archetype is a hypothesis that purports to identify the complete morphological toolkit the arctiid moths have used to generate their phenotypic diversity. Like the nymphalid groundplan, it omits detailed pattern elements (Nijhout, 1991; Otaki, 2012) such as venous stripes and stochastic peppering, and instead shows only a series of five homologous pattern elements. Following the lead of early butterfly color pattern researchers (Schwanwitsch, 1924; Süffert, 1927), we have attempted to



demonstrate the plausibility of the archetype using comparative morphological mapping. An analysis of the inter- and intra-specific variation documented in museum collections allows for inferences to be drawn about the location of the homologous pattern elements in the six most common types of arctiid wing patterns (Figure 6).



RESULTS

The arctiid moths exhibit some of the most pronounced intraspecific wing pattern variation found in the Lepidoptera (Figure 1). To give just a few examples, within a given species it is possible to identify variation in background coloration, the number of primary elements, and the overall morphological structure the pattern. Looking across species, the dorsal forewing patterns generally fall into one of three broad phenotypic classes: spotted, banded, and bold (Figure 2). In species with spotted patterns, the intervenous elements that compose the presumptive homologous characters take the form of a highly discretized series of spots arranged in columns that run anterior-posterior across the wing. Spotted patterns can be further decomposed into two sub-types: (a) “single spot” phenotypes where all spots are homogenous in their coloration, and (b) “concentric spot” phenotypes, in which a large halo-like ring surrounds a spot of a different color.

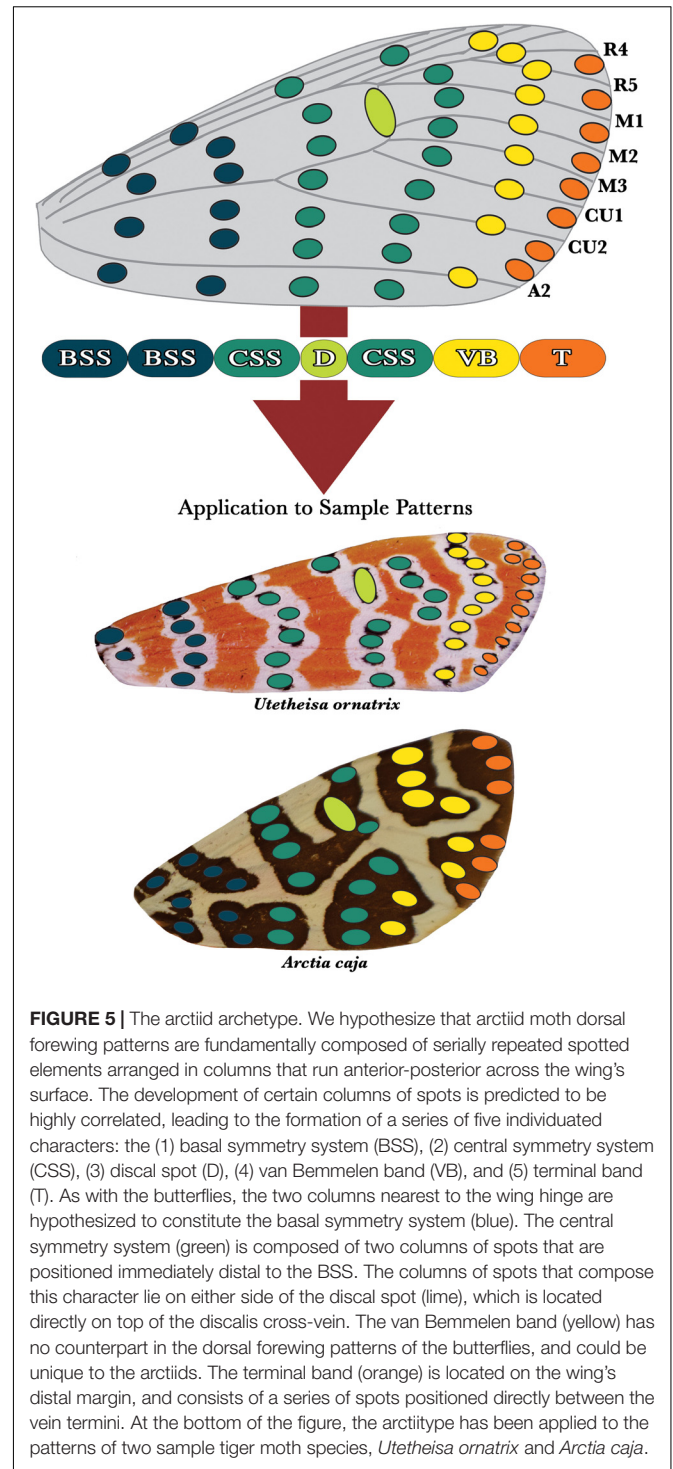
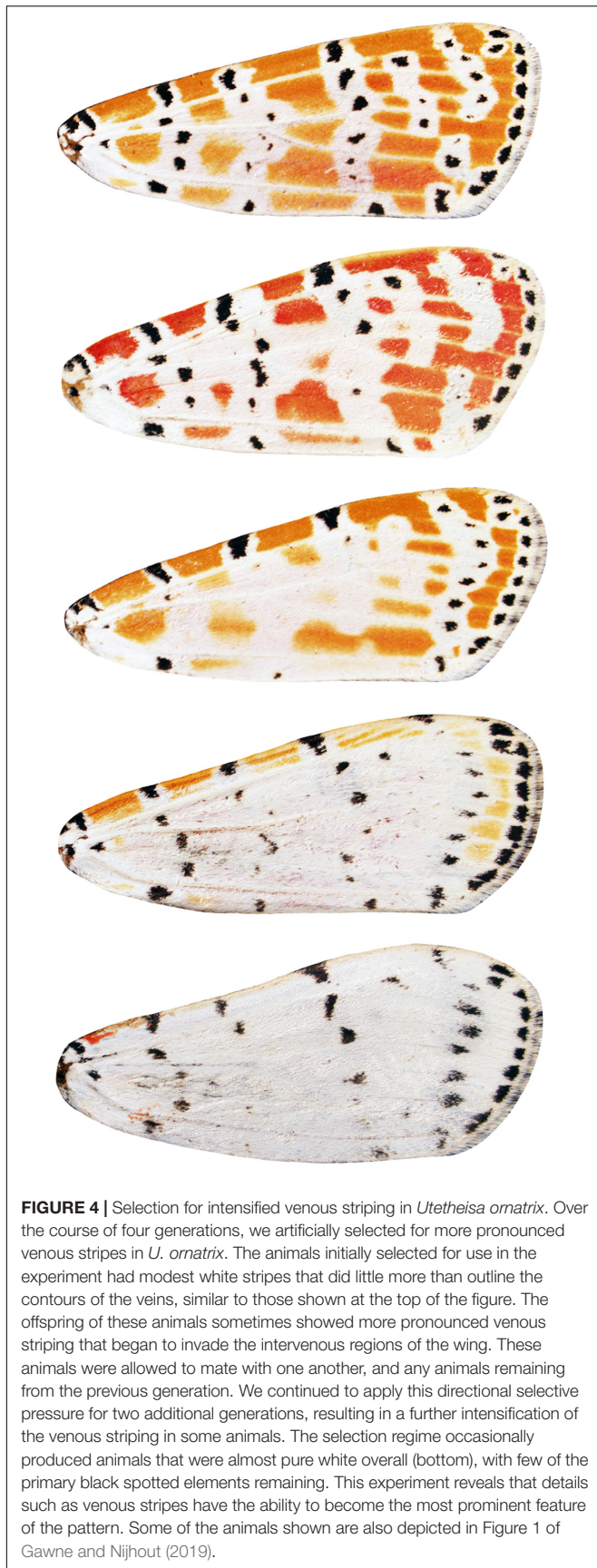
As with the spotted patterns, banded phenotypes can be separated into two varieties. In species that exhibit “continuous bands,” a series of semi-parallel stripes run mostly uninterrupted across the wing's surface in an anterior-posterior direction. In the “broken band” sub-type, the columnar banded patterns exhibit more regular or pronounced interruptions, especially along the CU2 vein. Bold patterns are the third class of phenotypes found in the arctiids. In most instances, this type of patterning appears to result from the fusion of characters, predominantly along the wing's proximo-distal axis. Bold phenotypes are characterized by the presence of contiguous melanic regions that are interspersed

with smaller islands of background coloration (Nijhout and Wray, 1988; Nijhout, 1991). For comparative purposes, we have divided bold patterns into the sub-classes “double” and “triple,” which are differentiated by the number of fused melanic regions present on the wing.

As shown in Figure 3, detailed elements such as venous striping, filled wing cells, ripples, and stochastic peppering can also be found in tiger moth wing patterns. Details are often overlaid on top of the primary pattern elements, but can also become the dominant feature of the pattern. Our artificial selection experiment with *U. ornatrix* (Figure 4) revealed that the venous striping which initially did little more than outline the contours of the wing veins could be intensified through the application of directional selective pressure. In no more than four generations, we were able to produce animals in which the formerly modest venous stripes expanded into the intervenous regions. The fact that details such as venous stripes can be upregulated in this way is important because it raises the possibility that some of the bold patterns observed in the tiger moths might be produced by the expansion of these elements, rather than the fusion of spots or bands that comprise the primary characters. This hypothesis is reinforced by an examination of other arctiid species (Figure 7).

It appears that the developmental processes responsible for generating venous stripes in the tiger moths are at least partially independent of the mechanisms that give rise to other pattern components. However, very little is known about the specific developmental pathways that produce the detailed elements of any lepidopteran color pattern, and as such we cannot be certain that the same ontogenetic processes are responsible for their formation in every individual or species. Because of this, we suggest that details must be treated as phenotypic analogies until their development is more thoroughly understood. This implies that the melanic peppering, venous striping, ripple patterns, etc. observed in one arctiid species should not be assumed to be homologous to similar details found in the patterns of other species (Nijhout, 1991).

The arctiid archetype is visualized in Figure 5. Like the nymphalid groundplan, this archetype has been derived from an analysis of intraspecific pattern variation, and interspecific pattern diversity. We suggest that the dorsal forewing patterns of the arctiids are produced from a morphological toolkit that consists of no more than five primary characters: (1) a basal symmetry system, (2) a central symmetry system, (3) a discal spot, and two distinct marginal characters we term the (4) van Bemmelen and (5) terminal bands. The basal symmetry system shown on the arctiidtype consists of two columns of spots that run side-by-side near the wing hinge. Similarly, the central symmetry system is composed of two columns of spots that lie on either side of the discal spot. As with the NGP (Nijhout, 1991, 2001), the discal spot depicted on the arctiid archetype is a relatively simple character that is always located on the discalis cross-vein. The van Bemmelen band is a unique trait that has no counterpart on the modern version of nymphalid groundplan. It is located immediately adjacent to the central symmetry system's distal band. This character is composed of smaller spotted elements (sometimes merged into a band-like



column) that can be located directly on top of the wing veins (Gawne and Nijhout, 2019). The final character shown on the arctiid archetype is the terminal band, which generally consists of a series of semi-discrete spots that are positioned between the vein termini along the wing's distal margin.

The basal and central symmetry systems shown on the arctiid type are likely homologs of their counterparts on the

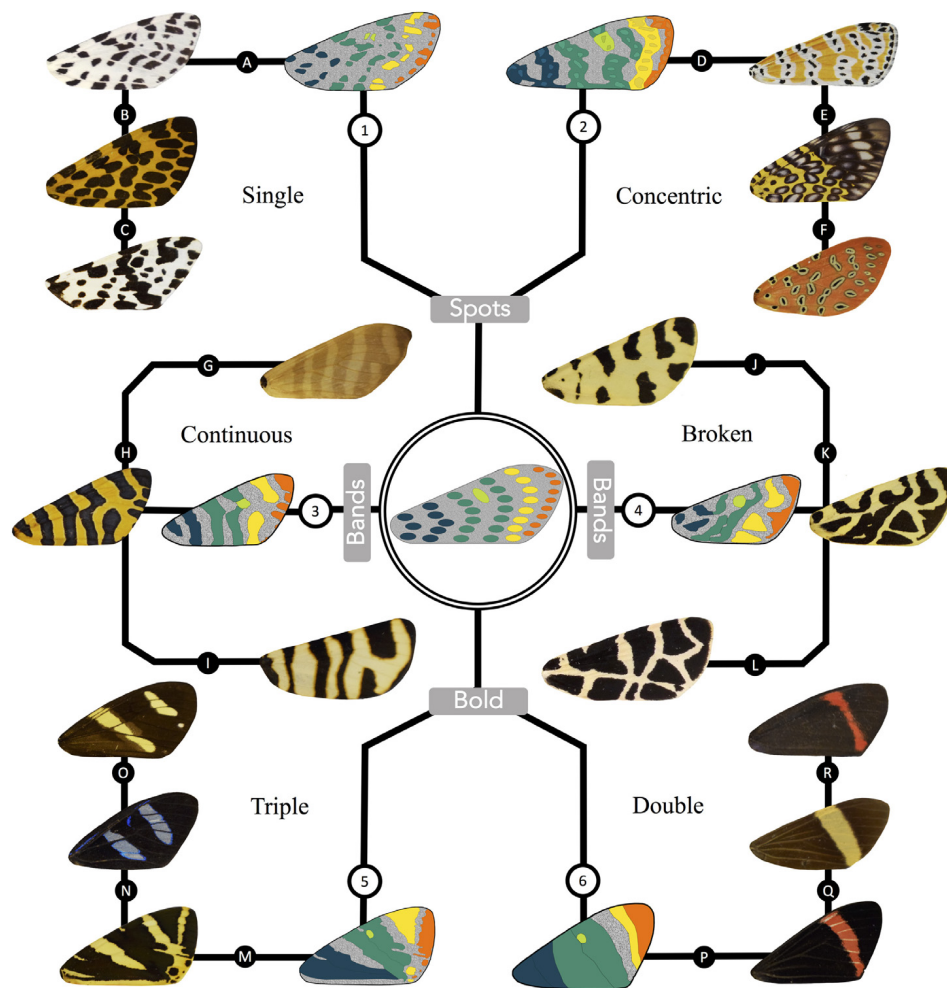


FIGURE 6 | A derivation of the primary types of arctiid patterning from the morphologically simple archetypal characters. The arctiid archetype is depicted in the center of the figure, with six surrounding branches that are representative of the various types of patterning found in the tiger moths. The basal symmetry system, central symmetry system, discal spot, van Bemmelen band, and terminal band (colored blue, green, lime, yellow and orange, respectively) have been identified in one sample species from each of the six types. The top set of branches show animals exhibiting the (1) single spot, and (2) concentric spot phenotypes. Continuous (3) and broken band (4) phenotypes are shown on the branches to the left and right of the archetypal pattern. Bold patterns are depicted at the bottom of the figure. The animals shown in branch (5) exhibit a “triple” bold phenotype, where the primary pattern elements have fused to form three more or less continuous patches. Branch (6) shows animals exhibiting the “double” bold phenotype. In patterns of this type, fusion of the primary characters is more complete, resulting in a pair of dark-colored regions on either side of an isolated island of background coloration. Specimens from the following species are represented in the figure. Group 1: (A) *Hypercompe permaculata*, (B) *Calpenia monilifera*, (C) *Eucereon coenobita*; Group 2: (D) *Utetheisa ornatrix*, (E) *Sebastia argus*, (F) *Argina argus*; Group 3: (G) *Elysia superbus*, (H) *Amphicallia thelwalli*, (I) *Eucharia festiva*; Group 4: (J) *Apantesis tigrina*, (K) *Preparctia romanovi*, (L) *Apantesis nevadensis*; Group 5: (M) *Callimorpha quadripunctaria*, (N) *Agyrtidia uranophila*, (O) *Chetone isse*; Group 6: (P) *Coreura fida*, (Q) *Napata jynx*, (R) *Coreura euchromioides*.

nymphalid groundplan (Gawne and Nijhout, 2019). However, the symmetry systems of the arctiotype differ from those of the NGP in that they are represented as discrete columns of spots, as opposed to fused bands (Gawne and Nijhout, 2019). This has been done to indicate that spotted elements produced by presumptive morphogenetic point sources are the most basic compositional unit of arctiid wing patterns. The banded and bold patterns found in the arctiids are emergent structures that result from the fusion of these smaller elements, as occurs, e.g., in the *Heliconius* butterflies (Nijhout and Wray, 1988).

In Figure 6, we have used the arctiotype to preliminarily identify homologous pattern elements in the six major classes

of tiger moth wing patterns. The archetype itself is shown in the center of this figure, and the pattern types (1) single spot, (2) concentric spot, (3) continuous band, (4) broken band, (5) triple bold, and (6) double bold are shown around its periphery. For each of these classes, we have illustrated the symmetry systems and other primary elements for the first specimen shown in the series. The presumptive pattern homologs have been color-coded to match the appropriate characters in the arctiotype, i.e., the basal symmetry system is shown in blue, the central symmetry system has been colored green, the discal spot is lime, the van Bemmelen band is yellow, and the terminal band is depicted in orange. Immediately adjacent to the stylized graphical figure



FIGURE 7 | Continued

FIGURE 7 | Bold patterns can be produced through the intensification of venous striping. The scheme depicted in **Figure 6** suggests that bold patterns are produced from the distal-proximal fusion of primary elements. However, examination of specimens found in museum collections seems to reveal that an alternative mechanism of bold pattern development also exists. For example, in the genus *Callopepla*, it appears that bold patterns can be produced through an intensification of venous striping, which has the ability to expand into the intervenous regions and produce continuous patches of melanic coloration. Specimens such as those shown exhibit venous striping which varies continuously in intensity, and a gives rise to a corresponding continuum of overall pattern “boldness” (Top to bottom: *Callopepla inachia*; *Callopepla grandis*; *Callopepla similis*; *Callopepla similis*).

shown for each type is a photograph of the focal wing it is based on, followed by two additional samples which belong to the same phenotypic class. As shown in **Figure 6**, the archiotype can be applied to a diverse array of tiger moth wing patterns in order to identify presumptive pattern homologs within and across species. The fact that the archetype can be used to identify homologous characters enables us to tame what initially appears to a bewildering amount of pattern diversity, and this in turn allows us to ask novel questions about how the dorsal forewing patterns of the arctiids develop and evolve.

DISCUSSION

The arctiid archetype described in the preceding sections is remarkably similar to a scheme proposed by the Dutch biologist van Bemmelen (1918). Van Bemmelen’s paper represents an important intellectual achievement, as it constitutes one of the first attempts to identify pattern homologies within the Lepidoptera, and subsequently use the presence of these characters to describe and systematize interspecific phenotypic diversity. Unfortunately, his groundbreaking study was almost immediately lost to history (but see Hegna, 2013; Hegna and Mappes, 2014). Within a decade of Van Bemmelen’s work, Schwanwitsch (1924) and Süffert (1927) independently published the comparative analysis of butterfly color patterns that eventually became encapsulated as the nymphalid groundplan (Nijhout, 1991). Although Schwanwitsch showed that his groundplan was also broadly applicable to the moths, neither he nor Süffert seems to have paid much attention to Van Bemmelen’s model, noting it merely as one of several prior works that pointed out the significance of crossbands as a fundamental organizing principle of lepidopteran color patterns. A decade later, Sokolow (1936) published a lengthy study of the evolution of color patterns in the Arctiidae, but his work was based on that of Schwanwitsch, and gave only a glancing nod to van Bemmelen’s study. Sokolow’s analysis was largely focused on what we have referred to as the “bold” patterns, and used Schwanwitsch’s coding nomenclature. Although we only became aware of van Bemmelen’s work recently, we had independently arrived at many of the same conclusions that he reached about the morphological structure of tiger moth dorsal forewing patterns [cf. (Nijhout, 1991) on the parallel and independent development of the NGP by Süffert and Schwanwitsch].

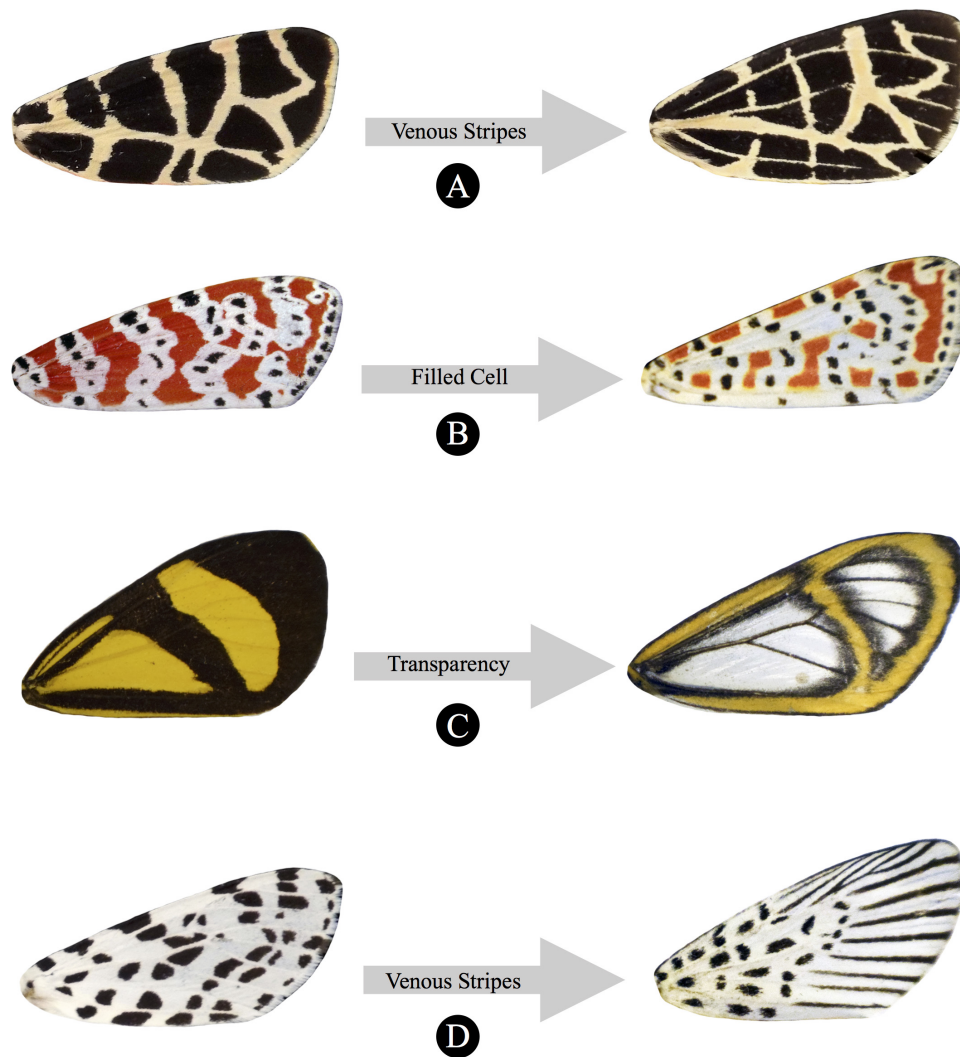


FIGURE 8 | The diversity observed in tiger moth wing patterns is not attributable solely to changes in the size, shape, and position of the five archetypical characters. Detailed elements can be added to dorsal forewing patterns, and their inclusion often radically changes its appearance. Row (A) shows how the inclusion of overlaid venous stripes can alter pattern morphology (left: *Apantesis nevadensis*; right: *Apantesis ornata*). Row (B) illustrates how a filled discal wing cell can significantly alter a color pattern's appearance (left: *Utetheisa ornatrix*; right: *Utetheisa pulchella*). As shown in row (C), the replacement of various pattern features with transparency can be a source of pattern diversity (left: *Ephestria melaxantha*; right: *Hyalurga fenestra*). With *H. fenestra*, the black primary characters have become transparent, but in some wasp-mimicking species, it appears that the background coloration has been modified to become transparent. Finally, the animals shown in row (D), demonstrate that the addition of venous stripes can change the overall structure and appearance of the pattern (left: *Hypercompe permaculata*; right: *Agrisius guttivitta*).

At first glance, the dorsal forewing patterns found in the tiger moths are almost bafflingly diverse – some have spots, while others have roughly parallel bands, or contiguous monochromatic patches of pigmentation, and the overall structure of a pattern can be radically altered through the addition of detailed elements (Figure 8). To complicate things even further, the incomplete fusion of primary pattern elements often produces isolated regions of background coloration that can easily be mistaken for novel characters (Figure 9). Like van Bemmelen, we have attempted to systematize the pattern diversity observed in the arctiids by taking a reductionist approach which assumes that the patterns of all these animals

are composed of a limited number of common compositional units: serially repeated spots arranged in columns [somewhat misleadingly referred to as “rows” by Gawne and Nijhout (2019)] that run anterior-posterior across the wing’s surface. Comparative analyses of pattern morphology using large numbers of specimens strongly suggests that the bands found in arctiid wing patterns are emergent “pseudocharacters” that arise as a byproduct when intervenous spots fuse with their anterior/posterior neighbors. Developmental studies have revealed that the intervenous spots found in lepidopteran color patterns are produced by diffusible morphogens that spread out from point sources located between the wing veins (Nijhout,

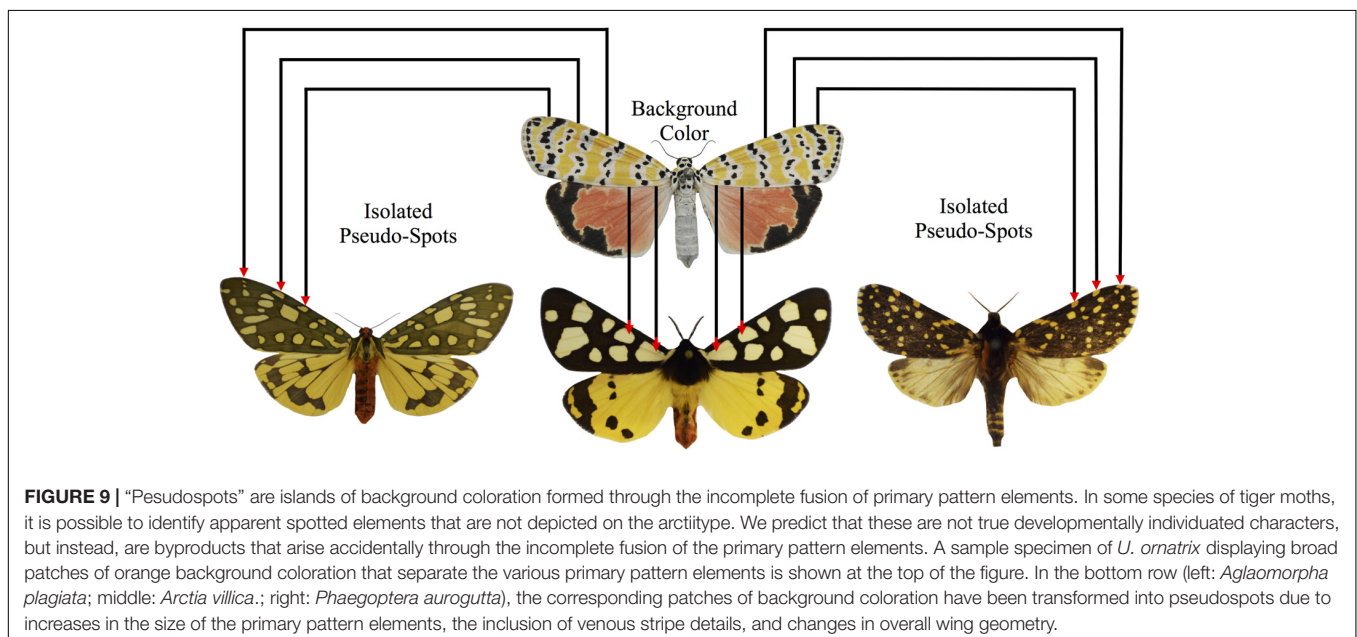
1991; French and Brakefield, 1992; Monteiro et al., 2001). This being so, banded phenotypes are predicted to be produced when: (a) the secretory cells which constitute the point sources produce a larger quantity of the morphogen, or (b) the receiving tissue becomes more sensitive to this signal, such that a lower concentration of the chemical suffices to initiate pigment synthesis in the scale cells.

We hypothesize that bold patterns arise in a similar manner. The continuous melanic regions observed in patterns of this type are produced when multiple columns of spots belonging to either the same or different systems, fuse with one another in a proximo-distal direction. Patterns of this type could be formed through changes in the way morphogens are secreted and received, but in addition, we predict that their development is dependent upon alterations to the wing's overall shape. Venous junction points on the wing provide positional information which functions to “anchor” the primary characters to one region of the wing, rather than another. If the distance between the junction points is reduced, then all else being equal with the morphogen point sources, we would expect the characters to fuse in a distal-proximal direction, and produce a bold pattern. Experimental studies of the relevant morphogens, and quantitative analyses of wing shape will be needed to confirm the validity of this hypothesis, which is readily testable using existing molecular, statistical, and image processing tools.

Like van Bemmelen, we believe that the identification of large-scale trends in tiger moth patterning is heavily dependent upon our ability to draw a distinction between the primary pattern elements depicted in **Figure 5**, and the remaining portion of the wing that is now commonly referred to as “background coloration” (Nijhout, 1991). The separation of ground and foreground is essential (**Figure 9**) because it enables researchers to communicate using a common language, and allows for experimental reproducibility. Failure to do this has often led

to semantic difficulties, especially with bold patterns such as those found in the *Heliconius* butterflies [(Nijhout and Wray, 1988), but see Jiggins (2017) for an alternative interpretation]. When a distinction between ground (background coloration) and foreground (pattern) is not made, it is extremely difficult to homologize pattern elements (Nijhout and Wray, 1988), and perhaps more importantly, there is a tendency to treat isolated patches of background color, e.g., the “Belem spot” and “Ecuador triangle” in *Heliconius*, as individuated characters (Nijhout et al., 1990; Brakefield and French, 1993; Morris et al., 2019) [see Van Valen (1982) for discussion of an analogous problem in comparative dental morphology]. In the tiger moths, the background coloration is often a bright color such as red, orange or yellow, and the primary pattern elements tend to be black, brown, or white. As the literature on tiger moth color patterns grows, it will be essential for researchers to agree on a common terminology, and then consistently stick with the conventional designations in order to avoid unproductive disagreements pertaining to the assignment of homologies.

Although we find ourselves to be in agreement with van Bemmelen on many issues, there is at least one important difference between his system and the archetype we have proposed. According to van Bemmelen, “the color-pattern of Arctiidae [can] be deduced from an ancestral fundamental form, in which a light ground is divided into seven fields by a corresponding number of transverse rows of dark spots” (van Bemmelen, 1918, p. 8). The pattern depicted on the arctiotype (**Figure 5**) is composed of seven columns of spots, and thus it would appear that we are in agreement with him on this issue. However, our analysis assumes that the discal spot is a unique character, whereas van Bemmelen combined this element with the central symmetry system, and counted certain discontinuous pattern elements twice. In many tiger moths, the distal portion of the central symmetry system is broken and “pierellized” (Nijhout,



1991) posterior to the cell formed by the M3 and CU1 veins, giving the impression that this unified character is actually two separate elements. A previously published study (Gawne and Nijhout, 2019) speaks against such an interpretation. Our comparative studies of tiger moth color patterns suggest that they composed of five primary characters which, in their more discretized states, divide the wing into a total of seven “fields”: (i) two columns that comprise the basal symmetry system, (ii) two columns that comprise the central symmetry system, (iii) a discal spot, and a pair of distal elements that we have designated the (iv) van Bemmelen, and (v) terminal bands.

As mentioned earlier, the basal symmetry system, central symmetry system, and discal spot are likely homologs of similarly designated pattern elements that have been identified in the butterflies (Nijhout, 1991), and other groups of moths (Schwanwitsch, 1956). This judgment is based on the spatial positioning of these elements on the wing—the basal symmetry system is located near the wing hinge, the discal spot is positioned on the discalis vein, and the central symmetry system lies on either side of the discal spot—and some relatively uncontroversial assumptions about the conservation of developmental mechanisms. That being said, there is no denying that the symmetry systems found in the arctiids are visually distinct from those present in the butterflies. As described above, the basal and central symmetry systems of tiger moth color patterns are likely produced by, and often manifest phenotypically as serially repeated spots. Conversely, in the butterflies these characters tend to take the form of continuous bi-colored bands that are mirror images of one another (Nijhout, 1991; Otaki, 2012). These phenotypic differences are significant, but do not in themselves provide evidence that the characters in question are non-homologous.

What matters in the ascription of homology are shared developmental mechanisms (Roth, 1984; Wagner, 2014), and like the pattern elements found in the tiger moths, butterfly symmetry systems are known to be produced by serially repeated intervenous morphogen point sources. We hypothesize that the gene regulatory networks that produce correlations in the development of the spots which jointly compose the basal symmetry system, central symmetry system, and discal spot are implicated in the formation of these characters in both the butterflies and tiger moths. Under such circumstances, these pattern elements would be homologs. To put the situation in slightly different terms, we are suggesting that the central symmetry system found in a tiger moth such as *U. ornatrix* and a focal species of butterfly represent different character states of the same underlying character identity (Wagner, 2014, 2016). Given that we currently have very little data on color pattern development in the tiger moths, it is difficult to make any sort of conclusive homology assignments, but the morphology and positioning of the various elements on the wing is highly suggestive, and we expect that molecular studies will eventually vindicate our claims of homology.

Although the tiger moths and butterflies have several common pattern elements, they also differ in at least two respects. Specifically, arctiid color patterns: (a) lack both border ocelli and parafoveal elements, and (b) contain two unique elements not

found in the patterns of the butterflies, i.e., the van Bemmelen band and the terminal band. The absence of the border ocelli is hardly surprising – these characters have never been documented outside of the Rhopalocera, and likely emerged as an evolutionary novelty within this clade (Oliver et al., 2014). Similarly, given that the parafoveal elements are thought to develop in conjunction with the border ocelli (Nijhout, 1991, 2017), the absence of the former characters is also predictable. What makes tiger moth color patterns interesting is not the absence of these elements, but the fact that they appear to have characters that are not present in the butterflies. The van Bemmelen band is of special interest developmentally because in species such as *U. ornatrix*, the spots that compose this character are located directly on top of the wing veins. In the butterflies, details such as venous stripes are positioned on the veins, but all of the primary characters except the discal spot develop exclusively in the interstitial region between the wing veins. It is possible that in the tiger moths the column of spots we have referred to as the van Bemmelen band (a primary character) is actually a series of truncated venous stripes (a detailed element). However, the fact that *U. ornatrix* and other species are known to develop non-truncated venous stripes (Figure 4) which sometimes occur in conjunction with the van Bemmelen band's co-located spots seems to speak against such an interpretation.

Additionally, there are species such as *Hypercompe permaculata* (Figure 2, Row A, left specimen) in which the apparent van Bemmelen band is composed of spots that are positioned *between* the wing veins. With this species, none of the primary pattern elements other than the discal spot are located on top of the veins. This could mean that the location of the spots comprising the van Bemmelen band is indeterminate, i.e., they have the potential to exist either on or between the wing veins. However, we believe that an alternative explanation is more plausible. In the butterflies, intervenous pattern elements are known to break apart, and disassociate from one another (Nijhout, 1991). This can mistakenly give the impression that a particular species or individual possesses a novel character(s). Knowing that this is possible in the Rhopalocera, we suggest that *H. permaculata* and similar species could lack a van Bemmelen band. Under such circumstances, the intervenous spots positioned where this structure would normally be are dissociated portions of either the distal band of the central symmetry system, or the neighboring terminal band. These, and other peculiarities associated with the van Bemmelen band are intriguing puzzles that represent an opportunity for future research.

One important issue that has not yet been discussed is the relationship between the arctiid archetype and the nymphalid groundplan. As mentioned earlier, the NGP is a conceptual tool that allows us to make sense of the pattern diversity observed in lepidopteran color patterns. When Schwanwitsch (1924) and Süffert (1927) were originally developing this model, they looked at a large number of nymphalid butterflies, and identified every novel pattern element they could find in this family. Each of these characters was subsequently depicted on the groundplan in order to allow for the identification of homologous elements

across the Nymphalidae. Later, Schwanwitsch (Schwanwitsch, 1956) would add additional characters to the NGP in order to make it more broadly applicable to other species of butterflies and moths, including the arctiids. When the NGP was resurrected in the 1970s, it was primarily in the context of developmental research of butterfly color patterns (Nijhout, 1991; Otaki, 2012) and accordingly, the archetype was modified yet again. This time, a number of elements found in the moths were removed, and new features such as the marginal and sub-marginal bands were added (Nijhout, 1991; Otaki, 2012).

The point in recounting all of this is to emphasize that the NGP is a theoretical construct that we are free to update in light of emerging data, or alter as we see fit in order to suit the needs of a particular research program. In its modern form, the NGP has been streamlined to allow for ease of use in the context of evolutionary and developmental studies of butterfly color patterns (Nijhout, 1991; Gawne and Nijhout, 2019). One way to make the NGP applicable to tiger moth wing patterns is to return to an earlier incarnation of the model which included the characters that Schwanwitsch (1956) referred to as “umbræ” and “externæ.” These pattern elements appear to correspond to the van Bemmelen and terminal bands described above, and it is worth emphasizing again that researchers such as Sokolow (1936) successfully applied an early version of the NGP to the arctiids. However, in order to avoid unnecessary confusion caused by the surplus of characters on this rendition of the groundplan, it seems more prudent to proceed by crafting a new model that depicts all and only those characters that are present in the dorsal forewing patterns of the tiger moths. The arctiid type fits this description, and allows for the identification of homologous pattern elements in the roughly 11,000 existing species of arctiids (Weller et al., 2009).

Before concluding, it is important to emphasize that the arctiid archetype we have presented is much more than a tool for understanding the morphological structure of arctiid wing patterns. To date, the vast majority of research on color pattern development has focused on the butterflies, and few would dispute that this emphasis has made it difficult to construct truly generalized ontogenetic models that are applicable to the Lepidoptera as a whole. The arctiid archetype has the potential to help remedy this situation by allowing us to accurately describe and quantify the results of experimental studies designed to interfere with the mechanisms that drive pattern development in these animals. Ecological and evolutionary research on arctiids such as *U. ornatrix* (Conner et al., 1981; Dussourd et al., 1988; LaMunyon and Eisner, 1993; Egan et al., 2016) and the wood tiger *Arctia plantaginis* (Nokelainen et al., 2012, 2014; Henze et al., 2018; Rojas et al., 2019) has been ongoing for decades, and the associated literature touches on a number of fundamentally

important theoretical issues in evolutionary biology, including aposematic signaling, mimetic resemblance, and sexual selection. Given that both *U. ornatrix* and *A. plantaginis* can be reared in the lab, an opportunity exists for developmental biologists to provide ontogenetic context for these innovative evolutionary studies. Moreover, since the arctiids are so species rich, new model systems chosen specifically for their heuristic value in developmental contexts are undoubtedly waiting to be introduced. Tiger moths are widely revered as aesthetic objects, but they are much more than visually striking curiosities. These animals are a scientific resource that remains almost completely untapped.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

RG and HN jointly conceived of the ideas presented, and contributed equally to the final manuscript.

FUNDING

This work was supported by the grant IOS-1557341 from the National Science Foundation.

ACKNOWLEDGMENTS

We thank Jameson Clarke, Laura Grunert, and Ken McKenna for their input and assistance during various stages of this project. Audiences at the National Center for Biological Sciences (NCBS) and the University of Jyväskylä provided stimulating feedback and helpful criticisms of the ideas presented. Comments from two anonymous *Frontiers* reviewers significantly improved the quality of the final manuscript. We are grateful to the curators and staff at the Smithsonian National Museum of Natural History, the Vienna Museum of Natural History, and the Hungarian Museum of Natural History for allowing us to use their collections. Much of this research was completed during a postdoctoral fellowship at the Konrad Lorenz Institute for Evolution and Cognition Research awarded to RG, who adds special thanks to the KLI staff/fellows and Katharina Aigner for their support during the final months of his stay.

REFERENCES

- Allen, W. L., Cuthill, I. C., Scott-Samuel, N. E., and Baddeley, R. (2011). Why the leopard got its spots: relating pattern development to ecology in felids. *Proc. R. Soc. B Biol. Sci.* 278, 1373–1380. doi: 10.1098/rspb.2010.1734
- Beldade, P., and Brakefield, P. M. (2002). The genetics and evo-devo of butterfly wing patterns. *Nat. Rev. Genet.* 3, 442–452. doi: 10.1038/nrg818
- Brakefield, P. M., and French, V. (1993). Butterfly wing patterns. *Acta Biotheor.* 41, 447–468.
- Caro, T., Izzo, A., Reiner, R. C., Walker, H., and Stankowich, T. (2014). The function of zebra stripes. *Nat. Commun.* 5, 1–10.

- Conner, W. E., Eisner, T., Vander Meer, R. K., Guerrero, A., and Meinwald, J. (1981). Precopulatory sexual interaction in an arctiid moth (*Utetheisa ornatrix*): role of a pheromone derived from dietary alkaloids. *Behav. Ecol. Sociobiol.* 9, 227–235. doi: 10.1007/bf00302942
- Dussourd, D. E., Ubik, K., Harvis, C., Resch, J., Meinwald, J., and Eisner, T. (1988). Biparental defensive endowment of eggs with acquired plant alkaloid in the moth *Utetheisa ornatrix*. *Proc. Natl. Acad. Sci. U.S.A.* 85, 5992–5996. doi: 10.1073/pnas.85.16.5992
- Egan, A. L., Hook, K. A., Reeve, H. K., and Iyengar, V. K. (2016). Polyandrous females provide sons with more competitive sperm: support for the sexy-sperm hypothesis in the rattlebox moth (*Utetheisa ornatrix*). *Evolution* 70, 72–81. doi: 10.1111/evo.12829
- French, V., and Brakefield, P. M. (1992). The development of eyespot patterns on butterfly wings: morphogen sources or sinks? *Development* 116, 103–109.
- Gawne, R., and Nijhout, H. F. (2019). Expanding the nymphalid groundplan's domain of applicability: pattern homologies in an arctiid moth (*Utetheisa ornatrix*). *Biol. J. Linn. Soc.* 126, 912–924. doi: 10.1093/biolinnean/bly193
- Hegna, R. (2013). *Geographic Variation in the Warning Signals of the Wood Tiger Moth (Parasemia plantaginis; Arctiidae)*. Jyväskylä: University of Jyväskylä.
- Hegna, R. H., and Mappes, J. (2014). Influences of geographic differentiation in the forewing warning signal of the wood tiger moth in Alaska. *Evol. Ecol.* 28, 1003–1017. doi: 10.1007/s10682-014-9734-7
- Henze, M. J., Lind, O., Mappes, J., Rojas, B., and Kelber, A. (2018). An aposematic colour-polymorphic moth seen through the eyes of conspecifics and predators—Sensitivity and colour discrimination in a tiger moth. *Funct. Ecol.* 32, 1797–1809. doi: 10.1111/1365-2435.13100
- Jiggins, C. D. (2017). *The Ecology and Evolution of Heliconius Butterflies*. Oxford: Oxford University Press.
- Jonathan, B. B. (1977). A unity underlying the different zebra striping patterns. *J. Zool.* 183, 527–539. doi: 10.1111/j.1469-7998.1977.tb04204.x
- LaMunyon, C. W., and Eisner, T. (1993). Postcopulatory sexual selection in an arctiid moth (*Utetheisa ornatrix*). *Proc. Natl. Acad. Sci. U.S.A.* 90, 4689–4692. doi: 10.1073/pnas.90.10.4689
- Martin, A., and Reed, R. D. (2014). Wnt signaling underlies evolution and development of the butterfly wing pattern symmetry systems. *Dev. Biol.* 395, 367–378. doi: 10.1016/j.ydbio.2014.08.031
- Mazo-Vargas, A., Concha, C., Livraghi, L., Massardo, D., Wallbank, R. W., Zhang, L., et al. (2017). Macroevolutionary shifts of WntA function potentiate butterfly wing-pattern diversity. *Proc. Natl. Acad. Sci. U.S.A.* 114, 10701–10706. doi: 10.1073/pnas.1708149114
- Monteiro, A. (2008). Alternative models for the evolution of eyespots and of serial homology on lepidopteran wings. *Bioessays* 30, 358–366. doi: 10.1002/bies.20733
- Monteiro, A., French, V., Smit, G., Brakefield, P. M., and Metz, J. A. (2001). Butterfly eyespot patterns: evidence for specification by a morphogen diffusion gradient. *Acta Biotheor.* 49, 77–88.
- Monteiro, A., Glaser, G., Stockslager, S., Glansdorp, N., and Ramos, D. (2006). Comparative insights into questions of lepidopteran wing pattern homology. *BMC Dev. Biol.* 6:52. doi: 10.1186/1471-213X-6-52
- Morris, J., Navarro, N., Rastas, P., Rawlins, L. D., Sammy, J., Mallet, J., et al. (2019). The genetic architecture of adaptation: convergence and pleiotropy in *Heliconius* wing pattern evolution. *Heredity* 123, 138–152. doi: 10.1038/s41437-018-0180-0
- Nijhout, H. F. (1978). Wing pattern formation in Lepidoptera: a model. *J. Exp. Zool.* 206, 119–136. doi: 10.1002/jez.1402060202
- Nijhout, H. F. (1991). *The Development and Evolution of Butterfly Wing Patterns*. Washington, DC: Smithsonian Institution.
- Nijhout, H. F. (2001). Elements of butterfly wing patterns. *J. Exp. Zool.* 291, 213–225. doi: 10.1002/jez.1099
- Nijhout, H. F. (2017). “The common developmental origin of eyespots and parafocal elements and a new model mechanism for color pattern formation,” in *Diversity and Evolution of Butterfly Wing Patterns*, eds T. Sekimura, and H. Nijhout (Singapore: Springer), 3–19. doi: 10.1007/978-981-10-4956-9_1
- Nijhout, H. F., and Wray, G. A. (1988). Homologies in the colour patterns of the genus *Heliconius* (Lepidoptera: Nymphalidae). *Biol. J. Linn. Soc.* 33, 345–365. doi: 10.1111/j.1095-8312.1988.tb00449.x
- Nijhout, H. F., Wray, G. A., and Gilbert, L. E. (1990). An analysis of the phenotypic effects of certain colour pattern genes in *Heliconius* (Lepidoptera: Nymphalidae). *Biol. J. Linn. Soc.* 40, 357–372. doi: 10.1111/j.1095-8312.1990.tb00545.x
- Nokelainen, O., Hegna, R. H., Reudler, J. H., Lindstedt, C., and Mappes, J. (2012). Trade-off between warning signal efficacy and mating success in the wood tiger moth. *Proc. R. Soc. B Biol. Sci.* 279, 257–265. doi: 10.1098/rspb.2011.0880
- Nokelainen, O., Valkonen, J., Lindstedt, C., and Mappes, J. (2014). Changes in predator community structure shifts the efficacy of two warning signals in *Arctiid* moths. *J. Anim. Ecol.* 83, 598–605. doi: 10.1111/1365-2656.12169
- Oliver, J. C., Beaulieu, J. M., Gall, L. F., Piel, W. H., and Monteiro, A. (2014). Nymphalid eyespot serial homologues originate as a few individualized modules. *Proc. R. Soc. B Biol. Sci.* 281:20133262. doi: 10.1098/rspb.2013.3262
- Otaki, J. M. (2012). Color pattern analysis of nymphalid butterfly wings: revision of the nymphalid groundplan. *Zool. Sci.* 29, 568–576. doi: 10.2108/zsj.29.568
- Rojas, B., Mappes, J., and Burdfield-Steel, E. (2019). Multiple modalities in insect warning displays have additive effects against wild avian predators. *Behav. Ecol. Sociobiol.* 73:37.
- Roth, V. L. (1984). On homology. *Biol. J. Linn. Soc.* 22, 13–29.
- Schachat, S. R., and Brown, R. L. (2015). Color pattern on the forewing of *Micropterix* (Lepidoptera: Micropterigidae): insights into the evolution of wing pattern and wing venation in moths. *PLoS One* 10:e0139972. doi: 10.1371/journal.pone.0139972
- Schachat, S. R., and Brown, R. L. (2016). Forewing color pattern in Micropterigidae (Insecta: Lepidoptera): homologies between contrast boundaries, and a revised hypothesis for the origin of symmetry systems. *BMC Evol. Biol.* 16:116. doi: 10.1186/s12862-016-0687-z
- Schachat, S. R., and Goldstein, P. Z. (2018). Acronictinae (Lepidoptera: Macroheterocera: noctuidae) demonstrate the variable role of wing venation in the evolution of the nymphalid groundplan. *Insect Syst. Divers.* 2:4.
- Schwanwitsch, B. (1924). “21. On the ground-plan of wing-pattern in nymphalids and certain other families of the rhopalocerous lepidoptera,” in *Proceedings of the Zoological Society of London*, (London: Wiley Online Library).
- Schwanwitsch, B. (1956). Color-pattern in Lepidoptera. *Entomologeskoje Obozrenie* 35, 530–546.
- Sokolow, G. (1936). Die Evolution der Zeichnung der Arctiidae. *Zool. Jb.* 61, 107–238.
- Stüffert, F. (1927). Zur vergleichende Analyse der Schmetterlingszeichnung. *Biol. Zbl.* 47, 385–413.
- van Bemmelen, J. (1918). The wing-marking of Arctiidae. *Koninklijke Nederlandse Akademie van Wetenschappen Proc. Ser. B Phys. Sci.* 20, 849–860.
- Van Valen, L. M. (1982). Homology and causes. *J. Morphol.* 173, 305–312. doi: 10.1002/jmor.1051730307
- Wagner, G. P. (2014). *Homology, Genes, and Evolutionary Innovation*. Princeton, NJ: Princeton university press.
- Wagner, G. P. (2016). What is homology thinking and what is it for? *J. Exp. Zool. B Mol. Dev. Evol.* 326, 3–8. doi: 10.1002/jez.b.22656
- Weller, S., DaCosta, M., Simmons, R., Dittmar, K., and Whiting, M. (2009). “Evolution and taxonomic confusion in Arctiidae,” in *Tiger Moths and Woolly Bears, Behavior, Ecology, and Evolution of the Arctiidae*, ed. W. E. Conner (New York, NY: Oxford University Press), 11–30.
- Zahiri, R., Holloway, J. D., Kitching, I. J., Lafontaine, J. D., Mutanen, M., and Wahlberg, N. (2012). Molecular phylogenetics of Erebididae (Lepidoptera, Noctuoidea). *Syst. Entomol.* 37, 102–124. doi: 10.1111/j.1365-3113.2011.00607.x

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2020 Gawne and Nijhout. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Convergent Evolution of Broadband Reflectors Underlies Metallic Coloration in Butterflies

Anna Ren^{1†}, Christopher R. Day^{1,2†}, Joseph J. Hanly¹, Brian A. Counterman³, Nathan I. Morehouse⁴ and Arnaud Martin^{1*}

¹ Department of Biological Sciences, The George Washington University, Washington, DC, United States, ² Epigenetics and Stem Cell Biology Laboratory, National Institute of Environmental Health Sciences, National Institutes of Health, Durham, NC, United States, ³ Department of Biological Sciences, Mississippi State University, Starkville, MS, United States, ⁴ Department of Biological Sciences, University of Cincinnati, Cincinnati, OH, United States

OPEN ACCESS

Edited by:

Marcus Kronforst,
The University of Chicago,
United States

Reviewed by:

Matthew Shawkey,
Ghent University, Belgium
Bodo Wilts,
Université de Fribourg, Switzerland
Zhengzhi Mu,
Jilin University, China

*Correspondence:

Arnaud Martin
arnaud@gwu.edu

[†]These authors have contributed
equally to this work

Specialty section:

This article was submitted to
Evolutionary Developmental Biology,
a section of the journal
Frontiers in Ecology and Evolution

Received: 15 March 2020

Accepted: 04 June 2020

Published: 30 June 2020

Citation:

Ren A, Day CR, Hanly JJ,
Counterman BA, Morehouse NI and
Martin A (2020) Convergent Evolution
of Broadband Reflectors Underlies
Metallic Coloration in Butterflies.
Front. Ecol. Evol. 8:206.
doi: 10.3389/fevo.2020.00206

Butterfly wings often display structural colors, which are the result of light reflection from chitinous nanostructures that adorn the wing scales. Amongst these structural colors are broadband metallic reflections, which have been previously linked to an ultrathin broadband reflector in the nymphalid butterfly *Argyrophorus argenteus*. To test if similar optical modes of broadband, specular reflectance have evolved in other butterfly taxa, we characterized the reflective scales of eight species from five Papilionoidea families using microspectrophotometry (MSP), light microscopy in reflected and transmitted modes, and scanning electron microscopy (SEM). In Nymphalidae, Pieridae, and Hesperidae, and Lycaenidae, we find that broadband specularity is due to spatial mixing of densely juxtaposed colorful reflectances that change across microscale distances (e.g., 1–3 μm). These seemingly convergent silver scales are unpigmented, show a continuous upper lamina with reduced windows, and consist of an air-cuticle sandwich of variable thickness, forming an undulatory thin-film. Strikingly, *Hypochrysops apelles* (Lycaenidae) shows a novel mode of silver reflectance with spatial color mixing occurring across the entire proximo-distal length of the scale ($> 100 \mu\text{m}$), transitioning from blue to red hues between the stem and the tip of the scales. Unlike the undulatory type, this reflector shows flat thin-films which also includes a multilayered lower lamina, responsible for selective color iridescence in other lycaenids or in sunset moths. Finally, the gold scales of *Anteros formosus* (Riodinidae) show mixed reflectance in the green-to-red range, seemingly produced by a thin film in the lower lamina. Our comparative study suggests that evolution of metallic broadband reflectance repeatedly involved spatial color mixing and unperforated upper laminae, and is accomplished using at least three types of ultrastructural modifications. Undulatory thin-film systems, based on geometric adjustments of the transverse profile of the upper lamina and scale lumen, are widespread and may have evolved repeatedly from more generic colorless scale morphologies, while lycaenid and riodinid broadband reflectors may be elaborations of pre-existing iridescent states.

Keywords: structural colors, specular reflectance, Lepidoptera, convergent evolution, cuticular ultrastructure

INTRODUCTION

Biological mirrors and tissues with metallic appearances have evolved in many forms across the tree of life (Land, 1972) – from fish skin (McKenzie et al., 1995; Levy-Lior et al., 2008; Jordan et al., 2012), to scarab beetles (Agez et al., 2017), squid eyes (Holt et al., 2011; Ghoshal et al., 2013), and even begonia leaves (Lee, 2009; Zhang et al., 2009). Rather than relying on pigments *per se*, these mirror-like tissue reflectances result from microscopic optical structures that backscatter a wide spectrum of continuous wavelengths. For instance, dermal layers of guanine crystals of random thickness and distribution form a disordered stack termed a chaotic reflector and generate the broadband reflectance that gives herring and sardines their silvery aspect (Denton and Land, 1971; Levy-Lior et al., 2008; Jordan et al., 2012). Metallic reflections are also common among arthropods, including the elytra of many species of beetles and the pupal casing of some butterflies (Neville, 1977; Steinbrecht et al., 1985; Berthier, 2007; Kinoshita, 2008; Biro and Vigneron, 2011; Agez et al., 2017). These examples of insect metallic colors rely on broadband reflectance from multiple layers of chitin with differing thicknesses, often termed chirped stacks (Kinoshita and Yoshioka, 2005; Biro and Vigneron, 2011). As in the chaotic reflector, those chirped mirrors are often hundreds of microns thick because they rely on the stacking of bilayers of dielectric materials with distinctive refractive indices (Deparis et al., 2006; Cook and Amir, 2016; Chiadini et al., 2017), typically from air or cytoplasm (low-index $1 < n_L < 1.33$), and chitin (high-index $1.53 < n_H < 1.56$), melanized chitin (high-index $1.56 < n_H < 1.8$), or guanine platelets ($1.46 < n_H < 1.85$) (Leertouwer et al., 2011; Jordan et al., 2012; Stavenga et al., 2015a). Reducing the thickness of a chirped mirror theoretically requires increasing the number and refractive index of the dielectric materials. In particular, the difference in refractive index between layers determines how much light is reflected at each interface. The smaller this difference, the less light is reflected, implying that chirped stacks relying on natural materials can only achieve reasonable reflectances using multilayers of 10 thin films or more (Land, 1972; Cook and Amir, 2016).

The wing scales of butterflies and moths (order: Lepidoptera) are typically 1–2 μm thick. In contrast with thick chirped and chaotic reflectors, they have evolved *ultrathin* broadband reflectors only requiring sub-micron thickness (Vukusic et al., 2008; Stavenga et al., 2012; Wilts et al., 2013), often by the mixing of colors across a reflective surface rather than producing broadband reflectance at all points on the surface as in chirped stacks. In the examples described to date, broad spectrum reflectance of the wing scales results from the spatial mixing of parallel strips of colors that run alongside the periodic ridges of the reflective scale side (Vukusic et al., 2008), and derive from thin-film interference between air and two chitin layers, called the lower and upper laminae. The color heterogeneity is produced by an undulatory thin-film interface, where the sawtooth-shaped cross-sectional profile of the scale and the varying thicknesses of the air layer result in the reflection of a wide range of colors across a single scale. Such color variation disappears from the far field scattering appearance

of these scales, where spatial mixing results in an overall broadband reflectance (Vukusic et al., 2008; Stavenga et al., 2012; Wilts et al., 2013).

Metallic reflectances are widespread across the Lepidoptera, suggesting that the morphologies underlying them may be relatively “accessible” in the evolutionary sense (e.g., achievable by simple changes to scale architecture). There are a number of ways that such transitions to metallic coloration might occur. The current literature provides a limited view of the broader evolutionary patterns of metallic coloration in butterflies. Prior studies indicate that silvery states may have evolved at least three times, in HesperIIDae (Ge et al., 2017), Nymphalidae (Vukusic et al., 2008), and Lycaenidae (Wilts et al., 2013), presumably via relatively simple departures from the traditional scale architecture to achieve the mechanism described above (Ghiradella, 2010). Those cases invariably display a highly reflective configuration under the form of ectopic lamination, i.e., an apparent covering of the micropores (sometimes called *windows* or *pepper-pots*) that perforate the upper lamina of less reflective scales. This kind of simplification of the upper lamina to a film-like sheet has been consistently observed in scanning electron micrographs of silver-metallic scales, including from nymphalid species of the Heliconiinae and Satyrinae sub-families (Simonsen, 2007; Giraldo, 2008; Vukusic et al., 2008; García-Barros and Meneguz, 2012; Dinwiddie et al., 2014), in the lycaenid *Curetis acuta* (Wilts et al., 2013; Liu et al., 2019), and in the hesperid *Carystoides escalantei* (Ge et al., 2017). In the polymorphic species *Argynnis niobe*, scales from flat white spots show more perforation of the upper lamina compared to scales from individuals with brighter, recognizably silver spots (Simonsen, 2007). Thus, silver scales could be a more reflective elaboration of white scales, perhaps by reduction of the upper lamina fenestration.

Alternatively, broad-spectrum reflectance could evolve from increased disorder in iridescent scales that are already highly reflective, i.e., iridescent scale types with a more narrow color profile. Referring to a classification of mechanisms of iridescence in butterflies (Ghiradella, 1989; Vukusic et al., 2000; Mouchet and Vukusic, 2018), silver scales could derive from “Type II” body-lamellae scales, especially the Type IIa scales (or *Urania*-type) that are the most common mode of iridescence in Lycaenidae (Lippert and Gentil, 1959; Schmidt and Paulus, 1970; Tilley et al., 2002; Biró et al., 2007). Unlike silver scales, Type IIa scales generate iridescent colors by internal multilayering (Ghiradella, 1989; Wilts et al., 2008; Yoshioka et al., 2013), and show a level of reflectance that is linearly proportional to the extent of upper lamina filling (Wilts et al., 2008). Whether this configuration can yield the spatial color mixing of heterogeneous colors and achieve broad reflectance spectra remains an open question. Finally, none of the previously profiled silver scale architectures shows “Type I” ridge-lamellae scales emblematic of blue *Morpho* butterflies, or “Type III” body-scattering structures that involve a porous upper lamina opening into crystal-like inclusions such as pigment granules or gyroid structures (Vukusic et al., 2000; Dolan et al., 2015; Singer et al., 2016; Wilts et al., 2017; Mouchet and Vukusic, 2018). We shall note, however, that there are no reported studies

of metallic coloration and its underlying structural causes in either the Pieridae or the Riodinidae, which is surprising in the latter case given the family's vernacular name of "metalmarks." The mechanisms of broadband reflectance in those clades thus remain unexplored.

In this study we sought to expand our comparative knowledge of the ultrastructures underlying specular (mirror-like), broad-spectrum reflectance in butterflies, or in other words, to explore the diversity of scales showing metallic coloration. Using a combination of transmitted and reflected light microscopy, electron microscopy, and microspectrophotometry, we describe the modalities of specular, broad-spectrum reflectance and derive insights into the convergent evolution of metallic colors across butterflies.

RESULTS

Silver Coloration Exists in Five Out of Seven Butterfly Families

To gain insights into the diversity of structures underlying metallic broadband reflectance in the wing scales of butterflies (superfamily Papilionoidea), we selected one or two representatives from five of the seven families in this lineage (**Figure 1A**): Nymphalidae (*Agraulis vanillae* and *Speyeria cybele*, sub-family: Heliconiinae), Lycaenidae (*Hypochrysops apelles*, sub-family: Theclinae and *Cigaritis lohita*, sub-family: Aphnaeinae), Riodinidae (*Anteros formosus*, sub-family: Riodininae), Pieridae (*Colias eurytheme* and *Zerene cesonia*, sub-family: Coliadinae), and Hesperidae (*Epargyreus clarus*, sub-family: Eudaminae). We found no clear case of silver reflectance in the Hedyliidae family, which includes only one extant genus. We also omitted Papilionidae, though it is noteworthy that certain morphs of the endangered species *Baronia brevicornis brevicornis* display silver patches with bright reflectance (Vazquez, 1987), and that broad-spectrum light-scattering scales with a diffuse white appearance have been described in *Graphium sarpedon* (Stavenga et al., 2010, 2012).

While the gold/silver metallic color elements of our specimens were always on the ventral side, this may be a trend rather than a rule, as illustrated by the nymphalid *Argyrothorus argenteus* that displays a completely silver dorsal side (Vukusic et al., 2008). Overall, this phylogenetic sampling may represent at least four cases of homoplasy, with at least one origin in each of the nymphalid, lycaenid and riodinid, pierid, and hesperid lineages, or more independent acquisitions of the specular, broad-spectrum reflectance. In contrast, we can assume a likely homology between silver scales found in the ventral hindwing silver blotches from "fritillary butterflies" of the Heliconiinae sub-family (here, *A. vanillae* and *S. cybele*), or in the ventral discal ocelli of Coliadinae (*C. eurytheme* and *Z. cesonia*); we thus sampled two species in each lineage to get insights on the range of divergence within silver scales of shared origin and context. The reflectance of all silver elements extended into the ultraviolet range, as shown by UV-photography excluding visible light wavelengths above 400 nm (**Figure 1B**).

Metallic Scales Are Unpigmented, Except in Lycaenidae and Riodinidae

We isolated single scales from metallic elements of the eight sampled species for further observations, with all subsequent images and analyses focused on silver elements from the hindwings (**Figure 1C**). In order to assess the relative contribution of pigmentation and structural thin-film interference to silver scale coloration, we used reflected-light microscopy in air (refractive index $n = 1$) and transmitted-light microscopy under clove oil immersion ($n = 1.53$), a medium that matches the refractive index of chitin and thus substantially reduces light diffraction (Mayor, 1806; Stavenga et al., 2015b; Thayer et al., 2020). When immersed in clove oil, silver scales from species belonging to Nymphalidae, Pieridae, and Hesperidae turned transparent, highlighting a lack of pigment (**Figures 1D,E**). In contrast, the metallic scales of the three lycaenid and riodinid species showed a small amount of light-brown pigmentation, likely melanin, which increases the refractive index of chitin and may thus change the reflectance of the scale (Land, 1972).

Metallic Scales Show Consistent Modifications of Their Upper Surfaces

We examined the exterior features of silver scales in scanning electron micrographs, and systematically compared them to adjacent non-reflective scale types. As in previous studies (see section "Introduction"), metallic scales showed extensive lamination, where the spaces between crossribs are filled rather than left empty, as found in less reflective scales (**Figure 2**). With the exception of the closely related *Z. cesonia* and *C. eurytheme*, all silver scales also showed an increase in inter-ridge distance (**Figures 3A,C**). Increasing the surface of light-reflecting upper laminae could be of functional importance, particularly in the two species where inter-ridge spacings are the most differentiated: silver scale ridge intervals are $1.5\times$ larger than in adjacent scales in *A. vanillae* (silver, mean = $2.29\text{ }\mu\text{m}$; black, mean = $1.45\text{ }\mu\text{m}$), and $2.4\times$ larger in *H. apelles* (silver, mean = $2.73\text{ }\mu\text{m}$; orange, mean = $1.16\text{ }\mu\text{m}$). Finally, all silver scales were wider than adjacent scales (**Figures 3B,D**), indicating that silver scales may be tuned to provide dense coverage on the wing and maximize the surface area of light reflectance. Thus, compared to light-absorbing, pigment-colored scales, metallic scales achieve higher reflectance by maximizing the opportunity for backscattering of incident light via three concomitant mechanisms: expansion of lamination/decrease in fenestration of upper wing scale surface, increase in ridge distance, and increase in overall scale width. In the next section, we investigate possible mechanisms underlying the broad spectrum reflectance of these specialized scales.

Undulatory Thin-Film Broadband Reflectors Are Widespread

In order to substantiate the broad reflectance of silver scales that can be inferred from direct observation and UV-photography, we used a microspectrophotometer to compare the reflectance spectra of metallic scales vs. adjacent, non-metallic scales from each species (**Figures 4A–E**). We focus on nymphalid, pierid, and

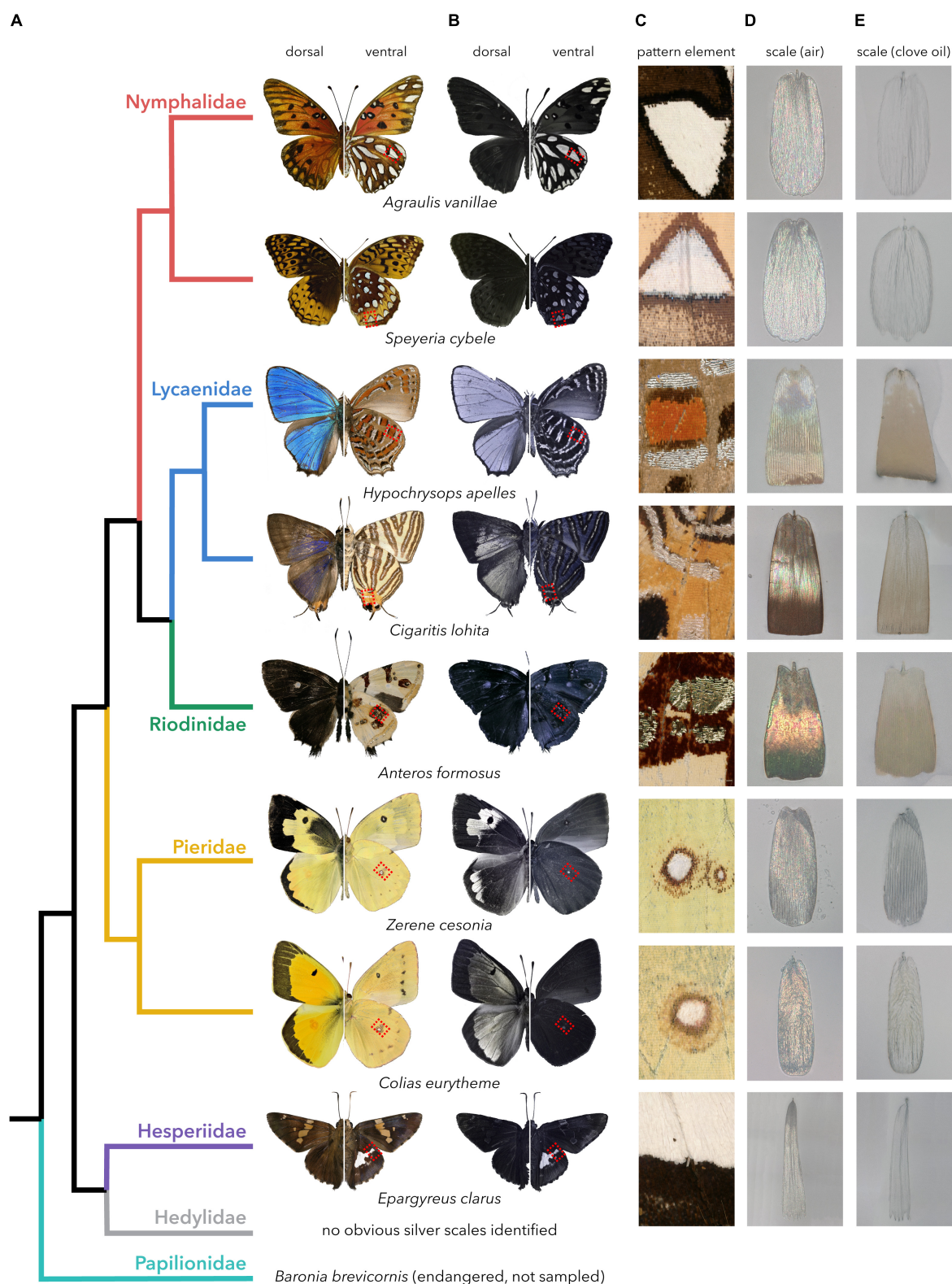


FIGURE 1 | Repeated occurrence of metallic scales in butterflies. **(A)** Phylogenetic relationships between the eight species sampled in this study (Espeland et al., 2018; Wiemers et al., 2019). **(B)** Ultraviolet (UV-B) photographs reveal reflectance in the non-visible range. **(C)** Magnified views of the reflective patterns obtained by a digital microscope, corresponding to red square insets in panels **(A,B)**. **(D,E)** Reflected-light microscopy of single silver or gold scales in air ($n = 1$) and transmitted-light in clove oil ($n = 1.53$).

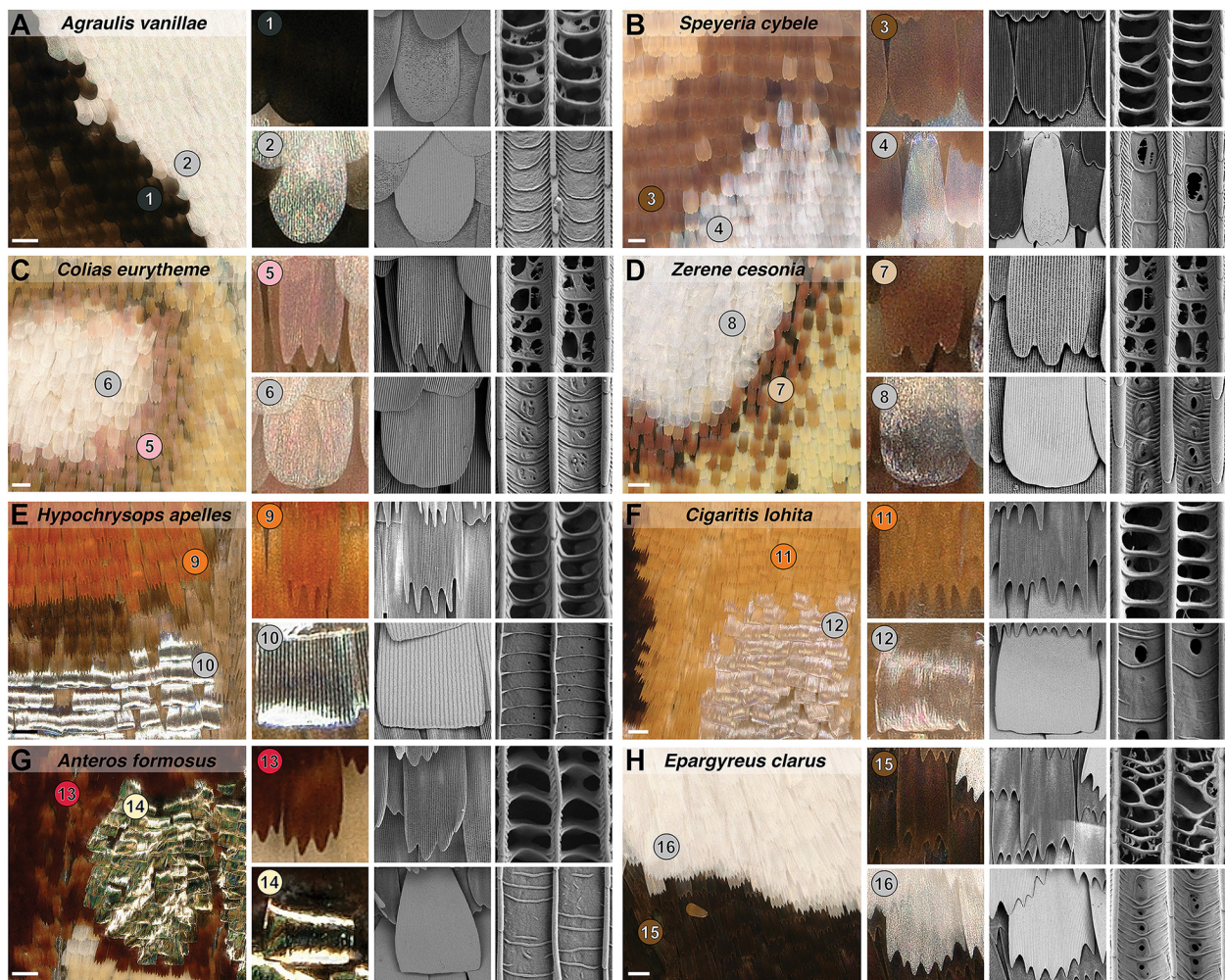


FIGURE 2 | Modified morphologies of metallic scales in five butterfly families. **(A,B)** Nymphalidae. **(C,D)** Pieridae. **(E,F)** Lycaenidae. **(G)** Riodinidae. **(H)** Hesperidae. Each panel shows a silver pattern imaged by digital light microscopy (left insets, scale bars = 100 μm), and close-up views of non-metallic (upper rows) vs. metallic (lower rows) scales under light and electron microscopy. The right columns show the ultrastructural details of the upper lamina, highlighting the fenestration of non-metallic scales vs. the smooth surface of reflective scales across two inter-ridge intervals. Numbered bullets mark scale types and species that are referred to in further figures.

hesperid samples first, and will address the more unusual optical morphologies observed in the lycaenid/riodinid clade samples in subsequent sections.

The unpigmented silver scales of *A. vanillae* and *S. cybele* (sub-family: Heliconiinae) produced a relatively flat reflectance spectra across the UV and visible range (300–700 nm) and extending into the infrared range (700–800 nm), consistent with previous results (Briscoe et al., 2010). Hesperid (*E. clarus*) and pierid (*C. eurytheme*, *Z. cesonia*) unpigmented scales showed a similar broad reflectance. The discal spot scales of *Z. cesonia* reflected less than half of the light reflected by laboratory-grade aluminum foil (**Figures 4E,F**), which is used here as a familiar point of comparison. Finally, all measurements of reflective surfaces showed more variation across measurements compared to adjacent pigmented scales, due to surface irregularities and the angle-dependency of light reflectance in the silver samples.

Next, we used reflected-light microscopy to test if those unpigmented silver scales would reproduce the pattern of spatial color mixing previously described in *A. argenteus* (sub-family: Satyrinae). In this species, color strips run parallel to the scale ridges as the sawtooth profile of the upper lamina imposes variation in the thickness of the subjacent air lumen (Vukusic et al., 2008; Mouchet and Vukusic, 2018). This ultrathin configuration produces dense rods of colors, each less than 1–3 μm thick, that alternate across the scale width. We found that similar stripes can be discerned in *A. vanillae* and *S. cybele* (**Figures 4G,H**), while in *E. clarus*, *Z. cesonia*, and *C. eurytheme*, individual colors are more speckled and pointillist (**Figures 4I–L**). Thus, all five species from the Nymphalidae, Pieridae, and Hesperidae achieve broad-spectrum reflectance via dense spatial color mixing spread across the scale surface.

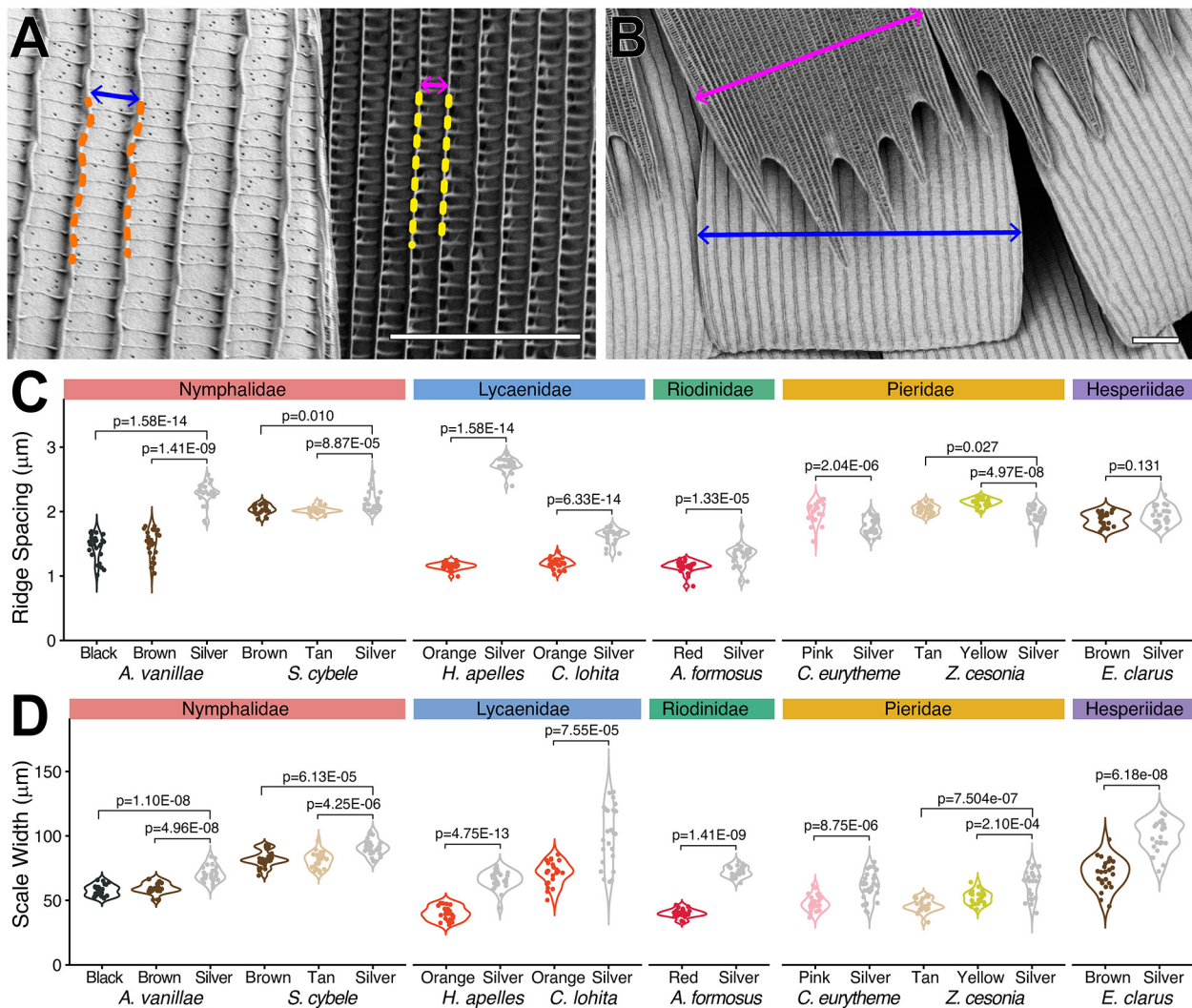
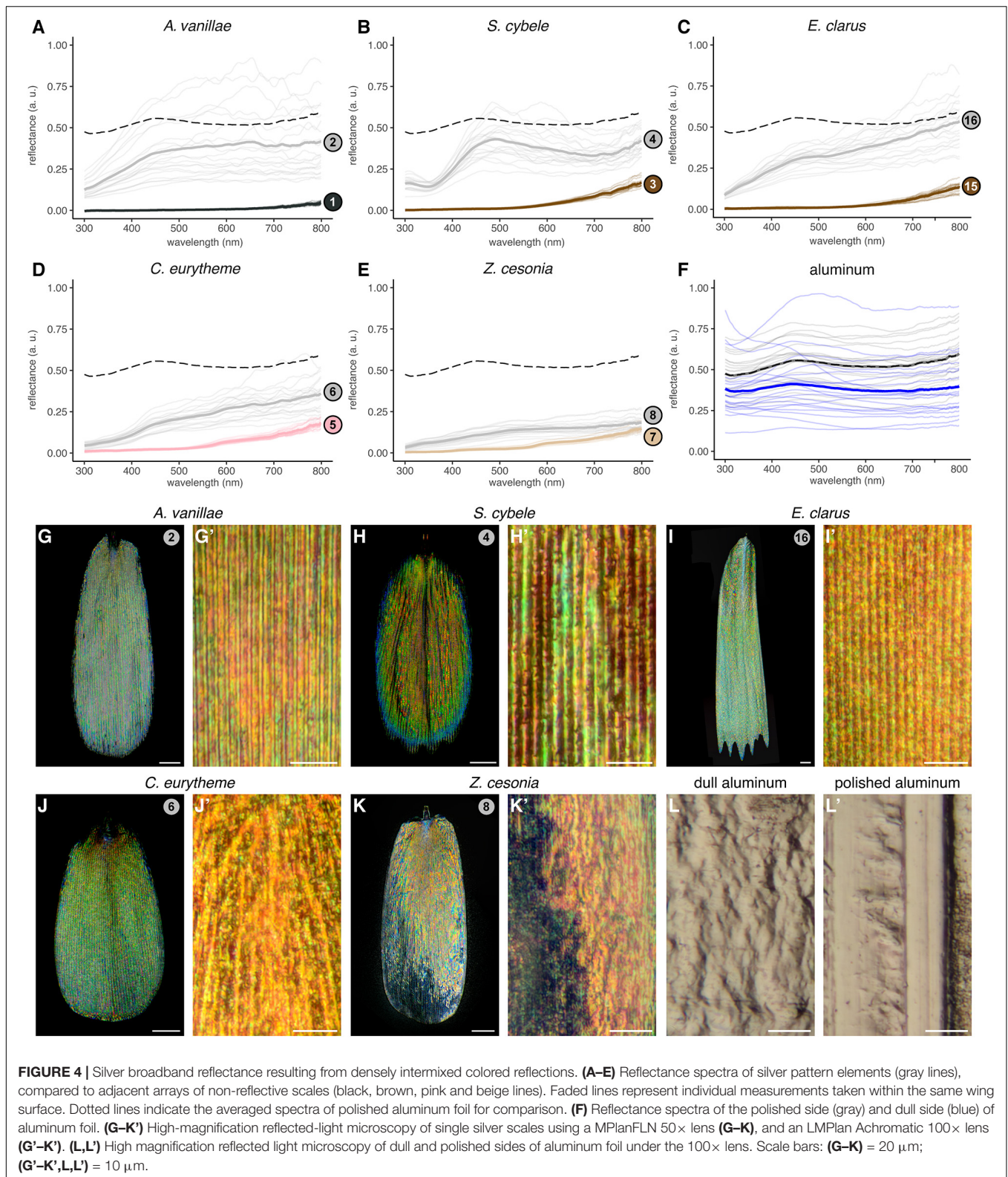


FIGURE 3 | Reflective scales tend to have larger ridge intervals and wider surfaces. **(A)** Measurement of ridge distance in the metallic (14) vs. orange scales (13) of *H. apelles*. **(B)** Measurement of maximal scale width in the metallic (14) vs. orange scales (13) of *H. apelles*. **(C)** Ridge distances across metallic vs. adjacent scale types from eight species ($N = 25$ scales per scale type). Each measurement corresponds to the Fourier mode, or average ridge interval per scale, extracted from the transversal pixel intensity across a single scale SEM. Pairwise differences show significant p -values following Mann-Whitney U tests. Pierid silver scales are the only metallic scales with smaller ridge intervals compared to adjacent scale types. **(D)** Maximal scale width distances across metallic vs. adjacent scale types from eight species ($N = 25$ scales per scale type). All metallic scales are enlarged compared to adjacent scales types (p -values, Mann-Whitney U tests). Scale bars, **(A,B)** = 10 μm . SEM image datasets are accessible on the Dryad online repository (Day et al., 2020).

Based on those observations, we hypothesized that these metallic scales would resemble the bilayered internal anatomy of *A. argenteus* scales, and examined the SEM profile of those scale samples after transversal cryofracture (Figure 5). We found that *A. vanillae*, *E. clarus*, *C. eurytheme*, and *Z. cesonia* share the serrated profile of *A. argenteus*, characterized by two laminae of relatively constant thickness and an internal air layer of varying thickness (“undulatory lumen”). *S. cybele* likewise has an undulating profile in its upper section, but with a flat lumen and an upper lamina of varied thickness that resembles “speed bumps,” due to local chitin thickening of the ridge area (Figure 5B). Of note, under near-field observation, *S. cybele* shows a single strip of color per ridge interval of 2–2.3 μm

(Figure 5H’), while in other nymphalids such as *A. vanillae* (Figure 5G’) and *A. argenteus* (Vukusic et al., 2008), multiple color transitions can be observed within a single ridge interval of comparable dimensions (Figure 3B). Thus, the *S. cybele* mode of undulation is structurally distinct from other examples (Figure 5F), and achieves similar broadband reflectance albeit through a coarser mode of spatial color mixing.

Nonetheless, the similarities between these morphologies suggest that the optical principles of an undulatory thin-film reflector are at play across these butterfly lineages, with wavelength-specific variation in light reflectance across the scale surface due to the periodic profiles of either the upper chitin layer or the scale lumen. We suggest the previous biophysical



characterization of a butterfly undulatory thin-film reflector (Vukusic et al., 2008) applies here, and infer that this general mechanism accounts for multiple cases of metallic iridescence

in Nymphalidae, Hesperidae, and Pieridae. Such reflectors may have evolved repeatedly by tuning pre-existing scale elements of the scale groundplan (Ghiradella, 2010) to create a undulatory

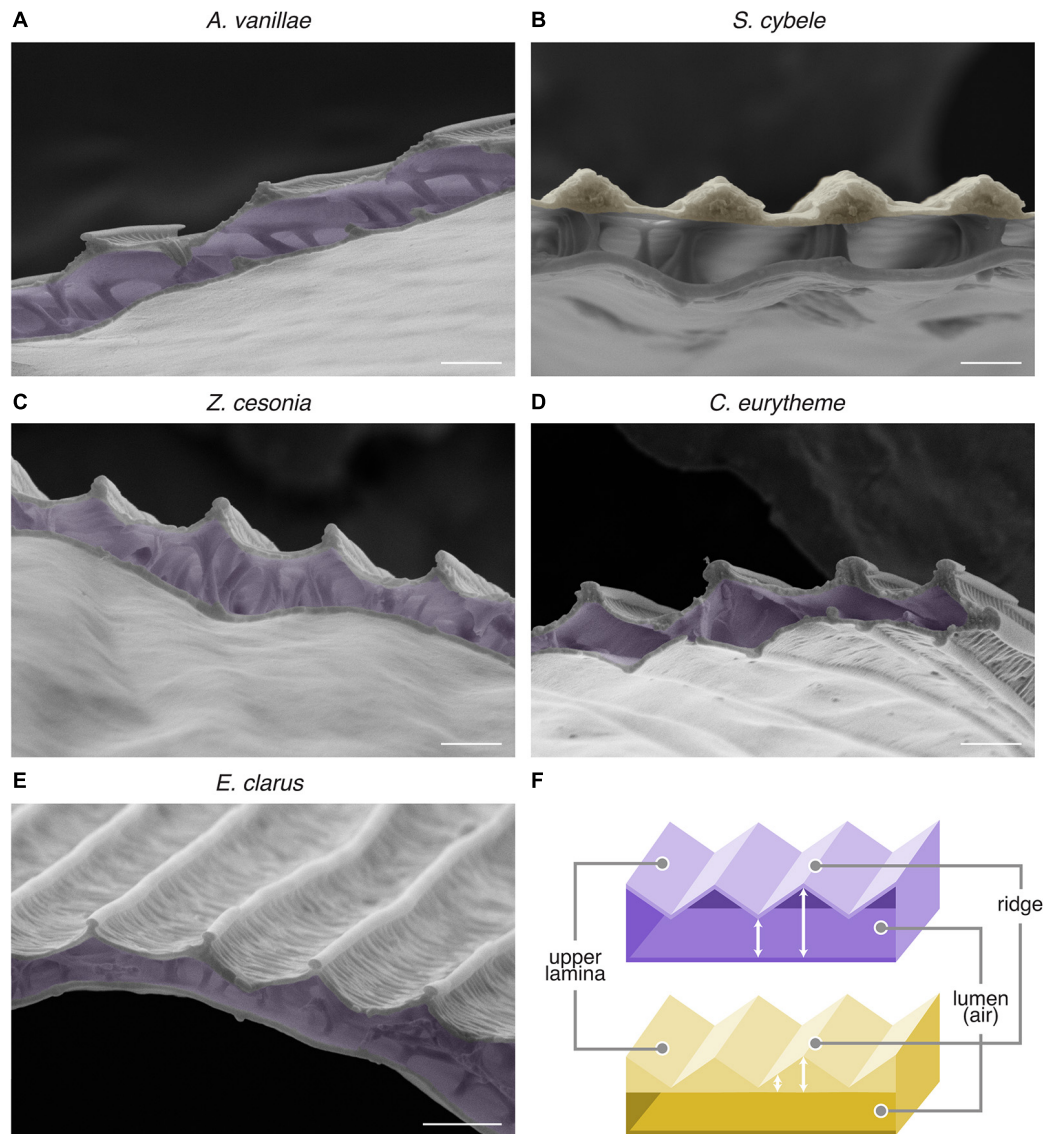


FIGURE 5 | Internal scale anatomy of nymphalid, pierid, and hesperid undulatory thin-film reflectors. **(A–E)** SEM of transversal sections of the metallic scales of *A. vanillae*, *S. cybele*, *Z. cesonia*, *C. eurytheme*, and *E. clarus* reveal the undulatory profile of the specular thin-film. False colors highlight the layer with the most consistent fluctuation in thickness, i.e., the air lumen (purple) in most cases, or for *S. cybele*, the thickened ridge regions (yellow). **(F)** Schematic representation of the two types of thin-film thickness undulation as observed in panels **(A,C–E)** (top, purple) and **(B)** (bottom, yellow). Scale bars: **(A–E)** = 1 μm .

thin-film system with a single inner air layer between the upper and lower laminae.

Two Modes of Broadband Reflectance in Lycaenidae

We also examined the features of silver scales from *C. lohita* and *H. apelles*, two lycaenid species, and from the gold scales of *A. formosus*, a species from the rioidinid sister lineage (Figure 6). All three types of metallic scales differed from other sub-families. The silver scales of *C. lohita* showed optical and structural features reminiscent of the undulatory thin-film configuration found in Nymphalidae, Hesperidae, and Pieridae in this study,

as well as in the specular scales of *Curetis acuta*, another lycaenid (Wilts et al., 2013; Liu et al., 2019). In particular the reflectance spectra, color speckling at microscopic levels, upper lamina external views, and transversal inner anatomies all resembled the hesperid and pierid samples (Figures 6A,D,G,H,M). Thus, the *C. lohita* reflector follows an undulatory thin-film architecture, providing yet another evolutionary occurrence of this category of mechanism in a fourth butterfly family.

In contrast, the silver scales of *H. apelles* showed a distinctive spectrum and a strikingly divergent mode of color mixing, with a continuous color transition, from blue to red, stemming from the base to the distal region of the scale (Figures 6B,E,I). This longitudinal gradient represents a novel, previously undescribed

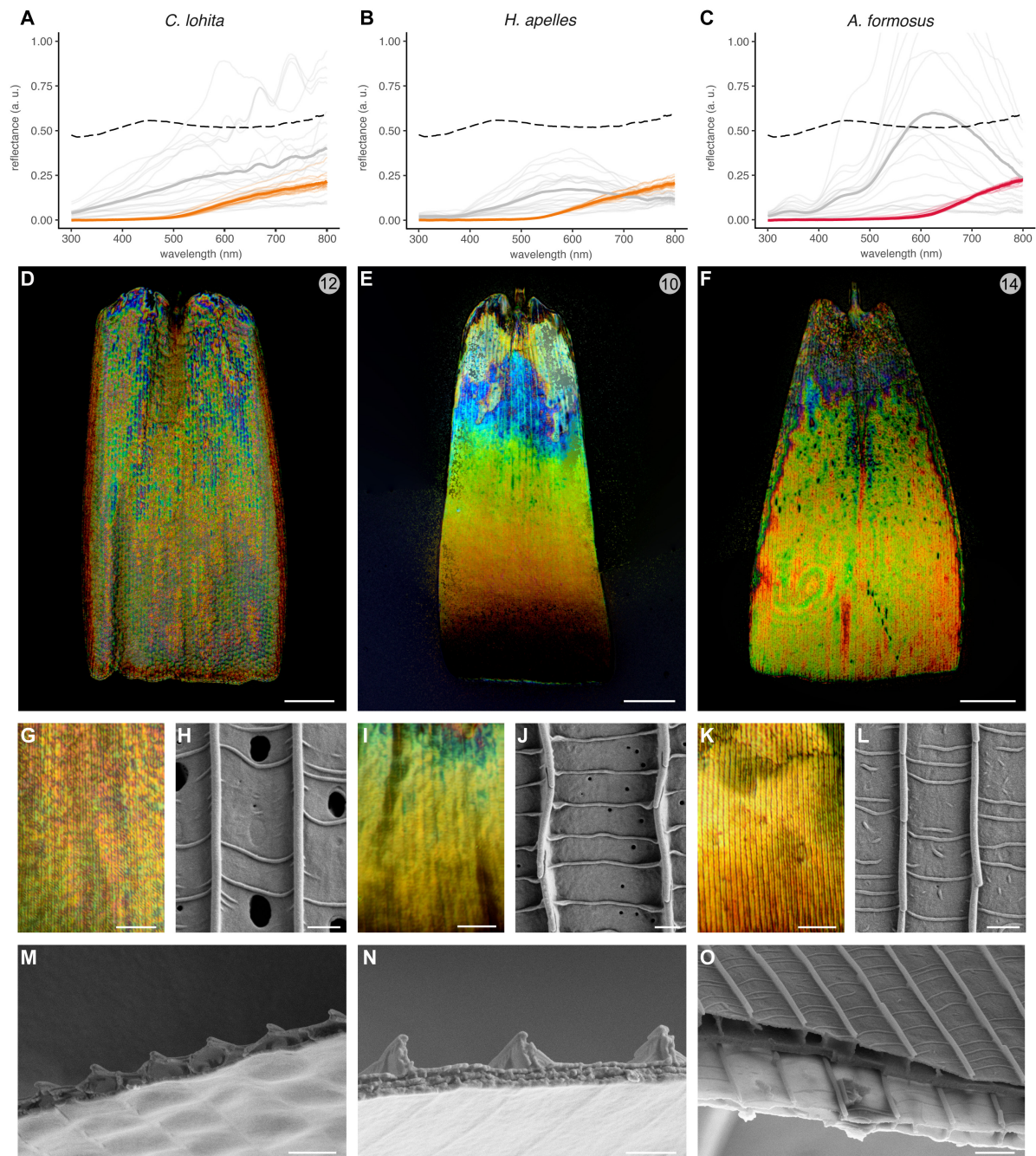


FIGURE 6 | Three distinct optical mechanisms drive broadband reflectance in the Lycaenidae + Riodinidae clade. **(A–C)** Averaged reflectance spectra of metallic pattern elements (gray lines), compared to an adjacent array of non-specular scales (orange and red lines). Faded lines represent individual measurements within the same wing surface. Dotted lines indicate the averaged spectra of polished aluminum foil for comparison. **(D–G,I,K)** High-magnification reflected-light microscopy of single metallic scales using a MPlanFLN 50× lens **(D–F)**, and an LMPlan Achromatic 100× lens **(G,I,K)**. **(H,J,L)** SEM top views of metallic scales. **(M–O)** SEM of transversal sections. In *A. formosus* **(O)**, more proximal regions of the scale (dark gray, upper) have thicker lower laminae than distal regions (light gray, lower). Scale bars, **(D–F)** = 20 μm; **(G,I,K)** = 10 μm, **(H,J,L,M–O)** = 1 μm.

mode of additive color mixing. Some of this color gradient is hinted at in our spectral measurements, which exhibited a pronounced peak in the long wavelengths due to the fact that the light paths of the MSP instrument are coaxial and normal to the

wing surface, and thus likely to measure the apex and orange-red portion of the scale.

The external aspect of the *H. apelles* upper surface is also peculiar, with large inter-ridge distances (**Figure 3B**) and periodic

crossribs that are perfectly perpendicular to the ridges, indicative of the complete flatness of the upper lamina (**Figure 6J**). The internal anatomy of cross-sectioned scales also revealed a divergent morphology, lacking the marks of transversal periodicity observed in undulatory thin-film types. Instead, the upper lamina and its underlying air layer are flat, and the ridges appear as thin vertical walls that are unlikely to provide sufficient light diffraction for producing dense arrays of colors. Another anatomical feature that varies in the longitudinal axis must thus explain the proximo-distal color gradient. Interestingly, the lower lamina shows a double-layering reminiscent of the Type-IIa multilayered thin films observed in other iridescent lycaenids (Lippert and Gentil, 1959; Schmidt and Paulus, 1970; Tilley et al., 2002; Biró et al., 2007) and in sunset moths (Prum et al., 2006; Yoshioka and Kinoshita, 2007; Yoshioka et al., 2008). To suggest an optical mixing for the observed color mixing, we sought to examine if thickness and periodicity parameters of this system varied in the longitudinal direction. Unfortunately, the fragility of those scales prevented us from properly cryo-fracturing those scales and imaging them in the sagittal plane with SEM. Thus, further characterization of this newly discovered broadband reflector will require alternative methods such as transmission electron microscopy (TEM), identification of the parameters that vary in the proximo-distal axis, and proper biophysical modeling.

A Simple Lower Lamina Thickness Likely Drives Gold Reflectance in a Riodinid

We further examined the gold scales of a riodinid, *A. formosus*. Microspectrophotometry shows remarkable brightness in the 500–800 nm green-to-infrared range (**Figure 6C**), and a lower reflectance in the UV-to-blue range (< 500nm), consistent with the gold rather than silver sheen of this reflector. Reflected microscopy reveals longitudinal stripes of green, yellow, and red colors, but unlike in the typical undulatory thin-films, we were unable to resolve such transitions across distance of 1–3 μm with a 100 $\times$ lens (**Figures 6F,K**). The *A. formosus* gold scale upper lamina and inner air layer, as observed in top and cross-sectional views (**Figures 6L,O**), also contradicts the role of an undulatory thin-film as defined above. In the absence of any other obvious optically relevant structures, the lower lamina of those scales is likely responsible for their bright reflectance. This is supported by the fact that in comparison, the subjacent scale fractured at a location $\sim 10 \mu\text{m}$ closer to the distal edge displays a significantly thinner lower lamina. Although this change in thickness appears to occur over a small proximo-distal distance, note that the transition from blue-violet to yellow-green color is similarly rapid (**Figure 6O**). Indeed, according to thin-film models that incorporate the refractive index of chitin, thicker laminae reflect shorter wavelengths (Stavenga et al., 2014). We thus expect the violet/blue base of the *A. formosus* scale to be thicker than the rest of the scale, which only shows green-yellow-red colors under the reflected light microscope (**Figures 6F,K**), and the green-to-infrared shifts responsible for the spectrally broad gold likely arise from microvariations in lower lamina thickness that we could not detect with SEM. Overall, these results suggest that the bright gold iridescence of the riodinid *A. formosus* scales derives from

a relatively simple reflector with a flat upper lamina and lumen, and a variable lower lamina. In contrast with the nymphalid butterflies that use lower laminae to reflect homogenous color spectra (Stavenga et al., 2014; Wasik et al., 2014; Thayer et al., 2020), we infer that local heterogeneities in those structures may underlie the spectral spread and spatial mixing of reflected colors in the green-to-red range.

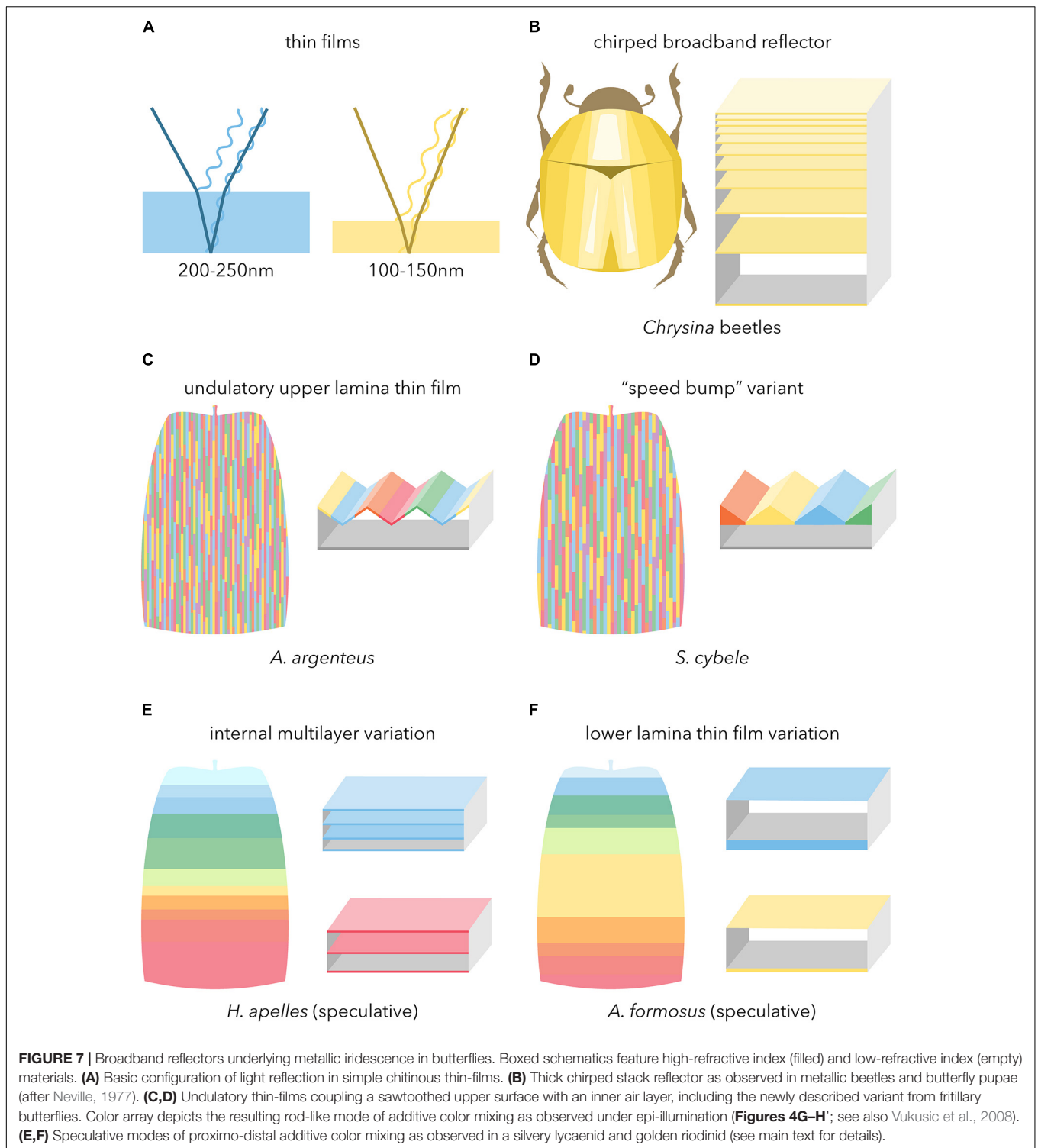
DISCUSSION

Metallic Coloration in Lepidoptera

We compared optical and ultrastructural structural aspects of metallic scales in five out of seven families of Papilionoidea (*sensu* Espeland et al., 2018), and identified several types of broadband reflectors that use distinct chitinous modifications to achieve additive color mixing, including two modes of metallic coloration that remain speculative before further biophysical characterization (**Figure 7**). While our phylogenetic sampling is currently too coarse for ancestral state reconstruction or detailed retracing of the evolution of metallic colorations on the butterfly phylogeny, we are starting to uncover general patterns of phenotypic convergence where sometimes similar, and sometimes novel ultrastructures achieve specular, broad-spectrum reflectance. Beyond butterflies, there is also a tremendous diversity of analogous metallic colorations in moths, including in Hepialidae (e.g., *Korscheltellus lupulinus*), Tortricidae (e.g., *Pelochrista ridingsana*), Crambidae (e.g., *Ramila ruficostalis*), Nolidae (e.g., the Mirror Moth *Titulcia confictella*), Cosmopterigidae (e.g., *Cosmopterix montisella*), and Saturniidae (e.g., *Attacus atlas*). In this section, we provide an overview of the different types of broadband reflectors that have evolved in Lepidoptera.

Undulatory Thin-Film Broadband Reflectors

The undulatory thin-film type described in *A. argenteus* is characterized by a continuous upper lamina, and an inner air layer of periodic thickness (Vukusic et al., 2008). This sawtoothed thin-film results in a mode of spatial color mixing where reflected wavelengths alternate across a 1–3 μm interval in the transversal axis. We recovered similar features in two other Nymphalidae, *A. vanillae* and *S. cybele*, with the nuance that the *S. cybele* thin-film showed dome shaped thickenings of the chitin layer at the level of ridges, rather than a sawtooth shaped lumen as observed in *A. vanillae* and our other samples (**Figures 7C,D**). This reinforces the previously made inference that silver patterns are a homoplasy within different “fritillary” butterflies of the subfamily Heliconiinae (Simonsen, 2007), specifically between tribe Argynnini such as *S. cybele*, and basal members of tribe Heliconiini such as *A. vanillae*. Second, Argynnini displays a variety of reflective scales that vary from dull white to shiny silver and that are similar in external ultrastructures (Simonsen, 2007), but likely vary slightly in their transversal geometry. A similar architecture and mode of additive color mixing has been well-described in the noctuid moth *Eudocima materna*, where apposition of mirror scales with pigmented scales yields



an angle-dependent reflective effect (Kelley et al., 2019). In this framework, specularity may be achieved by efficient angular reflections in the inter-ridge intervals and spatial color mixing, while more dull states could result from profiles that scatter more light. The evolution of undulatory broadband reflectors may proceed by increases in the amplitude of light-scattering

thin-films, notably from scales in a full white state where the upper lamina is unperforated. If this holds true in Heliconiinae, then this mode of convergence in silver iridescence may extend to Satyrinae (Vukusic et al., 2008; García-Barros and Meneguz, 2012), and other butterfly families as we documented here in Hesperidae, Pieridae, and one out of our two Lycaenidae samples

(*C. lohita*). In any case, the hollow lepidopteran *bauplan* scale type (Ghiradella, 2010) that is necessary to yield the undulatory thin-film seemingly extends to some early diverging branches of the lepidopteran phylogeny (Simonsen, 2001). We predict that the most distant case of convergent undulatory thin-film may have occurred in silver-reflective Hepialidae such as the silver-spotted ghost moth (*Leto venus*), since reflective white scales in this family show a bilayered architecture reminiscent of the butterflies and skippers studied here (Simonsen, 2002).

Proximo-Distal Color Mixing in the Multilayered Scales of Lycaenids

We discovered a new mode of broad-reflectance in *H. apelles* where the visible color spectrum is selectively reflected across the proximo-distal axis of the scale, from violet and blue colors at the base, to red at the tip (Figures 6E,I). This rainbow transition in the near field, likely coupled to scale stacking, produces broadband reflectance in the far field. What can we infer about the ultrastructural basis of this proximodistal color mixing? In *H. apelles*, the multilayered lower lamina (Figure 6N) is reminiscent of body-lamellae scale iridescence that are widespread in Lycaenidae (Lippert and Gentil, 1959; Schmidt and Paulus, 1970; Tilley et al., 2002; Biró et al., 2007; Wilts et al., 2008). In those butterflies, additional chitin layers increase reflectance, meaning that the multilayered lower lamina contributes to the brightness of the scales (Wilts et al., 2008). Sunset moths also show analogously multilayered structures, and changes in the thickness and spacing of their internal layers on the order of 10–50 nm are responsible for shifts as drastic as pale blue to orange (Yoshioka and Kinoshita, 2007; Yoshioka et al., 2008, 2013; Imafuku et al., 2012). In addition, melanin is present in the distal portion of the scale and absent at the base, perhaps increasing chitin refractive index or even forming a gradient that could contribute to spectral spread (Land, 1972). While we could not resolve here the mechanisms responsible for the continuous color transition from blue to red hues, we extrapolate that microvariations in layering and refractivity drive this shift along single scales (Figure 7E).

From an evolutionary perspective, one may postulate that the multilayered state of lycaenid and other body-lamellae (“Type IIa”) color iridescent scales (Ghiradella, 1989; Vukusic et al., 2000; Mouchet and Vukusic, 2018) can prime the evolution of broadband reflectance. In *Hypochrysops* spp., the iridescent blue scales widespread across the dorsal surface are composed of a lower lamina of 7–8 layers and a perforated upper lamina (Ingram and Parker, 2008) while we have observed three layers in the ventral silver scales. Transition to the silver state would then require a filling of the perforations in the upper lamina, and merging of internal layers to produce a variety of chitin layer thicknesses and spacings along the proximo-distal axis.

Tuning of Lower Lamina in the Gold Scales of a Riodinid

The optical and structural basis of color mixing in the specular scales of metalmarks butterflies (Riodinidae) has remained undescribed, and our data from the gold scales of *A. formosus*

showed an original configuration. These were the brightest among all the species sampled here, but their ultrastructure simply consisted of a flat, mostly featureless upper lamina, a flat air lumen, and a similarly flat lower lamina. We propose that a rapid and pronounced reduction in lower lamina thickness is responsible for the abrupt shift from blue-violet to yellow-green in the most proximal third of the scale. This thick (200–250 nm) region appearing blue is consistent with models and existing examples of thin-films (Stavenga et al., 2014; Thayer et al., 2020), and likewise a drop to 100–150 nm produces red and yellow (Thayer et al., 2020). We extrapolate then that microvariations in lower lamina thickness are likely responsible for the reds, yellows, and greens in the majority of the scale (Figure 7F), but we were unable to generate interpretable sagittal sections by cryofracture due to scale fragility. Most intriguing, it remains unclear how *A. formosus* achieved brightness levels that exceeded the reflectance of our specular reflectance standard (measurements > 1.00 in Figure 6C), a level of gold iridescence that is only achieved in other insects with much thicker stacks of chitin (Steinbrecht et al., 1985; Seago et al., 2009; Biro and Vigneron, 2011). While we have described here the “metalmark” (riodinid) system superficially, a better understanding of how such seemingly flat, simple, and ultrathin sandwich of chitin and air can yield an intensely specular output will be of particular interest.

Broadband Microrib Gratings

A fourth type of metallic reflectance mechanism has been described in the literature, but was not observed in this study. We dub “microrib grating” a type of 2D diffraction grating made of dense herringbone crossrib arrays, and where upper and lower laminae are apposed (“fused scales”), forming a single thin film without a lumen. It has been linked to silver/gold iridescence in the skipper butterfly *Carystoides escalantei* (Ge et al., 2017). Outside of the butterfly/skipper lineage, it has been best characterized in the Micropterigidae *Micropterix calthella* (Kilchoer et al., 2019), where the fused lamina acts as a bronze/gold thin-film reflector whose specularly is enhanced by the diffractive scattering of the overlying microribs (D’Alba et al., 2019). Other likely examples include the Noctuidae moths *Diachrysia (Plusia) balluca* and *Trichoplusia orichalcea* (Ghiradella, 1991; Brink et al., 1995). Scales from *Adela reaumurella* (Adelidae) and *Stigmella malella* (Nepticulidae) show fused laminae and herringbone microribs that are consistent with the metallic sheens of these moths (van Eldijk et al., 2018). The white reflective scales of *Paysandisia archon* (Castniidae) display this arrangement (Stavenga et al., 2018). In Papilionidae, the reflective component of the glass scales of *G. sarpedon* is also due to a microrib grating, with membranal bilin pigments restricting the reflectance spectrum to a blue-green hue (Stavenga et al., 2010, 2012). In the basal Papilionidae *Baronia brevicornis*, an electron micrograph also suggests a microrib grating arrangement that is indicative of a reflective type (Simonsen et al., 2011), but it is unclear if the image was taken from a silver morph of this polymorphic species (Vazquez, 1987). Herringbone patterns and fused laminae are characteristic of early diverging lepidopteran lineages (Kristensen, 1970;

Simonsen, 2001; Deparis et al., 2006) and in accordance with these authors, we extrapolate that the microrib diffraction grating could be a sophistication of the lepidopteran scale ancestral state, but it is also noteworthy that a convergent scale morphology and microrib grating mechanism have been linked to gold iridescence in Collembola, suggesting the optical trick may extend across many hexapod lineages that also bear cuticular scales (D'Alba et al., 2019). In any case, we gather from the literature that diffraction gratings consisting of dense microribs and apposed laminae are likely common and ancient enhancers of metallic coloration in Lepidoptera.

CONCLUSION

Metallic appearances require spectral spread that result from increased reflectance variance in the geometry of the scale thin-films. The literature has pinpointed 2D diffraction gratings, characterized by dense herringbone microribs and apposed lower laminae, as a common theme for metallic reflections. Our study indicates that in butterflies and beyond, a bright output requires an unperforated upper lamina, and that broadband reflectance is also often reached by transversal undulations of the upper chitinous section and air lumen (Figures 7D–G). This configuration may be a simple derivation from traditional scale types including depigmented, white light-scattering scales. Proximo-distal thickness gradients in the lower section of the scale (simple or multilayered lower laminae) appear as another mode of broadband reflectance as alluded in Lycaenidae and Riodinidae, a phenomenon that will require further comparative studies and biophysical characterization.

MATERIALS AND METHODS

Butterflies

A. vanillae larvae were obtained from Shady Oak Farms (Florida, United States) and reared on *Passiflora suberosa* or *Passiflora incarnata* until adult emergence. Specimens of *S. cybele*, *C. eurytheme*, *Z. cesonina*, and *E. clarus* were collected from wild populations in the vicinity of Silver Spring (Maryland, United States), Mason Neck (Virginia, United States), Starkville (Mississippi, United States), and Washington (District of Columbia, United States), respectively. Specimens of *H. apelles* (orig. Papua – New Guinea), *C. (Spindasis) lohita* (orig. Bali, Indonesia), and *A. formosus* (orig. Peru) were imported from online retailers with appropriate collection and transit permits.

Color and UV Macro-Photography

Pinned specimens were imaged in the visible range using a Nikon D5300 camera mounted with a Micro-Nikkor 105mm f/2.8G lens on a StackShot rail and focused-stacked using the Helicon Remote and Helicon Focus software. A Keyence VHX-5000 digital microscope was used to generate stitched high-resolution images of wing patterns using a VH-Z00T

lens at 50× magnification and a VH-Z100T lens at the 300× magnification. UV-photography was performed under the illumination of GE BlackLight 13-Watt T3 Spiral Light Bulbs, using a full-spectrum converted Panasonic G3 camera, mounted with a Kyoei-Kuribayashi 35mm F3.5 lens on a helicoid focusing adapter, and stacked Hoya U-330 and Schott BG39 1.5 mm glass filters eliminating the visible and infrared wavelengths above 400 nm.

Single-Scale Light Microscopy

Individual scales were removed from spread butterflies and positioned on a glass slide with an eyelash tool, and stitch-imaged with a Keyence VHX-5000 microscope in reflected light mode and a VH-Z100R lens at 1000× magnification. All scales were imaged in full coaxial lighting except for *H. apelles*, which required additional ring lighting for best balance of resolution and color features. The same scales were then immersed in clove oil, mounted with a coverslip, and imaged in transmission mode. For reflective microscopy, single scales were imaged with an Olympus BX53M microscope in Reflected Bright-Field mode, mounted with an Olympus MPlanFLN 50×/NA 0.80 objective and an SC50 color camera, before focus-stacking with the Olympus Stream software, and XY-stitching in Adobe Photoshop. For reflective microscopy at higher-magnifications, the same scales were imaged on a trinocular AmScope ME580-2L metallurgical microscope mounted with a Nikon D5300 camera, a Varimag II camera adapter at 3.5× magnification setting, and an LMPlan Achromatic 100×/NA 0.8 long working distance objective. Optimal balance of resolution and color features were obtained under polarized light with a field diaphragm at maximal shutting position, and an aperture diaphragm on 60–80% shut position.

Spectral Measurements

Reflectance spectra were measured from a 250 μm^2 region of the surface of intact wing sections, and thus represent the combined reflectance of cover scales, underlying ground scales, and wing membrane (Stavenga et al., 2014). Reflectance measurements were taken using a custom-built microspectrophotometer (20/20PV, CRAIC Technologies, Inc., San Dimas, CA, United States) equipped with a 5× UV-vis objective (LMU-5X-NUV, Thorlabs, Inc., Newton, NJ, United States). Samples were illuminated with a xenon arc lamp (XBO 75 W/2, OSRAM GmbH, Munich, Germany), with the light path oriented normal to the wing surface and coaxial with the axis of light collection. Reflectance spectra were calculated in relation to a high-reflectivity specular reflectance standard (STAN-SSH-NIST, Ocean Optics, Inc., Dunedin, FL, United States). For each scale type, individual spectra were the average of 25 spectra measured consecutively (integration time: 200–350 ms, total measurement time per average spectra: 5–8.75 s). In order to provide a familiar point of comparison, measurements were also taken on the polished and dull side of aluminum foil (0.018 mm, Fisherbrand), and resulted in spectra consistent with other studies (Vukusic et al., 2008; Pozzobon et al., 2020).

Scanning Electron Microscopy (SEM)

For surface imaging of scales of distinct identities, wing patterns of interest were excised and mounted on SEM stubs with double-sided carbon tape, and color imaged under the Keyence VHX-5000 microscope for registration of scale type. Samples were sputter-coated with two 12.5 nm layers of gold for improving sample conductivity, with the second layer applied after tilting the stub by 45°. SEM images were acquired on a FEI Teneo LV SEM, using secondary electrons (SE) and an Everhart-Thornley detector (ETD) using a beam energy of 2.00 kV, beam current of 25 pA, and a 10 μ s dwell time. Individual images were stitched using the Maps 3.10 software (Thermo Fisher Scientific).

To minimize charging for high magnification views of scale surface morphology, individual scales were collected by brushing the surface of the wing with an eyelash tool, then dusted onto an SEM stub with double-sided carbon tape. Stubs were sputter-coated with one 12.5 nm layer of gold, and imaged at 2.00 kV/25 pA with a 10 μ s dwell time. One sample per species was imaged, and all the SEM images used for morphometric analysis are accessible on the Dryad online repository (Day et al., 2020).

For documenting internal scale anatomy, silver pattern elements were excised and cryo-fractured following previous recommendations (Wasik et al., 2014; Matsuoka and Monteiro, 2018; Thayer et al., 2020). Briefly, wing sections were submerged in liquid nitrogen, immediately placed silver-side down onto a silicon wafer, and cut with a fresh ceramic-coated microtome blade. Alternatively, excised wing sections were placed silver-side down onto a silicon wafer and secured with foam board, glassine, and a binder clip before submersion in liquid nitrogen and cutting as previously described. After allowing to dry, individual cut scales were placed using an eyelash tool on copper tape, such that the cut edges were approximately parallel to and overhanging the tape edge. The copper tape was bent to 90° and placed on a stub so that the scales' cut edges faced upwards, i.e., normal to the stub surface, and secured with additional copper tape. The stubs were sputter-coated with a 10–12.5 nm layer of gold, and imaged at 5.00 kV/6.3 pA and a 10 μ s dwell time.

Morphometric Analysis

Morphometric measurements of scale widths and ridge distances were carried out on 25 scales of each color from eight

species, using a custom semi-automated R pipeline that derives ultrastructural parameters from large SEM images (Day et al., 2019). Briefly, ridge spacing was assessed by Fourier transforming intensity traces of the ridges acquired from the FIJI software (Schindelin et al., 2012). Scale width was directly measured in FIJI by manually tracing a line, orthogonal to the ridges, at the section of maximal width.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

AR and CD equally performed all the imaging and experiments, and contributed to the figures and data analysis. JH, BC, and NM contributed to the data analysis and intellectual support. AM supervised the project and wrote the manuscript with input from all co-authors. All authors contributed to the article and approved the submitted version.

FUNDING

This research was funded by the National Science Foundation award IOS-1755329 to AM and BC, and a Masters student fellowship allocated to AR by the GWU Minor in Sustainability.

ACKNOWLEDGMENTS

We thank Huimin Chen for advice and support with image analysis, Rod Eastwood for assisting with butterfly identification, and Christine Brantner and Anastas Popratiloff for their technical assistance at the GW Nanofabrication and Imaging Center. Additionally we thank Amruta Tendolkar for providing eyelashes for manipulations of single scales, Rachel Thayer and Marshall Nakatani for insights on cryo-fracturing techniques, and John Lill for providing the *S. cybele* specimen from his personal collection.

REFERENCES

- Agez, G., Bayon, C., and Mitov, M. (2017). Multiwavelength micromirrors in the cuticle of scarab beetle *Chrysina gloriosa*. *Acta Biomater.* 48, 357–367. doi: 10.1016/j.actbio.2016.11.033
- Berthier, S. (2007). *Iridescences: The Physical Colors Of Insects*. Berlin: Springer.
- Biró, L. P., Kertész, K., Vértessy, Z., Márk, G. I., Bálint, Z., Lousse, V., et al. (2007). Living photonic crystals: butterfly scales—nanostructure and optical properties. *Mater. Sci. Eng. C* 27, 941–946. doi: 10.1016/j.msec.2006.09.043
- Biro, L. P., and Vigneron, J.-P. (2011). Photonic nanoarchitectures in butterflies and beetles: valuable sources for bioinspiration. *Laser Photon. Rev.* 5, 27–51. doi: 10.1002/lpor.200900018
- Brink, D. J., Smit, J. E., Lee, M. E., and Möller, A. (1995). Optical diffraction by the microstructure of the wing of a moth. *Appl. Opt.* 34, 6049–6057.
- Briscoe, A. D., Bybee, S. M., Bernard, G. D., Yuan, F., Sison-Mangus, M. P., Reed, R. D., et al. (2010). Positive selection of a duplicated UV-sensitive visual pigment coincides with wing pigment evolution in heliconius butterflies. *Proc. Natl. Acad. Sci. U.S.A.* 107, 3628–3633. doi: 10.1073/pnas.0910085107
- Chiadini, F., Fiumara, V., and Scaglione, A. (2017). Design of bioinspired chirped reflectors using a genetic algorithm. *Bioinspirat. Biomimet. Bioreplicat.* 2017:101620T.
- Cook, C. Q., and Amir, A. (2016). Theory of chirped photonic crystals in biological broadband reflectors. *Optica* 3, 1436–1439.
- D'Alba, L., Wang, B., Vanthournout, B., and Shawkey, M. D. (2019). The golden age of arthropods: ancient mechanisms of colour production in body scales. *J. R. Soc. Interf.* 16:20190366.
- Day, C. R., Hanly, J. J., Ren, A., and Martin, A. (2019). Sub-micrometer insights into the cytoskeletal dynamics and ultrastructural diversity of butterfly wing scales. *Dev. Dyn.* 248, 657–670. doi: 10.1002/dvdy.63
- Day, C. R., Hanly, J. J., Ren, A., and Martin, A. (2020). *Data from: Convergent Evolution of Broadband Reflectors Underlies Metallic Colorations*

- in *Butterflies*, v3, *Dryad, Dataset*. Available online at: <https://doi.org/10.5061/dryad.2fqz612mc>
- Denton, E. J., and Land, M. F. (1971). Mechanism of reflexion in silvery layers of fish and cephalopods. *Proc. R. Soc. Lond. Ser. B Biol. Sci.* 178, 43–61. doi: 10.1098/rspb.1971.0051
- Deparis, O., Vandenbem, C., Rassart, M., Welch, V. L., and Vigneron, J.-P. (2006). Color-selecting reflectors inspired from biological periodic multilayer structures. *Optics Exp.* 14, 3547–3555.
- Dinwiddie, A., Null, R., Pizzano, M., Chuong, L., Krup, A. L., Tan, H. E., et al. (2014). Dynamics of F-actin prefigure the structure of butterfly wing scales. *Dev. Biol.* 392, 404–418. doi: 10.1016/j.ydbio.2014.06.005
- Dolan, J. A., Wilts, B. D., Vignolini, S., Baumberg, J. J., Steiner, U., and Wilkinson, T. D. (2015). Optical properties of gyroid structured materials: from photonic crystals to metamaterials. *Adv. Opt. Mater.* 3, 12–32. doi: 10.1002/adom.201400333
- Espeland, M., Breinholt, J., Willmott, K. R., Warren, A. D., Vila, R., Toussaint, E. F., et al. (2018). A comprehensive and dated phylogenomic analysis of butterflies. *Curr. Biol.* 28, 770–778.
- García-Barros, E., and Meneguz, M. (2012). Estructura de las escamas ventrales de las alas de *Coenonympha Hübner*, [1819], con especial referencia a *C. pamphilus* (L., 1758) y su morfotipo lyllus (Esper, 1805) (Lepidoptera: Nymphalidae). *SHILAP Rev. Lepidopterol.* 40, 279–293.
- Ge, D., Wu, G., Yang, L., Kim, H.-N., Hallwachs, W., Burns, J. M., et al. (2017). Varying and unchanging whiteness on the wings of dusk-active and shade-inhabiting *Carystoides escalantei* butterflies. *Proc. Natl. Acad. Sci. U.S.A.* 114, 7379–7384. doi: 10.1073/pnas.1701017114
- Ghiradella, H. (1989). Structure and development of iridescent butterfly scales: lattices and laminae. *J. Morphol.* 202, 69–88. doi: 10.1002/jmor.1052020106
- Ghiradella, H. (1991). Light and color on the wing: structural colors in butterflies and moths. *Appl. Opt.* 30, 3492–3500.
- Ghiradella, H. (2010). Insect cuticular surface modifications: scales and other structural formations. *Adv. Insect Physiol.* 38, 135–180. doi: 10.1016/s0065-2806(10)38006-4
- Ghoshal, A., DeMartini, D. G., Eck, E., and Morse, D. E. (2013). Optical parameters of the tunable Bragg reflectors in squid. *J. R. Soc. Interf.* 10:20130386. doi: 10.1098/rsif.2013.0386
- Giraldo, M. A. (2008). *Butterfly Wing Scales: Pigmentation and Structural Properties*. Thesis, University of Groningen. doi: 10.1098/rsif.2013.0386
- Holt, A. L., Sweeney, A. M., Johnsen, S., and Morse, D. E. (2011). A highly distributed Bragg stack with unique geometry provides effective camouflage for Loliginid squid eyes. *J. R. Soc. Interf.* 8, 1386–1399. doi: 10.1098/rsif.2010.0702
- Imafuku, M., Kubota, H. Y., and Inouye, K. (2012). Wing colors based on arrangement of the multilayer structure of wing scales in lycaenid butterflies (Insecta: Lepidoptera). *Entomol. Sci.* 15, 400–407. doi: 10.1111/j.1479-8298.2012.00525.x
- Ingram, A. L., and Parker, A. R. (2008). A review of the diversity and evolution of photonic structures in butterflies, incorporating the work of John Huxley (The Natural History Museum, London from 1961 to 1990). *Philos. Trans. R. Soc. B Biol. Sci.* 363, 2465–2480. doi: 10.1098/rstb.2007.2258
- Jordan, T. M., Partridge, J. C., and Roberts, N. W. (2012). Non-polarizing broadband multilayer reflectors in fish. *Nat. Photon.* 6:759. doi: 10.1038/nphoton.2012.260
- Kelley, J. L., Tatarnic, N. J., Schröder-Turk, G. E., Endler, J. A., and Wilts, B. D. (2019). A dynamic optical signal in a nocturnal moth. *Curr. Biol.* 29, 2919–2925.
- Kilchoer, C., Steiner, U., and Wilts, B. D. (2019). Thin-film structural coloration from simple fused scales in moths. *J. R. Soc. Interf. Focus* 9:20180044. doi: 10.1098/rsfs.2018.0044
- Kinoshita, S. (2008). *Structural Colors In The Realm Of Nature*. Singapore: World Scientific.
- Kinoshita, S., and Yoshioka, S. (2005). Structural colors in nature: the role of regularity and irregularity in the structure. *Chemphyschem* 6, 1442–1459. doi: 10.1002/cphc.200500007
- Kristensen, N. P. (1970). Morphological observations on the wing scales in some primitive *Lepidoptera* (Insecta). *J. Ultrastruct. Res.* 30, 402–410. doi: 10.1016/s0022-5320(70)80071-5
- Land, M. F. (1972). The physics and biology of animal reflectors. *Prog. Biophys. Mol. Biol.* 24, 75–106. doi: 10.1016/0079-6107(72)90004-1
- Lee, D. W. (2009). “Plant tissue optics: micro- and nanostructures,” in *Proceedings of the Biomimetics and Bioinspiration*, San Diego, CA.
- Leertouwer, H. L., Wilts, B. D., and Stavenga, D. G. (2011). Refractive index and dispersion of butterfly chitin and bird keratin measured by polarizing interference microscopy. *Opt. Exp.* 19, 24061–24066.
- Levy-Lior, A., Pokroy, B., Levavi-Sivan, B., Leiserowitz, L., Weiner, S., and Addadi, L. (2008). Biogenic guanine crystals from the skin of fish may be designed to enhance light reflectance. *Cryst. Growth Design* 8, 507–511. doi: 10.1021/cg0704753
- Lippert, W., and Gentil, K. (1959). Über lamellare feinstrukturen bei den schillerschuppen der schmetterlinge vom urania- und morpho- typ. *Zeitschrift Morphol. Ökol. Tiere* 48, 115–122. doi: 10.1007/bf00407836
- Liu, X., Wang, D., Yang, Z., Zhou, H., Zhao, Q., and Fan, T. (2019). Bright silver brilliancy from irregular microstructures in butterfly *Curetis acuta* moore. *Adv. Opt. Mater.* 7:1900687. doi: 10.1002/adom.201900687
- Matsuoka, Y., and Monteiro, A. (2018). Melanin pathway genes regulate color and morphology of butterfly wing scales. *Cell Rep.* 24, 56–65. doi: 10.1016/j.celrep.2018.05.092
- Mayor, A. G. (1806). *The Development of the Wing Scales and Their Pigment in Butterflies and Moths*. Cambridge, MA: Harvard College.
- McKenzie, D. R., Yin, Y., and McFall, W. D. (1995). Silvery fish skin as an example of a chaotic reflector. *Proc. R. Soc. Lond. Ser. A Math. Phys. Sci.* 451, 579–584. doi: 10.1098/rspa.1995.0144
- Mouchet, S. R., and Vukusic, P. (2018). Structural colours in lepidopteran scales. *Adv. Insect Physiol.* 54, 1–53. doi: 10.1016/bs.aip.2017.11.002
- Neville, A. C. (1977). Metallic gold and silver colours in some insect cuticles. *J. Insect Physiol.* 23, 1267–1274. doi: 10.1016/0022-1910(77)90069-5
- Pozzobon, V., Levasseur, W., Do, K.-V., Palant, B., and Perré, P. (2020). Household aluminum foil matte and bright side reflectivity measurements: application to a photobioreactor light concentrator design. *Biotechnol. Rep.* 25:e00399. doi: 10.1016/j.btre.2019.e00399
- Prum, R. O., Quinn, T., and Torres, R. H. (2006). Anatomically diverse butterfly scales all produce structural colours by coherent scattering. *J. Exp. Biol.* 209, 748–765. doi: 10.1242/jeb.02051
- Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., et al. (2012). Fiji: an open-source platform for biological-image analysis. *Nat. Methods* 9:676. doi: 10.1038/nmeth.2019
- Schmidt, K., and Paulus, H. (1970). Die feinstruktur der flügel-schuppen einiger *Lycaeniden* (Insecta, Lepidoptera). *Zeitschrift Morphol. Tiere* 66, 224–241. doi: 10.1007/bf00280735
- Seago, A. E., Brady, P., Vigneron, J.-P., and Schultz, T. D. (2009). Gold bugs and beyond: a review of iridescence and structural colour mechanisms in beetles (Coleoptera). *J. R. Soc. Interf.* 6, S165–S184.
- Simonsen, T. J. (2001). The wing vestiture of the non-ditrysian *Lepidoptera* (Insecta). Comparative morphology and phylogenetic implications. *Acta Zool.* 82, 275–298. doi: 10.1046/j.1463-6395.2001.00089.x
- Simonsen, T. J. (2002). Wing scale covering supports close relationship between *Callipielus* and *Dalaca*, austral South American ghost moths (Lepidoptera: Hepialidae). *Stud. Neotrop. Fauna Environ.* 37, 65–69. doi: 10.1076/snf.37.1.65.2117
- Simonsen, T. J. (2007). Comparative morphology and evolutionary aspects of the reflective under wing scale-pattern in fritillary butterflies (Nymphalidae: Argynniini). *Zool. Anzeiger A J. Compar. Zool.* 246, 1–10. doi: 10.1016/j.jcz.2005.04.005
- Simonsen, T. J., Zakharov, E. V., Djernaes, M., Cotton, A. M., Vane-Wright, R. I., and Sperling, F. A. (2011). Phylogenetics and divergence times of *Papilioninae* (Lepidoptera) with special reference to the enigmatic genera *Teinopalpus* and *Meandrusa*. *Cladistics* 27, 113–137. doi: 10.1111/j.1096-0031.2010.00326.x
- Singer, A., Boucheron, L., Dietze, S. H., Jensen, K. E., Vine, D., McNulty, I., et al. (2016). Domain morphology, boundaries, and topological defects in biophotonic gyroid nanostructures of butterfly wing scales. *Sci. Adv.* 2:e1600149. doi: 10.1126/sciadv.1600149
- Stavenga, D. G., Giraldo, M. A., and Leertouwer, H. L. (2010). Butterfly wing colors: glass scales of *Graphium sarpedon* cause polarized iridescence and enhance blue/green pigment coloration of the wing membrane. *J. Exp. Biol.* 213, 1731–1739. doi: 10.1242/jeb.041434

- Stavenga, D. G., Leertouwer, H. L., Meglič, A., Drašlar, K., Wehling, M. F., Piriš, P., et al. (2018). Classical lepidopteran wing scale colouration in the giant butterfly-moth *Paysandisia archon*. *PeerJ* 6:e4590. doi: 10.7717/peerj.4590
- Stavenga, D. G., Leertouwer, H. L., Osorio, D. C., and Wilt, B. D. (2015a). High refractive index of melanin in shiny occipital feathers of a bird of paradise. *Light Sci. Appl.* 4:e243. doi: 10.1038/lsa.2015.16
- Stavenga, D. G., Matsushita, A., and Arikawa, K. (2015b). Combined pigmentary and structural effects tune wing scale coloration to color vision in the swallowtail butterfly *Papilio xuthus*. *Zool. Lett.* 1:14.
- Stavenga, D. G., Leertouwer, H. L., and Wilt, B. D. (2014). Coloration principles of nymphaline butterflies—thin films, melanin, ommochromes and wing scale stacking. *J. Exp. Biol.* 217, 2171–2180. doi: 10.1242/jeb.098673
- Stavenga, D. G., Matsushita, A., Arikawa, K., Leertouwer, H. L., and Wilt, B. D. (2012). Glass scales on the wing of the swordtail butterfly *Graphium sarpedon* act as thin film polarizing reflectors. *J. Exp. Biol.* 215, 657–662. doi: 10.1242/jeb.066902
- Steinbrecht, R. A., Mohren, W., Pulker, H. K., and Schneider, D. (1985). Cuticular interference reflectors in the golden pupae of danaine butterflies. *Proc. R. Soc. Lond. Ser. B Biol. Sci.* 226, 367–390. doi: 10.1098/rspb.1985.0100
- Thayer, R. C., Allen, F. I., and Patel, N. H. (2020). Structural color in *Junonia* butterflies evolves by tuning scale lamina thickness. *eLife* 9:e52187.
- Tilley, R. J. D., Eliot, J. N., and Yoshimoto, H. (2002). Scale microstructure and its phylogenetic implications in lycaenid butterflies (Lepidoptera, Lycaenidae). *Lepidoptera Sci.* 53, 153–180.
- van Eldijk, T. J., Wappler, T., Strother, P. K., van der Weijst, C. M., Rajaei, H., Visscher, H., et al. (2018). A Triassic-Jurassic window into the evolution of Lepidoptera. *Sci. Adv.* 4:e1701568. doi: 10.1126/sciadv.1701568
- Vazquez, L. G. (1987). *Baronia brevicornis* Salvin y sus formas (Lepidoptera: Papilionidae-Baroniinae). México: National Autonomous University of Mexico.
- Vukusic, P., Kelly, R., and Hooper, I. (2008). A biological sub-micron thickness optical broadband reflector characterized using both light and microwaves. *J. R. Soc. Interf.* 6, S193–S201.
- Vukusic, P., Sambles, J. R., and Ghiradella, H. (2000). Optical classification of microstructure in butterfly wing-scales. *Photon. Sci. News* 6, 61–66.
- Wasik, B. R., Liew, S. F., Lilien, D. A., Dinwiddie, A. J., Noh, H., Cao, H., et al. (2014). Artificial selection for structural color on butterfly wings and comparison with natural evolution. *Proc. Natl. Acad. Sci. U.S.A.* 111, 12109–12114. doi: 10.1073/pnas.1402770111
- Wiemers, M., Chazot, N., Wheat, C. W., Schweiger, O., and Wahlberg, N. (2019). A complete time-calibrated multi-gene phylogeny of the European butterflies. *bioRxiv* [Preprint], doi: 10.1101/844175
- Wilt, B. D., Leertouwer, H. L., and Stavenga, D. G. (2008). Imaging scatterometry and microspectrophotometry of lycaenid butterfly wing scales with perforated multilayers. *J. R. Soc. Interf.* 6, S185–S192.
- Wilt, B. D., Piriš, P., Arikawa, K., and Stavenga, D. G. (2013). Shiny wing scales cause spec (tac) ular camouflage of the angled sunbeam butterfly, *Curetis acuta*. *Biol. J. Linnean Soc.* 109, 279–289. doi: 10.1111/bij.12070
- Wilt, B. D., Zubiri, B. A., Klatt, M. A., Butz, B., Fischer, M. G., Kelly, S. T., et al. (2017). Butterfly gyroid nanostructures as a time-frozen glimpse of intracellular membrane development. *Sci. Adv.* 3:e1603119. doi: 10.1126/sciadv.1603119
- Yoshioka, S., and Kinoshita, S. (2007). Polarization-sensitive color mixing in the wing of the Madagascan sunset moth. *Opt. Exp.* 15, 2691–2701.
- Yoshioka, S., Nakano, T., Nozue, Y., and Kinoshita, S. (2008). Coloration using higher order optical interference in the wing pattern of the Madagascan sunset moth. *J. R. Soc. Interf.* 5, 457–464. doi: 10.1098/rsif.2007.1268
- Yoshioka, S., Shimizu, Y., Kinoshita, S., and Matsuhana, B. (2013). Structural color of a lycaenid butterfly: analysis of an aperiodic multilayer structure. *Bioinspirat. Biomimet.* 8:e045001.
- Zhang, Y., Hayashi, T., Hosokawa, M., Yazawa, S., and Li, Y. (2009). Metallic lustre and the optical mechanism generated from the leaf surface of *Begonia rex* Putz. *Sci. Hortic.* 121, 213–217. doi: 10.1016/j.scienta.2009.01.030

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2020 Ren, Day, Hanly, Counterterman, Morehouse and Martin. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Wnt Genes in Wing Pattern Development of Coliadinae Butterflies

Jennifer Fenner^{1,2}, Caleb Benson², Luis Rodriguez-Caro^{2,3}, Anna Ren⁴, Riccardo Papa^{5,6}, Arnaud Martin⁴, Federico Hoffmann⁷, Ryan Range¹ and Brian A. Counterman^{2*}

¹ Department of Biological Sciences, College of Sciences and Mathematics, Auburn University, Auburn, AL, United States,

² Department of Biological Sciences, Mississippi State University, Starkville, MS, United States, ³ Division of Biological Sciences, College of Humanities and Sciences, University of Montana, Missoula, MT, United States, ⁴ Department of Biological Sciences, Columbian College of Arts and Sciences, The George Washington University, Washington, DC, United States, ⁵ Department of Biology, College of Natural Sciences, University of Puerto Rico—Rio Piedras, San Juan, Puerto Rico, ⁶ Molecular Sciences and Research Center, University of Puerto Rico, San Juan, Puerto Rico, ⁷ Department of Biochemistry, Molecular Biology, Entomology, and Plant Pathology, College of Agriculture and Life Sciences, Mississippi State University, Starkville, MS, United States

OPEN ACCESS

Edited by:

Marcus Kronforst,
The University of Chicago,
United States

Reviewed by:

Sean Mullen,
Boston University, United States
Luana S. Maroja,
Williams College, United States

*Correspondence:

Brian A. Counterman
bcounterman@biology.msstate.edu

Specialty section:

This article was submitted to
Evolutionary Developmental Biology,
a section of the journal
Frontiers in Ecology and Evolution

Received: 09 March 2020

Accepted: 29 May 2020

Published: 30 June 2020

Citation:

Fenner J, Benson C,
Rodriguez-Caro L, Ren A, Papa R,
Martin A, Hoffmann F, Range R and
Counterman BA (2020) Wnt Genes
in Wing Pattern Development
of Coliadinae Butterflies.
Front. Ecol. Evol. 8:197.
doi: 10.3389/fevo.2020.00197

Wnt genes are major developmental genes highly conserved across all animals. Yet, our understanding of the Wnt gene repertoire and their functions is still largely incomplete. In Lepidoptera, Wnt genes have been implicated in wing pattern development. For example, WntA has been shown as a driver of wing pattern diversification in nymphalid butterflies. In this study, we characterize the Wnt gene repertoire in *Zerene cesonia* (Family: Pieridae), which diverged from nymphalids ~51 million years ago, to determine if Wnt genes may have a conserved role in wing development across distant butterfly lineages. We first show that Wnt gene content is highly conserved across butterflies, but that there is strikingly different expression across the eight Wnt genes during wing development of *Z. cesonia* and nymphalid butterflies. Surprisingly, while four Wnt genes (Wnt1, 6, 7, and 11) are expressed during wing development in *Z. cesonia*, the renowned nymphalid wing patterning gene WntA was undetected. However, despite the differences in Wnt gene expression, Heparin injections yielded a similar disruption in wing color pattern development in both *Z. cesonia* and nymphalid butterflies. Interestingly, these assays also resulted in the loss of structurally based ultraviolet (UV) wing patterns in *Z. cesonia*. Collectively, the study shows that pierids maintained the highly conserved nature of Wnt gene content in Lepidoptera, but might deploy these genes very differently from nymphalid butterflies during wing pattern development.

Keywords: pigmentation, ultraviolet, Lepidoptera, RNA-seq, wing development

INTRODUCTION

Wnt genes are responsible for a variety of vital developmental processes and are highly conserved across animals. From axis formation in embryogenesis to insect wing development, Wnt genes are a major part of the developmental toolkit. There are 13 subfamilies of Wnt ligand genes that code for a set of secreted glycoproteins rich in cysteine. The transduction of these Wnt ligands is controlled by the canonical Wnt/ β -catenin pathway and the non-canonical Wnt/JNK planar cell polarity (PCP) and Wnt/Ca²⁺ pathways (Rao and Kühl, 2010). The generalized function of Wnt

genes in these pathways has been studied in detail over the past century (Nusse, 2012); however, the explicit function and contribution of each *Wnt* gene throughout development remains poorly understood, and most of what is known comes from model organisms such as mice and *Drosophila*. Attempts to extrapolate *Wnt* gene function from one model organism to another species have proven difficult, not only due to differences in the *Wnt* gene repertoires among species, but also the potential functions of different *Wnt* genes interacting in multiple *Wnt* pathways (Murat et al., 2010; Range, 2018). To better understand the role of *Wnt* ligands in the evolution of developmental processes, a systematic analysis of *Wnt* gene content and activity is needed in clades where *Wnt* ligands may have played an important role in morphological diversification.

Studies of the characterization and function of *Wnt* signaling among arthropods are limited outside of *Drosophila*. Among arthropod species that have been examined, the numbers of *Wnt* gene content vary from six in *Acyrtosiphon pisum* to nine in *Tribolium castaneum* (Ding et al., 2019). Among holometabolous insects, there are eight *Wnt* genes that are most commonly found: *Wnt1*, *Wnt5*, *Wnt6*, *Wnt7*, *Wnt9*, *Wnt10*, *Wnt11*, and *WntA*. *Drosophila* lacks only *WntA*; the functions of its seven *Wnt* genes include axis patterning in embryogenesis, control of the sexual dimorphic development of reproductive organs, development of neuroblast identity, and wing formation (Murat et al., 2010). Lepidoptera typically harbor a repertoire of eight *Wnt* genes, *Wnt1*, *Wnt5*, *Wnt6*, *Wnt7*, *Wnt9*, *Wnt10*, *Wnt11*, and *WntA* that are expressed in various tissues and stages (Ding et al., 2019; Holzem et al., 2019). In all arthropods (Murat et al., 2010) and in other animal phyla (Somorjai et al., 2018) *Wnt9–Wnt1–Wnt6–Wnt10* are arranged in a tandem array, forming a syntenic block of paralogs. Not only are these genes linked on a single chromosome, but some (*Wnt1–Wnt6–Wnt10*) also share similarities in expression patterns on the wing discs of wing-spotted fruit flies, *Drosophila guttifera* (Koshikawa et al., 2015) and the *Euphydryas chalcedon* butterfly (Martin and Reed, 2014). Such consistent expression patterns between diptera and lepidoptera suggest a possible ancestral role of these *Wnts* in wing development. In addition, *Wnt7–Wnt5–WntA* form a syntenic arrangement of genes that is conserved across insects (Murat et al., 2010), and seem to originate from an ancestral *Wnt5–7* cluster that pre existed at the root of Bilateria (Somorjai et al., 2018). Finally, *Wnt11* is not located within either lepidopteran *Wnt* cluster, but is on another chromosome separate from the two main clusters (Ding et al., 2019).

Recently, *Wnt* genes have been implicated in the development and evolution of color patterns on butterfly wings. Early studies in *Heliconius* butterflies used genetic mapping, gene expression assays, and pharmacological manipulation to identify *WntA* as the gene responsible for major differences in melanic forewing patterns across the genus (Martin et al., 2012). Recently, the functional role of *WntA* in wing pattern development was shown in twelve *Heliconius* species using CRISPR gene-editing (Concha et al., 2019). Further, the role of *WntA* in wing pattern development has also been explored across other nymphalid butterflies, which all showed unique changes in wing pattern, including the white vein contour markings of Monarch wings

(Gallant et al., 2014; Martin and Reed, 2014; Mazo-Vargas et al., 2017). Beyond this work in nymphalid butterflies, little is known about the importance of the *Wnt* landscape in various aspects of insect development (Murat et al., 2010).

Within Lepidoptera, the most well-characterized *Wnt* gene beside *WntA*, is *Wnt1*, for its role in color pattern development. *Wnt1*, designated as *wingless* when it was first discovered in *Drosophila*, is responsible for the development of imaginal discs, and is therefore vital for the formation of both wings and eyes (Baker, 1988). *Wnt1* is perhaps most extensively studied of the *Wnt* genes due to its role in segment polarity (Baker, 1987). In the silkworm, *Bombyx mori*, *Wnt1* has likely retained this ancestral role, as *Wnt1* knockouts with CRISPR/Cas9 reveal its necessity during embryogenesis (Edayoshi et al., 2015; Zhang et al., 2015). In nymphalid butterflies, *Wnt1* retains its expected embryonic expression (Holzem et al., 2019) and is also expressed in the peripheral tissue of the developing wings (Macdonald et al., 2010), as in other holometabolous insects (Tomoyasu et al., 2009). Within those developing wing tissues, butterfly *Wnt1* also pre-patterns the “Discalis” ground plan elements on the wing across several lepidopteran lineages (Martin and Reed, 2010). In at least one nymphalid butterfly, *Wnt6* and *Wnt10* wing disc expression is concurrent with *Wnt1* in both the peripheral tissue and discal pattern elements of the larval wing disk (Martin and Reed, 2014); an overlap that is consistent with the previously described functional redundancy between these three paralogs in *Drosophila* and *Tribolium* (Bolognesi et al., 2008; Murat et al., 2010). Quantitative PCR profiles of the silkworm *Wnt* repertoire also suggest that *Wnt1*, *Wnt6*, *Wnt7*, *Wnt10*, and *WntA* are all expressed in larval wing discs (Ding et al., 2019). Similarly, an RNA-seq study of pupal wing development in passionvine butterflies revealed expression of *Wnt1*, *Wnt5*, *Wnt6*, *Wnt7*, and *WntA* (Hanly et al., 2019). To our knowledge, *Wnt9* and *Wnt11* have not been shown to be expressed during lepidopteran wing development, and *Wnt9* (*syn. DWnt4*) has been implicated in differences in male and female reproductive development in *Drosophila* (Cohen et al., 2002; Murat et al., 2010).

Here, we characterize the repertoire of *Wnt* genes in a pierid butterfly, *Zerene cesonia*, and explore their expression and potential role during wing pattern development. *Z. cesonia* is a Coliadinae butterfly, which are characterized by their strikingly bright yellow wings that contrast sharply with black margins often found on the wing edge. They are also noted for the bright ultraviolet (UV) reflectance of male wings, produced by specialized nano-structures on yellow pterin scales in the medial region of the forewing (Fenner et al., 2019). Among Colidinae species, UV brightness is the top indicator of male mating success (Papke et al., 2007) and we are interested in whether *Wnt* genes may play similar roles in pattern development in *Z. cesonia* as to what has been observed in other butterflies.

Pierid butterflies diverged from better studied Nymphalidae, which includes Monarchs and *Heliconius* butterflies, ~51 million years ago, and from silkmoths > 95 million years ago, providing a comparative framework in Lepidoptera to explore *Wnt* evolution and function in wing development (Kawahara et al., 2019). Using an RNA-seq approach, we detect the expression of four out of eight *Wnt* genes during the wing development of *Z. cesonia*.

Interestingly, we find different *Wnt* genes are expressed in *Z. cesonia* relative to nymphalid wings, including no expression of *WntA*, despite its involvement in wing pattern development of several nymphalid species. Using pharmaceutical assays similar to studies of nymphalids, we observed a similar expansion of melanic scales across the forewing in two species of pierid butterflies, *Z. cesonia* and its close relative *Colias eurytheme*. Importantly, we also observed a loss of the UV reflective pattern in *Z. cesonia*. Our findings suggest differences in the way the *Wnt* repertoire is used for color patterning during the wing development of pierids and nymphalids.

MATERIALS AND METHODS

Gene Expression Assays

RNA was extracted using TRIzol from developing wing tissue of male *Z. cesonia* from fifth instar larvae and every day of pupation till emergence (i.e., days 1–6). A minimum of three males were used for each stage (with a maximum of five replicates on day 4 of pupation). Total RNA was extracted and mRNA was isolated using oligo(dT) magnetic beads from NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB, Ipswich, MA, United States). cDNA was prepared using a modified version of the Smart-seq2 protocol (Picelli et al., 2014) and libraries were prepared using a Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA, United States). Libraries were sequenced as 150 base pair paired-end reads, at an average of 18.9 million reads per library (minimum of 13.2 million reads and maximum of 28.19 million reads). RNA-seq reads were preprocessed using AfterQC (Chen et al., 2017) and Trimmomatic (Bolger et al., 2014). Reads were then mapped to gene models from the reference genome using Bowtie2 and gene expression was estimated using RSEM (Li and Dewey, 2011) with default parameters. *Wnt* expression profiles were identified for *Wnt1/wingless*, *Wnt6*, *Wnt7*, and *Wnt11*, but no RNA-seq reads mapped to *WntA*, *Wnt10*, *Wnt5*, or *Wnt9*. Expression profiles are reported as mean FPKM (fragments per kilobase of transcript per million mapped reads) values across individuals for each developmental stage.

Gene Content Analyses

We used a combination of bioinformatic approaches to identify the repertoire of *Wnt* genes in representative Lepidoptera, using the *Drosophila melanogaster* *wnt* repertoire as a reference. We first surveyed the Ensembl Metazoa database (Howe et al., 2020) for the full set of *wnt* paralogs in *B. mori*, *Danaus plexippus*, *Heliconius melpomene*, and *Melitaea cinxia*. We then used BLAST (Altschul et al., 1990) to search for the *Wnt* paralogs in *Bicyclus anynana*, *Pieris rapae*, and *Vanessa tameamea*, which are available at NCBI. *Wnt* genes for *Pieris napi* and *Phoebis sennae* were acquired using BLAST tools and available genome assemblies at lepbases.org (Challi et al., 2016). *Wnt* genes were identified in *Z. cesonia* using the annotated genome assembly v.1.0 (NCBI Accession JAAMXH000000000) (Rodriguez-Caro et al., 2020) and confirmed through sequence similarity searches and alignments with BLAST and MUMmer3.23 (Altschul et al., 1990; Kurtz et al., 2004). A *Zerene eurydice*, the sister species

to *Z. cesonia*, individual (collected in San Bernardino, CA, United States) was sequenced (150 bp paired end Illumina reads, 6 Gb data yielding 20X coverage) and reads were mapped to the *Z. cesonia* genome assembly v1.0. *Z. eurydice* *Wnt* gene sequences were extracted using the *Z. eurydice* genotypes. For *C. eurytheme*, a 10X Genomics linked-read library was constructed for a single individual (collected in Osborne, MS, United States) according to protocol (10X Genomics, San Francisco, CA, United States) and sequenced by Novogene yielding 10.6 Gb of sequence data, which was assembled with Supernova (Weisenfeld et al., 2017). Assembly scripts and information are available through a github repository¹. To identify the *Wnt* genes sequences in *C. eurytheme*, BLAST and MUMmer were used to search and align *Z. cesonia* *Wnt* gene sequences with the 10X assembly.

Phylogenetic Analyses

We aligned the *Wnt* paralogs for silkworm (*B. mori*), four species of nymphalids (*B. anynana*, *H. melpomene*, *Junonia coenia*, *V. tameamea*), and five species of pierid butterflies (*C. eurytheme*, *Phoebis sennae*, *P. napi*, *P. rapae*, *Z. cesonia*, and *Z. eurydice*) based on their conceptual amino acid translation using MUSCLE (Edgar, 2004) as implemented in MEGAX (Kumar et al., 2018). We then reconstructed phylogenetic relationships among lepidopteran *Wnt* genes to resolve orthology. We inferred phylogenies in a maximum likelihood framework using IQ-Tree ver 1.6.6 (Nguyen et al., 2015) in the implementation available from the IQ-Tree web server (Trifinopoulos et al., 2016) last accessed on December 2019, selecting the best-fitting model of substitution identified with the ModelFinder subroutine from IQ-Tree (Kalyaanamoorthy et al., 2017). Support for the nodes was evaluated with the Shimodaira–Hasegawa approximate likelihood-ratio test (SH-aLRT), the aBayes test (Anisimova et al., 2011) and 1000 pseudoreplicates of the ultrafast bootstrap procedure (Hoang et al., 2018). We then removed the *D. melanogaster* sequences and used the resulting maximum likelihood tree to estimate differences in selective pressure among the lepidopteran *wnt* paralogs as evidenced by differences in the ratio of the rate of non-synonymous substitution per non-synonymous site (dN) to the rate of synonymous substitution per synonymous site (dS) using the codon-based maximum likelihood approach proposed by Goldman and Yang (1994) as implemented in PAML ver 4.9j (Yang, 2007). We explored variation in the dN/dS ratio among the different lepidopteran *wnt* paralogs using the branch models implemented in the program codeML from PAML.

Pharmaceutical Assays

Zerene cesonia females were collected from a local Mississippi prairie (33°30'36.98"N, 88°44'14.57"W). Females were allowed to oviposit eggs on *Dalea purpurea*. Upon hatching, larvae were raised on an artificial diet (Shelby et al., 2020) under 16:8 photoperiod and 28°C conditions. A laboratory colony was then maintained over several generations under these conditions. At 12 h after pupation, *Z. cesonia* individuals (*N* = 25) were injected with either 0.5 µg of heparin sodium salt (Sigma–Aldrich) diluted

¹github.com/bcount/Colias10X

in water, or with water as a control, and *C. eurytheme* ($N = 5$) were injected with 0.25 μg of heparin. Injections were conducted with a 10 μl Hamilton syringe with a 26-gauge needle into the right side in the interstice that separates the baso-posterior sections of the developing wings. The outer pupal casing was sterilized with ethanol prior to injections and the pupa were hung in a pupal chamber until emergence. Individuals were preserved for photography and microscopy following eclosion.

Photography and Microscopy

Individuals were photographed using an UV-IR converted Nikon D7000 camera with an AF-S Micro Nikkor 105 mm Lens for visual color images. UV color images used the same lens and camera but with the addition of a BAADER U-Filter, 60 nm IR, and VIS filter. Scanning electron microscopy was performed on a JEOL JSM-6500F FE-SEM at 5 kV. Wing sections of *Z. cesonia* and specimens of *Heliconius sara* from Martin et al. (2012) were mounted on aluminum stubs with silver paste and coated with 15 nm of platinum.

RESULTS

Wnt Gene Repertoire and Evolution in Butterflies

The *Wnt* gene content is conserved across all Lepidopteran genomes studied. Our phylogenetic analyses grouped the Lepidopteran *Wnt* paralogs into eight strongly supported clades that correspond to previously described *Wnt* genes in nymphalid butterflies (*Wnt1/wingless*, *Wnt5*, *Wnt6*, *Wnt7*, *Wnt9*, *Wnt10*, *Wnt11*, and *WntA*). In the *Z. cesonia* assembly v1.0, the eight *Wnt* genes are distributed in clusters on three chromosomes: *Wnt 9-1-6-10* on chromosome 1, *Wnt A-5-7* on chromosome 25, and *Wnt11* on chromosome 11 (Figure 1 and Supplementary Table S1). This was not the case for *Wnt11* and *WntA*, which have been secondarily lost in the genome of *D. melanogaster*. The *D. melanogaster Wnt7* paralog was placed sister to the clade that included the lepidoptera *Wnt11* and *Wnt7* paralogs, likely as a result of deep divergence among these *Wnt* paralogs.

Our analyses of patterns of molecular evolution failed to find significant differences in estimates of synonymous and non-synonymous substitution rates (ω) among the different lepidoptera paralogs. The model that assigns a single estimate of ω to all codons in the alignment along all branches in the tree could not be rejected in any of our tests (Supplementary Table S2), with very low value for this parameter ($\omega < 0.01$). These findings suggest *Wnt* genes are evolving very slowly in Lepidoptera and are likely under stringent purifying selection due to their highly important and conserved functions during organismal development.

Wnt Gene Expression During Wing Development

Expression of *Wnt* genes during wing development of *Z. cesonia* was similar and divergent from patterns described in other lepidopteran species. Fifth instar larval wing disks showed strong

expression of *Wnt1* and *Wnt6*, consistent with their known expression in lepidoptera wing peripheral tissues (Macdonald et al., 2010; Martin and Reed, 2010, 2014) as well as in the *Drosophila* wing disk dorso-ventral boundary (Janson et al., 2001). In *Z. cesonia*, both genes showed decreased, but detectable levels during the pupal stages, which may be related to color patterning in the first 2 days after puparium formation (APF). *Wnt10* is co-expressed with *Wnt1* and *Wnt6* in *D. guttifera* and in the nymphalid *E. chalcodon* (Martin and Reed, 2014; Koshikawa et al., 2015), but was not recovered in our pierid dataset. *Wnt7* was expressed in both the larval stage and the first pupal day, as previously reported in two *Heliconius* species (Hanly, 2017). Following this initial peak, expression sharply decreased peaked again later during pupal development starting at the day 2 stage before rising again shortly at 5–6 days APF. *Wnt11* has been associated with Wnt/JNK PCP pathway in mammals (Uysal-Onganer and Kypta, 2012), and its expression was detected during mid-pupal stages when scale cytoskeletal structures are forming. However, *Wnt11* transcript abundance was at least twofold lower than any other *Wnt* gene suggesting expression may be very spatially and/or time restricted during wing development (Figure 2). Most noteworthy, is the lack of *WntA* expression, which is involved in wing pattern development among all the nymphalid butterflies assessed so far (Mazo-Vargas et al., 2017). For those *Wnt* genes not detected from the wing RNA-seq data, we also surveyed RNA-seq data from adult head and thorax previously generated to help annotate the *Z. cesonia* genome, and we were able to confirm expression of *Wnt5*, *Wnt10*, and *WntA*, but not *Wnt9*, suggesting the expression of *Wnt9* is restricted to specific tissues and stages as hinted from other studies (Bolognesi et al., 2008; Murat et al., 2010; Chen et al., 2016).

Pharmaceutical Assays Implicate Morphogens in Wing Pattern Development

Early studies using the pharmaceutical Heparin, which can bind to *Wnt* ligands and facilitate their secretion and transport, induced a possible *Wnt* gain-of-function effect in butterfly wing patterns. These Heparin injection individuals expressed phenotypes that exhibited an extension of the basal, discal, and external symmetry systems (Serfas and Carroll, 2005). Pupal injection of Heparin in coliadinae butterflies produced melanic expansions that were reminiscent of the effects of Heparin in other butterflies (Serfas and Carroll, 2005; Martin et al., 2012; Sourakov, 2018). Heparin caused the apical melanic wing region to expand broadly across dorsal forewings in *Z. cesonia* females. In males, dorsal forewings showed only moderate levels of melanization but underwent instead a complete loss of the UV patterns. The dorsal hindwing melanic border was expanded in females, but unaffected in males. Both males and females exhibited ectopic expression of melanin patterning on the ventral fore and hindwings, with particularly strong melanic coloration on the apical region of the forewing. A similar trend of forewing melanin expansion was observed in *C. eurytheme* Heparin injected female individuals (Figure 3). Only a single

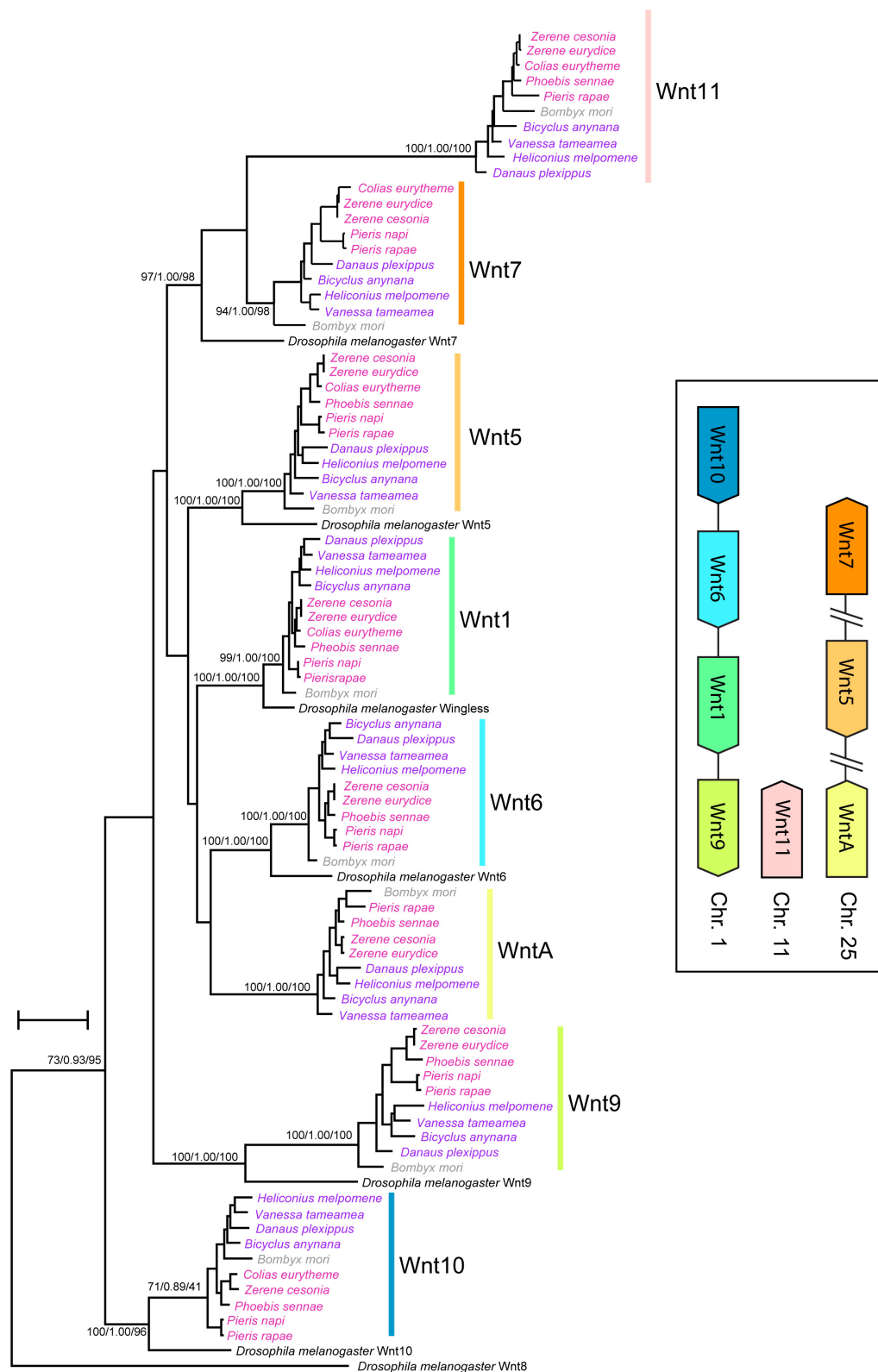


FIGURE 1 | *Wnt* gene content and synteny in butterfly genomes. Phylogenetic analyses reconstruct clades for each of eight *Wnt* genes present in lepidoptera genomes. Inset shows chromosomal linkage and order of *Wnt* genes in *Z. cesononia* genome assembly (**Supplementary Table S1**), which is known to vary within clusters across Lepidoptera (Holzem et al., 2019).

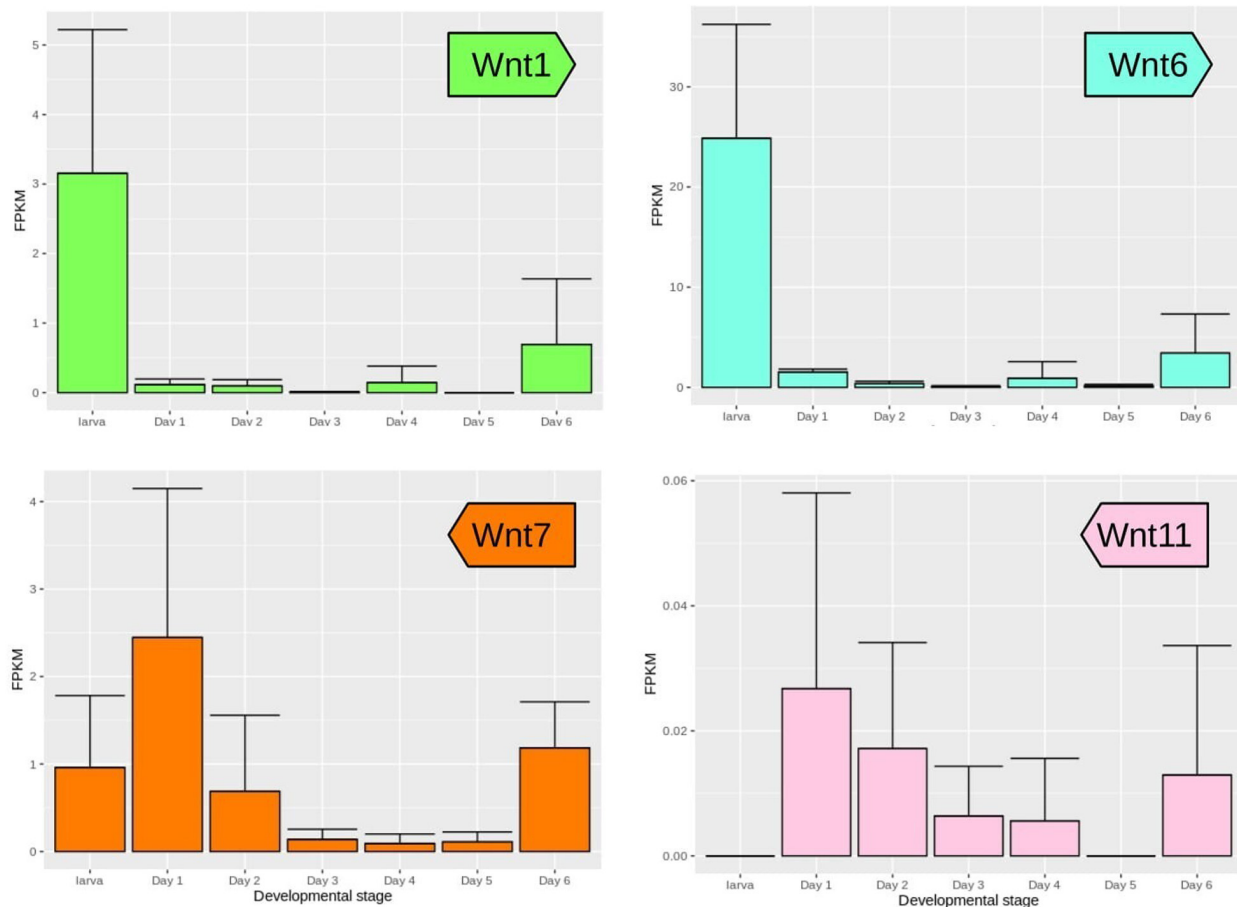


FIGURE 2 | *Wnt* gene expression during wing development of *Z. cesonina*. Mean and standard error of expression levels among replicates from larval fifth instar through pupal wing development. Note the scale of FPKM (fragments per kilobase of transcript per million mapped reads) on y-axes differ among genes, due to large differences in expression levels.

male *C. eurytheme* injected with Heparin survived, but did not eclose properly, therefore we were unable to assay Heparin's impact on UV reflectance in *Colias*. These experiments show that Heparin will perturb both pigment and structural coloration in Coliadinae butterflies in a manner similar to nymphalids, despite the lack of *WntA* expression. They also reveal sexually dimorphic effects of heparin that were unexpected. It is plausible that those differences highlight intrinsic divergence in the female and male patterning architecture, a hypothesis that will require further investigation but establish *Z. cesonina* as an interesting model system for developmental studies of sex-specific color patterning.

Inspection of scales on *Z. cesonina* wings revealed broad organizational and scale shape changes (Figure 3). In the expanded melanic region of Heparin injected individuals, the melanic scales had their characteristic shapes and structures, in place of yellow scales that would typically be present. Heparin injected males that lost UV patterning did develop cover scales that appear to have the tightly organized and stacked lamellae ridges responsible for reflecting UV. However, these scales had highly reduced widths, exposing much of the ground scales that lack the UV-reflecting structures. The result was a weakly

reflective UV patch on the forewings of Heparin injected male *Z. cesonina* (Figure 3). Scale size was highly reduced among Heparin treated individuals across multiple wing regions and both sexes (Figure 3B), suggesting that the Heparin had a systemic impact on scale development across the wings. This microscopic inspection of the impacts of Heparin demonstrates clear changes in scale structure that had not been previously documented in nymphalids.

Microscopic examination of Heparin treated *Heliconius* butterflies (Family: Nymphalidae) did not reveal the systemic scale reduction observed across the wings of *Z. cesonina*, suggesting that Heparin may affect scale development differently in the two families of butterflies. In *H. sara*, Heparin induced changes in both pigment and scale structure across the wing, with a complete loss of yellow scales. The black and yellow scales of *H. sara* are distinguishable by the shape and organization of their lamellar ridges, as seen at 5000X in Figure 4. SEM imaging revealed that Heparin induced melanic scales (Figure 4) had the expected ridge architecture of wildtype melanic scales. Similar changes in both pigment and scale structure were also observed in the CRISPR-edits of *WntA* among *Heliconius*

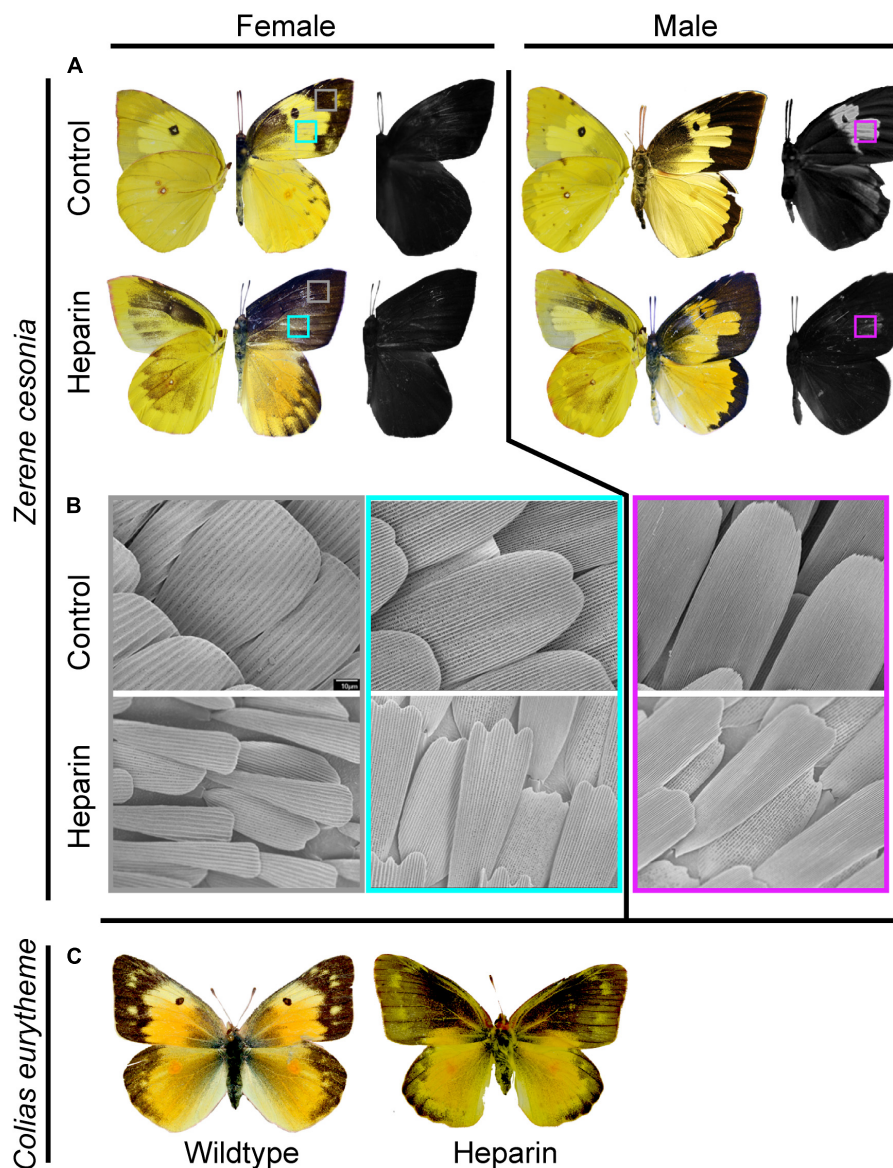


FIGURE 3 | Heparin impacts pigment and structurally based colors on *Z. cesonia* wings. **(A)** Heparin treated female *Z. cesonia* showed an increase in melanization across wings and Heparin treated males lost UV reflectance. Left side wings show ventral side and right side wings show dorsal. **(B)** SEM imaging (1000X) of scales from control and Heparin treated wings shows difference in scale morphology and organization. **(C)** Female *C. eurytheme* dorsal wings showed melanistic expansions with Heparin treatments.

butterflies (Concha et al., 2019), although the mutant color patterns typically altered in more narrow wing regions, since heparin is thought to also affect other Wnt ligands (Martin and Reed, 2014; Mazo-Vargas et al., 2017). In *Z. cesonia*, we did not see a concordant change in pigment and structure across the wing. Instead, melanic patterns expanded as expected and scale structures were altered across all color of scales (Figure 3). The melanic scales of Heparin treated female wings had an ultrastructure that was distinct from both black and yellow scales of control wings, with triple pronged leading edges that are not commonly found across *Z. cesonia* wings (Figure 3, blue box).

It is worth noting the large difference in Heparin dosage among nymphalid and pierid butterfly species; for example, 5 μg of Heparin was required to observe wing pattern changes in *Heliconius*, while only 0.5 μg was used in *Z. cesonia*, and higher dosage always resulted in pupal mortality ($n = 25$). This suggests that pierids may have a more sensitive response to Heparin, possibly due to Heparin binding to different ligands in *Z. cesonia* than in *H. sara*. Collectively, these Heparin results show that although Heparin appeared to produced similar wing pattern phenotypes across butterfly families, the effects of Heparin on scale development and dosage responses differ dramatically. Collectively, these findings suggest that targets of Heparin impact

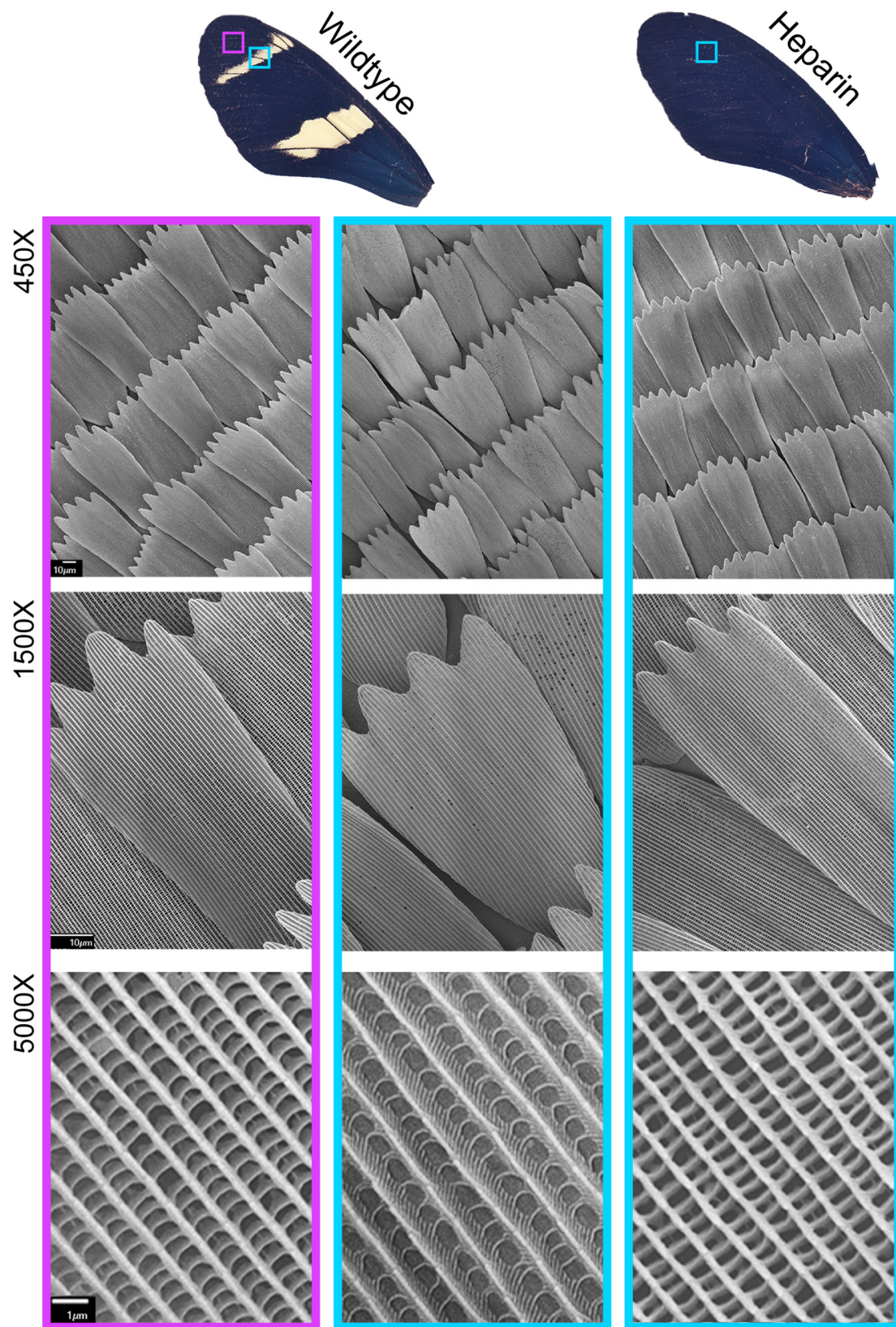


FIGURE 4 | Heparin impacts both scale pigmentation and structure in *H. sara*. Wings from Martin et al. (2012). Heparin injections result in yellow-to-blue/black scale color shifts, and SEM imaging reveals concordant changes in ultrastructure (5000X).

both scale pigmentation and structure across nymphalid and pierid butterfly wings, but also indicate that pierids may use a different set of Wnts or other signaling molecules to pre-pattern wing coloration.

DISCUSSION

Wnts in Butterfly Wing Pattern: Conservation and Divergence

Wnt gene content, clustering, and amino acid sequence appear to be highly conserved among pierids, as well as other Lepidoptera. However, the deployment of these genes appears to be highly divergent among different butterfly lineages. Interestingly several *Wnt* genes displayed divergent expressions between developing wings of nymphalid and pierid butterflies. The expression profiles for *Wnt* genes in *Z. cesonia* clearly showed that they do not deploy the same *Wnt* genes as nymphalids in wing pattern development.

Most surprising was the lack of *WntA* expression during wing development in *Z. cesonia*, despite its importance in wing pattern development of nymphalid butterflies (Mazo-Vargas et al., 2017; Concha et al., 2019). In nymphalids, *WntA* expression in larval wing discs prefigures adult wing elements and diminishes during the first days AFP (Martin et al., 2012; Martin and Reed, 2014). *Z. cesonia* completely lacked *WntA* expression, suggesting that this pierid lacks the *WntA*-positive stripe system of nymphalids called the Central Symmetry System (CSS). Of note, classic work in comparative morphology has established that this pattern is divergent and unusually reduced in Pieridae (Schwanwitsch, 1956) and it will be interesting to further investigate if *WntA* expression has been completely lost in additional pierids to test for generalization. It is possible that *WntA* may be expressed in levels too low to detect in the *Z. cesonia* transcriptome, with an average of 18.9 million reads per library, but if so such low levels of expression, it is unlikely that *WntA* would be serving the same vital function in *Z. cesonia* as it is in nymphalid butterflies where it is more highly expressed.

Differences in *Wnt* gene deployment during wing development among lepidoptera lineages likely result from changes in regulatory regions of the *Wnt* genes. Across Lepidoptera, there is evidence of strong purifying selection on *Wnt* gene amino acid sequences, demonstrating that both *Wnt* gene content and sequences are highly conserved (Martin et al., 2012; Mazo-Vargas et al., 2017; Holzem et al., 2019). In *Heliconius* butterflies, differences in the spatial expression of *WntA* across developing wings are associated with genetic changes in non-coding regions near *WntA*. Van Belleghem et al. (2017) speculate that these reflect putative *cis*-regulatory elements (CREs) that are responsible for initiating *WntA* expression in narrow wing regions that correspond to pattern elements in the adult wings. Similarly, Van Belleghem et al. identified CREs near the gene *optix* that were associated with red color patterning in *Heliconius*, which (Lewis et al., 2019) recently used CRISPR to edit the CREs and demonstrate their function in red pattern development. We suggest that evolution of CREs near the *Wnt* gene clusters is likely responsible for differences in *Wnt* gene deployment and pattern divergence of lepidoptera wings.

Wnt Pathway Involved in Structural Coloration

Heparin treatments consistently lead to an expansion of melanism across butterfly wings. However, if Heparin is impacting melanism via the Wnt pathway, it is likely affecting different Wnt ligands in different butterfly species. In nymphalid butterflies, *WntA* has been shown to be spatially expressed in wing regions that melanism expands and is a likely candidate target of Heparin treatments. However, in *Z. cesonia* wing discs, *WntA* is not expressed, and at the time of Heparin injections only *Wnt1*, *Wnt6*, and possibly *Wnt7* ligands appear to be present in wing tissue. Also different from nymphalid butterflies, the expansion of melanism was greatest in the anterior portion of the wing in *Z. cesonia* and *C. eurytheme*, where in nymphalids, melanic expansion largely occurred in elements of the CSS of the wing (Martin et al., 2012). Lastly, we note that the melanic response to Heparin was sex dependent, with females exhibiting greater expansions than males. This sexual dimorphism has not been observed in nymphalids; however, in species such as *Heliconius*, sexual dimorphism in wing color pattern is rare. Among pierids, wing color patterns are often sexually dimorphic, most notably in UV reflective patterns which are often present in males, but not females, as is found in *Z. cesonia* (Silberglied and Taylor, 1973; Stella et al., 2018; Fenner et al., 2019). Interestingly, in *Drosophila* *Wnt7* expression is sexually dimorphic and regulated by *doublesex* during embryonic gonad development of male specific pigment cells (Deshpande et al., 2016). Collectively, the gene expression and Heparin treatment results suggest that *Wnt7* could be a candidate ligand involved in sexually dimorphic wing development in a pierid butterfly.

Previous studies using Heparin assays have largely focused on pattern level changes across the wing, here we show how Heparin impacts the development of the wing scales. The Heparin assays suggest that the Wnt pathway may play a role in the development of structural as well as pigment coloration. Although much is known about the genes responsible for pigment colors such as melanin, little is known about the pathways responsible for generating structural colors (Zhang et al., 2017). Here we show that males lose the ability to produce their UV patterns, but not due to cell fate changes, but do to overall scale shape changes. In nymphalid butterflies, *WntA* has recently been implemented as a modulator of both scale structure and color (Concha et al., 2019). Since *WntA* is not expressed in *Z. cesonia*, but both scale and pattern phenotypes were altered, this suggests that the Wnt pathway in general may play an important role in the development of both scale coloration and overall structure.

Multiple Pathways Involved in Wing Pattern Development

To date, there are at least three different Wnt signaling pathways: the canonical Wnt/beta pathway, the non-canonical Wnt/JNK (Wnt/PCP), and the Wnt/Ca²⁺ pathway (Nusse, 2012). In the last decade, it has become evident that two or more of these Wnt signaling pathways often signal within the same cell or

territories to influence cell fate (Range et al., 2013; Range, 2018; Martínez-Bartolomé and Range, 2019). Here we characterized the expression of four *Wnt* genes in *Z. cesonia* throughout pupal wing development. As stated above, our Heparin injections showed perturbations in both scale color, which is likely due to transcriptional regulation, and scale structure, which is due to subcellular changes in the actin-myosin cytoskeleton. In many cases, the non-canonical Wnt signaling pathways are involved in morphogenetic processes; therefore, it is possible that multiple Wnt pathways are activated during wing development. We realize that individual Wnts can activate canonical and/or non-canonical pathways depending on the cellular context; it is interesting to note that *Wnt11* has been shown to activate the Wnt/JNK pathway in *Drosophila* development (Uysal-Onganer and Kypta, 2012). Although the expression of *Wnt11* is low during wing development in *Z. cesonia*, its peak expression occurs during days 2 and 3 when scale formation is taking place. On the other hand, *Wnt7* expression during wing development, like *WntA*, has only been seen in butterfly wings and thus the Wnt pathways they may initiate remain unresolved. Together, the handful of *Wnt* genes expressed suggest that multiple Wnt pathways could be concordantly regulating various aspects of wing development. Further characterization needs to be conducted in order to address which Wnt pathways are deployed during wing development in *Z. cesonia*.

In addition, other signaling pathways are likely involved and may contribute to the heparin-induced wing changes. Heparin binding is not limited to the Wnt family and impact the diffusion of other signaling genes, such as *fibroblast growth factor* (*FGF*), *Hedgehog* (*Hh*), and *transforming growth factor- β* (*TGF β*) families. In *Z. cesonia* *Hh*, *TGF- β activated kinase 1* (*Tak1*), *branchless* (*FGF* family), and *decapentaplegic* (*dpp*) (*FGF* family) are all expressed during wing development and could have been impacted by heparin treatments. In *Junonia* butterflies, *Hh* has been implicated in eyespot pattern development (Tong et al., 2012); however, the morphogens *Tak1*, *branchless*, and *dpp* have yet to be investigated in another butterfly species. Therefore, although *Wnt* genes have played a major role in Nymphalid wing pattern development and diversification (Concha et al., 2019), the results of this paper suggest that Pierids express *Wnts* differently and further characterization is needed for other families of morphogens that may be involved in wing patterning.

REFERENCES

- Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990). Basic local alignment search tool. *J. Mol. Biol.* 215, 403–410. doi: 10.1016/s0022-2836(05)80360-2
- Anisimova, M., Gil, M., Dufayard, J.-F., Dessimoz, C., and Gascuel, O. (2011). Survey of branch support methods demonstrates accuracy, power, and robustness of fast likelihood-based approximation schemes. *Syst. Biol.* 60, 685–699. doi: 10.1093/sysbio/syr041
- Baker, N. E. (1987). Molecular cloning of sequences from wingless, a segment polarity gene in *Drosophila*: the spatial distribution of a transcript in embryos. *EMBO J.* 6, 1765–1773. doi: 10.1002/j.1460-2075.1987.tb02429.x
- Baker, N. E. (1988). Transcription of the segment-polarity gene wingless in the imaginal discs of *Drosophila*, and the phenotype of a pupal-lethal wg mutation. *Development* 102, 489–497.

DATA AVAILABILITY STATEMENT

The names of the repository/repositories and accession number(s) can be found at: NCBI [BIOPROJECT PRJNA587792], GitHub [https://github.com/bcount/Colias10X].

AUTHOR CONTRIBUTIONS

JF and BC designed the study, conducted the pharmaceutical assays, and wrote the manuscript draft. JF collected microscopy and wing image data and prepared RNA-seq libraries. CB and LR-C conducted RNA-seq analyses. RP provided sequencing of the RNA-seq libraries. AR conducted pharmaceutical assays for *C. eurytheme*. FH conducted phylogenetic and molecular evolution analyses. AM and RR contributed to experimental design and data interpretation. All authors contributed to preparation and editing of the manuscript.

FUNDING

This research was funded by the National Science Foundation awards IOS-1755329 to AM and BC, OIA-1736026 to RP, FH, RR, and BC, IOS-1656389 to RP and AM, and Texas Ecolabs awards to BC.

ACKNOWLEDGMENTS

We thank Jared Cole for assistance with RNA-seq library preparation, and Danilo Gusmao Ferreira Souto for assistance with Heparin injections and butterfly rearing. We thank the MSU I²AT for technical assistance with SEM. We also thank the University of Puerto Rico, Rio Piedras Sequencing and Genomics Facility, and the UPR High Performance Computing Facility for assistance with the generation and analyses of RNA-seq data.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fevo.2020.00197/full#supplementary-material>

- Bolger, A. M., Lohse, M., and Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 30, 2114–2120. doi: 10.1093/bioinformatics/btu170
- Bolognesi, R., Beermann, A., Farzana, L., Wittkopp, N., Lutz, R., Balavoine, G., et al. (2008). *Tribolium* Wnts: evidence for a larger repertoire in insects with overlapping expression patterns that suggest multiple redundant functions in embryogenesis. *Dev. Genes Evol.* 218, 193–202. doi: 10.1007/s00427-007-0170-3
- Challi, R. J., Kumar, S., Dasmahapatra, K. K., Jiggins, C. D., and Blaxter, M. (2016). Lepbase: the Lepidopteran genome database. *bioRxiv* [Preprint]. doi: 10.1101/056994
- Chen, S., Huang, T., Zhou, Y., Han, Y., Xu, M., and Gu, J. (2017). AfterQC: automatic filtering, trimming, error removing and quality control for fastq data. *BMC Bioinformatics* 18:80. doi: 10.1186/s12859-017-1469-3

- Chen, Z., Zhu, J. Y., Fu, Y., Richman, A., and Han, Z. (2016). Wnt4 is required for ostia development in the *Drosophila* heart. *Dev. Biol.* 413, 188–198. doi: 10.1016/j.ydbio.2016.03.008
- Cohen, E. D., Mariol, M.-C., Wallace, R. M. H., Weyers, J., Kamberov, Y. G., Pradel, J., et al. (2002). DWnt4 regulates cell movement and focal adhesion kinase during *Drosophila* ovarian morphogenesis. *Dev. Cell* 2, 437–448. doi: 10.1016/s1534-5807(02)00142-9
- Concha, C., Wallbank, R. W. R., Hanly, J. J., Fenner, J., Livraghi, L., Rivera, E. S., et al. (2019). Interplay between developmental flexibility and determinism in the evolution of mimetic heliconius wing patterns. *Curr. Biol.* 23, 3996.e4–4009.e4. doi: 10.1016/j.cub.2019.10.010
- Deshpande, G., Nouri, A., and Schedl, P. (2016). Wnt signaling in sexual dimorphism. *Genetics* 202, 661–673. doi: 10.1534/genetics.115.177857
- Ding, X., Liu, J., Zheng, L., Song, J., Li, N., Hu, H., et al. (2019). Genome-wide identification and expression profiling of wnt family genes in the silkworm, *Bombyx mori*. *Int. J. Mol. Sci.* 20:1221. doi: 10.3390/ijms20051221
- Edayoshi, M., Yamaguchi, J., and Fujiwara, H. (2015). Protruding structures on caterpillars are controlled by ectopic Wnt1 expression. *PLoS One* 10:e0121736. doi: 10.1371/journal.pone.0121736
- Edgar, R. C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* 32, 1792–1797. doi: 10.1093/nar/gkh340
- Fenner, J., Rodriguez-Caro, L., and Counterman, B. (2019). Plasticity and divergence in ultraviolet reflecting structures on Dogface butterfly wings. *Arthropod. Struct. Dev.* 51, 14–22. doi: 10.1016/j.asd.2019.06.001
- Gallant, J. R., Imhoff, V. E., Martin, A., Savage, W. K., Chamberlain, N. L., Pote, B. L., et al. (2014). Ancient homology underlies adaptive mimetic diversity across butterflies. *Nat. Commun.* 5, 1–10.
- Goldman, N., and Yang, Z. (1994). A codon-based model of nucleotide substitution for protein-coding DNA sequences. *Mol. Biol. Evol.* 11, 725–736.
- Hanly, J. (2017). *Developmental Basis of Wing Pattern Diversity in Heliconius Butterflies*. Doctoral thesis, Cambridge University, Cambridge.
- Hanly, J. J., Wallbank, R. W. R., Owen McMillan, W., and Jiggins, C. D. (2019). Conservation and flexibility in the gene regulatory landscape of heliconiine butterfly wings. *EvoDevo* 10:15. doi: 10.1186/s13227-019-0127-4
- Hoang, D. T., Vinh, L. S., Flouri, T., Stamatakis, A., von Haeseler, A., and Minh, B. Q. (2018). MPBoot: fast phylogenetic maximum parsimony tree inference and bootstrap approximation. *BMC Evol. Biol.* 18:11. doi: 10.1186/s12862-018-1131-3
- Holzem, M., Braak, N., Brattström, O., McGregor, A. P., and Breuker, C. J. (2019). Wnt gene expression during early embryogenesis in the nymphalid butterfly *Bicyclus anynana*. *Front. Ecol. Evol.* 7:468. doi: 10.3389/fevo.2019.00468
- Howe, K. L., Contreras-Moreira, B., De Silva, N., Maslen, G., Akanni, W., Allen, J., et al. (2020). Ensembl Genomes 2020-enabling non-vertebrate genomic research. *Nucleic Acids Res.* 48, D689–D695.
- Janson, K., Cohen, E. D., and Wilder, E. L. (2001). Expression of DWnt6, DWnt10, and DFz4 during *Drosophila* development. *Mech. Dev.* 103, 117–120. doi: 10.1016/s0925-4773(01)00323-9
- Kalyaanamoorthy, S., Minh, B. Q., Wong, T. K. F., von Haeseler, A., and Jermin, L. S. (2017). ModelFinder: fast model selection for accurate phylogenetic estimates. *Nat. Methods* 14, 587–589. doi: 10.1038/nmeth.4285
- Kawahara, A. Y., Plotkin, D., Espeland, M., Meusemann, K., Toussaint, E. F. A., Donath, A., et al. (2019). Phylogenomics reveals the evolutionary timing and pattern of butterflies and moths. *Proc. Natl. Acad. Sci. U.S.A.* 116, 22657–22663. doi: 10.1073/pnas.1907847116
- Koshikawa, S., Giorgianni, M. W., Vaccaro, K., Kassner, V. A., Yoder, J. H., Werner, T., et al. (2015). Gain of cis-regulatory activities underlies novel domains of wingless gene expression in *Drosophila*. *Proc. Natl. Acad. Sci. U.S.A.* 112, 7524–7529. doi: 10.1073/pnas.1509022112
- Kumar, S., Stecher, G., Li, M., Knyaz, C., and Tamura, K. (2018). MEGA X: molecular evolutionary genetics analysis across computing platforms. *Mol. Biol. Evol.* 35, 1547–1549. doi: 10.1093/molbev/msy096
- Kurtz, S., Phillippy, A., Delcher, A. L., Smoot, M., Shumway, M., Antonescu, C., et al. (2004). Versatile and open software for comparing large genomes. *Genome Biol.* 5:R12.
- Lewis, J. J., Geltman, R. C., Pollak, P. C., Rondem, K. E., Van Belleghem, S. M., Hubisz, M. J., et al. (2019). Parallel evolution of ancient, pleiotropic enhancers underlies butterfly wing pattern mimicry. *Proc. Natl. Acad. Sci. U.S.A.* 116, 24174–24183. doi: 10.1073/pnas.1907068116
- Li, B., and Dewey, C. N. (2011). RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics* 12:323. doi: 10.1186/1471-2105-12-323
- Macdonald, W. P., Martin, A., and Reed, R. D. (2010). Butterfly wings shaped by a molecular cookie cutter: evolutionary radiation of lepidopteran wing shapes associated with a derived Cut/wingless wing margin boundary system. *Evol. Dev.* 12, 296–304. doi: 10.1111/j.1525-142x.2010.00415.x
- Martin, A., Papa, R., Nadeau, N. J., Hill, R. I., Counterman, B. A., Halder, G., et al. (2012). Diversification of complex butterfly wing patterns by repeated regulatory evolution of a Wnt ligand. *Proc. Natl. Acad. Sci. U.S.A.* 109, 12632–12637. doi: 10.1073/pnas.1204800109
- Martin, A., and Reed, R. D. (2010). Wingless and aristaless2 define a developmental ground plan for moth and butterfly wing pattern evolution. *Mol. Biol. Evol.* 27, 2864–2878. doi: 10.1093/molbev/msq173
- Martin, A., and Reed, R. D. (2014). Wnt signaling underlies evolution and development of the butterfly wing pattern symmetry systems. *Dev. Biol.* 395, 367–378. doi: 10.1016/j.ydbio.2014.08.031
- Martínez-Bartolomé, M., and Range, R. C. (2019). A biphasic role of non-canonical Wnt16 signaling during early anterior-posterior patterning and morphogenesis of the sea urchin embryo. *Development* 146:dev168799. doi: 10.1242/dev.168799
- Mazo-Vargas, A., Concha, C., Livraghi, L., Massardo, D., Wallbank, R. W. R., Zhang, L., et al. (2017). Macroevolutionary shifts of WntA function potentiate butterfly wing-pattern diversity. *Proc. Natl. Acad. Sci. U.S.A.* 114, 10701–10706. doi: 10.1073/pnas.1708149114
- Murat, S., Hopfen, C., and McGregor, A. P. (2010). The function and evolution of Wnt genes in arthropods. *Arthropod. Struct. Dev.* 39, 446–452. doi: 10.1016/j.asd.2010.05.007
- Nguyen, L.-T., Schmidt, H. A., von Haeseler, A., and Minh, B. Q. (2015). IQ-TREE: a fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. *Mol. Biol. Evol.* 32, 268–274. doi: 10.1093/molbev/msu300
- Nusse, R. (2012). Wnt signaling. *Cold Spring Harb. Perspect. Biol.* 4:a011163. doi: 10.1101/cshperspect.a011163
- Papke, R., Darrell, S., Kemp, J., and Rutowski, R. L. (2007). Multimodal signalling: structural ultraviolet reflectance predicts male mating success better than pheromones in the butterfly *Colias eurytheme* L. (Pieridae). *Anim. Behav.* 73, 47–54. doi: 10.1016/j.anbehav.2006.07.004
- Picelli, S., Björklund, A. K., Reinius, B., Sagasser, S., Winberg, G., and Sandberg, R. (2014). Tn5 transposase and tagmentation procedures for massively scaled sequencing projects. *Genome Res.* 24, 2033–2040. doi: 10.1101/gr.177881.114
- Range, R. C. (2018). Canonical and non-canonical Wnt signaling pathways define the expression domains of Frizzled 5/8 and Frizzled 1/2/7 along the early anterior-posterior axis in sea urchin embryos. *Dev. Biol.* 444, 83–92. doi: 10.1016/j.ydbio.2018.10.003
- Range, R. C., Angerer, R. C., and Angerer, L. M. (2013). Integration of canonical and noncanonical Wnt signaling pathways patterns the neuroectoderm along the anterior-posterior axis of sea urchin embryos. *PLoS Biol.* 11:e1001467. doi: 10.1371/journal.pbio.1001467
- Rao, T. P., and Kühl, M. (2010). An updated overview on Wnt signaling pathways: a prelude for more. *Circ. Res.* 106, 1798–1806. doi: 10.1161/circresaha.110.219840
- Rodriguez-Caro, L., Fenner, J., Benson, C., Van Belleghem, S. M., and Counterman, B. A. (2020). Genome assembly of the dogface butterfly *Zerene cesonia*. *Genome Biol. Evol.* 12, 3580–3585. doi: 10.1093/gbe/evz254
- Schwanwitsch, B. N. (1956). Wing pattern of pierid butterflies (Pieridae, Lepidoptera), Ent Obozr, Moscow. *Entomol. Obozrenie* 35, 285–301.
- Serfas, M. S., and Carroll, S. B. (2005). Pharmacologic approaches to butterfly wing patterning: sulfated polysaccharides mimic or antagonize cold shock and alter the interpretation of gradients of positional information. *Dev. Biol.* 287, 416–424. doi: 10.1016/j.ydbio.2005.09.014
- Shelby, E. A., Fenner, J., and Counterman, B. A. (2020). Impact of artificial diet on development and coloration in *Zerene cesonia*. *J. Lepid. Soc.* 74, 65–72. doi: 10.18473/lepi.74i2.a1
- Silberglied, R. E., and Taylor, O. R. (1973). Ultraviolet differences between the sulphur butterflies, *Colias eurytheme* and *C. philodice*, and a possible isolating mechanism. *Nature* 241, 406–408. doi: 10.1038/241406a0
- Somorjai, I. M. L., Martí-Solans, J., Diaz-Gracia, M., Nishida, H., Imai, K. S., Escrivá, H., et al. (2018). Wnt evolution and function shuffling in liberal and conservative chordate genomes. *Genome Biol.* 19:98.

- Sourakov, A. (2018). Scientific Note: The Emperor's new clothes: radical transformation of the wing pattern in *Asterocampa clyton* caused by heparin. *Trop. Lepid. Res.* 28, 29–31.
- Stella, D., Faltýnek Fric, Z., Rindoš, M., Kleisner, K., and Pecháček, P. (2018). Distribution of ultraviolet ornaments in colias butterflies (Lepidoptera: Pieridae). *Environ. Entomol.* 47, 1344–1354. doi: 10.1093/ee/nvy111
- Tomoyasu, Y., Arakane, Y., Kramer, K. J., and Denell, R. E. (2009). Repeated co-options of exoskeleton formation during wing-to-elytron evolution in beetles. *Curr. Biol.* 19, 2057–2065. doi: 10.1016/j.cub.2009.11.014
- Tong, X., Lindemann, A., and Monteiro, A. (2012). Differential involvement of Hedgehog signaling in butterfly wing and eyespot development. *PLoS One* 7:e51087. doi: 10.1371/journal.pone.0051087
- Trifinopoulos, J., Nguyen, L.-T., von Haeseler, A., and Minh, B. Q. (2016). W-IQ-TREE: a fast online phylogenetic tool for maximum likelihood analysis. *Nucleic Acids Res.* 44, W232–W235. doi: 10.1093/nar/gkw256
- Uysal-Onganer, P., and Kypta, R. M. (2012). Wnt11 in 2011—the regulation and function of a non-canonical Wnt. *Acta Physiol.* 204, 52–64. doi: 10.1111/j.1748-1716.2011.02297.x
- Van Belleghem, S. M., Rastas, P., Papanicolaou, A., Martin, S. H., Arias, C. F., Supple, M. A., et al. (2017). Complex modular architecture around a simple toolkit of wing pattern genes. *Nat. Ecol. Evol.* 1:52.
- Weisenfeld, N. I., Kumar, V., Shah, P., Church, D. M., and Jaffe, D. B. (2017). Direct determination of diploid genome sequences. *Genome Res.* 27, 757–767. doi: 10.1101/gr.214874.116
- Yang, Z. (2007). PAML 4: phylogenetic analysis by maximum likelihood. *Mol. Biol. Evol.* 24, 1586–1591. doi: 10.1093/molbev/msm088
- Zhang, L., Martin, A., Perry, M. W., van der Burg, K. R. L., Matsuoka, Y., Monteiro, A., et al. (2017). Genetic basis of melanin pigmentation in butterfly wings. *Genetics* 205, 1537–1550. doi: 10.1534/genetics.116.196451
- Zhang, Z., Aslam, A. F. M., Liu, X., Li, M., Huang, Y., and Tan, A. (2015). Functional analysis of *Bombyx* Wnt1 during embryogenesis using the CRISPR/Cas9 system. *J. Insect Physiol.* 79, 73–79. doi: 10.1016/j.jinsphys.2015.06.004

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2020 Fenner, Benson, Rodriguez-Caro, Ren, Papa, Martin, Hoffmann, Range and Counterman. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



ebony Affects Pigmentation Divergence and Cuticular Hydrocarbons in *Drosophila americana* and *D. novamexicana*

Abigail M. Lamb¹, Zinan Wang^{2,3}, Patricia Simmer¹, Henry Chung^{2,3} and Patricia J. Wittkopp^{1,4*}

¹ Department of Molecular, Cellular, and Developmental Biology, University of Michigan, Ann Arbor, MI, United States,

² Department of Entomology, Michigan State University, East Lansing, MI, United States, ³ Ecology, Evolutionary Biology, and Behavior Program, Michigan State University, East Lansing, MI, United States, ⁴ Department of Ecology and Evolutionary Biology, University of Michigan, Ann Arbor, MI, United States

OPEN ACCESS

Edited by:

Ricardo Mallarino,
Princeton University, United States

Reviewed by:

Daniel Matute,
The University of North Carolina
at Chapel Hill, United States
Nicolas Haupaix,
Collège de France, France

*Correspondence:

Patricia J. Wittkopp
wittkopp@umich.edu

Specialty section:

This article was submitted to
Evolutionary Developmental Biology,
a section of the journal
Frontiers in Ecology and Evolution

Received: 27 February 2020

Accepted: 25 May 2020

Published: 30 June 2020

Citation:

Lamb AM, Wang Z, Simmer P,
Chung H and Wittkopp PJ (2020)
ebony Affects Pigmentation
Divergence and Cuticular
Hydrocarbons in *Drosophila*
americana and *D. novamexicana*.
Front. Ecol. Evol. 8:184.
doi: 10.3389/fevo.2020.00184

Drosophila pigmentation has been a fruitful model system for understanding the genetic and developmental mechanisms underlying phenotypic evolution. For example, prior work has shown that divergence of the *tan* gene contributes to pigmentation differences between two members of the virilis group: *Drosophila novamexicana*, which has a light yellow body color, and *D. americana*, which has a dark brown body color. Quantitative trait locus (QTL) mapping and expression analysis has suggested that divergence of the *ebony* gene might also contribute to pigmentation differences between these two species. Here, we directly test this hypothesis by using CRISPR/Cas9 genome editing to generate *ebony* null mutants in *D. americana* and *D. novamexicana* and then using reciprocal hemizyosity testing to compare the effects of each species' *ebony* allele on pigmentation. We find that divergence of *ebony* does indeed contribute to the pigmentation divergence between species, with effects on both the overall body color as well as a difference in pigmentation along the dorsal abdominal midline. Motivated by recent work in *D. melanogaster*, we also used the *ebony* null mutants to test for effects of *ebony* on cuticular hydrocarbon (CHC) profiles. We found that *ebony* affects CHC abundance in both species, but does not contribute to qualitative differences in the CHC profiles between these two species. Additional transgenic resources for working with *D. americana* and *D. novamexicana*, such as *white* mutants of both species and *yellow* mutants in *D. novamexicana*, were generated in the course of this work and are also described. Taken together, this study advances our understanding of loci contributing to phenotypic divergence and illustrates how the latest genome editing tools can be used for functional testing in non-model species.

Keywords: CRISPR, Cas9, melanin, nanos, abdominal pigmentation, genome editing, virilis group

INTRODUCTION

Insect pigmentation is a well-studied trait that displays a variety of phenotypic differences within and between species (Wittkopp et al., 2003a; Kronforst et al., 2012). These differences have evolved over a wide range of divergence times and in a great diversity of ecological contexts. Differences in insect pigmentation often appear to be ecologically relevant, correlating with geographic and climatic factors and playing a role in phenomena such as mate recognition, camouflage, thermoregulation, and water balance (True, 2003; Wittkopp and Beldade, 2009). Studies of pigmentation differences within the genus *Drosophila* have emerged as a productive model for studying the evolution of development, exploiting the diversity of phenotypes as well as genetic tools available for working with *Drosophila* and a long history of research into the genetic and biochemical mechanisms controlling pigmentation development (Wittkopp et al., 2003a; Massey and Wittkopp, 2016; Rebeiz and Williams, 2017). Indeed, since the early 2000s, the genetic bases of dozens of pigmentation differences have been identified in varying levels of detail. Strikingly, in every case where a causal role has been directly attributed to a specific gene, the mechanism of change has been found to be a *cis*-regulatory change that affects gene expression rather than a change in the protein's function (Massey and Wittkopp, 2016). These case studies have also identified multiple independent instances of divergent expression for some pigmentation genes, suggesting that these genes are particularly tractable routes for the evolution of pigmentation in this genus (Massey and Wittkopp, 2016).

Changes in *cis*-regulatory sequences are thought to be a common mechanism of developmental evolution because they tend to be less pleiotropic than changes in protein function (Wray et al., 2003; Carroll, 2005). For example, a *cis*-regulatory change might alter a gene's expression in only a single tissue or a single point in development whereas changing its protein function is expected to impact the organism everywhere that protein is expressed. Genes controlling pigmentation development in *Drosophila* might be especially likely to evolve using this mechanism because the proteins encoded by these genes are also required for other biological functions. For example, genes required for pigment synthesis have also been shown to affect mating success, circadian rhythm, vision, and innate immunity (Nappi and Christensen, 2005; True et al., 2005; Suh and Jackson, 2007; Wittkopp and Beldade, 2009; Takahashi, 2013; Massey et al., 2019a). The pigmentation biosynthesis genes *ebony* and *tan* have also been found to affect the profiles of cuticular hydrocarbons on adult flies, which are hydrophobic lipids on the surface of insect cuticle that are involved in chemical communication, mate recognition, and water balance (Chung et al., 2014; Chung and Carroll, 2015; Massey et al., 2019b).

Here, we investigate genetic changes contributing to the evolution of novel body color in *D. novamexicana*. This species has evolved a much lighter and more yellow body color than its sister species *D. americana* during the approximately 400,000 years since these species diverged from their most recent common ancestor (Figure 1; Caletka and McAllister, 2004; Morales-Hojas et al., 2008). *D. novamexicana* and *D. americana*

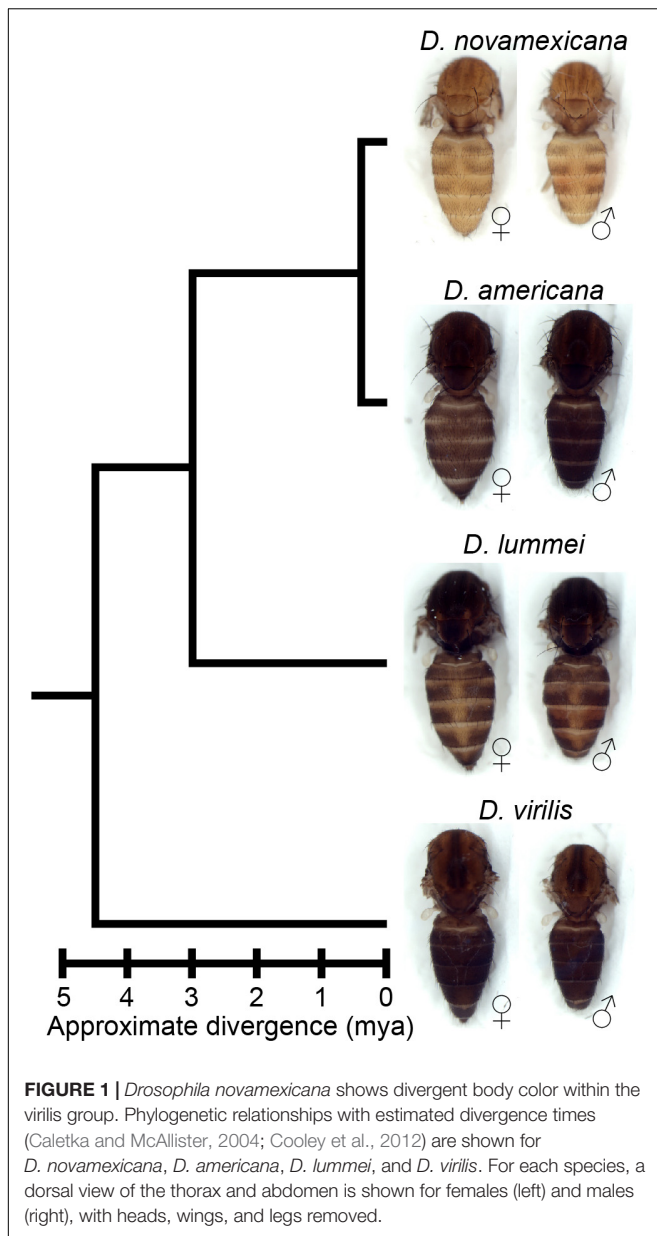
show signs of reproductive isolation (Patterson and Stone, 1949; Ahmed-Braimah and McAllister, 2012), but they are interfertile and can produce viable, fertile F₁ hybrids in the laboratory, allowing genetic analysis (Wittkopp et al., 2003b, 2009). Prior genetic mapping has identified two quantitative trait loci (QTL) that together account for ~87% of the pigmentation difference between *D. novamexicana* and *D. americana* (Wittkopp et al., 2009). Fine mapping and transgenic analysis revealed that the QTL of smaller effect was driven by divergence at *tan* (Wittkopp et al., 2009), a gene that encodes a hydrolase that catalyzes the conversion of N-B-alanyl dopamine (NBAD) to dopamine, a precursor for dark melanin pigment (True et al., 2005). The QTL of larger effect was linked to an inverted region containing the candidate gene *ebony*, but the presence of the inversion prevented fine mapping to separate the effects of *ebony* from linked loci (Wittkopp et al., 2009). *ebony* encodes a synthetase that catalyzes the conversion of dopamine into NBAD, a precursor for light yellow pigments (Koch et al., 2000), which is the opposite of the reaction catalyzed by Tan. *ebony* has also been shown to have expression differences between *D. novamexicana* and *D. americana* caused by *cis*-regulatory divergence (Cooley et al., 2012).

Despite these data suggesting that *ebony* contributes to pigmentation divergence between *D. novamexicana* and *D. americana*, the phenotypic effects of sequence divergence at *ebony* have not been demonstrated. Here, we show that divergence at *ebony* does indeed contribute to pigmentation divergence between these two species. We use CRISPR/Cas9 genome editing to mutate *ebony* in both species and use these mutant genotypes to directly test *ebony*'s contribution to pigmentation divergence through reciprocal hemizyosity testing (Stern, 2014). We find that the *D. novamexicana* *ebony* allele causes lighter pigmentation throughout the body than the *D. americana* *ebony* allele. We also find that allelic divergence at *ebony* is primarily responsible for a spatial difference in abdominal pigmentation between these species: the *D. novamexicana* *ebony* allele causes the absence of dark melanin along the dorsal midline of the abdomen seen in *D. novamexicana*. Finally, we show that *ebony* affects the cuticular hydrocarbon (CHC) profiles in *D. americana* and *D. novamexicana*, but does not contribute to the qualitative differences in CHC profiles seen between species. Taken together, our data show the power of using CRISPR/Cas9 genome editing to test functional hypotheses about evolutionary mechanisms. In addition, resources generated and lessons learned in the course of this work are expected to help other researchers perform CRISPR/Cas9 genome editing in *D. americana*, *D. novamexicana* and other *Drosophila* species.

MATERIALS AND METHODS

Fly Stocks and Husbandry

The following fly lines were used in this study: *D. americana* "A00" (National Drosophila Species Stock Center number 15010-0951.00), *D. novamexicana* "N14" (National Drosophila Species Stock Center number 15010-1031.14), *D. lummei*



(National *Drosophila* Species Stock Center number 15010-1011.08), *D. virilis* (National *Drosophila* Species Stock Center number 15010-1051.87), *D. melanogaster* $y^1 M\{w[+mC] = nos-Cas9.P\}ZH-2A w^*$ (Bloomington *Drosophila* Stock Center number 54591), and *D. melanogaster* Canton-S. All flies were reared on standard cornmeal medium at 23–25°C with a 12:12 hour light:dark cycle.

Transgenesis and CRISPR Mutant Generation in *D. americana* and *D. novamexicana*

To the best of our knowledge, prior to this work, the only trans-formation of *D. americana* or *D. novamexicana* resulted from the insertion of a piggyBac transgene (Wittkopp et al., 2009).

We therefore first used the CRISPR/Cas9 system to generate *white* mutants in both species to test the feasibility of CRISPR genome modification and to create lines that are easier to screen for common transformation markers that drive expression of fluorescent proteins or restore red pigmentation in the eyes by restoring *white* function. We successfully generated *white* mutant N14 and A00 lines used as transgenic hosts for future work, by injecting single guide RNAs (sgRNAs) targeting coding sequences in *white* conserved between *D. novamexicana* and *D. americana* in the second and third exons and screening for the loss of red eye pigment in male offspring of injected females (**Supplementary Figure 1**); *white* is on the X chromosome and thus only present in a single copy in males. These same guide RNAs were also used in *Drosophila virilis* to cut *white* and integrate an attP landing site potentially useful for site-directed transgene insertion (Lachowiec and Wittkopp, unpublished data), although the PhiC31 system does not seem to work well in *D. virilis* (Stern et al., 2017). For all CRISPR experiments, sgRNAs were *in vitro* transcribed from DNA templates using Invitrogen T7 MEGascript Transcription Kit according to protocol described by Bassett et al. (2013). Oligonucleotides used to generate sgRNAs are listed in **Supplementary Table 1**. After transcription, sgRNAs were purified using RNA Clean and Concentrator 5 kit (Zymo Research), eluted with nuclease-free water, and quantified with Qubit RNA BR Assay Kit (Thermo Fisher Scientific). For CRISPR injections, sgRNAs were mixed with purified Cas9 protein (PNA Bio #CP01) with a final injected concentration of 0.05% phenol red to visualize the injection mix. CRISPR injections were performed in-house, using previously described methods (Miller et al., 2002).

To try to increase efficiency of CRISPR mutagenesis in these species, we next sought to generate transgenic lines expressing Cas9 in the germlines of *white* mutant *D. americana* (A00) and *D. novamexicana* (N14) flies using piggyBac transgenesis (Horn and Wimmer, 2000). Based on prior reports that the *nanos* (*nos*) promoter and 3'UTR drive expression in the germline of *Drosophila virilis* (Holtzman et al., 2010), a close relative of *D. americana* and *D. novamexicana*, we amplified the *nos-Cas9-nos* transgene from the pnos-Cas9-nos plasmid (Addgene #62208; Port et al., 2014) using Phusion High Fidelity Polymerase (NEB) with tailed primers and cloned the amplicon into pBac{3XP3-ECFPafm} (Horn and Wimmer, 2000) digested with *AscI* and *Bsu36I* restriction enzymes using Gibson Assembly Master Mix (NEB). Primers are included in **Supplementary Table 1**. We confirmed the insert was correctly incorporated and free of PCR-induced errors by Sanger sequencing. We sent the *white* mutant lines of *D. americana* A00 and *D. novamexicana* N14 that we generated to Rainbow transgenic services for piggyBac transgenesis¹ and screened offspring of injected adults for expression of the enhanced cyan fluorescent protein (ECFP) in the eye using a Leica MZ6 stereoscope equipped with a Kramer Scientific Quad Fluorescence Illuminator. Transformants were obtained from injections into *D. novamexicana* (N14) (PCR verified), but not from injections into *D. americana* (A00), despite multiple attempts.

¹www.rainbowgene.com

All subsequent CRISPR injections in *D. novamexicana* were performed using flies homozygous for the *nos-Cas9-nos* transgene, some with and some without the inclusion of commercially available Cas9 protein in the injection mix. CRISPR mutants were only obtained from injections containing the commercially available Cas9 protein, however, suggesting that the *nos-Cas9-nos* transgene might not drive expression of Cas9 in the germline of *D. novamexicana*. To test this hypothesis, we used western blotting to examine Cas9 protein expression in 3 transformed *D. novamexicana* N14 lines with independent insertions of the piggyBac transgene and in a *D. melanogaster* transgenic line carrying the original *pnos-Cas9-nos* transgene (Bloomington Drosophila Stock Center line 54591, transformed with Addgene plasmid #62208, Port et al., 2014). These experiments showed that the *nos-Cas9-nos* transgene in *D. novamexicana* N14 flies does not express Cas9 protein in the ovaries (**Supplementary Figure 2**). This conclusion was further supported when injection of sgRNAs targeting the *yellow* gene into the *D. novamexicana* line carrying the *nos-Cas9-nos* transgene also only produced *yellow* mutants when the Cas9 protein was co-injected with the sgRNAs (**Supplementary Figure 3**). Ability of the *nos* promoter to drive germline expression in the closely related species *D. virilis* has also been found to be variable among transgenic lines (Hannah McConnell, Aida de la Cruz, and Harmit Malik, personal communication), suggesting that other promoters should be used in the future to drive reliable germline expression in the virilis group.

To generate *ebony* mutant *D. americana* (A00) and *D. novamexicana* (N14), we synthesized five sgRNAs targeting conserved sites in the first coding exon of *ebony*. Because *ebony* is located on an autosome and *ebony* loss-of-function mutant alleles are generally considered recessive in *D. melanogaster* (Thurmond et al., 2019), we did not expect to be able to identify *ebony* mutants by simply screening progeny of injected flies for mutant phenotypes as we did for *white* and *yellow*. We therefore co-injected a donor plasmid containing the sequence of an eye-specific red fluorescent protein marker (3XP3-RFP) flanked by *ebony* sequences that could be inserted into *ebony* via homology-directed repair and used to screen for *ebony* mutants. Although we observed RFP expression in larvae injected with the homology-directed repair donor fragment, indicating that the reporter gene was functional in these species, injected individuals did not produce any offspring with red fluorescent eyes, suggesting that the donor plasmid was not integrated in the germline of injected individuals. Because non-homologous end joining occurs more frequently than homology directed repair following double-strand breaks (Liu et al., 2018), we also tried to identify flies that might be heterozygous for an *ebony* mutant allele by closely inspecting all offspring of injected (G_0) flies for any subtle changes in pigmentation. Specifically, we collected and mated (G_1) offspring of injected flies with any noticeably darker pigmentation, keeping them grouped by G_0 parent of origin. As further described in the results, we were ultimately able to identify homozygous *ebony* mutants among progeny from these $G_1 \times G_1$ crosses of relatively dark flies derived from two independent

D. novamexicana G_0 flies and one *D. americana* G_0 fly. Sanger sequencing these flies confirmed they were homozygous for *ebony* alleles containing deletions. We then crossed the mutated *ebony* alleles back into wild-type backgrounds of each parental species to generate homozygous *ebony* mutant lines with wild-type red eyes.

Western Blotting

For *ebony* western blotting, proteins were extracted from stage P14/15 pupae, identified by the following characteristics: black pigmentation present in wings and bristles, meconium visible in abdomen (Cooley et al., 2012). For each sample, five pupae were homogenized in 100 μ L of homogenization buffer (125 mM Tris pH 6.8, 6% SDS, 2.5X Roche cOmplete protease inhibitor cocktail, EDTA-free), then centrifuged for 15 min at 15000 rcf, and the supernatant transferred to a fresh tube with an equal volume of 2X Laemmli buffer (125 mM Tris pH 6.8, 6% SDS, 0.2% glycerol, 0.25% bromophenol blue, 5% Beta-mercaptoethanol).

For Cas9 western blotting, protein was extracted from ovaries dissected in ice cold PBS from the following lines: untransformed N14 *white* mutants (host line), three independently transformed lines of N14 *white* carrying the pBac{3XP3-ECFPafm-nosCas9nos} transgene, transgenic *D. melanogaster* carrying the *pnos-Cas9-nos* transgene, and wild-type (Canton-S) *D. melanogaster*. For *D. novamexicana* samples, we collected ovaries from 10 sexually mature flies, whereas for *D. melanogaster* samples, we collected ovaries from 18 sexually mature flies. Different numbers of flies were used for the two species because of differences in body size. In each case, ovaries were placed into microcentrifuge tubes on ice, spun down briefly in a tabletop centrifuge, and excess PBS was removed and replaced with 20 μ L of homogenization buffer. Samples were then treated as described for *ebony* western blots above. A positive control Cas9 sample was made by diluting purified Cas9 protein (PNA Bio CP01) in homogenization buffer, and mixing with 2X Laemmli buffer to a final concentration of 2.5 ng/ μ L.

Samples were heated at 95°C for 10 min before loading into 7.5% Mini-PROTEAN® TGX™ Precast Protein Gels (Bio-Rad) and running at 150V for approximately 90 min at 4°C in 1X tris-glycine running buffer. Separate gels were run for *ebony* and Cas9 blots. Samples were loaded in the following volumes: 35 μ L per pupa sample, 30 μ L per ovary sample, 10 μ L of Cas9 positive control (25 ng protein), 5 μ L PageRuler prestained protein ladder (Thermo Fisher Scientific). Gels were transferred onto PVDF membrane in tris-glycine transfer buffer, 10% MeOH, 0.01% SDS at 100 V for 1 h with stirring on ice at 4°C. Membranes were blocked in 3% nonfat dry milk in TBST for 30 min at RT with shaking, then divided in half using the prestained ladder as a guide just below the 100 kDa mark for the Cas9 membrane and just below the 70 kDa mark for the *ebony* membrane. The lower molecular weight halves of the membranes were placed in solutions containing primary antibodies to detect the protein used as a loading control (tubulin or lamin), whereas the halves of the membranes containing the higher molecular weight proteins were placed in solutions containing primary antibody solutions against the protein of

interest (Ebony or Cas9), each diluted in 3% nonfat dry milk in TBST. In all cases, membranes were incubated with the primary antibodies overnight at 4°C. Primary antibody solutions for *ebony* included rabbit anti-*ebony* 1:300 (Wittkopp et al., 2002) and rabbit anti- α -tubulin 1:5000 (Abcam ab52866) as a loading control. Primary antibody solutions for Cas9 included mouse anti-Cas9 1:1000 (Novus NBP2-36440) and mouse anti-lamin 1:200 (DHSB adl67.10) as a loading control. Membranes were washed in TBST and transferred to secondary antibody solutions diluted in 3% nonfat milk in TBST for 2 h at RT. The following secondary antibodies were used: donkey anti-rabbit HRP 1:5000 (Amersham na934) or goat anti-mouse HRP 1:5000 (abcam ab97023). Membranes were finally washed in TBST and developed with SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific) and imaged using a Licor Odyssey FC imaging system.

Fly Crosses for Reciprocal Hemizygoty Testing and Cuticular Hydrocarbon Analysis

To generate F_1 hybrids carrying only one (*D. americana* or *D. novamexicana*) functional *ebony* allele, wild-type and *ebony* mutant flies from each species were collected as virgins and aged in vials for at least 12 days to reach sexual maturity and verify virgin female status by absence of larvae. Crosses were all set on the same batch of food on the same day and placed at 25°C. For most crosses, 4 virgin females and 4 males were used; however, 8 virgin females and 8 males were used in interspecific crosses with *D. novamexicana* females because of reduced mating success in these crosses. After 3 days, adult flies from these crosses that would be used for cuticular hydrocarbon (CHC) analysis were transferred to new vials with a fresh batch of food. Offspring from the first set of vials were used for imaging and pigmentation analysis, while offspring from the second set of vials were used for CHC analysis. Flies used for pigmentation phenotyping were aged 5–7 days after eclosion and preserved in 10% glycerol in ethanol before imaging (Wittkopp et al., 2011).

Imaging of Fly Phenotypes

Insect specimens were imaged using a Leica DC480 camera attached to a Leica MZ16F stereoscope equipped with a ring light attachment and Leica KL 1500 LCD lamp. Images were captured using Leica DC Twain software version 5.1.1 run through Adobe Photoshop CS6 version 13.0 X32. Prior to imaging, pupal cases and wings were mounted on slides in PVA mounting medium (BioQuip). Thorax, abdomen, and whole-body specimens were prepared from age-matched, preserved flies as described in the previous section. For imaging, thorax, abdomen, and whole-body specimens were submerged in 100% ethanol in custom wells composed of white oven-cured polymer clay (Sculpey).

Because the color of specimens spanned a wide range across genotypes, exposure was optimized for each sample type (e.g., whole body, thorax, abdomen, wing, pupal case) individually by placing specimens from the two phenotypic extremes in the same frame and adjusting exposure to avoid

over-exposing the lightest flies while capturing as much detail as possible from the darkest flies. Exposure time, lighting, white balance, background, and zoom were kept identical across all images of single tissue type. Minor color adjustments to improve visibility of phenotypes were performed simultaneously across all raw images of the same sample type in a single combined document using Photoshop CC 2019, ensuring that all images presented for direct comparisons were adjusted identically.

Cuticular Hydrocarbon Analyses

CHCs for each cross were extracted from 5-day-old females by soaking the flies for 10 min in 200 μ l hexane containing hexacosane (C26; 25 ng/ μ l) as an internal standard. Eight replicates were prepared for each cross. Extracts were directly analyzed by the GC/MS (7890A, Agilent Technologies Inc., Santa Clara, CA, United States) coupled with a DB-17ht column 30 m by 0.25 mm (i.d.) with a 0.15 μ m film thickness (Agilent Technologies Inc., Santa Clara, CA, United States). Mass spectra were acquired in Electron Ionization (EI) mode (70 eV) with Total Ion Mode (TIM) using the GC/MS (5975C, Agilent Technologies Inc., Santa Clara, CA, United States). The peak areas were recorded by MassHunter software (Agilent Technologies Inc., Santa Clara, CA, United States). Helium was the carrier gas at 0.7 ml/min and the GC thermal program was set as follows: 100°C for 4 min, 3°C/min to 325°C. Straight-chain compounds were identified by comparing retention times and mass spectra with authentic standard mixture (C6–C40) (Supelco® 49452-U, Sigma-Aldrich, St. Louis, MO, United States). Methyl-branched alkanes, alkenes, dienes and trienes were then identified by a combination of their specific fragment ions on the side of functional groups (methyl branch or double bonds) and retention times relative to linear-chain hydrocarbon standards. Each individual CHC peak was quantified by normalizing its peak area to the peak area of the internal C26 standard, converting each CHC peak area to ng/fly using the known internal standard concentration of 1000 ng/fly. Welch's *t*-tests with a Benjamini–Hochberg correction for multiple testing (Benjamini and Hochberg, 1995) were used to compare CHC amounts between pairs of genotypes. Because the effect of *ebony* on individual CHC abundance in *D. melanogaster* was recently shown to increase with CHC chain length (Massey et al., 2019b), we also compared the effects of *ebony* loss of function on different chain-lengths of CHCs. Eight biological replicates of homozygous *ebony* null measurements were divided by the mean measurement of the eight replicates of the matched *ebony* heterozygote for each individual CHC. The ratio of *ebony* null to heterozygote CHC abundance was plotted against CHC chain length. The relative effects of *D. americana* versus *D. novamexicana* *ebony* in a common F_1 hybrid background (described as $F_1[e^A/e^-]$ and $F_1[e^N/e^-]$, respectively), were also compared in this manner, with the replicates of the $F_1[e^A/e^-]$ divided by the mean $F_1[e^N/e^-]$ measurement for each CHC. We used Spearman's rank correlation (Spearman's rho) to test the relationship between CHC chain length and the effect of *ebony* on CHC abundance. The threshold for statistical significance was set at $\alpha = 0.05$.

for all tests. Datafile and R code used for this analysis are provided in **Supplementary Files 1, 2**, respectively.

RESULTS AND DISCUSSION

The reciprocal hemizygosity test is a powerful strategy for identifying genes with functional differences that contribute to phenotypic divergence [reviewed in Stern (2014)]. This test is performed by comparing the phenotypes of two hybrid genotypes that are genetically identical except for which allele of the candidate gene is mutated. Any phenotypic differences observed between these two genotypes are attributed to divergence of the candidate gene. Applying this test to identify functional differences between species requires loss-of-function (null) mutant alleles in both species and the ability for the species to cross and produce F_1 hybrids. Consequently, in order to use this strategy to test *ebony* for functional divergence between *D. novamexicana* and *D. americana*, we first needed to generate *ebony* null mutant alleles in both species.

Generating *ebony* Mutants in *D. americana* and *D. novamexicana* Using CRISPR/Cas9

We generated *ebony* null mutants in *D. novamexicana* and *D. americana* by using CRISPR/Cas9 to target double-strand breaks to five conserved sites within the first coding exon of *ebony*. As described more fully in the “Materials and Methods” section, we injected embryos of *white* mutants from both species with purified Cas9 protein and sgRNAs targeting all five sites simultaneously. BLAST searches showed that all of the sgRNAs targets were at least 5 bp different from all other sequences in genomes from two different strains of *D. americana*. Prior work in *D. melanogaster* has shown that heritable off-target mutations were never recovered in sequences with 3 or more mismatches to the sgRNA (Ren et al., 2014). To make it easier to identify *ebony* mutant alleles, we also injected a donor plasmid that would allow homology directed repair to integrate a transgene expressing red fluorescent protein in the fly’s eyes, but no progeny of injected flies were observed to express this transformation marker. However, we reasoned that although we were unable to insert a marker at *ebony*, the CRISPR machinery may still have induced double-strand breaks in the target sequence, and *ebony* mutants could have been generated by non-homologous end-joining resulting in deletions or insertions. Therefore, we also searched for *ebony* mutants by looking for changes in body pigmentation.

In *D. melanogaster*, *ebony* loss-of-function mutants have a much darker appearance than wild-type flies because they are unable to produce yellow sclerotin, causing an increase in production of black and brown melanins (Wittkopp et al., 2002). *D. melanogaster* *ebony* mutant alleles are commonly described as recessive to wild-type *ebony* alleles (Thurmond et al., 2019); however, in some genetic backgrounds, flies heterozygous for an *ebony* mutant allele are slightly darker than wild-type flies (Thurmond et al., 2019). Because *D. novamexicana* has such a light yellow body color (Figure 2A), we thought it possible

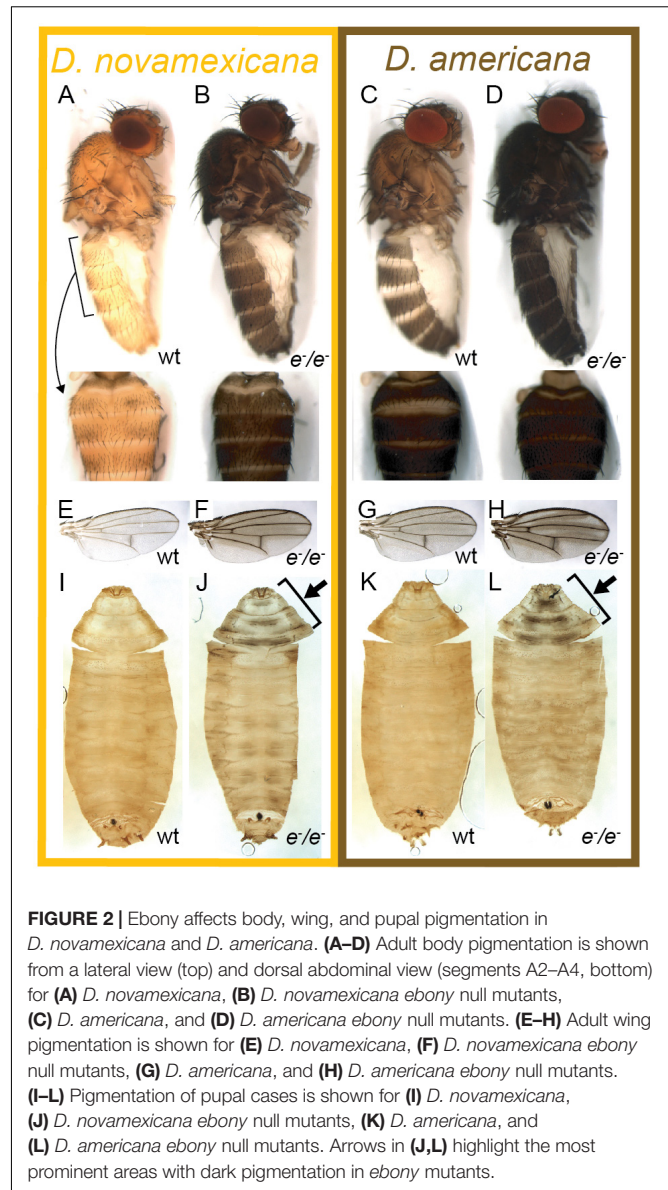


FIGURE 2 | Ebony affects body, wing, and pupal pigmentation in *D. novamexicana* and *D. americana*. (A–D) Adult body pigmentation is shown from a lateral view (top) and dorsal abdominal view (segments A2–A4, bottom) for (A) *D. novamexicana*, (B) *D. novamexicana* *ebony* null mutants, (C) *D. americana*, and (D) *D. americana* *ebony* null mutants. (E–H) Adult wing pigmentation is shown for (E) *D. novamexicana*, (F) *D. novamexicana* *ebony* null mutants, (G) *D. americana*, and (H) *D. americana* *ebony* null mutants. (I–L) Pigmentation of pupal cases is shown for (I) *D. novamexicana*, (J) *D. novamexicana* *ebony* null mutants, (K) *D. americana*, and (L) *D. americana* *ebony* null mutants. Arrows in (J,L) highlight the most prominent areas with dark pigmentation in *ebony* mutants.

that flies heterozygous for an *ebony* mutant allele might also show a detectable darkening of pigmentation; we were less optimistic about being able to detect heterozygous *ebony* mutants based on pigmentation in *D. americana* because its wild-type pigmentation is already very dark (Figure 2C). Nonetheless, we sorted through the progeny of injected *D. novamexicana* and *D. americana* flies, isolating any individuals that seemed to have darker pigmentation than their siblings and allowing these relatively dark flies to freely mate in vials segregated by injected parents, keeping individual “founder” mutations separate.

Two of the vials of darker pigmented *D. novamexicana* flies produced pupae with an unusual black pattern on the anterior end of the pupal case (Figure 2J). We moved these pupae to new vials and found that black-patterned pupae from both “founder” vials developed into adults with the much darker than wild-type body color expected for

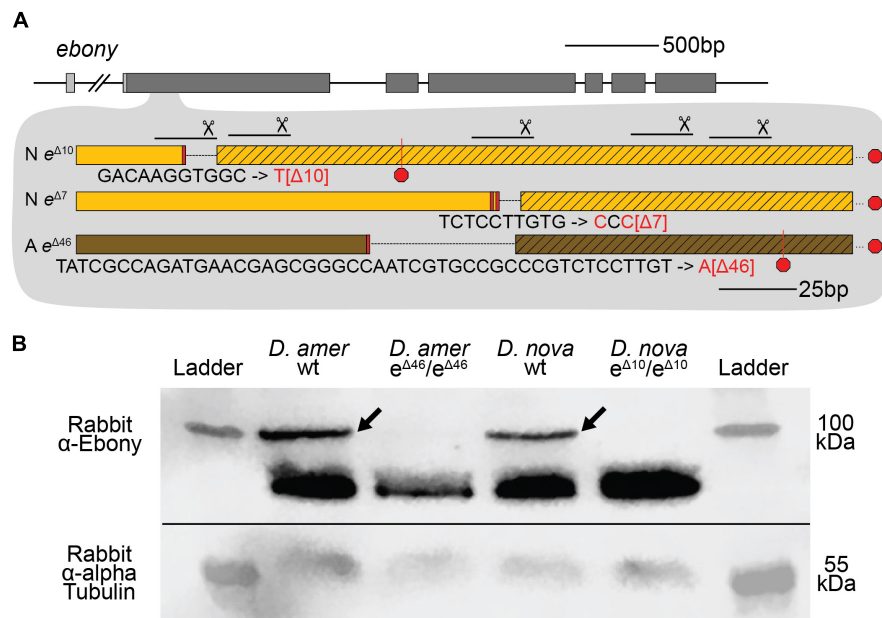


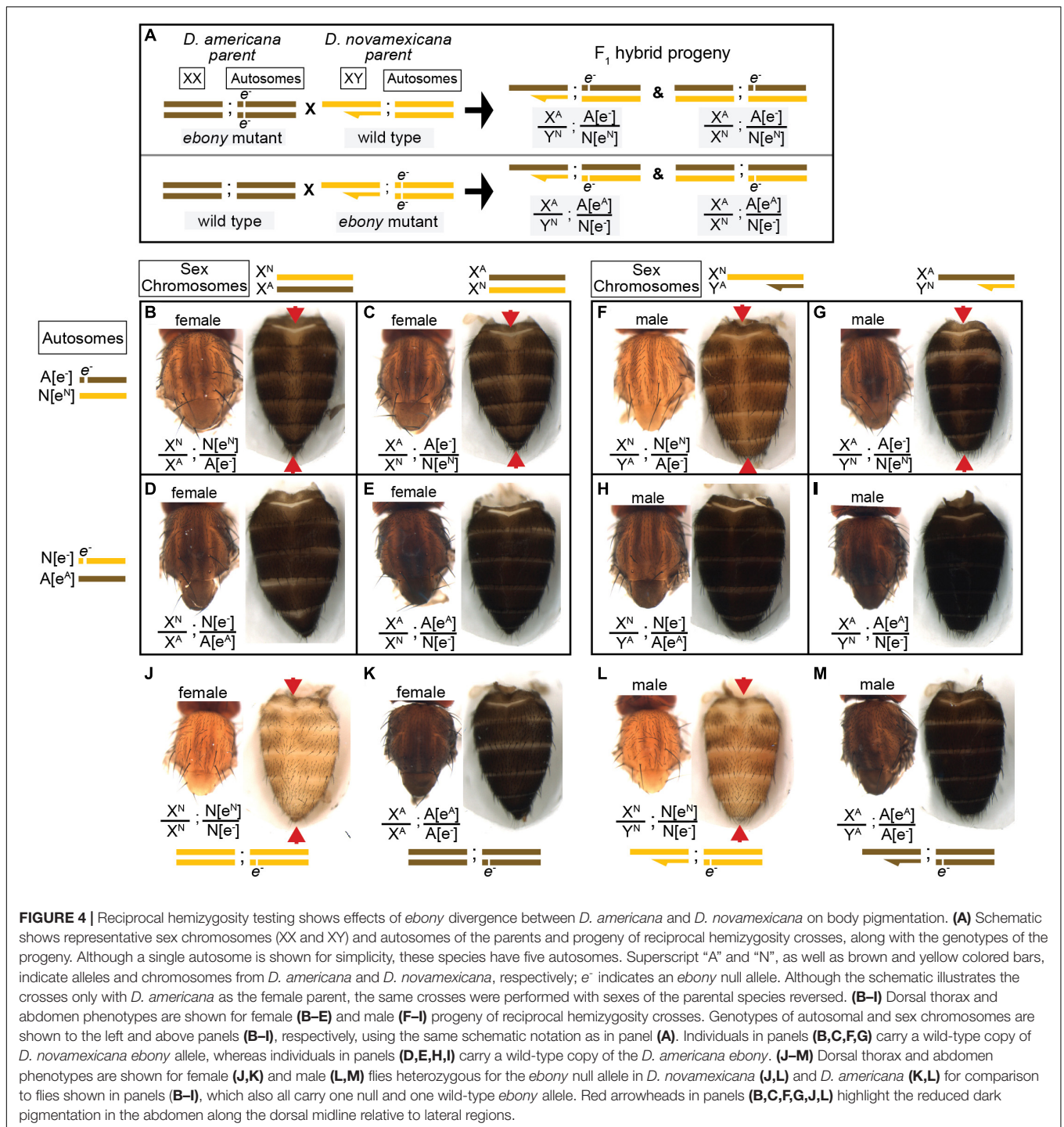
FIGURE 3 | CRISPR/Cas9-induced mutations created null alleles of the *D. novamexicana* and *D. americana* *ebony* genes. **(A)** A schematic of the *ebony* gene is shown with gray boxes indicating exons; coding sequence is indicated in the darker shade of gray. Locations of the five guide RNAs targeting the second exon of *ebony* are shown with solid lines below scissor symbols. Mutations observed in the two *ebony* mutants ($e^{\Delta 10}$ and $e^{\Delta 7}$) isolated in *D. novamexicana* ("N") and the one *ebony* mutant ($e^{\Delta 46}$) isolated in *D. americana* ("A") are shown. All three alleles included deletions that caused frameshifts. **(B)** Western blotting showed that the *D. americana* $e^{\Delta 46}$ and *D. novamexicana* $e^{\Delta 10}$ mutants (lanes 2 and 4, respectively), lacked a ~100 kDa protein (arrows) recognized by an antibody raised against *D. melanogaster* Ebony protein (Wittkopp et al., 2002) that is present in wild-type (wt) *D. americana* and *D. novamexicana* (lanes 1 and 3, respectively). Relative abundance of total protein loaded into each lane can be seen by the relative intensities of the shorter proteins also detected by the Ebony antibody (Wittkopp et al., 2002) as well as the relative intensities of ~55 kDa bands detected by an antibody recognizing alpha Tubulin (Abcam ab52866). The solid black line shows where the membrane was cut prior to incubation with primary antibodies during the western blotting procedure; the top half was incubated with anti-Ebony antibodies whereas the bottom half was incubated with anti-Tubulin antibodies. The two halves were realigned by hand for imaging, using the shape of the cut and the ladder staining as a guide. An un-annotated image of this blot is shown in **Supplementary Figure 4**.

homozygous *ebony* mutants in *D. novamexicana* (Figures 2A,B). Because pigmentation of the pupal case is very similar between *D. novamexicana* and *D. americana* (Figures 2I,K, Ahmed-Braimah and Sweigart, 2015), we also searched for pupae with similar pigmentation marks in the vials containing progeny of darker flies descended from injected *D. americana*. We found such pupae in only one of the *D. americana* vials (Figure 2L). Flies emerging from these pupal cases also showed darker pigmentation than wild-type *D. americana* (Figures 2C,D), as expected for homozygous *ebony* mutants, but this difference was much more subtle than in *D. novamexicana* (Figures 2A,B). Flies from both species emerging from pupal cases with abnormal pigmentation also showed increased levels of dark melanins in wings in a pattern similar to that seen in *D. melanogaster* *ebony* mutants (Figures 2E–H, Wittkopp et al., 2002), further suggesting that they were homozygous for *ebony* mutant alleles. Crossing putative homozygous *ebony* mutants from the same species to each other resulted in true-breeding lines of *D. americana* and *D. novamexicana* presumed to be homozygous for *ebony* mutant alleles.

To determine whether these true-breeding lines were indeed homozygous for *ebony* mutant alleles, we used Sanger sequencing to search for changes in the *ebony* sequence in the region

targeted for double strand breaks with CRISPR/Cas9. We found that the presumed *ebony* mutant lines of both species harbored deletions corresponding to the locations of sgRNA target sites in the first coding exon, with the two *D. novamexicana* mutant lines carrying deletions of 7 and 10 bases and the *D. americana* mutant line carrying a deletion of 46 bases (Figure 3A). Each of these mutations is expected to cause frameshifts, leading to multiple early stop codons. Further experiments described in this study using *D. novamexicana* *ebony* mutants were conducted with the 10 base deletion line, and any further description of *ebony* null *D. novamexicana* refers to this line.

To further assess whether these mutations caused null alleles, we used western blotting to examine the expression of the Ebony protein during late pupal stages when adult pigmentation is developing and the *ebony* gene is expressed in the developing abdomen (Wittkopp et al., 2002; Cooley et al., 2012). We performed western blots on protein extracts from P14/P15 stage pupae of both wild-type and homozygous *ebony* mutant flies of both *D. americana* and *D. novamexicana* using an antibody against *D. melanogaster* *ebony* (Wittkopp et al., 2002). This antibody recognizes a 94 kDa protein consistent with the predicted molecular weight of Ebony in pupal protein extracts from wild-type lines of both *Drosophila*



melanogaster and *Drosophila biarmipes*, but does not produce a 94 kDa band in pupal protein extracts of either *e1* or *In(3R)eAFA* *ebony* mutant lines of *D. melanogaster* (Wittkopp et al., 2002). Wild-type extracts of both *D. americana* and *D. novamexicana* produced presumptive Ebony bands while extracts from flies homozygous for *ebony* deletions did not produce a 94 kDa band for either species (**Figure 3B**). The nature of the frameshift deletions as well as the western

blot evidence together show that these *ebony* mutations cause null alleles.

ebony* Divergence Contributes to Body Color Differences Between *D. novamexicana* and *D. americana

We used the homozygous *ebony* mutant *D. novamexicana* and *D. americana* lines to perform a reciprocal hemizygosity test

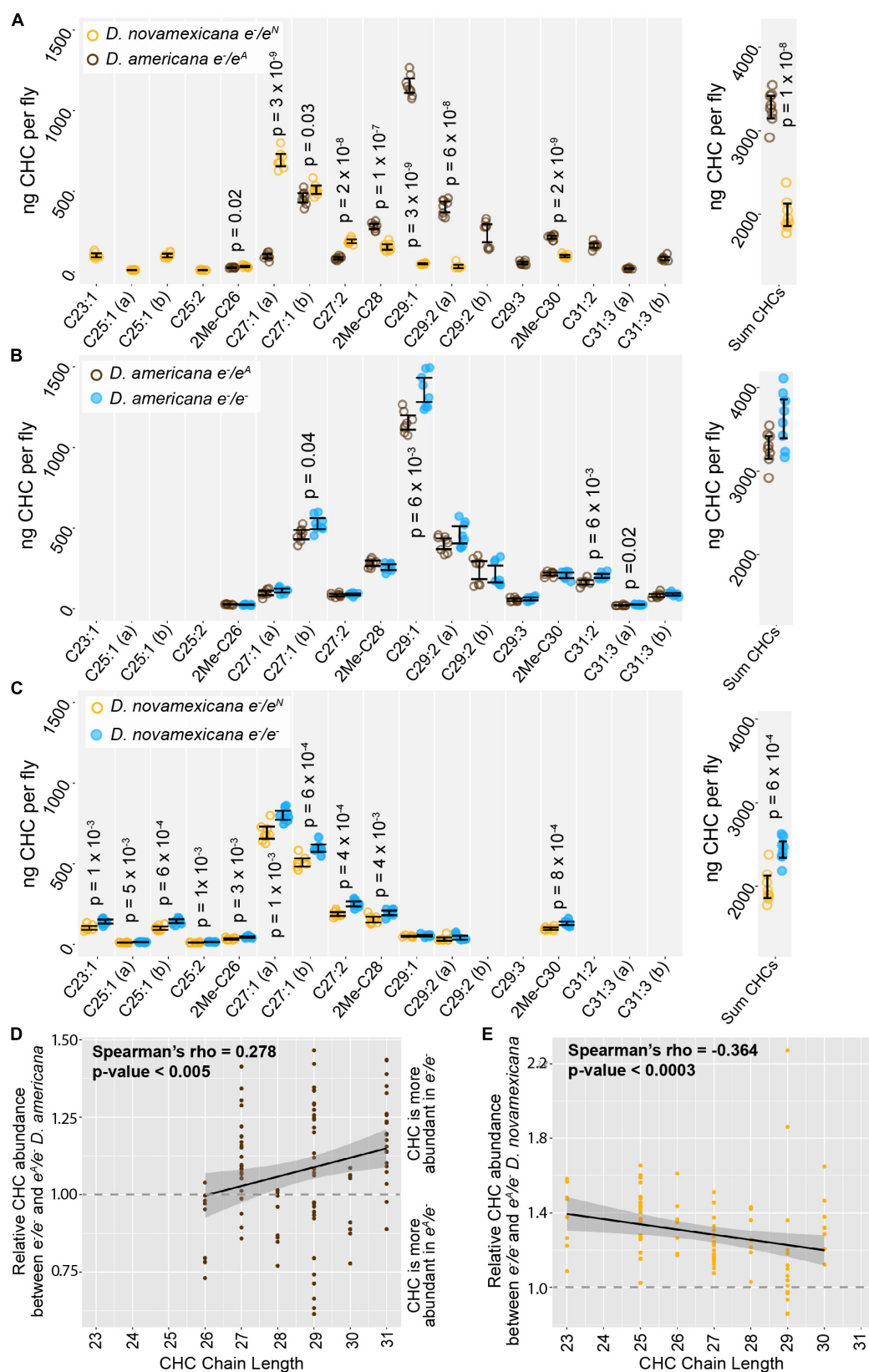


FIGURE 5 | Continued

FIGURE 5 | Cuticular hydrocarbons (CHCs) are affected by ebony and differ between *D. americana* and *D. novamexicana*. **(A–C)** Abundance of individual CHC compounds (ng/fly) and summed CHCs extracted from female flies are plotted for the following genotypes: **(A)** *D. americana* and *D. novamexicana*, each heterozygous for an *ebony* null (e^-) allele, **(B)** *D. americana* heterozygous and homozygous for an *ebony* null allele, **(C)** *D. novamexicana* heterozygous and homozygous for an *ebony* null allele. Eight biological replicates are shown for each genotype, with error bars representing 95% confidence intervals. For each comparison, the p -value from a Welch's t -test with a Benjamini–Hochberg multiple test correction ($\alpha = 0.05$) is shown when a significant difference in abundance was detected for a CHC present in both genotypes being compared. CHCs are shown from left to right with increasing chain length (represented by “C” followed by the chain length) with double-bond and methyl-branched structures indicated by notations after the colon or before the “C”, respectively. For example, C25:1 represents a 25-carbon monoene, C25:2 represents a 25-carbon diene, and 2Me-C28 represents a 28-carbon alkene with a methyl branch at the second carbon. **(D–E)** Abundance of each CHC in *ebony* null mutants relative to flies heterozygous for the *ebony* null allele is plotted by carbon chain length for **(D)** *D. americana* and **(E)** *D. novamexicana*. Black trendlines in panels **(D–E)** show linear regressions, with shaded areas representing the standard error and both Spearman's rho and p -values indicated on each plot.

by crossing *ebony* mutant *D. novamexicana* (e^-/e^-) to wild-type *D. americana* (e^A/e^A) and *ebony* mutant *D. americana* (e^-/e^-) to wild-type *D. novamexicana* (e^N/e^N) (**Figure 4A**). In order to observe the effects of the two species' *ebony* alleles in the presence of each species X chromosome, we conducted sets of reciprocal crosses (i.e., swapping the genotypes of the male and female parents). Female F_1 hybrids from reciprocal crosses are genetically identical except for the parent of origin of their one functional *ebony* allele (e^N or e^A). F_1 hybrid females carrying a functional *D. novamexicana* *ebony* allele ($F_1[e^N/e^-]$) developed a lighter body color than F_1 hybrid females carrying a functional *D. americana* *ebony* allele ($F_1[e^A/e^-]$) (**Figures 4B,C** vs. **Figures 4D,E**). These data demonstrate for the first time that functional divergence between the *D. novamexicana* and *D. americana* *ebony* alleles contributes to divergent body color between these two species.

To determine how *ebony* divergence interacts with divergent loci on the X-chromosome, we also compared the body color of male progeny from these reciprocal crosses. Like the F_1 hybrid females, these F_1 hybrid males differ for the parent of origin for their one functional *ebony* allele (e^A or e^N); however, they also differ for the parent of origin of all X-linked genes. Prior work has shown that divergence on the X-chromosome, particularly divergence in non-coding sequences of the *tan* gene, also contributes to differences in body color between *D. novamexicana* and *D. americana* (Wittkopp et al., 2003b, 2009). As expected, we found that body color differed between males carrying alternate species' X chromosomes (**Figure 4F** vs. **Figure 4G** and **Figure 4H** vs. **Figure 4I**) as well as between males carrying the same X chromosome but different species' functional *ebony* alleles (**Figure 4F** vs. **Figure 4H** and **Figure 4G** vs. **Figure 4I**). Consistent with prior findings demonstrating that divergence in the QTL containing *ebony* explained more of the difference in pigmentation than divergence at X-linked genes, we found that males with functional *D. americana* *ebony* alleles had the darkest phenotypes, regardless of their X-chromosome genotype (**Figures 4F–I**).

ebony* Divergence Also Contributes to a Difference in Abdominal Pigment Patterning Between *D. novamexicana* and *D. americana

Although the divergent overall body color is the most striking difference in pigmentation between *D. novamexicana* and

D. americana, there is also a difference in the distribution of pigments along the dorsal midline of the abdomen between these two species (**Figure 1**). This difference is also visible in individuals of both species heterozygous for an *ebony* null allele (**Figures 4J–M**). Prior work has shown that the absence of dark pigments seen in this region of *D. novamexicana* is dominant in F_1 hybrids to the presence of dark pigments seen in this region of *D. americana* (Wittkopp et al., 2003b). In addition, genetic mapping of this trait between *D. novamexicana* and *D. virilis* (which has a dark midline region similar to *D. americana*) has shown that the chromosome including *ebony* (chromosome 2) has a large effect on this trait (Spicer, 1991). We found that *D. novamexicana* *ebony* mutants showed even pigmentation across the width of each abdominal segment (**Figure 2B**), demonstrating that *ebony* is required for the development of lighter pigmentation along the dorsal midline in wild-type *D. novamexicana* (**Figure 2A**). In addition, comparing the pigmentation of this abdominal dorsal midline region between F_1 hybrid flies of both sexes from the reciprocal crosses described above (**Figure 4**) showed that divergence at *ebony* contributes to this trait difference between *D. novamexicana* and *D. americana*. Specifically, we observed less dark pigments in the dorsal midline region of the abdomen in F_1 hybrid individuals inheriting the wild-type *D. novamexicana* *ebony* allele ($F_1[e^N/e^-]$, **Figures 4B,C,F,G**) than the *D. americana* *ebony* allele ($F_1[e^A/e^-]$) (**Figures 4D,E,H,I**). Males carrying a functional *D. novamexicana* *ebony* allele ($F_1[e^N/e^-]$) showed reduced pigmentation in the dorsal midline relative to the lateral regions regardless of the origin of their X chromosome (**Figures 4F,G**), indicating that divergent loci on the X-chromosome (including *tan*) do not affect the presence of this phenotype.

Cuticular Hydrocarbon Profiles Differ Between *D. americana* and *D. novamexicana* and Are Affected by *ebony* Expression but Not Ebony Divergence

ebony expression was recently found to affect the relative abundance of cuticular hydrocarbons (CHCs) in *D. melanogaster* (Massey et al., 2019b). In addition, variation in *ebony* expression was also shown to correlate with variation in CHC profiles among natural isolates of *D. melanogaster* (Massey et al., 2019b). CHC profiles have been shown to vary among virilis group species as well, including between *D. novamexicana* and

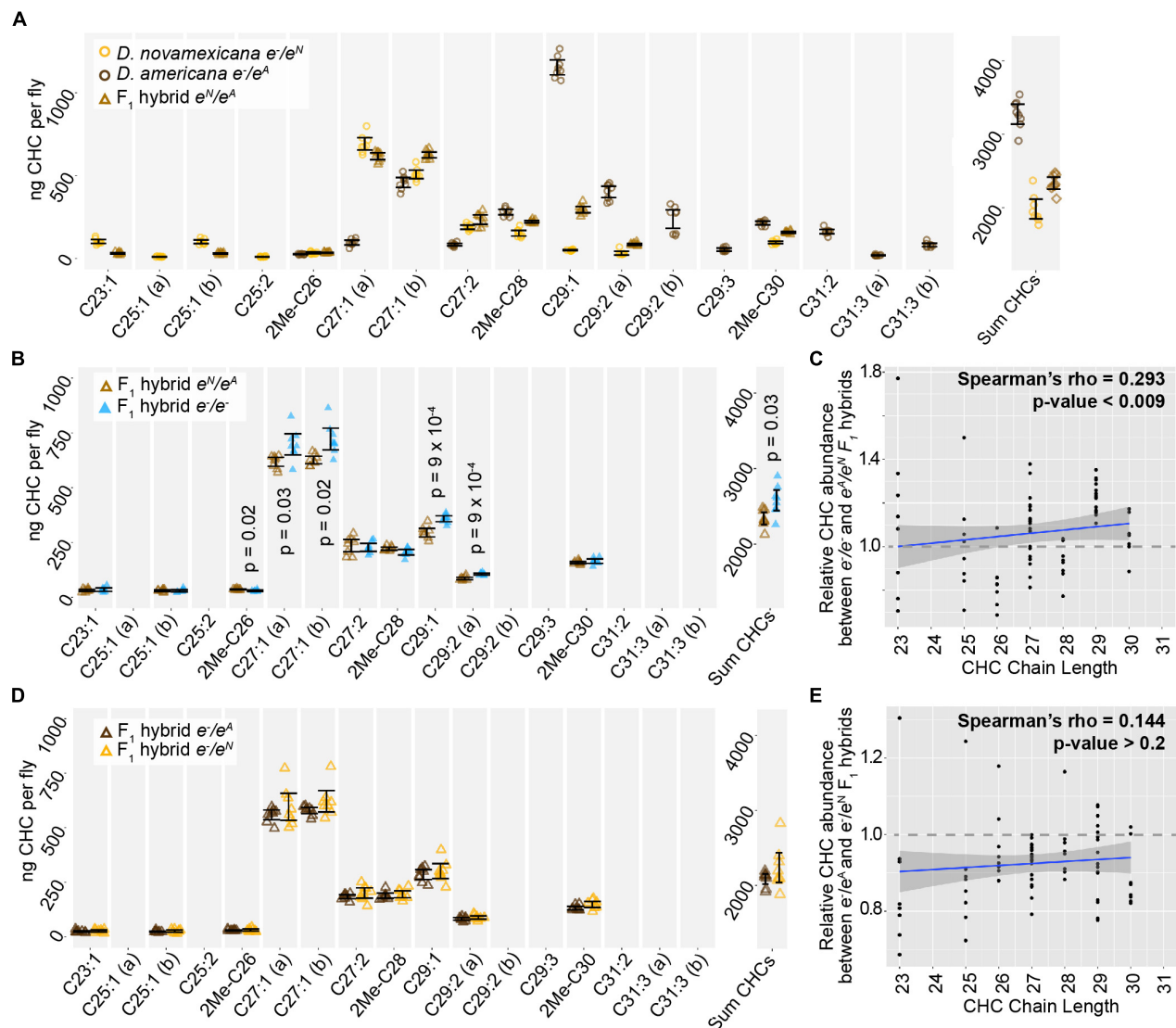


FIGURE 6 | *ebony* does not contribute to divergence of CHCs between *D. americana* and *D. novamexicana*. **(A)** Abundance of individual CHC compounds (ng/fly) and summed CHCs extracted from female flies are plotted for *D. americana* and *D. novamexicana* *ebony* heterozygotes as well as F_1 hybrids heterozygous for wild-type alleles of *ebony*. **(B–C)** CHCs from F_1 hybrids homozygous for *ebony* null alleles are compared to CHCs from F_1 hybrids with wild-type *D. americana* and *D. novamexicana* *ebony* alleles, showing the absolute abundance of individual and summed CHC compounds **(B)** as well as the relative abundance of CHCs by carbon chain length **(C)**. In panel **(B)**, p -values are shown from a Welch's t -test with a Benjamini–Hochberg multiple test correction ($\alpha = 0.05$) when a significant difference in abundance was detected for a CHC present in both genotypes. **(D–E)** CHC profiles are plotted for reciprocal F_1 hybrids that differ only by which wild-type *ebony* allele they carry, either *D. americana* (e^A) or *D. novamexicana* (e^N), with absolute abundance of individual and summed CHCs shown in **(D)** and relative abundance of CHCs by chain length shown in **(E)**. No p -values are shown in **(D)** because no CHCs showed a statistically significant difference in abundance between the two F_1 hybrid genotypes from the reciprocal hemizygosity test (Welch's t -test with Benjamini–Hochberg multiple test correction, $p > 0.05$ for each CHC). In panels **(C,E)**, blue trendlines show linear regressions, with shaded areas representing the standard error and both Spearman's ρ and p -values indicated on each plot. In all panels, data from eight replicate flies is shown for each genotype.

the strain of *D. americana* used in this study (Bartelt et al., 1986). We therefore asked whether differences in *ebony* might contribute to differences in CHC profiles between these two species using a reciprocal hemizygosity test. Because this test compares phenotypes of reciprocal hemizygotes that each carry a single functional copy of *ebony*, we also examined CHC profiles in *D. americana* and *D. novamexicana* flies with a single functional copy of *ebony*. We found that *D. novamexicana*

flies hemizygous for *ebony* contained a distribution of CHCs biased toward shorter chain hydrocarbons relative to CHCs extracted from *D. americana* flies hemizygous for *ebony*. Prior work found similar profiles of CHCs for wild type *D. americana* and *D. novamexicana* (Bartelt et al., 1986): in both studies, CHCs with a chain length of 25 or fewer were only present in *D. novamexicana*, whereas multiple CHCs with a chain length greater than 30 were only present in *D. americana* (Figure 5A).

These data suggest that the wild-type *ebony* allele has a dominant effect on CHC profiles.

In order to test whether *ebony* affects CHCs in these species, we compared CHCs extracted from homozygous *ebony* mutants to those extracted from *ebony* heterozygotes. In both species, the loss of *ebony* function had no qualitative effect on which CHCs were produced by either species, but increased the abundance of some CHCs in both *D. americana* and *D. novamexicana* (Figures 5B,C). Because *ebony* loss-of-function mutants in *D. melanogaster* were recently shown to preferentially increase the abundance of long chain CHCs (Massey et al., 2019b), we compared relative abundance of individual CHCs between *ebony* null and heterozygous samples and plotted the results against CHC chain length (Figures 5D,E). We observed a similar pattern to *D. melanogaster* in *D. americana*, with *ebony* loss-of-function increasing the abundance of longer chain CHCs more strongly (Figure 5D). In *D. novamexicana*, we observed the opposite pattern, however: CHCs with shorter chain lengths showed greater increases in abundance in *ebony* null mutants (Figure 5E). The reason for this difference in how *ebony* affects CHCs in *D. americana* and *D. novamexicana* remains unclear, but might have to do with the different levels of *tan* expression in these two species (Cooley et al., 2012) given that *tan* was also shown to affect CHC profiles in *D. melanogaster* (Massey et al., 2019b). Specifically, *tan* expression is lower in *D. novamexicana* pupae relative to *D. americana* (Cooley et al., 2012), and *tan* loss-of-function has been shown to preferentially increase the abundance of shorter chain CHCs in *D. melanogaster* (Massey et al., 2019b). Further experiments exploring the mechanisms underlying CHC production in these species may provide more insight into the contrasting effects of *ebony* on CHCs in *D. americana* and *D. novamexicana*.

We also examined the CHC profiles of female F₁ hybrids produced by crossing *D. americana* females with *D. novamexicana* males. We found that these F₁ hybrid females showed a CHC profile that was distinct from both species, but more similar to *D. novamexicana* (Figure 6A): it contained some of the short chain CHCs unique to *D. americana* (Figure 6A). As seen for both species, eliminating *ebony* function in F₁ hybrids by making them homozygous for *ebony* null alleles caused an increase in abundance of some CHCs but did not alter which CHCs were present (Figure 6B). Longer chain CHCs were more likely to show increased abundance than shorter chain CHCs (Figure 6C), but this relationship was not as strong as that seen for *D. americana* (Figure 5D). To determine whether divergence between the *D. americana* and *D. novamexicana* *ebony* alleles affected CHCs profiles, we compared CHCs extracted from females from the reciprocal hemizyosity test. These flies have only one functional *ebony* allele (*D. americana* or *D. novamexicana*) in the F₁ hybrid genetic background. The CHC profiles from these flies were not significantly different from each other (Figures 6D,E), indicating that allelic divergence at *ebony* does not have a detectable effect on CHCs in this species pair.

CONCLUSION

Identifying the genes responsible for phenotypic differences between species remains a significant challenge for evolutionary biology. This task is especially challenging when a gene contributing to phenotypic divergence is located in a region of the genome inverted between species, which precludes recombination-based mapping. Such is the case for the *ebony* gene in *D. americana* and *D. novamexicana*. Prior work suggested that *ebony* might contribute to differences in overall body color between these two species (Wittkopp et al., 2009; Cooley et al., 2012), but its location in an inversion made it difficult to directly test this hypothesis. In this study, we overcame this hurdle by using CRISPR/Cas9 genome editing to generate null mutants for *ebony* in *D. americana* and *D. novamexicana*, and then using these mutants to perform a reciprocal hemizyosity test (Stern, 2014), which directly compares the effects of the two species' alleles on pigmentation. We found that divergence at *ebony* does indeed contribute to differences in body color between *D. americana* and *D. novamexicana*.

Characterizing the phenotypes of *D. americana* and *D. novamexicana* *ebony* mutants, as well as flies from the reciprocal hemizyosity test, also identified effects of *ebony* on other phenotypes. For example, we found that differences in the activity of *ebony* alleles between *D. americana* and *D. novamexicana* are responsible for the absence of dark pigmentation seen along the dorsal abdominal midline of *D. novamexicana* but not *D. americana*. This trait has previously been described as derived in *D. novamexicana* (Spicer, 1991); however, we see a similar dorsal midline lightening in at least some lines of *D. lummei* (see Figure 1), another member of the virilis group, suggesting that the dorsal midline activity of *ebony* existed prior to the divergence of *D. americana* and *D. novamexicana*. An unexpected change in pupal pigment patterning was also seen in *D. americana* and *D. novamexicana* *ebony* null mutants. Although *ebony* is known to affect pupal case development in *D. melanogaster* (Sherald, 1980), its loss causes a pale white pupa color rather than the dark pigmentation we see in *D. americana* and *D. novamexicana* *ebony* null mutants. Because *ebony* is required for the production of yellow pigments, the dark markings seen in *ebony* mutant pupal cases likely result from expression of an enzyme required for synthesis of dark pigments, such as *tan*. Finally, we found that *ebony* null mutants showed significant changes in the abundance of some CHCs in each species, but divergence of *ebony* did not contribute to differences in the CHC profiles seen between species. These observations illustrate how *cis*-regulatory changes can cause divergence of some, but not all, traits affected by a pleiotropic gene.

Observations reported in this work were made possible by the ability to manipulate the *D. americana* and *D. novamexicana* genomes with CRISPR/Cas9 genome editing. While this technology has great potential for allowing functional hypothesis testing in species that have not historically been considered genetic model systems, this work was not always straightforward. We hope that the detailed descriptions of our genome editing efforts provided in the Materials and “Materials and Methods”

section of this paper will be helpful for other researchers striving to manipulate the genomes of non-model species.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

AL and PW conceived of the experiments and wrote the manuscript. AL, PS, and ZW performed the experiments. HC and PJ provided funding, advice, and oversight. All authors contributed to the article and approved the submitted version.

FUNDING

This work was funded by the National Institutes of Health (Grant Nos. 1R35GM118073 and 1R01GM089736) awarded to PW; National Science Foundation Graduate Research Fellowship Program (Grant No. DGC 1256260) and National Institute

of Health training grant: “Michigan Predoctoral Training in Genetics” (Grant No. T32-GM07544) to AL; and startup funding provided by the Michigan State University AgBioResearch to HC.

ACKNOWLEDGMENTS

We thank Arnaud Martin (George Washington University) as well as Kathy Vaccarro and other members of Sean Carroll's laboratory (University of Wisconsin) for advice on CRISPR/Cas9 genome editing and *Drosophila* injections, respectively; Hannah McConnell, Aida de la Cruz, and Harmit Malik (Fred Hutchinson Cancer Research Center) for sharing their experience working with the *nanos* promoter in *Drosophila virilis*; and the Bloomington *Drosophila* Stock Center as well as the National *Drosophila* Species Stock Center for maintaining and providing fly stocks.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fevo.2020.00184/full#supplementary-material>

REFERENCES

- Ahmed-Braimah, Y. H., and McAllister, B. F. (2012). Rapid evolution of assortative fertilization between recently allopatric species of *Drosophila*. *Int. J. Evol. Biol.* 2012:285468. doi: 10.1155/2012/285468
- Ahmed-Braimah, Y. H., and Sweigart, A. L. (2015). A single gene causes an interspecific difference in pigmentation in *Drosophila*. *Genetics* 200, 331–342. doi: 10.1534/genetics.115.174920
- Bartelt, R. J., Arnold, M. T., Schaner, A. M., and Jackson, L. L. (1986). Comparative analysis of cuticular hydrocarbons in the *Drosophila virilis* species group. *Comp. Biochem. Physiol.* 83, 731–742. doi: 10.1016/0305-0491(86)90138-0
- Bassett, A. R., Tibbit, C., Ponting, C. P., and Liu, J.-L. (2013). Highly efficient targeted mutagenesis of *Drosophila* with the CRISPR/Cas9 System. *Cell Rep.* 4, 220–228. doi: 10.1016/j.celrep.2013.06.020
- Benjamini, Y., and Hochberg, Y. (1995). Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R. Stat. Soc. Ser. B* 57, 289–300. doi: 10.1111/j.2517-6161.1995.tb02031.x
- Caletka, B. C., and McAllister, B. F. (2004). A genealogical view of chromosomal evolution and species delimitation in the *Drosophila virilis* species subgroup. *Mol. Phylogenet. Evol.* 33, 664–670. doi: 10.1016/j.ympev.2004.08.007
- Carroll, S. B. (2005). Evolution at two levels: on genes and form. *PLoS Biol.* 3:e245. doi: 10.1371/journal.pbio.0030245
- Chung, H., and Carroll, S. B. (2015). Wax, sex and the origin of species: dual roles of insect cuticular hydrocarbons in adaptation and mating. *BioEssays* 37, 822–830. doi: 10.1002/bies.201500014
- Chung, H., Loehlin, D. W., Dufour, H. D., Vaccarro, K., Millar, J. G., and Carroll, S. B. (2014). A single gene affects both ecological divergence and mate choice in *Drosophila*. *Science* 343, 1148–1151. doi: 10.1126/science.1249998
- Cooley, A. M., Shefner, L., McLaughlin, W. N., Stewart, E. E., and Wittkopp, P. J. (2012). The ontogeny of color: developmental origins of divergent pigmentation in *Drosophila americana* and *D. novamexicana*. *Evol. Dev.* 14, 317–325. doi: 10.1111/j.1525-142X.2012.00550.x
- Holtzman, S., Miller, D., Eisman, R., Kuwayama, H., Niimi, T., and Kaufman, T. (2010). Transgenic tools for members of the genus *Drosophila* with sequenced genomes. *Fly (Austin)* 4, 349–362. doi: 10.4161/fly.4.4.13304
- Horn, C., and Wimmer, E. A. (2000). A versatile vector set for animal transgenesis. *Dev. Genes Evol.* 210, 630–637. doi: 10.1007/s004270000110
- Koch, P. B., Behnecke, B., Weigmann-Lenz, M., and Ffrench-Constant, R. H. (2000). Insect pigmentation: activities of β -alanyldopamine synthase in wing color patterns of wild-type and melanic mutant swallowtail butterfly *Papilio glaucus*. *Pigment Cell Res.* 13, 54–58. doi: 10.1111/j.0893-5785.2000.130811.x
- Kronforst, M. R., Barsh, G. S., Kopp, A., Mallet, J., Monteiro, A., Mullen, S. P., et al. (2012). Unraveling the thread of nature's tapestry: the genetics of diversity and convergence in animal pigmentation. *Pigment Cell Melanoma Res.* 25, 411–433. doi: 10.1111/j.1755-148X.2012.01014.x
- Liu, M., Rehman, S., Tang, X., Gu, K., Fan, Q., Chen, D., et al. (2018). Methodologies for improving HDR efficiency. *Front. Genet.* 9:691. doi: 10.3389/fgene.2018.00691
- Massey, J. H., Akiyama, N., Bien, T., Dreisewerd, K., Wittkopp, P. J., Yew, J. Y., et al. (2019b). Pleiotropic effects of *ebony* and *tan* on pigmentation and cuticular hydrocarbon composition in *Drosophila melanogaster*. *Front. Physiol.* 10:518. doi: 10.3389/fphys.2019.00518
- Massey, J. H., Chung, D., Siwanowicz, I., Stern, D. L., and Wittkopp, P. J. (2019a). The *yellow* gene influences *Drosophila* male mating success through sex comb melanization. *eLife* 8:e49388. doi: 10.7554/eLife.49388
- Massey, J. H., and Wittkopp, P. J. (2016). The genetic basis of pigmentation differences within and between *Drosophila* species. *Curr. Top. Dev. Biol.* 119, 27–61. doi: 10.1016/bs.ctdb.2016.03.004
- Miller, D. F. B., Holtzman, S. L., and Kaufman, T. C. (2002). Customized microinjection glass capillary needles for P-element transformations in *Drosophila melanogaster*. *Biotechniques* 33, 366–375. doi: 10.2144/02332rr03
- Morales-Hojas, R., Vieira, C. P., and Vieira, J. (2008). Inferring the evolutionary history of *Drosophila americana* and *Drosophila novamexicana* using a multilocus approach and the influence of chromosomal rearrangements in single gene analyses. *Mol. Ecol.* 17, 2910–2926. doi: 10.1111/j.1365-294X.2008.03796.x
- Nappi, A. J., and Christensen, B. M. (2005). Melanogenesis and associated cytotoxic reactions: applications to insect innate immunity. *Insect Biochem. Mol. Biol.* 35, 443–459. doi: 10.1016/j.ibmb.2005.01.014
- Patterson, J. T., and Stone, W. S. (1949). The relationship of *novamexicana* to the other members of the *virilis* group. *Univ. Texas Publ.* 4920, 7–17.
- Port, F., Chen, H. M., Lee, T., and Bullock, S. L. (2014). Optimized CRISPR/Cas tools for efficient germline and somatic genome engineering in *Drosophila*.

- Proc. Natl. Acad. Sci. U.S.A. 111, E2967–E2976. doi: 10.1073/pnas.1405500111
- Rebeiz, M., and Williams, T. M. (2017). Using *Drosophila* pigmentation traits to study the mechanisms of cis-regulatory evolution. *Curr. Opin. Insect Sci.* 19, 1–7. doi: 10.1016/j.cois.2016.10.002
- Ren, X., Yang, Z., Xu, J., Sun, J., Mao, D., Hu, Y., et al. (2014). Enhanced specificity and efficiency of the CRISPR/Cas9 system with optimized sgRNA parameters in *Drosophila*. *Cell Rep.* 9, 1151–1162. doi: 10.1016/j.celrep.2014.09.044
- Sherald, A. F. (1980). Sclerotization and coloration of the insect cuticle. *Experientia* 36, 143–146. doi: 10.1007/BF01953696
- Spicer, G. S. (1991). The genetic basis of a species-specific character in the *Drosophila virilis* species group. *Genetics* 128, 331–337.
- Stern, D. L. (2014). Identification of loci that cause phenotypic variation in diverse species with the reciprocal hemizyosity test. *Trends Genet.* 30, 547–554. doi: 10.1016/j.tig.2014.09.006
- Stern, D. L., Crocker, J., Ding, Y., Frankel, N., Kappes, G., Kim, E., et al. (2017). Genetic and transgenic reagents for *Drosophila simulans*, *D. mauritiana*, *D. yakuba*, *D. santomea*, and *D. virilis*. *G3* 7, 1339–1347. doi: 10.1534/g3.116.038885
- Suh, J., and Jackson, F. R. (2007). *Drosophila ebony* activity is required in glia for the circadian regulation of locomotor activity. *Neuron* 55, 435–447. doi: 10.1016/j.neuron.2007.06.038
- Takahashi, A. (2013). Pigmentation and behavior: potential association through pleiotropic genes in *Drosophila*. *Genes Genet. Syst.* 88, 165–174. doi: 10.1266/ggs.88.165
- Thurmond, J., Goodman, J. L., Strelets, V. B., Attrill, H., Gramates, L. S., Marygold, S. J., et al. (2019). FlyBase 2.0: the next generation. *Nucleic Acids Res.* 47, D759–D765. doi: 10.1093/nar/gky1003
- True, J. R. (2003). Insect melanism: the molecules matter. *Trends Ecol. Evol.* 18, 640–647. doi: 10.1016/j.tree.2003.09.006
- True, J. R., Yeh, S. D., Hovemann, B. T., Kemme, T., Meinertzhagen, I. A., Edwards, T. N., et al. (2005). *Drosophila tan* encodes a novel hydrolase required in pigmentation and vision. *PLoS Genet.* 1:e63. doi: 10.1371/journal.pgen.0010063
- Wittkopp, P. J., and Beldade, P. (2009). Development and evolution of insect pigmentation: genetic mechanisms and the potential consequences of pleiotropy. *Semin. Cell Dev. Biol.* 20, 65–71. doi: 10.1016/j.semcdb.2008.10.002
- Wittkopp, P. J., Carroll, S. B., and Kopp, A. (2003a). Evolution in black and white: genetic control of pigment patterns in *Drosophila*. *Trends Genet.* 19, 495–504. doi: 10.1016/S0168-9525(03)00194-X
- Wittkopp, P. J., Smith-Winberry, G., Arnold, L. L., Thompson, E. M., Cooley, A. M., Yuan, D. C., et al. (2011). Local adaptation for body color in *Drosophila americana*. *Heredity (Edinb)* 106, 592–602. doi: 10.1038/hdy.2010.90
- Wittkopp, P. J., Stewart, E. E., Arnold, L. L., Neidert, A. H., Haerum, B. K., Thompson, E. M., et al. (2009). Intraspecific polymorphism to interspecific divergence: genetics of pigmentation in *Drosophila*. *Science* 326, 540–544. doi: 10.1126/science.1176980
- Wittkopp, P. J., True, J. R., and Carroll, S. B. (2002). Reciprocal functions of the *Drosophila* yellow and ebony proteins in the development and evolution of pigment patterns. *Development* 129, 1849–1858.
- Wittkopp, P. J., Williams, B. L., Selegue, J. E., and Carroll, S. B. (2003b). *Drosophila* pigmentation evolution: divergent genotypes underlying convergent phenotypes. *Proc. Natl. Acad. Sci. U.S.A.* 100, 1808–1813. doi: 10.1073/pnas.0336368100
- Wray, G. A., Hahn, M. W., Abouheif, E., Balhoff, J. P., Pizer, M., Rockman, M. V., et al. (2003). The evolution of transcriptional regulation in eukaryotes. *Mol. Biol. Evol.* 20, 1377–1419. doi: 10.1093/molbev/msg140

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2020 Lamb, Wang, Simmer, Chung and Wittkopp. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Diversity of UV Reflection Patterns in Odonata

Ryo Futahashi*

Bioproduction Research Institute, National Institute of Advanced Industrial Science and Technology (AIST), Tsukuba, Japan

OPEN ACCESS

Edited by:

Marcus Kronforst,
The University of Chicago,
United States

Reviewed by:

Doekele G. Stavenga,
University of Groningen, Netherlands
David Outomuro,
University of Cincinnati, United States

*Correspondence:

Ryo Futahashi
ryo-futahashi@aist.go.jp

Specialty section:

This article was submitted to
Evolutionary Developmental Biology,
a section of the journal
Frontiers in Ecology and Evolution

Received: 29 February 2020

Accepted: 02 June 2020

Published: 09 July 2020

Citation:

Futahashi R (2020) Diversity of UV
Reflection Patterns in Odonata.
Front. Ecol. Evol. 8:201.
doi: 10.3389/fevo.2020.00201

Dragonflies and damselflies (Odonata) are large-eyed diurnal insects that exhibit a variety of color patterns on their wings and/or bodies. Because Odonata can perceive light with wavelengths extending from ultraviolet (UV) to red, the color patterns with UV reflection can be visually recognized by each other. The UV reflection patterns of Odonata have been investigated by using a UV camera and optically measuring the reflectance of body and wing surfaces. The UV reflection of Odonata can be classified into three classes, namely, iridescent UV reflection, wax-based UV reflection, and UV reflection by epidermal granules. In this review, I present UV images of these three classes and representative examples of reflectance. Among the Odonata with colorful iridescent wings consisting of multilayer structures, some species (e.g., *Chalcopteryx scintillans*) predominantly reflect UV light, whereas other species (e.g., *Calopteryx japonica*) mostly reflect light at wavelengths above 400 nm. Whitish wax (called pruinescence) on the body and/or wing surface strongly reflects light including UV, due to the light-scattering fine structures produced by the wax. The chemical composition of the dragonfly's abdominal UV-reflective wax differs from previously identified waxes of other organisms. The blue-tailed damselflies *Ischnura* species exhibit immature-adult-specific UV reflection by the pteridine pigment-based selective light scattering and absorption. Iridescent coloration and wax-based color changes are generally important for mate recognition and male-male competition in Odonata, although the ecological importance of UV reflection remains largely unknown except for few examples in damselflies.

Keywords: Odonata, dragonfly, damselfly, color, UV reflection, multilayer, wax, pigment

INTRODUCTION

Dragonflies and damselflies (Odonata) are large-eyed diurnal insects, and adults exhibit a variety of color patterns on their wings and/or bodies. These color patterns often represent sexual color dimorphism, color transition via sexual maturation, and intraspecific color polymorphisms within the same sex (Tillyard, 1917; Corbet, 1999; Bybee et al., 2016; Futahashi, 2016). Both dragonflies (suborders Anisozygoptera and Anisoptera) and damselflies (suborder Zygoptera) possess a remarkably large number of visual opsin genes (Futahashi et al., 2015; Suvorov et al., 2017), and electrophysiological studies have shown that Odonata species can perceive light with wavelengths extending from ultraviolet (UV) to red (Meinertzhagen et al., 1983; Yang and Osorio, 1991; Labhart and Nilsson, 1995; Huang et al., 2014; Futahashi et al., 2015), suggesting that the UV reflection patterns can be visually recognized by each other.

The UV reflection pattern of Odonata has been classically investigated using a UV camera, and it has been reported that several Libellulidae dragonflies coated with a whitish wax consistently exhibit UV reflection (Robey, 1975; Silberglied, 1979; Harris et al., 2011; Futahashi et al., 2019). Hilton (1986) comprehensively investigated UV reflection using dried specimens preserved in a museum. Even though 343 species belonging to 16 families were investigated, UV reflection was detected only in one damselfly species, namely *Euphaea amphicyana* (Euphaeidae). As pointed out in this paper, one of the reasons that UV reflection was not observed in almost all the species was that their body color was changed and the surface wax was destroyed by acetone treatment for preparing the dried specimens. Harris et al. (2011) used fresh specimens of 10 species belonging to 4 families and found that the UV reflection pattern often differed between species and sexes, although it was difficult to evaluate the intensity of the UV reflection due to the low UV sensitivity of the equipment.

Aside from the observation using a UV camera, optical measurements of the reflectance of wing and body surfaces have been conducted, and UV reflection has been detected in several Odonata species (Robertson, 1984; Schultz and Fincke, 2009; Guillermo-Ferreira et al., 2014, 2015, 2019; Nixon et al., 2015, 2017; Futahashi et al., 2019; Henze et al., 2019). The UV reflection of Odonata can be classified into the following three classes: iridescent UV reflection, wax-based UV reflection, and UV reflection by epidermal granules. In this review, I show UV photographs of these three classes taken with a high-sensitivity camera (Sony A7S, IDAS UV-VIS mod) and a UV filter (IDAS-U) in the field, as well as representative examples of reflectance from previous reports. I also aim to review the mechanism underlying the UV reflection and its ecological significance.

EXAMPLES AND MECHANISMS OF IRIDESCENT UV REFLECTION

Some Odonata species have colorful iridescent wings and/or bodies (**Figures 1A–C**), and the spectral reflectance and cuticular structures of the iridescent coloration have been reported (Fitzstephens and Getty, 2000; Vukusic et al., 2004; Hariyama et al., 2005; Schultz and Fincke, 2009; Kuitunen and Gorb, 2011; Stavenga et al., 2012; Nixon et al., 2013, 2015; Guillermo-Ferreira et al., 2014, 2015, 2019; Appel et al., 2015). In the iridescent coloration of the wings, some species (e.g., *Chalcopteryx scintillans*, Polythoridae) predominantly reflect UV light (**Figures 2A,B**), whereas other species (e.g., *Calopteryx japonica*, Calopterygidae, **Figures 1C,D**) mostly reflect light at wavelengths above 400 nm (**Figure 2B**; Stavenga et al., 2012; Guillermo-Ferreira et al., 2019). In the flutterer dragonfly *Rhyothemis fuliginosa* (Libellulidae), bluish male wings strongly reflected UV light (**Figure 1A**), while greenish female wings scarcely reflected UV light (**Figure 1B**). Like other insects, the difference in reflectance of the iridescent coloration is primarily attributed to the difference in the multilayer structure that contains several layers with alternating high and low electron density near the wing surface. The resulting reflected

wavelength is dependent on several factors, such as the thickness of the dense layers, the interval between layers, the refractive index of the layers, and the angles of refraction in layers (Kinoshita and Yoshioka, 2005; Stavenga et al., 2011). In general, a thinner multilayer interval produces shorter peaks in the reflected light. In the black-winged damselfly *Calopteryx maculata* (Calopterygidae), the iridescent coloration of male wings can be manipulated experimentally by the changes in diet. The bluish males (resulting from higher food consumption) have thinner layers than the greenish males (Fitzstephens and Getty, 2000). In the damselfly *Matronoides cyaneipennis* (Calopterygidae), males have bluish iridescent coloration on the dorsal hindwings and greenish iridescent coloration on the ventral hindwings (Nixon et al., 2013). There are five layers at both surfaces of the wings; the only difference is that the second layer of the dorsal surface is thinner than that of the ventral surface. Theoretically modeled reflectance shows that this small difference results in a significant change in the peak reflection wavelength (Nixon et al., 2013).

In the flutterer dragonfly *Rhyothemis resplendens* (Libellulidae), the multilayer structure is obviously wrinkled, which causes the light to be scattered at a broader angle than the unwrinkled multilayer structure (Nixon et al., 2015). In addition, the surface wax plays an important role in color tuning and angle-dependent light scattering (Kuitunen and Gorb, 2011; Guillermo-Ferreira et al., 2015, 2019). In the neotropical morpho dragonfly *Zenithoptera lanei* (Libellulidae), the surface wax of the dorsal wings increases brightness and reduces chroma (Guillermo-Ferreira et al., 2015). In the Amazonian damselfly *C. scintillans*, the surface wax of the dorsal wings provides specific angle-dependent light scattering and antireflective properties (Guillermo-Ferreira et al., 2019).

EXAMPLES AND MECHANISMS OF WAX-BASED UV REFLECTION

A wide variety of Odonata species are partly coated with whitish or pale bluish wax (called pruinescence) on body surfaces of the abdomen, thorax, head, and wings (**Figures 1E–G, 2A**). Wax secreted from numerous fine ducts penetrating the cuticle layer increases during adult maturation and is generally well-developed in males than in females, resulting in wax-based, sex-specific color changes (Tillyard, 1917; Jacobs, 1955; Robey, 1975; Gorb, 1995; Corbet, 1999; Futahashi et al., 2019). In general, mature males secrete dense wax predominantly on the dorsal region, whereas mature females secrete pruinose wax mostly on the ventral region and often become slightly whitish with aging (Gorb, 1995; Harris et al., 2011; Futahashi et al., 2019). In the suborder Zygoptera, species with whitish wax are found in most families, whereas in the suborder Anisoptera, species with conspicuous whitish wax are found predominantly in the family Libellulidae (Corbet, 1999). River-dwelling dragonfly species (e.g., Epiophlebiidae, Gomphidae, Chlorogomphidae, Cordulegastridae, and Macromiidae) scarcely secrete whitish wax with a few exceptions like *Gomphurus lynnae* (Gomphidae) (Paulson, 1983; **Figure 2A**). Weak pruinose wax on the ventral

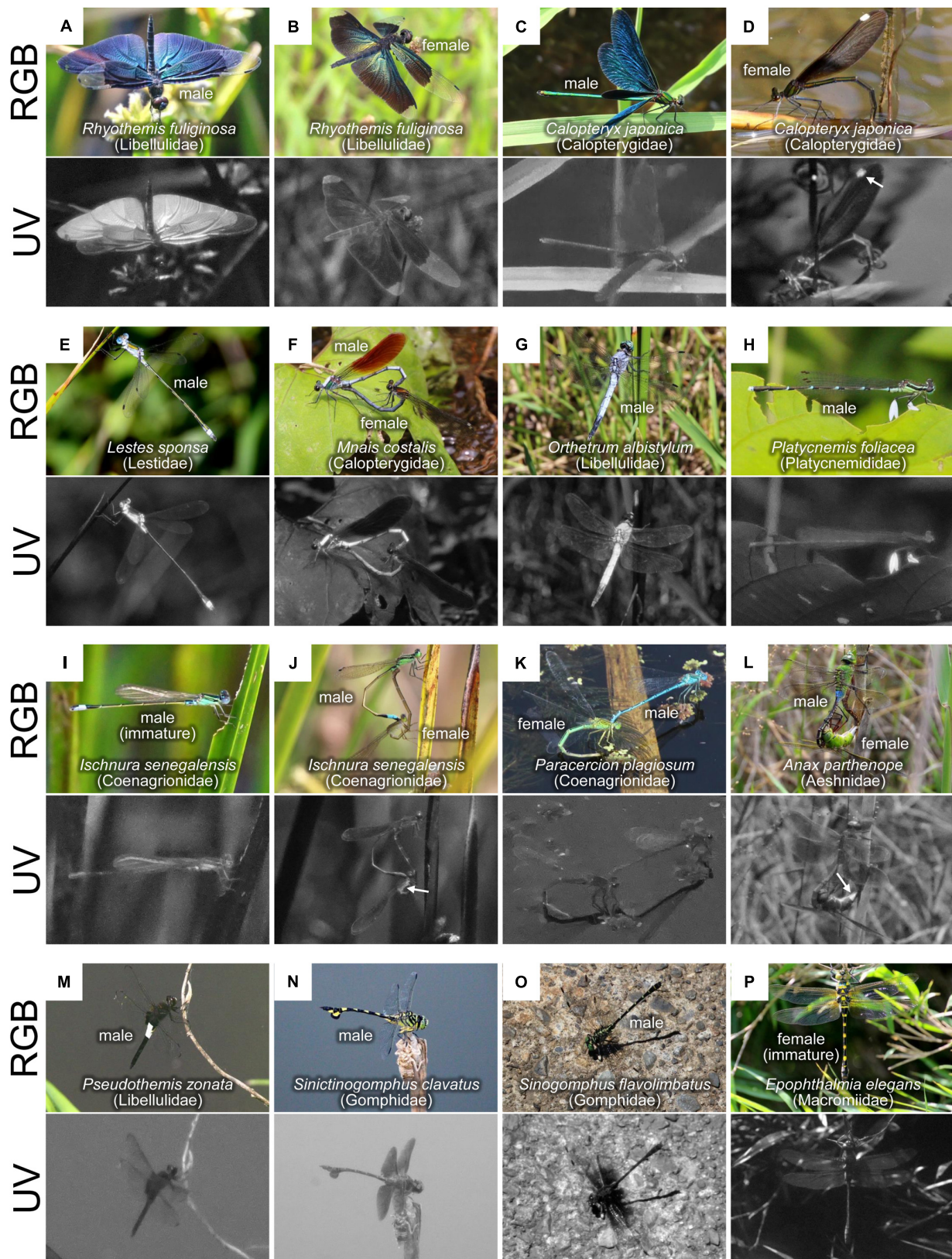


FIGURE 1 | Color pattern diversity in dragonflies as visualized in red, green, blue (RGB), and ultraviolet (UV) light. **(A–P)** Each image was photographed normally (upper) or through a UV filter (lower) in the field. Sex and species names are provided for each image.

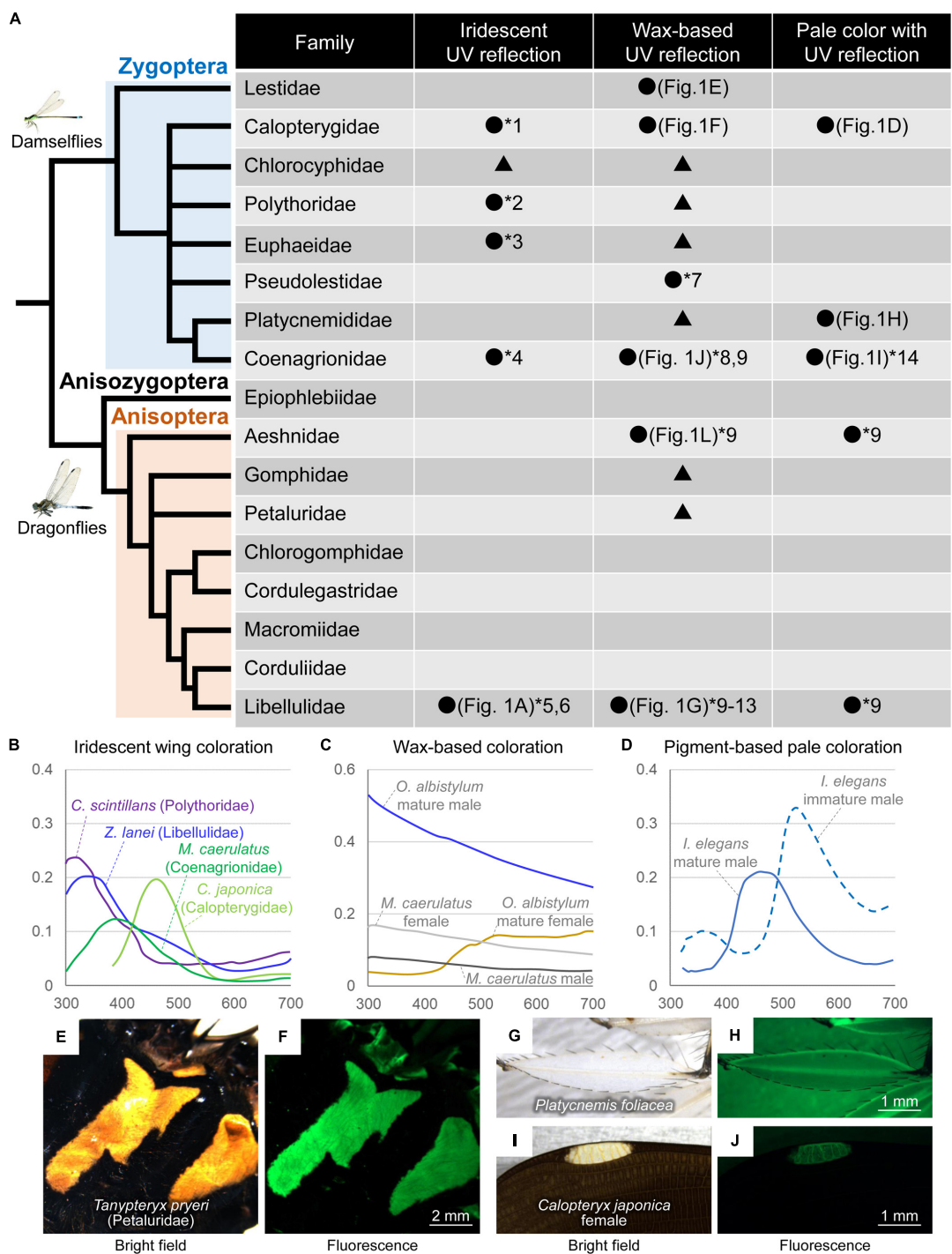


FIGURE 2 | (A) Summary of UV reflection patterns in Odonata. The Odonata phylogeny is modified from Bybee et al. (2016). Black circles and numbers indicate that UV reflection has been confirmed due to reflectance and/or UV images as follows: *1, Guillermo-Ferreira et al., 2014 Reflectance; *2, Guillermo-Ferreira et al., 2019 Reflectance; *3, Hilton, 1986 UV images; *4, Schultz and Fincke, 2009 Reflectance; *5, Nixon et al., 2015 Reflectance; *6, Guillermo-Ferreira et al., 2015 Reflectance; *7, Nixon et al., 2017 Reflectance; *8, Schultz and Fincke, 2009 Reflectance; *9, Harris et al., 2011 UV images; *10, Robey, 1975 UV images; *11, Silberglied, 1979 UV images; *12, Robertson, 1984 Reflectance; *13, Futahashi et al., 2019 Reflectance and UV images; *14, Henze et al., 2019 Reflectance. Black triangles indicate that iridescent coloration and/or wax-based whitish color are recognized although UV reflection has not been investigated. **(B)** Examples of reflectance of iridescent dorsal wing coloration based on Schultz and Fincke (2009), Stavenga et al. (2012), and Guillermo-Ferreira et al. (2015, 2019). **(C)** Examples of reflectance of wax-based coloration based on Schultz and Fincke (2009) and Futahashi et al. (2019). **(D)** Examples of reflectance of pigment-based thoracic pale coloration based on Henze et al. (2019). **(E–J)** Bright field and green fluorescence images of yellow-white coloration in Odonata. **(E,F)** Thorax of *Tanypteryx pryeri* male. **(G,H)** An enlarged region of a hindleg of *P. foliacea* male. **(I,J)** Pseudopterostigma of *C. japonica* female.

thorax and abdomen is detected in several families, such as Aeshnidae (Harris et al., 2011; **Figures 1J,L**, arrows). The surface wax can be removed by rubbing and dissolves completely in chloroform (Schultz and Fincke, 2009; Guillermo-Ferreira et al., 2015, 2019; Futahashi et al., 2019).

In Libellulidae dragonflies, the abdominal surface wax strongly reflects both UV and visible light (Robey, 1975; Silberglied, 1979; Robertson, 1984; Harris et al., 2011; Futahashi et al., 2019). UV photographs confirmed that the whitish surface wax caused UV reflection in all examined species (**Figures 1E–G**). Based on its fine structure, the abdominal surface wax can be classified into two types, lamellar type (detected in Lestidae and Libellulidae) and filamentous type (detected in Calopterygidae and Euphaeidae) (Gorb, 1995). The sizes of wax lamellae on the abdomen differ between the dorsal and ventral regions as well as between species and sexes (Gorb, 1995; Futahashi et al., 2019). The whitish color of the wax-secreted region temporarily blackens when light scattering is disturbed by the application of acetone, while it irreversibly blackens when the wax is removed by chloroform treatment, suggesting that wax-based fine structures are responsible for the whitish color with UV reflection (Futahashi et al., 2019). The whitish wax also appears on the wing surface of several Odonata species, forming micropillar or filamentous structures (Gorb et al., 2000; Schultz and Fincke, 2009; Guillermo-Ferreira et al., 2015, 2019; Nixon et al., 2017). In the neotropical helicopter damselfly *Megaloprepus caerulatus* (Coenagrionidae, formerly known as Pseudostigmatidae), white bands on the wings are coated with longer and thicker structures compared to the bluish iridescent and hyaline wing regions (Schultz and Fincke, 2009). Chloroform treatment removes the wax on the wing surface, turning white color into transparent (Schultz and Fincke, 2009).

The reflectance of the dorsal abdomen of Libellulidae species shows that mature males with whitish wax represent a spectrum that decreases gradually from 300 to 700 nm (**Figure 2C**; Robertson, 1984; Futahashi et al., 2019). Meanwhile, the dorsal abdomen of mature females (without the pruinose wax) exhibits little UV reflection and mainly reflects light at wavelengths above 500 nm (**Figure 2C**; Futahashi et al., 2019). The reflectance of the whitish wing markings shows a flat spectrum (Schultz and Fincke, 2009; Xu and Fincke, 2015; Nixon et al., 2017). In *M. caerulatus*, sex-specific white bands appear on the wings, and white bands on females exhibit stronger reflection than those on males (**Figure 2C**; Schultz and Fincke, 2009; Xu and Fincke, 2015).

In the Libellulidae species, chemical analysis has revealed that very long-chain aldehydes and very long-chain methyl ketones are the major components of the abdominal wax. The chemically synthesized compound of very long-chain methyl ketone, which is the main UV-reflective wax component of mature males of *Orthetrum albistylum* (Libellulidae) (**Figure 1G**), spontaneously forms light-scattering fine structures with strong UV reflection (Futahashi et al., 2019), suggesting that the secreted very long-chain methyl ketones play a crucial role in UV reflection. The comparative transcriptome in different stages, sexes, and abdominal regions of *O. albistylum* has revealed that the *ELOVL17* gene, a member of the elongation of the very long-chain fatty acid (ELOVL) protein family,

is specifically expressed in the region where very long-chain methyl ketones are present, suggesting that this gene is involved in the production of very long-chain methyl ketones (Futahashi et al., 2019). Wax-based UV reflection has been also reported in several other organisms (Byers, 1975; Pope, 1979); however, the chemical composition of dragonfly abdominal wax differs from previously identified waxes of other organisms. Because the wax-based body color changes in the Libellulidae dragonflies are likely to have evolved independently of those in other Odonata (**Figure 2A**), further research is needed to determine whether the wax component and the genes involved in wax production are the same in other Odonata species.

EXAMPLES AND MECHANISMS OF PIGMENT-BASED PALE COLOR WITH UV REFLECTION

Recently, another type of UV reflection has been reported, namely, UV reflection by epidermal granules containing pteridine pigments. UV reflection is generally weak or undetectable in the blue-green, yellow, and white thoracic and abdominal markings of most Odonata species (**Figures 1H–P**; Hilton, 1986). Notably, UV reflection has been sometimes confirmed in pale-colored regions without whitish wax (**Figure 2A**; Harris et al., 2011; Henze et al., 2019). For example, in the blue-tailed damselflies *Ischnura* species (Coenagrionidae), remarkable UV reflection has been recognized in the blue-green thoracic regions of males and andromorphic females only during the immature adult stage, and UV reflection decreases rapidly as they mature (**Figures 1I,J**, **2D**; Henze et al., 2019). Such non-iridescent light blue-green coloration of dragonflies can be explained by coherent scattering due to light-scattering granules containing pteridine pigments in the distal layer of the epidermis and light-absorbing granules containing ommochrome pigments in the proximal layer of the epidermis (Veron, 1974; Prum et al., 2004; Henze et al., 2019). Some pteridine pigments (e.g., leucopterin and xanthopterin) absorb light predominantly in the UV range, and the amount and composition of pteridine pigments cause sex- and species-specific differences in UV reflection in the wings of pierid butterflies (Makino et al., 1952; Stavenga et al., 2004; Rutowski et al., 2005; Giraldo and Stavenga, 2007; Morehouse et al., 2007). In *Ischnura* damselflies, reflectance at different adult developmental stages is associated with changes in pteridine pigments (probably erythropterin and xanthopterin) within epidermal nanospheres (Henze et al., 2019). The calculated reflectance spectra show that erythropterin-containing nanospheres reflect light with two peaks in the green and UV wavelength range, whereas xanthopterin-containing nanospheres reflect light in the blue wavelength range (Henze et al., 2019), corresponding to the reflectance of immature or mature males, respectively (**Figure 2D**).

In males of the featherleg damselfly *Platycnemis foliacea* (Platycnemididae), UV reflection was observed on the white petal-like enlarged regions of the legs, although the central lines of the white legs, whitish thoracic, and abdominal

markings scarcely reflected UV light (**Figure 1H**). Moreover, UV reflection was also prominent at the white spot called the pseudopterstigma on the female wings of *C. japonica* (Calopterygidae) (**Figure 1D**, arrow). Yellowish pigments in Odonata have been considered to be pteridine pigments (Krieger, 1954; Vilela et al., 2017), and pteridine pigments often emit strong fluorescence under UV light (Kayser, 1985). Fluorescence signals were generally observed with yellowish markings (**Figures 2E,F**), the edge and central line of the white legs of *P. foliacea* (**Figures 2G,H**), and the wing veins of the pseudopterstigma region of *C. japonica* (**Figures 2I,J**), probably due to pteridine pigments. Meanwhile, the fluorescence signals were weak in the UV-reflective regions, such as white petal-like regions on the legs of *P. foliacea* (**Figures 2G,H**) and the whitish wing membranes in the pseudopterstigma of *C. japonica* females (**Figures 2I,J**), suggesting that UV-reflective white colors are probably due to substances other than pteridine pigments. Identification of pigments in various Odonata species deserves future study.

ECOLOGICAL SIGNIFICANCE OF UV REFLECTION AND ABSORPTION

Since individuals of Odonata species visually recognize each other, body color patterns are generally important for mate recognition and male–male competition. Species with iridescent wings are found mainly in Zygoptera species (**Figure 2A**). This is likely because Zygoptera species fly more slowly than Anisoptera species (Rüppell, 1989), and species with colored wings often pause their wings during encounters (Hilfert-Rüppell and Rüppell, 2013). Also, courtship behavior is found predominantly in Zygoptera species (e.g., Calopterygidae), as males usually display their iridescent wings to females and rival males (Waage, 1973, 1988; Siva-Jothy, 1999; Hariyama et al., 2005; Nixon et al., 2013; Günther et al., 2014). The reflectance patterns suggest that the UV reflection seems to be important for some Odonata species, such as *C. scintillans* and *Z. lanei* (**Figure 2B**). In *C. scintillans*, the winners of territorial fights have higher values for the wing pigmentation and the hue of the wing's dorsal surface (Guillermo-Ferreira et al., 2019). In *Z. lanei*, males with distal half of their dorsal wings painted black reduce aggression from rivals, suggesting that iridescent wing coloration is important for male-male competition (Guillermo-Ferreira et al., 2015).

In *M. caerulatus*, both males and females have UV-blue iridescent markings and UV-reflective white bands on their wings (**Figures 2B,C**), although the position of white bands differs between sexes. Behavioral experiments suggest that the white band pattern on females helps males to distinguish potential mates from rivals (Schultz and Fincke, 2009). When UV reflection is artificially suppressed by using a UV-absorbing sunscreen agent, males with white bands that are less UV-reflective are more likely to lose territorial fights, although the UV reflectivity of the female's white bands does not affect the male's mate recognition (Xu and Fincke, 2015). In the damselfly *Mnesarete pudica* (Calopterygidae), both

mature males and mature females have UV-reflective coloration on their wings. Behavioral experiments with reducing UV reflection on their wings show that UV reflection enhances both sexual and aggressive responses, while male-specific red wing pigmentation is important for mate recognition (Guillermo-Ferreira et al., 2014).

Wax-based abdominal color changes are also important for mate recognition and male–male competition in many Odonata species (Jacobs, 1955; Robey, 1975; Corbet, 1999), although it remains unclear whether UV reflection itself is important. Moreover, a wax-based whitish color may help thermoregulation and provide protection against UV-induced damage by reflecting UV radiation (Paulson, 1983; Ubukata, 1985; Futahashi et al., 2019). Species that prefer sunny habitats tend to show stronger UV reflection, like mature males of *O. albistylum* (**Figures 1G, 2C**; Paulson, 1983; Futahashi et al., 2019). Given that most dragonfly species with ventral wax in females mate in sunny places without flying (**Figure 1F**; Sugimura et al., 2001; Ozono et al., 2017), it seems plausible that the UV-reflective wax in females protects the ventral abdomen, including ovaries, from UV damage (Futahashi et al., 2019).

Compared with iridescent coloration and the wax-based whitish color, little study has been conducted about the pigment-dependent differences in UV reflection. It should be noted that males of *I. elegans* prefer mature females with little UV reflection to immature females (Van Gossum et al., 2001). Males of *P. foliacea* display their enlarged white legs to females, as well as to rival males, and *C. japonica* males first grab white pseudopterstigmata on the female's wings when connecting with a female (Sugimura et al., 2001; Ozono et al., 2017). The importance of UV reflection in these behaviors should be confirmed experimentally, like *M. caerulatus* and *M. pudica*.

Other than reflection, UV light is absorbed by pigments or transmitted due to absence of pigments. Hilton (1986) reported that the wings transmitted UV light without absorbing or reflecting it in about half of the species examined, and yellow, orange, and brown wing markings generally exhibited UV absorption (**Figures 1B–D,E,M–O**). Notably, conspicuous UV absorption has been observed in the almost hyaline (very faint yellow to human eyes) wings in some dragonflies, especially in Gomphidae species (**Figures 1N,O**; Hilton, 1986). Also, these UV absorption patterns often differ between sexes and species. For example, in the dragonfly *Phanogomphus hodgesi* (Gomphidae), UV light is absorbed in male wings and transmitted in female wings, although both males and females have similar hyaline wings (Hilton, 1986). In the male of *Sinogomphus flavolimbatus* (Gomphidae), a UV absorption pattern was detected at the base of the wings (**Figure 1O**), which is reminiscent of a UV-absorbing flower nectar guide. These examples suggest that sex- and species-specific UV absorption should also be considered for examining the importance of body color.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

FUNDING

The author work was supported by JSPS KAKENHI Grant Numbers JP26660276, JP26711021, JP18H02491, JP18H04893, and JP19H03287.

REFERENCES

- Appel, E., Heepe, L., Lin, C. P., and Gorb, S. N. (2015). Ultrastructure of dragonfly wing veins: composite structure of fibrous material supplemented by resilin. *J. Anat.* 227, 561–582. doi: 10.1111/joa.12362
- Bybee, S., Cordoba-Aguilar, A., Duryea, M. C., Futahashi, R., Hansson, B., Lorenzo-Carballa, M. O., et al. (2016). Odonata (dragonflies and damselflies) as a bridge between ecology and evolutionary genomics. *Front. Zool.* 13:46. doi: 10.1186/s12983-016-0176-7
- Byers, J. R. (1975). Tyndall blue and surface white of tent caterpillars, *Malacosoma* spp. *J. Insect Physiol.* 21, 401–415. doi: 10.1016/0022-1910(75)90033-5
- Corbet, P. S. (1999). *Dragonflies, Behavior and Ecology of Odonata*. Ithaca: Cornell University Press.
- Fitzstephens, D. M., and Getty, T. (2000). Colour, fat and social status in male damselflies, *Calopteryx maculata*. *Anim. Behav.* 60, 851–855. doi: 10.1006/anbe.2000.1548
- Futahashi, R. (2016). Color vision and color formation in dragonflies. *Curr. Opin. Insect Sci.* 17, 32–39. doi: 10.1016/j.cois.2016.05.014
- Futahashi, R., Kawahara-Miki, R., Kinoshita, M., Yoshitake, K., Yajima, S., Arikawa, K., et al. (2015). Extraordinary diversity of visual opsin genes in dragonflies. *Proc. Natl. Acad. Sci. U.S.A.* 112, E1247–E1256.
- Futahashi, R., Yamahama, Y., Kawaguchi, M., Mori, N., Ishii, D., Okude, G., et al. (2019). Molecular basis of wax-based color change and UV reflection in dragonflies. *eLife* 8:e43045.
- Giraldo, M. A., and Stavenga, D. G. (2007). Sexual dichroism and pigment localization in the wing scales of *Pieris rapae* butterflies. *Proc. Biol. Sci.* 274, 97–102. doi: 10.1098/rspb.2006.3708
- Gorb, S. N. (1995). Scanning electron microscopy of pruinosity in Odonata. *Odonatologica* 24, 225–228.
- Gorb, S. N., Kesel, A., and Berger, J. (2000). Microsculpture of the wing surface in Odonata: evidence for cuticular wax covering. *Arthropod. Struct. Dev.* 29, 129–135. doi: 10.1016/S1467-8039(00)00020-7
- Guillermo-Ferreira, R., Bispo, P. C., Appel, E., Kovalev, A., and Gorb, S. N. (2015). Mechanism of the wing coloration in the dragonfly *Zenithoptera lanei* (Odonata: Libellulidae) and its role in intraspecific communication. *J. Insect Physiol.* 81, 129–136. doi: 10.1016/j.jinsphys.2015.07.010
- Guillermo-Ferreira, R., Bispo, P. C., Appel, E., Kovalev, A., and Gorb, S. N. (2019). Structural coloration predicts the outcome of male contests in the Amazonian damselfly *Chalcopteryx scintillans* (Odonata: Polythoridae). *Arthropod Struct. Dev.* 53:100884. doi: 10.1016/j.asd.2019.100884
- Guillermo-Ferreira, R., Therézio, E. M., Gehlen, M. H., Bispo, P. C., and Marletta, A. (2014). The role of wing pigmentation, UV and fluorescence as signals in a Neotropical damselfly. *J. Insect Behav.* 27, 67–80. doi: 10.1007/s10905-013-9406-4
- Günther, A., Hilfert-Rüppell, D., and Rüppell, G. (2014). Reproductive behaviour and the system of signalling in *Neurobasis chinensis* - a kinematic analysis. *Int. J. Odonatol.* 17, 31–52. doi: 10.1080/13887890.2014.881305
- Hariyama, T., Hironaka, M., Horiguchi, H., and Stavenga, D. G. (2005). “The leaf beetle, the jewel beetle, and the damselfly; insects with a multilayered show case,” in *Structural Colors in Biological Systems: Principles and Applications*, eds T. Shimozawa and T. Hariyama (Osaka: Osaka University Press), 153–176.
- Harris, W. E., Forman, D. W., Battell, R. D., Battell, M. T. R., Nelson, A. K., and Brain, P. F. (2011). Odonata colour: more than meets the eye? *Int. J. Odonatol.* 14, 281–289.
- Henze, M. J., Lind, O., Wilts, B. D., and Kelber, A. (2019). Pterin-pigmented nanospheres create the colours of the polymorphic damselfly *Ischnura elegans*. *J. R. Soc. Interface* 16:20180785. doi: 10.1098/rsif.2018.0785
- Hilfert-Rüppell, D., and Rüppell, G. (2013). Do coloured-winged damselflies and dragonflies have flight kinematics different from those with clear wings? *Int. J. Odonatol.* 16, 119–134. doi: 10.1080/13887890.2013.763332
- Hilton, D. F. J. (1986). A survey of some Odonata for ultraviolet patterns. *Odonatologica* 15, 335–345.
- Huang, S. C., Chiou, T. H., Marshall, J., and Reinhard, J. (2014). Spectral sensitivities and color signals in a polymorphic damselfly. *PLoS One* 9:e87972. doi: 10.1371/journal.pone.0087972
- Jacobs, M. E. (1955). Studies on territorialism and sexual selection in dragonflies. *Ecology* 36, 566–586. doi: 10.2307/1931296
- Kayser, H. (1985). “Pigments,” in *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, eds G. A. Kerkut and L. I. Gilbert (New York, NY: Pergamon), 367–415.
- Kinoshita, S., and Yoshioka, S. (2005). Structural colors in nature: the role of regularity and irregularity in the structure. *Chem. Phys. Chem.* 6, 1442–1459. doi: 10.1002/cphc.200500007
- Krieger, F. (1954). Untersuchungen über den Farbwechsel der Libellenlarven. *Z. vergl. Physiology* 36, 352–366. doi: 10.1007/bf00345026
- Kuitunen, K., and Gorb, S. N. (2011). Effects of cuticle structure and crystalline wax coverage on the coloration in young and old males of *Calopteryx splendens* and *Calopteryx virgo*. *Zoology* 114, 129–139. doi: 10.1016/j.zool.2011.01.003
- Labhart, T., and Nilsson, D. E. (1995). The dorsal eye of the dragonfly *Sympetrum*: specializations for prey detection against the blue sky. *J. Comp. Physiol. A* 176, 437–453.
- Makino, K., Satoh, K., Koike, M., and Ueno, N. (1952). Sex in *Pieris rapae* L. and the pteridin content of their wings. *Nature* 170, 933–934. doi: 10.1038/170933a0
- Meinertzhagen, I. A., Menzel, R., and Kahle, G. (1983). The identification of spectral receptor types in the retina and lamina of the dragonfly *Sympetrum rubicundulum*. *J. Comp. Physiol.* 15, 295–310. doi: 10.1007/bf00623906
- Morehouse, N. I., Vukusic, P., and Rutowski, R. (2007). Pterin pigment granules are responsible for both broadband light scattering and wavelength selective absorption in the wing scales of pierid butterflies. *Proc. Biol. Sci.* 274, 359–366. doi: 10.1098/rspb.2006.3730
- Nixon, M. R., Orr, A. G., and Vukusic, P. (2013). Subtle design changes control the difference in colour reflection from the dorsal and ventral wing-membrane surfaces of the damselfly *Matronoides cyaneipennis*. *Opt. Express* 21, 1479–1488.
- Nixon, M. R., Orr, A. G., and Vukusic, P. (2015). Wrinkles enhance the diffuse reflection from the dragonfly *Rhyothemis resplendens*. *J. R. Soc. Interface* 12:20140749. doi: 10.1098/rsif.2014.0749
- Nixon, M. R., Orr, A. G., and Vukusic, P. (2017). Covert linear polarization signatures from brilliant white two-dimensional disordered wing structures of the phoenix damselfly. *J. R. Soc. Interface* 14:20170036. doi: 10.1098/rsif.2017.0036
- Ozono, A., Kawashima, I., and Futahashi, R. (2017). *Dragonflies of Japan*, 3rd Edn. Tokyo: Bunichi-Sogo Syuppan, Co Ltd.
- Paulson, D. R. (1983). A new species of dragonfly *Gomphus (Gomphunis) lynnae* sp. nov. from the Yakima River, Washington, with notes on pruinosity in Gomphidae (Anisoptera). *Odonatologica* 12, 59–70.
- Pope, R. D. (1979). Wax production by coccinellid larvae (Coleoptera). *Syst. Entomol.* 4, 171–196. doi: 10.1111/j.1365-3113.1979.tb00632.x
- Prum, R. O., Cole, J. A., and Torres, R. H. (2004). Blue integumentary structural colours in dragonflies (Odonata) are not produced by incoherent Tyndall scattering. *J. Exp. Biol.* 207, 3999–4009. doi: 10.1242/jeb.01240
- Robertson, H. M. (1984). Pruinosity in odonates reflects UV. *Nolul. Odonatol.* 2, 68–69.
- Robey, C. W. (1975). Observations on breeding behavior of *Pachydiplax longipennis* (Odonata: Libellulidae). *Psyche* 82, 89–96.
- Rüppell, G. (1989). Kinematic analysis of symmetrical flight manoeuvres of Odonata. *J. Exp. Biol.* 144, 13–42.
- Rutowski, R. L., Macedonia, J. M., Morehouse, N., and Taylor-Taft, L. (2005). Pterin pigments amplify iridescent ultraviolet signal in males of the orange sulphur butterfly, *Colias eurytheme*. *Proc. Biol. Sci.* 272, 2329–2335. doi: 10.1098/rspb.2005.3216

ACKNOWLEDGMENTS

I would like to thank Tohru Usui for technical advice on taking UV photographs, and Genta Okude, Mizuko Osanai-Futahashi, and two reviewers for helpful comments of the manuscript.

- Schultz, T., and Fincke, O. (2009). Structural colours create a flashing cue for sexual recognition and male quality in a Neotropical giant damselfly. *Funct. Ecol.* 23, 724–732. doi: 10.1111/j.1365-2435.2009.01584.x
- Silberglied, R. E. (1979). Communication in the ultraviolet. *Ann. Rev. Ecol. Syst.* 10, 373–398.
- Siva-Jothy, M. T. (1999). Male wing pigmentation may affect reproductive success via female choice in a calopterygid damselfly (Zygoptera). *Behaviour* 136, 1365–1377. doi: 10.1163/156853999500776
- Stavenga, D. G., Leertouwer, H. L., Hariyama, T., De Raedt, H. A., and Wilts, B. D. (2012). Sexual dichromatism of the damselfly *Calopteryx japonica* caused by a melanin-chitin multilayer in the male wing veins. *PLoS One* 7:e49743. doi: 10.1371/journal.pone.0049743
- Stavenga, D. G., Stowe, S., Siebke, K., Zeil, J., and Arikawa, K. (2004). Butterfly wing colours: scale beads make white pierid wings brighter. *Proc. R. Soc. B* 271, 1577–1584. doi: 10.1098/rspb.2004.2781
- Stavenga, D. G., Wilts, B. D., Leertouwer, H. L., and Hariyama, T. (2011). Polarized iridescence of the multilayered elytra of the Japanese jewel beetle, *Chrysochroa fulgidissima*. *Philos. Trans. R. Soc. Lond. B* 366, 709–723. doi: 10.1098/rstb.2010.0197
- Sugimura, M., Ishida, S., Kojima, K., Ishida, K., and Aoki, T. (2001). *Dragonflies of the Japanese Archipelago in Color*. Sapporo: Hokkaido Univ Press.
- Suvorov, A., Jensen, N. O., Sharkey, C. R., Fujimoto, M. S., Bodily, P., Wightman, H. M., et al. (2017). Opsins have evolved under the permanent heterozygote model: insights from phylotranscriptomics of Odonata. *Mol. Ecol.* 26, 1306–1322. doi: 10.1111/mec.13884
- Tillyard, R. J. (1917). *The Biology of Dragonflies*. Cambridge, MA: Cambridge Univ Press.
- Ubukata, H. (1985). The Odonata collected at Mt. Taisetsu in 1984, with a discussion on the pruinescent female of *Orthetrum albistylum speciosum*. *Silvicola* 3, 23–26.
- Van Gossum, H., Stoks, R., and De Bruyn, L. (2001). Discriminative mate choice in relation with female maturation in *Ischnura elegans* (Odonata: Coenagrionidae). *Int. J. Odonatol.* 4, 83–91. doi: 10.1080/13887890.2001.9748161
- Veron, J. E. (1974). Physiological colour changes in Odonata eyes. A comparison between eye and epidermal chromatophore pigment migrations. *J. Insect Physiol.* 20, 1491–1505. doi: 10.1016/0022-1910(74)90081-x
- Vilela, D. S., Tosta, T. A. A., Rodrigues, R. R., Del-Claro, K., and Guillermo-Ferreira, R. (2017). Colours of war: visual signals may influence the outcome of territorial contests in the tiger damselfly, *Tigriagrion aurantinigrum*. *Biol. J. Linn. Soc.* 121, 786–795. doi: 10.1093/biolinnean/blx024
- Vukusic, P., Wootton, R. J., and Sambles, J. R. (2004). Remarkable iridescence in the hindwings of the damselfly *Neurobasis chinensis chinensis* (Linnaeus) (Zygoptera: Calopterygidae). *Proc. Biol. Sci.* 271, 595–601. doi: 10.1098/rspb.2003.2595
- Waage, J. K. (1973). Reproductive behavior and its relation to territoriality in *Calopteryx maculata* (Beauvois) (Odonata: Calopterygidae). *Behaviour* 47, 240–256. doi: 10.1163/156853973x00094
- Waage, J. K. (1988). Confusion over residency and the escalation of damselfly territorial disputes. *Anim. Behav.* 36, 586–595. doi: 10.1016/s0003-3472(88)80030-7
- Xu, M., and Fincke, O. M. (2015). Ultraviolet wing signal affects territorial contest outcome in a sexually dimorphic damselfly. *Anim. Behav.* 101, 67–74. doi: 10.1016/j.anbehav.2014.12.018
- Yang, E. C., and Osorio, D. (1991). Spectral sensitivities of photoreceptors and lamina monopolar cells in the dragonfly, *Hemicordulia tau*. *J. Comp. Physiol. A* 169, 663–669.

Conflict of Interest: The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2020 Futahashi. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Avian Pigment Pattern Formation: Developmental Control of Macro- (Across the Body) and Micro- (Within a Feather) Level of Pigment Patterns

Masafumi Inaba and Cheng-Ming Chuong*

Department of Pathology, Keck School of Medicine, University of Southern California, Los Angeles, CA, United States

OPEN ACCESS

Edited by:

Ricardo Mallarino,
Princeton University, United States

Reviewed by:

Evan P. Kingsley,
Harvard Medical School,
United States
Pedro Martinez,
University of Barcelona, Spain

*Correspondence:

Cheng-Ming Chuong
cmchuong@usc.edu

Specialty section:

This article was submitted to
Evolutionary Developmental Biology,
a section of the journal
Frontiers in Cell and Developmental
Biology

Received: 04 March 2020

Accepted: 22 June 2020

Published: 10 July 2020

Citation:

Inaba M and Chuong C-M (2020)
Avian Pigment Pattern Formation:
Developmental Control of Macro-
(Across the Body) and Micro- (Within
a Feather) Level of Pigment Patterns.
Front. Cell Dev. Biol. 8:620.
doi: 10.3389/fcell.2020.00620

Animal color patterns are of interest to many fields, such as developmental biology, evolutionary biology, ethology, mathematical biology, bio-mimetics, etc. The skin provides easy access to experimentation and analysis enabling the developmental pigment patterning process to be analyzed at the cellular and molecular level. Studies in animals with distinct pigment patterns (such as zebrafish, horse, feline, etc.) have revealed some genetic information underlying color pattern formation. Yet, how the complex pigment patterns in diverse avian species are established remains an open question. Here we summarize recent progress. Avian plumage shows color patterns occurring at different spatial levels. The two main levels are macro- (across the body) and micro- (within a feather) pigment patterns. At the cellular level, colors are mainly produced by melanocytes generating eumelanin (black) and pheomelanin (yellow, orange). These melanin-based patterns are regulated by melanocyte migration, differentiation, cell death, and/or interaction with neighboring skin cells. In addition, non-melanin chemical pigments and structural colors add more colors to the available palette in different cell types or skin regions. We discuss classic and recent tissue transplantation experiments that explore the avian pigment patterning process and some potential molecular mechanisms. We find color patterns can be controlled autonomously by melanocytes but also non-autonomously by dermal cells. Complex plumage color patterns are generated by the combination of these multi-scale patterning mechanisms. These interactions can be further modulated by environmental factors such as sex hormones, which generate striking sexual dimorphic colors in avian integuments and can also be influenced by seasons and aging.

Keywords: morphogenesis, melanocyte, regional specificity, integument, skin appendages, color, stripes, Evo-Devo

INTRODUCTION

Many animals exhibit color patterns on their skin surface, which serve the function for inter- and intra-species communications. These patterns can be either highly vivid to draw attention from others in their local environment or drab to provide camouflage so they can blend into their environment. How these color patterns form is a fundamental question of scientific interest.

Pigment patterns have been studied in different animals using different approaches. In zebrafish, relatively simple pigment patterns and strong genetics allowed scientists to discover genes related to pigment patterning and cell-cell interactions that govern patterning (Watanabe and Kondo, 2015; Irion et al., 2016; Patterson and Parichy, 2019). In mice, genetic studies have revealed many pigment-related mutants (Bennett and Lamoreux, 2003). Recent progress of genetic and genome-wide analysis led to the identification of several important genes involved in pigment patterning in non-model animals, such as the cheetah, horse, and chipmunks (Kaelin et al., 2012; Imsland et al., 2016; Mallarino et al., 2016).

Although the complex pigment patterns and accessibility for analyses make birds a fascinating model to study morphogenesis, little is known about patterning mechanisms. Here we summarize recent results from our and other's studies. Bird's colorations emerge in their skin and/or feathers as striped, spotted, and more complex patterns, which provide us with an excellent platform to study biological pattern formation. Birds have a variety of ways of producing colors (several types of pigments and structural color) as well as a multiscale hierarchy in their macro- (across body/domains) to micro- (within feathers) pattern distributions. The largest macro-pigment pattern covers the whole-body pattern (**Figure 1A**). Most birds exhibit a specific color on a specific region, such as the head, back, wing, and tail. This implies that a long-range patterning mechanism enables the specification of each domain across the whole body. Each domain often exhibits regular pigment patterns, such as stripes with different sizes (**Figure 1B**). Lastly, the pigment patterns within a feather form micro-patterns (**Figure 1C**). So far, many attempts to understand the mechanism of avian pigment patterning have been performed, especially in different domains and within feather patterns, from the viewpoint of genetics, developmental biology, or mathematical biology. Domestic fowl genetics has brought understanding into the molecular basis of pigmentation (Kerje et al., 2003; Ishishita et al., 2018; Andersson et al., 2020). Developmental biology approaches using transplantation of embryonic tissues have revealed the important interactions between pigment cells and skin tissues that contribute to pigment patterns (Haupaix et al., 2018; Inaba et al., 2019b). In this review, we discuss the known mechanisms of avian pigmentation and how the pigmentation is regulated to make spatial patterns at different scales (skin domain and feather level). We also present possible future directions that can help unravel the complex avian pigment patterns.

MOLECULAR MECHANISM OF COLORATIONS: MELANIN, NON-MELANIN CHEMICAL PIGMENT, AND STRUCTURAL COLORS

Bird colorations are derived from pigments and structural colors. The pigments are chemical substances that directly absorb and reflect specific wavelengths of light. Birds exploit different groups of pigments: melanins, produced by melanocytes, are the main

determinant of the skin and feather color (Galván and Solano, 2016). There are two melanin types: eumelanin (black) and pheomelanin (yellow to orange). Switching melanin type and the regulation of melanin density play an important role in forming pigment patterns in a wide range of animals (Hoekstra, 2006). There are also chemical pigments that are not derived from melanocytes. The non-melanin pigments include carotenoids (yellow, orange, red, and purple), pterins (yellow and red), porphyrins (red, brown, and green), or psittacofulvins (yellow and red) (Lucas and Stettenheim, 1972; Galván and Solano, 2016; Lopes et al., 2016; Cooke et al., 2017).

In addition, structural colors are also important factors that confer shiny colors on birds. Generally, structural colors occur by the interaction of light with nanoscale structures, such as melanosomes or keratin filaments in feathers (Yoshioka and Kinoshita, 2002; Zi et al., 2003; Maia et al., 2012). A combination of chemical and structural colors can expand the variation of colors displayed as seen in parrots (Cooke et al., 2017). In this minireview, we will focus more on the control of melanin pigments.

In vertebrates, melanins are produced by melanocytes (Schartl et al., 2016). Melanocytes in the skin are derived from neural crest cells (NCCs), while those in the retina (retinal pigment epithelium) are derived from optic neuroepithelium. Melanocyte progenitor cells (melanoblasts) express microphthalmia-associated transcription factor (MITF), a master regulator of melanocyte development. During maturation, melanocytes express tyrosinase (TYR) and tyrosinase-related protein 1 (TYRP1), enzymes that produce melanin pigment. Melanocytes contain organelles called melanosomes where melanins are synthesized and accumulated. Mature melanosomes are transferred to adjacent keratinocytes through melanocyte dendrites. So far, different models of melanosome transfer have been proposed (Wu and Hammer, 2014). A recent study using live imaging of chicken embryonic skin revealed that melanosomes are transferred by vesicles derived from the melanocyte plasma membrane (Tadokoro et al., 2016).

The expression of melanins can be modulated by many factors. For hormonal regulation of melanogenesis, the melanocortin pathway has been well studied (Wolf Horrell et al., 2016). Melanocortins, including adrenocorticotrophic hormone (ACTH) and several types of melanocyte-stimulating hormone (MSH), are produced in the pituitary gland and bind to the melanocortin 1 receptor (MC1R), which is a G protein-coupled receptor expressed by melanocytes. Binding of α MSH to the MC1R increases cellular cyclic adenosine monophosphate (cAMP), leading to eumelanin synthesis. The agouti signaling protein (ASIP) is a secreted peptide that inhibits MC1R signaling by antagonizing α MSH binding. Binding of ASIP to MC1R leads to preferential pheomelanin synthesis. Pheomelanin production also requires cysteine uptake into melanocytes via the xCT transporter (Chintala et al., 2005). Cysteine availability may be affected by environmental oxidative stress (Galván et al., 2017), leading to color changes. ASIP is preferentially expressed in the ventral skin, and its level and spatial domain can affect the survival fitness

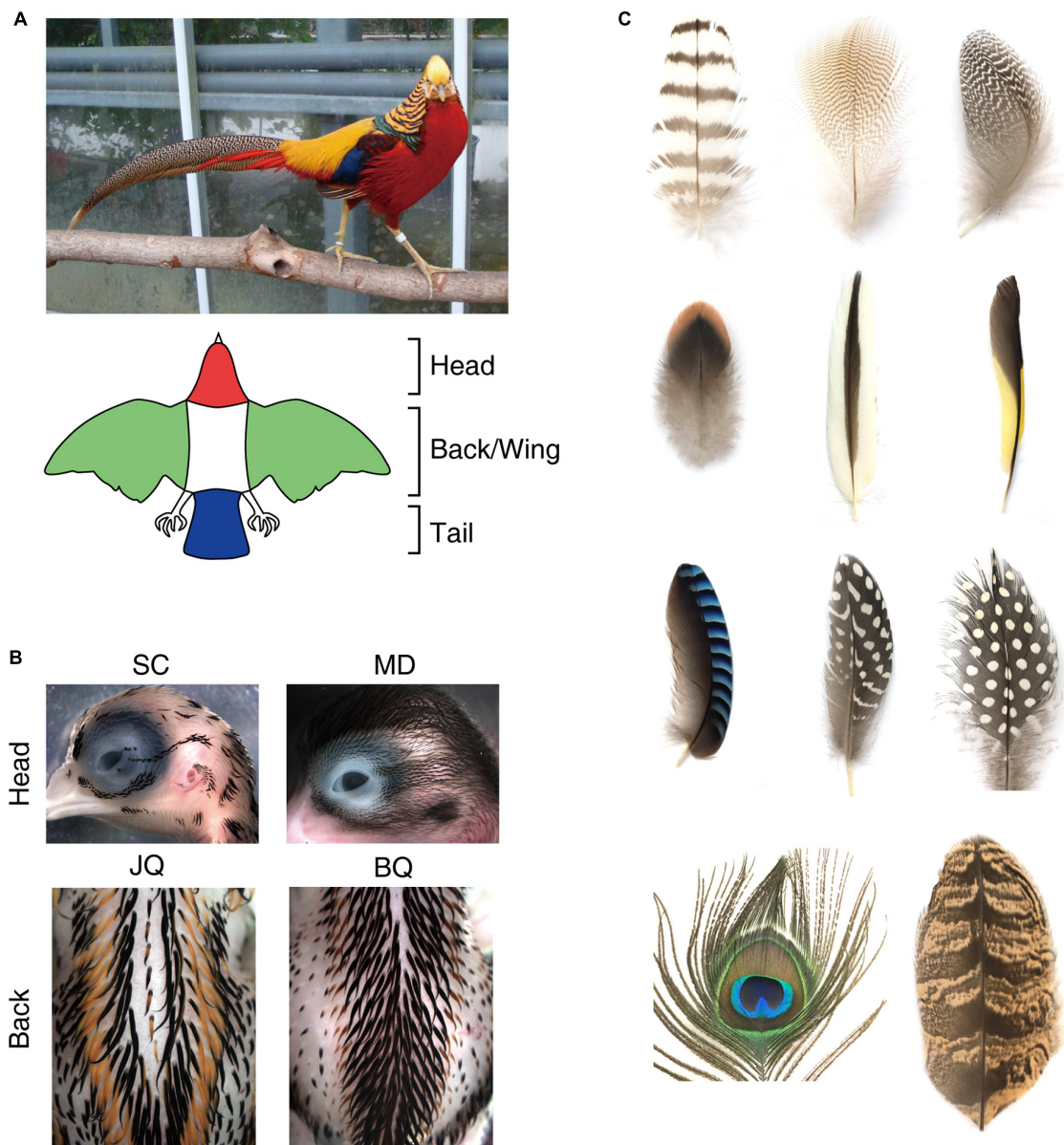


FIGURE 1 | Macro- (across the body or within a skin domain) and micro (within a feather) color patterns in the bird. **(A)** Color patterns across the whole body. Upper: a male golden pheasant. Lower: a schematic drawing showing a typical whole color pattern in birds. **(B)** Pigment patterns within domains. Upper: pigment patterns on the head in an E12 Sicilian Buttercup chicken (SC) and E16 Mallard duck (MD). Lower: distinct pigment stripe patterns in E10 Japanese quail (JQ) back skin and E13 Bobwhite quail (BQ). **(C)** Diversity of pigment patterns within a feather. Photos by M. Inaba.

of wild animals (Millar et al., 1995; Manceau et al., 2011; Ceinos et al., 2015).

MACRO-PATTERNS: COLOR PATTERNS ACROSS THE WHOLE BODY AND WITHIN A SKIN DOMAIN

Bird pigment patterns across the whole body include stripes, spots, patches, or more complex patterns. This is achieved at the whole-body level (Figure 1A), either by pigment patterns

in a skin domain (Figure 1B), within a feather (Figure 1C), or a combination of these two locations. Here, we focus on the pigment pattern arising in the skin domain. Interestingly, striped patterns are the most prominent pigment patterns observed in the bird's skin domain. Stripes often emerge in embryonic skin domains, as seen in the head and back skin (Figure 1B). The patterns of stripes vary among related species, implying that they share a common pattern control mechanism with minor genetic changes that function in a modulatory capacity.

Galliform birds such as guineafowl, pheasant, chicken, and quail often show stripe patterns on their back skin at embryonic

and juvenile stages. During development, melanoblasts migrate along the dorsoventral pathway between the epidermis and dermis and eventually incorporate into the epidermal layer (**Figure 2A**). Prior to melanin synthesis, melanoblasts are committed to producing eumelanin, which is marked by expression of glutathione s-transferase (Nataf et al., 1993, 1995; Niwa et al., 2002). While the mechanism of this specification has not been discerned yet, this process can be regulated by melanocytes autonomously or modulated by surrounding tissues. Therefore, the molecular mechanism of pigment patterning must clarify the role of each tissue in this process.

Tissue interactions can be studied utilizing tissue grafting techniques between different avian species. In classical experiments using chicken-quail chimera, when Japanese quail neural crest was transplanted into White Leghorn chickens which show no pigmentation, a quail-like striped pigment pattern appeared in the host chicks (Kinutani and Le Douarin, 1985). This result suggests that melanocytes likely have information that guides pigment patterning. In another study, Japanese quail wing buds prior to melanocyte immigration were transplanted into guineafowl embryos (Richardson et al., 1991). This experiment placed guineafowl melanocytes surrounded by quail tissues (epidermis and dermis). The expected results predicted two possible outcomes: (1) if the host shows a guineafowl-like pigment pattern, it would suggest that melanocytes of different genotypes can respond differently to environmental cues shared among different species. (2) If the host shows a quail-like pigment pattern, it would suggest that a prepattern guiding pigment patterning is present in environmental tissues (epidermis or dermis). The result of this transplantation produced the latter result. Such hetero-species tissue recombination analysis should be valuable to identify the molecular cues needed for pigment patterning.

Recently, somite transplantation experiments among galliform birds showed that longitudinal stripe patterns developing on embryonic back skin correlated with dermal ASIP expression patterns. Hence, pigment stripe patterning was determined by the somite species of origin (Haupaix et al., 2018; Haupaix and Manceau, 2020) (**Figure 2A**). Loss-of-function mutant quails with a shortened ASIP expression period display a thinner yellow stripe width, whereas the gain-of-function mutant with a prolonged-expression period expands the yellow stripe width. Furthermore, by grafting Japanese quail somites into pheasant embryos, the quail specific ASIP expression pattern was found to be induced in the host. These results led the authors to suggest that the stripe pattern is determined by the regulation of ASIP in dermal cells derived from somites. Yet how these dermal cells generate the striped agouti expression pattern was not addressed yet.

Although somites can play an important role in bird stripe patterning, other tissues may contribute to the patterning in different ways. Quail melanocytes purified in *in vitro* culture were transplanted into White Leghorn chickens. Interestingly, host chickens initially express ASIP in longitudinal stripes but ASIP disappears soon after E6 (Inaba et al., 2019b). In the chicken host, quail melanocytes form periodic pigment patterns without directionality along the longitudinal axis. This suggests that melanocytes still have an ability to form periodic patterns

autonomously but the stripe directionality is largely dependent on the striped ASIP expression pattern. The stripe width is regulated not only by levels of ASIP but also by melanocyte interactions in a connexin-dependent manner. Connexins can comprise gap junctions that directly connect neighboring cells, enabling cell-cell communications via small molecules, such as ions and second messengers. Connexin40 is expressed in both eumelanin and pheomelanin producing melanocytes. Because the melanocytes contact each other in the skin with filopodia, connexin40 is expected to comprise gap junctional channels between melanocytes (**Figure 2A**). Over-expression of connexin40 in quail melanocytes leads to the expansion of yellow regions with a corresponding reduction of black regions. In contrast, inhibition of connexin40 by expressing a dominant negative form reduced the yellow and expanded the black regions.

It should be noted that the role of gap junctions in stripe patterning has been studied in zebrafish before. Zebrafish stripes are formed by pigment cell interactions that satisfy the Turing-type patterning (reaction-diffusion system) requirement (Nakamasu et al., 2009; Inaba et al., 2012; Hamada et al., 2014). A recent transgenic zebrafish study showed that unidirectional current flow between melanocytes mediated by gap junctions is sufficient to produce stripe patterning (Usui et al., 2019). In the future, it is important to understand how gap junction communication is involved in generating the self-organizing pigment patterning process.

Taken together, ASIP expression may present a prepattern that guides the global orientation of stripes, while self-organizing melanocytes involve the local patterning of stripes. Such global and local patterning mechanisms are often exploited combinatorially to achieve robust and plastic development, e.g., feather bud arrangement (Bailleul et al., 2019; Ho et al., 2019; Inaba et al., 2019a). In studying the patterning process, one has to bear in mind that both autonomous and non-autonomous mechanisms may be used together or in different contexts, and that a pattern (e.g., stripes) can be generated by multiple mechanisms.

MICRO-PATTERNS: COLOR PATTERNS WITHIN A FEATHER

Pigment patterning within a feather vane is fascinating, as spots, stripes, chevrons, rings, or combinations of these patterns can be formed (**Figure 1C**). Although feathers are cyclically replaced to maintain their functions throughout a lifetime, they can retain identical pigment patterns in each feather generation. On the other hand, feather pigment patterns can be modulated in response to intrinsic and extrinsic factors. The feather pigment pattern varies depending on the characteristics of the region where feathers can grow (tract, pterylae). This suggests that the patterning mechanism is influenced by positional information that determines body regional specificity. Further, sexual maturation can alter feather morphology, texture, or pigment patterns to attract a mate. The sex hormones indeed determine the feather phenotypes; however, the molecular mechanisms that convert the hormonal signal to feather coloring remain to be elucidated (Widelitz et al., 2019).

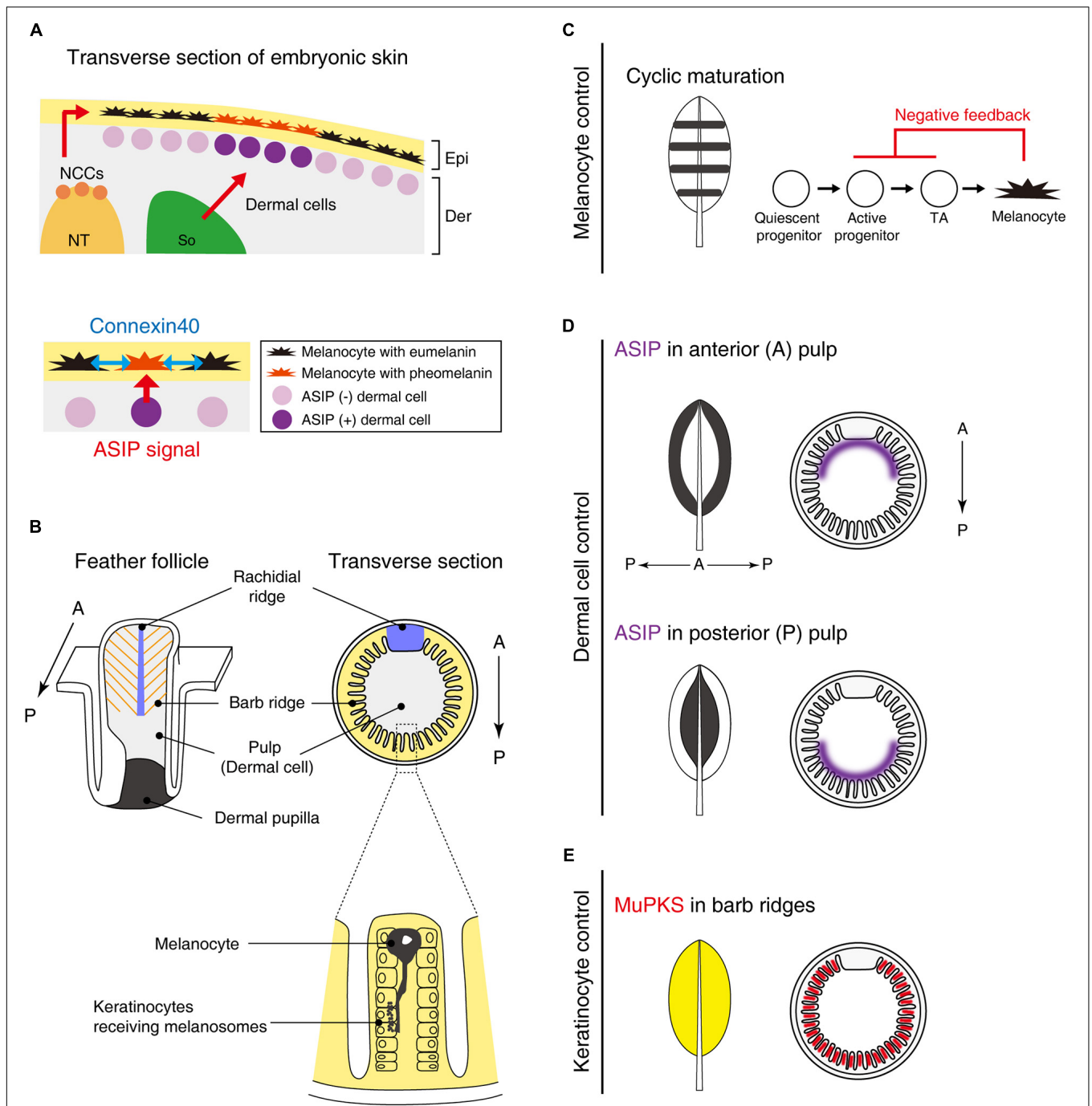


FIGURE 2 | Putative molecular mechanisms in macro- and micro-pigment pattern formation. **(A)** The embryonic development of Japanese quail skin (Left). Neural crest cells (NCCs) delaminate from the dorsal part of the neural tube (NT). Some NCCs differentiate into melanoblasts and emigrate to their destination in the epidermis (Epi). A part of the somite (So) differentiates to dermis (Der). Dermal cells in the vicinity of melanocytes secrete ASIP to switch the melanin synthesis pathway from eumelanin to pheomelanin. Dermal cell–melanocyte interactions confer striped pigment patterns (Lower) (Haupaix et al., 2018). In addition, melanocytes interact with each other via connexin40 (blue arrows) to refine the size of the pigmentation stripes (Inaba et al., 2019b). **(B)** Schematic drawing of a feather follicle. Melanocytes are located within barb ridges and transport its melanosome into adjacent keratinocytes (Lin et al., 2013). **(C–E)** Schematic drawing showing that interactions between melanocytes and their environments (dermis/epidermis) form a variety of feather pigment patterns. **(C)** Melanocyte intrinsic banded pigment pattern. Cyclic maturation of the progenitor cells gives rise to banded pigment patterns on the feather vane. To accomplish this oscillation, negative feedback from mature melanocytes to active progenitors and transit amplifying cells (TA) is assumed. **(D)** Dermal cell instructive pigment pattern. ASIP is expressed in either side of the anterior or posterior pulp comprised of dermal cells, leading edge, or central pigmented patterns (Lin et al., 2013). **(E)** Feather keratinocytes can confer the pigmentation. MuPKS, polyketide synthase, is expressed in barb ridges and deposits yellow psittacofulvin pigments (Cooke et al., 2017).

To appreciate patterning within a feather, we need to appreciate the topology of the platform (Lucas and Stettenheim, 1972; Yu et al., 2004). The follicle structure (**Figure 2B**) is formed by the invagination of feather primordia toward the dermal layer. The feather follicle epithelial sheet periodically invaginates along the circumference of the follicle, forming future feather branches called barb ridges. The anterior part of the epithelial sheet is specialized within the rachidial ridge for future feather shaft development. The follicles contain dermal cells: those in the distal follicle become pulp and those in the proximal follicle become the dermal papilla, which plays a critical role in feather regeneration. In the resting phase of feather development, melanoblasts are localized in the feather follicle papilla ectoderm. During the growth phase, melanoblasts are located at the lower bulge zone, close to the feather follicle base. Melanoblasts migrate upward into barb ridges and then differentiate into mature pigmented melanocytes (Lin et al., 2013). Mature melanocytes located in barb ridges transport their melanosomes to keratinocytes to form the micro-pigment patterns (Lucas and Stettenheim, 1972).

At least two major mechanisms to achieve unpigmented (white color) under the control of melanocyte activity have been investigated (Lin et al., 2013). The first is the presence/absence of melanoblasts. White Leghorn chickens completely lose the progenitor cells at a late stage of embryogenesis (Jimbow et al., 1974), resulting in entirely white feathers. When melanoblasts are controlled spatiotemporally, more complex pigment patterns emerge. Barred Plymouth Rock chickens, which have mutations in the tumor suppressor gene, *CDKN2A* (Hellström et al., 2010), display banded pigment patterns. The *cis*-regulatory mutations upregulate the expression of *CDKN2A*, which leads to melanoblasts reduction, not apoptosis. The melanocyte maturation zone cyclically travels from the posterior to the anterior side during feather growth from the distal to the proximal end. This cyclic presence/absence of melanoblasts driven by a negative feedback system results in the black/white banded patterns oriented along the proximal-distal axis (**Figure 2C**).

The second mechanism is the control of melanocyte melanin synthesis by adjacent dermal cells (**Figure 2D**). The pulp derived from dermal cells heterogeneously expresses ASIP. When ASIP expression is localized in the anterior side of feather pulp, the central feather exhibits a white color while the feather edge displays a black color. How this polarized ASIP expression in pulp fibroblasts is regulated is unknown, but we know it is a sexually dimorphic process (Lin et al., 2013). Administration of estrogen to male chickens can induce a female-specific ASIP expression pattern that causes female-like pigmentation (Oribi et al., 2012). Through sex-hormone regulation, birds can reset feather colors and forms by molting and regenerating new feathers at different life stages (Widelitz et al., 2019).

In addition to the roles played by melanocytes and dermal cells, the keratinocytes also play a role in pigmentation (**Figure 2E**). One example is polyketide synthase (MuPKS), an enzyme that produces yellow polyene pigments called psittacofulvins. MuPKS is expressed in keratinocytes of barb ridges during follicle formation, which confers yellow pigmentation on budgerigar feathers (Cooke et al., 2017).

FUTURE DIRECTIONS

Avian color patterns provide a wonderful patterning model that acts at different spatial scales (**Figure 1**). In this minireview, we covered some classical studies, recent experiments examining tissue interactions, and lines of experimentation providing some molecular insights into pigment patterning (**Figure 2**). With the advance of current technologies, we expect a new synthesis will help reveal the organizing principles of complex patterns. We think further advances will need to include the following approaches.

- (1) Cell and developmental biology approaches: More analyses of melanocyte stem cell status, proliferation, migration and differentiation, and their interactions with adjacent tissues within the embryo and regenerating feather follicles (Lin et al., 2013; Haupaix and Manceau, 2020) will help discern cellular mechanisms that underlie pigment patterning. This will also be aided by higher resolution time-lapse imaging analyses of cell interactive behaviors.
- (2) Systems biology approaches: Recent advances in the genome-wide analysis would enable us to detect more novel genes related to pigment patterns both from domestic and wild birds. Bulk and single-cell transcriptomics, ATAC, Chip-seq provide a new possibility to understand epigenetic changes and fate specification which can be further evaluated with promoter-reporter analyses for melanogenesis pathway members and factors that activate them.
- (3) Genetic approaches: Some chicken variants show unique color patterns and can provide clues to the patterning process (Cooke et al., 2017; Andersson et al., 2020).
- (4) Develop a biological database patterning model: Previously, mathematical modeling based on a reaction-diffusion system has provided diverse pigment patterns that simulate the real feather pigment patterns (Prum and Williamson, 2002). While mathematical models help to highlight possible patterning principles, it is important to examine the actual biological processes that generate pigment patterns and synthesize a model based on experimental results. With these investigations, we hope to advance our understanding of the development, regeneration, and evolution of feather forms and pigment patterns (Chen et al., 2015).

AUTHOR CONTRIBUTIONS

MI and C-MC wrote the review. MI made figures. Both authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by NIH AR 047364 and AR 060306. MI was supported by the Human Frontier Science Program

(LT001148/2015-L). The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

REFERENCES

- Andersson, L., Bed'hom, B., Chuong, C.-M., Inaba, M., Okimoto, R., and Tixier-Boichard, M. (2020). "The genetic basis for pigmentation phenotypes," in *Advances In Poultry Genetics And Genomics*, 1st Edn, eds S. E. Aggrey, H. Zhou, M. Tixier-Boichard, and D. D. Rhoads (Philadelphia: Burleigh Dodds Science Publishing).
- Bailleul, R., Curantz, C., Desmarquet-Trin Dinh, C., Hidalgo, M., Touboul, J., and Manceau, M. (2019). Symmetry breaking in the embryonic skin triggers directional and sequential plumage patterning. *PLoS Biol.* 17:e3000448. doi: 10.1371/journal.pbio.3000448
- Bennett, D. C., and Lamoreux, M. L. (2003). The color loci of mice—a genetic century. *Pigment. Cell Res.* 16, 333–344. doi: 10.1034/j.1600-0749.2003.00067.x
- Ceinos, R. M., Guillot, R., Kelsh, R. N., Cerdá-Reverter, J. M., and Rotllant, J. (2015). Pigment patterns in adult fish result from superimposition of two largely independent pigmentation mechanisms. *Pigment Cell Melanoma Res.* 28, 196–209. doi: 10.1111/pcmr.12335
- Chen, C.-F., Foley, J., Tang, P.-C., Li, A., Jiang, T. X., Wu, P., et al. (2015). Development, regeneration, and evolution of feathers. *Annu. Rev. Anim. Biosci.* 3, 169–195. doi: 10.1146/annurev-animal-022513-114127
- Chintala, S., Li, W., Lamoreux, M. L., Ito, S., Wakamatsu, K., Sviderskaya, E. V., et al. (2005). Slc7a11 gene controls production of pheomelanin pigment and proliferation of cultured cells. *Proc. Natl. Acad. Sci. U.S.A.* 102, 10964–10969. doi: 10.1073/pnas.0502856102
- Cooke, T. F., Fischer, C. R., Wu, P., Jiang, T.-X., Xie, K. T., Kuo, J., et al. (2017). Genetic mapping and biochemical basis of yellow feather pigmentation in budgerigars. *Cell* 171, 427.e2–439.e2. doi: 10.1016/j.cell.2017.08.016
- Galván, I., Inácio, Á., Romero-Haro, A. A., and Alonso-Alvarez, C. (2017). Adaptive downregulation of pheomelanin-related Slc7a11 gene expression by environmentally induced oxidative stress. *Mol. Ecol.* 26, 849–858. doi: 10.1111/mec.13952
- Galván, I., and Solano, F. (2016). Bird integumentary melanins: biosynthesis, forms, function and evolution. *Int. J. Mol. Sci.* 17:520. doi: 10.3390/ijms17040520
- Hamada, H., Watanabe, M., Lau, H. E., Nishida, T., Hasegawa, T., Parichy, D. M., et al. (2014). Involvement of Delta/Notch signaling in zebrafish adult pigment stripe patterning. *Development* 141, 318–324. doi: 10.1242/dev.099804
- Haupaix, N., Curantz, C., Bailleul, R., Beck, S., Robic, A., and Manceau, M. (2018). The periodic coloration in birds forms through a prepattern of somite origin. *Science* 361:1216. doi: 10.1126/science.aar4777
- Haupaix, N., and Manceau, M. (2020). The embryonic origin of periodic color patterns. *Dev. Biol.* 460, 70–76. doi: 10.1016/j.ydbio.2019.08.003
- Hellström, A. R., Sundström, E., Gunnarsson, U., Bed'Hom, B., Tixier-Boichard, M., Honaker, C. F., et al. (2010). Sex-linked barring in chickens is controlled by the CDKN2A/B tumour suppressor locus. *Pigment Cell Melanoma Res.* 23, 521–530. doi: 10.1111/j.1755-148X.2010.00700.x
- Ho, W. K. W., Freem, L., Zhao, D., Painter, K. J., Woolley, T. E., Gaffney, E. A., et al. (2019). Feather arrays are patterned by interacting signalling and cell density waves. *PLoS Biol.* 17:e3000132. doi: 10.1371/journal.pbio.3000132
- Hoekstra, H. E. (2006). Genetics, development and evolution of adaptive pigmentation in vertebrates. *Heredity* 97, 222–234. doi: 10.1038/sj.hdy.6800861
- Imsland, F., McGowan, K., Rubin, C.-J., Henegar, C., Sundström, E., Berglund, J., et al. (2016). Regulatory mutations in TBX3 disrupt asymmetric hair pigmentation that underlies Dun camouflage color in horses. *Nat. Genet.* 48, 152–158. doi: 10.1038/ng.3475
- Inaba, M., Harn, H. I.-C., and Chuong, C.-M. (2019a). Turing patterning with and without a global wave. *PLoS Biol.* 17:e3000195. doi: 10.1371/journal.pbio.3000195
- Inaba, M., Jiang, T.-X., Liang, Y.-C., Tsai, S., Lai, Y.-C., Widelitz, R. B., et al. (2019b). Instructive role of melanocytes during pigment pattern formation of the avian skin. *Proc. Natl. Acad. Sci. U.S.A.* 116, 6884–6890. doi: 10.1073/pnas.1816107116
- Inaba, M., Yamanaka, H., and Kondo, S. (2012). Pigment pattern formation by contact-dependent depolarization. *Science* 335:677. doi: 10.1126/science.1212821
- Irion, U., Singh, A. P., and Nüsslein-Volhard, C. (2016). The developmental genetics of vertebrate color pattern formation: lessons from zebrafish. *Curr. Top. Dev. Biol.* 117, 141–169. doi: 10.1016/bs.ctdb.2015.12.012
- Ishishita, S., Takahashi, M., Yamaguchi, K., Kinoshita, K., Nakano, M., Nunome, M., et al. (2018). Nonsense mutation in PMEL is associated with yellowish plumage colour phenotype in Japanese quail. *Sci. Rep.* 8:16732. doi: 10.1038/s41598-018-34827-4
- Jimbow, K., Szabo, G., and Fitzpatrick, T. B. (1974). Ultrastructural investigation of autophagocytosis of melanosomes and programmed death of melanocytes in White Leghorn feathers: a study of morphogenetic events leading to hypomelanosis. *Dev. Biol.* 36, 8–23. doi: 10.1016/0012-1606(74)90187-0
- Kaelin, C. B., Xu, X., Hong, L. Z., David, V. A., McGowan, K. A., Schmidt-Küntzel, A., et al. (2012). Specifying and sustaining pigmentation patterns in domestic and wild cats. *Science* 337, 1536–1541. doi: 10.1126/science.1220893
- Kerje, S., Lind, J., Schütz, K., Jensen, P., and Andersson, L. (2003). Melanocortin 1-receptor (MC1R) mutations are associated with plumage colour in chicken. *Anim. Genet.* 34, 241–248. doi: 10.1046/j.1365-2052.2003.00991.x
- Kinutani, M., and Le Douarin, N. M. (1985). Avian spinal cord chimeras. I. Hatching ability and posthatching survival in homo- and heterospecific chimeras. *Dev. Biol.* 111, 243–255. doi: 10.1016/0012-1606(85)90449-x
- Lin, S. J., Foley, J., Jiang, T. X., Yeh, C. Y., Wu, P., Foley, A., et al. (2013). Topology of feather melanocyte progenitor niche allows complex pigment patterns to emerge. *Science* 340, 1442–1445. doi: 10.1126/science.1230374
- Lopes, R. J., Johnson, J. D., Toomey, M. B., Ferreira, M. S., Araujo, P. M., Melo-Ferreira, J., et al. (2016). Genetic basis for red coloration in birds. *Curr. Biol.* 26, 1427–1434. doi: 10.1016/j.cub.2016.03.076
- Lucas, A. M., and Stettenheim, P. R. (eds) (1972). "Avian anatomy – integument," in *Agricultural Handbook*, Vol. 362, (Washington, DC: Agricultural Research Services), 1–750.
- Maia, R., Macedo, R. H. F., and Shawkey, M. D. (2012). Nanostructural self-assembly of iridescent feather barbules through depletion attraction of melanosomes during keratinization. *J. R. Soc. Interface* 9, 734–743. doi: 10.1098/rsif.2011.0456
- Mallarino, R., Henegar, C., Mirasierra, M., Manceau, M., Schradin, C., Vallejo, M., et al. (2016). Developmental mechanisms of stripe patterns in rodents. *Nature* 539, 518–523. doi: 10.1038/nature20109
- Manceau, M., Domingues, V. S., Mallarino, R., and Hoekstra, H. E. (2011). The developmental role of Agouti in color pattern evolution. *Science* 331, 1062–1065. doi: 10.1126/science.1200684
- Millar, S. E., Miller, M. W., Stevens, M. E., and Barsh, G. S. (1995). Expression and transgenic studies of the mouse agouti gene provide insight into the mechanisms by which mammalian coat color patterns are generated. *Development* 121, 3223–3232.
- Nakamasu, A., Takahashi, G., Kanbe, A., and Kondo, S. (2009). Interactions between zebrafish pigment cells responsible for the generation of Turing patterns. *Proc. Natl. Acad. Sci. U.S.A.* 106, 8429–8434. doi: 10.1073/pnas.0808622106
- Nataf, V., Mercier, P., De Néchard, B., Guillemot, J. C., Capdevielle, J., Lapointe, F., et al. (1995). Melanoblast/melanocyte early marker (MelEM) is a glutathione S-transferase subunit. *Exp. Cell Res.* 218, 394–400. doi: 10.1006/excr.1995.1171
- Nataf, V., Mercier, P., Ziller, C., and Le Douarin, N. M. (1993). Novel markers of melanocyte differentiation in the avian embryo. *Exp. Cell Res.* 207, 171–182. doi: 10.1006/excr.1993.1177
- Niwa, T., Mochii, M., Nakamura, A., and Shiojiri, N. (2002). Plumage pigmentation and expression of its regulatory genes during quail development—histochemical analysis using Bh (black at hatch) mutants. *Mech. Dev.* 118, 139–146. doi: 10.1016/s0925-4773(02)00256-3
- Oribe, E., Fukao, A., Yoshihara, C., Mendori, M., Rosal, K. G., Takahashi, S., et al. (2012). Conserved distal promoter of the agouti signaling protein (ASIP)

ACKNOWLEDGMENTS

We thank Dr. R. B. Widelitz for editing the text.

- gene controls sexual dichromatism in chickens. *Gen. Comp. Endocrinol.* 177, 231–237. doi: 10.1016/j.ygcen.2012.04.016
- Patterson, L. B., and Parichy, D. M. (2019). Zebrafish pigment pattern formation: insights into the development and evolution of adult form. *Annu. Rev. Genet.* 53, 505–530. doi: 10.1146/annurev-genet-112618-043741
- Prum, R. O., and Williamson, S. (2002). Reaction-diffusion models of within-feather pigmentation patterning. *Proc. Biol. Sci.* 269, 781–792. doi: 10.1098/rspb.2001.1896
- Richardson, M. K., Hornbruch, A., and Wolpert, L. (1991). Pigment patterns in neural crest chimeras constructed from quail and guinea fowl embryos. *Dev. Biol.* 143, 309–319. doi: 10.1016/0012-1606(91)90082-E
- Schartl, M., Larue, L., Goda, M., Bosenberg, M. W., Hashimoto, H., and Kelsh, R. N. (2016). What is a vertebrate pigment cell? *Pigment Cell Melanoma Res.* 29, 8–14. doi: 10.1111/pcmr.12409
- Tadokoro, R., Murai, H., Sakai, K.-I., Okui, T., Yokota, Y., and Takahashi, Y. (2016). Melanosome transfer to keratinocyte in the chicken embryonic skin is mediated by vesicle release associated with Rho-regulated membrane blebbing. *Sci. Rep.* 6:38277. doi: 10.1038/srep38277
- Usui, Y., Aramaki, T., Kondo, S., and Watanabe, M. (2019). The minimal gap-junction network among melanophores and xanthophores required for stripe pattern formation in zebrafish. *Development* 146:dev181065. doi: 10.1242/dev.181065
- Watanabe, M., and Kondo, S. (2015). Is pigment patterning in fish skin determined by the Turing mechanism? *Trends Genet.* 31, 88–96. doi: 10.1016/j.tig.2014.11.005
- Widelitz, R. B., Lin, G.-W., Lai, Y.-C., Mayer, J. A., Tang, P.-C., Cheng, H.-C., et al. (2019). Morpho-regulation in diverse chicken feather formation: integrating branching modules and sex hormone-dependent morpho-regulatory modules. *Dev. Growth Differ.* 61, 124–138. doi: 10.1111/dgd.12584
- Wolf Horrell, E. M., Boulanger, M. C., and D’Orazio, J. A. (2016). Melanocortin 1 receptor: structure, function, and regulation. *Front. Genet.* 7:95. doi: 10.3389/fgene.2016.00095
- Wu, X., and Hammer, J. A. (2014). Melanosome transfer: it is best to give and receive. *Curr. Opin. Cell Biol.* 29, 1–7. doi: 10.1016/j.ceb.2014.02.003
- Yoshioka, S., and Kinoshita, S. (2002). Effect of macroscopic structure in iridescent color of the peacock feathers. *Forma* 17, 169–181.
- Yu, M., Yue, Z., Wu, P., Wu, D.-Y., Mayer, J.-A., Medina, M., et al. (2004). The biology of feather follicles. *Int. J. Dev. Biol.* 48, 181–191. doi: 10.1387/ijdb.031776my
- Zi, J., Yu, X., Li, Y., Hu, X., Xu, C., Wang, X., et al. (2003). Coloration strategies in peacock feathers. *Proc. Natl. Acad. Sci. U.S.A.* 100, 12576–12578. doi: 10.1073/pnas.2133313100

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2020 Inaba and Chuong. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Molecular Evolution and Developmental Expression of Melanin Pathway Genes in Lepidoptera

Muktai Kuwalekar[†], Riddhi Deshmukh[†], Ajay Padvi and Krushnamegh Kunte*

National Centre for Biological Sciences, Tata Institute of Fundamental Research, Bengaluru, India

OPEN ACCESS

Edited by:

Marcus Kronforst,
The University of Chicago,
United States

Reviewed by:

Heather Hines,
Pennsylvania State University (PSU),
United States
Simon Martin,
The University of Edinburgh,
United Kingdom

*Correspondence:

Krushnamegh Kunte
krushnamegh@ncbs.res.in

[†]These authors have contributed
equally to this work

Specialty section:

This article was submitted to
Evolutionary Developmental Biology,
a section of the journal
Frontiers in Ecology and Evolution

Received: 12 March 2020

Accepted: 19 June 2020

Published: 14 July 2020

Citation:

Kuwalekar M, Deshmukh R,
Padvi A and Kunte K (2020) Molecular
Evolution and Developmental
Expression of Melanin Pathway
Genes in Lepidoptera.
Front. Ecol. Evol. 8:226.
doi: 10.3389/fevo.2020.00226

Pigmentation is involved in a wide array of biological functions across insect orders, including body patterning, thermoregulation, and immunity. The melanin pathway, in particular, has been characterized in several species. However, molecular evolution of the genes involved in this pathway is poorly explored. We traced the molecular evolution of six melanin pathway genes in 53 species of Lepidoptera covering butterflies and moths, and representing over 100 million years of diversification. We compared the rates of synonymous and non-synonymous substitutions within and between these genes to study the signatures of selection at the level of individual sites, genes, and branches of the gene tree. We found that molecular evolution of all six genes was governed by strong purifying selection. Yet, a number of sites showed signs of being under positive selection, including in the highly conserved domain regions of three genes. Further, we traced the expression of these genes across developmental stages, tissues, and sexes in the *Papilio polytes* butterfly using a developmental transcriptome dataset. We observed that the expression patterns of the genes in *P. polytes* largely reflected their known tissue-specific function in other species. The expression of sequentially acting genes in the melanin pathway was correlated. Interestingly, melanin pathway genes also showed a sexually dimorphic pattern of developmental heterochrony, i.e., females showed prominent upregulation of melanin pathway genes in pre-pupal stage compared to males, while males showed prominent upregulation in 9-day pupal wings compared to females. Our evolutionary and developmental analyses suggest that the vast diversity of wing patterning and pigmentation in Lepidoptera may have evolved despite largely constrained sequence evolution, with potential contribution from differential developmental expression of genes in a highly conserved pathway.

Keywords: melanization, pigmentation, developmental heterochrony, butterfly wing coloration, wing patterns

INTRODUCTION

The evolution of color patterns contributes to the striking diversity of lifeforms. Coloration is a critical adaptation that impacts an organism's intra- and inter-specific communication and interactions. In several cases, the emergence of sexual dimorphism and polymorphism is also linked to the evolution and differential expression of pigmentation genes (Wittkopp et al., 2009;

Miyazaki et al., 2014; Yassin et al., 2016). Among Lepidoptera, wing coloration and patterning are remarkably diverse, which have shaped several adaptations such as aposematism, crypsis, mimicry, and thermoregulation (True et al., 2005; Hegna et al., 2013; Olofsson et al., 2013; Kronforst and Papa, 2015; Nadeau, 2016; Deshmukh et al., 2018; van't Hof et al., 2019). In insects, melanins, ommochromes, and pterins are three major biosynthesized pigments deposited in developing wing scales (Wittkopp and Beldade, 2009). Melanin pathway and its components—being involved in a wide range of physiological processes—are conserved across insects. For instance, increased melanization in high-altitude moths aids in thermoregulation, resulting in a trade-off between warning coloration and hindwing melanization (Hegna et al., 2013). Melanization is also associated with immunity, wound healing, and protection from both ultraviolet light and parasitoids (Yassine et al., 2012; Bilandžija et al., 2017). Melanin and related pigments—such as DOPA-melanin, dopamine-melanin, NBAD sclerotin, and NADA sclerotin—contribute to the production of black, brown, and yellow coloration (Wright, 1987; Koch et al., 2000a; Zhang et al., 2017a). The availability of genetic manipulation techniques in non-model systems has allowed elucidation of the role of melanin pathway genes in color adaptations (Zhang and Reed, 2017; Zhang et al., 2017a; Matsuoka and Monteiro, 2018). While the developmental mechanisms underlying pigmentation and patterning are being extensively studied, the evolutionary trajectories of these pigmentation genes remain poorly explored.

We chose six melanin pathway genes—*tan*, *black*, *ebony*, *pale* (tyrosine hydroxylase), *arylalkylamine N-acetyltransferase 1* (*aaNAT*), and *DOPA decarboxylase* (*DDC*)—whose developmental function in Lepidoptera coloration is well characterized (Zhang et al., 2017a; Matsuoka and Monteiro, 2018). The functions and phenotypic effects of these genes are summarized in **Figure 1** and **Supplementary Table S1**. Melanin synthesis occurs by a branched pathway with tyrosine and uracil as precursors which are shunted into the pathway by *pale* and *DDC* (Zhang et al., 2017a). *black* and *ebony* are involved in the synthesis of N- β -alanyl dopamine (NBAD), which is responsible for yellow coloration, while *tan* breaks down NBAD to dopamine, thus countering the synthesis of NBAD and the activity of *ebony*. *aaNAT* facilitates the formation of colorless cuticles essential for wing pigmentation (**Figure 1** and **Supplementary Table S1**). These genes are thus crucial for melanin production and its deposition on Lepidoptera wings. The pigments observed in adults start appearing on the wings and body tissues during late pupal stages, and black melanin is usually the last one to be deposited (Koch et al., 1998; Wittkopp and Beldade, 2009; Matsuoka and Monteiro, 2018). In this study, we trace the molecular evolution of these six genes in 53 species across nine superfamilies of Lepidoptera (total over 100 million years of divergence) and additionally characterize their expression patterns across various developmental stages and tissues of *Papilio polytes* to address the following questions: (i) What selection pressures have shaped the evolution of these genes in the Lepidoptera? (ii) Do these genes show differential activity in sexually dimorphic species? and (iii) Do they have similar expression patterns

during larval pigmentation and adult wing patterning and pigmentation?

MATERIALS AND METHODS

Gene Sequences and Multiple Sequence Alignment

We downloaded whole genome sequences of 53 species of Lepidoptera from GenBank¹, LepBase², and GigaDB³, which best represented different families. We selected six genes which are central to pigmentation and patterning pathways, namely *tan*, *black*, *ebony*, *pale* (tyrosine hydroxylase), *aaNAT* (Arylalkylamine N-acetyltransferase 1), and *DDC* (dopa decarboxylase) (Wright, 1987; Zhang et al., 2017a; Matsuoka and Monteiro, 2018). We performed exon-wise NCBI local tBLASTn to locate these genes within genomes of the selected species (**Supplementary Table S2**). For all the genes, we chose the longest isoform for performing tBLASTn as it incorporates maximum sequence information for the given gene. Using these gene coordinates, we extracted respective genes from the genome using an in-house python script. We chose the hits with the highest similarity scores to avoid misidentification. The downloaded genome sequences showed no evidence of duplication for any of the six melanin pathway genes. Several genome files (such as those of *Bicyclus anynana*, *P. polytes*, *Papilio xuthus*, and *Bombyx mori*) had annotated two copies of *aaNAT* and *black*, but they shared low similarity scores (~40%) and were therefore not considered as duplicates in our analysis. We performed multiple sequence alignment of each gene with MEGA X MUSCLE aligner with codon alignment option (Kumar et al., 2018). We included homologous gene sequences from Diptera as outgroups for respective genes.

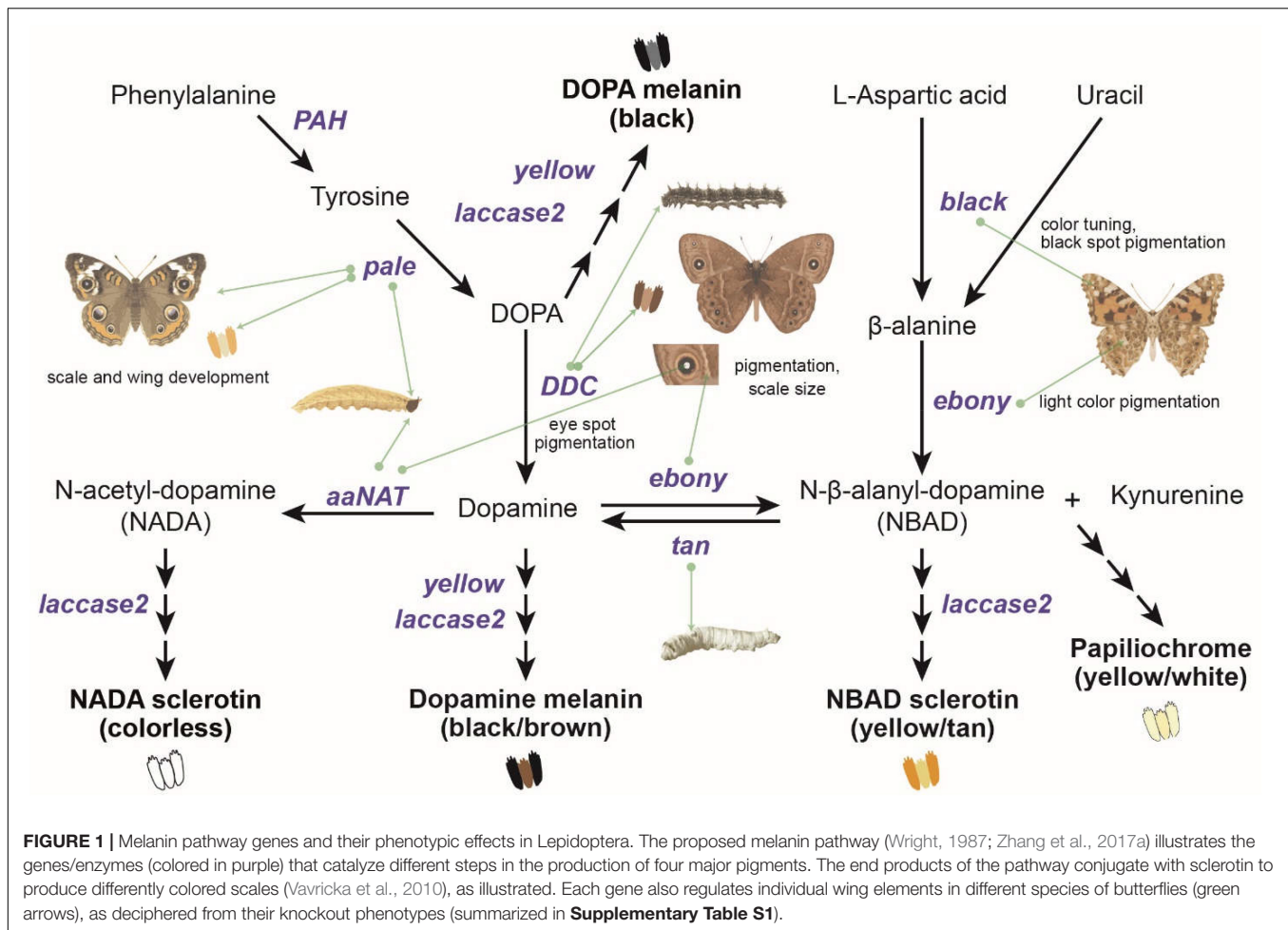
Gene Tree Construction and Phylogenetic Analysis

We constructed a species-level phylogeny with two mitochondrial (*cytochrome c oxidase I*, *acetyl-CoA acetyltransferase*) and ten nuclear genes (*elongation factor 1-alpha*, *wingless*, *ribosomal protein S5*, *ribosomal protein S2*, *isocitrate dehydrogenase*, *glyceraldehyde-3-phosphate dehydrogenase*, *malate dehydrogenase*, *catalase*, *CAD*, *ribosomal protein S27a/hairy cell leukemia*) that are used commonly in phylogenetic reconstruction (Wahlberg and Wheat, 2008). We performed multiple sequence alignment on these 13 markers using the codon aligner PRANK v150803 (Löytynoja and Goldman, 2005). For construction of species and gene trees, we calculated the best partition scheme and corresponding sequence evolution model using Partition Finder 2.1.1 (Lanfear et al., 2016). We chose greedy algorithm and Mr. Bayes model of evolution. We selected Bayesian information criterion to compare and choose from the best-fit models. We used a split

¹<http://www.ncbi.nlm.nih.gov>

²<http://www.lepbases.org>

³<http://www.re3data.org>



frequency below 0.01 to assess stationarity and to set the burn-in in Mr. Bayes and then built a consensus tree using the remaining trees (Ronquist et al., 2012).

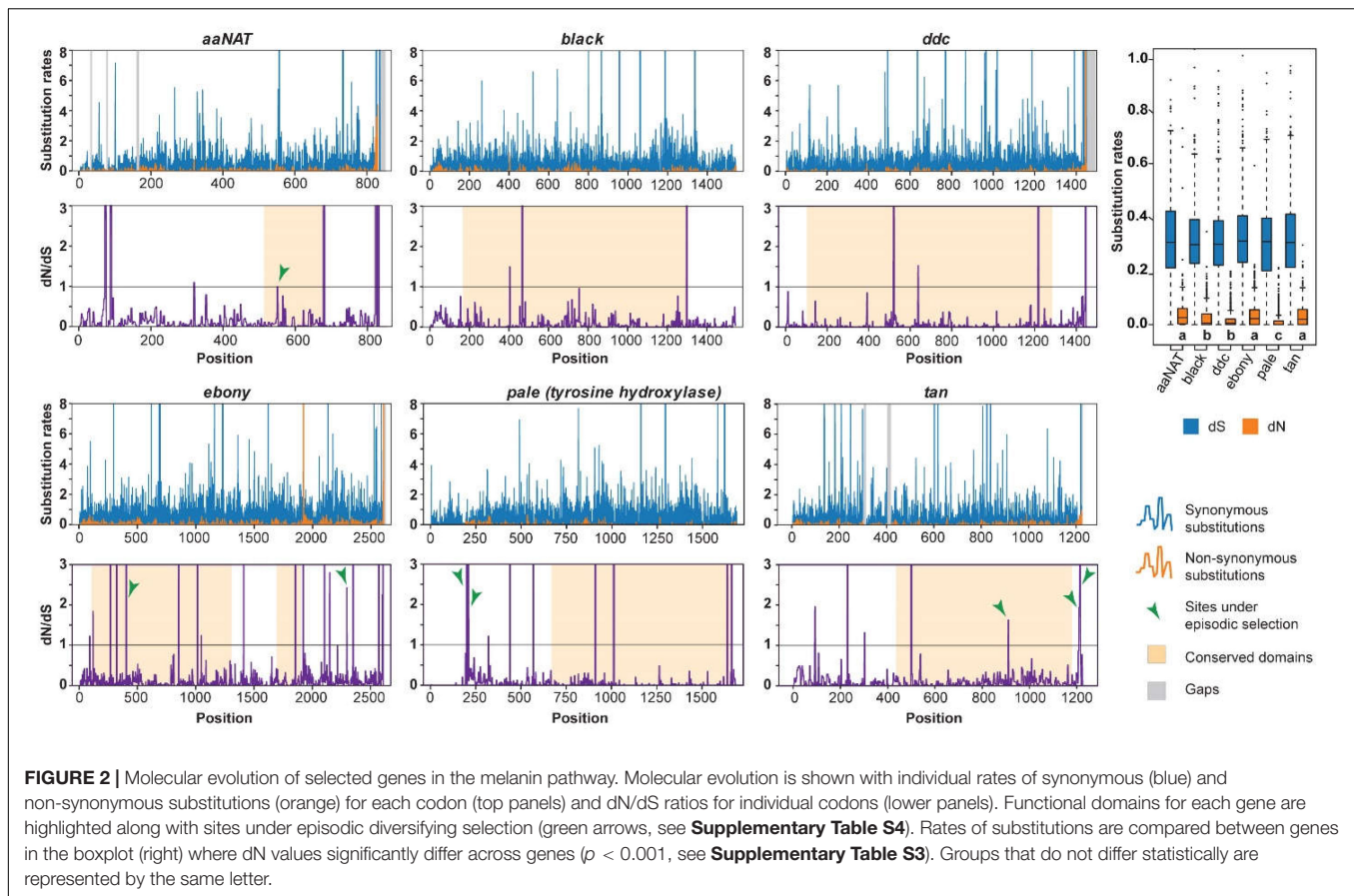
Determination of Synonymous (dS) and Non-synonymous (dN) Substitution Rates

To calculate site-wise synonymous and non-synonymous substitution rates of each gene, we used their respective MUSCLE alignments and the fixed effect likelihood (FEL) method with a maximum-likelihood (ML) approach (Kosakovsky and Frost, 2005). We plotted and compared codon-wise dN, dS, and dN/dS values for each gene (**Figure 2**) and performed a Kruskal-Wallis test followed by Dunn's test with Bonferroni correction in R (Derek et al., 2020). We also identified sites that have been subjected to pervasive diversification ($dN/dS > 1$) or purifying selection ($dN/dS < 1$) within every gene using FEL. We calculated global omega (R value, which represents dN/dS for the entire sequence) for respective alignments using AnalyzeCodonData function of HyPhy 2.3.14 (Pond et al., 2005; Kosakovsky et al., 2020; **Table 1**). For comparison, we also calculated R for genes used in species-level phylogenetic

reconstruction. To identify conserved domains in the given proteins, we used the Conserved Domain Database (CDD) and the CD-Search Tool (Marchler-Bauer et al., 2012). We separately performed FEL analysis and estimated global omega for these domains to compare gene-wide and domain-wide signatures of selection (**Figure 2** and **Table 1**).

Branch-Level, Gene-Wide, and Site-Wise Assessment of Molecular Evolution

We used aBSREL (adaptive Branch-Site Random Effects Likelihood) to test for positive selection along each gene tree (Kosakovsky et al., 2011; Smith et al., 2015). We performed aBSREL analysis in exploratory mode which tests for positive selection along a branch of the gene tree and corrects for multiple testing. aBSREL models site and branch-level omega values but does not test for site-level selection. We used BUSTED to investigate gene-level positive selection. It calculates site-specific evidence ratios and combines this information from sites with weak positive selection to infer selection at the gene level. We carried out MEME (Mixed Effects Model of Evolution) which takes ML approach to test whether individual sites have been subjected to episodic positive or diversifying selection ($dN/dS > 1$). We performed FEL, MEME,



BUSTED, and aBSREL on the Datamonkey Adaptive Evolution Server (Weaver et al., 2018), processing the data using in-house scripts.

Sample Collection, RNA Extraction, and Transcriptome Sequencing for Quantification of Expression

We bred greenhouse populations of *P. polytes* from mated wild-caught females. We maintained larvae at $28 \pm 4^\circ\text{C}$ on lemon (*Citrus* sp.) and curry plants (*Murraya koenigii*) and adults on Birds Choice^{RM} butterfly nectar. We preserved *P. polytes* at different stages of metamorphosis in TRIzol^{RM}. We collected eggs at 2, 10, and 24 h, and at 3 days after oviposition, and pooled five eggs for each sample to get sufficient amount of RNA. We sampled larvae at 1st, 3rd, and 5th instars. For RNA extraction, we used gutted bodies of 1st and 3rd instar larvae and dissected wing disks from 5th instar larvae and pre-pupae to get three tissue types—forewings, hindwings, and gutted body. We collected pupae at 3, 6, and 9 days after pupation and dissected them to separate the forewings, hindwings, abdomen, thorax, and head. Several pigmentation pathway genes participate in transport and phototransduction in insect eyes (True et al., 2005; Ziegler et al., 2013). To account for altered sensitivity and visual perception due to the mating status of the butterflies, if any, we collected unmated

and mated adults separately and further dissected them to separate abdomen, head, eyes, and thorax. We used pure-breeding mimetic lines for this study. We sampled eggs, 1st and 3rd instar larvae in triplicates, 5th instar larvae, pre-pupae, and pupae in duplicates for males and females each, and adults in quadruplicates for each sex.

We extracted RNA from preserved tissues using the chloroform-isopropanol-based extraction method. We prepared libraries using TruSeq[®] RNA Sample Preparation Kit v2 and used Qubit fluorometric quantification. We also used Bioanalyzer to check library profiles. For transcriptome sequencing, we used 2×100 PE runs on Illumina HiSeq 2500 and obtained ~20 million reads for each sample. We performed quality check on these reads using FastQC, trimmed them using Trimmomatic, and aligned them to *P. polytes* reference genome (Nishikawa et al., 2015) using STAR and HISAT2 aligners. We chose the alignments showing higher mapping percentages. We used HTSeq for obtaining raw counts and edgeR pipeline for analyzing and plotting gene expression for all the genes. To determine the effect of sex on expression, we performed a principal component analysis (PCA) in R using the normalized counts for five melanin pathway genes. We excluded *tan* as it did not show sexual dimorphism in its expression profile. We also performed pairwise correlation tests in R (Pearson correlation) to test similarities of expression profiles for all six melanin pathway genes.

TABLE 1 | Molecular evolution of melanin pathway genes in Lepidoptera.

A.						
Gene	<i>aaNAT</i>	<i>black</i>	<i>DDC</i>	<i>ebony</i>	<i>pale</i>	<i>tan</i>
Number of species	37	44	41	39	38	36
Sequence length in nucleotides	852	1545	1503	2622	1689	1277
Percentage of sites with pervasive purifying selection	79.9	89.1	88.02	88.1	88.45	86.06
Percentage of sites with pervasive diversifying selection	0	0	0	0	0	0
Number of branches under episodic diversifying selection	0	2	0	11	2	3
Evidence for gene-wide episodic diversifying selection	Yes	Yes	No	Yes	Yes	No
Number of sites under episodic selection	1	0	0	2	2	3
Global omega value for the gene	0.09	0.06	0.04	0.08	0.04	0.08
Omega value for conserved domain 1	0.07	0.05	0.03	0.07	0.02	0.07
Omega value for conserved domain 2	–	–	–	0.05	–	–
B.						
Gene	Global omega value for the gene					
<i>Cytochrome c oxidase I</i>	0.03					
<i>Acetyl-CoA acetyltransferase</i>	0.03					
<i>Elongation factor 1 – alpha</i>	0.017					
<i>Wingless</i>	0.03					
<i>Ribosomal Protein S5</i>	0.02					
<i>Ribosomal protein S2</i>	0.03					
<i>Isocitrate dehydrogenase</i>	0.02					
<i>Glyceraldehyde-3-phosphate dehydrogenase</i>	0.03					
<i>Malate dehydrogenase</i>	0.03					
<i>Catalase</i>	0.04					
<i>CAD</i>	0.02					
<i>Ribosomal protein S27a/hairy cell leukemia</i>	0.006					

(A) A summary of tests of selection at the levels of individual sites, genes and branches. All the genes studied contained a single conserved domain, except for two domains in *ebony*. (B) Global omega values for genes used in reconstruction of the species tree for taxa used in this study.

RESULTS

Melanin Pathway Genes Are Single-Copy Conserved Genes Under Strong Purifying Selection in Lepidoptera

We reconstructed gene trees for each of the six genes (Supplementary Figure S1). Each gene tree was well supported, and it essentially mirrored the species tree in terms of species relationships and divergence, except *aaNAT*, which had poor resolution with a high degree of polytomy. Absence of long

branches and broad similarity with the species tree suggest that these genes have evolved with species divergence and do not seem to have undergone rapid evolution that would be incongruent with the species tree in any lineage.

We plotted dN/dS values for each site in each gene (Figure 2). The molecular evolution of these genes was characterized by predominantly synonymous substitutions, suggesting constrained evolution (dN/dS values for most sites were well below 1, with mean dN/dS value for each gene ranging from 0.04 to 0.10). Rates for gene-wide synonymous substitutions were similar, but non-synonymous rates were significantly different between three groups of genes: *black* and *DDC*; *ebony*, *tan* and *aaNAT*; and *pale* (Dunn's test, $p < 0.001$) (Supplementary Table S3). Overall, >80% sites showed signatures of pervasive purifying selection ($p < 0.05$), but no sites showed signatures of pervasive diversifying selection in any melanin pathway genes (Table 1).

All the genes had a single functional domain except for *ebony*, which contained two (Figure 2). *R* values for the six genes ranged between 0.04 and 0.09 and were found to be consistently lower for functional domains compared to the gene-wide values (Table 1A). Similarly, *R* values for genes used in species-level phylogenetic reconstruction were considerably lower and ranged between 0.006 and 0.04. We observed that the non-synonymous substitution rate was lowest in *pale*, followed by *black* and *DDC* (Figure 2, $p < 0.05$). Since *DDC*, *black*, and *pale* are essential for the synthesis of dopamine—a precursor for multiple pathways including neuromodulation, immune functions, and the circadian cycle (Basu and Dasgupta, 2000; Allen et al., 2011; Shang et al., 2011)—the low dN/dS as well as global *R* values were expected for these genes. On the other hand, *tan*, *aaNAT*, and *ebony* yielded high *R* values, indicating that they may have evolved under relatively relaxed selection (Figure 2 and Table 1).

Melanin Pathway Genes Show Signs of Episodic Positive Selection

In spite of the overall constrained molecular evolution of the six genes, eight sites showed signatures of episodic diversifying selection ($p < 0.05$), including some in the otherwise highly conserved domain regions (Figure 2, Table 1, and Supplementary Table S4). Interestingly, although *pale* generally shows very constrained molecular evolution similar to *DDC* and *black*, it also contained two of the eight sites under episodic positive selection. While BUSTED could not detect gene-wide episodic diversifying selection in *DDC* and *tan*, aBSREL found evidence for it in all the genes except *DDC*.

Melanin Pathway Genes Show Stage-Specific, Often Sexually Dimorphic Expression

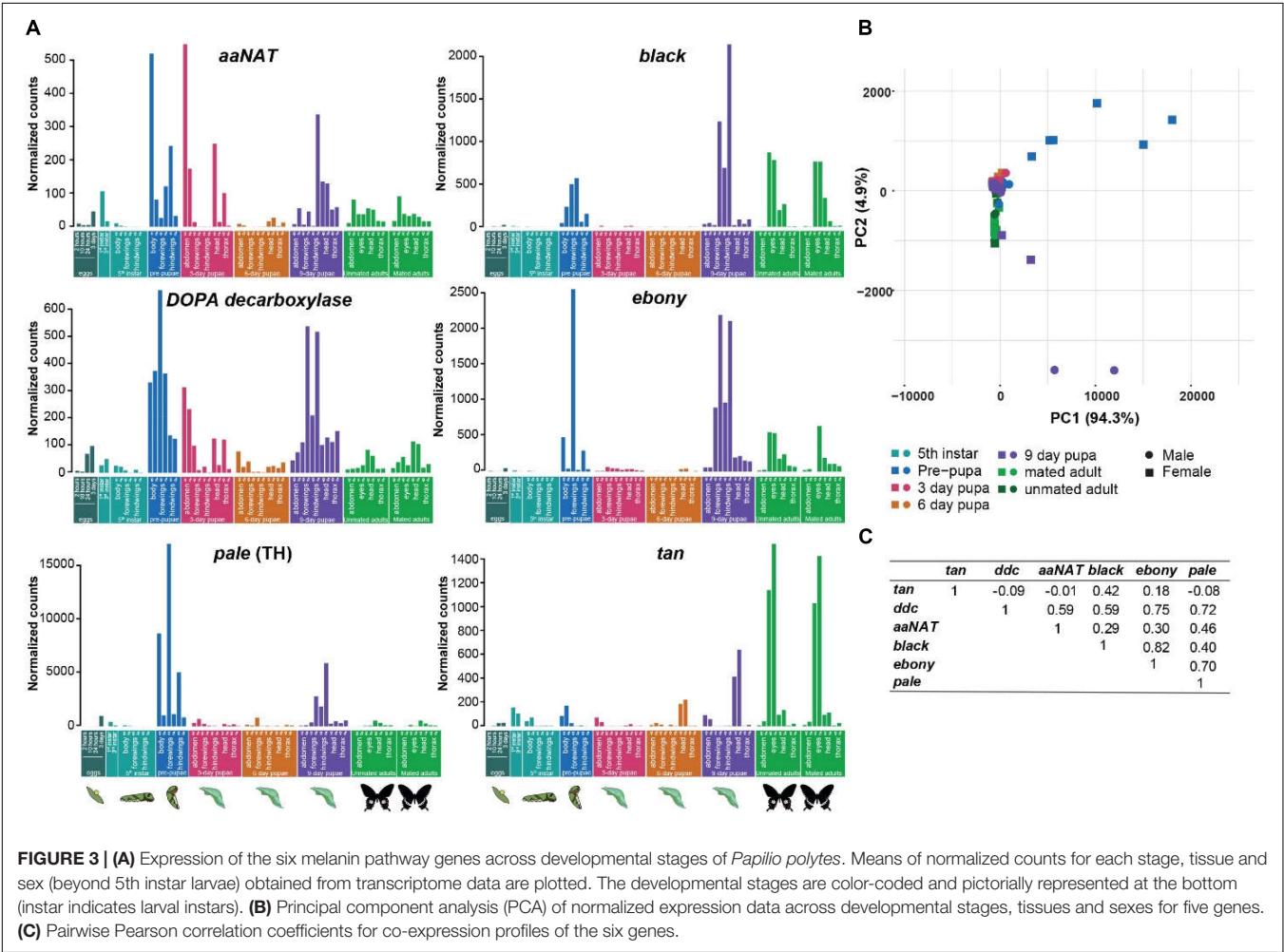
We traced the expression of the six genes across different developmental stages, tissues, and sexes in the developmental transcriptome of *P. polytes*. Broadly, we identified two groups with similar expression patterns across stages and tissues: *aaNAT*,

DDC, and *pale* formed one group, while *black* and *ebony* formed another group (Figure 3). Since *pale*, *DDC*, and *aaNAT* convert tyrosine to N-acetyl-dopamine in successive reactions with no intermediates (Figure 1), we expected similar activity patterns of these genes. *aaNAT*, *DDC*, and *pale* showed similar activity across stages with a peak in the pre-pupal stage (Figure 3 and Supplementary Figure S2), suggesting a more general function than melanization-specific activity. Although *ebony* combines the products of *black* and *DDC* to produce NBAD (Figure 1), its expression pattern was similar to that of *black*, not *DDC*. This suggested that *ebony* activity was more dependent on *black* than on *DDC*. We tested this possibility with an analysis of co-expression between these genes, which indeed showed that the expression profiles of *ebony* and *black* were most strongly correlated compared to any other pairs among these six genes (Figure 3C). Interestingly, *DDC* showed a strong positive correlation with all the other genes except *tan*, which can be attributed to the central function of *DDC* in the melanin pathway (Figure 3C), whose products feed into several branches. These correlations need to be experimentally verified.

Unlike any of the other genes, *tan* had a remarkably distinct pattern of expression across development. It showed

little to no expression in wings but it was expressed in the 1st and 3rd instar larvae, suggesting possible involvement in larval melanization. Expression of *tan*, *black*, and *ebony* in adult eyes and head may have resulted from their role in photo-transduction. For example, *tan* is involved in metabolism of neurotransmitters in photoreceptors, while *ebony* and *black* play a role in signal transduction in the optic lobe (True et al., 2005; Ziegler et al., 2013). Because of this, we further tested whether mated and unmated females show differential expression since female visual systems may switch from mate choice to host plant search after mating. However, expression of these genes was similar in unmated and mated females as well as in males (Figure 3B and Supplementary Table S5).

Finally, to test for sexual dimorphism in the expression patterns of the melanin pathway genes, we performed a PCA using normalized counts of five genes excluding *tan*. Most stages and tissues had low expression of melanin pathway genes, which caused most of the points to cluster around zero. Two stages that stood out—pre-pupal tissues and 9-day pupal wings—showed sexual dimorphism. Melanin pathway genes were upregulated in female pre-pupae and male



wings of 9-day pupae, indicating a sex-specific developmental heterochrony in activity of these genes in developing pupae of *P. polytes*.

DISCUSSION

The melanin pathway is complex and versatile, with products of intermediately placed genes feeding into cross-pathways, performing diverse biological functions (**Figure 1**). The feasibility of genetic manipulation in non-model organisms has made it possible to understand the extent of role played by each gene in pigment production in a wide range of organisms (Mazo-Vargas et al., 2017; Zhang et al., 2017a,b; Connahs et al., 2019). We supplement this understanding by studying the molecular evolution of six genes (*tan*, *black*, *ebony*, *pale*, *aaNAT*, and *DDC*) in the melanin pathway that have prominent roles in color production in butterflies and moths (Koch, 1995; Koch et al., 2000a,b; Futahashi and Fujiwara, 2005; Futahashi et al., 2010; Zhang et al., 2017a; Matsuoka and Monteiro, 2018). With each gene governing a generalized or specialized step, the pathway experiences varied constraints at different levels. We estimated synonymous and non-synonymous substitution rates for each gene using sequence data and tested for signatures of selection. Our study shows that these six genes are highly conserved, despite 100 million years of evolutionary divergence across the sampled Lepidopteran superfamilies (Misof et al., 2014). However, the degree of conservation varies across genes, and most of them even show some signatures of episodic and positive selection.

Our selection analysis revealed that all the melanin pathway genes studied here show signatures of strong purifying selection. The dearth of non-synonymous substitutions across these genes indicates a lack of pervasive diversifying selection at a sequence level, although the developmental regulation of these genes produces dissimilar color patterns across species (Wittkopp et al., 2009; Miyazaki et al., 2014; Yassin et al., 2016). While testing for episodic diversifying selection at the levels of individuals sites, branches and genes, *aaNAT*, *black*, *ebony*, *pale*, and *tan*, but not *DDC*, showed evidence of diversifying selection. Similarly, we did not find any site-level evidence for diversifying selection in *black*. These differential signatures of selection across the genes are consistent with their estimated non-synonymous substitutions, which shows that *black* and *DDC* have followed more constrained evolutionary trajectories compared to the other four genes. The slight difference between domain-wide and gene-wide *R* values suggests that these six genes are nearly as conserved as their functional domains, and in some cases, this may be a result of the domain spanning a large portion of the gene. The melanin pathway genes showed slightly higher *R* values compared to the genes used for phylogenetic reconstruction, reiterating their high degree of conservation. Our study does not offer insights into the functional roles of these sites under diversifying selection. However, it does point out potential developmental genetic targets for future manipulative experiments. The functional

roles of these sites may also become clear using protein structural analysis.

We obtained a few contradictory results in our selection analysis with the three methods used. For example, gene-level analysis using BUSTED inferred the presence of episodic diversifying selection in *black*, but MEME could not detect individual sites under selection. This may be because BUSTED is capable of combining information from sites with weak signals of positive selection to infer presence of selection at the whole-gene level, unlike MEME, which detects individual sites and might therefore underestimate sites under selection at the gene level. Similarly, we performed branch-level tests of diversifying selection with aBSREL, whose exploratory module is less likely to detect selection compared to targeted testing (Kosakovsky et al., 2011; Smith et al., 2015). This may have resulted in *aaNAT* not showing branch-level selection despite showing site-level and gene-level diversifying selection using other methods.

We explored the spatio-temporal activity of melanin pathway genes in *P. polytes* using a developmental transcriptome dataset. The first four instars of *P. polytes* larvae mimic bird droppings with black, brown, and white coloration on their bodies. We expected to detect expression of melanin pathway genes in the eggs and 1st and 3rd larval instars, similar to what we see in 9-day old pupae. However, we did not find substantial larval expression of any of the six genes (except *tan*) even though knockout phenotypes in previous studies have reported a lack of larval pigmentation (Zhang et al., 2017a; Matsuoka and Monteiro, 2018). It is possible that the low expression that we detected suffices in triggering melanization, or we may have missed the transient stage where melanin pathway genes are upregulated in early development. Exploring the expression patterns of other genes in the pathway, such as *laccase2* and *yellow*, may help corroborate either hypothesis. The expression patterns of genes also showed signs of sex-specific developmental heterochrony in melanin production, with expression during pupal development peaking early in females (pre-pupae) and late in males (9-day pupae), as corroborated by PCA. While this could be an artifact of sampling, in some cases (especially between male and female pre-pupae and 9-day old pupae), the difference is quite stark and observed across five of the six genes to be attributed to sampling error. It is possible that males and females differentially invest in melanin production and immunity (or other functions that require melanin pathway intermediates), which would also explain the heterochrony we observed. However, this needs further investigation. With the additional complexity of mimicry in this species, and the use of only mimetic females in this study, the effect of mimicry-related wing-pattern reorganization on melanin pathway genes remains to be explored and could perhaps be linked to the developmental heterochrony.

Melanin pathway genes such as *tan*, *ebony*, and *yellow* are involved in the evolution of color pattern-related sexual dimorphism and polymorphism (Wittkopp et al., 2009; Miyazaki et al., 2014; Yassin et al., 2016), suggesting that these genes have the potential to evolve novel functions despite evolutionarily constrained sequences. Many butterflies and moths exhibit

sexual dimorphism and/or female polymorphism, so they are good system to study signatures of selection in such clades. This would also require information regarding functional allelic variation and duplicated copies of these genes, which we did not encounter in the genes and species that we studied. Alternatively, production of certain color patterns is associated with changes in proximal and/or distal *cis* regulatory regions which govern gene expression. For instance, gain of novel transcription factor binding sites in the *cis*-regulatory region of *yellow* has influenced wing pattern evolution in *Drosophila biarmipes* (Gompel et al., 2005). Examining the regulatory regions of such highly conserved genes and tracing the molecular evolution of upstream regulators might help detect sites that influence color pattern-related sexual dimorphism or polymorphism. Furthermore, selective manipulation of protein-coding versus *cis*-regulatory regions of melanin pathway genes might also help dissect the relative contributions of these genetic elements in pigment production. We have excluded *yellow* gene family from our study even though substantial work has been done on this in flies and butterflies (Arnoult et al., 2013; Miyazaki et al., 2014; Camino et al., 2015; Zhang et al., 2017a; Matsuoka and Monteiro, 2018). We were unable to identify sequences of this gene family from the downloaded genomes with sufficient confidence due to issues with sequence similarity scores. However, the evolution of this gene family has been explored previously (Ferguson et al., 2011).

Our work demonstrates that molecular sequences of the six melanin pathway genes are highly conserved across the vastly diverse Lepidoptera, yet they show some signatures of positive selection. The pattern of conservation and divergence of these genes may be a direct outcome of their functions. Such studies on molecular evolution of genes associated with ecological adaptations (Ferguson et al., 2011; Hughes, 2011; Baral et al., 2019) can help establish baselines against which evolutionary patterns of other genes could be compared. We also explored the developmental expression of these genes across tissues and sexes, which recapitulates what we observe in adult stages, but does not provide insights into larval pigmentation. Our work provides a framework in which broad comparisons can be made across genes and species to understand the genetics and evo-devo of complex pathways that produce the remarkable color patterns displayed by insects.

REFERENCES

- Allen, J. M., Van Kummer, B. H., and Cohen, R. W. (2011). Dopamine as an anorectic neuromodulator in the cockroach *Rhyarobia maderae*. *J. Exp. Biol.* 214, 3843–3849. doi: 10.1242/jeb.062430
- Arnoult, L., Su, K. F. Y., Manoel, D., Minervino, C., Magriña, J., Gompel, N., et al. (2013). Emergence and diversification of fly pigmentation through evolution of a gene regulatory module. *Science* 339, 1423–1426. doi: 10.1126/science.1233749
- Baral, S., Gandhimathi, A., Deshmukh, R., and Kunte, K. (2019). Genetic architecture and sex-specific selection govern modular, male-biased evolution of *doublesex*. *Sci. Adv.* 5:eau3753. doi: 10.1126/sciadv.aau3753
- Basu, S., and Dasgupta, P. S. (2000). Dopamine, a neurotransmitter, influences the immune system. *J. Neuroimmunol.* 102, 113–124. doi: 10.1016/S0165-5728(99)00176-9
- Bilandžija, H., Laslo, M., Porter, M. L., and Fong, D. W. (2017). Melanization in response to wounding is ancestral in arthropods and conserved in albino cave species. *Sci. Rep.* 7:17148. doi: 10.1038/s41598-017-17471-2

DATA AVAILABILITY STATEMENT

The raw RNA seq data are deposited in the NCBI SRA database (BioProject PRJNA634605, Accession nos. SAMN15001929 to SAMN15002046; <https://dataview.ncbi.nlm.nih.gov/object/PRJNA634605?reviewer=mf4q72fcfvq67spjf5gl1sduop>). The accession numbers for genomes used in the molecular evolutionary analysis are provided in **Supplementary Table S2**.

AUTHOR CONTRIBUTIONS

RD and KK designed the study. MK and AP downloaded the sequence data and performed the molecular evolution analysis. RD performed the developmental transcriptome sequencing and analysis. MK and RD prepared the figures and wrote the manuscript. KK conceived and directed the project. All authors contributed to the article and approved the submitted version.

FUNDING

This work was partially funded by an NCBS Research Grant to KK, support of the Department of Atomic Energy, Government of India, under project nos. 12-R&D-TFR-5.04-0800 and 12-R&D-TFR-5.04-0900, and a CSIR Shyama Prasad Mukherjee Fellowship to RD.

ACKNOWLEDGMENTS

We thank Atharva Khotpal, Bhavanarayeni R., Sai Guha, and Sarvesh Menon for assistance in downloading and analyzing genome sequences, Saurav Baral and Vinod Shankar for help with molecular evolution analysis, Sergei L. Kosakovsky Pond for valuable inputs on HyPhy, NCBS Sequencing Facility for transcriptome sequencing, and NCBS Greenhouse Facility for butterfly breeding facilities.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fevo.2020.00226/full#supplementary-material>

- Camino, E. M., Butts, J. C., Ordway, A., Vellky, J. E., Rebeiz, M., and Williams, T. M. (2015). The evolutionary origination and diversification of a dimorphic gene regulatory network through parallel innovations in *cis* and *trans*. *PLoS Genet.* 11:e1005136. doi: 10.1371/journal.pgen.1005136
- Connahs, H., Tlili, S., van Creijl, J., Loo, T. Y. J., Banerjee, T., Das, et al. (2019). Activation of butterfly eyespots by Distal-less is consistent with a reaction-diffusion process. *Development* 146:dev169367. doi: 10.1242/dev.169367
- Derek, O., Powell, W., and Dinno, A. (2020). *FSA: Fisheries Stock Analysis*. Available online at: <https://github.com/droglenc/FSA> (accessed February 2020).
- Deshmukh, R., Baral, S., Gandhimathi, A., Kuwalekar, M., and Kunte, K. (2018). Mimicry in butterflies: co-option and a bag of magnificent developmental genetic tricks. *WIREs Dev. Biol.* 7:e291. doi: 10.1002/wdev.291
- Ferguson, L. C., Green, J., Surridge, A., and Jiggins, C. D. (2011). Evolution of the insect yellow gene family. *Mol. Biol. Evol.* 28, 257–272. doi: 10.1093/molbev/msq192
- Futahashi, R., Banno, Y., and Fujiwara, H. (2010). Caterpillar color patterns are determined by a two-phase melanin gene pre-patterning process: new evidence from *tan* and *laccase2*. *Evol. Dev.* 12, 157–167. doi: 10.1111/j.1525-142X.2010.00401.x
- Futahashi, R., and Fujiwara, H. (2005). Melanin-synthesis enzymes coregulate stage-specific larval cuticular markings in the swallowtail butterfly, *Papilio xuthus*. *Dev. Genes Evol.* 215, 519–529. doi: 10.1007/s00427-005-0014-y
- Gompel, N., Prud'homme, B., Wittkopp, P. J., Kassner, V. A., and Carroll, S. B. (2005). Chance caught on the wing: cis-regulatory evolution and the origin of pigment patterns in *Drosophila*. *Nature* 433, 481–487. doi: 10.1038/nature03235
- Hegna, R. H., Nokelainen, O., Hegna, J. R., and Mappes, J. (2013). To quiver or to shiver: increased melanization benefits thermoregulation, but reduces warning signal efficacy in the wood tiger moth. *Proc. R. Soc. B* 280:20122812. doi: 10.1098/rspb.2012.2812
- Hughes, A. L. (2011). Runaway evolution of the male-specific exon of the *doublesex* gene in Diptera. *Gene* 472, 1–6. doi: 10.1016/j.gene.2010.10.015
- Koch, B. P., Behnecke, B., Weigmann-Lenz, M., and Ffrench-Constant, R. H. (2000a). Insect pigmentation: activities of beta-alanyldopamine synthase in wing color patterns of wild-type and melanic mutant swallowtail butterfly *Papilio glaucus*. *Pigment cell Res.* 13(Suppl. 8), 54–58. doi: 10.1111/j.0893-5785.2000.130811.x
- Koch, B. P., Behnecke, B., and Ffrench-Constant, R. H. (2000b). The molecular basis of melanism and mimicry in a swallowtail butterfly. *Curr. Biol.* 10, 591–594. doi: 10.1016/S0960-9822(00)00494-2
- Koch, B. P., Keys, D. N., Rocheleau, T., Aronstein, K., Blackburn, M., Carroll, S. B., et al. (1998). Regulation of *dopa decarboxylase* expression during colour pattern formation in wild-type and melanic tiger swallowtail butterflies. *Development* 125, 2303–2313.
- Koch, P. B. (1995). Colour pattern specific melanin synthesis is controlled by ecdysteroids via *dopa decarboxylase* in wings of *Precis coenia* (Lepidoptera: Nymphalidae). *Eur. J. Entomol.* 92, 161–167.
- Kosakovsky, P. S. L., and Frost, S. D. W. (2005). Not so different after all: a comparison of methods for detecting amino acid sites under selection. *Mol. Biol. Evol.* 22, 1208–1222. doi: 10.1093/molbev/msi105
- Kosakovsky, P. S. L., Murrell, B., Fourment, M., Frost, S. D. W., Delpont, W., and Scheffler, K. (2011). A random effects branch-site model for detecting episodic diversifying selection. *Mol. Biol. Evol.* 28, 3033–3043. doi: 10.1093/molbev/msr125
- Kosakovsky, P. P. L., Poon, A. F. Y., Velazquez, R., Weaver, S., Hepler, N. L., Murrell, B., et al. (2020). HyPhy 2.5—A customizable platform for evolutionary hypothesis testing using phylogenies. *Mol. Biol. Evol.* 37, 295–299. doi: 10.1093/molbev/msz197
- Kronforst, M. R., and Papa, R. (2015). The functional basis of wing patterning in *Heliconius* butterflies: the molecules behind mimicry. *Genetics* 200, 1–19. doi: 10.1534/genetics.114.172387
- Kumar, S., Stecher, G., Li, M., Knyaz, C., and Tamura, K. (2018). MEGA X: molecular evolutionary genetics analysis across computing platforms. *Mol. Biol. Evol.* 35, 1547–1549. doi: 10.1093/molbev/msy096
- Lanfear, R., Frandsen, P. B., Wright, A. M., Senfeld, T., and Calcott, B. (2016). PartitionFinder 2: new methods for selecting partitioned models of evolution for molecular and morphological phylogenetic analyses. *Mol. Biol. Evol.* 34, 772–773. doi: 10.1093/molbev/msw260
- Löytynoja, A., and Goldman, N. (2005). An algorithm for progressive multiple alignment of sequences with insertions. *Proc. Natl. Acad. Sci. U.S.A.* 102, 10557–10562. doi: 10.1073/pnas.0409137102
- Marchler-Bauer, A., Zheng, C., Chitsaz, F., Derbyshire, M. K., Geer, L. Y., Geer, R. C., et al. (2012). CDD: conserved domains and protein three-dimensional structure. *Nucleic Acids Res.* 41, D348–D352. doi: 10.1093/nar/gks1243
- Matsuoka, Y., and Monteiro, A. (2018). Melanin pathway genes regulate color and morphology of butterfly wing scales. *Cell Rep.* 24, 56–65. doi: 10.1016/j.celrep.2018.05.092
- Mazo-Vargas, A., Concha, C., Livraghi, L., Massardo, D., Wallbank, R. W. R., Zhang, L., et al. (2017). Macroevolutionary shifts of *WntA* function potentiate butterfly wing-pattern diversity. *Proc. Natl. Acad. Sci. U.S.A.* 114, 10701–10706. doi: 10.1073/pnas.1708149114
- Misof, B., Liu, S., Meusemann, K., Peters, R. S., Donath, A., Mayer, C., et al. (2014). Phylogenomics resolves the timing and pattern of insect evolution. *Science* 346, 763–767.
- Miyazaki, S., Okada, Y., Miyakawa, H., Tokuda, G., Cornette, R., Koshikawa, S., et al. (2014). Sexually dimorphic body color is regulated by sex-specific expression of *yellow* gene in ponerine ant *Diacamma* sp. *PLoS One* 9:e92875. doi: 10.1371/journal.pone.0092875
- Nadeau, N. J. (2016). Genes controlling mimetic colour pattern variation in butterflies. *Curr. Opin. Insect Sci.* 17, 24–31. doi: 10.1016/j.cois.2016.05.013
- Nishikawa, H., Iijima, T., Kajitani, R., Yamaguchi, J., Ando, T., Suzuki, Y., et al. (2015). A genetic mechanism for female-limited Batesian mimicry in *Papilio* butterfly. *Nat. Genet.* 47, 1–7. doi: 10.1038/ng.3241
- Olofsson, M., Lovlie, H., Tibblin, J., Jakobsson, S., and Wiklund, C. (2013). Eyespot display in the peacock butterfly triggers antipredator behaviors in naïve adult fowl. *Behav. Ecol.* 24, 305–310. doi: 10.1093/beheco/ars167
- Pond, S. L. K., Frost, S. D. W., and Muse, S. V. (2005). HyPhy: hypothesis testing using phylogenies. *Bioinformatics* 21, 676–679. doi: 10.1093/bioinformatics/bti079
- Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D. L., Darling, A., Höhna, S., et al. (2012). MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Syst. Biol.* 61, 539–542. doi: 10.1093/sysbio/sys029
- Shang, Y., Haynes, P., Pérez, N., Harrington, K. I., Guo, F., Pollack, J., et al. (2011). Imaging analysis of clock neurons reveals light buffers the wake-promoting effect of dopamine. *Nat. Neurosci.* 14, 889–895. doi: 10.1038/nn.2860
- Smith, M. D., Wertheim, J. O., Weaver, S., Murrell, B., Scheffler, K., and Kosakovsky, P. S. L. (2015). Less is more: an adaptive branch-site random effects model for efficient detection of episodic diversifying selection. *Mol. Biol. Evol.* 32, 1342–1353. doi: 10.1093/molbev/msv022
- True, J. R., Yeh, S.-D., Hovemann, B. T., Kemme, T., Meinertzhagen, I. A., Edwards, T. N., et al. (2005). *Drosophila tan* encodes a novel hydrolase required in pigmentation and vision. *PLoS Genet.* 1:e63. doi: 10.1371/journal.pgen.0010063
- van't Hof, A. E., Reynolds, L. A., Yung, C. J., Cook, L. M., and Saccheri, I. J. (2019). Genetic convergence of industrial melanism in three geometrid moths. *Biol. Lett.* 15:20190582. doi: 10.1098/rsbl.2019.0582
- Vavricka, C. J., Christensen, B. M., and Li, J. (2010). Melanization in living organisms: a perspective of species evolution. *Protein Cell* 1, 830–841. doi: 10.1007/s13238-010-0109-8
- Wahlberg, N., and Wheat, C. W. (2008). Genomic outposts serve the phylogenomic pioneers: designing novel nuclear markers for genomic DNA extractions of Lepidoptera. *Syst. Biol.* 57, 231–242. doi: 10.1080/10635150802033006
- Weaver, S., Shank, S. D., Spielman, S. J., Li, M., Muse, S. V., and Kosakovsky, P. S. L. (2018). Datamonkey 2.0: a modern web application for characterizing selective and other evolutionary processes. *Mol. Biol. Evol.* 35, 773–777. doi: 10.1093/molbev/msx335
- Wittkopp, P. J., and Beldade, P. (2009). Development and evolution of insect pigmentation: genetic mechanisms and the potential consequences of pleiotropy. *Semin. Cell Dev. Biol.* 20, 65–71. doi: 10.1016/j.semcdb.2008.10.002

- Wittkopp, P. J., Stewart, E. E., Arnold, L. L., Neidert, A. H., Haerum, B. K., Thompson, E. M., et al. (2009). Intraspecific polymorphism to interspecific divergence: genetics of pigmentation in *Drosophila*. *Science* 326, 540–544. doi: 10.1126/science.1176980
- Wright, T. R. F. (1987). The genetics of biogenic amine metabolism, sclerotization, and melanization in *Drosophila melanogaster*. *Adv. Genet.* 24, 127–222. doi: 10.1016/S0065-2660(08)60008-5
- Yassin, A., Bastide, H., Chung, H., Veuille, M., David, J. R., and Pool, J. E. (2016). Ancient balancing selection at *tan* underlies female colour dimorphism in *Drosophila erecta*. *Nat. Commun.* 7:10400. doi: 10.1038/ncomms10400
- Yassine, H., Kamareddine, L., and Osta, M. A. (2012). The mosquito melanization response is implicated in defense against the entomopathogenic fungus *Beauveria bassiana*. *PLoS Pathog.* 8:e1003029. doi: 10.1371/journal.ppat.1003029
- Zhang, L., Martin, A., Perry, M. W., van der Burg, K. R. L., Matsuoka, Y., Monteiro, A., et al. (2017a). Genetic basis of melanin pigmentation in butterfly wings. *Genetics* 205, 1537–1550. doi: 10.1534/genetics.116.196451
- Zhang, L., Mazo-Vargas, A., and Reed, R. D. (2017b). Single master regulatory gene coordinates the evolution and development of butterfly color and iridescence. *Proc. Natl. Acad. Sci. U.S.A.* 114, 10707–10712. doi: 10.1073/pnas.1709058114
- Zhang, L., and Reed, R. D. (2017). “A practical guide to CRISPR/Cas9 genome editing in Lepidoptera,” in *Diversity and Evolution of Butterfly Wing Patterns*, eds T. Sekimura and H. F. Nijhout (Singapore: Springer), 155–172. doi: 10.1007/978-981-10-4956-9_8
- Ziegler, A. B., Brüsselbach, F., and Hovemann, B. T. (2013). Activity and coexpression of *Drosophila* black with ebony in fly optic lobes reveals putative cooperative tasks in vision that evade electroretinographic detection. *J. Comp. Neurol.* 521, 1207–1224. doi: 10.1002/cne.23247

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2020 Kuwarekar, Deshmukh, Padvi and Kunte. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Expression of Multiple *engrailed* Family Genes in Eyespots of *Bicyclus anynana* Butterflies Does Not Implicate the Duplication Events in the Evolution of This Morphological Novelty

Tirtha Das Banerjee^{1*}, Diane Ramos² and Antónia Monteiro^{1,3*}

¹ Department of Biological Sciences, Faculty of Science, National University of Singapore, Singapore, Singapore,

² Department of Natural Sciences, Daemen College, Amherst, NY, United States, ³ Science Division, Yale-NUS College, Singapore, Singapore

OPEN ACCESS

Edited by:

Marcus Kronforst,
University of Chicago, United States

Reviewed by:

Elizabeth Jenness Duncan,
University of Leeds, United Kingdom
Ralf Janssen,
Uppsala University, Sweden

*Correspondence:

Tirtha Das Banerjee
tirtha_banerjee@u.nus.edu
Antónia Monteiro
antoniamonteiro@nus.edu.sg

Specialty section:

This article was submitted to
Evolutionary Developmental Biology,
a section of the journal
Frontiers in Ecology and Evolution

Received: 01 April 2020

Accepted: 22 June 2020

Published: 24 July 2020

Citation:

Banerjee TD, Ramos D and
Monteiro A (2020) Expression
of Multiple *engrailed* Family Genes
in Eyespots of *Bicyclus anynana*
Butterflies Does Not Implicate
the Duplication Events in the Evolution
of This Morphological Novelty.
Front. Ecol. Evol. 8:227.
doi: 10.3389/fevo.2020.00227

Gene duplication events often create genetic redundancy that can either lead to the appearance of pseudogenes or, instead, create opportunities for the evolution of novel proteins that can take on new functions. One of the genes which has been widely studied with respect to gene duplication is *engrailed* (*en*). *En*-family proteins are expressed in a morphological novelty, eyespots (in the center and in the outer gold ring), in the African squinting bush brown butterfly *Bicyclus anynana*, as well as in a more conserved pattern, the posterior compartment of a wing. In the present study, we used whole-genome sequencing and transcriptome data to show the presence of three *en*-family genes and their differential expression on the pupal wings of *B. anynana* using *in situ* hybridization. The results suggest two duplication events of *en*-family genes, the first evidence of a two-fold duplication in the Lepidoptera. We propose that all copies initially had posterior wing compartment expression and all copies subsequently gained a novel expression domain associated with eyespot centers. Two copies secondarily lost the posterior compartment expression, and one copy alone gained the outer ring expression domain. By dating the origin of both duplication events, however, we conclude that they predate the origin of eyespots by at least 60 mya, and hence our data does not support the retention of the multiple *en* gene duplicates in the genome via their involvement with the novel eyespot evolutionary innovation.

Keywords: gene duplication, *in situ* hybridization, eyespots, *engrailed*, *invected*, *Bicyclus anynana*

INTRODUCTION

Gene duplication plays an essential role in the evolution of novel proteins, and this process has also been proposed to lead to the evolution of major morphological innovations such as flowers; the chambers of the heart; and brains, bones, and cartilage in vertebrates (Theißen, 2001; Olson, 2006; Conant and Wolfe, 2008; Wagner and Lynch, 2010; Zhang et al., 2015). When genes duplicate, there is initially genetic redundancy which allows one of the copies to quickly accumulate mutations and become converted into a pseudogene (Lynch et al., 2001; Zhang, 2003). However, genetic redundancy can also lead to opportunity. When gene duplicates remain intact during the

evolutionary processes this usually means one of three things: (1) that either the double dose of RNA and/or protein increased fitness (Zhang, 2003); (2) that each copy divided the original functions and/or expression domains of the protein among themselves, a process known as sub-functionalization that reduces the total number of functions taken over by each copy and lessens pleiotropy (Force et al., 1999); (3) or that one of the copies gained a novel function, known as neo-functionalization (Force et al., 1999). Examples include sub-functionalization of the gene *engrailed* (*en*) in zebrafish, which resulted in the differential expression of the copies in the pectoral appendage, and in neurons (Force et al., 1999); and neo-functionalization of eosinophil-derived neurotoxin (EDN) and eosinophil cationic protein (ECP) in humans and old world monkeys from EDN, where after the duplication ECP developed a novel anti-pathogenic function (Zhang et al., 1998). Neo-functionalization has been of particular interest since the origin of novel gene duplicates has been loosely associated with the origin of evolutionary innovations (Conant and Wolfe, 2008; Wagner and Lynch, 2010), such as with beetle horns (Zattara et al., 2016), human vision (Yokoyama and Yokoyama, 1989), and betalain pigments in plants (Brockington et al., 2015).

The gene *engrailed* (*en*) has been heavily studied with respect to gene duplications (Force et al., 1999). Duplication of *en* in several independent metazoan lineages has generated a family of related genes, varying from one to four copies, where the paralogs display varying degrees of divergence in expression and function (Peterson et al., 1998; Abzhanov and Kaufman, 2000; Gibert et al., 2000; Damen, 2002; Marie and Blagburn, 2003; Peel et al., 2006).

In addition to its conserved function in segmentation during invertebrate embryogenesis (Manzanares et al., 1993; Brown et al., 1994; Fujioka et al., 2002), *en* is also associated with morphological novelties in several species. For instance, *en* is involved in neurogenesis (Patel et al., 1989), in determining the fate of glial and neuronal cells in grasshopper median neuroblast (Condrón et al., 1994), in axonal targeting in centipedes (Whittington et al., 1991), and in the patterning of insect wing veins (Guillén et al., 1995; Banerjee and Monteiro, 2020). In addition, while no function is yet known, *en* is expressed in precursor cells that build a mollusc's shell (Nederbragt et al., 2002), in the tentacles of the cephalopod *Sepia officinalis* (Baratte and Bonnaud, 2009), in bacteriocytes of the aphid, *Acyrtosiphon pisum* (Braendle et al., 2003), and in the eyespots of saturniid moths (Monteiro et al., 2006) and nymphalid butterflies (Keys et al., 1999; Brunetti et al., 2001).

Eyespots in butterfly wings are novel traits that appear to be specified by old transcription factors and signaling pathways, deployed in novel ways (Keys et al., 1999; Brunetti et al., 2001; Oliver et al., 2012; Monteiro, 2015). Using an antibody that detects a variety of Engrailed proteins (Patel et al., 1989), Keys et al. (1999) found that En-family proteins are expressed in both conserved and novel domains in butterfly wings. En-family proteins maintained their conserved expression domain, and likely their role, in establishing the posterior compartment of each embryonic body segment including that of the wings during the larval stage (Carroll et al., 1994; Keys et al., 1999; Banerjee and Monteiro, 2020). However, one or more of the genes encoding En or its paralog Invested (Inv) were also expressed in the

eyespot centers, in late larval and pupal wings, and in a pattern mapping to the ring of gold scales during the pupal stage of wing development in *Bicyclus anynana* butterflies (Brakefield et al., 1996; Brunetti et al., 2001). A detailed investigation of the number of *en* family gene copies present in the genome of *butterflies*, however, and of the expression domains of each paralog in eyespot patterns is still missing.

Here we provide additional insights on the association between the retention of duplicated genes and their involvement in the origin of novel traits by detailing the expression domain of multiple *en* copies in *B. anynana* wings, asking whether one or more copies are exclusively associated with the eyespot patterns, and testing whether the gene duplication events are closely associated with the origin of eyespots. We report the presence of three *en*-family genes in *B. anynana*, two of which contain all the five major domains of En-family proteins (Peel et al., 2006) and one contains four of these domains. We used sequencing data, to analyze the phylogenetic relationship between the copies, and whole-mount *in situ* hybridizations to examine the expression of all three copies in the pupal wings. Two of the copies are closely related to each other and have a single expression domain on the pupal wing, in the eyespot centers, while the other copy displays three wing expression domains, in the posterior compartment, in the eyespot centers, and in the golden ring. The timing between the last *en*-family gene duplication and the origin of eyespots, however, spans at least 60 mya. We discuss the implications of our data on the mechanisms of evolution of these morphological novelties.

MATERIALS AND METHODS

Rearing *Bicyclus anynana*

B. anynana butterflies were reared in the lab at 27°C, 60% relative humidity, and 12–12 h day–night cycle. Larvae were fed young corn leaves and adults were fed mashed bananas.

Mining for *en* Paralogs and Splice Variants

The sequences of *en*-family genes and splice variants of *B. anynana* were obtained via whole genome sequencing data [*Bicyclus anynana* v1.2 (Nowell et al., 2017)], transcriptome [*B. anynana*; eyespot (Özsu and Monteiro, 2017) and pupal brain (Macias-Munoz et al., 2016)], and 5' and 3' RACE (Rapid Amplification of cDNA Ends). Protein sequences of En and Inv of *D. melanogaster* were obtained from FlyBase. En-family protein sequences of other Lepidopteran species were obtained from Lepbase (Challis et al., 2016), NCBI, EMBL and transcriptome data (Ferguson et al., 2010; Daniels et al., 2014; Connahs et al., 2016) and analyzed for conserved common and paralog-specific domains.

Sequence Alignment and Phylogenetic Tree Construction

Nucleotide sequence alignment was carried out using ClustalW (Thompson et al., 1994) with the default parameters in 'SLOW/ACCURATE' pairwise alignment option in GenomeNet

and visualized in Geneious v10.1.3 (Kearse et al., 2012). Nucleotides were converted into protein sequence via NCBI ORF finder. The protein sequences were aligned via ClustalW (Thompson et al., 1994) with gap open cost: 10, and gap extend cost 0.1; and afterward, manually edited and visualized in Geneious v10.1.3 (Kearse et al., 2012).

A maximum likelihood tree was inferred using RAxML v8.1.20 ran with model PROTGAMMAJTT and default parameters with 100 bootstraps (Stamatakis, 2014) using ETE v3.1.1 (Huerta-Cepas et al., 2016) implemented on GenomeNet. The same tree topology was also obtained using the following other methods and software:

- (a) PhyML v20160115 ran with model JTT and parameters: -f m -nclases 4 -alpha e -pinv e -o tlr -bootstrap 100 (Guindon et al., 2010), using ETE3 v3.1.1 (Huerta-Cepas et al., 2016);
- (b) fasttree with slow NNI and MLACC = 3 (to make the maximum-likelihood NNIs more exhaustive) (Price et al., 2009) using ETE v3.1.1 (Huerta-Cepas et al., 2016); and
- (c) geneious tree builder using Jukes-Cantor as the genetic distance model, UPGMA as tree build method, and 1000 bootstraps (Kearse et al., 2012).

Probe Design of the *en*-Family Genes

Sequence used for *en* probe: A 628 bp region in the exon 1 of *en* CDS was chosen for designing a probe. This region is absent in *inv* and *inv-like* and does not align well with these genes (~35% bp similarity) (Supplementary Figure S5).

Sequences used for *inv* and *inv-like* probes: The regions used for probe preparation are Exon 1 of *inv*, which is not present in *inv-like*, and the 5' UTR of *inv-like*, which is not present in *inv*. The sequence used for the *inv* probe has 24.9% identical bps to that of the *en* gene sequence, and 36.03% identical bps to that of the *inv-like* gene sequence in their overlapping regions. The sequence used for the *inv-like* probe has 30.2% identical bps to that of the *en* gene sequence, and 36.8% identical bps to that of the *inv* gene sequence in the overlapping region (Supplementary Figure S5). This level of sequence similarity is lower than that observed between highly unrelated genes such as *en* and *optix*, which share 41.2% identical sequences. The presence of a very low sequence identity and a small overlap as observed in Supplementary Figure S5 makes it unlikely for the two probes to produce results due to cross-reaction.

Control stainings: *inv-like* and *inv* are mostly identical in the CDS region. Due to this limitation, we used a 182 bps long region in the 5' UTR of *inv-like* (Figure 1A) which is small in size and can produce non-specific stainings. To test for presence of non-specific stainings we performed *inv-like* sense stainings using a probe prepared with the same sequence (Supplementary Figures S1p,q). Sense stainings were also performed for *en* and *inv* (Supplementary Figures S1d-f,j-l).

Prior to probe preparation, amplified fragments of each paralog were sequenced and aligned to ensure that the correct amplicons were used for *in situ* hybridization.

In situ Hybridization

One to two days old pupal wings, timed for moment of pupation using a time-lapse camera, were dissected in 1X PBS at room temperature based on a previously published protocol (Banerjee and Monteiro, 2020) and transferred into 1X PBST with 4% formaldehyde for 30–35 min. Afterward, the wings were washed three times in 1X PBST and treated with 20 mg/ml proteinase K (NEB, P8107S) in 1 ml 1X PBST and then with 2 mg/ml glycine in 1X PBST. Wings were then washed three times in 1X PBST and gradually transferred into a prehybridization buffer (composition in Supplementary Table S4) and incubated at 60°C for 1 h prior to transfer into hybridization buffer (composition in Supplementary Table S4) supplemented with 100 ng/μl of probe. The wings were incubated in hybridization buffer at 60°C for 16–20 h and washed five times with prehybridization buffer at 60°C to remove any unbound probe. To prevent cross-hybridization of the probes, experiments with each probe were performed on separate days. Afterward, wings were brought to room temperature and gradually transferred to 1X PBST and then incubated in block buffer (composition in Supplementary Table S4) for 1 h. 1:3000 dilution of anti-Digoxigenin was then added and incubated for 1 h followed by five washes with block buffer. The wings were then transferred to an alkaline-phosphatase buffer (composition in Supplementary Table S4) supplemented with NBT/BCIP. Imaging was done under a Leica DMS1000 microscope.

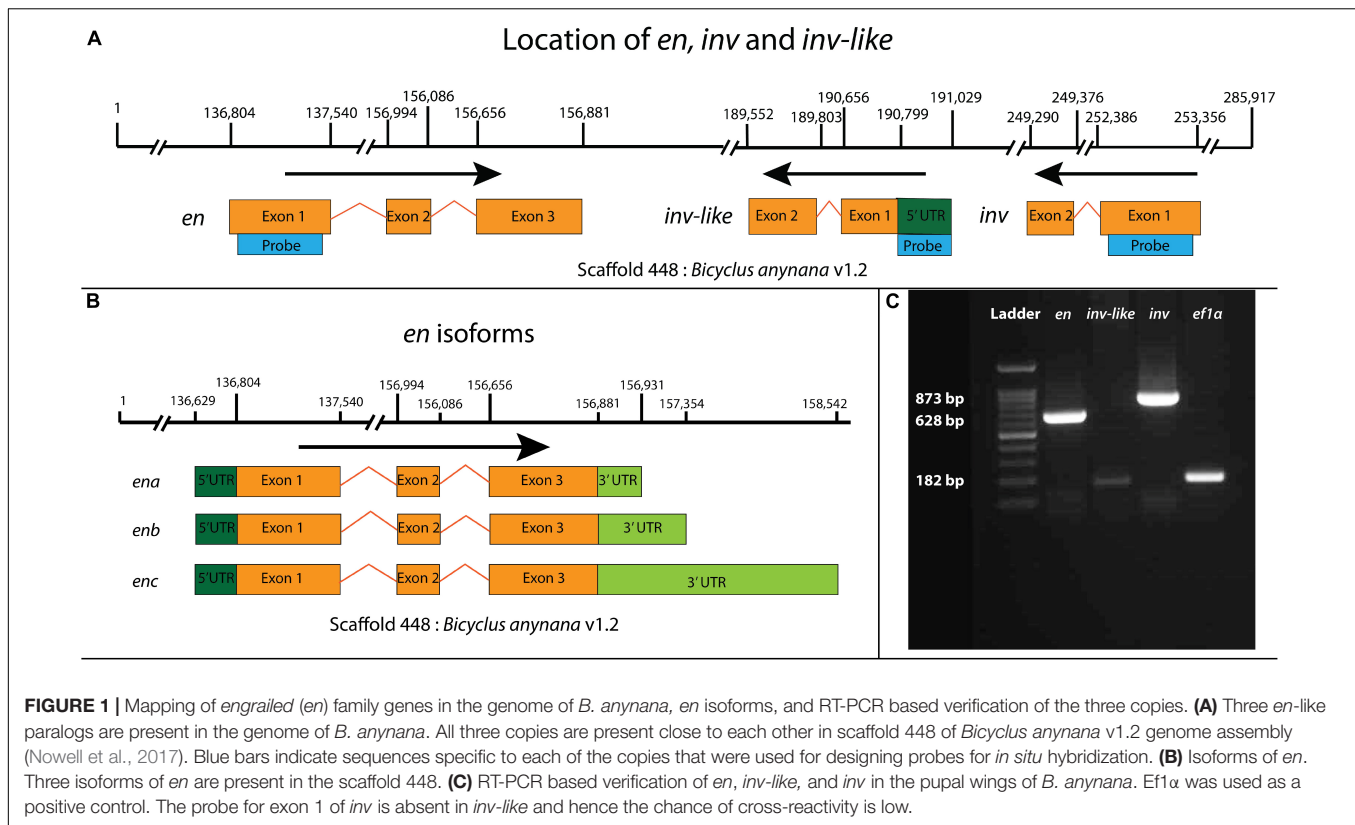
Antibody Staining

One to two days old pupal wings, timed using a time-lapse camera, were dissected in 1X PBS and immediately transferred into a fixation buffer supplemented with 4% formaldehyde at room temperature (composition in Supplementary Table S5) for 30–35 min. Wings were then moved to ice and washed four times with 1X PBS and transferred to block buffer for 1 day (composition in Supplementary Table S5). The next day wings were incubated in primary antibodies against En/Inv [1:15, mouse 4F11, a gift from Patel et al. (1989)], followed by anti-mouse AF488 (Invitrogen, #A28175) secondary antibody. Wings were then washed and mounted on an inhouse mounting media (composition in Supplementary Table S5) and imaged under an Olympus fv3000 confocal microscope.

RESULTS

Three *en*-Family Copies Are Present in the Genome of *Bicyclus anynana*

Whole-genome sequencing (Nowell et al., 2017) and transcriptomic data (Macias-Munoz et al., 2016; Özsü and Monteiro, 2017) revealed three *en*-family genes present in the genome of *B. anynana* within a single scaffold. We named the first copy *engrailed* (*en*) based on the presence of specific sequence domains described below. It has three exons and three splice variants. The 5' UTR of the splice variants are the same, but the 3' UTR region has different lengths across the three variants. The sequences are present between 136,804 and 158,542 bps



of Scaffold 448: *Bicyclus anynana* v1.2 (Figures 1A,B). The transcript is translated into a 352 aa sequence containing all the five conserved domains of En-family proteins and the two En specific domains (Hui et al., 1992) (Figure 2A). The second copy we named *invected* (*inv*). It has two exons present between 249,290 and 235,356 bps of the same scaffold (Figure 1A). The transcript is translated into a 479 aa sequence containing all the five conserved domains of En-family proteins and the Inv specific LVSG and RS domains (Hui et al., 1992; Peel et al., 2006) (Figure 2A). The third copy we named *invected-like* (*inv-like*). It has two exons with a 5' UTR as identified using a pupal brain (Macias-Munoz et al., 2016) and an eyespot-specific transcriptome (Özsu and Monteiro, 2017) (see **Supplementary File** for sequence). The sequence is present between 189,552 and 191,029 bps of the same scaffold (Figure 1A). The transcript is translated into a 125 aa sequence containing four domains of En-family proteins with high similarity to Inv (Figure 2A and results below for details).

Conserved Domains of En-Family Proteins in *B. anynana*

En and Inv proteins contain all five domains of high conservation previously identified for En-family proteins, while Inv-like contains three full domains and one partial domain (Figure 2A) (Logan et al., 1992). The function of most of these regions has been determined: EH1 and EH2 are binding sites for the transcriptional co-repressor Groucho (Tolkunova et al., 1998) and the co-activator Exd (Peltenburg and Murre, 1996)

respectively, EH4 is the homeodomain (Fjose et al., 1985) and EH5 represses En targets (Han and Manley, 1993). The function of EH3 is not known. The EH1 domain of En and Inv are 13 aa each and share 61.5% identity. The EH2 domain of En and Inv are 19 and 21 aa respectively and share 90.5% identity, while Inv-like has only three aa from this domain. The EH3 domain is highly divergent among species and paralogs. En has 11 aa, while Inv and Inv-like have 15 aa each for this domain, respectively. The EH4 domain is a 60 aa homeodomain region which is highly conserved in the three paralogs. Inv and Inv-like are 100% identical in this domain while En has 95% identity with the other two. The EH5 domain is an 18 aa region which is also highly conserved. Inv and Inv-like are 100% identical in this region while En shares 94.4% identity with the other two (Figure 2B).

Phylogenetic Analysis of En-Family Proteins in Lepidoptera

Phylogenetic analysis of the highly conserved sequences from the EH2 to EH5 domains of En-family proteins from nine lepidopteran species using RaxML (Randomly Accelerated Maximum likelihood) (Stamatakis, 2014) resulted in the clustering of all En sequences as a sister lineage to Inv and Inv-like sequences in Lepidoptera (Figure 2C). The same results were obtained using PhyML (Guindon et al., 2010) (Supplementary Figure S2), fasttree (Price et al., 2009) (Supplementary Figure S3), and Geneious Tree builder (Kearse et al., 2012) (Supplementary Figure S3). The protein sequences of Inv and Inv-like at the conserved EH2 to EH5 have 98.9% similarity;

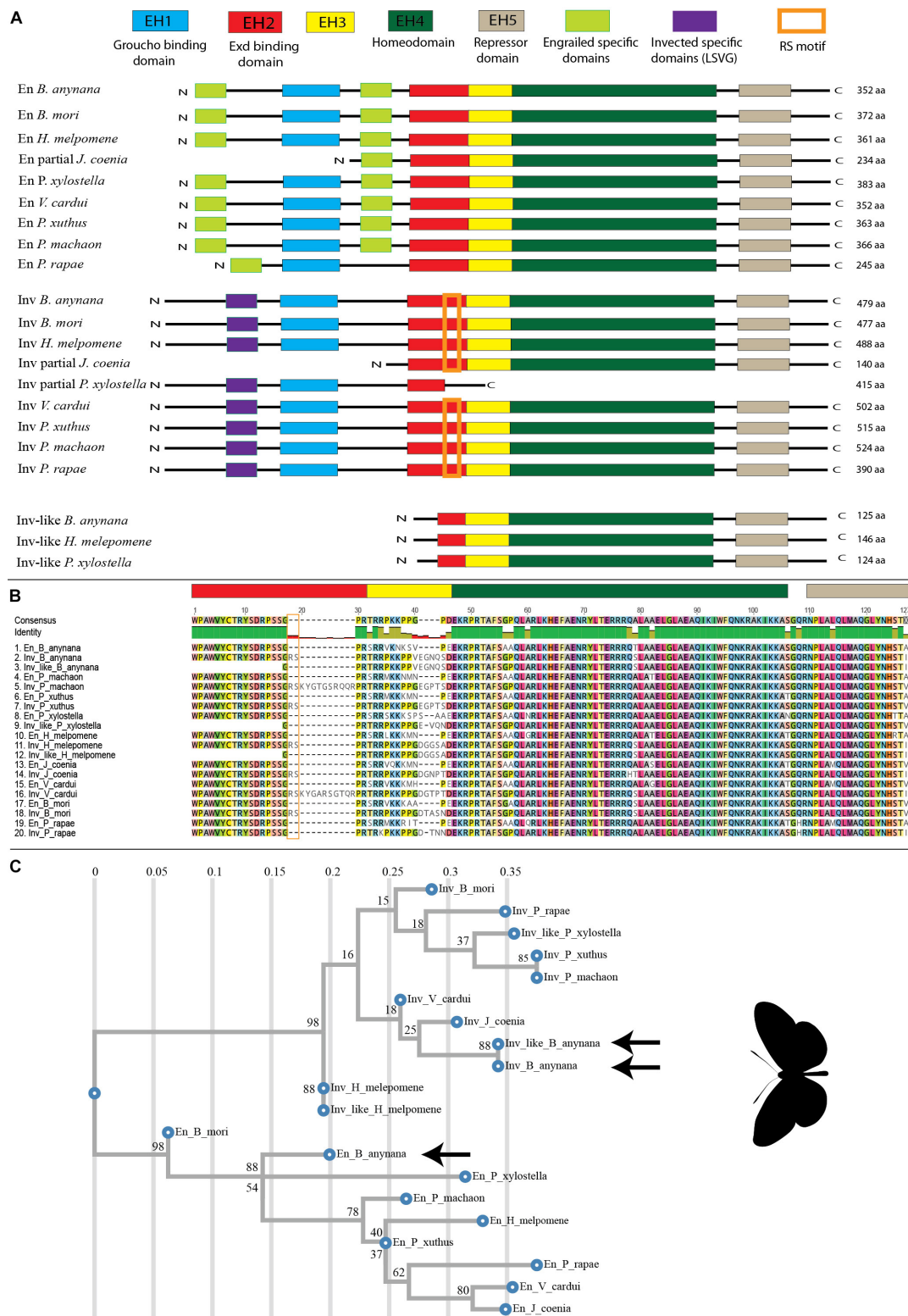


FIGURE 2 | Alignment and Phylogenetic tree of En-family proteins in Lepidoptera. **(A)** En-family proteins have five conserved domains named EH1 to EH5. En and Inv contain all the five En-family domains along with gene-specific domains, while Inv-like only contains three complete domains (EH3 to EH5) and one partial domain (EH2). **(B)** Alignment of En-family proteins around the conserved EH2-EH5. **(C)** Phylogenetic tree of En-family protein created using RAxML (Stamatakis, 2014). En proteins cluster together; and Inv, and Inv-like cluster together. Arrows point to the *B. anynana* paralogs. The values on the top axis represent mean number of amino acid substitutions per site and the node values represent branch support calculated based on 100 bootstraps.

while *En* has 90.1% similarity to *Inv*, and 91.2% similarity to *Inv*-like. Furthermore, the nucleotide sequences of *inv* and *inv-like* are 98.25% similar; while *en* is 80.07% similar to *inv*, and 81.81% similar to *inv-like* at the conserved EH4 and EH5 regions (Supplementary Figure S5 and Supplementary Table S1). The presence of highly conserved sequences between *Inv* and *Inv*-like compared to *En* indicate that *inv* and *inv-like* underwent a more recent duplication.

Expression of *en* Paralogs in the Pupal Wings of *B. anynana*

In situ hybridization results indicate expression differences between the three *en* paralogs in the pupal wings of *B. anynana*. *en* is expressed in the posterior compartment of the wing, in the eyespot centers, and in the golden ring area surrounding each center (Figures 3d–f; Supplementary Figures S1a–c). *inv* and *inv-like*, however, are only present in the eyespot centers (Figures 3i–n and Supplementary Figures S1g–i,m–o).

DISCUSSION

Duplication of *en* Paralogs in Holometabolous Insects

Recent research proposed that a common ancestor to all Lepidoptera has undergone a large-scale genome duplication that is consistent with a whole-genome duplication, while hexapod ancestors to both Diptera and Lepidoptera have undergone two other large scale bursts of gene duplication (Li et al., 2018). These duplications are believed to play important roles in the evolution of hexapods by increasing genetic redundancy and freeing up redundant copies to evolve new functions (Li et al., 2018; Doyle and Coate, 2019). The duplication of *en* into *en* and *inv* happened to a common ancestor to all hexapods including the holometabolous orders Diptera (containing *Drosophila*) and Lepidoptera (butterflies and moths) (Peel et al., 2006). Two of the families within the Lepidoptera, including Pieridae (containing *Pieris rapae*), and Nymphalidae (*Junonia coenia*, *Vanessa cardui*, and *B. anynana*) have butterflies whose genomes and transcriptomes have at least two copies of *en*-family genes (Daniels et al., 2014; Challis et al., 2016; Connahs et al., 2016) with characteristic *En*-family conserved domains. *Inv* has the conserved RS and LSVG motifs present across holometabolous insects (Peel et al., 2006), and *En* proteins one or both *En*-specific motifs (Figure 2A, see Supplementary File for sequences and motifs).

The gene phylogeny suggested that the duplication of a common ancestor into *inv* and *inv-like* might have occurred independently in at least three different lineages: *B. anynana*, *Heliconius melpomene*, and *Plutella xylostella* (Figure 2C). This phylogenetic pattern, of *Inv*-like clustering with its paralog *Inv* in two of these lineages (*H. melpomene* and *B. anynana*) before clustering with its other orthologs (*Inv*-like), can also derive from the phenomenon of gene conversion (discussed below). Independent evidence using genomic copy position and orientation suggests that the duplication of these genes likely

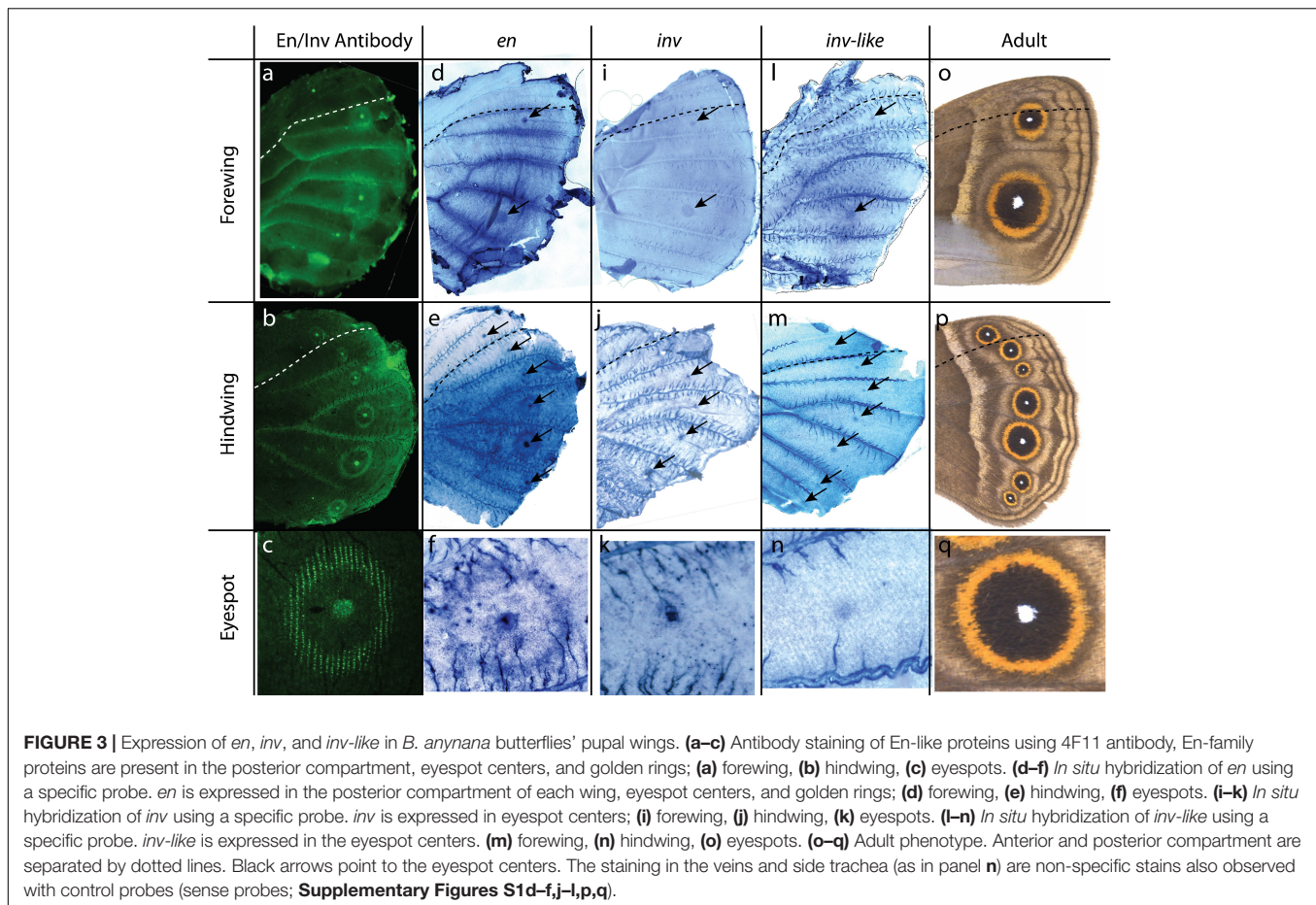
happened to a lineage ancestral to the divergence of all three species. The three *en*-like paralogs from *P. xylostella* map to contig unitig_1988 of *P. xylostella* pacbioV1 genome (Challis et al., 2016); and the same three paralogs from *H. melpomene* map to scaffold Hmel207002 of *H. melpomene* Hmel2 genome (Davey et al., 2015; Challis et al., 2016). All three paralogs map next to each other and in the same genomic orientation as in *B. anynana* (Supplementary Figures S6, S7, Supplementary Table S1, and Figure 1A; sequence in Supplementary File). The conserved genomic architecture suggests that this gene duplication event happened only once in a common ancestor to the three lineages, rather than in the three different lineages independently, and this must have happened at least 130 mya, which is an estimated date for the split of *P. xylostella* moths from the two butterflies obtained from a recent phylogenomic study that included fossil calibrations (Kawahara et al., 2019).

Concerted Evolution of *En*-Family Proteins in *B. anynana*

En-family proteins seem to have evolved under concerted evolution in *B. anynana* (as well as in *H. melpomene*). Gene duplicates that undergo concerted evolution are often found in close physical proximity and maintain high sequence similarity due to unequal crossing over and frequent gene conversion (Zhang, 2003). Due to concerted evolution high sequence similarity is maintained between *En*-family members which has often led to misleading phylogenetic trees (Abzhanov and Kaufman, 2000; Marie and Bacon, 2000; Peel et al., 2006). In particular, higher sequence similarity is maintained between paralogs within a species when compared to similarity between orthologs across species, giving a false phylogenetic signal of multiple duplication events having happened independently in each lineage (Liao, 1999). Specific conserved LSVG and RS motifs in *Inv* paralogs, however, appear to be immune to gene conversion allowing researchers to conclude that the divergence of *en* and *inv* happened before the divergence of hexapods (Peel et al., 2006). We have identified both LSVG and RS motifs in *B. anynana* and other Lepidopteran species (Figures 2A,B) but also observed clear signs of concerted evolution in that *B. anynana* *Inv* has more sequence similarity to *B. anynana* *En* than to *Inv* from *D. melanogaster* (Diptera) (see Supplementary File for sequence). The same phenomenon of gene conversion is also likely happening to *Inv* and *Inv*-like within Lepidoptera, as discussed above.

Overlapping and Distinct Expression Patterns of *en* Paralogs in the Pupal Wings of *B. anynana*

Studies on *en*-family genes in butterflies have been primarily carried out using the 4F11 monoclonal antibody that binds to a conserved domain of the translated *En* and *Inv* proteins of *D. melanogaster* (Patel et al., 1989). These *En*-family proteins are expressed in the posterior compartment and eyespot centers of larval and pupal wings (Keys et al., 1999; Banerjee and Monteiro, 2020) and in the cells that will differentiate the golden ring



of the eyespots in pupal wings of *B. anynana* (Brunetti et al., 2001), but until now it was not clear how many copies of *en-like* genes and transcripts were contributing to the observed multiple protein expression domains in this species. We aimed to find if one or more copies of *en-like* genes were exclusively associated with the novel eyespot color patterns and if these novel expression domains might have contributed to the preservation of *en* paralogs in the *B. anynana* genome.

In situ hybridization against each *en* paralog in the present study indicates that they are expressed in different patterns in the pupal wings of *B. anynana*. The protein expression patterns are likely the result of the expression of all three *en* paralogs (**Figure 3**). The expression in the posterior wing compartment and in the golden ring areas is specific to *en*, while all three paralogs are expressed in the eyespot centers.

Previous work established that both *en* and *inv* are expressed in the posterior compartment of butterflies during larval wing development (Carroll et al., 1994; Banerjee and Monteiro, 2020), however, we show here that later in development only *en* is present in the posterior compartment of pupal wings. No data is currently available for the expression of *inv-like* during the larval stages. Previous studies using *in situ* hybridization showed that *en* in *B. anynana* is homogeneously expressed in the posterior compartment, while *inv* is present in the

upper posterior compartment in *B. anynana* (Banerjee and Monteiro, 2020) and *Junonia coenia* (Carroll et al., 1994). In the pupal stage, we only observed the expression of *en* in the posterior compartment (**Figures 3d–f** and **Supplementary Figures S1a–c**). The loss of *inv* in the posterior compartment during pupal wing development is most likely because this gene is no longer needed to perform its earlier larval function of setting up posterior veins (Banerjee and Monteiro, 2020). Venation patterning happens during the early fifth instar (Banerjee and Monteiro, 2020), where both *en* and *inv* are likely involved in activating other downstream diffusible signals such as Hedgehog and Bone Morphogenic Proteins that position the veins (Banerjee and Monteiro, 2020). Late pupal expression of *en* in the wings of *D. melanogaster*, however, is involved in the maintenance of the posterior compartment, as mutations of *en* at these late stage leads to an enlarged posterior compartment with venation defects (Garcia-Bellido and Santamaria, 1972; Lawrence and Morata, 1976; Blair, 1992). The maintenance of *en* expression in pupal wings of butterflies, suggests that this gene may be playing a similar role.

The expression of all three *B. anynana* *en*-family genes in the eyespot centers indicates that all three paralogs might have a redundant function in the differentiation or signaling

from the eyespot center which sets the rings surrounding it, but this requires functional evidence (**Figure 3**) (Beldade and Brakefield, 2002; Monteiro, 2015). Many lineages of nymphalid butterflies lost expression of En-family protein in the eyespot center (as evidenced by lack of protein expression detected by the 4F11 antibody (Oliver et al., 2012)) and yet they develop eyespots. This suggests that En-family proteins might not be necessary for eyespot center differentiation and/or signaling (Oliver et al., 2012).

The expression of *en* in the golden ring area indicates that this gene alone might play a role in its differentiation, but again this requires functional validation. *B. anynana goldeneye* spontaneous mutants, where the golden ring extends inward into the area of the central black scales in the eyespots, display an equally extended expression of En-family proteins into that area (Saenko et al., 2008) but it is still unclear whether *en*, or some other gene equally affected by the *goldeneye* mutation is actually responsible for the change in scale colors in the central disk.

Alternative 3' UTR Splicing of *en* in *B. anynana*

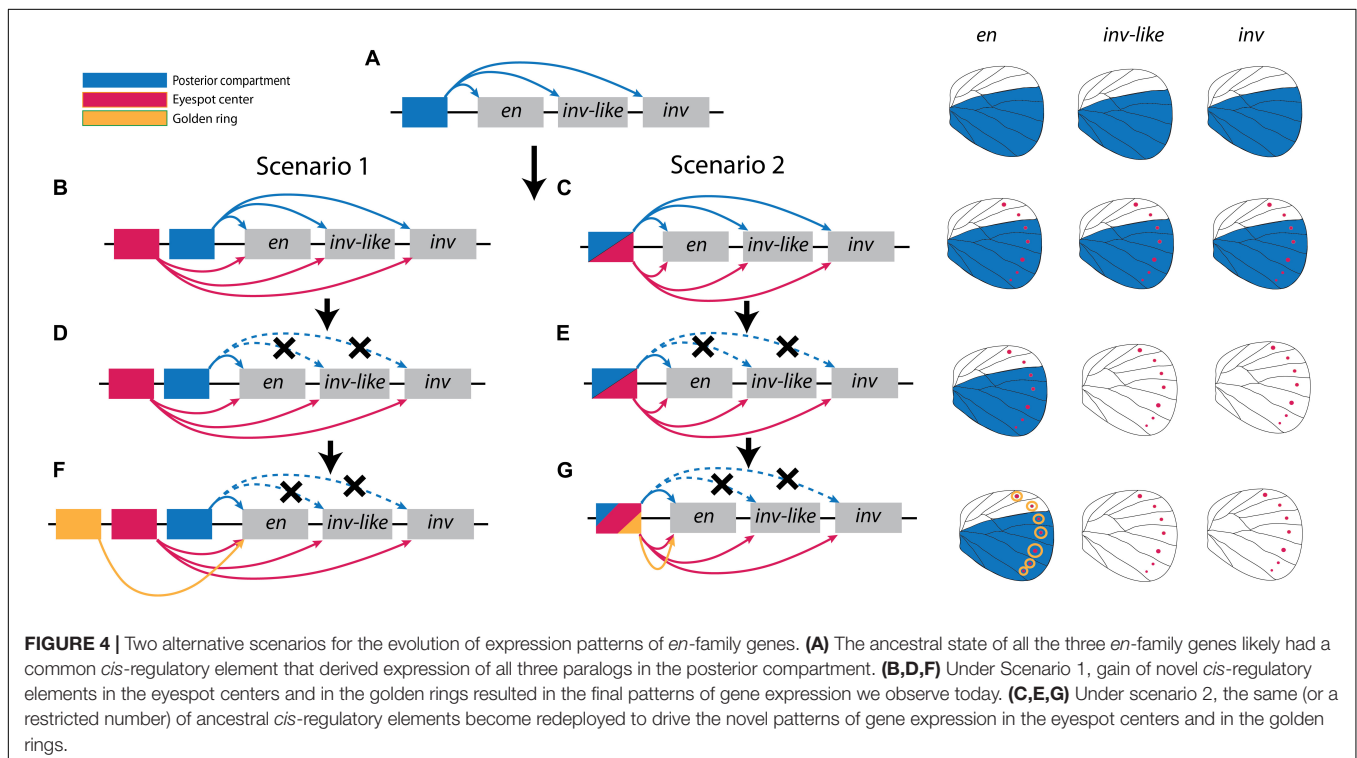
The different isoforms of *en* with varying 3' UTR length could be involved in controlling the level of proteins produced and their localization; and hence, they might also be playing a role in the differential expression of this paralog compared to *inv* and *inv-like*. Alternative 3' UTR lengths are achieved by the post-transcriptional process known as alternative cleavage and polyadenylation (APA) (Elkon et al., 2013). These 3' UTRs are known to control expression level and localization of proteins (Mayr and Bartel, 2009; Elkon et al., 2013; Mariella et al.,

2019). Shorter 3' UTRs are usually translated at higher rates, leading to higher protein levels (Mayr and Bartel, 2009). Future studies could focus on understanding the spatial and temporal expression of the alternative 3' UTR of *en* to investigate if they have diverged from each other.

en Paralogs and Eyespot Evolution

En-family proteins are differentially expressed in Nymphalid butterflies (Brunetti et al., 2001; Oliver et al., 2012). Eyespots are believed to have originated in a lineage sister to the sub-family Danainae, and now revised to have originated around 70 mya (Kawahara et al., 2019), via co-option which resulted in the expression of a set of genes in the eyespot centers, one of which includes En/Inv (Oliver et al., 2012). However, after the duplication, many lineages lost expression of En-family proteins in the eyespot centers (Oliver et al., 2012). *V. cardui*, for example, has expression in the golden rings but not in the eyespot centers (Brunetti et al., 2001), while the closely related *J. coenia* has expression of En-family proteins in the eyespot centers and the surrounding black scales, but not in the golden ring (Brunetti et al., 2001).

The *in situ* hybridization data on the pupal wings of *B. anynana* (**Figure 3**) reveal that two of the *en* paralogs (*inv* and *inv-like*) either lost two ancestral expression domains (posterior compartment and gold ring) or, alternatively, that one paralog (*en*) gained novel expression domains at these two locations after the duplication event. Given that *inv* is expressed in the posterior compartment of larval wings of *B. anynana*, but is absent in pupal wings, it might be more parsimonious to propose that *inv* might have had both



larval and pupal wing expression but subsequently lost its pupal expression.

We propose two equally parsimonious scenarios for the evolution of expression domains of *en* paralogs (Figure 4). The two scenarios are not exclusive. In both scenarios, the ancestral copy of all the present paralogs in *B. anynana* already had expression in the posterior compartment of the wing because this is the pattern of expression for these genes in *D. melanogaster* in the pupal stage (Blair, 1992), which shared a common ancestor with Lepidoptera around 290 mya (Misof et al., 2014) (Figure 4A). This would be facilitated if all copies shared a common set of regulatory elements, as is observed for the regulation of *en* and *inv* in *D. melanogaster* (Gustavson et al., 1989; Cheng et al., 2014). After eyespots originated, around 70 mya (Oliver et al., 2012) (this is a revised estimate based on a new phylogenetic study (Kawahara et al., 2019)), all paralogs gained a novel expression domain in the eyespot centers. This might have happened in a few different ways. A novel *cis*-regulatory element might have evolved in a genomic region that affects the expression of all *en*-copies simultaneously in the eyespot center (Scenario 1, Figure 4B), or a pre-existent *cis*-regulatory element that was already enhancing all copies simultaneously might have been re-used in a novel location (eyespot centers) due to the co-option of a gene regulatory network to that novel location, containing *en/inv* as genes positioned within the co-opted network (Monteiro and Podlaha, 2009; Monteiro and Gupta, 2016) (Scenario 2, Figure 4C). Subsequently, in both scenarios, the expression of *inv* and *inv-like* in the posterior compartment is lost (Figures 4D,E). Finally, either a new *cis*-regulatory element driving *en* expression in the golden ring is gained (Scenario 1, Figure 4F), or a pre-existent *cis*-regulatory element is reused for the novel *en* expression in the golden ring, again via gene network co-option (Scenario 2, Figure 4G). It is, however, also possible that the expression of *en* in association with the gold ring is gained in all three copies at once and subsequently lost in the *inv/inv-like* copies (not shown).

An important point to make here is that the timing of the first duplication event of *en* into *en* and *inv* paralogs happened around 480 mya or earlier (Peel et al., 2006; Misof et al., 2014), and the duplication *inv* into *inv* and *inv-like* happened no more recently than around 130 mya (see “Discussion” section above). Both of these duplications happened much earlier than the origin of eyespots, estimated to be around 70 mya (Oliver et al., 2012, 2014; Kawahara et al., 2019). This suggests that the duplication and maintenance of *en*-like paralogs in the genome of *B. anynana* as well as in the genome of other Lepidoptera is not particularly well associated with the origin of eyespots.

Future studies focused on the expression pattern of the *en* paralogs in model species such as *J. coenia* and *V. cardui*, as well as on the sequencing of open chromatin region surrounding the genes in *B. anynana* using techniques such as FAIRE and ATAC, will help us identify how the expression of *en*-like genes and their *cis*-regulatory elements diversified in the context of eyespot evolution. Functional studies will also be important to discover whether *en*-family

members are actually involved in the regulation of eyespots in nymphalid butterflies.

CONCLUSION

The present study provides the first evidence of the expression of multiple gene duplicates associated with the development of eyespots, a trait novel to nymphalid butterflies (Monteiro, 2015). By studying gene transcripts rather than conserved domains of *en/inv* protein we found that *en* paralogs are expressed in both overlapping and distinct domains. We proposed two models for the evolution of the expression pattern in *en* paralogs, where either loss or gain of novel expression domains resulted in differential expression of the paralogs in the wings on *B. anynana*. Furthermore, we showed that the duplication of the three genes happened much earlier than the origin of eyespots. Hence, this study does not lend support to the idea that these particular gene duplicates were retained in Lepidopteran genomes due to their involvement in the development of this particular evolutionary novelty. Future studies focused on understanding the interaction of *en* paralogs with each other as well as other known genes that play roles in eyespot development will continue to illuminate the evo-devo of eyespot patterns.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

AUTHOR CONTRIBUTIONS

TB, DR, and AM wrote the manuscript. TB performed bioinformatic analysis, *in situ* hybridization, and phylogenetic analysis. TB and AM performed antibody stainings. DR found the splice variants of *en*. TB, DR, and AM analyzed and visualized the results. All authors read and agreed to the final version of the manuscript.

FUNDING

This work was supported by the Ministry of Education, Singapore grant MOE2015-T2-2-159, the National Research Foundation, Singapore Investigatorship award NRF-NRFI05-2019-0006, and the Department of Biological Sciences LHK fund GL 710221. TB was supported by a Yale-NUS scholarship.

ACKNOWLEDGMENTS

We would like to thank Nipam Patel for the anti-Engrailed/Invected 4F11 antibody and Heidi Connahs for help with the transcriptome analysis. We would like to thank DBS-CBIS confocal facility for access to confocal microscopes.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fevo.2020.00227/full#supplementary-material>

FIGURE S1 | Replicates of *en*, *inv*, *inv-like* and control *in situ* hybridization staining in *B. anynana* pupal wings. **(a–c)** Pupal hindwings stained with probe against *en*. The transcript is present in the posterior compartment, in the eyespot centers and in the eyespot golden ring area. **(g–i)** Pupal hindwings stained with probe against *inv*. The transcript is present only in the eyespot centers **(m–o)** Pupal hindwings stained with probe against *inv-like*. The transcript is present only in the eyespot centers. **(d–f)** Pupal wings stained with *en* sense probe (control). **(j–l)** Pupal wings stained with *inv* sense probe. **(p,q)** Pupal wings stained with *inv-like* sense probe. Non-specific staining is observed in the veins and trachea. Anterior-posterior compartments are separated by dotted lines. Black arrows point to eyespot centers.

FIGURE S2 | Phylogenetic tree of En-family proteins in Lepidoptera created using PhyML (Maximum likelihood. See “Materials and Methods” in the main text for details). The tree clusters En together; and Inv and Inv-like together. Vertical lines indicate mean number of amino acid substitutions per site. Numbers above branches represent branch support calculated based on 100 bootstraps.

FIGURE S3 | Phylogenetic tree of En-family proteins in Lepidoptera created using fasttree (Maximum likelihood. See the “Materials and Methods” section in the main text for details). The tree clusters En together; and Inv and Inv-like together.

Vertical lines indicate mean number of amino acid substitutions per site. Numbers above branches represents branch support.

FIGURE S4 | Phylogenetic tree of En-family proteins in Lepidoptera created using Geneious tree builder (Jukes-Cantor as the genetic distance model, and UPGMA as tree build method). The tree clusters En together; and Inv and Inv-like together. Numbers above branches represent branch support calculated based on 1000 bootstraps.

FIGURE S5 | DNA sequence alignment of *en*, *inv* and *inv-like* and the regions used for probe design. Sequence used for *inv* probe has 24.9% identical bps to that of the *en* sequence, and 36.03% identical bps to that of the *inv-like* sequence. Sequence used for *inv-like* probe has 30.2% identical bps to that of the *en* sequence, and 36.8% identical bps to that of the *inv* sequence.

FIGURE S6 | Mapping of *en*, *inv* and *inv-like* in the genome of *P. xylostella* (pacbio1). Location of the transcripts was obtained from Lepbase.

FIGURE S7 | Mapping of *en*, *inv* and *inv-like* in the genome of *H. melpomene melpomene* (Hmel2). Location of the transcripts was obtained from Lepbase.

TABLE S1 | En scaffold features of *B. anynana*, *H. melpomene melpomene*, and *P. xylostella*.

TABLE S2 | Similarity matrix of highly conserved regions (EH4 and EH5) of *en*, *inv* and *inv-like* nucleotide sequences in **Supplementary Figure S5**.

TABLE S3 | Primer table.

TABLE S4 | *In situ* hybridization buffers.

TABLE S5 | Immunohistochemistry buffers.

REFERENCES

- Abzhanov, A., and Kaufman, T. C. (2000). Evolution of distinct expression patterns for *engrailed* paralogues in higher crustaceans (Malacostraca). *Dev. Genes Evol.* 210, 493–506. doi: 10.1007/s004270000090
- Banerjee, T. D., and Monteiro, A. (2020). Dissection of larval and pupal wings of *Bicyclus anynana* butterflies. *Methods Protoc.* 3:5. doi: 10.3390/mps3010005
- Banerjee, T. D., and Monteiro, A. (2020). Molecular mechanism underlying venation patterning in butterflies. *bioRxiv*. [Preprint]. doi: 10.1101/2020.01.02.892760
- Baratte, S., and Bonnaud, L. (2009). Evidence of early nervous differentiation and early catecholaminergic sensory system during sepia officinalis embryogenesis. *J. Comp. Neurol.* 549, 539–549. doi: 10.1002/cne.22174
- Beldade, P., and Brakefield, P. M. (2002). The genetics and evo-devo of butterfly wing patterns. *Nature* 3, 442–452. doi: 10.1038/nrg818
- Blair, S. S. (1992). Engrailed expression in the anterior lineage compartment of the developing wing blade of *Drosophila*. *Development* 115, 21–33.
- Braendle, C., Miura, T., Bicke, R., Shingleton, A. W., Kambhampati, S., Stern, D. L., et al. (2003). Developmental origin and evolution of bacteriocytes in the aphid-Buchnera symbiosis. *PLoS Biol.* 1:21. doi: 10.1371/journal.pbio.0000021
- Brakefield, P. M., Gates, J., Keys, D., Kesbeke, F., Wijngaarden, P. J., Monteiro, A., et al. (1996). Development, plasticity and evolution of butterfly eyespot patterns. *Nature* 384, 236–242. doi: 10.1038/384236a0
- Brockington, S. F., Yang, Y., Fernando, G.-H., Covshoff, S., Hibberd, J. M., Sage, R. F., et al. (2015). Lineage-specific gene radiations underlie the evolution of novel betalain pigmentation in Caryophyllales. *New Phytol.* 207, 1170–1180. doi: 10.1111/nph.13441
- Brown, S. J., Patel, N. H., and Denell, R. E. (1994). Embryonic expression of the single tribolium engrailed homology. *Dev. Genet.* 15, 7–18.
- Brunetti, C. R., Selegue, J. E., Monteiro, A., French, V., Brakefield, P. M., and Carroll, S. B. (2001). The generation and diversification of butterfly eyespot color patterns. *Curr. Biol.* 11, 1578–1585. doi: 10.1016/s0960-9822(01)00502-4
- Carroll, S. B., Gates, J., Keys, D. N., Paddock, S. W., Panganiban, G. E., Selegue, J. E., et al. (1994). Pattern formation and eyespot determination in butterfly wings. *Science* 265, 109–114. doi: 10.1126/science.7912449
- Challis, R. J., Kumar, S., Dasmahapatra, K. K., Jiggins, C. D., and Blaxter, M. (2016). Lepbase: The Lepidopteran genome database. *bioRxiv*. [Preprint]. doi: 10.1101/056994
- Cheng, Y., Brunner, A., Kremer, S., Devido, S., Stefaniuk, C. M., and Kassis, J. A. (2014). Co-regulation of invected and engrailed by a complex array of regulatory sequences in *Drosophila*. *Dev. Biol.* 395, 131–143. doi: 10.1016/j.ydbio.2014.08.021
- Conant, G. C., and Wolfe, K. H. (2008). Turning a hobby into a job: how duplicated genes find new functions. *Nat. Rev. Genet.* 9, 938–950. doi: 10.1038/nrg2482
- Condron, B. G., Patel, N. H., and Zinn, K. (1994). Engrailed controls glial/neuronal cell fate decisions at the midline of the central nervous system. *Neuron* 13, 541–554. doi: 10.1016/0896-6273(94)90024-8
- Connahs, H., Rhen, T., and Simmons, R. B. (2016). Transcriptome analysis of the painted lady butterfly, *Vanessa cardui* during wing color pattern development. *BMC Genomics* 17, 1–16.
- Damen, W. G. M. (2002). Parasegmental organization of the spider embryo implies that the parasegment is an evolutionary conserved entity in arthropod embryogenesis. *Development* 129, 1239–1250.
- Daniels, E. V., Murad, R., Mortazavi, A., and Reed, R. D. (2014). Extensive transcriptional response associated with seasonal plasticity of butterfly wing patterns. *Mol. Ecol.* 23, 6123–6134. doi: 10.1111/mec.12988
- Davey, J., Chouteau, M., Barker, S. L., Maroja, L. S., Baxter, S. W., Simpson, F., et al. (2015). Major improvements to the heliconius melpomene genome assembly used to confirm 10 chromosome fusion events in 6 million years of butterfly evolution. *G3*, 6 695–708. doi: 10.1534/g3.115.023655
- Doyle, J. J., and Coate, J. E. (2019). Polyploidy, the nucleotype, and novelty: the impact of genome doubling on the biology of the cell. *Int. J. Plant Sci.* 180, 1–52. doi: 10.1086/700636
- Elkon, R., Ugalde, A. P., and Agami, R. (2013). Alternative cleavage and polyadenylation: extent, regulation and function. *Nat. Rev. Genet.* 14, 496–506. doi: 10.1038/nrg3482
- Ferguson, L., Lee, S. F., Chamberlain, N., Nadeau, N., Joron, M., Baxter, S., et al. (2010). Characterization of a hotspot for mimicry: assembly of a butterfly wing transcriptome to genomic sequence at the HmYb/Sb locus. *Mol. Ecol.* 19, 240–254. doi: 10.1111/j.1365-294x.2009.04475.x

- Fjose, A., McGinnis, W. J., and Gehring, W. J. (1985). Isolation of a homoeo box-containing gene from the engrailed region of *Drosophila* and the spatial distribution of its transcripts. *Nature* 1, 284–289. doi: 10.1038/313284a0
- Force, A., Lynch, M., Pickett, F. B., Amores, A., Yan, Y. L., and Postlethwait, J. (1999). Preservation of duplicate genes by complementary, degenerative mutations. *Genetics* 151, 1531–1545.
- Fujioka, M., Yusibova, G. L., Patel, N. H., Brown, S. J., and Jaynes, J. B. (2002). The repressor activity of Even-skipped is highly conserved, and is sufficient to activate engrailed and to regulate both the spacing and stability of parasegment boundaries. *Development* 129, 4411–4421.
- Garcia-Bellido, A., and Santamaria, P. (1972). Developmental analysis of the wing disc in the mutant engrailed of *Drosophila melanogaster*. *Genetics* 72, 87–104.
- Gibert, J. M., Mouchel-Vielh, E., Quéinnec, E., and Deutsch, J. S. (2000). Barnacle duplicate *engrailed* genes: divergent expression patterns and evidence for a vestigial abdomen. *Evol. Dev.* 2, 194–202. doi: 10.1046/j.1525-142x.2000.00059.x
- Guillén, I., Mullor, J. L., Capdevila, J., Sánchez-Herrero, E., Morata, G., and Guerrero, I. (1995). The function of *engrailed* and the specification of *Drosophila* wing pattern. *Development* 121, 3447–3456.
- Guindon, S., Jean-François, D., Lefort, V., Anisimova, M., Hordijk, W., and Gascue, O. (2010). New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0. *Syst. Biol.* 59, 307–321. doi: 10.1093/sysbio/syq010
- Gustavson, E., Goldsborough, A. S., Ali, Z., and Kornberg, T. B. (1989). The *Drosophila* engrailed and invected genes: partners in regulation. *Expr. Funct.* 906, 893–906.
- Han, K., and Manley, J. L. (1993). Functional domains of the *Drosophila* engrailed protein. *EMBO J.* 12, 2723–2733. doi: 10.1002/j.1460-2075.1993.tb05934.x
- Huerta-Cepas, J., Serra, F., and Bork, P. (2016). ETE 3: reconstruction, analysis, and visualization of phylogenomic data. *Mol. Biol. Evol.* 33, 1635–1638. doi: 10.1093/molbev/msw046
- Hui, C. C., Matsuno, K., Ueno, K., and Suzuki, Y. (1992). Molecular characterization and silk gland expression of Bombyx engrailed and invected genes. *Proc. Natl. Acad. Sci. U.S.A.* 89, 167–171. doi: 10.1073/pnas.89.1.167
- Kawahara, A. Y., Plotkin, D., Espeland, M., Meusemann, K., Toussaint, E. F. A., Donath, A., et al. (2019). Phylogenomics reveals the evolutionary timing and pattern of butterflies and moths. *Proc. Natl. Acad. Sci. U.S.A.* 116, 22657–22663. doi: 10.1073/pnas.1907847116
- Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., et al. (2012). Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* 28, 1647–1649. doi: 10.1093/bioinformatics/bts199
- Keys, D. N., Lewis, D. L., Selegue, J. E., Pearson, B. J., Goodrich, L. V., Johnson, R. L., et al. (1999). Recruitment of a hedgehog regulatory circuit in butterfly eyespot evolution. *Science* 283, 532–534. doi: 10.1126/science.283.5401.532
- Lawrence, P. A., and Morata, G. (1976). Compartments in the wing of *Drosophila*: a study of the engrailed gene. *Dev. Biol.* 50, 321–337. doi: 10.1016/0012-1606(76)90155-x
- Li, Z., Tiley, G. P., Galuska, S. R., Reardon, C. R., Kidder, T. I., Rundell, R. J., et al. (2018). Multiple large-scale gene and genome duplications during the evolution of hexapods. *Proc. Natl. Acad. Sci. U.S.A.* 115:253609.
- Liao, D. (1999). Concerted evolution: molecular mechanism and biological implications. *Am. J. Hum. Genet.* 64, 24–30. doi: 10.1086/302221
- Logan, C., Hanks, M. C., Noble-Topham, S., Nallainathan, D., Provart, N. J., and Joyner, A. L. (1992). Cloning and sequence comparison of the mouse, human, and chicken engrailed genes reveal potential functional domains and regulatory regions. *Dev. Genet.* 13, 345–358. doi: 10.1002/dvg.1020130505
- Lynch, M., O'Hely, M., Walsh, B., and Force, A. (2001). The probability of preservation of a newly arisen gene duplicate. *Genetics* 159, 1789–1804.
- Macias-Munoz, A., Smith, G., Monteiro, A., and Briscoe, A. D. (2016). Transcriptome-wide differential gene expression in bicyclus anynana butterflies: female vision-related genes are more plastic. *Mol. Biol. Evol.* 33, 79–92. doi: 10.1093/molbev/msv197
- Manzanares, M., Marco, R., and Garesse, R. (1993). Genomic organization and developmental pattern of expression of the engrailed gene from the brine shrimp *Artemia*. *Development* 118, 1209–1219.
- Marie, B., and Bacon, J. P. (2000). Two engrailed-related genes in the cockroach: cloning, phylogenetic analysis, expression and isolation of splice variants. *Dev. Genes Evol.* 210, 436–448. doi: 10.1007/s004270000082
- Marie, B., and Blagburn, J. M. (2003). Differential roles of engrailed paralogs in determining sensory axon guidance and synaptic target recognition. *J. Neurosci.* 23, 7854–7862. doi: 10.1523/JNEUROSCI.23-01-07854.2003
- Mariella, E., Marotta, F., Grassi, E., Gilotto, S., and Provero, P. (2019). The length of the expressed 3' UTR is an intermediate molecular phenotype linking genetic variants to complex diseases. *Front. Genet.* 10:714. doi: 10.3389/fgene.2019.00714
- Mayr, C., and Bartel, D. P. (2009). Widespread shortening of 3'UTRs by alternative cleavage and polyadenylation activates oncogenes in cancer cells. *Cell* 138, 673–684. doi: 10.1016/j.cell.2009.06.016
- Misof, B., Liu, S., Meusemann, K., Peters, R. S., Donath, K., Mayer, C. U., et al. (2014). Phylogenomics resolves the timing and pattern of insect evolution. *Science* 346, 763–767.
- Monteiro, A. (2015). Origin, development, and evolution of butterfly eyespots. *Annu. Rev. Entomol.* 60, 253–271. doi: 10.1146/annurev-ento-010814-020942
- Monteiro, A., Glaser, G., Stockslager, S., Glansdorp, N., and Ramos, D. (2006). Comparative insights into questions of lepidopteran wing pattern homology. *BMC Dev. Biol.* 6:52.
- Monteiro, A., and Gupta, M. D. (2016). *Identifying Coopted Networks and Causative Mutations in the Origin of Novel Complex Traits. Current Topics in Developmental Biology*. Berlin: Elsevier Inc, doi: 10.1016/bs.ctdb.2016.03.003
- Monteiro, A., and Podlaha, O. (2009). Wings, horns, and butterfly eyespots: how do complex traits evolve? *PLoS Biol.* 7:e37. doi: 10.1371/journal.pbio.1000037
- Nederbragt, A. J., Loon, E. V., and Dictus, W. J. A. G. (2002). Expression of patella vulgata orthologs of engrailed and dpp-BMP2 / 4 in adjacent domains during molluscan shell development suggests a conserved compartment boundary mechanism. *Dev. Biol.* 355, 341–355. doi: 10.1006/dbio.2002.0653
- Nowell, R. W., Elsworth, B., Oostra, V., Zwaan, B. J., Wheat, C. W., and Saastamoinen, M. (2017). A high-coverage draft genome of the mycalesine butterfly *Bicyclus anynana*. *Gigascience* 6, 1–7.
- Oliver, J. C., Beaulieu, J. M., Gall, L. F., Piel, W. H., and Monteiro, A. (2014). Nymphalid eyespot serial homologues originate as a few individualized modules. *Proc. R. Soc. B Biol. Sci.* 281:20133262. doi: 10.1098/rspb.2013.3262
- Oliver, J. C., Tong, X. L., Gall, L. F., Piel, W. H., and Monteiro, A. A. (2012). Single origin for nymphalid butterfly eyespots followed by widespread loss of associated gene expression. *PLoS Genet* 8:e1002893. doi: 10.1371/journal.pgen.1002893
- Olson, E. N. (2006). Gene regulatory networks in the evolution and development of the heart. *Science* 313, 1922–1927. doi: 10.1126/science.1132292
- Özsu, N., and Monteiro, A. (2017). Wound healing, calcium signaling, and other novel pathways are associated with the formation of butterfly eyespots. *BMC Genomics* 18, 1–14.
- Patel, N. H., Martin-Blanco, E., Coleman, K. G., Poole, S. J., Ellis, M. C., Kornberg, T. B., et al. (1989). Expression of engrailed proteins in arthropods, annelids, and chordates. *Cell* 58, 955–968. doi: 10.1016/0092-8674(89)90947-1
- Peel, A. D., Telford, M. J., and Akam, M. (2006). The evolution of hexapod engrailed-family genes: evidence for conservation and concerted evolution. *Proc. R. Soc. B Biol. Sci.* 273, 1733–1742. doi: 10.1098/rspb.2006.3497
- Peltenburg, L. T., and Murre, C. (1996). Engrailed and Hox homeodomain proteins contain a related Pbx interaction motif that recognizes a common structure present in Pbx. *EMBO J.* 15, 3385–3393. doi: 10.1002/j.1460-2075.1996.tb00704.x
- Peterson, M. D., Popadić, A., and Kaufman, T. C. (1998). The expression of two engrailed-related genes in an apterygote insect and a phylogenetic analysis of insect engrailed-related genes. *Dev. Genes Evol.* 208, 547–557. doi: 10.1007/s004270050214
- Price, M. N., Dehal, P. S., and Arkin, A. P. (2009). Fasttree: computing large minimum evolution trees with profiles instead of a distance matrix. *Mol. Biol. Evol.* 26, 1641–1650. doi: 10.1093/molbev/msp077
- Saenko, S. V., French, V., Brakefield, P. M., and Beldade, P. (2008). Conserved developmental processes and the formation of evolutionary novelties: examples from butterfly wings. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 363, 1549–1555.

- Stamatakis, A. (2014). RAXML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* 30, 1312–1313. doi: 10.1093/bioinformatics/btu033
- Theissen, G. (2001). Development of floral organ identity: stories from the MADS house. *Curr. Opin. Plant Biol.* 4, 75–85. doi: 10.1016/s1369-5266(00)00139-4
- Thompson, J. D., Higgins, D. G., and Gibson, T. J. (1994). CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22, 4673–4680. doi: 10.1093/nar/22.22.4673
- Tolkunova, E. N., Fujioka, M., Kobayashi, M., Deka, D., and Jaynes, J. B. (1998). Two distinct types of repression domain in engrailed: one interacts with the groucho corepressor and is preferentially active on integrated target genes. *Mol. Cell. Biol.* 18, 2804–2814. doi: 10.1128/mcb.18.5.2804
- Wagner, G. P., and Lynch, V. J. (2010). Hox cluster duplications and the opportunity for evolutionary novelties. *Curr. Biol.* 20, 14603–14606. doi: 10.1073/pnas.2536656100
- Whittington, P. M., Meier, T., and King, P. (1991). Segmentation, neurogenesis and formation of early axonal pathways in the centipede, *Ethmostigmus rubripes* (Brandt). *Roux's Arch. Dev. Biol.* 199, 349–363. doi: 10.1007/bf01705928
- Yokoyama, S., and Yokoyama, R. (1989). Molecular evolution of human visual pigment genes. *Mol. Biol. Evol.* 6, 186–197.
- Zattara, E. E., Busey, H. A., Linz, D. M., Tomoyasu, Y., and Moczek, A. P. (2016). Neofunctionalization of embryonic head patterning genes facilitates the positioning of novel traits on the dorsal head of adult beetles. *Proc. R. Soc. B Biol. Sci.* 283:20160824. doi: 10.1098/rspb.2016.0824
- Zhang, J. (2003). Evolution by gene duplication: an update. *Trends Ecol. Evol.* 18, 292–298. doi: 10.1016/s0169-5347(03)00033-8
- Zhang, J., Rosenberg, H. F., and Nei, M. (1998). Positive darwinian selection after gene duplication in primate ribonuclease genes. *Proc. Natl. Acad. Sci. U.S.A.* 95, 3708–3713. doi: 10.1073/pnas.95.7.3708
- Zhang, S., Zhang, J. S., Zhao, J., and He, C. (2015). Distinct subfunctionalization and neofunctionalization of the B-class MADS-box genes in *Physalis floridana*. *Planta* 241, 387–402. doi: 10.1007/s00425-014-2190-3

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2020 Banerjee, Ramos and Monteiro. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



From Patterning Genes to Process: Unraveling the Gene Regulatory Networks That Pattern *Heliconius* Wings

W. Owen McMillan^{1*†}, Luca Livraghi^{1,2†}, Carolina Concha^{1†} and Joseph J. Hanly^{1,3†}

¹ Smithsonian Tropical Research Institute, Gamboa, Panama, ² Department of Zoology, University of Cambridge, Cambridge, United Kingdom, ³ Department of Biological Sciences, The George Washington University, Washington, DC, United States

OPEN ACCESS

Edited by:

Mark Rebeiz,
University of Pittsburgh, United States

Reviewed by:

Sean Mullen,
Boston University, United States
Jeffrey M. Marcus,
University of Manitoba, Canada

*Correspondence:

W. Owen McMillan
mcmillano@si.edu

[†]These authors have contributed
equally to this work

Specialty section:

This article was submitted to
Evolutionary Developmental Biology,
a section of the journal
Frontiers in Ecology and Evolution

Received: 04 March 2020

Accepted: 16 June 2020

Published: 24 July 2020

Citation:

McMillan WO, Livraghi L,
Concha C and Hanly JJ (2020) From
Patterning Genes to Process:
Unraveling the Gene Regulatory
Networks That Pattern *Heliconius*
Wings. *Front. Ecol. Evol.* 8:221.
doi: 10.3389/fevo.2020.00221

Butterfly wing patterns have emerged as exceptional model systems with which to link the developmental and genetic processes that generate morphological variation with the ecological and evolutionary processes that shape this variation in natural populations. Among butterflies, research on species within the genus *Heliconius* has provided remarkable opportunities to explore how phenotypic diversity is generated within the context of an extraordinary adaptive radiation. Wing pattern diversity among the 48 species and hundreds of intraspecific variants arose within the last 12–14 million years and includes striking pattern convergence between distantly related species, as well as marked pattern divergence between closely related populations and species. Here, we synthesize recent research aimed at gaining a mechanistic understanding of how this variation is generated. This research integrates decades of controlled crossing experiments, and the discovery of major wing patterning genes (*optix*, *aristaless1*, *WntA* and *cortex*) with recent functional genetic manipulation using CRISPR/Cas9 targeted mutagenesis. The emerging data provides a rich framework with which to explore the repeatability of evolution, particularly within the context of how natural selection acts on divergent gene regulatory networks to generate both highly convergent, as well as highly divergent phenotypes. Overall, the functional data show that the gene regulatory networks underlying pattern variation diverge rapidly in *Heliconius*; yet these networks retain enough flexibility so that natural selection can drive the evolution of nearly identical patterns from different developmental genetic starting points. Moreover, for the first time this research is starting to illuminate the links between the genetic changes modulating pattern variation and how they influence the larger gene networks that are ultimately responsible for patterning a butterfly wing. There are still large gaps in our understanding, but current research priorities are well laid out and experimental methodologies are in place to address them. The challenge is to synthesize diverse research strategies into a cohesive picture of morphological evolution.

Keywords: *Heliconius*, morphological evolution, CRISPR/Cas9, cell fate, patterning loci

“...on these expanded membranes, nature writes, as on a tablet, the story of the modifications of species.” Henry Walter Bates, in “Naturalist on the river Amazons” (1864).

BACKGROUND AND OVERVIEW

On the 28th of May 1848, Henry Walter Bates, then only 23 years old, stepped off the boat in Para, Brazil with his friend Alfred Russell Wallace. Bates was to spend the next 11 years exploring the Amazon Basin and his collections, particularly his butterfly collections, provided some of the most visually intuitive examples of evolution by natural selection (Bates, 1864). During his explorations, Bates was struck by the remarkable similarity in the vivid wing patterns of *Heliconius* and other butterfly and moth species and how these patterns would change rapidly as he traveled across the region, as if “touched by an enchanter’s wand” (Bates, 1879). His insights led to the development of mimicry theory, whereby natural selection drives the evolution of phenotypic similarity (Pasteur, 1982). In the intervening 150 years, research has started to identify the genetic and developmental processes responsible for the evolution of butterfly wing patterns generally, including the mimetic wing patterns of *Heliconius*. For this reason, the study of butterfly wing patterns continues to speak to research questions at the forefront of biology today, much as it did during that early dawn of evolutionary theory.

Here we review recent research into the genetic and developmental basis of wing pattern change. We focus on the vivid wing patterns of *Heliconius*, which are used both as aposematic warning signals and as conspecific mating cues (Benson, 1972; Mallet et al., 1990; Jiggins et al., 2001; Kapan, 2001; Chouteau et al., 2017; Merrill et al., 2019). This combination of natural and sexual selection is associated with remarkable variation in wing patterns both within and between species, including numerous cases of parallel evolution of similar wing patterns between distantly related species (**Figure 1**). Classical crossing work done in the 1950s and 1960s by Beebe, Crane, Sheppard, Turner, and others (Turner, 1972; Sheppard et al., 1985; Beebe, 2005) established that pattern variation within a specific *Heliconius* species had a relatively simple genetic basis, with most variation explained by a small number of autosomal loci. Later, linkage mapping and positional cloning demonstrated that the same suite of major patterning loci are modulating pattern variation broadly across the genus and that variation around these loci is responsible for both convergent change between species and divergent change within species (Joron et al., 2006; Kapan et al., 2006; Kronforst et al., 2006; Huber et al., 2015). The advent of more powerful genomic technologies culminated in the identification of specific genes that modulate pattern variation across the genus (Reed et al., 2011; Martin et al., 2012; Nadeau et al., 2016; Westerman et al., 2018). These loci join a growing list shown to play a general role in wing pattern variation in butterflies and moths (**Table 1**).

The discovery of “wing patterning” genes in *Heliconius* allows us to make broad general conclusions about how morphological diversity is generated. Foremost, patterning loci identified in *Heliconius* have also been shown to affect pattern variation broadly across butterflies and even moths. For example, the gene *cortex* appears to be a major target of evolution across the lepidoptera (Nadeau et al., 2016; van’t Hof et al., 2016). In *Heliconius*, the locus acts across both wing surfaces and

underlies variation in white, yellow, orange, and black pattern elements (Joron et al., 2006). *Cortex* has also been implicated in pattern variation in other butterflies (VanKuren et al., 2019), the peppered moth *Biston betularia* and other geometrids (van’t Hof et al., 2016, 2019), as well as the silkworm *Bombyx mori* (Ito et al., 2016). Secondly, nearly all the patterning genes thus far identified are highly conserved at the amino acid level and are used extensively throughout embryonic and larval development, and later simply redeployed on a developing wing to affect pattern variation. The transcription factor *optix*, for example, was first discovered in *Drosophila*, where ectopic expression of the gene in the developing antennae generates ectopic eyes (Seimiya and Gehring, 2000). It is also expressed in the *Drosophila* pupal wing and is involved with vein patterning there (Organista et al., 2015). In *Heliconius*, *optix* is expressed in the optic lobe (Martin et al., 2014), where it presumably plays a role in neural development. However, in *Heliconius*, *optix* has also evolved new expression domains and is expressed in the developing pupal wing from about 60 h after pupation, where its expression perfectly prefigures red pattern variation on *Heliconius* wings (Reed et al., 2011). Thirdly, pattern variation is produced mostly by regulating when and where these genes are expressed during development. The gene *WntA* is a member of the Wnt family of signaling ligands and has similarly evolved new patterning roles, with dramatic shifts in *WntA* expression fueling pattern variation in *Heliconius* (Martin et al., 2012; Concha et al., 2019) and other nymphalid butterflies (Gallant et al., 2014; Martin and Reed, 2014; Mazo-Vargas et al., 2017).

This review synthesizes recent research aimed at gaining a more mechanistic understanding of how wing color pattern variation in *Heliconius* is generated. We touch only briefly on the biology and ecology of the group that provides the context for understanding their remarkable pattern evolution. This aspect of *Heliconius* has been reviewed many times (see Supple et al., 2014; Kronforst and Papa, 2015), culminating in a recent book (Jiggins, 2017). Similarly, we do not review the body of research that led to the identification of the four major patterning genes. This was a sustained effort going back more than 50 years, which, coupled with ideas from evolutionary developmental genetics (Brakefield and French, 1999; Nijhout, 2001; Carroll, 2008), allowed the remarkable progress that has been made over the last 3 years. Rather, we focus on the research that is using the discovery of key patterning loci to begin to unravel the regulatory networks that lay at the heart of pattern diversity. Specifically, we outline how one can cast the origin of pattern diversity on a butterfly wing as a basic question of scale cell fate, and then discuss how recent experiments on *Heliconius* are beginning to illuminate the role that major patterning loci play in the determination of scale cell fate. Finally, we highlight the research challenges and opportunities moving forward.

WING PATTERNS AS A MODEL FOR THE EVOLUTION OF SCALE CELL FATE

The identification of the patterning loci that have been the targets of natural selection in *Heliconius* is a major research

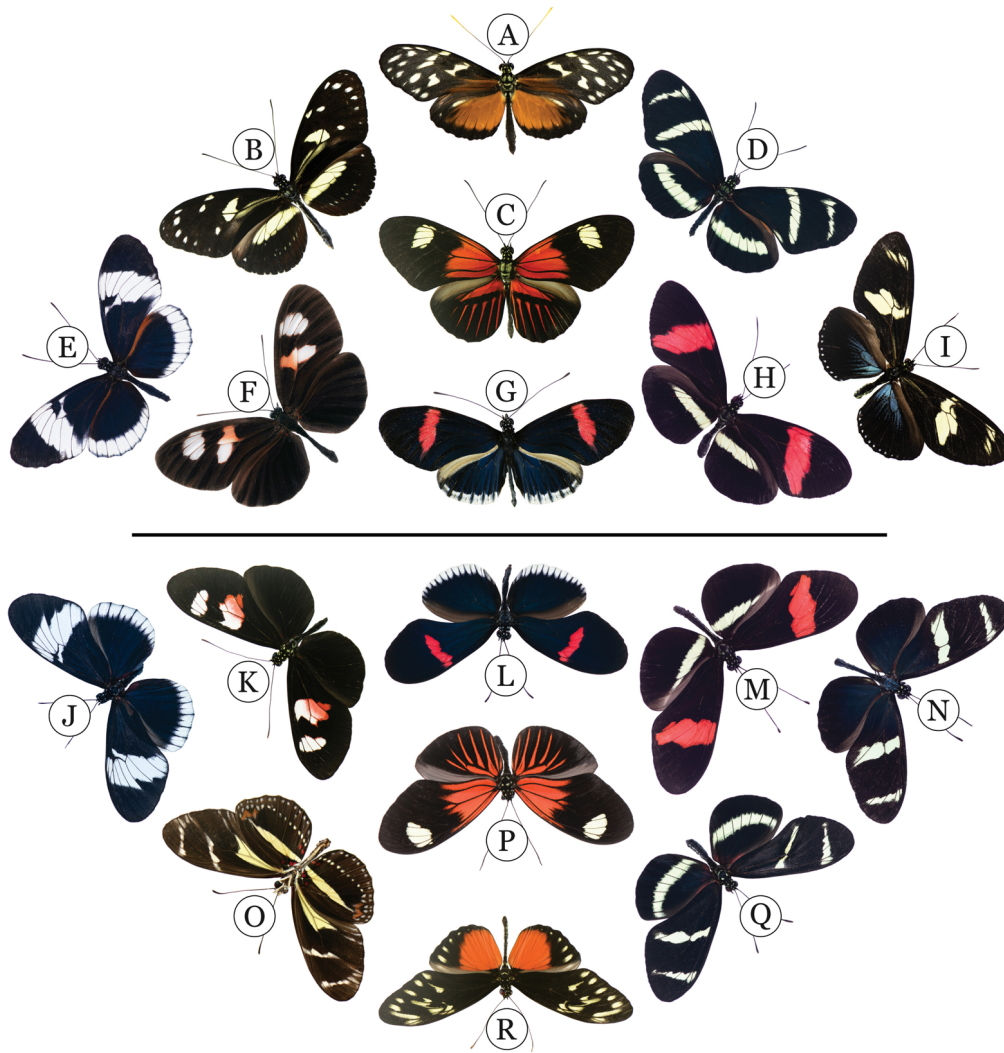


FIGURE 1 | Evolution's canvas: Wing pattern mimicry among species in the *melpomene/cydnosilvaniform* clade (MCS) (above line) and the *erato/sapho/sara* clade (ESS) (below line). The two lineages diverged from each other about 12 MYA (Kozak et al., 2015), yet have recently evolved a complement of identically patterned wings. Species in the MCS clade are: (A) *H. hecale zuleika*, (B) *H. atthis*, (C) *H. melpomene ecuadorensis*, (D) *H. pachinus*, (E) *H. cydnos chioneus*, (F) *H. m. plesseni*, (G) *H. m. cythera*, (H) *H. m. rosina*. Species in the ESS clade are: (J) *H. sapho sapho*, (K) *H. erato notabilis*, (L) *H. e. cyrbia*, (M) *H. e. demophoon*, (N) *H. sara sara*, (O) *H. peruvianus*, (P) *H. e. etylus*, (Q) *H. hewitsoni*, (R) *H. hecalesia formosus*. *Heliconius doris* (I) is from a third clade that falls between the ESS and MCS lineages.

step; however, it is important not to lose sight of the fact that genes do not make phenotypes directly (Gawne et al., 2018). Rather the products of genes interact with each other, and with parts of the genome and the environment to form complex webs of interactions that unfold through development (Rudel and Sommer, 2003). In the case of butterfly wing patterns, phenotype is realized in the patterned differentiation of wing epithelial cells into specific scale cell color types (Figure 2). Understanding how butterfly wing patterns form will require us to dissect how these cell types are specified, how they become spatially arranged, and how they differentiate.

The wing patterns of butterflies and moths are composed of overlapping wing scales, which are homologous to insect bristles (Overton, 1966; Galant et al., 1998; Zhou et al., 2009).

Each scale cell generally produces a single pigment and has an elaborate nanostructure. It is the combination of pigment and ultrastructural properties of the scale cells that creates the wing colors we observe (Nijhout, 1991). The fantastic array of complex patterns that we find in nature are generated by varying how different scale cell types are arranged across the wing surface. Importantly, the development of pattern on a butterfly wing is largely devoid of processes such as cell migration which complicate inference, and the developing wing tissue can be mapped accurately onto the corresponding adult phenotype (Nijhout, 1991). We therefore propose that the process of coloring a butterfly wing can be broken into a series of spatially programmed decisions, where progenitor cells acquire specific fates through the interplay of cell signaling and differential gene

TABLE 1 | Wing genes that have been identified by linkage or association as involved with development of wing color or pattern in butterflies and moths.

Gene	Described function	Evidence	References
WntA	Ligand in the Wnt signaling pathway. Conserved function in wing pattern induction across nymphalids and between color pattern elements in <i>Heliconius</i> .	Linkage studies: <i>Heliconius</i> , (various species) Association studies: <i>Heliconius</i> , (various species) <i>Limnitis</i> Expression studies: <i>H. melpomene</i> , <i>H. erato</i> , <i>H. numata</i> , <i>A. vanillae</i> , <i>J. coenia</i> , <i>V. cardui</i> , <i>Limnitis</i> Perturbation studies: <i>Heliconius</i> sp. (protein and regulatory elements), <i>Junonia</i> , <i>Agraulis</i> , <i>Vanessa</i> (Protein)	Martin et al., 2012; Mazo-Vargas et al., 2017; Concha et al., 2019
cortex	Unknown function, paralog of <i>cdc20</i> family. Conserved function in wing patterning across the lepidoptera, frequently associated with switch between melanistic and non-melanistic scale types.	Linkage studies: <i>Heliconius</i> , <i>Biston</i> , <i>Phigalia</i> , <i>Odontoptera</i> , (geometrid moths), <i>Bombyx mori</i> Association studies: <i>Heliconius</i> , <i>Pieris napi</i> , <i>Papilio</i> Expression studies: <i>H. melpomene</i> , <i>H. erato</i> , <i>H. numata</i> Perturbation studies: <i>H. melpomene</i> , <i>H. erato</i> , <i>H. hecale</i>	Nadeau et al., 2016; van't Hof et al., 2016; Saenko et al., 2019; Livraghi et al., 2020
optix	Transcription factor, induces ommochrome pigmentation in several nymphalids as well as structural color in <i>Junonia coenia</i> . In <i>Heliconius</i> , <i>Vanessa</i> and <i>Agraulis</i> , specifies red color pattern elements.	Linkage studies: <i>Heliconius</i> (various species) Association studies: <i>Heliconius</i> (various species) Expression studies: <i>H. melpomene</i> , <i>H. erato</i> , <i>H. numata</i> , <i>A. vanillae</i> , <i>J. coenia</i> , <i>V. cardui</i> Perturbation studies: <i>Heliconius</i> sp. (protein and regulatory elements), <i>Junonia</i> , <i>Agraulis</i> , <i>Vanessa</i> (Protein)	Reed et al., 2011; Martin et al., 2014; Zhang et al., 2017b; Lewis et al., 2019
aristaleless1	Transcription factor with a lepidoptera-specific duplication. Controls switch between white and yellow scales in <i>Heliconius</i> wings, likely through regulation of 3-OHK synthesis.	Linkage studies: <i>Heliconius cydno</i> Association studies: <i>Heliconius</i> sp. Expression studies: <i>Heliconius</i> , <i>Limnitis arthemis</i> , <i>Spodoptera ornithogalli</i> , <i>Ephesia kuehniella</i> Perturbation studies: <i>H. cydno</i>	Martin and Reed, 2010; Westerman et al., 2018
yellow	Gene involved with pigment biosynthesis. Associated with color differences between morphs of <i>D. chrysippus</i> .	Association studies: <i>Danaus chrysippus</i> Perturbation studies: <i>V. cardui</i> , <i>B. anynana</i> , <i>Papilio xuthus</i>	Zhang et al., 2017a; Matsuoka and Monteiro, 2018; Martin et al., 2020
<i>arrow</i>	Co-receptor of the Frizzled-family of Wnt pathway receptors, associated with pattern development in <i>D. chrysippus</i> .	Association studies: <i>Danaus chrysippus</i>	Martin et al., 2020
<i>Shank3</i>	Mediates cycling of Frizzled 2 to the membrane	Association studies: <i>Hesperia colorado</i>	Cong et al., 2019
<i>Sox102F</i>	Transcription factor which regulates the Wnt pathway. Associated with pattern development in <i>Hypolimnas</i>	Association studies: <i>Hypolimnas</i>	VanKuren et al., 2019
Doublesex	Sex-determination factor, involved with sexually dimorphic patterns in <i>Papilio</i> butterflies	Linkage studies: <i>Papilio</i> sp. Perturbation studies: <i>Papilio</i> sp.	Kunte et al., 2014
BarH1	Transcription factor, responsible for alternative life history strategy 'alba' in <i>Colias</i> butterflies	Linkage studies: <i>Colias croceus</i> Expression studies: <i>Colias croceus</i> Perturbation studies: <i>Colias croceus</i>	Woronik et al., 2019
<i>Domeless</i>	JAK-STAT pathway receptor, neighboring cortex and within the <i>H. numata</i> supergene.	Linkage studies: <i>Heliconius</i> Expression studies: <i>H. numata</i> , <i>H. melpomene</i> , <i>H. erato</i> ,	Saenko et al., 2019; Livraghi et al., 2020
<i>Washout</i>	Actin-interacting protein, neighboring cortex and within the <i>H. numata</i> supergene.	Linkage studies: <i>Heliconius</i> Expression studies: <i>H. melpomene</i> , <i>H. erato</i>	Saenko et al., 2010; Livraghi et al., 2020
<i>Ventral veins lacking</i>	Transcription factor involved with patterning the <i>Drosophila</i> wing, associated with forewing band shape differences in <i>Heliconius</i>	Association studies: <i>Heliconius erato</i>	Van Belleghem et al., 2017

Genes for which functional validation exists are shown in bold.

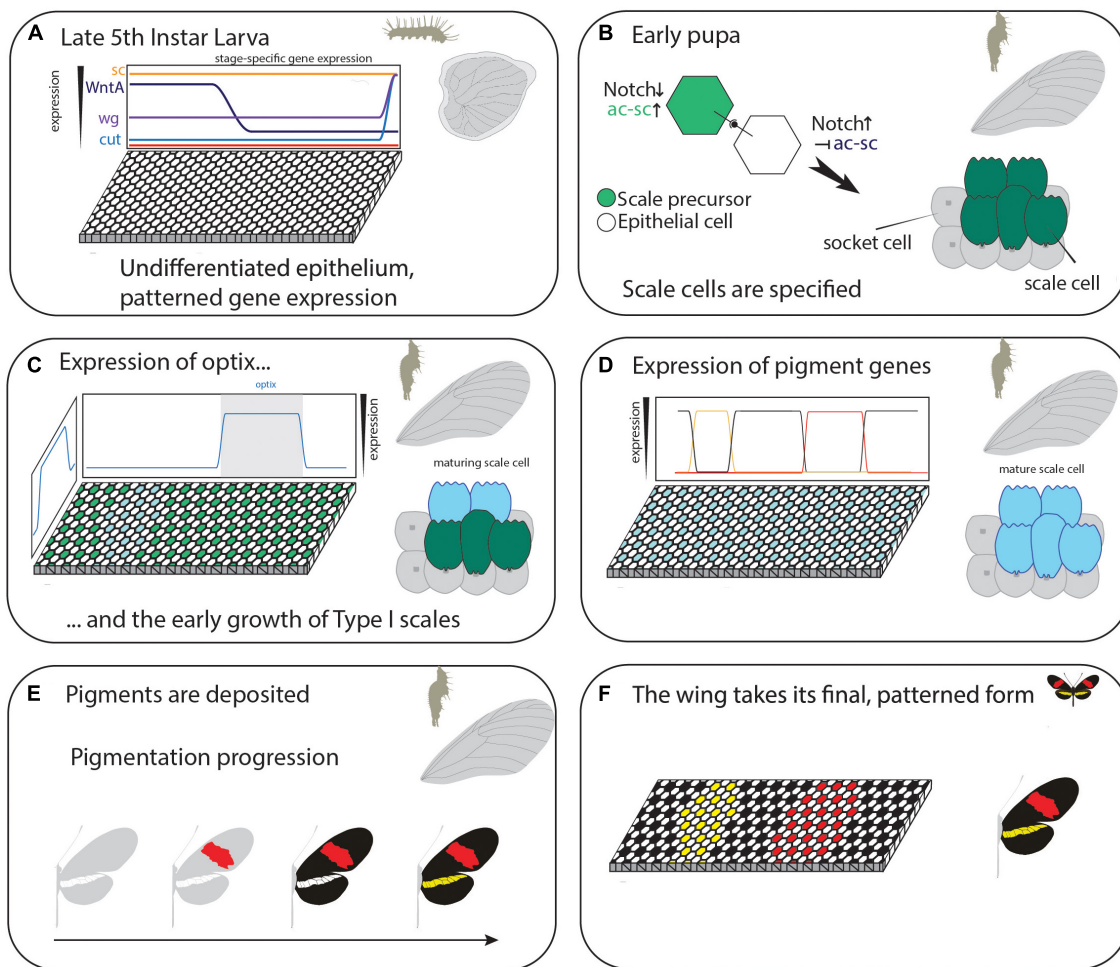


FIGURE 2 | Pattern formation on a butterfly wing: **(A)** In the late larval stage, the presumptive wing imaginal disk is patterned with lacunae (which will form the veins) indicating that the spatial arrangement of the wing has already been patterned. Aside from this, the wing consists of two opposing sheets of undifferentiated epithelial cells, each attached to the basement membrane. Unlike in *Drosophila*, this wing disk can be straightforwardly mapped to the adult wing and will not undergo major morphometric rearrangement. The expression domains of several transcription factors and signaling molecules are known at this stage in butterflies (summarized in Hanly et al., 2019), and notably, despite scale cells having not been specified yet, the ligand *WntA* is already expressed in domains that prefigure adult wing pattern. **(B)** At pupation, the wing disk expands rapidly. During the first 18h of pupal development, scale precursor cells differentiate from their neighbors by notch-delta mediated lateral inhibition (Reed, 2004). Each scale precursor is surrounded by six non-scale epithelial cells, equating to a 3:1 epithelia to scale ratio in the wing. These scale precursor then undergo a series of divisions and apoptosis events that result in a scale cell, which will form the blade of the scale and a socket cell, which will form a supportive structure to hold the scale in place; this indicates homology between the butterfly scale and the *D. melanogaster* bristle, which is supported by the expression of achaete-scute in the scale precursor cell. This process also requires the presence of planar cell polarity in the tissue. **(C)** After the generation of the scale and socket cell, the scale begins to send out a large, actin-driven cytoplasmic protrusion which will form the blade of the scale. In *Heliconius*, the presumptive Type I scales begin this process earlier than their neighbors, which indicates that there is developmental heterochrony between different scale cell types. The expression of additional genes have by now been patterned to prefigure adult wing pattern; for example the gene *optix* is expressed specifically in cells that will become Type III red scales in *Heliconius*. **(D)** Selector genes such as *optix* and *aristless1* in turn induce the expression of pigment biosynthesis in specific regions across the developing wing. **(E)** In mid to late pupal development, scale cell differentiation occurs across the wing, including morphological differentiation and pigment synthesis and deposition. Pigment deposition occurs in a specific temporal manner, with red ommochrome appearing first, followed by melanin and finally deposition of 3-OHK (yellow) in Type I scales. **(F)** This results in the adult wing pattern at eclosion.

expression (Figure 2). Tweaks to these developmental decisions lead to the variation in wing patterns seen both within and between the 20,000 species of butterflies (Kristensen et al., 2007).

The process of pattern formation occurs throughout wing development and is linked to the processes underlying scale cell development. In the final instar of butterfly larvae, developing wing disks increase their size through cell proliferation and begin

to acquire the shape and venation that is characteristic of the adult wing (Figure 2). At this point the wings are composed of a basal lamina straddled by a single cell epithelial layer on both the ventral and dorsal surfaces (Greenstein, 1972; Ghiradella and Radigan, 1976; Nardi and Magee-Adams, 1986; Dinwiddie et al., 2014). Developing cells at this stage are still composed of undifferentiated epithelia, but the inductive signals required

for scale cell identity are being laid out through the expression of transcription factors and signaling molecules (summarized in Hanly et al., 2019). It is during the pupal stage that the morphological development of the wing scales begins. Shortly after pupation, Notch signaling induces scale cell differentiation through lateral inhibition resulting in an array of aligned, parallel cell rows (Reed and Serfas, 2004). Cytological studies have shown that these cells in turn undergo two rounds of cell division promoting a neural lineage and a scale cell lineage (Stossberg, 1938). Neural precursor cells undergo apoptosis, whereas the scale cell precursors undergo a second round of cell division forming a socket cell from which a scale cell arises through actin bundle elongation (Dinwiddie et al., 2014). During pupal development, the scale cells grow dramatically in size and after about 60 h, scale cells fated to become a specific color can often be distinguished (Reed et al., 2008). In our model, during this period all scale cells must acquire a cell identity through the patterned expression of major patterning genes, which in turn will trigger the expression of further differentiation factors or terminal effector genes. Cells must also communicate with each other so that the correct edges and boundaries to delimit pattern elements are appropriately arranged. By 100 h of pupal development, we know that all scale cells are undergoing actin-dependent laminar extension and depositing cuticle, including scale type-specific gene expression of pigment synthesis pathways. By this stage, the wing patterning process is complete.

***Heliconius* AS A MODEL FOR UNDERSTANDING THE GENE REGULATORY NETWORKS (GRNs) RESPONSIBLE FOR PATTERN FORMATION**

The extraordinary diversity in wing patterns among the 48 *Heliconius* species arose within the last 12–14 million years (Kozak et al., 2015) and includes remarkable pattern convergence between distantly related species. Pattern matching is perhaps best exemplified by convergent change in two of the major lineages within the genus - the *H. erato/sapho/sara* (ESS) and the *H. melpomene/cydno/silvaniform* (MCS) species groups (see Figure 1). The two lineages diverged from a common ancestor early in the formation of the genus and have been evolving separately for the past 12 million years, diverging in many aspects of their ecology and reproductive biology (Jiggins, 2017). Nonetheless, within the last 2.5–4.5 million years the groups have converged on a similar complement of wing pattern types, including a number of cases of near-perfect wing pattern mimicry. Although hybridization and introgression play a role in the evolution of novel patterns within the ESS and MCS clades (Edelman et al., 2019), there is no evidence for hybridization between the two groups.

Within this evolutionary backdrop, much of the wing pattern variation in *Heliconius* is created by differences in the distribution of only three major scale cell types. Type I yellow scales

contain the ommochrome precursor 3-hydroxykynurenine (3-OHK) (Brown, 1967); whereas Type I white scales lack pigment and the color is the result of the scale cell morphology (i.e., structural) (Gilbert et al., 1988). Type II scale cells are pigmented with melanin and Type III scale cells contain the ommochrome pigments xanthommatin and dihydroxanthommatin (Gilbert et al., 1988). The timing of scale cell enlargement and maturation differs between scale types, with Type I scale cells maturing first, followed by Type III scale cells. Scale cells fated to become melanin (Type II) mature last (Aymone et al., 2013). Pigment development largely follows differences in scale cell maturation. Scales fated to become yellow or white are evident as colorless patches beginning at about 6 days after pupation. At the same developmental time point, red-fated scale cells are orange and progressively darken to red over the next 24 h. After the red pigments have matured, melanin scales develop in a 'wave' emanating from the center of the wing; whereas yellow-fated scales remain colorless until a few hours before adult emergence. These changes in scale cell color are associated with upregulation of genes involved in the uptake and synthesis of pigment, some of which show strong pattern specific expression, while others do not (Reed et al., 2008; Ferguson and Jiggins, 2009; Hines et al., 2012).

The process of scale cell differentiation provides a useful context to begin to understand the function of the major patterning loci that have been identified in *Heliconius* (Figure 3). The patterning genes *WntA* and *cortex* are expressed during larval wing development well before scale cells have begun to form (Martin et al., 2012; Nadeau et al., 2016). These genes provide key aspects of the positional information signals to downstream pigment and scale cell maturation pathways. Later in pupal development (about 40 h after pupation), the Type I scale cells begin to undergo laminar extension before their Type II and III neighbors (Gilbert et al., 1988; Reed et al., 2008; Aymone et al., 2013). Type III scales mature next and begin by expressing the transcription factor *optix*, signaling cells to produce the red ommochrome pigments (Reed et al., 2011). Type II cells contain melanins, which are laid down later in pupal development. The white/yellow difference between Type I scale cells is due to the presence or absence of the ommochrome precursor 3-OHK. This color switch appears to be regulated by the differential expression of *aristaless1* (Westerman et al., 2018), with the uptake of 3-OHK from the pupal hemolymph occurring right before adult emergence (Reed et al., 2008).

FROM PATTERNS TO PROCESS – A MODEL OF WING PATTERN EVOLUTION

Substantial insights into the function that major switch genes play in pattern variation has been gained with the recent development of CRISPR/Cas9 technology in *Heliconius* (Mazo-Vargas et al., 2017; Zhang et al., 2017b; Westerman et al., 2018; Concha et al., 2019; Lewis et al., 2019) (Figure 3). For the first time, researchers can perform functional assays by inducing targeted mutations in coding regions as well as putative regulatory elements in order to observe the resulting mutant wing pattern phenotypes (reviewed

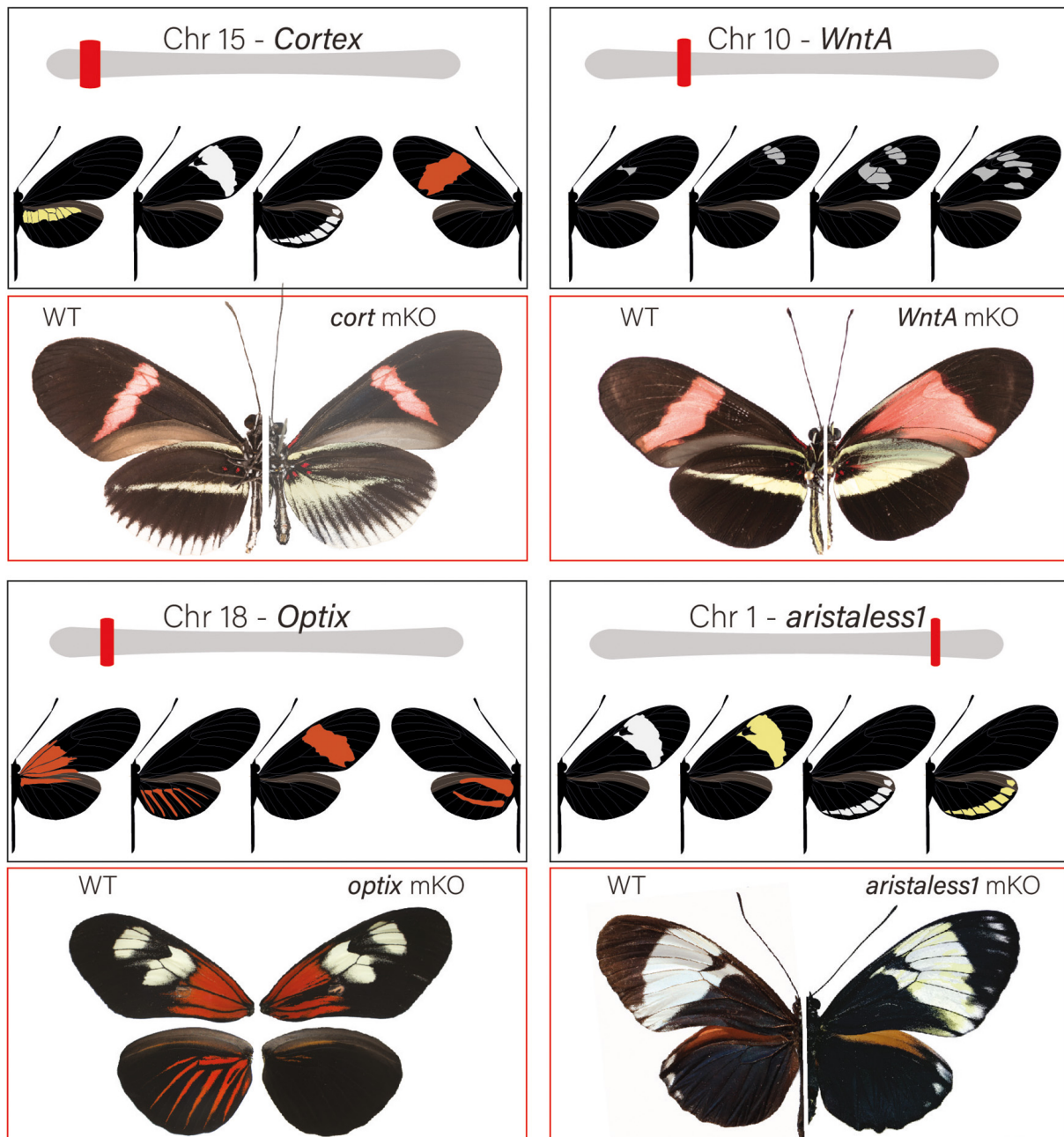


FIGURE 3 | Major patterning switch genes in *Heliconius* butterflies: Most of the color pattern variation in *Heliconius* is the result of 4 major effect loci. Loci are unlinked and allelic variation around them is associated with both convergent and divergent change among species. Over the past decade, loci have been positionally cloned and genotype $\times$ phenotype, expression and, most recently, KOs with CRISPR/Cas9 have identified specific patterning genes. The gene *cortex* is located on chromosome 15 and variation around the gene is associated with differences in the yellow hindwing bar, forewing band color, and the white margin and ventral color phenotypes among different geographic morphs of *H. erato* and *H. melpomene*. In *H. numata*, variation around the region that contains *cortex* controls all variation in wing patterns and is responsible for differences in yellow, orange, and black pattern elements among sympatric color pattern morphs (Nadeau et al., 2016; Saenko et al., 2019). *cortex* KO results in the appearance of Type I scales across the wing surface of *Heliconius* butterflies (see Livraghi et al., 2020). The signaling ligand *WntA* is on chromosome 10 and variation around the locus is associated with differences in forewing band size, shape and position in *H. erato* and other *Heliconius* species. KO results in the expansions of color pattern elements in proximal wing regions in *H. erato*, but generates a wide variety of phenotypic effects across the *Heliconius* radiation (Concha et al., 2019). The gene *optix* is on chromosome 18 and variation around this gene is responsible for variation in red color pattern elements. *optix* KOs result in the transformation of red Type III scales to melanic Type II scales. Finally, variation around the gene *aristaless1* on chromosome 1 modulates the presence or absence of yellow pigment in Type I scales. *Aristaless1* KOs result in the development of yellow scales suggesting that the gene represses synthesis/deposition of the yellow pigment, 3-hydroxy-L-kynurenine.

in Livraghi et al., 2018). This research is illuminating the roles that the four major patterning genes play in pattern variation, how they interact during development to influence scale cell fate, and how gene patterning networks evolve within the context of adaptive phenotypic change.

Two major patterning genes, *WntA* and *cortex*, are expressed early in wing development and act to establish positional information for the downstream expression of scale cell selector genes. Under a simple model (Figure 4), *cortex* acts as a “master” regulator of scale cell identity, where scales switch between Type I and Type II/III scales depending on the expression of *cortex*. A certain threshold of *cortex* expression may initiate a differentiation cue that sets up a permissive environment for either melanization (*optix* negative) or red pigment synthesis (*optix* positive). In contrast, cells below that threshold become either yellow or white depending on the expression of *aristaless1*, which represses the pathway leading to the synthesis and deposition of 3-OHK pigment (Westerman et al., 2018). Allelic variation in the expression of *cortex* is responsible for variation in white, yellow, and red/orange patches across the radiation (Figure 3). However, the expression of the gene appears to be more complex than initially envisioned. Although previous expression studies using *in situ* hybridization have shown a strong correlation between the position of *cortex* mRNA in wing disks of fifth instar larvae and distal black pattern elements in the adult wings of *H. numata* and *H. melpomene* (Nadeau et al., 2016; Saenko et al., 2019), the color switches induced by *cortex* knockouts (KOs) do not seem to act in a pattern specific way in *H. erato* and *H. melpomene* (Livraghi et al., 2020). Instead, *cortex* KOs result in ectopic switches from melanic (Type II) and red/brown/orange (Type III) into white/yellow (Type I) scale cells across the whole surface of both wings in *H. erato* and *H. melpomene*. The switch is associated with both a shift in scale cell color and nanostructure. Thus, the loss of function of *cortex* causes a wing scale cell to change its identity and fully acquire the identity of a Type I scale cell. The broad effects of *cortex* across the wings of *Heliconius* underscore the potential for this gene to generate much wider pattern variation than other major effects genes. This wide range of effects is exemplified by the polymorphic *H. numata*, where variation in yellow, white, black and orange elements all map to this locus (Joron et al., 2006; Saenko et al., 2010; Nadeau et al., 2016).

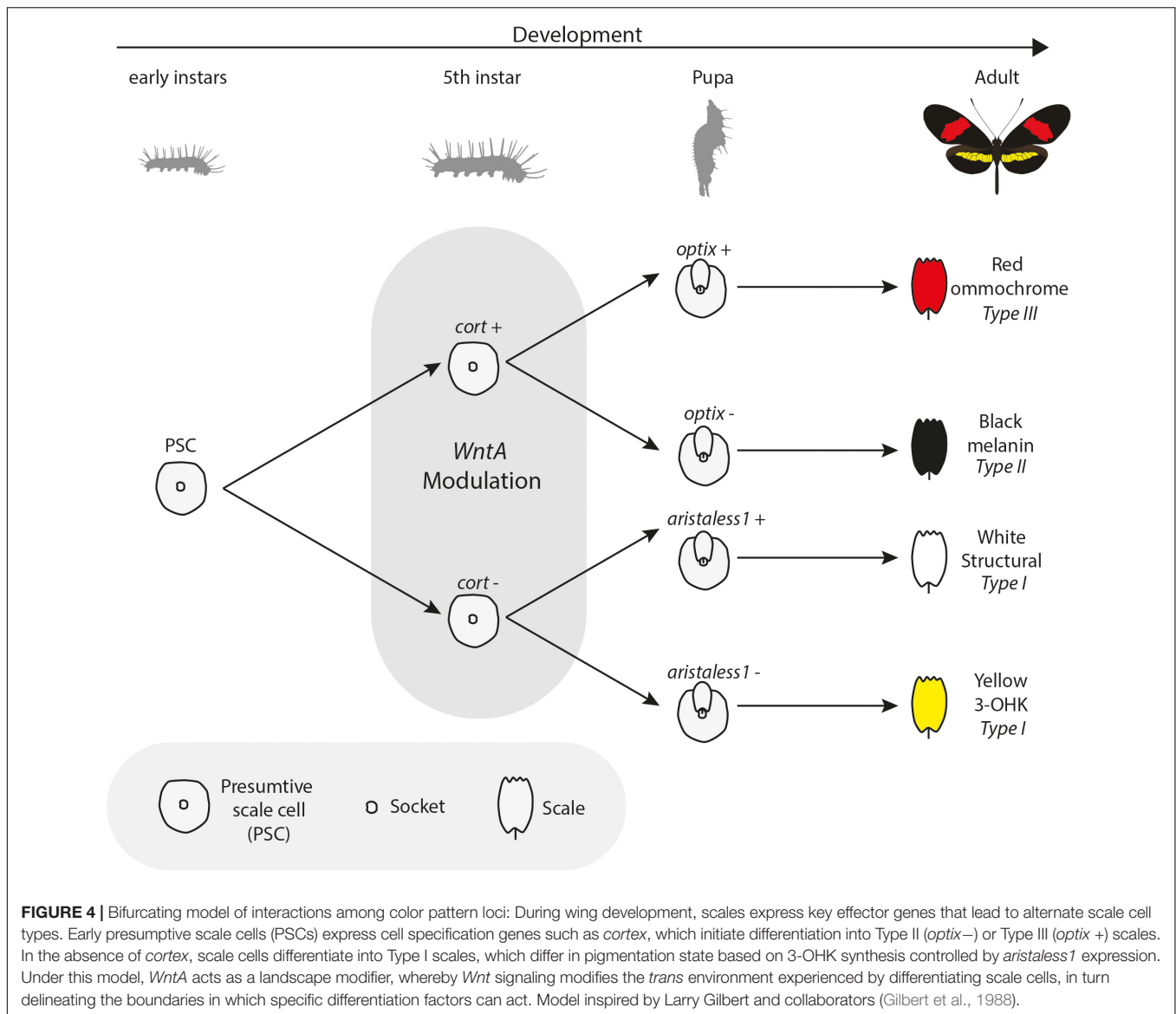
If *cortex* is acting as a “on-off” switch among scale cell types, the signaling ligand *WntA* might be best conceptualized as modifying the overall regulatory landscape used to provide the positional information for the establishment of boundaries between scale cell types. In contrast to *cortex*, *WntA* effects are limited to specific wing pattern elements and the extent of pattern induction across the wing varies both within and between species in the ESS/MCS radiation (Figure 5). The key role of *WntA* in orchestrating scale cell identity is illustrated by the observation that the shift in scale cell fate happens in every possible direction—from black to red, yellow, and white; as well as from any given color to black, and also from red to yellow scales (Figure 5A). Thus, *WntA* appears to regulate scale cell fate in a more flexible manner than *cortex*. Furthermore, unlike *cortex* where scale cells revert to Type I in *cortex* KO cells, the exact scale cell

phenotype induced by a *WntA* signal is highly context-dependent (Concha et al., 2019). The complexity of these interactions is best demonstrated in the different responses of *optix* to *WntA* KOs in divergent color pattern morphs of *H. erato* (Figure 5B). Within a given genetic background, *WntA* KOs result in an upregulation of *optix* expression and concomitant development of red scales. However, the opposite effect is observed in yellow-banded morphs of *H. erato*, where the existing yellow patterns expand, even across red scales which are known to express *optix* in wild-type butterflies.

The other two major patterning genes identified in *Heliconius*, *optix* and *aristaless1*, are both transcription factors that are expressed later in wing scale cell development in response to signals laid down either directly or indirectly by *WntA* and *cortex*. The transcription factor *optix* is expressed at about 72 h after pupation in Type III scale cells that are fated to become red. Functional knockout of this gene in *H. erato* results in the loss of red color patterns and is associated with changes in scale cell morphology (Zhang et al., 2017b). Consistent with a role in switching between ommochrome and melanin synthesis, an RNAseq experiment comparing *optix* mutant and wild type wings in two species of butterflies, *Vanessa cardui* and *Junonia coenia*, showed that *optix*, either directly or indirectly, regulates the expression of many known pigmentation genes involved in ommochrome and melanin synthesis (Zhang et al., 2017b). Lastly, the homeodomain transcription factor *aristaless1* causes a change in Type I scale cells and promotes the development of structural white scales by repressing the synthesis or uptake of yellow 3-OHK (Westerman et al., 2018). The *aristaless* gene is duplicated in Lepidoptera (Martin and Reed, 2010) and in divergent color pattern races of *H. cydno* there is a strong peak of differentiation associated with the presence/absence of white coloration in a putative *cis*-regulatory region midway between the two *aristaless* genes, *aristaless1* and *aristaless2*. Both genes are differentially expressed in the forewings of white and yellow *H. cydno* morphs, with *aristaless1* strongly upregulated late in wing development of the white morph. Consistent with a role in patterning, functional knockout of *aristaless1* in white morphs results in the transformation of white scale cells into yellow scale cells (Figure 3 and Westerman et al., 2018).

CHANCE AND CONTINGENCY ON THE *Heliconius* WING

In *Heliconius*, there are many replicated cases of mimetic convergence between species within the ESS and MCS clades (see Figure 1 for some examples), providing a powerful context to understand how patterning networks co-evolve to produce variation. One unresolved question is the extent to which similar or distinct mechanisms have generated convergent phenotypes – in other words, are phenotypic repeats based on parallel reiterations of the same process, or do distinct developmental pathways lead to the same outcome? One of the more intriguing conclusions to emerge from comparative studies between species in the two *Heliconius* clades is how labile the GRNs responsible for patterning a *Heliconius* wing are



(Concha et al., 2019). *WntA* knockouts in co-mimetic species of *Heliconius* result in very different phenotypes, demonstrating that the underlying GRNs controlling mimicry have diverged substantially between the two major clades (Concha et al., 2019; **Figure 5C**). Therefore, while *WntA* has been the repeated target of selection, its role in establishing pattern elements varies greatly between co-mimics. This fact is perhaps best demonstrated by comparing the KO phenotype between the two co-mimics *H. hewitsoni* (ESS clade) and *H. pachinus* (MCS clade). The two co-occur in forests of Panama and Costa Rica and have independently evolved identical wing patterns. However, the *WntA* KO forewing phenotypes are very different from each other, with *WntA* affecting pattern from the base of the forewing to nearly the tip in *H. hewitsoni*, whereas, the KO phenotype in *H. pachinus* causes a switch from melanistic (Type III) to yellow Type I scale cells much more distally. In addition, knocking out *WntA* in *H. pachinus* does not

affect the black melanistic patch across the midsection of the forewing (**Figure 5C**).

The *WntA* knockout effects across the radiation highlight rapid divergence in the developmental landscape responsible for patterning a *Heliconius* wing; including changes in *cis*-regulatory variation around the locus, divergence in the interpretation of *WntA* signals by downstream developmental pathways, and variable epistatic interactions between *WntA* and other patterning loci (Concha et al., 2019). This finding is consistent with a recent study showing considerable differences in the expression patterns of transcription factors and signaling molecules between the two co-mimetic species *H. e. demophoon* and *H. m. rosina* (Hanly et al., 2019). Together these findings reinforce a growing appreciation that gene regulatory networks can diverge rapidly (True and Haag, 2001) and create a complex set of historical contingencies that we are only beginning to appreciate (see Kittelmann et al., 2018; Sorrells et al., 2018; Ellison

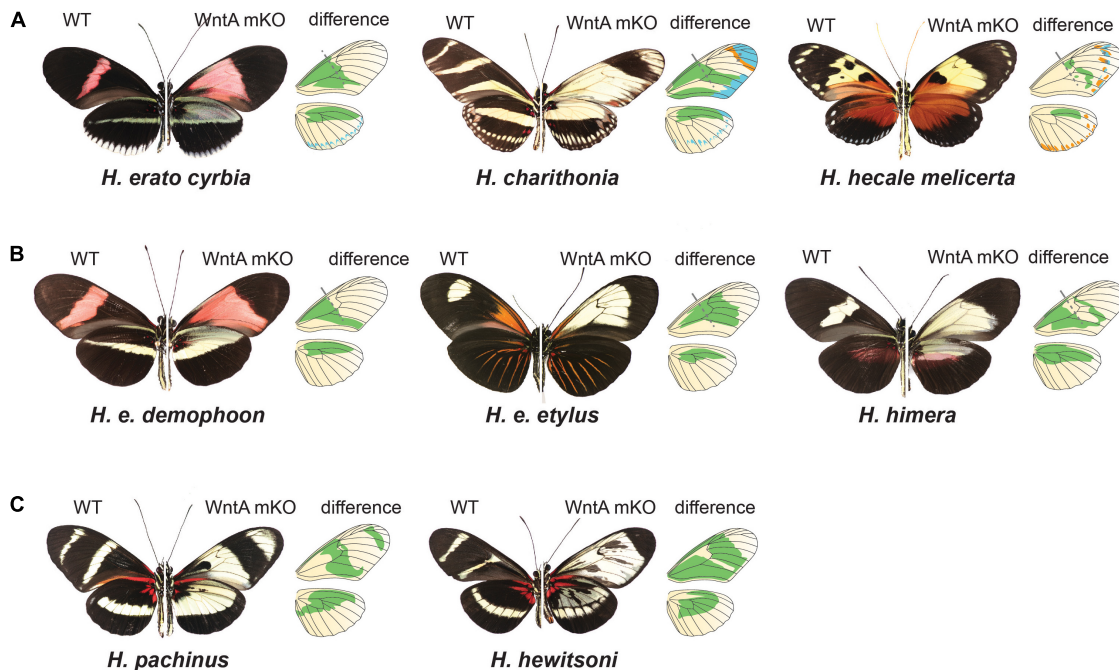


FIGURE 5 | Chance and contingency on a *Heliconius* wing: CRISPR/Cas9 experiments demonstrate the pervasive role that *WntA* plays in orchestrating pattern variation and underscores how rapidly the underlying GRNs shaping wing patterns can diverge. **(A)** *WntA* seems to be broadly involved in establishing pattern boundaries across the wing and causes shifts in the ultimate scale cell fate in every possible direction. **(B)** *WntA* KO effects are highly context specific within *H. erato* color pattern races. While the same proximal elements are affected, the color switch varies depending on genetic background. **(C)** The underlying GRNs controlling mimicry have diverged substantially between the MCS/ESS lineages. This is exemplified by the co-mimetic species *H. pachinus* and *H. hewitsoni*. *WntA* KO effects in *H. hewitsoni* affect pattern across most of the forewing, except the most distal regions. By contrast, *H. pachinus* KO effects extend more distally, and forewing band regions remain unchanged. For each *Heliconius* wild type and mutant image, we show a drawing of the wings, where green represents the wing color pattern domains that are changed by knocking out the *WntA* gene. In **(A)**, light blue represents changes in distal patterns and orange represents an extension of black patterns.

and Bachtrog, 2019). On a *Heliconius* wing, this divergence creates extraordinary flexibility for natural selection to drive the rapid evolution of highly convergent wing patterns in unpredictable ways.

CURRENT CHALLENGES

While the functional genomic work on major wing patterning genes is beginning to provide a picture of the molecular events underlying the process of scale cell differentiation, there are many unresolved questions regarding the details of this process.

How Are Patterning Signals Propagated to Alter Scale Cell Fate?

The major wing patterning loci in *Heliconius* are “evolutionary hotspots” and have been repeatedly targeted at both micro and macro-evolutionary scales to produce pattern variation. However, wing pattern development is likely to involve the coordinated expression of hundreds of genes. At the most basic level, we currently have little understanding of how *WntA* or *cortex* signals are propagated and interpreted by developing scale cells. Indeed, *cortex* does not conform to the typical paradigm of a wing patterning locus (*sensu*; Martin and Courtier-Orgogozo,

2017). Unlike the other wing patterning genes (Table 1), *cortex* is neither a conserved transcription factor nor a component of a known cell signaling pathway. Rather, the gene shows the closest homology to the *cdc20/fizzy* family of cell-cycle regulators (Nadeau et al., 2016). In *Drosophila*, Fizzy proteins are known to accumulate at kinetochores and centrosomes and regulate APC/C activity through the degradation of cyclins, which has been shown to prevent mitotic entry (Raff et al., 2002; Meghini et al., 2016). Specifically, expression of *fizzy-related* in *Drosophila* follicle cells induces cell endocycling and results in polyploidy, and *cortex* has been shown to interact with the APC/C and downregulate cyclins during female meiosis (Schaeffer et al., 2004; Shcherbata et al., 2004; Pesin and Orr-Weaver, 2007; Swan and Schüpbach, 2007). The *Heliconius* ortholog also localizes to the nucleus in developing pupal wings, suggesting it may also be interacting with the APC/C in developing butterfly scales (Livrighi et al., 2020). Other work has shown a correlation between scale cell ploidy and pigmentation state where increased ploidy coincides with darker scales (Cho and Nijhout, 2013; Iwata and Otaki, 2016) and *cortex* may thus be modulating pigmentation state by varying endocycle rates across the wing. Alternatively, *cortex* may be acting by modulating the developmental rate of scale cells during wing development. Melanic scales develop slower than the other scale cell types with melanin being synthesized

and deposited later in wing development (Gilbert et al., 1988; Reed et al., 2008; Aymone et al., 2013). By controlling the faster or slower development of the scales, *cortex* may be modulating the final pigmentation state of a scale cell. This hypothesis of “developmental heterochrony” to explain the mechanism of butterfly wing color patterning was proposed 20 years ago (Koch et al., 2000) without gaining much supporting evidence over the years.

The mechanism of how the locus containing *cortex* affects pattern variation is probably complex. Although *cortex* is clearly involved in determining scale cell identity, there are other genes that are physically linked to *cortex* that might also play a role in patterning. Most notably, the genes *domeless* and *washout* show pattern specific expression in both *H. melpomene* and *H. numata* (Nadeau et al., 2016; Saenko et al., 2019). *Domeless* encodes a transmembrane receptor of the JAK/STAT signaling pathway (Brown et al., 2001) while *washout* is involved in actin polymerization through the activation of the Arp2/3 complex (Liu et al., 2009; Verboon et al., 2015), raising the interesting possibility that it may be involved in the formation of adult scales through the control of actin bundle elongation (Dinwiddie et al., 2014). It is therefore possible that multiple linked genes around *cortex* contribute to wing patterning evolution in *Heliconius*. Theory predicts that in some cases the linking of such genes might be favored by evolution (Charlesworth and Charlesworth, 1976), leading to the emergence of “supergenes”: tightly linked functional genetic elements that allow a switch between phenotypes to be maintained in a stable local polymorphism (Joron et al., 2011; Le Poul et al., 2014). Indeed, there is some indication that such a system is acting to control polymorphism in *H. numata*, where all wing pattern variation is controlled by inversions surrounding *cortex*, *domeless* and *washout* (Joron et al., 2011; Nadeau et al., 2016; Saenko et al., 2019). However, we currently have no functional validation for other genes at this locus, nor evidence for interactions between them.

WntA, in contrast, is a classic cell signaling molecule belonging to the family of Wnt signaling ligands. However, we also currently lack an understanding of how *WntA* signals are transduced into a coordinated scale differentiation program. Wnt signaling may act through the canonical pathway, by which β -catenin is permitted to translocate to the nucleus and act as a transcriptional cofactor (reviewed in Collu et al., 2014), or alternatively it may signal through a non-canonical modality such as the planar cell polarity pathway (PCP) (Lawrence and Casal, 2018), or by a third mechanism such as Wnt-calcium signaling (Agrawal and Hasan, 2015). Understanding the mechanism by which *WntA* causes patterned scale cell differentiation will be an important first step in unraveling the larger gene regulatory networks that pattern the wings.

How Does New Regulatory Variation Evolve?

One idea to emerge from developmental biology is that loci that have become the nexus of morphological change, so called hotspot genes, are often associated with complex *cis*-regulatory variation (Stern and Orgogozo, 2009). This is certainly

true of the major patterning loci identified in *Heliconius*. Expression, association, and CRISPR studies have all revealed that *cis*-regulatory variation is important in driving wing patterning differences. Nonetheless, we still don't understand how new regulatory regions evolve and what role, if any, ancient regulatory architecture plays in wing pattern variation. Association studies in *Heliconius* suggest that there is a high degree of modularity with regards to the *cis*-regulatory elements controlling color pattern variation (Supple et al., 2013; Wallbank et al., 2016; Enciso-Romero et al., 2017; Van Belleghem et al., 2017). For example, different red color pattern elements in *H. melpomene* and *H. erato* are associated with discrete genomic intervals in non-coding regions around *optix* (Wallbank et al., 2016; Van Belleghem et al., 2017). A similar modular architecture is also observed for variation in forewing band shape (*WntA*) and the presence or absence of a yellow hindwing bar (*cortex*) within *H. erato* (Van Belleghem et al., 2017). The idea is that new wing pattern phenotypes can evolve rapidly by simply reshuffling these discrete regulatory regions. In the MCS lineage, for example, the phylogenetic patterns are consistent with at least four instances of between species hybridization that allowed these elements to cross species boundaries, caused pattern change, and probably promoted speciation in the group (Wallbank et al., 2016).

A model where “enhancer shuffling” drives morphological differentiation, however, was not strongly supported by recent work that has more directly probed the relationship between regulatory and phenotypic variation (Lewis et al., 2019). Here, the authors identified and excised a number of putative *cis*-regulatory regions in genomic intervals associated with red pattern variation in *H. erato* using a combination of epigenetic profiling and genotype $\times$ phenotype analysis. Surprisingly, they found that deletions of a single *cis*-regulatory element were highly pleiotropic and affected red pattern elements in both the forewing and hindwing. The level of pleiotropy observed suggests that more constraints are acting at color pattern loci than previously hypothesized and this is difficult to reconcile under a model where a single regulatory region affects a single pattern feature. A possible resolution to this apparent conflict between the association and functional data may be explained by the idiosyncratic properties of phenotypes segregating in *Heliconius* hybrid zones, whereby a single locus can act in a modular fashion in localized population structures; however, because of an ever shifting *trans*-regulatory landscape, excising a specific enhancer after patterns have become established and genetic backgrounds have diverged is not sufficient to induce the predicted phenotype (see Lewis and Van Belleghem, unpublished, for a discussion of this topic). At this point, we clearly need more functional studies that assess the evidence for and against modularity in driving the evolution of *Heliconius* wing patterns.

How Labile Is Pattern Variation on the Wing Across the Lepidoptera?

In *Heliconius*, much of the pattern variation across the genus is driven by variation at homologous genes. However, even in this relatively recent radiation, developmental systems drift

appears to be shifting the wiring of the underlying GRNs (Concha et al., 2019). Expanding functional studies across the lepidoptera will be necessary to start addressing questions relating to the evolutionary constraints acting across GRNs as a whole. Moreover, while mapping studies across *Heliconius* consistently implicate four major patterning genes, variation in other loci across the genome also have quantitative effects on pattern variation (Papa et al., 2013). Indeed, more recent mapping experiments show that structural coloration in *Heliconius* is not achieved using any of the known patterning loci and that different loci are used to produce structural colors in the two distantly related co-mimics *H. erato* and *H. melpomene* (Parnell et al., 2018; Brien et al., 2019). Also, while *cortex* has been repeatedly mapped in within-species pattern variation, recent studies have begun to identify other genes, not previously identified in *Heliconius*, as responsible for pattern variation (Table 1). In this light, it will be very interesting to determine if these other conserved components of GRNs underlying wing patterning in other lepidoptera contribute to pattern variation in *Heliconius*.

While individual components have been identified through evolutionary approaches, very few regulatory connections have been resolved, and we still don't know the broad applicability of discoveries made in *Heliconius* to other butterflies or moths. From an evolutionary perspective, *Heliconius* wings represent a highly derived type of a more ancestral patterning system called the Nymphalid Ground Plan (NGP) (Nijhout and Wray, 1988). In this sense, *Heliconius* wings may not represent the best system with which to address questions relating to the conservation of GRNs in other lepidoptera, as it is difficult to draw homologies between color pattern elements at larger evolutionary scales. For example, while *WntA* has been implicated in color pattern evolution across both *Heliconius* and other nymphalids (Martin et al., 2012; Gallant et al., 2014; Van Belleghem et al., 2017), the pattern elements it affects vary substantially. In species displaying NGP-like patterns, *WntA* functions as an organizing factor of Central and External Symmetry Systems in at least three distantly related nymphalids (Mazo-Vargas et al., 2017), suggesting a conserved role across large evolutionary scales. On the other hand, it is noteworthy that all of the four major patterning loci discovered in *Heliconius* affect pattern in other butterflies and moths. In this light, the apparent flexibility in the GRNs that we are observing in *Heliconius* may reflect the nature of wing patterning networks generally. Thus, while a particular set of conserved loci might have a function in generating pattern variation, how they generate that variation may differ substantially among lineages. For example, the eyespot patterns that are found in many nymphalid butterflies appear to have evolved once; but the gene regulatory networks underlying these patterns have diverged significantly over the past 65 million years such that the eyespots of different species are controlled by different sets of genes (Oliver et al., 2012).

LINKING GENOTYPE TO PHENOTYPE

In multicellular organisms, phenotype is ultimately realized in the patterned differentiation of cells into

specific functional types. In this respect, butterfly wings have a unique combination of features which make them an ideal system to uncover how changes in a DNA sequence are translated and interpreted through development to generate variation: a relatively simple developmental system, large phenotypic diversity, and experimental tractability.

There is clearly much to be discovered, but the last few years have witnessed remarkable progress in our understanding of pattern formation on butterfly wings. Research priorities are clearly laid out and technical improvements, including techniques for characterizing the developmental landscape of protein–DNA interactions (Park, 2009; Rao et al., 2014; Buenrostro et al., 2015; Lewis et al., 2016, 2019) and the expression profiles of individual cells (Kulkarni et al., 2019), coupled with more efficient functional manipulation provide us with the necessary instruments to further uncover the gene regulatory networks underlying wing pattern variation. In this respect, one of the key technical limitations facing *Heliconius* is the lack of transgenic tools. Our current use of CRISPR as a functional tool has been limited to the analysis of mosaic loss-of-function mutants, where only a subset of cells carry the desired mutation on the adult wing. While this can be a strength, allowing the analysis of the effects of highly pleiotropic genes and permitting the observation of effects at clone boundaries, in many cases this makes it difficult to determine the extent to which a given induced mutation affects pattern across the wing. These shortcomings could be bypassed by developing technologies that allow researchers to routinely produce gain-of-function mutations; as well as those that would allow better spatiotemporal control over genetic manipulations. For example, by placing reporter constructs under specific inducible elements, the ability of a putative *cis*-regulatory element to drive expression in the tissue of interest could be directly visualized (Tong et al., 2014). Nonetheless, if we stay on pace we will have come a long way toward fulfilling the prophecy made by Bates (1864) and “the study of butterflies—creatures selected as the types of airiness and frivolity—instead of being despised, will someday be valued as one of the most important branches of Biological science.”

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

WM was supported by the Smithsonian Tropical Research Institute and the Smithsonian Scholarly Studies Program. LL was supported by BBSRC BB/R007500/1. JH was supported by the NSF award IOS-1656553. CC was supported by the Smithsonian Institution Biodiversity Genomics Fellowship and NSF IOS-1656389.

ACKNOWLEDGMENTS

We would like to thank the Butterfly Ecology and Evolution Research group at STRI, as well as the Butterfly Genetics group at the University of Cambridge, the Martin Lab at GWU and

the Papa Lab at UPR-RP for constructive conversations that helped to formulate this review. In addition, we thank Marcus Kronforst, Bob Reed, and Steven Van Belleghem for providing images of butterflies, and two reviewers for their helpful feedback on this manuscript.

REFERENCES

- Agrawal, T., and Hasan, G. (2015). Maturation of a central brain flight circuit in *Drosophila* requires Fz2/Ca2+ signaling. *eLife* 4:e07046. doi: 10.7554/eLife.07046.001
- Aymone, A. C. B., Valente, V. L. S., and de Araújo, A. M. (2013). Ultrastructure and morphogenesis of the wing scales in *Heliconius erato* phyllis (Lepidoptera: Nymphalidae): what silvery/brownish surfaces can tell us about the development of color patterning? *Arthropod. Struct. Dev.* 42, 349–359. doi: 10.1016/j.asd.2013.06.001
- Bates, H. W. (1864). *The Naturalist on the River Amazons: A Record of Adventures, Habits of Animals, Sketches of Brazilian and Indian Life, and Aspects of Nature under the Equator, During Eleven Years of Travel*. London: J. Murry. Available online at: <https://www.biodiversitylibrary.org/page/13494275#page/9/mode/1up>
- Bates, H. W. (1879). Reservations on Mullerian mimicry. *Trans. Ent. Soc. Lond.* xxviii–xxix.
- Beebe, W. (2005). Polymorphism in reared broods of *Heliconius* butterflies from Surinam and Trinidad. *Zoologica* 40, 139–143.
- Benson, W. W. (1972). Natural selection for Müllerian mimicry in *Heliconius erato* in Costa Rica. *Science* 176, 936–939. doi: 10.1126/science.176.4037.936
- Brakefield, P. M., and French, V. (1999). Butterfly wings: the evolution of development of colour patterns. *Bioessays* 21, 391–401. doi: 10.1002/(sici)1521-1878(199905)21:5<391::aid-bies6>3.0.co;2-q
- Brien, M. N., Enciso-Romero, J., Parnell, A. J., Salazar, P. A., Morochz, C., Chala, D., et al. (2019). Phenotypic variation in *Heliconius erato* crosses shows that iridescent structural colour is sex-linked and controlled by multiple genes. *Interface Focus* 9:20180047. doi: 10.1098/rsfs.2018.0047
- Brown, K. S. (1967). Chemotaxonomy and chemomimicry: the case of 3-hydroxykynurenine. *Syst. Zool.* 16, 213–216.
- Brown, S., Hu, N., and Hombria, J. C. (2001). Identification of the first invertebrate interleukin JAK/STAT receptor, the *Drosophila* gene *domeless*. *Curr. Biol.* 11, 1700–1705. doi: 10.1016/s0960-9822(01)00524-3
- Buenrostro, J. D., Wu, B., Chang, H. Y., and Greenleaf, W. J. (2015). ATAC-seq: a method for assaying chromatin accessibility genome-wide. *Curr. Protoc. Mol. Biol.* 109:21.29.1–21.29.9. doi: 10.1002/0471142727.mb2129s109
- Carroll, S. B. (2008). Evo-devo and an expanding evolutionary synthesis: a genetic theory of morphological evolution. *Cell* 134, 25–36. doi: 10.1016/j.cell.2008.06.030
- Charlesworth, D., and Charlesworth, B. (1976). Theoretical genetics of Batesian mimicry II. *Evol. Supergenesis* 55, 305–324. doi: 10.1016/s0022-5193(75)80082-8
- Cho, E. H., and Nijhout, H. F. (2013). Development of polyploidy of scale-building cells in the wings of *Manduca sexta*. *Arthropod. Struct. Dev.* 42, 37–46. doi: 10.1016/j.asd.2012.09.003
- Chouteau, M., Llaurens, V., Piron-Prunier, F., and Joron, M. (2017). Polymorphism at a mimicry supergene maintained by opposing frequency-dependent selection pressures. *Proc. Natl. Acad. Sci.* 114, 8325–8329. doi: 10.1073/pnas.1702482114
- Collu, G. M., Hidalgo-Sastre, A., and Brennan, K. (2014). Wnt-Notch signaling crosstalk in development and disease. *Cell. Mol. Life Sci.* 71, 3553–3567. doi: 10.1007/s00018-014-1644-x
- Concha, C., Wallbank, R. W. R., Hanly, J. J., Fenner, J., Livraghi, L., Rivera, E. S., et al. (2019). Interplay between developmental flexibility and determinism in the evolution of mimetic *Heliconius* wing patterns. *Curr. Biol.* 29, 3996.e–4009.e. doi: 10.1016/j.cub.2019.10.010
- Cong, Q., Shen, J., Zhang, J., Li, W., Kinch, L. N., Calhoun, J. V., et al. (2019). Genomics reveals the origins of ancient specimens. *bioRxiv* [Preprint] doi: 10.1101/752121
- Dinwiddie, A., Null, R., Pizzano, M., Chuong, L., Leigh Krup, A., Ee Tan, H., et al. (2014). Dynamics of F-actin prefigure the structure of butterfly wing scales. *Dev. Biol.* 392, 404–418. doi: 10.1016/j.ydbio.2014.06.005
- Edelman, N. B., Frandsen, P. B., Miyagi, M., Clavijo, B., Davey, J., Dikow, R. B., et al. (2019). Genomic architecture and introgression shape a butterfly radiation. *Science* 366, 594–599. doi: 10.1126/science.aaw2090
- Ellison, C., and Bachtrog, D. (2019). Contingency in the convergent evolution of a regulatory network: dosage compensation in *Drosophila*. *PLoS Biol.* 17:e3000094. doi: 10.1371/journal.pbio.3000094
- Enciso-Romero, J., Pardo-Diaz, C., Martin, S. H., Arias, C. F., Linares, M., McMillan, W. O., et al. (2017). Evolution of novel mimicry rings facilitated by adaptive introgression in tropical butterflies. *Mol. Ecol.* 26, 5160–5172. doi: 10.1111/mec.14277
- Ferguson, L. C., and Jiggins, C. D. (2009). Shared and divergent expression domains on mimetic *Heliconius* wings. *Evol. Dev.* 11, 498–512. doi: 10.1111/j.1525-142X.2009.00358.x
- Galant, R., Skeath, J. B., Paddock, S., Lewis, D. L., and Carroll, S. B. (1998). Expression pattern of a butterfly achaete-scute homolog reveals the homology of butterfly wing scales and insect sensory bristles. *Curr. Biol.* 8, 807–813. doi: 10.1016/s0960-9822(98)70322-7
- Gallant, J. R., Imhoff, V. E., Martin, A., Savage, W. K., Chamberlain, N. L., Pote, B. L., et al. (2014). Ancient homology underlies adaptive mimetic diversity across butterflies. *Nat. Commun.* 5:4817. doi: 10.1038/ncomms5817
- Gawne, R., McKenna, K. Z., and Nijhout, H. F. (2018). Unmodern synthesis: developmental hierarchies and the origin of phenotypes. *Bioessays* 40:1600265. doi: 10.1002/bies.201600265
- Ghiradella, H., and Radigan, W. (1976). Development of butterfly scales. II. Struts, lattices and surface tension. *J. Morphol.* 150, 279–297. doi: 10.1002/jmor.1051500202
- Gilbert, L. E., Forrest, H. S., Schultz, T. D., and Harvey, D. J. (1988). Correlations of ultrastructure and pigmentation suggest how genes control development of wing scales of *Heliconius* butterflies. *J. Res. Lepidoptera* 26, 141–160.
- Greenstein, M. E. (1972). The ultrastructure of developing wings in the giant silkworm, *Hyalophora cecropia*. II. Scale-forming and socket-forming cells. *J. Morphol.* 136, 23–51. doi: 10.1002/jmor.1051360103
- Hanly, J. J., Wallbank, R. W. R., McMillan, W. O., and Jiggins, C. D. (2019). Conservation and flexibility in the gene regulatory landscape of heliconiine butterfly wings. *Evodevo* 10, 1–15. doi: 10.1186/s13227-019-0127-4
- Hines, H. M., Papa, R., Ruiz, M., Papanicolaou, A., Wang, C., Nijhout, H. F., et al. (2012). Transcriptome analysis reveals novel patterning and pigmentation genes underlying *Heliconius* butterfly wing pattern variation. *BMC Genomics* 13:288. doi: 10.1186/1471-2164-13-288
- Huber, B., Whibley, A., Poul, Y. L., Navarro, N., Martin, A., Baxter, S., et al. (2015). Conservatism and novelty in the genetic architecture of adaptation in *Heliconius* butterflies. *Heredity* 114, 515–524. doi: 10.1038/hdy.2015.22
- Ito, K., Katsuma, S., Kuwazaki, S., Jouraku, A., Fujimoto, T., Sahara, K., et al. (2016). Mapping and recombination analysis of two moth colour mutations, Black moth and Wild wing spot, in the silkworm *Bombyx mori*. *Heredity* 116, 52–59. doi: 10.1038/hdy.2015.69
- Iwata, M., and Otaki, J. M. (2016). Spatial patterns of correlated scale size and scale color in relation to color pattern elements in butterfly wings. *J. Insect Physiol.* 85, 32–45. doi: 10.1016/j.jinsphys.2015.11.013
- Jiggins, C. D. (2017). *The Ecology and Evolution of Heliconius Butterflies*. Oxford: Oxford University Press.
- Jiggins, C. D., Naisbit, R. E., Coe, R. L., and Mallet, J. (2001). Reproductive isolation caused by colour pattern mimicry. *Nature* 411, 302–305. doi: 10.1038/35077075
- Joron, M., Frezal, L., Jones, R. T., Chamberlain, N. L., Lee, S. F., Haag, C. R., et al. (2011). Chromosomal rearrangements maintain a polymorphic supergene controlling butterfly mimicry. *Nature* 477, 203–206. doi: 10.1038/nature10341
- Joron, M., Papa, R., Beltrán, M., Chamberlain, N., Mavárez, J., Baxter, S., et al. (2006). A conserved supergene locus controls colour pattern diversity in *Heliconius* butterflies. *PLoS Biol.* 4:e303. doi: 10.1371/journal.pbio.0040303

- Kapan, D. D. (2001). Three-butterfly system provides a field test of müllerian mimicry. *Nature* 409, 338–340. doi: 10.1038/35053066
- Kapan, D. D., Flanagan, N. S., Tobler, A., Papa, R., Reed, R. D., Gonzalez, J. A., et al. (2006). Localization of Müllerian mimicry genes on a dense linkage map of *Heliconius erato*. *Genetics* 173, 735–757. doi: 10.1534/genetics.106.057166
- Kittlmann, S., Buffry, A. D., Franke, F. A., Almudi, I., Yoth, M., Sabaris, G., et al. (2018). Gene regulatory network architecture in different developmental contexts influences the genetic basis of morphological evolution. *PLoS Genet.* 14:e1007375. doi: 10.1371/journal.pgen.1007375
- Koch, P. B., Behnecke, B., and ffrrench-Constant, R. H. (2000). The molecular basis of melanism and mimicry in a swallowtail butterfly. *Curr. Biol.* 10, 591–594. doi: 10.1016/S0960-9822(00)00494-2
- Kozak, K. M., Wahlberg, N., Neild, A. F. E., Dasmahapatra, K. K., Mallet, J., and Jiggins, C. D. (2015). Multilocus species trees show the recent adaptive radiation of the mimetic *Heliconius* butterflies. *Syst. Biol.* 64, 505–524. doi: 10.1093/sysbio/syv007
- Kristensen, N. P., Scibke, M. J., and Karsholt, O. (2007). Lepidoptera phylogeny and systematics: the state of inventorying moth and butterfly diversity. *Zootaxa* 1668, 699–747. doi: 10.11646/zootaxa.1668.1.30
- Kronforst, M. R., Kapan, D. D., and Gilbert, L. E. (2006). Parallel genetic architecture of parallel adaptive radiations in mimetic *Heliconius* butterflies. *Genetics* 174, 535–539. doi: 10.1534/genetics.106.059527
- Kronforst, M. R., and Papa, R. (2015). The functional basis of wing patterning in *Heliconius* butterflies: the molecules behind mimicry. *Genetics* 200, 1–19. doi: 10.1534/genetics.114.172387
- Kulkarni, A., Anderson, A. G., Merullo, D. P., and Konopka, G. (2019). Beyond bulk: a review of single cell transcriptomics methodologies and applications. *Curr. Opin. Biotechnol.* 58, 129–136. doi: 10.1016/j.copbio.2019.03.001
- Kunte, K., Zhang, W., Tenger-Trolander, A., Palmer, D. H., Martin, A., Reed, R. D., et al. (2014). *doublesex* is a mimicry supergene. *Nature* 507, 229–232. doi: 10.1038/nature13112
- Lawrence, P. A., and Casal, J. (2018). Planar cell polarity: two genetic systems use one mechanism to read gradients. *Development* 145:dev168229. doi: 10.1242/dev.168229
- Le Poul, Y., Whibley, A., Chouteau, M., Prunier, F., Llaurens, V., and Joron, M. (2014). Evolution of dominance mechanisms at a butterfly mimicry supergene. *Nat. Commun.* 5:5644. doi: 10.1038/ncomms6644
- Lewis, J. J., Geltman, R. C., Pollak, P. C., Rondem, K. E., Van Belleghem, S. M., Hubisz, M. J., et al. (2019). Parallel evolution of ancient, pleiotropic enhancers underlies butterfly wing pattern mimicry. *Proc. Natl. Acad. Sci.* 116, 24174–24183. doi: 10.1073/pnas.1907068116
- Lewis, J. J., van der Burg, K. R. L., Mazo-Vargas, A., and Reed, R. D. (2016). ChIP-Seq-annotated *Heliconius erato* genome highlights patterns of cis-regulatory evolution in Lepidoptera. *Cell Rep.* 16, 2855–2863. doi: 10.1016/j.celrep.2016.08.042
- Liu, R., Abreu-Blanco, M. T., Barry, K. C., Linardopoulou, E. V., Osborn, G. E., and Parkhurst, S. M. (2009). Wash functions downstream of Rho and links linear and branched actin nucleation factors. *Development* 136, 2849–2860. doi: 10.1242/dev.035246
- Livraghi, L., Hanly, J. J., Loh, L. S., Ren, A., Warren, I. A., Concha, C., et al. (2020). The gene cortex controls scale colour identity in *Heliconius*. *bioRxiv* [Preprint] doi: 10.1101/2020.05.26.116533
- Livraghi, L., Martin, A., Gibbs, M., Braak, N., Arif, S., and Joron, M. (2018). CRISPR/Cas9 as the key to unlocking the secrets of butterfly wing pattern development and its evolution. *Adv. Insect Physiol.* 54, 85–115. doi: 10.1016/bs.aip.2017.11.001
- Mallet, J., Barton, N., Lamas, G., Santisteban, J., Muedas, M., and Eeley, H. (1990). Estimates of selection and gene flow from measures of cline width and linkage disequilibrium in *Heliconius* hybrid zones. *Genetics* 124, 921–936.
- Martin, A., and Courtier-Ordogozo, V. (2017). “Morphological evolution repeatedly caused by mutation in signaling ligand genes,” in *Diversity and Evolution of Butterfly Wing Patterns*, eds T. Sekimura and H. F. Nijhout (Singapore: Springer Singapore), 1–54. doi: 10.1007/978-981-10-4956-9
- Martin, A., McCulloch, K. J., Patel, N. H., Briscoe, A. D., Gilbert, L. E., and Reed, R. D. (2014). Multiple recent co-options of Optix associated with novel traits in adaptive butterfly wing radiations. *Evodevo* 5:7. doi: 10.1186/2041-9139-5-7
- Martin, A., Papa, R., Nadeau, N. J., Hill, R. I., Counterman, B. A., Halder, G., et al. (2012). Diversification of complex butterfly wing patterns by repeated regulatory evolution of a Wnt ligand. *Proc. Natl. Acad. Sci. U.S.A.* 109, 12632–12637. doi: 10.1073/pnas.1204800109
- Martin, A., and Reed, R. D. (2010). *wingless* and *aristaless2* define a developmental ground plan for moth and butterfly wing pattern evolution. *Mol. Biol. Evol.* 27, 2864–2878. doi: 10.1093/molbev/msq173
- Martin, A., and Reed, R. D. (2014). Wnt signaling underlies evolution and development of the butterfly wing pattern symmetry systems. *Dev. Biol.* 395, 367–378. doi: 10.1016/j.ydbio.2014.08.031
- Martin, S. H., Singh, K. S., Gordon, I. J., Omufwoko, K. S., Collins, S., Warren, I. A., et al. (2020). Whole-chromosome hitchhiking driven by a male-killing endosymbiont. *PLoS Biol.* 18:e3000610. doi: 10.1371/journal.pbio.3000610
- Matsuoka, Y., and Monteiro, A. (2018). Melanin pathway genes regulate color and morphology of butterfly wing scales. *Cell Rep.* 24, 56–65. doi: 10.1016/j.celrep.2018.05.092
- Mazo-Vargas, A., Concha, C., Livraghi, L., Massardo, D., Wallbank, R. W. R., Zhang, L., et al. (2017). Macroevolutionary shifts of *WntA* function potentiate butterfly wing-pattern diversity. *Proc. Natl. Acad. Sci. U.S.A.* 114, 10701–10706. doi: 10.1073/pnas.1708149114
- Meghini, F., Martins, T., Tait, X., Fujimitsu, K., Yamano, H., Glover, D. M., et al. (2016). Targeting of *Fzr/Cdh1* for timely activation of the APC/C at the centrosome during mitotic exit. *Nat. Commun.* 7, 12607–12617. doi: 10.1038/ncomms12607
- Merrill, R. M., Rastas, P., Martin, S. H., Melo, M. C., Barker, S., Davey, J., et al. (2019). Genetic dissection of assortative mating behavior. *PLoS Biol.* 17:e2005902. doi: 10.1371/journal.pbio.2005902
- Nadeau, N. J., Pardo-Diaz, C., Whibley, A., Supple, M. A., Saenko, S. V., Wallbank, R. W. R., et al. (2016). The gene cortex controls mimicry and crypsis in butterflies and moths. *Nature* 534, 106–110. doi: 10.1038/nature17961
- Nardi, J. B., and Magee-Adams, S. M. (1986). Formation of scale spacing patterns in a moth wing. *Dev. Biol.* 116, 278–290. doi: 10.1016/0012-1606(86)90131-4
- Nijhout, H. F. (1991). *The Development and Evolution of Butterfly Wing Patterns*. Washington, DC: Smithsonian Institution Press, doi: 10.18984/yadoriga.1998.177_2
- Nijhout, H. F. (2001). Elements of butterfly wing patterns. *J. Exp. Zool.* 291, 213–225. doi: 10.1002/jez.1099
- Nijhout, H. F., and Wray, G. A. (1988). Homologies in the colour patterns of the genus *Heliconius* (Lepidoptera: Nymphalidae). *Biol. J. Linnean Soc.* 33, 345–365. doi: 10.1111/j.1095-8312.1988.tb00449.x
- Oliver, J. C., Tong, X.-L., Gall, L. F., Piel, W. H., and Monteiro, A. (2012). A single origin for Nymphalid butterfly eyespots followed by widespread loss of associated gene expression. *PLoS Genet.* 8:e1002893. doi: 10.1371/journal.pgen.1002893
- Organista, M. F., Martín, M., de Celis, J. M., Barrio, R., López-Varea, A., Esteban, N., et al. (2015). The spalt transcription factors generate the transcriptional landscape of the *Drosophila melanogaster* wing pouch central region. *PLoS Genet.* 11:e1005370. doi: 10.1371/journal.pgen.1005370
- Overton, J. (1966). Microtubules and microfilaments in morphogenesis of the scale cells of *Ephestia kuehniella*. *J. Cell Biol.* 29, 293–293.
- Papa, R., Kapan, D. D., Counterman, B. A., Maldonado, K., Lindstrom, D. P., Reed, R. D., et al. (2013). Multi-allelic major effect genes interact with minor effect QTLs to control adaptive color pattern variation in *Heliconius erato*. *PLoS One* 8:e57033. doi: 10.1371/journal.pone.0057033
- Park, P. J. (2009). ChIP-seq: advantages and challenges of a maturing technology. *Nat. Rev. Genet.* 10, 669–680. doi: 10.1038/nrg2641
- Parnell, A. J., Bradford, J. E., Curran, E. V., Washington, A. L., Adams, G., Brien, M. N., et al. (2018). Wing scale ultrastructure underlying convergent and divergent iridescent colours in mimetic *Heliconius* butterflies. *J. R. Soc. Interf.* 15:20170948. doi: 10.1098/rsif.2017.0948
- Pasteur, G. (1982). A classificatory review of mimicry systems. *Ann. Rev. Ecol. Syst.* 13, 169–199. doi: 10.1146/annurev.es.13.110182.001125
- Pesin, J. A., and Orr-Weaver, T. L. (2007). Developmental role and regulation of cortex, a meiosis-specific anaphase-promoting complex/cyclosome activator. *PLoS Genet.* 3:e202. doi: 10.1371/journal.pgen.0030202
- Raff, J. W., Jeffers, K., and Huang, J.-Y. (2002). The roles of *Fzy/Cdc20* and *Fzr/Cdh1* in regulating the destruction of cyclin B in space and time. *J. Cell Biol.* 157, 1139–1149. doi: 10.1083/jcb.200203035
- Rao, S. S. P., Huntley, M. H., Durand, N. C., Stamenova, E. K., Bochkov, I. D., Robinson, J. T., et al. (2014). A 3D map of the human genome at kilobase

- resolution reveals principles of chromatin looping. *Cell* 159, 1665–1680. doi: 10.1016/j.cell.2014.11.021
- Reed, R. D. (2004). Evidence for Notch-mediated lateral inhibition in organizing butterfly wing scales. *Dev. Genes Evol.* 214, 43–46. doi: 10.1007/s00427-003-0366-0
- Reed, R. D., McMillan, W. O., and Nagy, L. M. (2008). Gene expression underlying adaptive variation in *Heliconius* wing patterns: non-modular regulation of overlapping *cinnabar* and *vermilion* prepatterning. *Proc. Biol. Sci.* 275, 37–45. doi: 10.1098/rspb.2007.1115
- Reed, R. D., Papa, R., Martin, A., Hines, H. M., Counterman, B. A., Pardo-Diaz, C., et al. (2011). *optix* drives the repeated convergent evolution of butterfly wing pattern mimicry. *Science* 333, 1137–1141. doi: 10.1126/science.1208227
- Reed, R. D., and Serfas, M. S. (2004). Butterfly wing pattern evolution is associated with changes in a Notch/Distal-less temporal pattern formation process. *Curr. Biol.* 14, 1159–1166. doi: 10.1016/j.cub.2004.06.046
- Rudel, D., and Sommer, R. J. (2003). The evolution of developmental mechanisms. *Dev. Biol.* 264, 15–37. doi: 10.1016/S0012-1606(03)00353-1
- Saenko, S. V., Brakefield, P. M., and Beldade, P. (2010). Single locus affects embryonic segment polarity and multiple aspects of an adult evolutionary novelty. *BMC Biol.* 8:111. doi: 10.1186/1741-7007-8-111
- Saenko, S. V., Chouteau, M., Piron-Prunier, F., Blugeon, C., Joron, M., and Llaurens, V. (2019). Unravelling the genes forming the wing pattern supergene in the polymorphic butterfly *Heliconius numata*. *Evodevo* 10, 16–12. doi: 10.1186/s13227-019-0129-2
- Schaeffer, V., Althausen, C., Shcherbata, H. R., Deng, W.-M., and Ruohola-Baker, H. (2004). Notch-dependent fuzzy-related/Hec1/Cdh1 expression is required for the mitotic-to-endocycle transition in *Drosophila* follicle cells. *Curr. Biol.* 14, 630–636. doi: 10.1016/j.cub.2004.03.040
- Seimiya, M., and Gehring, W. J. (2000). The *Drosophila* homeobox gene *optix* is capable of inducing ectopic eyes by an *eyeless*-independent mechanism. *Development* 127, 1879–1886.
- Shcherbata, H. R., Althausen, C., Findley, S. D., and Ruohola-Baker, H. (2004). The mitotic-to-endocycle switch in *Drosophila* follicle cells is executed by Notch-dependent regulation of G1/S, G2/M and M/G1 cell-cycle transitions. *Development* 131, 3169–3181. doi: 10.1242/dev.01172
- Sheppard, P. M., Turner, J. R. G., Brown, K. S. Jr., Benson, W. W., and Singer, M. A. (1985). Genetics and the evolution of Mullerian mimicry in *Heliconius* butterflies. *Philos. Trans. R. Soc. Lond.* 308, 433–610. doi: 10.1098/rstb.2010.0410
- Sorrells, T. R., Johnson, A. N., Howard, C. J., Britton, C. S., Fowler, K. R., Feigerle, J. T., et al. (2018). Intrinsic cooperativity potentiates parallel cis-regulatory evolution. *eLife* 7:e37563. doi: 10.7554/eLife.37563
- Stern, D. L., and Orgogozo, V. (2009). Is genetic evolution predictable? *Science* 323, 746–751. doi: 10.1126/science.1158997
- Stossberg, M. (1938). Die zellvorgänge bei der entwicklung der flügel-schuppen von *Ephesia kühniella* Z. *Zeitschrift Morphol. Okol. Tiere* 34, 173–206. doi: 10.2307/43261719
- Supple, M., Papa, R., Counterman, B., and McMillan, W. O. (2014). The genomics of an adaptive radiation: insights across the *Heliconius* speciation continuum. *Adv. Exp. Med. Biol.* 781, 249–271. doi: 10.1007/978-94-007-7347-9_13
- Supple, M. A., Hines, H. M., Dasmahapatra, K. K., Lewis, J. J., Nielsen, D. M., Lavoie, C., et al. (2013). Genomic architecture of adaptive color pattern divergence and convergence in *Heliconius* butterflies. *Genome Res.* 23, 1248–1257. doi: 10.1101/gr.150615.112
- Swan, A., and Schüpbach, T. (2007). The Cdc20 (Fzy)/Cdh1-related protein. Cort, cooperates with Fzy in cyclin destruction and anaphase progression in meiosis I and II in *Drosophila*. *Development* 134, 891–899. doi: 10.1242/dev.02784
- Tong, X., Hrycaj, S., Podlaha, O., Popadic, A., and Monteiro, A. (2014). Over-expression of *Ultrabithorax* alters embryonic body plan and wing patterns in the butterfly *Bicyclus anynana*. *Dev. Biol.* 394, 357–366. doi: 10.1016/j.ydbio.2014.08.020
- True, J. R., and Haag, E. S. (2001). Developmental system drift and flexibility in evolutionary trajectories. *Evol. Dev.* 3, 109–119. doi: 10.1046/j.1525-142x.2001.003002109.x
- Turner, J. R. G. (1972). The genetics of some polymorphic forms of the butterflies *Heliconius melpomene* (Linnaeus) and *H. erato* (Linnaeus). II. The hybridization of subspecies of *H. melpomene* from Surinam and Trinidad. *Zoologica* 52:125.
- Van Belleghem, S. M., Rastas, P., Papanicolaou, A., Martin, S. H., Arias, C. F., Supple, M. A., et al. (2017). Complex modular architecture around a simple toolkit of wing pattern genes. *Nat. Ecol. Evol.* 1:52. doi: 10.1038/s41559-016-0052
- Van Kuren, N. W., Massardo, D., Nallu, S., and Kronforst, M. R. (2019). Butterfly mimicry polymorphisms highlight phylogenetic limits of gene reuse in the evolution of diverse adaptations. *Mol. Biol. Evol.* 36, 2842–2853. doi: 10.1093/molbev/msz194
- van't Hof, A. E., Campagne, P., Rigden, D. J., Yung, C. J., Lingley, J., Quail, M. A., et al. (2016). The industrial melanism mutation in British peppered moths is a transposable element. *Nature* 534, 102–105. doi: 10.1038/nature17951
- van't Hof, A. E., Reynolds, L. A., Yung, C. J., Cook, L. M., and Saccheri, I. J. (2019). Genetic convergence of industrial melanism in three geometrid moths. *Biol. Lett.* 15:20190582. doi: 10.1098/rsbl.2019.0582
- Verboon, J. M., Rincon-Arango, H., Werwie, T. R., Delrow, J. J., Scalzo, D., Nandakumar, V., et al. (2015). Wash interacts with lamin and affects global nuclear organization. *Curr. Biol.* 25, 804–810. doi: 10.1016/j.cub.2015.01.052
- Wallbank, R. W. R., Baxter, S. W., Pardo-Diaz, C., Hanly, J. J., Martin, S. H., Mallet, J., et al. (2016). Evolutionary novelty in a butterfly wing pattern through enhancer shuffling. *PLoS Biol.* 14:e1002353. doi: 10.1371/journal.pbio.1002353
- Westerman, E. L., Van Kuren, N. W., Massardo, D., Tenger-Trolander, A., Zhang, W., Hill, R. L., et al. (2018). *Aristaless* controls butterfly wing color variation used in mimicry and mate choice. *Curr. Biol.* 28, 3469.e–3474.e. doi: 10.1016/j.cub.2018.08.051
- Woronik, A., Tunström, K., Perry, M. W., Neethiraj, R., Stefanescu, C., de la Paz Celorio-Mancera, M., et al. (2019). A transposable element insertion is associated with an alternative life history strategy. *Nat. Commun.* 10:5757. doi: 10.1038/s41467-019-13596-13592
- Zhang, L., Martin, A., Perry, M. W., van der Burg, K. R. L., Matsuoka, Y., Monteiro, A., et al. (2017a). Genetic basis of melanin pigmentation in butterfly wings. *Genetics* 205, 1537–1550. doi: 10.1534/genetics.116.196451
- Zhang, L., Mazo-Vargas, A., and Reed, R. D. (2017b). Single master regulatory gene coordinates the evolution and development of butterfly color and iridescence. *Proc. Natl. Acad. Sci. U.S.A.* 114, 10707–10712. doi: 10.1073/pnas.1709058114
- Zhou, Q., Yu, L., Shen, X., Li, Y., Xu, W., Yi, Y., et al. (2009). Homology of dipteran bristles and lepidopteran scales: requirement for the *Bombyx mori* achaete-scute homologue *ASH2*. *Genetics* 183, 619–627. doi: 10.1534/genetics.109.102848

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2020 McMillan, Livraghi, Concha and Hanly. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Mechanisms of Change: A Population-Based Perspective on the Roles of Modularity and Pleiotropy in Diversification

James J. Lewis^{1*} and Steven M. Van Belleghem^{2*}

¹ Baker Institute for Animal Health, Cornell University, Ithaca, NY, United States, ² Department of Biology, University of Puerto Rico – Río Piedras Campus, San Juan, Puerto Rico

OPEN ACCESS

Edited by:

Marie Manceau,
Collège de France, France

Reviewed by:

Ariel D. Chipman,
The Hebrew University of Jerusalem,
Israel

Sebastian Kittelmann,
Oxford Brookes University,
United Kingdom

*Correspondence:

James J. Lewis
jjl336@cornell.edu
Steven M. Van Belleghem
VanBelleghemSteven@hotmail.com

Specialty section:

This article was submitted to
Evolutionary Developmental Biology,
a section of the journal
Frontiers in Ecology and Evolution

Received: 23 December 2019

Accepted: 24 July 2020

Published: 14 August 2020

Citation:

Lewis JJ and Van Belleghem SM
(2020) Mechanisms of Change:
A Population-Based Perspective on
the Roles of Modularity and Pleiotropy
in Diversification.
Front. Ecol. Evol. 8:261.
doi: 10.3389/fevo.2020.00261

Developmental modularity has long been viewed as a hierarchical organization that facilitates evolution through modification or reuse of preexisting modules. More recently, developmental modularity has been proposed as a mechanism capable of driving rapid evolution of novel color pattern phenotypes between closely related taxa. In this scenario, recombination between modular *cis*-regulatory elements (CREs) generates novel phenotypes by shuffling genetic variation at preexisting color pattern modules into new arrangements. Recent functional evidence from *Drosophila* flies and *Heliconius* butterflies, however, provides a series of examples in which CREs function in multiple developmental contexts and are thus highly pleiotropic. The potential prevalence of pleiotropy in CRE function could be a barrier to the proposed importance of CRE modules as a mechanism for rapid evolutionary change. Here we review the concept of developmental modularity, some examples that suggest developmental modularity underlies pattern evolution, and recent evidence that indicates modular CREs may be less common than previously expected. This leads us to suggest that alternative, non-modular hypotheses should be considered alongside proposals of modular CREs. We then propose the concept of evolutionary modularity as a specific alternative to developmental modularity when discrete, seemingly modular, phenotypes occur in hybridizing taxa. We suggest that evolutionary modularity provides a potentially important pathway for exchange of phenotypic elements between hybridizing taxa independent of the underlying developmental architecture.

Keywords: modularity, pleiotropy, *cis*-regulatory element, *Heliconius*, color pattern, hybridization, evolutionary modularity

INTRODUCTION

Diversification of animal coloration has been often used as a model for the genetics and ecology of adaptive evolution. In numerous taxa, adaptive color pattern diversity has repeatedly mapped to relatively few genomic loci. Bird plumage coloration (Toews et al., 2016; Campagna et al., 2017), cryptic hair pigmentation in mice (Steiner et al., 2007; Manceau et al., 2011), and aposematic color patterns in *Heliconius* butterflies (Reed et al., 2011; Martin et al., 2012; Nadeau et al., 2016; Westerman et al., 2018) are just a few key examples where the loci that differentiate

color morphs include only a handful of developmental genes. Consistent with the well-known trend that gene regulatory mechanisms evolve faster than coding sequence change, many of the loci driving differentiation of adaptive coloration show the strongest signal of divergence between morphs at non-coding loci presumed to capture *cis*-regulatory variants. “Combinatorial” adaptation – the restructuring of existing genetic variation in cases of rapid diversification – has become a common observation in studies of diversification and adaptive radiation (Marques et al., 2019). That is, genomic comparisons often show signals suggesting that combinations of extant alternative alleles at closely linked loci underlie the diversity seen in these color patterns. In some cases of combinatorial evolution, the architecture of *cis*-regulatory adaptation has been coined as modular and modularity of *cis*-regulatory elements (CREs) has been suggested as a potent genetic architecture to explain the rapidly evolving diversity found in these systems (Wallbank et al., 2016; Campagna et al., 2017; Van Belleghem et al., 2017).

Recent studies on the genetic basis of *Drosophila* morphology and *Heliconius* butterfly wing color pattern evolution provide the first lines of evidence that putative *cis*-regulatory modules may, instead, be pleiotropic and non-modular. Here we review the case for modular elements inferred from genomic comparisons, then consider how recent counterexamples question the ubiquity of CRE modularity. We propose that, while exchange of modular elements may indeed underlie the transfer of adaptive phenotypes, recombination of developmental modules should not be the only hypothesis considered. Instead, a genetic model combining hybrid zone homogenization of *trans* acting factors with evolutionary modules can reconcile the apparent modular exchange of alternate alleles with developmental pleiotropy and non-modular genetics.

THE CASE FOR EXCHANGE OF MODULAR ELEMENTS AS A MECHANISM OF DIVERSIFICATION

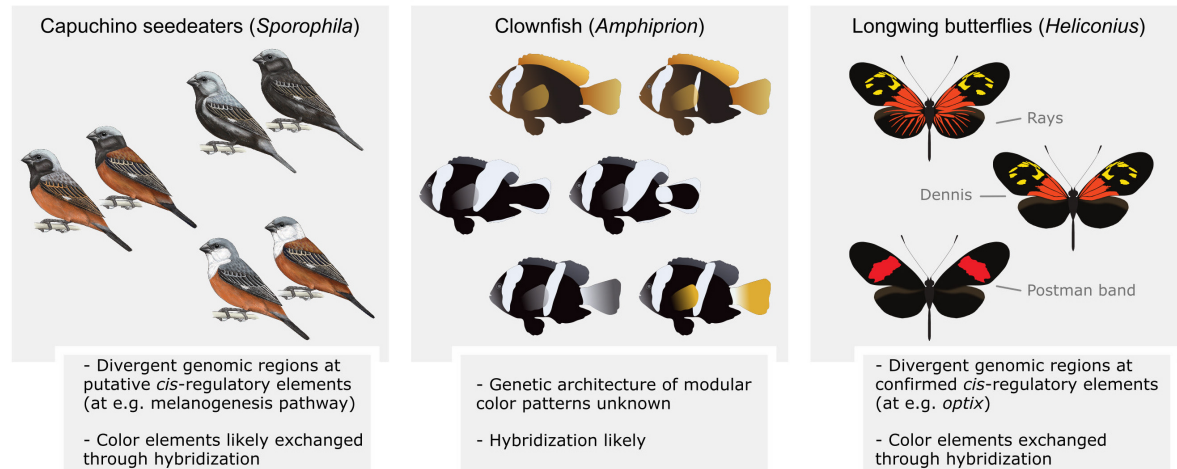
Modularity has long been a mainstay of developmental biology and evo-devo. Examples of modular genetic mechanisms that underlie trait development, such as melanin patterning across *Drosophila* species (Rebeiz et al., 2009) and *Hox* gene expression domains (Kuratani, 2009), have demonstrated the importance of modular architectures in determining organismal form. More recently, comparative and evolutionary genomics have begun to propose modularity as a mechanism capable of facilitating phenotypic diversification (e.g., Hughes and Leips, 2017; Van Belleghem et al., 2017; Mason et al., 2020). Modularity as a combinatorial mechanism of diversification has been associated with exchange of modular CREs, such as enhancer, promoter, insulator, and silencer elements. In this model of diversification, transfer of autonomous CREs via hybridization and recombination of specific genomic loci from one population to another allows for swapping of discrete phenotypic elements to generate new phenotypes from ancestral genetic components (Wallbank et al., 2016; Van Belleghem et al., 2017).

The concept of a “module” or “modular element” is critical to grasping the evidence for modularity as a mechanism of evolution. Despite our focus here on modular CREs, modularity is not specific to non-coding regulatory elements, and thus we aim for a more general definition that fits both non-coding and coding loci alike. While the abstract concept “modularity” is a difficult term to define, autonomy of function (i.e., module components mostly function independently; von Dassow et al., 2000; Schlosser, 2004; Wagner et al., 2007; Monteiro and Podlaha, 2009; Espinosa-Soto and Wagner, 2010; Lacquaniti et al., 2013; Melo et al., 2016) and sufficiency (i.e., modules include all novel elements necessary to induce a phenotype; Monteiro and Podlaha, 2009; Arnoult et al., 2013; Henry et al., 2015; Koshikawa, 2015; Merrill et al., 2019) are common requirements of developmental modules and we continue this practice here. For this perspective, we adopt the definition of a developmental module as: *A genomic locus or set of loci sufficient to semi-autonomously induce a phenotype when activated in any common genetic background within a species.* Developmental modularity then refers to a developmental system consisting of one or more distinct modules [e.g., induced eyeless expression is sufficient to produce ectopic eyes in non-retinal tissues (Weasner et al., 2009)]. This can be contrasted against non-modular architectures, such as seen in many polygenic traits (e.g., Gudbjartsson et al., 2008) and additive *cis*-regulatory control of non-specific gene expression (e.g., Fulco et al., 2016).

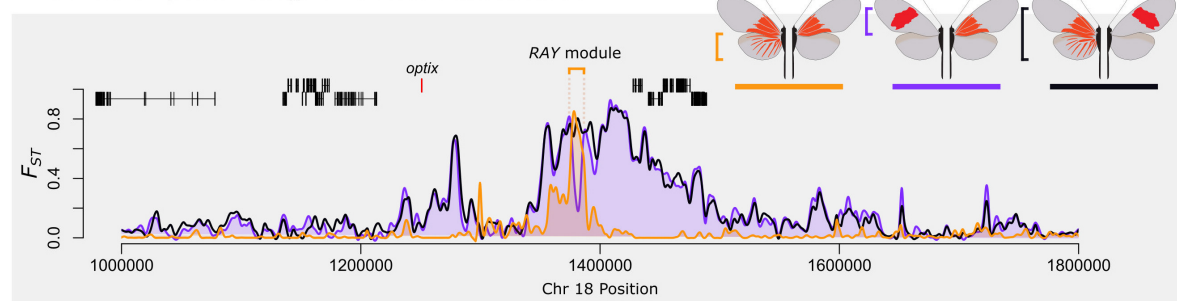
When considered as a mechanism for producing novel adaptive phenotypes, modular loci seem most likely to underlie variation in phenotypes with discrete pattern elements, such as bird plumage patches, fish scale pigmentation, or butterfly color pattern elements. Specific examples indicative of recombination of modular elements include capuchino seedeaters in Argentina (Campagna et al., 2017) and North American warblers (Toews et al., 2016; Figure 1A). In both species groups, discrete plumage color pattern elements appear to be exchanged via hybridization and the strongest signal of genomic differentiation almost exclusively maps to intergenic, putatively *cis*-regulatory, loci. A modular mechanism of pattern diversification has been proposed in cichlids (Maan and Sefc, 2013), and stripe variation among clownfishes (Litsios and Salamin, 2014; Salis et al., 2018) appears modular as well (Figure 1A). Consistent with modular stripe evolution in fish, CRE differences at the gene *csf1* can explain the transitions between striped and non-striped zebrafish species (Patterson et al., 2014). Direct evidence for the mechanism of phenotypic exchange in fish and birds is sparse, however, and studies have limited speculation on the role of modular loci in generating novel color pattern phenotypes. Nonetheless, closely related, hybridizing taxa that appear to exchange color pattern elements to produce novel phenotypes provide an ideal scenario for evolution via modular genomic loci.

Perhaps the best argument for adaptive evolution via transfer of modular CREs comes from *Heliconius* hybrid zones in two co-mimetic species. In both *Heliconius erato* and *Heliconius melpomene*, regional butterfly populations converge on the same mimicry-related phenotypes to form local morphs with discrete aposematic color pattern elements. In the *H. melpomene* clade,

A Proposed examples of modular color pattern variation



B 'Modular' sequence divergence in *Heliconius* butterflies



C Hypothetical examples of CRE module shuffling in *Heliconius* butterflies

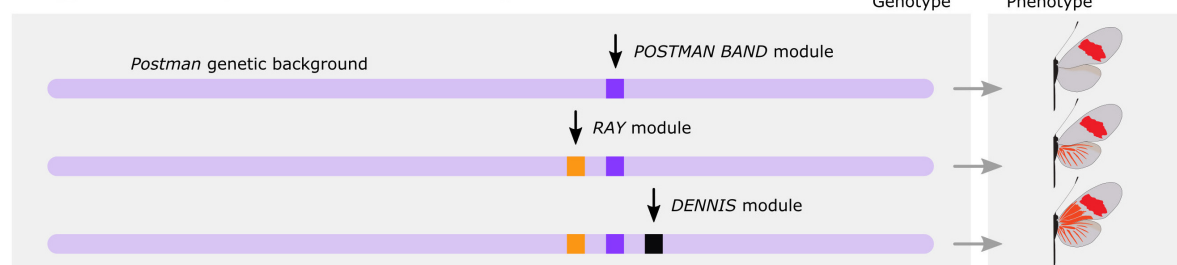


FIGURE 1 | Examples of putative modular color pattern diversity. **(A)** Examples of potential exchange of modular phenotypes via recombination include plumage patterns in capuchino seedeaters (left, reprinted from Campagna et al., 2017), white stripes in clownfish (middle, modified from Salis et al., 2018), and red wing color patterns in longwing butterflies (right). **(B)** Genomic differentiation (F_{ST}) between red wing pattern morphs in *Heliconius erato* at the *optix* locus (data from hybrid zones in French Guiana; Van Belleghem et al., 2017). Orange: Differentiation between radiate (R) and dennis (D) morphs; Purple: Differentiation between postman (P) and dennis (D) morphs; and Black: Differentiation between radiate (R) and postman (P) morphs. A single locus, called Ray module, shows a putative *cis*-regulatory module controlling differentiation between the radiate and dennis morphs. **(C)** Model of CRE module shuffling assuming these divergent loci function as developmental modules. In this case, different combinations of CRE modules would generate modular alternative phenotypes. Note however that the proposed phenotypes with both a red forewing band and dennis/rays on the right have not been found in a homozygous state in nature and suggest that recombination has not occurred (F1 hybrids can have these phenotypes due to dominance effects of *optix*).

some subspecies have evolved to contain partial phenotypes completely present in neighboring subspecies. This includes, for example, the sole presence of rayed hindwing or red forewing triangle patterns in the absence of the other element (Wallbank et al., 2016). This scenario repeats in *H. erato*, where butterfly morphs again form narrow hybrid zones in which discrete red wing pattern phenotypes appear to be exchanged between morphs (Van Belleghem et al., 2017; **Figure 1A**). Importantly,

in both species, the exchange of red pattern elements: (A) maps back to a *cis*-regulatory region distal to the "switch" gene *optix*, and (B) associates with recombination of specific genomic haplotypes at these loci that transfer via recombination between neighboring populations with shared wing color pattern elements (**Figure 1B**). In cases similar to *Heliconius* wing patterns, where hybridization and recombination of extant alleles can produce phenotype diversity, exchange of modular elements is an

attractive hypothesis that posits a simple mechanistic process by which new adaptive phenotypes can be rapidly gained or lost.

CRE PLEIOTROPY AND NON-MODULAR GENE REGULATION

While indirect evidence for recombination of modular CREs between geographically close and genetically related taxa is abundant, recent studies in *Drosophila*, cell lines, and *Heliconius* butterflies suggest pervasive CRE pleiotropy and interdependence. These results present several counterexamples to key principles that support phenotype modularity derived from modular CREs. We consider evidence against the following two principles: (A) CREs are independent and highly tissue-specific and that (B) recombination of existing CREs is more important for rapid evolutionary change compared to *de novo* mutations. We discuss these findings and their consequences for CRE and phenotypic modularity in more detail below.

The first principle favoring exchange of modular CREs can be stated as: genes are frequently found to be highly pleiotropic, where a single gene often affects multiple characteristics, while *cis*-regulatory loci are often assumed to be highly tissue-specific (Prud'homme et al., 2007; Carroll, 2008). This principle provides a foundation for the exchange of phenotype-specific CREs without any corresponding alteration of fitness from undesirable pleiotropic effects. In support of this principle, many early studies of trait evolution found that evolution of one or two enhancer elements underlies variation in phenotypes. Yet recent evidence suggests that *cis*-regulatory loci may often be substantially more pleiotropic than initially expected. The overall prevalence of enhancer pleiotropy has been well covered by Sabaris et al. (2019) and the potential interpretations of “pleiotropy” in Paaby and Rockman (2013). For this perspective, we adopt the standard genetic definition of pleiotropy, where a locus is pleiotropic if it affects multiple characteristics. Some aspects of CRE biology that suggest pleiotropic elements may frequently play an important role in generating novel traits are worth considering further.

For example, pioneering work by the ENCODE project found that CRE availability, an accessible chromatin state important for CRE activity, tends to be maintained through cell lineages (Sergachis et al., 2013). Thus, enhancers activated in earlier cell types are often available for use through much of the remainder of development. It is likely, then, that these elements could be reutilized or co-opted by evolutionary processes to drive new expression patterns instead of generating a suite of novel CREs *de novo* (Monteiro and Podlaha, 2009). Important for evolution, CREs active in multiple tissues or during extended periods of development show increased conservation between taxa and provide an evolutionarily stable set of pre-wired regulatory loci (Lewis et al., 2016; Fish et al., 2017). The availability of accessible CREs in many tissues – a likely precursor for the evolution of enhancer pleiotropy – thus appears frequent enough to be found in meta-analyses of CREs. But does this context result in actual enhancer pleiotropy? The recent discovery of pleiotropic enhancers associated with development of leg bristles and trichome patterning explicitly demonstrates that pleiotropic

CREs targeting developmental genes can and do underlie more than one important traits in *Drosophila* (Nagy et al., 2018; Preger-Ben Noon et al., 2018).

The second principle guiding the prediction of modular CRE transfer is that recombination of extant CRE modules is a logical mechanism for the rapid introduction of new alleles into a population while mitigating deleterious effects likely to occur with coding sequence variation (Prud'homme et al., 2007; Wallbank et al., 2016). While this is undoubtedly true, past studies suggest that transfer of modular elements should not necessarily be the default assumption. In many cases, such as loss of stickleback spines (Chan et al., 2010) and horizontal stripes in cichlids (Kratochwil et al., 2018), adaptive trait evolution is driven by loss of function mutations when an organism is exposed to a novel environment. Adaptive loss of phenotype requires no assumptions regarding the modularity of a trait, as a simple deletion can be sufficient to break the regulatory architecture that underlays trait development in both modular and non-modular scenarios (Prud'homme et al., 2007). In the gain of function case, numerous studies have highlighted the relatively rapid rate of *cis*-regulatory evolution (e.g., Villar et al., 2015; Lewis et al., 2016). Multiple studies have shown that mutations within enhancer elements drive variation in complex phenotypes (e.g., Gompel et al., 2005; Nagy et al., 2018). Similarly, convergent evolution can occur from independent mutations at the same loci, such as the repeated evolution of warning coloration in bumblebees (Tian et al., 2019). Thus, recombination of existing CREs is not required for rapid evolutionary change. Conditional on the number of CREs, distance between loci, and strength of selection against partial recombinants, evolution via directional selection on novel variants or some alternate process may potentially be faster than precise exchange of multiple CRE modules.

Evidence of pleiotropic enhancers and non-modular evolution of novel phenotypes does not reject the transfer of modular CREs. Instead, these counterexamples and arguments suggest we need additional studies to explicitly test for adaptation via exchange of putatively modular elements. Fortunately, recent work on the evolution of mimicry phenotypes in *Heliconius* provides the perfect case study for how evolution of non-modular genetic architectures may drive variation in apparently modular traits.

HELICONIUS WING PATTERNS AS A CASE STUDY IN NON-MODULAR PHENOTYPE EVOLUTION

Heliconius wing patterns have been proposed as a key example of how modular *cis*-regulatory alleles can generate novel color pattern adaptations (Mallet and Clarke, 1989; Wallbank et al., 2016; Van Belleghem et al., 2017). In *Heliconius erato*, red wing pattern phenotypes appear to be shuffled between hybridizing populations to produce morphs with various combinations of these pattern components. The entire list of hybrid zones described in Van Belleghem et al. (2017) is extensive, so we focus here on a particularly clear example of apparent modularity.

In French Guiana and Suriname, three red pattern morphs – named radiate, dennis, and postman (**Figure 1A**) – form a complex hybrid zone. Dennis, which has the forewing pattern of the radiate morph and the hindwing pattern of the postman, appears by eye to be a simple shuffling of these two other phenotypes via recombination of forewing and hindwing pattern-associated alleles. Consistent with this, DNA sequence analysis of these morphs has shown that a single locus downstream of the red switch gene *optix* is the only site that differentiates red wing phenotypes between the radiate and dennis morphs (Van Belleghem et al., 2017; **Figure 1B**). When adding the postman phenotype to this comparison, we see that the locus differentiating dennis from radiate morphs contains the postman allele, rather than the radiate allele. This suggests a simple evolutionary mechanism where a presumptive postman hindwing allele (lacking the Rays module) has been recombined into the radiate haplotype, resulting in the loss of the hindwing rays to produce the dennis phenotype. Thus, the evolution of the dennis morph appears to be a clear example of modular CRE transfer to create a novel phenotype. CRE modules have been similarly suggested for the other red color pattern elements, including a module for the red Dennis and Band color pattern elements (**Figure 1C**).

Recent findings, however, suggest that the origin and subsequent evolution of the radiate morph is much more complicated than expected from a few simple modular CREs (Lewis et al., 2019). These results show that at least five distinct *cis*-regulatory loci drive adaptive evolution of the radiate mimicry phenotype. Consistent with the maintenance of CRE availability through cell-lineages, wing CREs rarely differ between forewings and hindwings (Lewis and Reed, 2018; van der Burg et al., 2019). Thus, the same CRE landscape is shared near *optix* by both forewing and hindwing color patterns (Lewis et al., 2019). Inconsistent with modularity, pattern associated CREs are all interdependent and pleiotropic – CRE mutants alter both the rays and the dennis components of the radiate morph (**Figure 2A**). While hybridization seems to have spread the radiate phenotype between multiple geographic populations, recombination of a single CRE is insufficient to produce a partial or complete radiate phenotype in a postman-like morph. Perhaps most surprisingly, deletion of one CRE showed the effects of both enhancer (phenotype suppression in the mutant) and silencer (phenotype activation in the mutant) activity in different wing sections. This underscores the long-standing view that epistatic effects, and thus the genetic background, can play an important part in how traits evolve (Phillips, 2008). The dual-modality of individual CREs in *Heliconius* is consistent with evidence from *Drosophila* that activating or repressing behavior from CREs can be context dependent (Gisselbrecht et al., 2019). Similarly, epistatic control of wing-pattern CREs is supported by recent observations in various *Heliconius* species that a single gene can modulate phenotype expression associated with other unlinked color pattern loci (Concha et al., 2019).

Taken together, pleiotropic CRE activity and indication of epistasis between color pattern loci runs counter to the developmental modularity of red color pattern CREs in *Heliconius erato*. These experiments can, specifically, reject the

hypothesis that a single locus is sufficient to induce wing pattern components in the absence of additional CRE alleles and the necessary genetic background. This work does, however, raise the question: Why do specific loci appear to control wing phenotypes in a modular fashion in genome sequence comparisons?

EVOLUTIONARY MODULARITY: A HYBRID ZONE MODEL

It is important to reconcile the apparent conflict between the genomic sequence comparisons and experimental data in our case study. It is our view that comparative genomic analyses indicating modular transfer of phenotype components capture an important aspect of adaptive evolution and phenotype stability in the face of gene flow. While this approach does not demonstrate developmental modularity, it can provide strong evidence for a similar concept – evolutionary modularity. *By evolutionary modules, we mean: Any locus sufficient to modulate the gain or loss of phenotype components in the local genetic context of two or more hybridizing populations.* Evolutionary modularity differs from developmental modularity by making no requirements for the autonomy or sufficiency of a module, and may be specific only to a single geographic region or pair of hybridizing taxa. That is, an evolutionary module may require a specific genetic background found only within specific geographic regions. This concept does not make any assumptions about the true developmental genetic architecture of a trait, but instead suggests that many architectures can be utilized in a modular fashion by evolutionary processes.

To parse out how evolutionary modularity would work, we return to our example of the East Amazon *Heliconius* hybrid zone. In the admixed genetic background of the hybrid zone, many combinations of pleiotropic and epistatic loci are likely to occur due to hybridization of “pure” parental phenotypes and recombination in hybrid and backcrossed offspring. When most genetic elements for a trait are homogenous among all three morphs, a single, variable locus may be sufficient to create a novel phenotype and modulate between wing pattern morphs. The apparent gain of a modular phenotype in the dennis morph can be explained as the product of this scenario: In the admixed genetic background of the hybrid zone, *trans*-acting factors are shared by both morphs and a single, *cis*-regulatory domain provides a module-like switch for swapping between phenotypes (**Figures 2B,C**). This single locus may be sufficient to maintain differentiation between the derived dennis morph and the radiate population, while more complicated differentiation patterns would separate radiate from postman. Thus, a single locus, insufficient for producing a phenotype in the absence of a specific genetic context, may act modular in localized population structures. This is different from the scenario of modular CRE shuffling presented in **Figure 1C**, as evolutionary modules require the interdependent regulatory genetic architecture to be present and may require additional *trans* factors.

The concept of evolutionary modularity points to an important feature of adaptive evolution: Evolutionary novelty arises at a specific time and place. The process of refining or separating phenotype components in derived taxa can be distinct

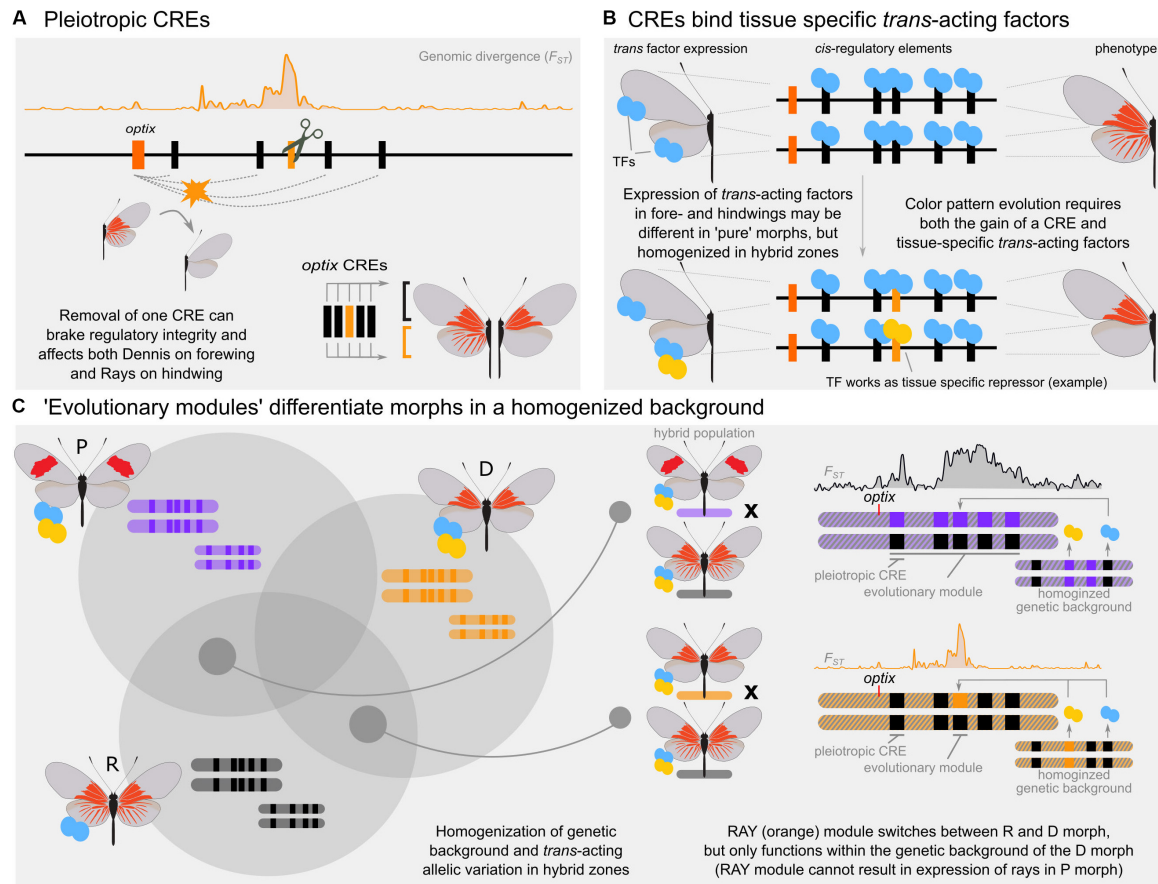


FIGURE 2 | Conceptual overview of evolutionary modularity. **(A)** CRISPR/Cas9 experiments of five putative CREs demonstrate that red wing patterns in *Heliconius erato* are controlled by an interdependent set of enhancers. Loss of any enhancer causes loss of red pattern phenotype in both fore- and hindwings and are pleiotropic (Lewis et al., 2019). **(B)** Hypothetical mechanism by which a novel, tissue specific phenotype (dennis) might evolve through the acquisition of both a CRE and a tissue-specific transcription factor. Blue and yellow symbols indicate tissue specific transcription factors (TFs) which may be unique to a "pure" population but homogenized in hybrid zones. The evolutionary module in this example includes a CRE that is accessible to TFs in both the fore- and hindwing, but may bind a TF unique to the hindwing in the dennis morph only. **(C)** Depiction of hypothesized evolutionary modularity in the East Amazon *H. erato* hybrid zone. Multiple unlinked alleles control divergence between all three morphs (Left). These loci become homogenized in narrow regions of hybridization, allowing a single locus to modulate wing pattern phenotype (Right). These homogenized loci can include *trans*-acting factors, which may not show any signal of differentiation in genomic hybrid zone comparisons.

from the processes that generate the ancestral form. This, in turn, suggests that individual hybrid zones – where modular phenotypes appear most likely – can be a breeding ground for phenotype diversity via evolutionary modularity from either modular or non-modular developmental landscapes.

PROSPECTS FOR FUTURE STUDIES OF ADAPTIVE PHENOTYPE EVOLUTION

Here we consider a single case study of whether modular CREs drive adaptation of novel phenotypes. Many more studies will be necessary before we can parse the relative significance of modular and non-modular genetic architectures for phenotypic novelty and diversification. Importantly, we are not suggesting that modular genetic elements cannot or do not underlie novel phenotypes. Our perspective simply

suggests that developmental modularity should not be the default assumption, even in cases where discrete phenotypes are swapped between hybridizing populations. The larger evolutionary implications of developmental CRE modularity, or the lack thereof, are substantial: modular CREs would favor adaptation via exchange of a few large-effect loci and the potential for simple adaptive introgression of genetic elements. If, however, our concept of evolutionary modularity accurately captures a common evolutionary scenario, we might expect to see an increasing number of studies mapping traits to oligogenic and polygenic adaptive architectures. Similarly, combinatorial adaptation would thus require multiple genetically distinct recombination events.

It will be important that future cases of putative developmental modularity be demonstrated with empirical assays, rather than assumed from sequence comparisons. While consideration of genetic background is not new, and is even quite common

in many studies of developmental genetics, we predict that analysis of genomic interactions and pleiotropic effects will be increasingly important during future studies of phenotypic adaptation. We also suspect, though only time will tell, that evolutionary modularity will be an important process in the production of novel phenotypes. As a deeper understanding of the genetic basis of adaptive evolution emerges, we anticipate that complex developmental architectures will repeatedly be processed in fairly simple evolutionary scenarios via hybridization and recombination to produce ecologically significant phenotypes.

AUTHOR CONTRIBUTIONS

JL and SV contributed equally to the presented ideas, writing of the manuscript, and making the figures. Both authors contributed to the article and approved the submitted version.

REFERENCES

- Arnoult, L., Su, K. F. Y., Manoel, D., Minervino, C., Magriña, J., Gompel, N., et al. (2013). Emergence and diversification of fly pigmentation through evolution of a gene regulatory module. *Science* 339, 1423–1426. doi: 10.1126/science.1233749
- Campagna, L., Repenning, M., Silveira, L. F., Fontana, C. S., Tubaro, P. L., and Lovette, I. J. (2017). Repeated divergent selection on pigmentation genes in a rapid finch radiation. *Sci. Adv.* 3:e1602404. doi: 10.1126/sciadv.1602404
- Carroll, S. B. (2008). Evo-devo and an expanding evolutionary synthesis: a genetic theory of morphological evolution. *Cell* 134, 25–36. doi: 10.1016/j.cell.2008.06.030
- Chan, Y. F., Marks, M. E., Jones, F. C., Villarreal, G., Shapiro, M. D., Brady, S. D., et al. (2010). Adaptive evolution of pelvic reduction in sticklebacks by recurrent deletion of a *Pitx1* enhancer. *Science* 327, 302–305. doi: 10.1126/science.1182213
- Concha, C., Wallbank, R. W. R., Hanly, J. J., Fenner, J., Livraghi, L., Rivera, E. S., et al. (2019). Interplay between developmental flexibility and determinism in the evolution of mimetic *Heliconius* wing patterns. *Curr. Biol.* 29, 3996.e4–4009.e4. doi: 10.1016/j.cub.2019.10.010
- Espinosa-Soto, C., and Wagner, A. (2010). Specialization can drive the evolution of modularity. *PLoS Comput. Biol.* 6:e1000719. doi: 10.1371/journal.pcbi.1000719
- Fish, A., Chen, L., and Capra, J. A. (2017). Gene regulatory enhancers with evolutionarily conserved activity are more pleiotropic than those with species-specific activity. *Genome Biol. Evol.* 9, 2615–2625. doi: 10.1093/gbe/evx194
- Fulco, C. P., Munschauer, M., Anyoha, R., Munson, G., Grossman, S. R., Perez, E. M., et al. (2016). Systematic mapping of functional enhancer-promoter connections with CRISPR interference. *Science* 354, 769–773. doi: 10.1126/science.aag2445
- Gisselbrecht, S. S., Palagi, A., Kurland, J. V., Rogers, J. M., Ozadam, H., Zhan, Y., et al. (2019). Transcriptional silencers in *Drosophila* serve a dual role as transcriptional enhancers in alternate cellular contexts. *Mol. Cell* 77, 324.e8–337.e8. doi: 10.1016/j.molcel.2019.10.004
- Gompel, N., Prud'homme, B., Wittkopp, P. J., Kassner, V. A., and Carroll, S. B. (2005). Chance caught on the wing: cis-regulatory evolution and the origin of pigment patterns in *Drosophila*. *Nature* 433, 481–487. doi: 10.1038/nature03235
- Gudbjartsson, D. F., Walters, G. B., Thorleifsson, G., Stefansson, H., Halldorsson, B. V., Zemanovich, P., et al. (2008). Many sequence variants affecting diversity of adult human height. *Nat. Genet.* 40, 609–615. doi: 10.1038/ng.122

FUNDING

JL was supported by the NASA 17-EXO-17-2-0112 and NSF DEB-1546049. SV was supported by the NSF EPSCoR RII Track-2 FEC (OIA 1736026) and in part by National Institutes of Health-NIGMS COBRE Phase 2 Award – Center for Neuroplasticity at the University of Puerto Rico (Grant No. 1P20GM103642). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

ACKNOWLEDGMENTS

We thank Leo Campagna and Vincent Laudet for providing us with images for capuchino seedeaters and clownfish, and Karin van der Burg, Arnaud Martin, and Joe Hanly for helpful comments.

- Henry, K. F., Kawashima, T., and Goldberg, R. B. (2015). A cis-regulatory module activating transcription in the suspensor contains five cis-regulatory elements. *Plant Mol. Biol.* 88, 207–217. doi: 10.1007/s11103-015-0308-z
- Hughes, K. A., and Leips, J. (2017). Pleiotropy, constraint, and modularity in the evolution of life histories: insights from genomic analyses. *Ann. N.Y. Acad. Sci.* 1389, 76–91. doi: 10.1111/nyas.13256
- Koshikawa, S. (2015). Enhancer modularity and the evolution of new traits. *Fly* 9, 155–159. doi: 10.1080/19336934.2016.1151129
- Kratochwil, C. F., Liang, Y., Gerwin, J., Woltering, J. M., Urban, S., Henning, F., et al. (2018). Agouti-related peptide 2 facilitates convergent evolution of stripe patterns across cichlid fish radiations. *Science* 362, 457–460. doi: 10.1126/science.aao6809
- Kuratani, S. (2009). Modularity, comparative embryology and evo-devo: Developmental dissection of evolving body plans. *Dev. Biol.* 332, 61–69. doi: 10.1016/j.ydbio.2009.05.564
- Lacquaniti, F., Ivanenko, Y. P., d'Avella, A., Zelik, K. E., and Zago, M. (2013). Evolutionary and developmental modules. *Front. Comput. Neurosci.* 7:61. doi: 10.3389/fncom.2013.00061
- Lewis, J. J., Geltman, R. C., Pollak, P. C., Rondem, K. E., Van Belleghem, S. M., Hubisz, M. J., et al. (2019). Parallel evolution of ancient, pleiotropic enhancers underlies butterfly wing pattern mimicry. *Proc. Natl. Acad. Sci. U.S.A.* 116, 24174–24183. doi: 10.1073/pnas.1907068116
- Lewis, J. J., and Reed, R. D. (2018). Genome-wide regulatory adaptation shapes population-level genomic landscapes in *Heliconius*. *Mol. Biol. Evol.* 36, 159–173. doi: 10.1093/molbev/msy209
- Lewis, J. J., van der Burg, K. R. L., Mazo-Vargas, A., and Reed, R. D. (2016). ChIP-seq-annotated *Heliconius erato* genome highlights patterns of cis-regulatory evolution in Lepidoptera. *Cell Rep.* 16, 2855–2863. doi: 10.1016/j.celrep.2016.08.042
- Litsios, G., and Salamin, N. (2014). Hybridisation and diversification in the adaptive radiation of clownfishes. *BMC Evol. Biol.* 14:245. doi: 10.1186/s12862-014-0245-5
- Maan, M. E., and Sefc, K. M. (2013). Colour variation in cichlid fish: developmental mechanisms, selective pressures and evolutionary consequences. *Semin. Cell Dev. Biol.* 24, 516–528. doi: 10.1016/j.semcdb.2013.05.003
- Mallet, J., and Clarke, C. (1989). The genetics of warning colour in Peruvian hybrid zones of *Heliconius erato* and *H. melpomene*. *Proc. R. Soc. London B Biol. Sci.* 236, 163–185. doi: 10.1098/rspb.1989.0019
- Manceau, M., Domingues, V. S., Mallarino, R., and Hoekstra, H. E. (2011). The developmental role of Agouti in color pattern evolution. *Science* 331, 1062–1065. doi: 10.1126/science.1200684
- Marques, D. A., Meier, J. I., and Seehausen, O. (2019). A combinatorial view on speciation and adaptive radiation. *Trends Ecol. Evol.* 34, 531–544. doi: 10.1016/j.tree.2019.02.008

- Martin, A., Papa, R., Nadeau, N. J., Hill, R. I., Counterman, B. A., Halder, G., et al. (2012). Diversification of complex butterfly wing patterns by repeated regulatory evolution of a *Wnt* ligand. *Proc. Natl. Acad. Sci. U.S.A.* 109, 12632–12637. doi: 10.1073/pnas.1204800109
- Mason, A. J., Margres, M. J., Strickland, J. L., Rokyta, D. R., Sasa, M., and Parkinson, C. L. (2020). Trait differentiation and modular toxin expression in palm-pitvipers. *BMC Genomics* 21:147. doi: 10.1186/s12864-020-6545-9
- Melo, D., Porto, A., Cheverud, J. M., and Marroig, G. (2016). Modularity: genes, development and evolution. *Annu. Rev. Ecol. Syst.* 47, 463–486. doi: 10.1146/annurev-ecolsys-121415-032409
- Merrill, R. M., Rastas, P., Martin, S. H., Melo, M. C., Barker, S., Davey, J., et al. (2019). Genetic dissection of assortative mating behavior. *PLoS Biol.* 17:e2005902. doi: 10.1371/journal.pbio.2005902
- Monteiro, A., and Podlaha, O. (2009). Wings, horns, and butterfly eyespots: how do complex traits evolve? *PLoS Biol.* 7:e1000037. doi: 10.1371/journal.pbio.1000037
- Nadeau, N. J., Pardo-Diaz, C., Whibley, A., Supple, M. A., Saenko, S. V., Wallbank, R. W. R., et al. (2016). The gene *cortex* controls mimicry and crypsis in butterflies and moths. *Nature* 534, 106–110. doi: 10.1038/nature17961
- Nagy, O., Nuez, I., Savisaar, R., Peluffo, A. E., Yassin, A., Lang, M., et al. (2018). Correlated evolution of two copulatory organs via a single *cis*-regulatory nucleotide change. *Curr. Biol.* 28, 3450.e13–3457.e13. doi: 10.1016/j.cub.2018.08.047
- Paaby, A. B., and Rockman, M. V. (2013). The many faces of pleiotropy. *Trends Genet.* 29, 66–73. doi: 10.1016/j.tig.2012.10.010
- Patterson, L. B., Bain, E. J., and Parichy, D. M. (2014). Pigment cell interactions and differential xanthophore recruitment underlying zebrafish stripe reiteration and *Danio* pattern evolution. *Nat. Commun.* 5:5299. doi: 10.1038/ncomms6299
- Phillips, P. C. (2008). Epistasis—the essential role of gene interactions in the structure and evolution of genetic systems. *Nat. Rev. Genet.* 9, 855–867. doi: 10.1038/nrg2452
- Preger-Ben Noon, E., Sabaris, G., Ortiz, D. M., Sager, J., Liebowitz, A., Stern, D. L., et al. (2018). Comprehensive analysis of a *cis*-regulatory region reveals pleiotropy in enhancer function. *Cell Rep.* 22, 3021–3031. doi: 10.1016/j.celrep.2018.02.073
- Prud'homme, B., Gompel, N., and Carroll, S. B. (2007). Emerging principles of regulatory evolution. *Proc. Natl. Acad. Sci. U.S.A.* 104(Suppl. 1), 8605–8612. doi: 10.1073/pnas.0700488104
- Rebeiz, M., Pool, J. E., Kassner, V. A., Aquadro, C. F., and Carroll, S. B. (2009). Stepwise modification of a modular enhancer underlies adaptation in a *Drosophila* population. *Science* 326, 1663–1667. doi: 10.1126/science.1178357
- Reed, R. D., Papa, R., Martin, A., Hines, H. M., Counterman, B. A., Pardo-Diaz, C., et al. (2011). *optix* drives the repeated convergent evolution of butterfly wing pattern mimicry. *Science* 333, 1137–1141. doi: 10.1126/science.1208227
- Sabaris, G., Laiker, I., Preger-Ben Noon, E., and Frankel, N. (2019). Actors with multiple roles: pleiotropic enhancers and the paradigm of enhancer modularity. *Trends Genet.* 35, 423–433. doi: 10.1016/j.tig.2019.03.006
- Salis, P., Roux, N., Soulat, O., Lecchini, D., Laudet, V., and Frédérick, B. (2018). Ontogenetic and phylogenetic simplification during white stripe evolution in clownfishes. *BMC Biol.* 16:90. doi: 10.1186/s12915-018-0559-7
- Schlosser, G. (2004). The role of modules in development and evolution. *Modul. Dev. Evol.* 1, 519–582.
- Steiner, C. C., Weber, J. N., and Hoekstra, H. E. (2007). Adaptive variation in beach mice produced by two interacting pigmentation genes. *PLoS Biol.* 5:e219. doi: 10.1371/journal.pbio.0050219
- Stergachis, A. B., Neph, S., Reynolds, A., Humbert, R., Miller, B., Paige, S. L., et al. (2013). Developmental fate and cellular maturity encoded in human regulatory dna landscapes. *Cell* 154, 888–903. doi: 10.1016/j.cell.2013.07.020
- Tian, L., Rahman, S. R., Ezray, B. D., Franzini, L., Strange, J. P., Lhomme, P., et al. (2019). A homeotic shift late in development drives mimetic color variation in a bumble bee. *Proc. Natl. Acad. Sci. U.S.A.* 116, 11857–11865. doi: 10.1073/pnas.1900365116
- Toews, D. P. L., Taylor, S. A., Vallender, R., Brelsford, A., Butcher, B. G., Messer, P. W., et al. (2016). Plumage genes and little else distinguish the genomes of hybridizing warblers. *Curr. Biol.* 26, 2313–2318. doi: 10.1016/j.cub.2016.06.034
- Van Belleghem, S. M., Rastas, P., Papanicolaou, A., Martin, S. H., Arias, C. F., Supple, M. A., et al. (2017). Complex modular architecture around a simple toolkit of wing pattern genes. *Nat. Ecol. Evol.* 1:0052. doi: 10.1038/s41559-016-0052
- van der Burg, K. R. L., Lewis, J. J., Martin, A., Nijhout, H. F., Danko, C. G., and Reed, R. D. (2019). Contrasting roles of transcription factors Spineless and EcR in the highly dynamic chromatin landscape of butterfly wing metamorphosis. *Cell Rep.* 27, 1027.e3–1038.e3. doi: 10.1016/j.celrep.2019.03.092
- Villar, D., Berthelot, C., Aldridge, S., Rayner, T. F., Lukk, M., Pignatelli, M., et al. (2015). Enhancer evolution across 20 mammalian species. *Cell* 160, 554–566. doi: 10.1016/j.cell.2015.01.006
- von Dassow, G., Meir, E., Munro, E. M., and Odell, G. M. (2000). The segment polarity network is a robust developmental module. *Nature* 406, 188–192. doi: 10.1038/35018085
- Wagner, G. P., Pavlicev, M., and Cheverud, J. M. (2007). The road to modularity. *Nat. Rev. Genet.* 8, 921–931. doi: 10.1038/nrg2267
- Wallbank, R. W. R., Baxter, S. W., Pardo-Diaz, C., Hanly, J. J., Martin, S. H., Mallet, J., et al. (2016). Evolutionary novelty in a butterfly wing pattern through enhancer shuffling. *PLoS Biol.* 14:e1002353. doi: 10.1371/journal.pbio.1002353
- Weasner, B. M., Weasner, B., Deyoung, S. M., Michaels, S. D., and Kumar, J. P. (2009). Transcriptional activities of the Pax6 gene *eyeless* regulate tissue specificity of ectopic eye formation in *Drosophila*. *Dev. Biol.* 334, 492–502. doi: 10.1016/j.ydbio.2009.04.027
- Westerman, E. L., VanKuren, N. W., Massardo, D., Tenger-Trolander, A., Zhang, W., Hill, R. I., et al. (2018). Aristaless controls butterfly wing color variation used in mimicry and mate choice. *Curr. Biol.* 28, 3469.e4–3474.e4. doi: 10.1016/j.cub.2018.08.051

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2020 Lewis and Van Belleghem. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Blue, Black, and Stripes: Evolution and Development of Color Production and Pattern Formation in Lizards and Snakes

Takeo Kuriyama¹, Arata Murakami^{2,3}, Matt Brandley⁴ and Masami Hasegawa^{2*}

¹ Institute of Natural and Environmental Sciences, University of Hyogo, Tamba, Japan, ² Laboratory of Geographical Ecology, Department of Biology, Faculty of Science, Toho University, Chiba, Japan, ³ Kaiyo High School, Gamagori, Japan, ⁴ School of Biological Sciences, The University of Sydney, Sydney, NSW, Australia

OPEN ACCESS

Edited by:

Marcus Kronforst,
The University of Chicago,
United States

Reviewed by:

David Pfennig,
The University of North Carolina
at Chapel Hill, United States
Ajeet Pratap Singh,
Novartis Institutes for BioMedical
Research, United States

*Correspondence:

Masami Hasegawa
mhase@bio.sci.toho-u.ac.jp

Specialty section:

This article was submitted to
Evolutionary Developmental Biology,
a section of the journal
Frontiers in Ecology and Evolution

Received: 13 December 2019

Accepted: 25 June 2020

Published: 21 August 2020

Citation:

Kuriyama T, Murakami A,
Brandley M and Hasegawa M (2020)
Blue, Black, and Stripes: Evolution
and Development of Color Production
and Pattern Formation in Lizards
and Snakes. *Front. Ecol. Evol.* 8:232.
doi: 10.3389/fevo.2020.00232

In order to understand molecular and genetic mechanism of color pattern formation, not only adult phenotypes but also processes and mechanisms of color production and pattern formation during embryonic and postembryonic stages should be described. The pigment cell based color production and pattern formation during embryogenesis were reviewed for the recent studies on lizards and snakes, by focusing on different color production mechanisms in terms of epidermal and dermal pigment cell architectures, and then discuss the genetic determinants of pattern formation considering both biologically relevant theoretical models which consider pigment cell specification, migration, and architecture differentiation. Clarifying the contributions of pigment cells and genetic factors improves our general understanding of reptilian color pattern evolution.

Keywords: pigment cells, embryogenesis, polymorphism, melanism, reptile

INTRODUCTION

Why do we study color pattern production mechanisms in squamate reptiles. Basically, animal color pattern is a longstanding and important topic in evolutionary biology in general, and squamates in particular, which exhibit a wide variety of color patterns such as warning signal of venomous coral snakes and its mimicry by the distantly related non-venomous snakes (**Figure 1A**), and convergent evolution of stripes and vivid blue tails by small lizards (**Figure 1B**), are important target of several agents of natural selection for effective visual signals to conspecifics for reproduction, to predators for avoidance, and to sometimes prey for feeding (e.g., Cooper and Greenberg, 1992; Pianka and Vitt, 2003; Stuart-Fox et al., 2008; McKinnon and Pierotti, 2010; Kronforst et al., 2012; Allen et al., 2013; Olsson et al., 2013). The rich diversity of reptiles, as a stem lineage of terrestrial vertebrates living in various ecosystems, provides an invaluable opportunity to study coloration by mainly focusing on functional mechanisms of adaptation for avoiding predation (Ruxton et al., 2018) such as aposematic coloration and coral snake mimicry (Wallace, 1867; Jackson et al., 1976; Pfennig et al., 2001), back ground matching for cryptic coloration (Poulton, 1890; Cott, 1940; Rosenblum et al., 2004), and predator avoidance by motion dazzle coloration (Thayer, 1909; Stevens et al., 2008; Murali et al., 2018; Kodandaramaiah et al., 2020). Yet, we could point out that here have been no recent reviews on the proximate mechanisms underlying various color patterns in reptiles, though several important studies in this topic have been published to date.



Our brief review attempt to fill this gap of proximate mechanisms of color pattern formation by extending the conceptual frame-work presented by Arnold (1983) into color pattern evolution (**Figure 2**). According to the Arnold's conceptual frame-work or rationale linking traits, performance and fitness of organisms, function of prey's color pattern and its performance should be measured by considering the interactions between sender and receiver of visual signals under a variety of light environment (Endler, 1978, 1992, 1993). Therefore, when we hope to understand function and performance of prey's color pattern from a predator's view (Endler, 1978), we have to know how prey produce color signal and how predator perceive prey coloration. Under the classic mathematical theory of communication by Shannon and Weaver (1948), we first simply ask how reptile skin is capable of producing a wide array of color signals (Kuriyama et al., 2006; Kikuchi et al., 2014), no matter how it is perceived by other organisms (**Figure 2**).

Admitting that predator's visual perception is strictly important for understanding predator avoidance function of prey's coloration (Endler, 1978), we would like to present a case study of lizard tail color which functions to deflect predator attacks from body to tail (Cooper and Vitt, 1985; Murali et al., 2018; Kodandaramaiah et al., 2020). Kuriyama et al. (2016a) showed that vividness of blue tail color was associated with the differences in color vision capabilities of lizard predator species, i.e., lizard tails with vivid blue reflectance evolved in communities with either weasel or snake predators, whereas, cryptic brown tail evolved independently on the islands where birds are the primary predator (Brandley et al., 2014). This review comes from our past effort of understanding mechanism of color pattern formation in reptiles, under Arnold's conceptual framework of evolutionary biology

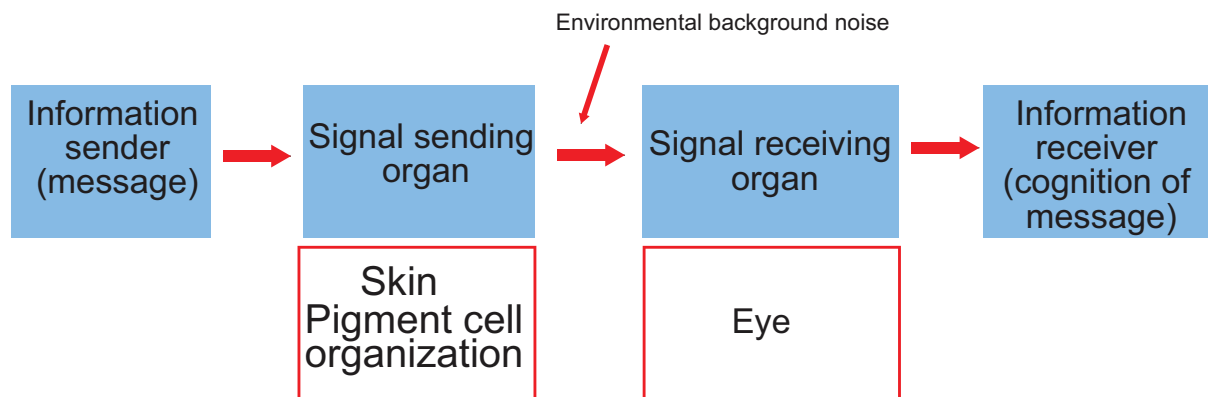
(Arnold, 1983), with intent to obtain broader perspective of understanding function and performance of color pattern in the complex prey-predator interactions (Endler, 1978; Hämäläinen et al., 2015).

Elucidating the spatial and vertical architecture of pigment cells within skin tissues is a fundamental step to understand color pattern formation (Kuriyama et al., 2006; Saenko et al., 2013; Mallarino et al., 2016). It is necessary to not just describe the composition and spatial arrangement of pigment cells in adults, but also to characterize the processes of pigment cell specification, migration, and spatial organization during embryonic and postembryonic development (Kelsh et al., 2009; Kuriyama et al., 2013; Murakami et al., 2016, 2017). These analyses can provide insight into gene regulatory networks (GRNs) involved in color pattern formation (Mallarino et al., 2016). However, our current understanding of the color pattern formation in vertebrates is primarily based on the studies of mouse for a simple single pigment cell type (Mills and Patterson, 2009) and zebrafish for multiple pigment cell types (e.g., Ziegler, 2003; Kelsh, 2004; Parichy, 2006; Kelsh et al., 2009; Greenhill et al., 2011; Parichy and Spiewak, 2015; Petratos et al., 2018), and little is known for the developmental mechanisms of color pattern in reptiles.

In this paper, we review recent advances in our understanding of the roles of pigment cells in the production of various skin colors and patterns in reptilian sauropsids (Kuriyama et al., 2006, 2013, 2016a,b; Murakami et al., 2017; Alibardi, 2011, 2012, 2013, 2015; Kikuchi and Pfennig, 2012; Saenko et al., 2013; Kikuchi et al., 2014; Murakami et al., 2016, 2017; Szydlowski et al., 2016; Kuriyama and Hasegawa, 2017; Jindřich et al., 2019). We approach this topic from the viewpoint of theoretical studies of pattern formation (Murray and Myerscough, 1991; Chang et al., 2009; Allen et al., 2013) and molecular studies of gene systems involved in generating color patterns (Rosenblum et al., 2004; Manceau et al., 2010; Saenko et al., 2015; Irizarry and Bryden, 2016; Iwanishi et al., 2018). Owing to the lack of extensive genomic resources, early studies relied on sequence analyses of a few candidate genes, such as *MC1R* (Rosenblum et al., 2004; Cox et al., 2013). Positional cloning to identify mutations or genomic intervals harboring causal mutations requires both controlled pedigrees to generate recombinant mapping populations and a dense and large set of genetic markers that co-segregate with the phenotype of interest. Therefore, Saenko et al. (2015) and Tzika et al. (2015) adopted an unbiased next-generation sequencing and exome assembly approach, with extensive genotyping and candidate gene sequencing.

PIGMENT CELL-BASED UNDERSTANDING OF COLOR AND PATTERN FORMATION

In reptiles, four basic types of pigment cells have been identified in the dermal skin: xanthophores, erythrophores, iridophores, and melanophores (Taylor and Hadley, 1970; Cooper and Greenberg, 1992; Morrison, 1995; Morrison et al., 1995; Bagnara and Matsumoto, 2006). The spatial arrangement and architecture of the pigment cells produce a variety of



Autotomizable lizard tail coloration against specific predators with different colour vision

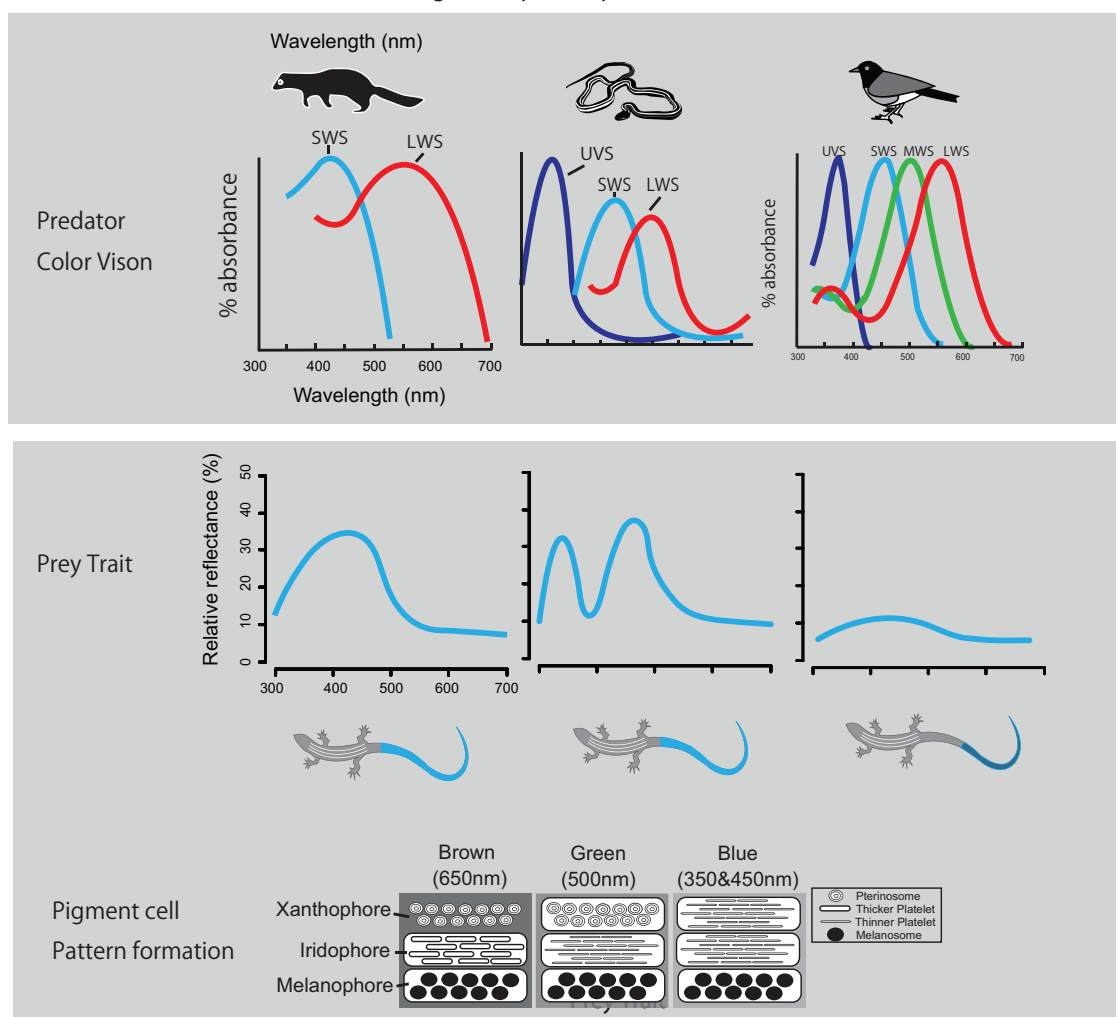


FIGURE 2 | Logical relationship between color producing pigment cell organization and visual signal perception by eye, according to the Shannon – Weaver model of communication (Shannon and Weaver, 1948). Layered organization of pigment cells act as signal transmitter, and eye of predator act as signal receiver.

skin colors in reptiles (e.g., Kuriyama et al., 2013, 2017; Saenko et al., 2013; Murakami et al., 2016), tuatara (Alibardi, 2012), crocodiles (Alibardi, 2011), and chelonians (Alibardi,

2013; Brejcha et al., 2019). These layered organization of pigment cells (xanthophores on top, iridophores in the middle, and melanophores on the bottom) is conserved in zebrafish

(Hirata et al., 2003) but not in mammals and birds (Olsson et al., 2013). An evolutionarily independent transition from the conserved core layering of multiple pigment cells to single melanophore layer is worth emphasizing, because it is well known but least understood phenomena in terms of genetic and molecular control (Bagnara et al., 1968; Grether et al., 2004; Hofreiter and Schöneberg, 2010).

For lizards and snakes, the color of a given patch of skin is determined not only by the static structural combination of pigment cells (Kuriyama et al., 2006), but also by dynamic interactions between chemical and physical parameters (Saenko et al., 2013; Teyssier et al., 2015). Xanthophores and erythrophores produce yellow to red coloration through the selective absorption of short wave length of light by pterinosomes containing pteridine and carotenoid vesicles in cells. Saenko et al. (2013) revealed that the pH or redox states reversibly change yellow xanthophores to red erythrophores and vice versa. Melanophores are light-absorbing pigment cells that produce black or brown colors. The melanophore density controls the lightness or darkness of skin. Iridophores are important light-reflecting cells containing light-reflecting platelets made of crystalline purines and pteridines, which generate structural colors by thin-layer interference and the scatter or diffraction of light from stacks of reflecting platelets (Huxley, 1968; Fujii, 1993; Bagnara and Matsumoto, 2006). The wavelength of light reflected by iridophores is determined by size, shape, orientation, number, and conformation of reflecting platelets and their cytoplasmic spacing (e.g., Land, 1972; Rohrich and Porter, 1972; Morrison, 1995; Morrison et al., 1995; Kuriyama et al., 2006, 2016a, 2017; Saenko et al., 2013). Rohrich (1974) noted that extensive filament networks in passive iridophores of *Anolis* lizards play a cytoskeletal function to maintain crystal sheets in their strict parallel array, while the motile filament system may mediate cellular changes by altering the array, spacing, or tilt of cellular crystals for rapidly changing colors due to dispersion/aggregation of pigment-containing organelles within dermal pigment cells. Recently, Teyssier et al. (2015) showed that chameleons can rapidly change their coloration through active tuning of a lattice of guanine nanocrystals within a superficial thick layer of dermal iridophores. Additionally, a group of iridophores with larger crystals layered in deeper dermis reflected the near-infrared range of light to produce red color. Therefore, the iridophores organized into two superposed layers is an evolutionarily novel mechanism functioning both for efficient camouflage and potentially avoiding passive thermal stress. Collectively, these complex interactions amongst pigment cells can not only produce various colors in the visible spectrum (Figure 3) but also provide various physiological functions.

Recently, studies of lizard body color pattern formation revealed that, a quasi-hexagonal lattice of skin scales, rather than individual chromatophore cells, established a green and black labyrinthine pattern of skin color in ocellated lizards, and that this pattern was produced a cellular automaton that dynamically computed the color states of individual mesoscopic skin scales to produce the corresponding macroscopic color pattern (Manukyan et al., 2017), indicating that cellular automata

can directly correspond to a continuous Turing reaction-diffusion system's processes generated by biological evolution. However, due to our little understandings of physiological mechanisms controlling environmentally derived pigments such as carotenoids (Kikuchi et al., 2014), this review did little consideration of phenotypic plasticity in color patterns of squamates, admitting that there exist important topic of behavioral ecology and physiology in squamate reptiles (Pérez i de Lanuza et al., 2014).

Blue Tail Coloration and Stripe Formation in Lizards

Blue coloration of the lizard is produced by the vertical organization of iridophores with thin platelets in the uppermost layer and melanophores in the basal layer (Figure 4; Kuriyama et al., 2006; Bagnara et al., 2007). In the lizards with blue colored tail, iridophores with thin platelets are found only in skin tissues in the tail (Kuriyama et al., 2006, 2016a). Although the precise border between iridophores with thin or thick platelets have not yet been specified, it is possible that iridophore types radically shift along the longitudinal axis of the trunk and tail. During the embryonic development, iridophores with thin platelets appear in a specific region of the tail, and this position well coincides with the boundary between the blue coloration and brown coloration after hatching (Figure 5). The factors controlling the locations of iridophores with thinner or thicker platelets differ among populations of the same species and among species within the genus (Kuriyama et al., 2016a). After hatching, juveniles with a vivid blue tail gradually shifted to the adult coloration with a uniform brown tail, and the loss of blue tail during ontogeny is due to the change in iridophores with thin platelets into iridophores with thicker platelets and the appearance of xanthophores (Kuriyama et al., unpublished).

For stripe pattern formation during embryogenesis of *P. latiscutatus*, melanophores initially appear before the appearance of iridophores and xanthophores to form stripe pattern (Kuriyama and Hasegawa, 2017), and iridophores subsequently occupy the area above the melanophore layers. Iridophores start to fill the dermal space that is not yet occupied by melanophores, resulting in a higher iridophore density in yellowish-white stripes than in the inter-stripe region. This observation suggests that mechanisms controlling the density gradient of melanophores during embryogenesis are crucial to understand GRNs involved in naturally occurring variation in stripe pattern formation.

The stripe pattern formation is a dynamic process also in the zebrafish (Singh et al., 2014), but the role of iridophores in pattern formation seems to differ between the zebrafish and the lizard. In the zebrafish, iridophore instead of melanophore proliferate, disperse and aggregate to organize the interstripe framework along the dorsoventral axis. However, it seems premature to conclude there is a fundamental difference between fish and reptiles, even though fish and lizard iridophores shared same iridophore types with thin and thick platelets.

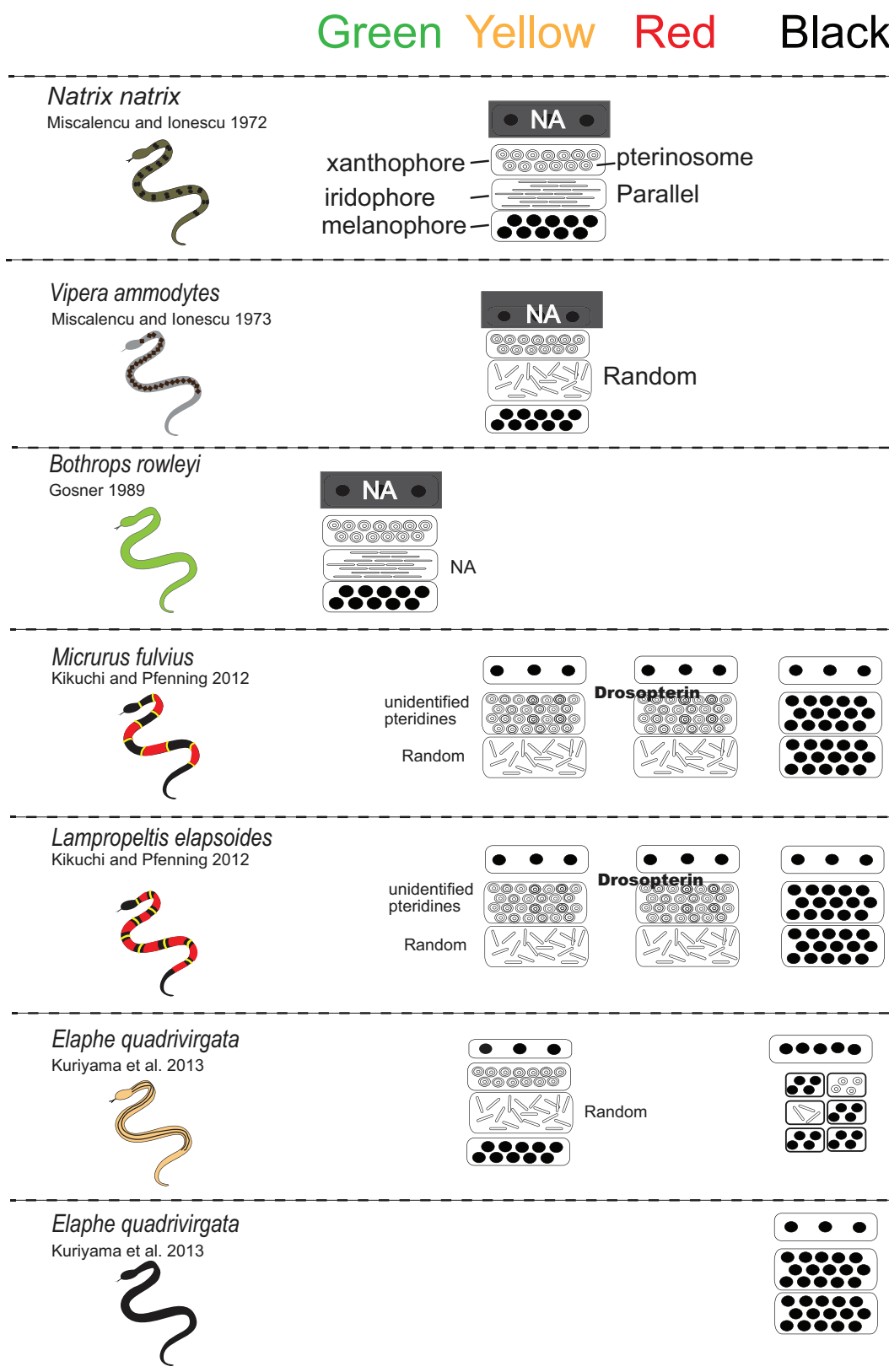


FIGURE 3 | A schematic drawing of the vertical combination and relative thickness of epidermal and dermal pigment cells producing different skin coloration in snakes.

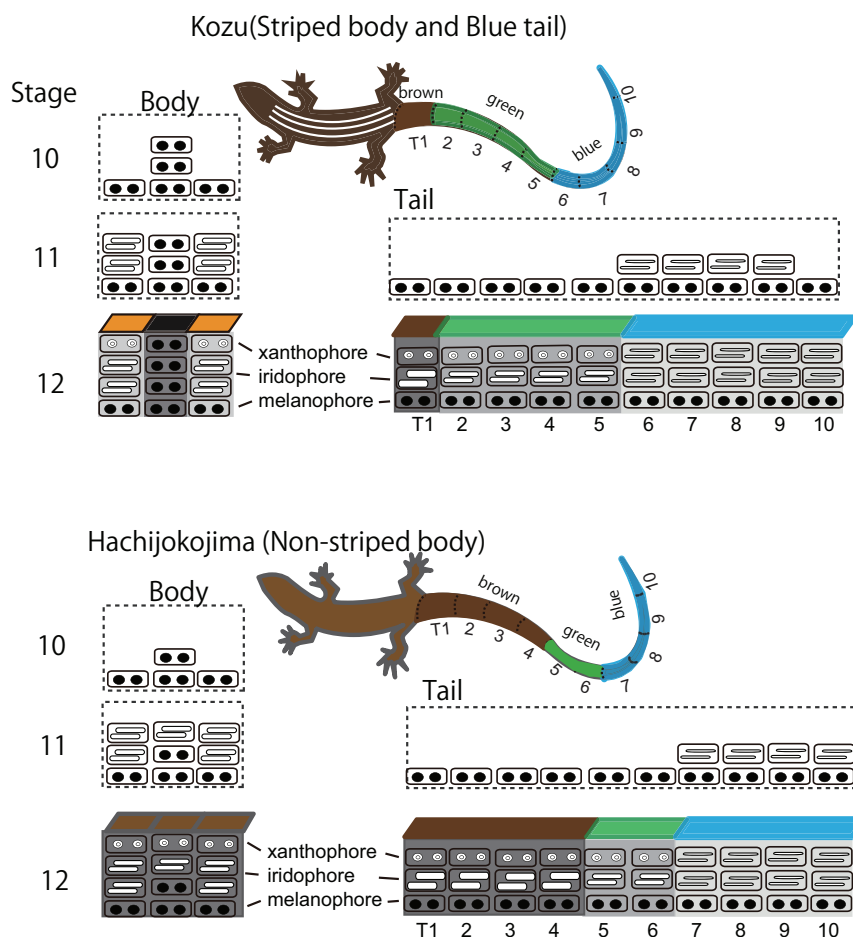


FIGURE 4 | A schematic drawing of stripe pattern and tail color formation during embryogenesis in *Plestiodon latiscutatus*. The melanophore density in the black background region was higher in lizards with vivid stripes in Koze island of Japan than in lizards with drab stripes in Hachijokojima island. Iridophores started to fill the dermal space that was not yet occupied by melanophores, which resulted in a higher iridophore density in stripes than in the inter-stripe regions. Iridophores with thin platelets reflecting blue structural coloration appeared at a specific region in the tail at stage 11. The position of the tail where iridophores emerged coincided with the boundary area separating anterior brown and green colored tail from posterior blue colored tail after hatching. Figure from Kuriyama and Hasegawa (2017) with permissions.



FIGURE 5 | Dorsolateral views of *Plestiodon latiscutatus* juveniles and full adult males from Koze and Hachijokojima. **(A)** A juvenile in Koze island of Japan has five yellowish-white longitudinal lines in the trunk and a green tail in the anterior half that transitions to blue in the posterior region. **(B)** A full adult male in Koze is almost uniformly brown. **(C)** A juvenile in Hachijokojima island of Japan is almost uniformly brown, except for a blue tail tip. **(D)** A full adult male in Hachijokojima is almost uniformly brown.

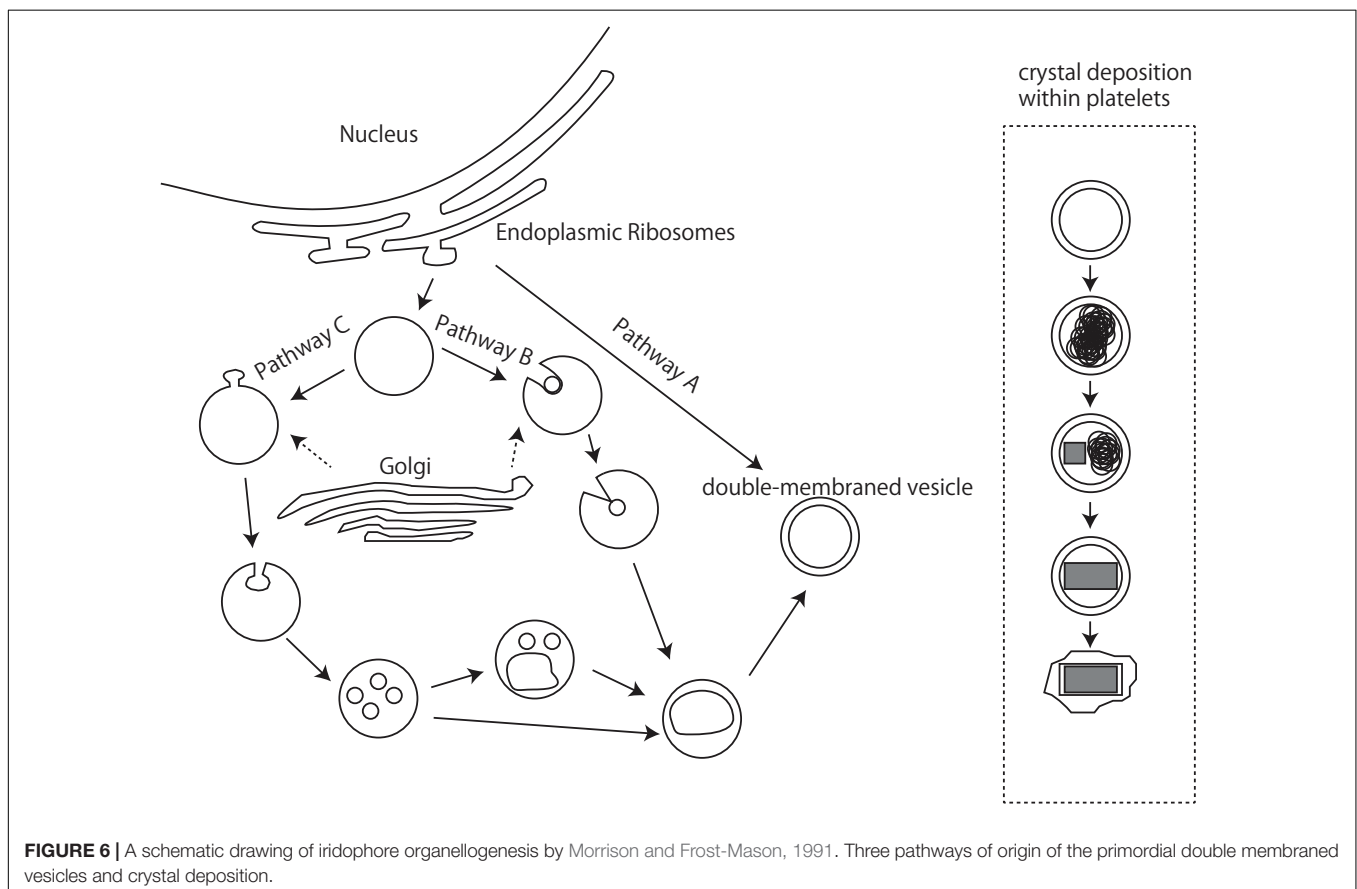
Iridophores differentiate from the NC cells during embryonic development (Bagnara et al., 1979), and the endoplasmic reticulum and Golgi apparatus, which produce platelets in iridophores, possibly play a certain role (Bagnara et al., 1979). Although the mechanisms controlling platelet thickness and orientation remain unknown, it seems possible to hypothesize that the initial volume of vesicles derived via budding from the endoplasmic reticulum and Golgi apparatus are responsible for the size of reflecting platelets (**Figure 6**). The process by which platelets are arranged is poorly understood at present, and additional observations of microtubule and/or microfilament formation in iridophores are needed; Rohrich (1974) suggested that extensive filament networks in iridophores have cytoskeletal functions to maintain crystal sheets in a strict parallel array, while a motile filament system may mediate cellular changes by altering the array, spacing, or tilt of cellular crystals during embryonic development. Further comparative studies of various iridophore types with parallel or random and thick or thin platelets will be indispensable for inferring the genetic basis of reflecting platelet formation.

Stripe Pattern Formation in Snakes

The Japanese four-lined snake *Elaphe quadrivirgata* is polymorphic with striped, pale-striped, non-striped, banded, and melanistic morphs (Mori et al., 2005;

Kuriyama et al., 2011, 2013). Vivid striped and non-striped morphs possess the same set of epidermal melanophores and dermal pigment cells (**Figure 7**, xanthophores at the top, iridophores in the middle, and melanophores at the bottom), but the spatial aggregation and concentrations of epidermal and dermal melanophores differ between the morphs in terms of the sharpness of the boundary between dark- and light-colored scales (Kuriyama et al., 2013). Even at hatching, stripe and non-stripe patterns are detectable (Murakami et al., 2014, 2016), indicating that the basic stripe pattern might be established during embryonic development (Murakami et al., 2016).

During embryonic development, the initial positions of melanophores do not correspond exactly to the positions of stripes (Murakami et al., 2017). Melanophores first appearing in the epidermis may form a precursor stripe pattern, and a region of highly dense melanophores in hatchlings follows these precursor stripes. An increase in the density gradient of epidermal melanophores proceeds the development of the stripe pattern due to dermal melanophores. A distinct stripe pattern is observed during late embryonic stages in association with the appearance of dermal melanophores. However, the higher density and the double stratum of dermal melanophores in the stripe region, which are characteristics of adult snakes (Kuriyama et al., 2013), are not developed by the time of hatching. Unlike the stripe pattern of *Plestiodon* lizards,



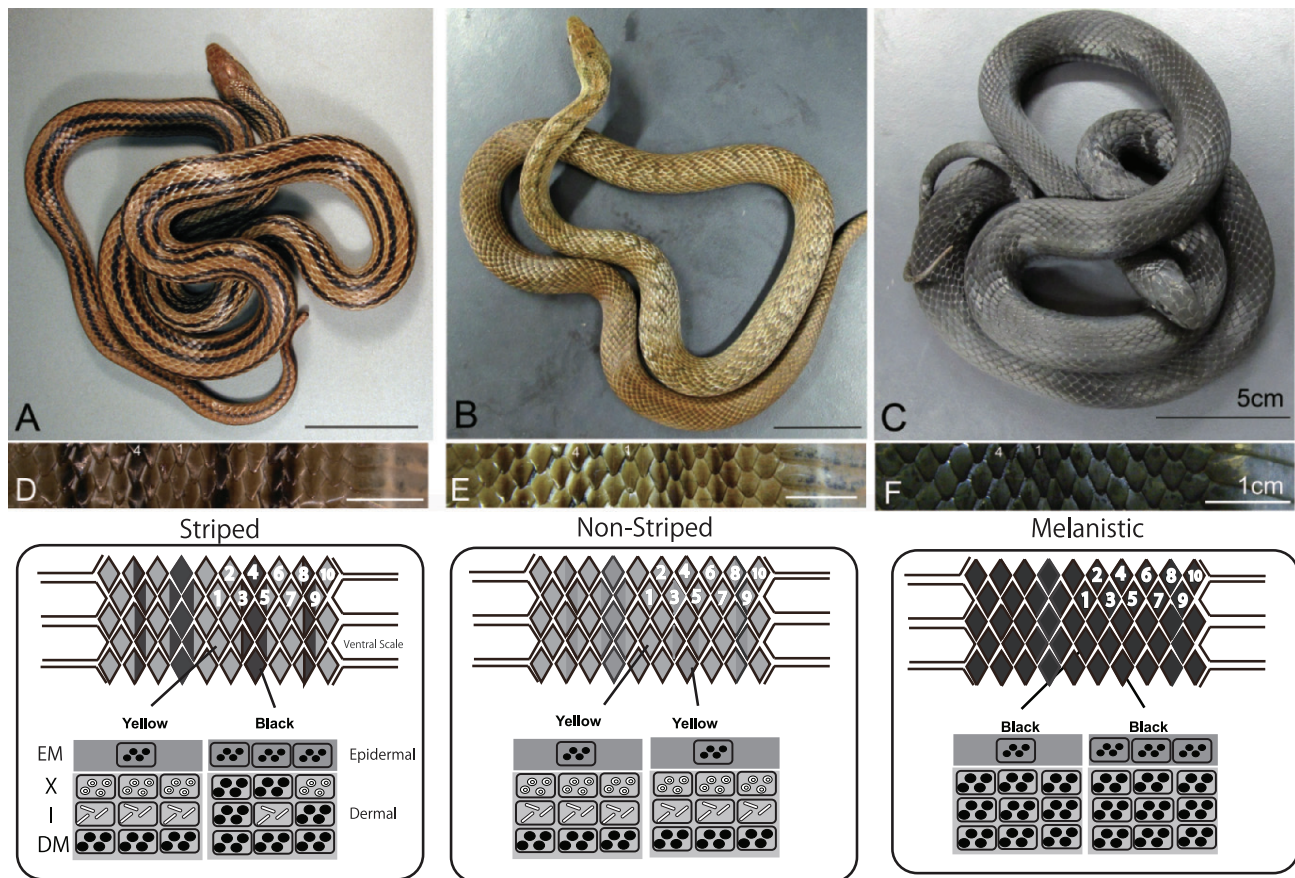


FIGURE 7 | Dorso-lateral views and vertical combination of pigment cells of three-color morphs of *Elaphe quadrigata*. **(A)** The striped morph with four vivid dark-brown longitudinal stripes over yellowish-brown back ground. **(B)** The non-striped morph with a uniform brown or light-brown ground color without a trace of the stripe pattern except the region just behind head. **(C)** The uniformly jet black melanistic morph with no sign of stripe pattern. **(D)** Magnified view of the dorsal surface of a striped morph. Two dorsal scales along the lateral direction were selected as standard landmark scales to describe stripedness (No. 1) and background coloration (No. 4). **(E)** Magnified view of the dorsal surface of a non-striped morph. **(F)** Magnified view of the dorsal surface of a melanistic morph. A schematic drawing of the vertical combination and relative thickness of epidermal and dermal pigment cells producing different skin coloration and color pattern in *Elaphe quadrigata*. Number, shape, and size of pigment cells and organelles are simplified and exaggerated. EM, epidermal melanophore; X, xanthophore; I, iridophore; DM, dermal melanophore. Figure from Kuriyama et al. (2013) with permissions.

which is fully developed at the time of hatching but decays during ontogeny (Kuriyama and Hasegawa, 2017), the snake stripe pattern continues to develop during ontogeny after hatching (Murakami et al., 2016). The density of dermal melanophores presumably increases to replace xanthophores and to form the double stratum in dark-brown scales during postembryonic growth.

Regarding the developmental timing of pigment cells in forming stripe pattern in snakes, there is a lesson from zebrafish. It was thought, for a long time, that melanophores are the first cell type to appear in the metamorphic skin from juvenile to adult zebrafish. Our observation did not pose question if melanophores are not the first cell type canalizing pattern formation in reptiles. However, advent of pigment-independent cell labeling techniques showed that xanthophores are the first cell type that cover the skin, followed by iridophores and then melanophores (Mahalwar et al., 2014). In order to determine that developmental timing

of pattern formation in snakes, one has to utilize pigment-independent markers of cell types, if earlier appearance of xanthophores prior to melanophores was controlled pattern formation in the snakes too.

Color Pattern Polymorphisms

Studies of the mode of inheritance of particular color patterns in polymorphic species are important for exploring standing genetic variation contributing to amelanistic or melanistic coloration and stripe pattern polymorphism (Blanchard and Blanchard, 1940; Bechtel, 1978; Zweifel, 1981; Bechtel and Whitecar, 1983; Bechtel and Bechtel, 1989; King, 2003). A pigment cell-based understanding of color polymorphism facilitates the identification of types of pigment cell architectures corresponding to gains or losses of function to produce color and pattern polymorphisms. Higher order mutations, such as insertions of transposable elements, translocations, inversions, and recombination events, may contribute substantially to

phenotypic variation, depending on their location in protein-coding regions or *cis*-regulatory DNA sequences (Wray, 2007; Wittkopp and Kalay, 2012). The precise roles of these events in the variation of color patterns in reptiles now have been revealing.

Albinism or amelanistic color variation was explained by a mutation that cause loss-of-function in *Phelsuma guttata* (Saenko et al., 2015). These mutations occur in the coding region of a cell surface protein receptor regulating inner cell pH. In particular, a retrotransposon insertion in an exon of *OCA*, similar to genetic variation in *MC1R* (responsible for the dysfunction of melanin synthesis), explains the whitish coloration in lizards living in white sand dunes (Rosenblum et al., 2004). A complete lack of tyrosinase gene due to a nonsense mutation in the coding region of the tyrosinase gene (*TYR*) in *Elaphe climacophora* (Iwanishi et al., 2018) explains another albinistic color variant. In these cases, color variation results from dysfunctions in melanin bio-synthesis. Interestingly, Saenko et al. (2015) hypothesized that even though melanin bio-synthesis is suppressed, melanophores without melanin can still play crucial roles in pattern formation.

Melanism is a typical variant of animal pigmentation (Kettlewell, 1973; Majerus, 1998; True, 2003), and reptilian melanism could be realized by one of two processes involving distinct pigment cell mechanisms: an increase in the relative amount of melanophores and a lack of pigment cells other than melanophores (Kuriyama et al., 2016b). As an example of an increase in the melanophore density in the skin layer, Morrison et al. (1995) studied the melanism of the iguanid lizard *Sceloporus undulatus erythrocheilus*, and revealed that melanophores are four times more abundant, producing darker skin than that of typical individuals. The complete dominance inheritance system with two alleles at one locus explain normal and melanistic morphs in *T. sirtalis* (King, 2003) and *E. quadrivirgata* (Tanaka, 2009). Because the melanistic morph has only epidermal and dermal melanophores and lacks dermal xanthophores and iridophores in *E. quadrivirgata*, it is reasonable to assume that the melanistic morph can be explained by the loss of function to differentiate xanthophores and iridophores. The underlying genetic alterations seem to occur in *cis*-regulatory elements within GRNs for pigment cell differentiation from chromatoblasts or melanophores to xanthophores and iridophores.

In the studies of striped and non-striped polymorphism, striped pattern is a dominant trait in the gopher snake *Pituophis melanoleucus* (Bechtel and Whitecar, 1983), the California king snake *Lampropeltis getula californiae* (Zweifel, 1981) and in the Japanese four-lined snake *E. quadrivirgata*. Because vivid striped and non-striped morphs possess the same set of epidermal and dermal pigment cells, Murakami et al. (2014) hypothesized that the alleles producing the striped morph were dominant under a co-dominance model of one locus with two alleles. Murakami et al. (2016, 2017) later proposed that the alleles of genes controlling the distribution and density of melanophores would additively influence individual variation in the vividness of stripes, and that the vividness of dorsal stripes is controlled by genes involved in the density gradient of melanophores at particular stages during

embryonic development. This inheritance mode of the stripe and non-stripe polymorphism is compatible with the simulation result for the cell-chemotaxis model showing that a stronger chemotactic response generates a vivid longitudinal stripe pattern (Murray and Myerscough, 1991).

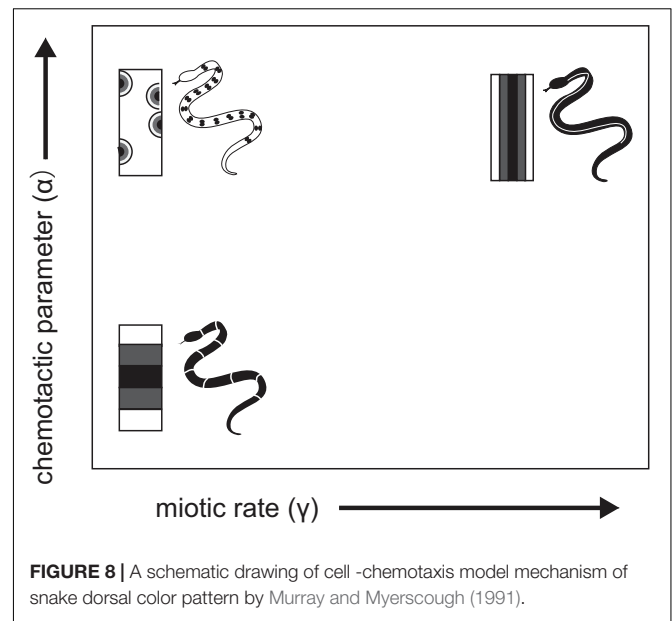
PIGMENT CELL SPECIFICATION GENE NETWORK AND EVOLUTION OF COLOR PATTERN FORMATION

To understand color pattern formation in reptiles with four types of melanocytes (i.e., epidermal melanophores, dermal melanophores, xanthophores, and iridophores), it seems instructive to consider GRNs controlling pattern formation and color production step by step. Pigment cells are differentiated from the NC, a multipotent and migratory cell population, along with other diverse derivatives, such as the facial skeleton and peripheral nervous system (Bagnara et al., 1979). Candidate GRNs for color pattern formation would include genes responsible for cell differentiation, proliferation, death, and localization (Chang et al., 2009; Olsson et al., 2013; Parichy and Spiewak, 2015). Therefore, in this section, we first summarize potential genetic mechanisms orchestrating pigment cell formation from NC cells, from the viewpoint of recognizing evolutionarily important GRNs and mutations in both coding and *cis*-regulatory DNA elements (Wray, 2007; Stern and Orgogozo, 2008; Wittkopp and Kalay, 2012). Later in this section, we consider theoretical analysis of pattern-generating mechanisms, such as cell-chemotaxis mechanisms (Oster and Murray, 1989; Murray and Myerscough, 1991), because it is promising to identify molecular components of the biological system by mathematical modeling to provide a basis for experimental studies and to refine and improve our knowledge of the mechanisms underlying pattern formation (Chang et al., 2009). Later in this section, potential roles of mutations in upstream GRNs for generating novel color phenotypes under domestication (Wilkins et al., 2014) will be reviewed for speculating evolutionary mechanism of color pattern evolution.

During embryonic development, Neural Crest cells undergo cytoskeletal rearrangements and morphological changes to lose cell-cell adhesion, allowing them to delaminate and emigrate from the neuroepithelium, with the concomitant acquisition of migratory ability, cell-surface receptors, metalloproteases, and adhesion molecules that allow them to respond properly to cell-cell interactions and environmental cues influencing migration pathways (reviewed in Sauka-Spengler and Bronner-Fraser, 2008). Although many kinds of GRNs have the potential to contribute to cell motility, cell-cell interactions, and cell population growth rates before the terminal differentiation of pigment cells, the core genetic mechanisms underlying the formation of NC cell derivatives are relatively uniform and conserved among vertebrate lineages. This is instructive for understanding characteristics of intermediate and terminally differentiated pigment cells essential for pattern formation. Based on zebrafish studies, the genes responsible for differentiation

from NC cells into pigment and neural cells have been identified (Pavan and Raible, 2012). The subsequent specification of pigment cells and development of pigment cells occur in temporal order from melanophores, xanthophores, and iridophores (Ziegler, 2003). The regulation of pigment cell differentiation involves protein synthesis; transcription factor (TF) binding to *cis*-regulatory DNA sequences; the promotion or suppression of target gene expression, subsequent expression of TFs, signaling molecules, and cell surface receptor proteins; regulation of standing or activated signal transduction pathways; and the sequential production of pigment synthesis enzymes and other essential products for specific pigment cells. In zebrafish, the core GRNs governing melanocyte specification have been established (Greenhill et al., 2011; Pavan and Raible, 2012), and iridophore GRNs have recently been established by a systems biology approach (Higdon et al., 2013; Petratou et al., 2018). GRNs for xanthophores have not yet been fully established, but the development and patterning of pteridine synthesis and the regulation of the pteridine pathway and of its patterning are largely known in zebrafish (Ziegler, 2003). Consequently, we have established components and logical maps for GRNs of melanophore, iridophore, and xanthophore differentiation in zebrafish and in reptiles, given the high conservation of core GRNs for the same set of pigment cells.

A theoretical model was developed that explicitly considers actual cell movement, chemically mediated cell–cell interactions, and growth of the integumental domain during the patterning process (Murray and Myerscough, 1991). Pattern-generating mechanisms such as reaction-diffusion models (Turing, 1952), however, are unable to generate many common snake patterns, and other models are similarly insufficient to mimic snake pattern. A cell chemotaxis model has been developed considering chromatoblasts, which are NC progenitors; chemotaxis may be a factor in the migration of chromatoblasts to the dermis. According to the model, chromatoblasts both respond to and produce their own chemoattractant to promote the localization of differentiated cells in certain regions of skin. The model thus formalizes cell density in a given region as the product of chemically mediated interactions among dispersive and aggregative effects on cell motility and independent cell population growth rates. The chemoattractant produced by chromatoblasts diffuse and decay. Murray and Myerscough (1991) showed through numerical simulations that longitudinal stripes are most likely to form when both the chemotactic response (α) and cell population growth rate (r) are extremely high (Figure 8). This corresponds to the rapid production or slow diffusion or decay of the chemoattractant in a confined integumental domain. The lateral stripe is likely to form when the chemotactic response is weak. Chemically mediated cell motility (chemotaxis) and cell–cell interactions considered in the model thus provide a hypothetical basis for recognizing candidate GRNs for pattern formation. Both cell–cell and cell–environment interactions are thought to have important roles in directing immature pigment cell to appropriate sites. Assuming that the final transformation to mature pigment cells occurs after NC progenitors migrate to certain regions, GRNs responsible for color pattern formation will be those involved in the



timing and speed of motility, sensitivity to chemical signals, cell population growth and death, and the strength of cell–cell and cell–environment interactions during migration from the NC to correct sites.

Deficiencies in upstream GRNs for melanophore specification are often related to skin cancer, neural diseases, or behavioral abnormalities (e.g., Jackson, 1994; Asher et al., 1996), implying that the evolution of color pattern would involve a balance between the removal of lethal mutations and neutral evolution or positive selection on non-lethal mutations in GRNs for the divergence or generation of novel color patterns. Along this line of thoughts, Wilkins et al. (2014) have hypothesized that the domestication syndrome in mammals such as a combination of behavioral tameness, pigmentation changes, and reduced facial skeleton and tooth size results predominantly from mild neural crest cell failure during embryonic development. Throat and ventral color polymorphism in lizards is often associated with behavioral, immune functional, or life history variation, which is advantageous or disadvantageous in an intraspecific social context, and color polymorphisms can evolve under the varieties of social and ecological selection regimes (Sinervo et al., 2001; Sinervo and Svensson, 2002), presumably due to mutations and subsequent correlational selection on GRNs affecting differentiation from NC cells to peripheral neurons and pigment cell progenitors upstream of GRNs for pigment cell differentiation. Similarly, non-lethal but likely adaptive mutations responsible for pattern formation may exist before the final transformation to mature pigment cells (Wilkins et al., 2014). The GRNs responsible for color pattern formation are expected to function in the timing and speed of motility, sensitivity to chemical signaling, cell population growth and death, and the strength of cell–cell and cell–environment interactions during migration from NC to correct sites (Sauka-Spengler and Bronner-Fraser, 2008), as discussed on the model of cell-chemotaxis (Murray and Myerscough, 1991).

CONCLUSION

According to the new perspective of the molecular mechanisms that regulate gene expression, we are increasingly appreciating that mutations affecting *cis*-regulatory sequences (as opposed to *trans*-regulatory sequences encoding TFs that bind to *cis*-regulatory sequences) are the most frequent cause of phenotypic divergence (Wray, 2007; Carroll, 2008; Stern and Orgogozo, 2008; Jones et al., 2012; Wittkopp and Kalay, 2012). Despite the significance in evolutionary and developmental biology, few studies have successfully connected changes in *cis*-regulatory and coding DNA with changes in pigment cells and color pattern variation. Advances in population genetic approaches for detecting either positive or balancing selection will facilitate the identification of genetic mechanisms underlying the fascinating adaptive color pattern divergence in reptiles.

Comparative studies for elucidating proximate mechanisms of color pattern convergence amongst the distantly related coral snake model and mimics (Kikuchi et al., 2014) and blue tail coloration amongst the closely related and distantly related groups of lizard species (Richmond and Reeder, 2002; Richmond, 2006) are the promising study projects to uncover gene regulatory networks (GRNs) for convergent phenotypic evolution. Under the extended conceptual framework linking GRN, developmental biology, morphology, performance and fitness, fruitful crops of evolutionary biology will be harvested from a sort of classic but long-term mark-release-recapture study of lizard and snake, associated with individually obtained genetic information, because lizards and snakes are particularly affordable animals for both field and laboratory studies (e.g., Sinervo and Svensson, 2002) for detecting natural selection played by the prey lizard and predator snakes.

REFERENCES

- Alibardi, L. (2011). Histology, ultrastructure, and pigmentation in the horny scales of growing crocodilians. *Acta Zool.* 92, 187–200. doi: 10.1111/j.1463-6395.2010.00469.x
- Alibardi, L. (2012). Cytology and localization of chromatophores in the skin of the Tuatara (*Sphenodon punctatus*). *Acta Zool.* 93, 330–337. doi: 10.1111/j.1463-6395.2011.00506.x
- Alibardi, L. (2013). Observations on the ultrastructure and distribution of chromatophores in the skin of chelonians. *Acta Zool.* 94, 222–232. doi: 10.1111/j.1463-6395.2011.00546.x
- Alibardi, L. (2015). Ultrastructural features of skin pigmentation in the lizard *Heloderma suspectum* with emphasis on xanto-melanophores. *Acta Zool.* 96, 154–159. doi: 10.1111/azo.12062
- Allen, W. L., Baddeley, R., Scott-Samuel, N. E., and Cuthill, I. C. (2013). The evolution and function of pattern diversity in snakes. *Behav. Ecol.* 24, 1237–1250. doi: 10.1093/beheco/art058
- Arnold, S. J. (1983). Morphology, performance and fitness. *Am. Zool.* 23, 347–361. doi: 10.1093/icb/23.2.347
- Asher, J. H., Harrison, R. W., Morell, R., Carey, M. L., and Fridman, T. B. (1996). Effects of Pax3 modifier genes on craniofacial morphology, pigmentation, and viability: a murine model of Waardenburg syndrome variation. *Genomics* 34, 285–298. doi: 10.1006/geno.1996.0289
- Bagnara, J. T., Fernandez, P. J., and Fujii, R. (2007). On the blue coloration of vertebrates. *Pigm. Cell Res.* 20, 14–26. doi: 10.1111/j.1600-0749.2006.00360.x
- Bagnara, J. T., and Matsumoto, J. (2006). “Comparative anatomy and physiology of pigment cells in nonmammalian tissues,” in *The Pigmentary System, Physiology*

AUTHOR CONTRIBUTIONS

TK and MH wrote the manuscript and prepared the figures. AM and MB reviewed the manuscript before submission. TK, MH, and AM discussed the scope of review before stating the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This review work was financially supported by the Japan Society for the Promotion of Science (JSPS) to MH (19H03307), and original field and laboratory studies were supported by the JSPS grants to MH (19570026, 21570024, 24570031, and 15H04426) and G. Morimoto (20770018), and the Sasakawa Scientific Research Grant from the Japan Science Society and the Fujiwara Natural History Foundation to TK.

ACKNOWLEDGMENTS

We thank H. Abe, S. Fukasawa, K. Furuse, H. Misawa, T. Mizuta, K. Osawa, T. Okamoto, H. Takahashi, H. Takeda, K. Tomizawa, H. Sanayama, H. Usuda, the members of the Laboratory of Geographical Ecology of Toho University, and T. Okamoto of Kyoto University for their field assistances, A. Mori, R. Fukuyama, and K. Fujishima for providing us the photos of coral snake and its mimics, and two reviewers for constructive criticism.

- and *Pathophysiology*, 2nd Edn, eds J. J. Nordlund, R. E. Boissy, V. J. Hearing, R. A. King, and J. Ortonne (New York, NY: Oxford University Press), 9–40.
- Bagnara, J. T., Matsumoto, J., Ferris, W., Frost, S., Turner, W., Tchen, T., et al. (1979). Common origin of pigment cells. *Science* 203, 410–415. doi: 10.1126/science.760198
- Bagnara, J. T., Taylor, J. D., and Hadley, M. E. (1968). The dermal chromatophore unit. *J. Cell Biol.* 38, 67–79. doi: 10.1083/jcb.38.1.67
- Bechtel, E. R., and Whitecar, T. (1983). Genetics of striping in the gopher snake, *Pituophis melanoleucus*. *J. Herpetol.* 17, 362–370.
- Bechtel, H. B. (1978). Color and pattern in snakes (Reptilia: Serpentes). *J. Herpetol.* 12, 521–532.
- Betctel, H. B., and Betchtel, E. R. (1989). Color mutations in the corn Snake (*Elaphe guttata guttata*): review and additional breeding data. *J. Hered.* 80, 272–276. doi: 10.1093/oxfordjournals.jhered.a110853
- Blanchard, F., and Blanchard, F. (1940). The inheritance of melanism in the garter snake *Thamnophis sirtalis sirtalis* (Linnaeus) and some further evidence of effective autumn mating. *Michigan Acad. Sci. Arts Lett.* 26, 177–193.
- Brandley, M. C., Kuriyama, T., and Hasegawa, M. (2014). Snake and bird predation drive the repeated convergent evolution of correlated life history traits and phenotype in the Izu Island scincid lizard (*Plestiodon latiscutatus*). *PLoS One* 9:e92233. doi: 10.1371/journal.pone.0092233
- Brejcha, J., Bataller, J. V., Bosáková, Z., Geryk, J., Havlíková, M., Kleisner, K., et al. (2019). Body coloration and mechanisms of colour production in Archelosauria: the case of deirocheline turtles. *R. Soc. open sci.* 6:190319. doi: 10.1101/556670

- Carroll, S. B. (2008). Evo-devo and an expanding evolutionary synthesis: a genetic theory of morphological evolution. *Cell* 134, 25–36. doi: 10.1016/j.cell.2008.06.030
- Chang, C., Wu, P., Baker, R. E., Maini, P. K., Alibardi, L., and Chuong, C. M. (2009). Reptile scale paradigm: Evo-Devo, pattern formation and regeneration. *Int. J. Dev. Biol.* 53, 813–826. doi: 10.1387/jdb.072556cc
- Cooper, W. E., and Greenberg, N. (1992). “Reptilian coloration and behavior,” in *Biology of the Reptilia. Physiology E, Hormones, Brain, and Behavior*, Vol. 18, eds C. Gans and D. Crews (Chicago, IL: University of Chicago Press), 298–422.
- Cooper, W. E., and Vitt, L. J. (1985). Blue tails and autotomy: enhancement of predation avoidance in juvenile skinks. *Z. Tierpsychol.* 70, 265–276. doi: 10.1111/j.1439-0310.1985.tb00518.x
- Cott, B. (1940). *Adaptive Coloration in Animals*. London: Methuen.
- Cox, C. L., Rabosky, A. R., and Chippindale, R. T. (2013). Sequence variation in the Mc1r gene for a group of polymorphic snakes. *Gene* 513, 282–286. doi: 10.1016/j.gene.2012.10.065
- Endler, J. A. (1978). A predator's view of animal color patterns. *Evol. Biol.* 11, 319–364. doi: 10.1007/978-1-4615-6956-5_5
- Endler, J. A. (1992). Signals, signal conditions, and the direction of evolution. *Am. Nat.* 139, s125–s153. doi: 10.1086/285308
- Endler, J. A. (1993). The color of light in forests and its implications. *Ecol. Monogr.* 63, 1–27. doi: 10.2307/2937121
- Fujii, R. (1993). Cytophysiology of fish chromatophores. *Int. Rev. Cytol.* 143, 191–255. doi: 10.1016/s0074-7696(08)61876-8
- Gosner, K. L. (1989). Histological notes on the green coloration of arboreal pit vipers: genus *Bothrops*. *J. Herpetol.* 23, 318–320.
- Greenhill, E. R., Rocco, A., Vibert, L., Nikaido, M., and Kelsh, R. N. (2011). An iterative genetic and dynamical modelling approach identifies novel features of the gene regulatory network underlying melanocyte development. *PLoS Genet.* 7:e1002265. doi: 10.1371/journal.pgen.1002265
- Grether, G. F., Kolluru, G. R., and Nersissian, K. (2004). Individual color patches as multicomponent signals. *Biol. Rev.* 79, 583–610. doi: 10.1017/s1464793103006390
- Hämäläinen, L., Valkonen, J., Mappes, J., and Rojas, B. (2015). Visual illusions in predator-prey interactions: birds find moving patterned prey harder to catch. *Anim. Cogn.* 18, 1059–1068. doi: 10.1007/s10071-015-0874-0
- Higdon, C. W., Mitra, R. D., and Johnson, S. L. (2013). Gene expression analysis of zebrafish melanocytes, iridophores, and retinal pigmented epithelium reveals indicators of biological function and developmental origin. *PLoS One* 8:e67801. doi: 10.1371/journal.pone.0067801
- Hirata, M., Nakamura, K., Kanemaru, T., Shibata, Y., and Kondo, S. (2003). Pigment cell organization in the hypodermis of zebrafish. *Dev. Dyn.* 227, 497–503. doi: 10.1002/dvdy.10334
- Hofreiter, M., and Schöneberg, T. (2010). The genetic and evolutionary basis of colour variation in vertebrates. *Cell. Mol. Life Sci.* 67, 2591–2603. doi: 10.1007/s00018-010-0333-7
- Huxley, A. F. (1968). A theoretical treatment of the reflection of light by multilayer structures. *J. Exp. Biol.* 48, 227–245.
- Irizarry, K. J. L., and Bryden, R. L. (2016). *In silico* analysis of gene expression network components underlying pigmentation phenotypes in the *Python* identified evolutionarily conserved clusters of transcription factor binding sites. *Adv. Bioinformatics* 2016:1286510.
- Iwanishi, S., Zaitzu, S., Shibata, H., and Nitasaka, E. (2018). An albino mutant of the Japanese rat snake (*Elaphe climacophora*) carries a nonsense mutation in the tyrosinase gene. *Genes Genet. Syst.* 93, 163–167. doi: 10.1266/ggs.18-00021
- Jackson, I. J. (1994). Molecular and developmental genetics of mouse coat color. *Annu. Rev. Genet.* 28, 189–217. doi: 10.1146/annurev.ge.28.120194.001201
- Jackson, J. F., Ingram, I. I. W., and Campbell, H. W. (1976). The dorsal pigmentation pattern of snakes as an antipredator strategy: a multivariate approach. *Am. Nat.* 110, 1029–1053. doi: 10.1086/283125
- Jindřich, B., Jose Vicente, B., Bosáková, Z., Geryk, J., Havlíková, M., Kleisner, K., et al. (2019). Body coloration and mechanisms of colour production in Archelosauria: the case of deirocheline turtles. *R. Soc. Open Sci.* 6:190319. doi: 10.1098/rsos.190319
- Jones, F. C., Grabherr, M. G., Chan, Y. F., Russell, P., Mauceli, E., Johnson, J., et al. (2012). The genomic basis of adaptive evolution in threespine stickleback. *Nature* 484, 55–61.
- Kelsh, R. N. (2004). Genetics and evolution of pigment patterns in fish. *Pigment Cell Res.* 17, 326–336. doi: 10.1111/j.1600-0749.2004.00174.x
- Kelsh, R. N., Harris, M. L., Colanesi, S., and Erickson, C. A. (2009). Stripes and belly-spots—A review of pigment cell morphogenesis in vertebrates. *Semin. Cell Dev. Biol.* 20, 90–104. doi: 10.1016/j.semcdb.2008.10.001
- Kettlewell, B. (1973). *The Evolution of Melanism: The Study of a Recurring Necessity, with Special Reference to Industrial Melanism in the Lepidoptera*. Oxford: Clarendon Press.
- Kikuchi, D. W., and Pfennig, D. W. (2012). A Batesian mimic and its model share color production mechanisms. *Curr. Zool.* 58, 658–667. doi: 10.1093/czoolo/58.4.658
- Kikuchi, D. W., Seymoure, B. M., and Pfennig, D. W. (2014). Mimicry's palette: widespread use of conserved pigments in the aposematic signals of snakes. *Evol. Dev.* 16, 61–67. doi: 10.1111/ede.12064
- King, R. B. (2003). Mendelian inheritance of melanism in the garter snake *Thamnophis sirtalis*. *Herpetologica* 59, 484–489. doi: 10.1655/02-93
- Kodandaramaiah, U., Palathingal, S., Kurup, G. P., and Murali, G. (2020). What makes motion dazzle markings effective against predation? *Behav. Ecol.* 31, 43–53.
- Kronforst, M. R., Barsh, G. S., Kopp, A., Mallet, J., Monteiro, A., Mullen, S. P., et al. (2012). Unraveling the thread of nature's tapestry: the genetics of diversity and convergence in animal pigmentation. *Pigment Cell Melanoma Res.* 25, 411–433. doi: 10.1111/j.1755-148x.2012.01014.x
- Kuriyama, T., Brandley, M. C., Katayama, A., Mori, A., Honda, M., and Hasegawa, M. (2011). A time-calibrated phylogenetic approach to assessing the phylogeography, colonization history and phenotypic evolution of snakes in the Japanese Izu Islands. *J. Biogeogr.* 38, 259–271. doi: 10.1111/j.1365-2699.2010.02403.x
- Kuriyama, T., Esashi, J., and Hasegawa, M. (2017). Light reflection from crystal platelets in iridophores determines green or brown skin coloration in *Takydromus* lizards. *Zoology* 121, 83–90. doi: 10.1016/j.zool.2016.11.003
- Kuriyama, T., and Hasegawa, M. (2017). Embryonic developmental process governing the conspicuousness of body stripes and blue tail coloration in the lizard *Plestiodon latiscutatus*. *Evol. Dev.* 19, 29–39. doi: 10.1111/ede.12214
- Kuriyama, T., Misawa, H., Miyaji, K., Sugimoto, M., and Hasegawa, M. (2013). Pigment cell mechanisms underlying dorsal color-pattern polymorphism in the Japanese four-lined snake. *J. Morphol.* 274, 1353–1364. doi: 10.1002/jmor.20182
- Kuriyama, T., Miyaji, K., Sugimoto, M., and Hasegawa, M. (2006). Ultrastructure of the dermal chromatophores in a lizard (Scincidae: *Plestiodon latiscutatus*) with conspicuous body and tail coloration. *Zool. Sci.* 23, 793–799. doi: 10.2108/zsj.23.793
- Kuriyama, T., Morimoto, G., Miyaji, K., and Hasegawa, M. (2016a). Cellular basis of anti-predator adaptation in a lizard with autotomizable blue tail against specific predators with different colour vision. *J. Zool.* 300, 89–98. doi: 10.1111/jzo.12361
- Kuriyama, T., Okamoto, T., Miyaji, K., and Hasegawa, M. (2016b). Iridophore- and xanthophore-deficient melanistic color variant of the lizard *Plestiodon latiscutatus*. *Herpetologica* 72, 189–195. doi: 10.1655/herpetologica-d-15-00022
- Land, M. F. (1972). The physics and biology of animal reflectors. *Prog. Biophys. Mol. Biol.* 24, 75–106. doi: 10.1016/0079-6107(72)90004-1
- Mahalwar, P., Walderich, B., Singh, A. P., and Nusslei-Volhard, C. (2014). Local reorganization of xanthophores fine-tunes and colors the striped pattern of zebrafish. *Science* 345, 1362–1364. doi: 10.1126/science.1254837
- Majerus, M. (1998). *Melanism: Evolution in Action*. Oxford: Oxford University Press.
- Mallarino, R., Henegar, C., Mirasierra, M., Manceau, M., Schradin, C., Vallejo, M., et al. (2016). Developmental mechanisms of stripe patterns in rodents. *Nature* 539, 518–523. doi: 10.1038/nature20109
- Manceau, M., Domingues, V. S., Linnen, C. R., Rosenblum, E. B., and Hoekstra, H. E. (2010). Convergence in pigmentation at multiple levels: mutations, genes and function. *Philos. Trans. R. Soc. B Biol. Sci.* 365, 2439–2450. doi: 10.1098/rstb.2010.0104
- Manukyan, L., Montandon, S. A., Fofonjka, A., Smirnov, S., and Milinkovitch, M. C. (2017). A living mesoscopic cellular automaton made of skin scales. *Nature* 544, 173–179. doi: 10.1038/nature22031

- McKinnon, J. S., and Pierotti, M. E. R. (2010). Colour polymorphism and correlated characters: genetic mechanisms and evolution. *Mol. Ecol.* 19, 5101–5125. doi: 10.1111/j.1365-294x.2010.04846.x
- Mills, M. G., and Patterson, L. B. (2009). Not just black and white: pigment pattern development and evolution in vertebrates. *Semin. Cell Dev. Biol.* 20, 72–81. doi: 10.1016/j.semcdb.2008.11.012
- Miscalencu, D., and Ionescu, M. D. (1972). Fine structure of dermal chromatophores in the *Natrix natrix* (L.) snake. *Anat. Anz.* 131, 470–475.
- Miscalencu, D., and Ionescu, M. D. (1973). Fine-structure of epidermis and dermal chromatophores in *Vipera ammodytes* (L.). *Acta Anat.* 86, 111–122. doi: 10.1159/000144113
- Mori, A., Tanaka, K., Moriguchi, H., and Hasegawa, M. (2005). Color variations in *Elaphe quadrivirgata* throughout Japan. *Bull. Herpetol. Soc. Jpn.* 2005, 22–38. doi: 10.14880/hrhgsj1999.2005.22
- Morrison, R. L. (1995). A transmission electron microscopic (TEM) method for determining structural colors reflected by lizard iridophores. *Pigment Cell Res.* 8, 28–36. doi: 10.1111/j.1600-0749.1995.tb00771.x
- Morrison, R. L., and Frost-Mason, S. K. (1991). Ultrastructure analysis of iridophore organogenesis in a lizard, *Sceloporus graciosus* (Reptilia, Phrynosomatidae). *J. Morphol.* 209, 229–239. doi: 10.1002/jmor.1052090209
- Morrison, R. L., Rand, M. S., and Frost-Mason, S. K. (1995). Cellular basis of color differences in three morphs of the lizard *Sceloporus undulatus erythrocheilus*. *Copeia* 1995, 397–408.
- Murakami, A., Hasegawa, M., and Kuriyama, T. (2014). Identification of Juvenile Color Morphs for Evaluating Heredity Model of Stripe/Non-stripe Pattern Polymorphism in Japanese four-lined snake *Elaphe quadrivirgata*. *Curr. Herpetol.* 33, 68–74. doi: 10.5358/hsj.33.68
- Murakami, A., Hasegawa, M., and Kuriyama, T. (2016). Pigment cell mechanism of postembryonic stripe pattern formation in the Japanese four-lined snake. *J. Morphol.* 277, 196–203. doi: 10.1002/jmor.20489
- Murakami, A., Hasegawa, M., and Kuriyama, T. (2017). Developmental mechanisms of longitudinal stripes in the Japanese four-lined snake. *J. Morphol.* 279, 27–36. doi: 10.1002/jmor.20750
- Murali, G., Merilait, S., and Kodandaramaiah, U. (2018). Grab my tail: evolution of dazzle stripes and colourful tails in lizards. *J. Evol. Biol.* 31, 1675–1688. doi: 10.1111/jeb.13364
- Murray, J. D., and Myerscough, M. R. (1991). Pigmentation pattern formation on snakes. *J. Theor. Biol.* 149, 339–360. doi: 10.1016/s0022-5193(05)80310-8
- Olsson, M., Stuart-Fox, D., and Ballen, C. (2013). Genetics and evolution of colour patterns in reptiles. *Semin. Cell Dev. Biol.* 24, 529–541. doi: 10.1016/j.semcdb.2013.04.001
- Oster, G. F., and Murray, J. D. (1989). Pattern formation models and developmental constraints. *J. Exp. Zool.* 251, 186–202. doi: 10.1002/jez.1402510207
- Parichy, D. M. (2006). Evolution of danio pigment pattern development. *Heredity* 97, 200–210. doi: 10.1038/sj.hdy.6800867
- Parichy, D. M., and Spiewak, J. E. (2015). Origins of adult pigmentation: diversity in pigment stem cell lineages and implications for pattern evolution. *Pigment Cell Melanoma Res.* 28, 31–50. doi: 10.1111/pcmr.12332
- Pavan, W. J., and Raible, D. W. (2012). Specification of neural crest into sensory neuron and melanocyte lineages. *Dev. Biol.* 366, 55–63. doi: 10.1016/j.ydbio.2012.02.038
- Pérez i de Lanuza, G., Carazo, P., and Font, E. (2014). Colours of quality: structural (but not pigment) coloration informs about male quality in a polychromatic lizard. *Anim. Behav.* 90, 73–81. doi: 10.1016/j.anbehav.2014.01.017
- Petratou, K., Subkhankulova, T., Lister, J. A., Rocco, A., Schwetlick, H., Kelsh, R. N., et al. (2018). A systems biology approach uncovers the core gene regulatory network governing iridophore fate choice from the neural crest. *PLoS Genet.* 14:e1007402. doi: 10.1371/journal.pgen.1007402
- Pfennig, D. W., Harcombe, W. R., and Pfennig, K. S. (2001). Frequency-dependent Batesian mimicry. *Nature* 410:323. doi: 10.1038/35066628
- Pianka, E. R., and Vitt, L. J. (2003). *Lizards: Windows to the Evolution of Diversity*. Berkeley, CA: University of California Press.
- Poulton, E. B. (1890). *The Colors of Animals*. London: Trübner.
- Richmond, J. Q. (2006). Evolutionary basis of parallelism in North American scincid lizards. *Evol. Dev.* 8, 477–490. doi: 10.1111/j.1525-142x.2006.00121.x
- Richmond, J. Q., and Reeder, T. W. (2002). Evidence for parallel ecological speciation in scincid lizards of the *Eumeces skiltonianus* species group (Squamata: Sincidae). *Evolution* 56, 1498–1513. doi: 10.1111/j.0014-3820.2002.tb01461.x
- Rohrlich, S. T. (1974). Fine structural demonstration of ordered arrays of cytoplasmic filaments in vertebrate iridophores, a comparative survey. *J. Cell Biol.* 62, 295–304. doi: 10.1083/jcb.62.2.295
- Rohrlich, S. T., and Porter, K. R. (1972). Fine structural observations relating to the production of color by the iridophores of a lizard, *Anolis carolinensis*. *J. Cell Biol.* 53, 38–52. doi: 10.1083/jcb.53.1.38
- Rosenblum, E. B., Hoekstra, H. E., and Nachman, M. W. (2004). Adaptive reptile color variation and the evolution of the *Mclr* gene. *Evolution* 58, 1794–1808. doi: 10.1111/j.0014-3820.2004.tb00462.x
- Ruxton, G. D., Allen, W. L., Speed, M. P., and Sherratt, T. N. (2018). *Avoiding Attack: the Evolutionary Ecology of Crypsis, Aposematism, and Mimicry*. Oxford: Oxford University Press.
- Saenko, S. V., Lamichhaney, S., Barrio, A. M., Rafati, N., Andersson, L., and Milinkovitch, M. C. (2015). Amelanism in the corn snake is associated with the insertion of an LTR-retrotransposon in the OCA2 gene. *Sci. Rep.* 5:17118.
- Saenko, S. V., Teyssier, J., van der Marel, D., and Milinkovitch, M. (2013). Precise co-localization of interacting structural and pigmentary elements generates extensive color pattern variation in *Phelsuma* lizards. *BMC Biol.* 11:105. doi: 10.1186/1741-7007-11-105
- Sauka-Spengler, T., and Bronner-Fraser, M. (2008). A gene regulatory network orchestrates neural crest formation. *Nat. Rev. Mol. Cell Biol.* 9, 557–568. doi: 10.1038/nrm2428
- Shannon, C. E. W., and Weaver, W. (1948). *The Mathematical Theory of Communication*. Champaign, IL: University of Illinois press.
- Sinervo, B., Bleay, C., and Adamopoulou, C. (2001). Social causes of correlational selection and the resolution of a heritable throat color polymorphism in a lizard. *Evolution* 55, 2040–2052. doi: 10.1111/j.0014-3820.2001.tb01320.x
- Sinervo, B., and Svensson, E. (2002). Correlational selection and the evolution of genomic architecture. *Heredity* 89, 329–338. doi: 10.1038/sj.hdy.6800148
- Singh, A. P., Schach, U., and Nüsslein-Volhard, C. (2014). Proliferation, dispersal and patterned aggregation of iridophores in the skin prefigure striped coloration of zebrafish. *Nat. Cell Biol.* 16, 604–611. doi: 10.1038/ncb2955
- Stern, D. L., and Orgogozo, V. (2008). The loci of evolution: how predictable is genetic evolution? *Evolution* 62, 2155–2177. doi: 10.1111/j.1558-5646.2008.00450.x
- Stevens, M., Yule, D. H., and Ruxton, G. D. (2008). Dazzle coloration and prey movement. *Proc. Biol. Sci.* 275, 2639–2643. doi: 10.1098/rspb.2008.0877
- Stuart-Fox, D., Moussalli, A., and Whiting, M. J. (2008). Predator-specific camouflage in chameleons. *Biol. Lett.* 4, 326–329. doi: 10.1098/rsbl.2008.0173
- Szydlowski, P., Madej, J. P., and Mazurkiewicz-Kania, M. (2016). Ultrastructure and distribution of chromatophores in the skin of the leopard gecko (*Eublepharis macularius*). *Acta Zool.* 97, 370–375. doi: 10.1111/azo.12132
- Tanaka, K. (2009). Function of color pattern and adaptive significance of color polymorphism in snakes. *Jpn. J. Herpetol.* 2009, 175–187.
- Taylor, J. D., and Hadley, M. E. Z. (1970). Chromatophores and color change in the lizard, *Anolis carolinensis*. *Zellforsch. Mikrosk. Anat.* 104, 282–294. doi: 10.1007/bf00309737
- Teyssier, J., Saenko, S. V., Van der Marel, D., and Milinkovitch, M. C. (2015). Photonic crystals cause active colour change in chameleons. *Nat. Commun.* 6:6368.
- Thayer, G. H. (1909). *Concealing-Coloration in the Animal Kingdom; An Exposition of the Laws of Disguise Through Color and Pattern: Being a Summary of Abbott H. Thayer's Discoveries*. New York, NY: Macmillan.
- True, J. R. (2003). Insect melanism: the molecules matter. *Trends Ecol. Evol.* 18, 640–647. doi: 10.1016/j.tree.2003.09.006

- Turing, A. M. (1952). The chemical basis for morphogenesis. *Philos. Trans. R. Soc. Lond. B* 237, 37–72.
- Tzika, A. C., Ullate-Agote, A., Grbic, D., and Milinkovitch, M. C. (2015). Reptilian transcriptome v2.0: an extensive resource for sauropsida genomics and transcriptomics. *Genome Biol. Evol.* 7, 1827–1841. doi: 10.1093/gbe/evv106
- Wallace, A. R. (1867). *Mimicry, and Other Protective Resemblances Among Animals*. (Vancouver: Read Books), 1–27.
- Wilkins, A. S., Wrangham, R. W., and Fitch, W. T. (2014). The “domestic syndrome” in mammals: a unified explanation based on neural crest cell behavior and genetics. *Genetics* 197, 795–808. doi: 10.1534/genetics.114.165423
- Wittkopp, P. J., and Kalay, G. (2012). Cis-regulatory elements molecular mechanisms and evolutionary processes underlying divergence. *Nat. Rev. Genet.* 13, 59–69. doi: 10.1038/nrg3095
- Wray, G. (2007). The evolutionary significance of cis-regulatory mutations. *Nat. Rev. Genet.* 8, 206–216. doi: 10.1038/nrg2063
- Ziegler, I. (2003). The pteridine pathway in Zebrafish: regulation and Specification during the Determination of Neural Crest Cell-Fate. *Pigment Cell Res.* 16, 172–182. doi: 10.1034/j.1600-0749.2003.00044.x
- Zweifel, R. G. (1981). Genetics of color pattern polymorphism in the California kingsnake. *J. Hered.* 72, 238–244. doi: 10.1093/oxfordjournals.jhered.a109487
- Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2020 Kuriyama, Murakami, Brandley and Hasegawa. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



On the Potential Role of the Neural Crest Cells in Integrating Pigmentation Into Behavioral and Physiological Syndromes

Luis M. San-Jose^{1*} and Alexandre Roulin²

¹ Laboratoire Évolution and Diversité Biologique, UMR 5174, CNRS, Université Toulouse III Paul Sabatier, Toulouse, France,

² Department of Ecology and Evolution, University of Lausanne, Lausanne, Switzerland

OPEN ACCESS

Edited by:

Marie Manceau,
Collège de France, France

Reviewed by:

Pedro Martinez,
University of Barcelona, Spain
Patricia Johnston Moore,
University of Georgia, United States

*Correspondence:

Luis M. San-Jose
luismsanjose@gmail.com

Specialty section:

This article was submitted to
Evolutionary Developmental Biology,
a section of the journal
Frontiers in Ecology and Evolution

Received: 29 February 2020

Accepted: 05 August 2020

Published: 02 September 2020

Citation:

San-Jose LM and Roulin A (2020)
On the Potential Role of the Neural
Crest Cells in Integrating Pigmentation
Into Behavioral and Physiological
Syndromes. *Front. Ecol. Evol.* 8:278.
doi: 10.3389/fevo.2020.00278

Melanin pigments color the integument of vertebrates with shades of rufous, gray, and black. In numerous wild species, melanin-based coloration associates to different behavioral, physiological, and morphological traits, yet the proximate basis of such associations remains largely unknown. The neural crest/domestication syndrome hypothesis (Wilkins et al., 2014) proposes that correlated changes in pigimentary, behavioral, physiological, and morphological traits of domestic species result from deficiencies affecting the number or development of neural crest cells in the embryo. Here, we review to what extent the neural crest cells might also explain the associations observed between melanin-based coloration and distinct traits in wild vertebrate populations. Genes involved in the development of the neural crest explain color differences in some vertebrate species, particularly, in cichlid fishes. Cells that originate from the embryo's neural crest cells play functions that have been previously associated with melanin-based coloration in wild vertebrates and we propose the potential mechanisms through which the association of melanin-based coloration with other traits can be the consequence of having a common origin at the neural crest cells. We encourage considering the influence of the neural crest as a new avenue of research, integrating developmental biology and evolutionary ecology to better understand why and how melanin-based coloration of vertebrates become associated to multiple phenotypic aspects.

Keywords: animal signals, aggressiveness, eco-evo-devo, behavior, melanism, pigmentation, phenotypic integration, trait associations

INTRODUCTION

Most vertebrates color their integuments with melanin pigments (Roy et al., 2020). When deposited in the skin, feathers, scales or hairs, melanins produce different shades of black, gray, and rufous colors (e.g., Stoddard and Prum, 2011). Coloration based on melanin pigments (hereafter referred to as melanin-based coloration, MbC) can play different biological functions such as protecting animals from UV radiation (Brenner and Hearing, 2008; Singaravelan et al., 2008),

promoting thermal adaptation by regulating heat gain (Clusella-Trullas et al., 2008), and mediating social and mating encounters in the case of melanin-based ornamental signals (Roulin, 2016).

Moreover, studies in wild vertebrate populations have repeatedly shown that the expression of MbC is associated with multiple aspects of an individual's phenotype including physiology (metabolism, stress response and stress hormone levels), immunology (inflammatory response and parasite load), morphology (body size and mass), behavior (aggressiveness, boldness, and exploratory behavior) and reproductive traits (sex hormone levels and fecundity) (reviewed in San-Jose and Roulin, 2018). Often, these associations have the same sign in different species. For instance, individuals with a darker or more extended MbC (i.e., entailing a higher deposition of melanin pigments, generally eumelanin) have been often found to be more aggressive and/or to have a higher social rank in different vertebrate taxa, including fishes (Schweitzer et al., 2015), amphibians (Reiter et al., 2014), testudines (Mafli et al., 2011), squamates (Plasman et al., 2015), birds (Santos et al., 2011), and mammals (Loehr et al., 2008).

The recurrent association of MbC with the same traits across vertebrates suggests the existence of shared developmental, genetic, and/or selective mechanisms that link the expression of MbC with other phenotypic aspects (Ducrest et al., 2008; Roulin and Ducrest, 2011; San-Jose and Roulin, 2018). Vertebrate MbC thus constitutes a good model system to address questions of general relevance for evolutionary biologists, particularly those regarding the evolution of the phenotype that acknowledge its multidimensional nature. The study of the proximate and ultimate causes underlying the association of MbC to other, disparate traits can offer insights into the molecular basis of trait correlations (Saltz et al., 2017), into how complex organisms evolve and adapt (Orr, 2000; Wagner et al., 2007; Melo et al., 2016) and how developmental and genetic constraints and trade-offs influence evolutionary trajectories, adaptation, and species differentiation (Smith et al., 1985; West-Eberhard, 2005; Mauro and Ghalambor, 2020).

With this review, our aim is to draw attention toward a new, potentially explanatory mechanism linking MbC with other phenotypic aspects in wild vertebrate populations. Research on domestic mammals has also encountered the recurrent association of MbC with different morphological and behavioral traits, a phenomenon already highlighted by Darwin and that was named the “domestication syndrome” (Wilkins et al., 2014; see also Sánchez-Villagra et al., 2015). Wilkins et al. (2014; see also Wilkins, 2019) proposed that many of the phenotypes often displayed by domestic mammals: depigmentation, floppy ears, smaller brains, reduced tooth size, higher fecundity, can be explained by deficiencies affecting the initial numbers and/or the capacity to migrate and differentiate of the cells in the neural crest, NC. Cells in the NC are the precursors of pigment cells, including melanocytes (*sensu* Schartl et al., 2016), as well as of different types of endocrine cells, mesenchymal cells, and neuron and glial cells (Douarin et al., 2008; Monsoro-Burq, 2015; Simões-Costa and Bronner, 2015; Rothstein et al., 2018; Etchevers et al., 2019). This shared origin increases the likelihood

that genetic and environmental factors affecting the NC cells' normal development result in parallel changes in the different cell types deriving from the NC and thus, on correlated changes in different phenotypic aspects (Wilkins et al., 2014). We explore here if the ideas of Wilkins et al. (2014) can be exported outside domestic mammals and used in future studies to explore the yet unknown causes linking MbC and multiple phenotypic aspects in wild vertebrate populations.

NEURAL CREST CELLS AND COLOR VARIATION IN WILD ANIMALS

The neural crest, NC, is a transitory structure exclusive to the vertebrates' embryo (Donoghue et al., 2008; Shimeld and Holland, 2000). It is a cord of cells that originates from the neural plate borders and arises approximately when the neural plates join to form the embryo's neural tube (Simões-Costa and Bronner, 2015; Etchevers et al., 2019; **Figures 1A,B**). Cells in the NC initially lie between the dorsal ectoderm and the neural tube but, as the embryo develops, NC cells migrate to different locations, settle, and differentiate, giving rise to a remarkably wide array of cell types (Rothstein et al., 2018; Etchevers et al., 2019; **Figure 1C**). The specific processes underlying NC cell migration and differentiation into melanocytes and other cells types can be found in several recent reviews (Thomas and Erickson, 2008; Kelsh et al., 2009; Mills and Patterson, 2009; Etchevers et al., 2019) as well as the latest advances in deciphering the intricate genetic network regulating each step of the NC development and differentiation (Simões-Costa and Bronner, 2015; Martik and Bronner, 2017; Hockman et al., 2019).

A first question is to understand the type of color variation that any factors affecting the NC cells might end up having in the expression of MbC and to what extent such NC-related variation mirrors the variation in MbC that we often observe and study in wild vertebrate populations. As shown by studies in the zebrafish, *Danio rerio*, variation in NC cells development can be expected to affect whether or not melanocytes are present in a given location of the body, and if present, in what numbers and whether they display a normal morphology (Kelsh et al., 1996). Thus, variation in NC cells development might potentially account for discrete or continuous variation in color patterns among individuals, differences in color intensity, or differences in the size of a color patch. These are all sources of variation in MbC that are often studied in wild populations (Luiselli et al., 1995; Horth, 2003; Bortolotti et al., 2006; Quesada and Senar, 2007; Gratten et al., 2008; Loehr et al., 2008; Kittilsen et al., 2009; Lepetz et al., 2009; Laucht et al., 2010; Roulin et al., 2010; Rosenblum et al., 2010; Mateos-Gonzalez and Senar, 2012; Chen et al., 2013; Kim et al., 2013; Saino et al., 2013; Reiter et al., 2014; Johnson and Fuller, 2015; McGlothlin et al., 2016; Tate et al., 2016; Evans and Gustafsson, 2017; Kappers et al., 2018; Romero-Diaz et al., 2019; San-Jose et al., 2019; Santostefano et al., 2019).

While variation at the level of NC development has the capacity to result in diverse and substantial changes in vertebrate coloration, evidence showing that NC cells underlie intraspecific color variation in wild species is still scarce.

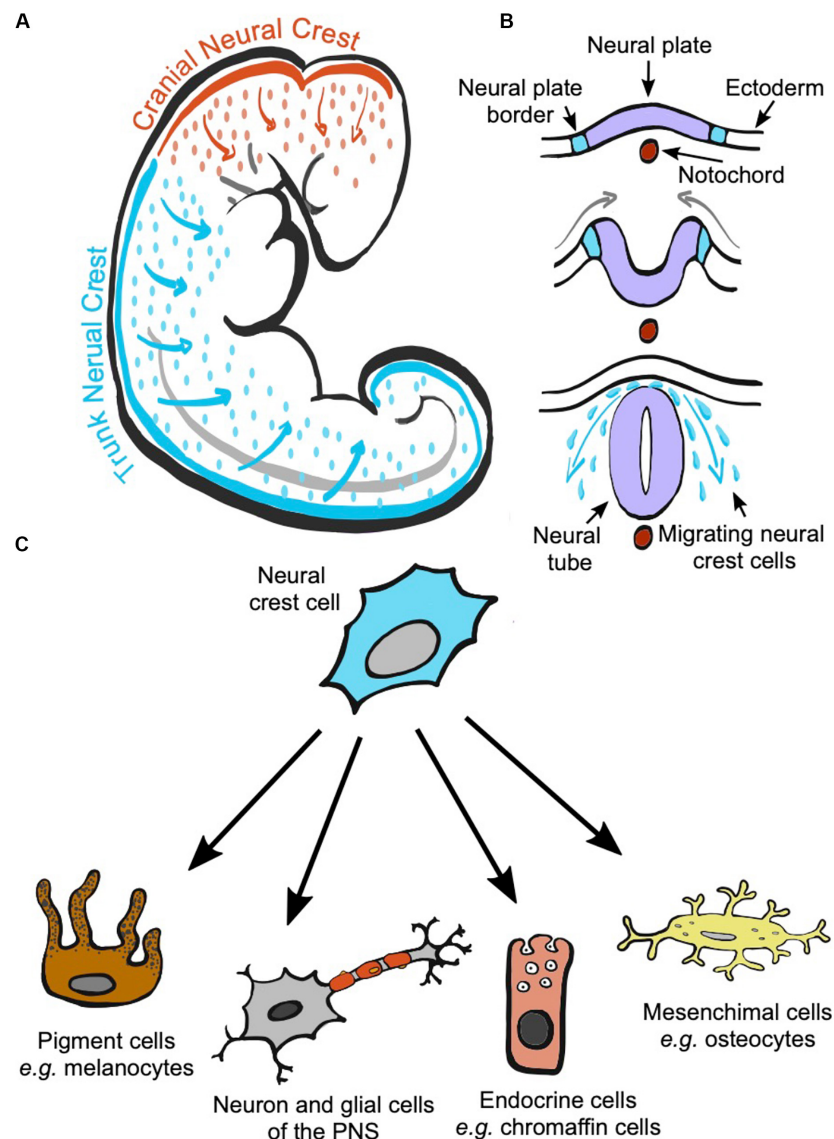


FIGURE 1 | The neural crest is a transitory structure of the vertebrate embryo that initially locates on the most dorsal part of the neural tube. During development, cranial and trunk neural crest cells migrate to different parts of the embryo depending on where they originated (A). The neural crest is formed from the borders of the neural plate. The neural plate folds forming the neural tube, and neural crest cells will delaminate from the neural plate and migrate from the space between the neural tube and the ectoderm to reach different locations in the body (B). During migration and at their arrival sites, neural crest cells commit and differentiate into a wide range of cell types (C).

Variation in NC cells development can have a genetic origin (Kelsh et al., 1996; Rothstein et al., 2018; Hovland et al., 2019), although environmental factors such as temperature, maternal nutritional status and oxidative stress can also affect NC development (Finnell et al., 2004; Običan et al., 2010; Xu et al., 2013; Dreier et al., 2014). To date, genetic studies in cichlid fish species of the lake Malawi offer the best evidence of the involvement of NC cells in producing color variation in wild species, particularly through *cis*-regulatory mutations in *Pax* genes. The expression of *Pax 3* and *Pax 7* genes is required for the formation of the neural crest and, together with other genes in the *Pax* family, they regulate NC cells proliferation,

migration, and specification into different cell types, including melanocytes (Minchin and Hughes, 2008; Monsoro-Burq, 2015; Hovland et al., 2019). In cichlid fishes, differential expression of the *Pax3a* gene would be implicated in continuous variation in the levels of melanophores and xanthophores (Albertson et al., 2014), whereas the upregulation of the gene *Pax7a* would underlie female blotch polymorphism in different cichlid species (Roberts et al., 2009), with different *Pax7a* alleles leading to changes in the number, size, and spread of melanophores (Roberts et al., 2017). Other genes such as *csf1r*, *edn3b*, and *ednrB1a*, which are actively involved in the migration of NC cells (Minchin and Hughes, 2008; Kelsh et al., 2009) have been

suggested to underlie the formation of the characteristic *egg spots* or *egg dummies* present in the anal fins of several cichlid species (Salzburger et al., 2007; Diepeveen and Salzburger, 2011; Santos et al., 2014). The genes *Ednrb1* and *Tfap2a* were found to be upregulated in the dark morph vs. the gold morph of the Midas cichlid, *Amphilophus citrinellus* (Henning et al., 2010).

Other key genes in the development of the NC cells, particularly involved in NC cells commitment and differentiation into melanocytes, have been suggested to underlie color differences between populations or species. In mouse, chicken and zebrafish, the expression of the *melanocyte inducing transcription factor*, *MITF* (*mitfa* in the zebrafish), is required for the production of mature melanocytes from the NC cells (Opdecamp et al., 1997; Goding, 2000; Johnson et al., 2011). In wild populations, *MITF* has been identified as a candidate gene underlying the characteristic color pattern of African wild dogs (*Lycaon pictus*) (Campana et al., 2016), and plumage color variation among populations of pied flycatchers (*Ficedula hypoleuca*) (Lehtonen et al., 2012). *MITF* might be also involved in plumage color differences between carrion and hooded crows (*Corvus corone corone* and *C. c. cornix*) (Poelstra et al., 2015). Reduced expression of the *Kit ligand* gene, which is involved in the survival and migration of melanoblasts (Kelsh et al., 2009), has been linked to reduced melanization of ventral body parts in some populations of threespine stickleback fish (*Gasterosteus aculeatus*).

NON-PIGMENTARY NEURAL CREST DERIVED TISSUES: FUNCTIONS AND LINKS WITH MELANIN-BASED COLORATION

In addition to pigment cells, the NC derivatives include different types of endocrine cells, mesenchymal cells, and neuron and glial cells (Douarin et al., 2008; Monsoro-Burq, 2015; Simões-Costa and Bronner, 2015; Rothstein et al., 2018; Etchevers et al., 2019). Here, we briefly review the main functions of these other cell types, highlighting if such functions also relate to phenotypic aspects observed in association with MbC in wild vertebrate populations.

Endocrine cells deriving from NC cells include adrenal medullary cells, carotid body glomus cells, and calcitonin-secreting cells of the ultimobranchial body (although the NC-cell origin of this later cell type has been questioned recently; Nilsson and Williams, 2016). Adrenal medullary cells, also known as chromaffin cells or pheochromocytes, are responsible for the synthesis and secretion of catecholamines (epinephrine, nor-epinephrine, and dopamine) as part of the sympatho-adrenal system (Kvetnansky et al., 2009). Catecholamines are released in response to stressors, which triggers different physiological and neuronal changes oriented to surmount the stressful conditions (e.g., increased heart rate, blood pressure, cellular metabolism, glucose levels in blood, among others). The association between MbC and stress response has been studied in different vertebrate species where darker individuals have been often found to be

more resistant to stress (reviewed in San-Jose and Roulin, 2018). The association between MbC and stress is mainly considered within the context of the hypothalamus-pituitary-adrenocortical system and thus in relation to the stress hormones released by the cortex and not by the medulla of the adrenal glands (Husak and Moore, 2008; Roulin, 2016). However, the potential implication of catecholamines and the sympatho-adrenal system in explaining why MbC associates to stress response deserves further attention, considering also the potential interactions with the hypothalamus-pituitary-adrenocortical system (e.g., the adrenal medulla may regulate hormone secretion by the adrenal cortex and *vice versa*: Gallo-Payet et al., 1987; Kvetnansky et al., 2009). The fact that catecholamines can induce color changes in vertebrates (Bagnara and Matsumoto, 2007; Zhang et al., 2020) increases the interest of investigating their role in linking coloration and stress response.

Epinephrine and nor-epinephrine levels are also related with behavioral traits such as aggressiveness, boldness, and social dominance (Koolhaas et al., 1999, 2010; Chichinadze, 2004; Vaz-Serrano et al., 2011), which are often associated with MbC (Ducrest et al., 2008; Santos et al., 2011; San-Jose and Roulin, 2018). Dopamine plays also an important role in regulating aggressive behaviors (Pavlov et al., 2012). However, dopamine does not cross the blood-brain barrier and thus the dopamine secreted by adrenal medulla is not expected to influence aggressiveness contrary to the dopamine secreted in brain tissues (Waltes et al., 2016). Peripheral dopamine (i.e., the dopamine secreted by the adrenal medulla among other tissues) is suspected to regulate metabolism, food intake, and body weight (Rubí and Maechler, 2010), which are also aspects that has been found to differ between individuals with distinct MbC.

Calcitonin-secreting cells of the ultimobranchial body produces calcitonin, the parathyroid hormone, which has a central role in regulating calcium and phosphorus levels (Naot et al., 2018). The link between MbC and calcium levels have received some attention in birds, where increased levels of calcium in the diet enhance MbC expression (McGraw, 2003, 2007, although see Stewart and Westneat, 2010) and where melanin-pigmented feathers have been observed to accumulate more calcium (Niecke et al., 1999, 2003). One study in barn owls (*Tyto alba*) also showed that calcium content in bones is higher in individuals presenting more eumelanin spots in their plumage, suggesting that a link between the expression of MbC and calcium physiology may exist (Roulin et al., 2006). A closer look on the association between MbC, calcitonin, and calcitonin-secreting cells may offer new insights into the links between MbC and calcium.

Carotid body glomus cells sense changes in oxygen tension and trigger the activation of the brainstem respiratory center to induce hyperventilation in response to hypoxemia (Lopez-Barneo et al., 2008). In some species, rapid change in skin coloration in response to temperature changes seems to be dependent on oxygen levels (de Velasco and Tattersall, 2008, although see also Rahn, 1956). However, we are not aware of studies directly showing that differences in MbC between individuals relate to differences in dealing with hypoxemia or hypoxia. Yet, we expect MbC and the response to low

oxygen levels to be co-selected in high altitude environments (Leaché et al., 2010; Reguera et al., 2014), where darker MbC are expected to be favored at low temperatures (Delhey, 2017).

NC gives rise to the neurons and the glial cells of the ganglia of the peripheral nervous system (PNS) (Jessen, 2004; Bhatheja and Field, 2006). The autonomous PNS promotes the release of catecholamines by the adrenal medulla in response to stress (the sympatho-adrenal system), whose potential link to MbC has been discussed lines above. The autonomous PNS participate in the stress response also through the sympatho-neural system (Del Giudice et al., 2011). The sympatho-neural system is involved in regulating the immune response (Jänig, 2014), also a phenotypic aspect that has been often associated with differences in MbC (Côte et al., 2018). Another aspect that has been associated often with MbC is sexual behavior, sex steroid concentration, and fecundity (San-Jose and Roulin, 2018). The PNS regulates the production of sex hormones *via* sympathetic innervation of the gonads (Lara et al., 2002; Daneri et al., 2013; Bronzi et al., 2015) while the parasympathetic system has been related to sexual arousal (Giuliano et al., 2002).

Finally, NC crest cells give rise to the mesenchymal cells that further differentiate into many of the numerous distinct cells that form the organs and tissues of the head of vertebrates. Thus, NC cells differentiate into osteocytes and chondrocytes forming the facial skeleton, and the inner ear, odontoblasts producing the dentine of the teeth, and muscle and cartilage cells of the face, among others (Kaucka et al., 2016; Etchevers et al., 2019). To the best of our knowledge, head morphology has been found in association to MbC in at least one study. San-Jose et al. (2017) found that common lizards, *Zootoca vivipara*, with a more melanized ventral coloration had a larger head and a stronger bite force even when controlling for body size differences.

POTENTIAL MECHANISMS OF COVARIATION

In the previous sections, we have reviewed that (i) genetic factors linked to NC development explain color variation in some wild vertebrate species and that (ii) non-pigmentary NC derived tissues participate in functions previously found to be associated with MbC in wild species. We argue that these two general observations justify considering the shared developmental origin of melanocytes and other NC derivatives as a potential linking mechanism underlying the associations of MbC with other phenotypic aspects in wild vertebrates; i.e., an hypothesis to be addressed in future studies.

Genetic or environmental factors affecting the proliferation, migration, and/or initial stages of differentiation of the NC cells may impact both coloration and the functions of other NC-derived tissues in post-embryonic stages. Such mechanism of covariation was pushed forward by Wilkins et al. (2014) as an explanation for the domestication syndrome. A direct example of such a mechanism can be found on recent findings showing how mutations at the gene *ADARI* pleiotropically disrupt the development of both melanocytes and Schwann cells (the major type of glial cell of the PNS: Gacem et al., 2020). The

potential for pleiotropic effects will depend on which steps of the NC development are affected by genetic or environmental factors. For instance, genetic changes as those discussed in relation to *Pax3/7* genes in cichlid fishes have the capacity of affecting multiple phenotypic traits besides MbC. The reason is that *Pax3/7* genes are key for the development of the NC before NC cells start to commit and differentiate into different cell types (Martik and Bronner, 2017). Thus, changes in the functioning of *Pax3/7* genes may have effects that can be *inherited* by different cell types. Contrarily, other genes such as *MITF* that may also associate to color differences in the wild have a lower potential for pleiotropic action. *MITF* regulates final stages of NC cells development: the differentiation of a population of NC cells into melanocytes (Martik and Bronner, 2017).

There are other mechanisms through which MbC and other phenotypic traits can become linked due to the functioning of other NC-derived tissues. A second mechanism of covariation might rely on the fact that multipotent NC stem cells persist in adult peripheral nerves. Such stem cells are able to leave the PNS nerves and differentiate into melanocytes (Singh et al., 2016; Dupin et al., 2018; Mehrotra et al., 2020). Thus, factors, either genetic or environmental, affecting this population of multipotent NC stem cells might have cascading effects on different tissues, linking the expression of MbC with other phenotypic traits. A third mechanism of covariation might be based on a direct regulation of pigmentation by the functioning of some NC-derived tissues. One example of such mechanism comes from the recent discovery of how acute stress triggers hair whitening in mammals (Zhang et al., 2020). In response to acute stress, the PNS releases norepinephrine through the sympathetic nerves that innervate melanocyte stem-cells in the hair bulb. This causes melanocyte stem-cells to rapidly proliferate and differentiate into mature melanocytes, which eventually extenuates the niche of melanocyte stem-cells and causes hair whitening. In reptiles, fishes and amphibians, catecholamines released by the adrenal medulla had been known for long to play an important role in regulating melanophore contraction and MbC (Bagnara and Matsumoto, 2007). As discussed above, this may constitute a direct link between pigmentation, stress response, and perhaps, behavior.

FINAL NOTES

Inspired by the ideas of Wilkins et al. (2014), we highlight here the potential role of the NC cells to drive associations between MbC and other phenotypic aspects in wild vertebrates. Such associations may result from other proposed mechanisms (reviewed in San-Jose and Roulin, 2018), among which, the pleiotropic action of the genes in the melanocortin system has received substantial attention (Ducrest et al., 2008). The genes in the melanocortin system regulate melanin synthesis as well as different aspects of behavior, morphology and physiology. As we review above, some of this aspects have also a developmental origin in the NC: stress response, metabolism, aggressiveness, sexual behavior, sex hormone levels, metabolism,

body weight, and immune response. We think that different mechanisms such as the NC and the melanocortin system do not necessarily constitute competing hypotheses as to how MbC becomes associated with other phenotypic traits. Contrarily, they may even act in a complementary way given that they are expected to associate to the same phenotypic aspects through distinct pathways (e.g., the NC cells associate to the stress response through the sympatho-adrenal and sympatho-neural systems and the melanocortin system through the hypothalamus-pituitary-adrenocortical system). Like the NC, the melanocortin system is specific of the vertebrate lineage, having its origin in early chordates (Cortés et al., 2014). We find remarkable as well as intriguing that both, the NC and the melanocortin systems constitute shared developmental and genetic mechanisms underlying the expression of similar phenotypic aspects of vertebrates. This raises the question of whether the co-expression of such traits: MbC, metabolism, stress response, sexual behavior might have had any selective advantage in the conditions in which vertebrate ancestors evolved. Or, contrarily, whether the fact that MbC and other phenotypic traits shared aspects of their development and regulation may be simply due to *historical contingencies*.

In current vertebrate populations, mechanisms such as the NC and the melanocortin system offer high potentiality for pleiotropic regulation of MbC and several other phenotypic traits. If genetic variation for such pleiotropic regulation exists selection can shape associations between MbC and phenotypic traits (Lande and Arnold, 1983). Acting in a correlational manner, selection on MbC and other traits can promote complex adaptations (Wagner and Altenberg, 1996), for instance, in ecological scenarios such as in low temperature habitats where having a darker pigmentation, a larger body size, a higher metabolism, a higher stress resistance might all be advantageous (San-Jose and Roulin, 2018). Plausible proximate mechanisms of covariation between MbC and other phenotypic traits also increase the likelihood for indirect selection driving trait associations as well as for the existence of developmental and genetic constraints to adaptation (Orr, 2000). By being shared across vertebrates, mechanisms of covariation such as the NC cells and the melanocortin system may help us understand why

and how similar association between MbC and other phenotypic traits can be recurrently found within (e.g., Santos et al., 2011) and across distinct vertebrate taxa (Eliason and Clarke, 2018).

Research on the potential role of the NC cells in linking different phenotypic aspects will thus offer insights into both the proximate and ultimate causes of associations between MbC and distinct phenotypic traits. Taxa such cichlid fish seem good model species to test for correlated changes between MbC and other traits with developmental roots in the NC and to investigate how selection might be jointly acting on MbC and associated traits. Cichlid fish are also a good system to investigate how NC cells and the melanocortin system interact and affect different traits (Dijkstra et al., 2017). The zebra fish, *Danio rerio*, is also a promising model system in controlled laboratory conditions, taking advantage of the different existing color mutants for NC-related genes (Singh and Nüsslein-Volhard, 2015). We expect that research on how the NC origin of MbC might result in associations of coloration with ecologically relevant traits will bring together developmental biology and evolutionary ecology to better understand the evolution of animal coloration.

AUTHOR CONTRIBUTIONS

LS-J and AR conducted the review and synthesis of the literature and wrote the manuscript. Both authors contributed to the article and approved the submitted version.

FUNDING

We acknowledge the Swiss National Science Foundation for funding (ref: 173178 to AR).

ACKNOWLEDGMENTS

We are grateful to Jessica Delhay, Adam S. Wilkins, and two reviewers for their constructive comments on earlier versions of our manuscript.

REFERENCES

- Albertson, R. C., Powder, K. E., Hu, Y., Coyle, K. P., Roberts, R. B., and Parsons, K. J. (2014). Genetic basis of continuous variation in the levels and modular inheritance of pigmentation in cichlid fishes. *Mol. Ecol.* 23, 5135–5150. doi: 10.1111/mec.12900
- Bagnara, J. T., and Matsumoto, J. (2007). “Comparative anatomy and physiology of pigment cells in nonmammalian tissues,” in *The Pigmentary System: Physiology and Pathophysiology*, eds J. J. Nordlund, R. E. Boissy, V. J. Hearing, R. A. King, W. S. Oetting, and J.-P. Ortonne (Oxford: Blackwell Publishing), 11–59. doi: 10.1002/9780470987100.ch2
- Bhatheja, K., and Field, J. (2006). Schwann cells: origins and role in axonal maintenance and regeneration. *Int. J. Biochem. Cell Biol.* 38, 1995–1999. doi: 10.1016/j.biocel.2006.05.007
- Bortolotti, G. R., Blas, J., Negro, J. J., and Tella, J. L. (2006). A complex plumage pattern as an honest social signal. *Anim. Behav.* 72, 423–430. doi: 10.1016/j.anbehav.2006.01.016
- Brenner, M., and Hearing, V. J. (2008). The protective role of melanin against UV damage in human skin. *Photochem. Photobiol.* 84, 539–549. doi: 10.1111/j.1751-1097.2007.00226.x
- Bronzi, C. D., Vega Orozco, A. S., Rodriguez, D., Rastrilla, A. M., Sosa, Z. Y., and Casais, M. (2015). Noradrenaline modulates the presence of gonadotropin-releasing hormone in ovary. The importance of its interrelation on the ovarian steroidogenesis and apoptosis on dioestrus II in rat. *J. Steroid Biochem. Mol. Biol.* 154, 39–46. doi: 10.1016/j.jsbmb.2015.06.005
- Campana, M. G., Parker, L. D., Hawkins, M. T. R., Young, H. S., Helgen, K. M., Szykman Gunther, M., et al. (2016). Genome sequence, population history, and pelage genetics of the endangered African wild dog (*Lycaon pictus*). *BMC Genomics* 17:1013. doi: 10.1186/s12864-016-3368-9
- Chen, I.-P., Symonds, M. R. E., Melville, J., and Stuart-Fox, D. (2013). Factors shaping the evolution of colour patterns in Australian agamid lizards (Agamidae): a comparative study: evolution of Colour Patterns. *Biol. J. Linn. Soc. Lond.* 109, 101–112. doi: 10.1111/bij.12030

- Chichinadze, K. N. (2004). Motor and neurochemical correlates of aggressive behavior in male mice. *Neurophysiology* 36, 262–269. doi: 10.1007/s11062-004-0005-z
- Clusella-Trullas, S., Terblanche, J. S., Blackburn, T. M., and Chown, S. L. (2008). Testing the thermal melanism hypothesis: a macrophysiological approach. *Funct. Ecol.* 22, 232–238. doi: 10.1111/j.1365-2435.2007.01377.x
- Cortés, R., Navarro, S., Agulleiro, M. J., Guillot, R., García-Herranz, V., Sánchez, E., et al. (2014). Evolution of the melanocortin system. *Gen. Comp. Endocrinol.* 209, 3–10. doi: 10.1016/j.ygcen.2014.04.005
- Côte, J., Boniface, A., Blanchet, S., Hendry, A. P., Gasparini, J., and Jacquin, L. (2018). Melanin-based coloration and host–parasite interactions under global change. *Proc. R. Soc. B Biol. Sci.* 285:20180285. doi: 10.1098/rspb.2018.0285
- Daneri, C., Orozco, A. V., Bronzi, D., Mohn, C., Rastrilla, A. M., and Sosa, Z. Y. (2013). Involvement of the ganglion cholinergic receptors in gonadotropin-releasing hormone, catecholamines, and progesterone release in the rat ovary. *Fertil. Steril.* 99, 2062–2070. doi: 10.1016/j.fertnstert.2013.02.037
- de Velasco, J. B., and Tattersall, G. J. (2008). The influence of hypoxia on the thermal sensitivity of skin coloration in the bearded dragon, *Pogona vitticeps*. *J. Comp. Physiol. B* 178, 867–875. doi: 10.1007/s00360-008-0274-8
- Del Giudice, M., Ellis, B. J., and Shirtcliff, E. A. (2011). The adaptive calibration model of stress responsivity. *Neurosci. Biobehav. Rev.* 35, 1562–1592. doi: 10.1016/j.neubiorev.2010.11.007
- Delhey, K. (2017). Gloger's rule. *Curr. Biol.* 27, R689–R691. doi: 10.1016/j.cub.2017.04.031
- Diepeveen, E. T., and Salzburger, W. (2011). Molecular characterization of two endothelin pathways in east african cichlid fishes. *J. Mol. Evol.* 73, 355–368. doi: 10.1007/s00239-012-9483-6
- Dijkstra, P. D., Maguire, S. M., Harris, R. M., Rodriguez, A. A., DeAngelis, R. S., Flores, S. A., et al. (2017). The melanocortin system regulates body pigmentation and social behaviour in a colour polymorphic cichlid fish. *Proc. R. Soc. B Biol. Sci.* 284:20162838. doi: 10.1098/rspb.2016.2838
- Donoghue, P. C. J., Graham, A., and Kelsh, R. N. (2008). The origin and evolution of the neural crest. *Bioessays* 30, 530–541. doi: 10.1002/bies.20767
- Douarin, N. M. L., Calloni, G. W., and Dupin, E. (2008). The stem cells of the neural crest. *Cell Cycle* 7, 1013–1019. doi: 10.4161/cc.7.8.5641
- Dreier, J. W., Andersen, A.-M. N., and Berg-Beckhoff, G. (2014). Systematic review and meta-analyses: fever in pregnancy and health impacts in the offspring. *Pediatrics* 133, e674–e688. doi: 10.1542/peds.2013-3205
- Ducrest, A.-L., Keller, L., and Roulin, A. (2008). Pleiotropy in the melanocortin system, coloration and behavioural syndromes. *Trends Ecol. Evol.* 23, 502–510. doi: 10.1016/j.tree.2008.06.001
- Dupin, E., Calloni, G. W., Coelho-Aguar, J. M., and Le Douarin, N. M. (2018). The issue of the multipotency of the neural crest cells. *Dev. Biol.* 444, S47–S59. doi: 10.1016/j.ydbio.2018.03.024
- Eliason, C. M., and Clarke, J. A. (2018). Metabolic physiology explains macroevolutionary trends in the melanic colour system across amniotes. *Proc. R. Soc. B Biol. Sci.* 285:20182014. doi: 10.1098/rspb.2018.2014
- Etchevers, H. C., Dupin, E., and Douarin, N. M. L. (2019). The diverse neural crest: from embryology to human pathology. *Development* 146:dev169821. doi: 10.1242/dev.169821
- Evans, S. R., and Gustafsson, L. (2017). Climate change upends selection on ornamentation in a wild bird. *Nat. Ecol. Evol.* 1:39. doi: 10.1038/s41559-016-0039
- Finnell, R. H., Shaw, G. M., Lammer, E. J., Brandl, K. L., Carmichael, S. L., and Rosenquist, T. H. (2004). Gene–nutrient interactions: importance of folates and retinoids during early embryogenesis. *Toxicol. Appl. Pharmacol.* 198, 75–85. doi: 10.1016/j.taap.2003.09.031
- Gacem, N., Kavou, A., Zerad, L., Richard, L., Mathis, S., Kapur, R. P., et al. (2020). ADAR1 mediated regulation of neural crest derived melanocytes and Schwann cell development. *Nat. Commun.* 11:198. doi: 10.1038/s41467-019-14090-5
- Gallo-Payet, N., Pothier, P., and Isler, H. (1987). On the presence of chromaffin cells in the adrenal cortex: their possible role in adrenocortical function. *Biochem. Cell Biol.* 65, 588–592. doi: 10.1139/o87-076
- Giuliano, F., Rampin, O., and Allard, J. (2002). Neurophysiology and pharmacology of female genital sexual response. *J. Sex Marital Ther.* 28, 101–121. doi: 10.1080/00926230252851230
- Goding, C. R. (2000). Mitf from neural crest to melanoma: signal transduction and transcription in the melanocyte lineage. *Genes Dev.* 14, 1712–1728. doi: 10.1101/gad.14.14.1712
- Gratten, J., Wilson, A. J., McRae, A. F., Beraldi, D., Visscher, P. M., Pemberton, J. M., et al. (2008). A localized negative genetic correlation constrains microevolution of coat color in wild sheep. *Science* 319, 318–320. doi: 10.1126/science.1151182
- Henning, F., Renz, A. J., Fukamachi, S., and Meyer, A. (2010). Genetic, comparative genomic, and expression analyses of the *Mclr* locus in the polychromatic midas cichlid fish (Teleostei, Cichlidae *Amphilophus* sp.) Species Group. *J. Mol. Evol.* 70, 405–412. doi: 10.1007/s00239-010-9340-4
- Hockman, D., Chong-Morrison, V., Green, S. A., Gavriouchkina, D., Candido-Ferreira, I., Ling, I. T. C., et al. (2019). A genome-wide assessment of the ancestral neural crest gene regulatory network. *Nat. Commun.* 10:4689. doi: 10.1038/s41467-019-12687-4
- Horth, L. (2003). Melanic body colour and aggressive mating behaviour are correlated traits in male mosquitofish (*Gambusia holbrooki*). *Proc. Biol. Sci. R. Soc.* 270, 1033–1040. doi: 10.1098/rspb.2003.2348
- Hovland, A. S., Rothstein, M., and Simoes-Costa, M. (2019). Network architecture and regulatory logic in neural crest development. *Wiley Interdiscip. Rev. Syst. Biol. Med.* 12:e1468. doi: 10.1002/wsbm.1468
- Husak, J. F., and Moore, I. T. (2008). Stress hormones and mate choice. *Trends Ecol. Evol.* 23, 532–534. doi: 10.1016/j.tree.2008.06.007
- Jänig, W. (2014). Sympathetic nervous system and inflammation: a conceptual view. *Auton. Neurosci.* 182, 4–14. doi: 10.1016/j.autneu.2014.01.004
- Jessen, K. R. (2004). Glial cells. *Int. J. Biochem. Cell Biol.* 36, 1861–1867. doi: 10.1016/j.biocel.2004.02.023
- Johnson, A. M., and Fuller, R. C. (2015). The meaning of melanin, carotenoid, and pterin pigments in the bluefin killifish, *Lucania goodei*. *Behav. Ecol.* 26, 158–167. doi: 10.1093/beheco/aru164
- Johnson, S. L., Nguyen, A. N., and Lister, J. A. (2011). mitfa is required at multiple stages of melanocyte differentiation but not to establish the melanocyte stem cell. *Dev. Biol.* 350, 405–413. doi: 10.1016/j.ydbio.2010.12.004
- Kappers, E. F., de Vries, C., Alberda, A., Forstmeier, W., Both, C., and Kempenaers, B. (2018). Inheritance patterns of plumage coloration in common buzzards *Buteo buteo* do not support a one-locus two-allele model. *Biol. Lett.* 14:20180007. doi: 10.1098/rsbl.2018.0007
- Kauka, M., Ivashkin, E., Gyllborg, D., Zikmund, T., Tesarova, M., Kaiser, J., et al. (2016). Analysis of neural crest-derived clones reveals novel aspects of facial development. *Sci. Adv.* 2:e1600060. doi: 10.1126/sciadv.1600060
- Kelsh, R. N., Brand, M., Jiang, Y. J., Heisenberg, C. P., Lin, S., Haffter, P., et al. (1996). Zebrafish pigmentation mutations and the processes of neural crest development. *Development* 123, 369–389.
- Kelsh, R. N., Harris, M. L., Colanesi, S., and Erickson, C. A. (2009). Stripes and belly-spots – a review of pigment cell morphogenesis in vertebrates. *Semin. Cell Dev. Biol.* 20, 90–104. doi: 10.1016/j.semcdb.2008.10.001
- Kim, S.-Y., Fargallo, J. A., Vergara, P., and Martinez-Padilla, J. (2013). Multivariate heredity of melanin-based coloration, body mass and immunity. *Heredity* 111, 139–146. doi: 10.1038/hdy.2013.29
- Kittilsen, S., Schjolden, J., Beitnes-Johansen, I., Shaw, J. C., Pottinger, T. G., Sørensen, C., et al. (2009). Melanin-based skin spots reflect stress responsiveness in salmonid fish. *Horm. Behav.* 56, 292–298. doi: 10.1016/j.yhbeh.2009.06.006
- Koolhaas, J. M., de Boer, S. F., Coppens, C. M., and Buwalda, B. (2010). Neuroendocrinology of coping styles: towards understanding the biology of individual variation. *Front. Neuroendocrinol.* 31:307–321. doi: 10.1016/j.yfrne.2010.04.001
- Koolhaas, J. M., Korte, S. M., De Boer, S. F., Van Der Vegt, B. J., Van Reenen, C. G., Hopster, H., et al. (1999). Coping styles in animals: current status in behavior and stress-physiology. *Neurosci. Biobehav. Rev.* 23, 925–935. doi: 10.1016/S0149-7634(99)00026-3
- Kvetnansky, R., Sabban, E. L., and Palkovits, M. (2009). Catecholaminergic systems in stress: structural and molecular genetic approaches. *Physiol. Rev.* 89, 535–606. doi: 10.1152/physrev.00042.2006
- Lande, R., and Arnold, S. J. (1983). The measurement of selection on correlated characters. *Evolution* 37, 1210–1226. doi: 10.1111/j.1558-5646.1983.tb00236.x
- Lara, H. E., Dorfman, M., Venegas, M., Luza, S. M., Luna, S. L., Mayerhofer, A., et al. (2002). Changes in sympathetic nerve activity of the mammalian ovary

- during a normal estrous cycle and in polycystic ovary syndrome: studies on norepinephrine release. *Microsc. Res. Tech.* 59, 495–502. doi: 10.1002/jemt.10229
- Laucht, S., Kempenaers, B., and Dale, J. (2010). Bill color, not badge size, indicates testosterone-related information in house sparrows. *Behav. Ecol. Sociobiol.* 64, 1461–1471. doi: 10.1007/s00265-010-0961-9
- Leaché, A. D., Helmer, D.-S., and Moritz, C. (2010). Phenotypic evolution in high-elevation populations of western fence lizards (*Sceloporus occidentalis*) in the Sierra Nevada Mountains. *Biol. J. Linn. Soc.* 100, 630–641. doi: 10.1111/j.1095-8312.2010.01462.x
- Lehtonen, P. K., Laaksonen, T., Artemyev, A. V., Belskii, E., Berg, P. R., Both, C., et al. (2012). Candidate genes for colour and vision exhibit signals of selection across the pied flycatcher (*Ficedula hypoleuca*) breeding range. *Heredity* 108, 431–440. doi: 10.1038/hdy.2011.93
- Lepetz, V., Massot, M., Chaine, A. S., and Clobert, J. (2009). Climate warming and the evolution of morphotypes in a reptile. *Glob. Change Biol.* 15, 454–466. doi: 10.1080/00031305.1989.10475662
- Loehr, J., Carey, J., Ylönen, H., and Suhonen, J. (2008). Coat darkness is associated with social dominance and mating behaviour in a mountain sheep hybrid lineage. *Anim. Behav.* 76, 1545–1553. doi: 10.1016/j.anbehav.2008.07.012
- Lopez-Barneo, J., Ortega-Saenz, P., Pardal, R., Pascual, A., and Piruat, J. I. (2008). Carotid body oxygen sensing. *Eur. Respir. J.* 32, 1386–1398. doi: 10.1183/09031936.00056408
- Luiselli, L., Monney, J.-C., and Capula, M. (1995). Correlates of melanism in a population of adders (*Vipera berus*) from the Swiss Alps and comparisons with other alpine populations. *Amphib. Reptil.* 16, 323–330. doi: 10.1163/156853895X00406
- Maffi, A., Wakamatsu, K., and Roulin, A. (2011). Melanin-based coloration predicts aggressiveness and boldness in captive eastern Hermann's tortoises. *Anim. Behav.* 81, 859–863. doi: 10.1016/j.anbehav.2011.01.025
- Martik, M. L., and Bronner, M. E. (2017). Regulatory logic underlying diversification of the neural crest. *Trends Genet.* 33, 715–727. doi: 10.1016/j.tig.2017.07.015
- Mateos-Gonzalez, F., and Senar, J. C. (2012). Melanin-based trait predicts individual exploratory behaviour in siskins, *Carduelis spinus*. *Anim. Behav.* 83, 229–232. doi: 10.1016/j.anbehav.2011.10.030
- Mauro, A. A., and Ghalambor, C. K. (2020). Trade-offs, pleiotropy, and shared molecular pathways: a unified view of constraints on adaptation. *Integr. Comp. Biol.* doi: 10.1093/icb/icaa056
- McGlothlin, J. W., Parker, P. G., Nolan, V., and Ketterson, E. D. (2016). Correlational selection leads to genetic integration of body size and an attractive plumage trait in dark-eyed juncos. *Evolution* 59, 658–671. doi: 10.1111/j.0014-3820.2005.tb01024.x
- McGraw, K. (2003). Melanins, metals, and mate quality. *Oikos* 102, 402–406. doi: 10.1034/j.1600-0579.2003.12513.x
- McGraw, K. J. (2007). Dietary mineral content influences the expression of melanin-based ornamental coloration. *Behav. Ecol.* 18, 137–142. doi: 10.1093/beheco/arl059
- Mehrotra, P., Tseropoulos, G., Bronner, M. E., and Andreadis, S. T. (2020). Adult tissue-derived neural crest-like stem cells: sources, regulatory networks, and translational potential. *Stem Cells Transl. Med.* 9, 328–341. doi: 10.1002/sctm.19-0173
- Melo, D., Porto, A., Cheverud, J. M., and Marroig, G. (2016). Modularity: genes, development, and evolution. *Annu. Rev. Ecol. Evol. Syst.* 47, 463–486. doi: 10.1146/annurev-ecolsys-121415-032409
- Mills, M. G., and Patterson, L. B. (2009). Not just black and white: pigment pattern development and evolution in vertebrates. *Semin. Cell Dev. Biol.* 20, 72–81. doi: 10.1016/j.semdb.2008.11.012
- Minchin, J. E. N., and Hughes, S. M. (2008). Sequential actions of Pax3 and Pax7 drive xanthophore development in zebrafish neural crest. *Dev. Biol.* 317, 508–522. doi: 10.1016/j.ydbio.2008.02.058
- Monsoro-Burq, A. H. (2015). PAX transcription factors in neural crest development. *Semin. Cell Dev. Biol.* 44, 87–96. doi: 10.1016/j.semdb.2015.09.015
- Naot, D., Musson, D. S., and Cornish, J. (2018). The activity of peptides of the calcitonin family in bone. *Physiol. Rev.* 99, 781–805. doi: 10.1152/physrev.00066.2017
- Niecke, M., Heid, M., and Krüger, A. (1999). Correlations between melanin pigmentation and element concentration in feathers of White-tailed Eagles (*Haliaeetus albicilla*). *J. Ornithol.* 140, 355–362. doi: 10.1007/BF01651032
- Niecke, M., Rothlaender, S., and Roulin, A. (2003). Why do melanin ornaments signal individual quality? Insights from metal element analysis of barn owl feathers. *Oecologia* 137, 153–158. doi: 10.1007/s00442-003-1307-3
- Nilsson, M., and Williams, D. (2016). On the origin of cells and derivation of thyroid cancer: C cell story revisited. *ETJ* 5, 79–93. doi: 10.1159/000447333
- Običan, S. G., Finnell, R. H., Mills, J. L., Shaw, G. M., and Scialli, A. R. (2010). Folic acid in early pregnancy: a public health success story. *FASEB J.* 24, 4167–4174. doi: 10.1096/fj.10-165084
- Opdecamp, K., Nakayama, A., Nguyen, M. T., Hodgkinson, C. A., Pavan, W. J., and Arnheiter, H. (1997). Melanocyte development in vivo and in neural crest cell cultures: crucial dependence on the Mitf basic-helix-loop-helix-zipper transcription factor. *Development* 124, 2377–2386.
- Orr, H. A. (2000). Adaptation and the cost of complexity. *Evolution* 54, 13–20. doi: 10.1111/j.0014-3820.2000.tb00002.x
- Pavlov, K. A., Chistiakov, D. A., and Chekhonin, V. P. (2012). Genetic determinants of aggression and impulsivity in humans. *J. Appl. Genet.* 53, 61–82. doi: 10.1007/s13353-011-0069-6
- Plasman, M., Reynoso, V. H., Nicolás, L., and Torres, R. (2015). Multiple colour traits signal performance and immune response in the Dickerson's collared lizard *Crotaphytus dickersonae*. *Behav. Ecol. Sociobiol.* 765–775. doi: 10.1007/s00265-015-1892-2
- Poelstra, J. W., Vijay, N., Hoepfner, M. P., and Wolf, J. B. W. (2015). Transcriptomics of colour patterning and coloration shifts in crows. *Mol. Ecol.* 24, 4617–4628. doi: 10.1111/mec.13353
- Quesada, J., and Senar, J. C. (2007). The role of melanin- and carotenoid-based plumage coloration in nest defence in the Great Tit. *Ethology* 113, 640–647. doi: 10.1111/j.1439-0310.2007.01364.x
- Rahn, H. (1956). The relationship between hypoxia, temperature, adrenalin release and melanophore expansion in the lizard, *Anolis carolinensis*. *Copeia* 1956, 214–217. doi: 10.2307/1440273
- Reguera, S., Zamora-Camacho, F. J., and Moreno-Rueda, G. (2014). The lizard *Psammotromus algeris* (Squamata: Lacertidae) is darker at high altitudes. *Biol. J. Linn. Soc.* 112, 132–141. doi: 10.1111/bj.12250
- Reiter, M., Anthony, C., and Hickerson, C.-A. M. (2014). Territorial behavior and ecological divergence in a polymorphic salamander. *Copeia* 2014, 481–488. doi: 10.1643/ce-13-154
- Roberts, R. B., Moore, E. C., and Kocher, T. D. (2017). An allelic series at pax7a is associated with colour polymorphism diversity in Lake Malawi cichlid fish. *Mol. Ecol.* 26, 2625–2639. doi: 10.1111/mec.13975
- Roberts, R. B., Ser, J. R., and Kocher, T. D. (2009). Sexual conflict resolved by invasion of a novel sex determiner in Lake Malawi cichlid fishes. *Science* 326, 998–1001. doi: 10.1126/science.1174705
- Romero-Diaz, C., Rivera, J. A., Ossip-Draho, A. G., Zúñiga-Vega, J. J., Vital-García, C., Hews, D. K., et al. (2019). Losing the trait without losing the signal: evolutionary shifts in communicative colour signalling. *J. Evol. Biol.* 32, 320–330. doi: 10.1111/jeb.13416
- Rosenblum, E. B., Römler, H., Schöneberg, T., and Hoekstra, H. E. (2010). Molecular and functional basis of phenotypic convergence in white lizards at White Sands. *Proc. Natl. Acad. Sci. U.S.A.* 107, 2113–2117. doi: 10.1073/pnas.0911042107
- Rothstein, M., Bhattacharya, D., and Simoes-Costa, M. (2018). The molecular basis of neural crest axial identity. *Dev. Biol.* 444, S170–S180. doi: 10.1016/j.ydbio.2018.07.026
- Roulin, A. (2016). Condition-dependence, pleiotropy and the handicap principle of sexual selection in melanin-based colouration. *Biol. Rev.* 91, 328–348. doi: 10.1111/brev.12171
- Roulin, A., Altwegg, R., Jensen, H., Steinsland, I., and Schaub, M. (2010). Sex-dependent selection on an autosomal melanin female ornament promotes the evolution of sex ratio bias. *Ecol. Lett.* 13, 616–626. doi: 10.1111/j.1461-0248.2010.01459.x
- Roulin, A., Dauwe, T., Blust, R., Eens, M., and Beaud, M. (2006). A link between eumelanism and calcium physiology in the barn owl. *Naturwissenschaften* 93, 426–430. doi: 10.1007/s00114-006-0128-8

- Roulin, A., and Ducrest, A.-L. (2011). Association between melanism, physiology and behaviour: a role for the melanocortin system. *Eur. J. Pharmacol.* 660, 226–233. doi: 10.1016/j.ejphar.2011.01.036
- Roy, A., Pittman, M., Saitta, E. T., Kaye, T. G., and Xu, X. (2020). Recent advances in amniote palaeocolour reconstruction and a framework for future research. *Biol. Rev.* 95, 22–50. doi: 10.1111/brv.12552
- Rubí, B., and Maechler, P. (2010). Minireview: new roles for peripheral dopamine on metabolic control and tumor growth: let's seek the balance. *Endocrinology* 151, 5570–5581. doi: 10.1210/en.2010-0745
- Saino, N., Canova, L., Costanzo, A., Rubolini, D., Roulin, A., and Möller, A. P. (2013). Immune and stress responses Covary with melanin-based coloration in the barn swallow. *Evol. Biol.* 40, 521–531. doi: 10.1007/s11692-013-9228-5
- Saltz, J. B., Hessel, F. C., and Kelly, M. W. (2017). Trait correlations in the genomics era. *Trends Ecol. Evol.* 32, 279–290. doi: 10.1016/j.tree.2016.12.008
- Salzburger, W., Braasch, I., and Meyer, A. (2007). Adaptive sequence evolution in a color gene involved in the formation of the characteristic egg-dummies of male haplochromine cichlid fishes. *BMC Biol.* 5:51. doi: 10.1186/1741-7007-5-51
- Sánchez-Villagra, M. R., Geiger, M., and Schneider, R. A. (2015). The taming of the neural crest: a developmental perspective on the origins of morphological covariation in domesticated mammals. *R. Soc. Open Sci.* 3:160107. doi: 10.1098/rsos.160107
- San-Jose, L. M., Huyghe, K., Schuerch, J., and Fitze, P. S. (2017). More melanized males bite stronger but run slower: potential performance trade-offs related to melanin-based coloration. *Biol. J. Linn. Soc.* 122, 184–196. doi: 10.1093/biolinnean/blx045
- San-Jose, L. M., and Roulin, A. (2018). Toward understanding the repeated occurrence of associations between melanin-based coloration and multiple phenotypes. *Am. Nat.* 192, 111–130. doi: 10.1086/698010
- San-Jose, L. M., Séchaud, R., Schalcher, K., Judes, C., Questiaux, A., Oliveira-Xavier, A., et al. (2019). Differential fitness effects of moonlight on plumage colour morphs in barn owls. *Nat. Ecol. Evol.* 3, 1331–1340. doi: 10.1038/s41559-019-09672
- Santos, E. S. A., Scheck, D., and Nakagawa, S. (2011). Dominance and plumage traits: meta-analysis and metaregression analysis. *Anim. Behav.* 82, 3–19. doi: 10.1016/j.anbehav.2011.03.022
- Santos, M. E., Braasch, I., Boileau, N., Meyer, B. S., Sauter, L., Böhne, A., et al. (2014). The evolution of cichlid fish egg-spots is linked with a cis-regulatory change. *Nat. Commun.* 5:5149. doi: 10.1038/ncomms6149
- Santostefano, F., Fanson, K. V., Endler, J. A., and Biro, P. A. (2019). Behavioral, energetic, and color trait integration in male guppies: testing the melanocortin hypothesis. *Behav. Ecol.* 30, 1539–1547. doi: 10.1093/beheco/arz109
- Schartl, M., Larue, L., Goda, M., Bosenberg, M. W., Hashimoto, H., and Kelsh, R. N. (2016). What is a vertebrate pigment cell? *Pigment Cell Melanoma Res.* 29, 8–14. doi: 10.1111/pcmr.12409
- Schweitzer, C., Motreuil, S., and Dechaume-Moncharmont, F.-X. (2015). Coloration reflects behavioural types in the convict cichlid, *Amatitlania siquia*. *Anim. Behav.* 105, 201–209. doi: 10.1016/j.anbehav.2015.04.024
- Shimeld, S. M., and Holland, P. W. H. (2000). Vertebrate innovations. *Proc. Natl. Acad. Sci. U.S.A.* 97, 4449–4452. doi: 10.1073/pnas.97.9.4449
- Simões-Costa, M., and Bronner, M. E. (2015). Establishing neural crest identity: a gene regulatory recipe. *Development* 142, 242–257. doi: 10.1242/dev.105445
- Singaravelan, N., Grishkan, I., Beharav, A., Wakamatsu, K., Ito, S., and Nevo, E. (2008). Adaptive melanin response of the soil fungus *Aspergillus niger* to UV radiation stress at “Evolution Canyon”, Mount Carmel, Israel. *PLoS One* 3:e2993. doi: 10.1371/journal.pone.0002993
- Singh, A. P., Dinwiddie, A., Mahalwar, P., Schach, U., Linker, C., Irion, U., et al. (2016). Pigment cell progenitors in Zebrafish Remain multipotent through metamorphosis. *Dev. Cell* 38, 316–330. doi: 10.1016/j.devcel.2016.06.020
- Singh, A. P., and Nüsslein-Volhard, C. (2015). Zebrafish stripes as a model for vertebrate colour pattern formation. *Curr. Biol.* 25, R81–R92. doi: 10.1016/j.cub.2014.11.013
- Smith, J. M., Burian, R., Kauffman, S., Alberch, P., Campbell, J., Goodwin, B., et al. (1985). Developmental constraints and evolution: a perspective from the mountain lake conference on development and evolution. *Q. Rev. Biol.* 60, 265–287. doi: 10.1086/414425
- Stewart, I. R. K., and Westneat, D. F. (2010). Dietary calcium negatively affects the size of a status signal in juvenile male house sparrows (*Passer domesticus*) El Calcio de la Dieta Afecta Negativamente el Tamaño de una Señal de Estatus en los Machos Jóvenes de *Passer domesticus*. *Auk* 127, 549–557. doi: 10.1525/auk.2010.10048
- Stoddard, M. C., and Prum, R. O. (2011). How colorful are birds? Evolution of the avian plumage color gamut. *Behav. Ecol.* 22, 1042–1052. doi: 10.1093/beheco/arr088
- Tate, G. J., Bishop, J. M., and Amar, A. (2016). Differential foraging success across a light level spectrum explains the maintenance and spatial structure of colour morphs in a polymorphic bird. *Ecol. Lett.* 19, 679–686. doi: 10.1111/ele.12606
- Thomas, A. J., and Erickson, C. A. (2008). The making of a melanocyte: the specification of melanoblasts from the neural crest. *Pigment Cell Melanoma Res.* 21, 598–610. doi: 10.1111/j.1755-148X.2008.00506.x
- Vaz-Serrano, J., Ruiz-Gomez, M. L., Gjøen, H. M., Skov, P. V., Huntingford, F. A., Øverli, Ø., et al. (2011). Consistent boldness behaviour in early emerging fry of domesticated Atlantic salmon (*Salmo salar*): decoupling of behavioural and physiological traits of the proactive stress coping style. *Physiol. Behav.* 103, 359–364. doi: 10.1016/j.physbeh.2011.02.025
- Wagner, G. P., and Altenberg, L. (1996). Complex adaptations and the evolution of evolvability. *Evolution* 50, 967–976. doi: 10.2307/2410639
- Wagner, G. P., Pavlicev, M., and Cheverud, J. M. (2007). The road to modularity. *Nat. Rev. Genet.* 8, 921–931. doi: 10.1038/nrg2267
- Waltes, R., Chiocchetti, A. G., and Freitag, C. M. (2016). The neurobiological basis of human aggression: a review on genetic and epigenetic mechanisms. *Am. J. Med. Genet. Part B Neuropsychiatr. Genet.* 171, 650–675. doi: 10.1002/ajmg.b.32388
- West-Eberhard, M. J. (2005). Developmental plasticity and the origin of species differences. *Proc. Natl. Acad. Sci. U.S.A.* 102, 6543–6549. doi: 10.1073/pnas.0501844102
- Wilkins, A. S. (2019). A striking example of developmental bias in an evolutionary process: the “domestication syndrome”. *Evol. Dev.* 22, 143–153. doi: 10.1111/ede.12319
- Wilkins, A. S., Wrangham, R. W., and Tecumseh Fitch, W. (2014). The “domestication syndrome” in mammals: a unified explanation based on neural crest cell behavior and genetics. *Genetics* 197, 795–808. doi: 10.1534/genetics.114.165423
- Xu, C., Li, X., Wang, F., Weng, H., and Yang, P. (2013). Trehalose prevents neural tube defects by correcting maternal diabetes-suppressed autophagy and neurogenesis. *Am. J. Physiol. Endocrinol. Metab.* 305, E667–E678. doi: 10.1152/ajpendo.00185.2013
- Zhang, B., Ma, S., Rachmin, I., He, M., Baral, P., Choi, S., et al. (2020). Hyperactivation of sympathetic nerves drives depletion of melanocyte stem cells. *Nature* 577, 676–681. doi: 10.1038/s41586-020-1935-3

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2020 San-Jose and Roulin. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.



Characterization of the Leucistic Texas Rat Snake *Pantherophis obsoletus*

Asier Ullate-Agote^{1,2,3} and Athanasia C. Tzika^{1,2,3*}

¹ Laboratory of Artificial and Natural Evolution (LANE), Department of Genetics and Evolution, University of Geneva, Geneva, Switzerland, ² SIB Swiss Institute of Bioinformatics, Lausanne, Switzerland, ³ Institute of Genetics and Genomics of Geneva (IGE3), University of Geneva, Geneva, Switzerland

OPEN ACCESS

Edited by:

Marcus Kronforst,
University of Chicago, United States

Reviewed by:

Florian Raible,
University of Vienna, Austria
Ajeet Pratap Singh,
Novartis Institutes for BioMedical
Research, United States

*Correspondence:

Athanasia C. Tzika
athanasia.tzika@unige.ch

Specialty section:

This article was submitted to
Evolutionary Developmental Biology,
a section of the journal
Frontiers in Ecology and Evolution

Received: 14 July 2020

Accepted: 15 January 2021

Published: 09 February 2021

Citation:

Ullate-Agote A and Tzika AC (2021)
Characterization of the Leucistic Texas
Rat Snake *Pantherophis obsoletus*.
Front. Ecol. Evol. 9:583136.
doi: 10.3389/fevo.2021.583136

Albinism and leucism are phenotypes resulting from impaired melanin pigmentation in the skin and skin appendages. However, melanin pigmentation of eyes remains unaffected in leucism. Here, using transmission electron microscopy, we show that the leucistic morph of the Texas rat snake (*Pantherophis obsoletus lindheimeri*) lacks both melanophores and xanthophores in its skin and exhibits a uniform ivory white color generated by iridophores and collagen fibers. In addition, we sequenced the full genome of a leucistic individual and obtained a highly-contiguous near-chromosome quality assembly of 1.69 Gb with an N50 of 14.5 Mb and an L50 of 29 sequences. Using a candidate-gene approach, we then identify in the leucistic genome a single-nucleotide deletion that generates a frameshift and a premature termination codon in the melanocyte inducing transcription factor (*MITF*) gene. This mutation shortens the translated protein from 574 to 286 amino acids, removing the helix-loop-helix DNA-binding domain that is highly conserved among vertebrates. Genotyping leucistic animals of independent lineages showed that not all leucistic individuals carry this single-nucleotide deletion. Subsequent gene expression analyses reveal that all leucistic individuals that we analyzed exhibit a significantly decreased expression of *MITF*. We thus suggest that mutations affecting the regulation and, in some cases, the coding sequence of *MITF*, the former probably predating the latter, could be associated with the leucistic phenotype in Texas rat snakes. *MITF* is involved in the development and survival of melanophores in vertebrates. In zebrafish, a classical model species for pigmentation that undergoes metamorphosis, larvae and adults of homozygous *mitfa* mutants lack melanophores, show an excess of iridophores and exhibit reduced yellow pigmentation. On the contrary, in the leucistic Texas rat snake, a non-metamorphic species, only iridophores persist. Our results suggest that fate determination of neural-crest derived melanophores and xanthophores, but not of iridophores, could require the expression of *MITF* during snake embryonic development.

Keywords: *MITF*, leucism, chromatophores, reptiles, melanophores, xanthophores, genome, pigmentation

INTRODUCTION

Vertebrates display a wide variety of adaptive color patterns on their skin and skin appendages. Coloration is involved in the thermoregulation of ectotherms, ultraviolet radiation protection and in intra- and inter-specific interactions, including sexual selection and mating, camouflage for predator evasion and as a warning of dangerous traits. Melanophores are found in all vertebrate lineages and they are the only chromatophores in birds and mammals. Melanophores synthesize melanin from tyrosine and store the resulting black/brown pigment in melanosomes, a type of lysosomal-related organelles (LROs) (Wasmeier et al., 2008). Albinism and leucism, two distinct phenotypes, result from impaired melanin pigmentation. Albinism is characterized by the absence (amelanism) or reduction (hypomelanism) of melanin pigment in the skin, skin appendages and eyes. Mutations causing this condition can affect either genes involved in melanin production *per se* or the biogenesis and maturation of melanosomes. Known variants in genes such as tyrosinase (*TYR*), oculocutaneous albinism II (*OCA2*), solute carrier family 45 member 2 (*SLC45A2*), and tyrosinase related protein 1 (*TYRP1*) are known to affect melanin production and cause oculocutaneous albinism in humans (Montoliu et al., 2014). On the other hand, mutations affecting melanosome biogenesis/maturation can impact on other LROs, hence, affecting different cell types and impacting other organs and functions. For example, in humans, mutations in subunits of the lysosomal-trafficking protein complexes cause the Hermansky-Pudlak syndrome (Wei and Li, 2013) and a non-functional lysosomal trafficking regulator (*LYST*) results in the Chédiak-Higashi syndrome (Barbosa et al., 1996). In both syndromes, decreased or absent pigmentation is associated with other symptoms, such as platelet abnormality and peripheral neuropathy. Recently, we have shown that the Lavender phenotype in corn snakes is likely caused by a mutation in *LYST* and impacts the formation of (i) pterinosomes in xanthophores, (ii) reflecting platelets of guanine crystals in iridophores, and (iii) melanosomes, resulting in lighter overall coloration without affecting the geometrical elements of the color (Ullate-Agote et al., 2020). Spontaneously-occurring albino phenotypes are widespread among vertebrates, from fish to snakes and mammals (e.g., Chang et al., 2006; Anistoroaei et al., 2013; Ullate-Agote et al., 2014; Saenko et al., 2015; Hart and Miller, 2017; Nakayama et al., 2017; Woodcock et al., 2017; Iwanishi et al., 2018).

In leucistic mammals, the eye pigmentation is preserved, while skin coloration is absent or reduced, resulting in a fully white or piebald phenotype (Reissmann and Ludwig, 2013). Apart from the retinal pigment epithelium (RPE), a one-cell layer derived from the optic neuroepithelium (Fuhrmann et al., 2014), all chromatophores originate from the neural crest. Note that the iris coloration can be affected in leucism because, while the back layer of the iris is an extension of the RPE and thus maintains its pigmentation, the front of the iris is populated by neural crest-derived chromatophores which are impacted in leucistic individuals (Bharti et al., 2006). Therefore, leucism is caused by mutations in genes involved in the migration, differentiation or survival of the neural crest-derived chromatophores during

development, rather than melanin production. Examples of such genes are the melanocyte inducing transcription factor (*MITF*), paired box 3 (*PAX3*), Sry-Box transcription factor 10 (*SOX10*), endothelin 3 (*EDN3*), and KIT proto-oncogene receptor tyrosine kinase (*KIT*). Mutations in all but *KIT* cause the Waardenburg syndrome in humans, in which pigmentation abnormalities are associated with hearing loss (Pingault et al., 2010).

The plethora of color skin patterns in squamates, i.e., snakes and lizards, originates from different combinations of chromatophore cells (Kuriyama et al., 2006, 2013; Saenko et al., 2013; Teyssier et al., 2015; Manukyan et al., 2017; Andrade et al., 2019; Ullate-Agote et al., 2020). In addition to melanophores, squamates exhibit xanthophores and erythrophores, jointly referred to as xanthophores. These are pigment-containing cells producing yellow and red coloration, respectively. The pigments are either pteridines, synthesized from purines and stored in pterinosomes (Ziegler, 2003), or carotenoids absorbed from the diet and stored in carotenoid vesicles. Color differences may also arise from variations in the pH of the organelle or redox state of the pigments within (Saenko et al., 2013). The third chromatophore type is the iridophore, a cell type that contains stacks of crystallized guanine forming reflective platelets, and which produce structural colors through light interference.

Albino snakes lack melanin in their skin and eyes, while preserving their yellow and red coloration and overall pattern (Ullate-Agote et al., 2014; Saenko et al., 2015; Iwanishi et al., 2018). However, it has been suggested that leucistic snakes are missing both xanthophores and melanophores, resulting in a uniform ivory white coloration generated by iridophores (Bechtel, 1991). On the other hand, leucistic axolotl (*Ambystoma mexicanum*) individuals, named “white,” have defects in the morphogenesis of both melanophores and xanthophores, whereas iridophores are absent (Woodcock et al., 2017). The endothelin 3 (*edn3*) transcripts, encoding a peptide that functions as a ligand for the Endothelin receptor type B, consistently lack exon 2 in “white” axolotl. The interaction of these two proteins is essential for the migration of the neural crest-derived cells (Baynash et al., 1994), and it is probably for this reason that all types of chromatophores are lacking in “white” axolotl.

Here, we focus on the Texas rat snake (*Pantherophis obsoletus lindheimeri*), a non-venomous colubrid found in central North America, characterized by black blotches on an orange background. Similarly to the closely related corn snake (*Pantherophis guttatus*), spontaneously-occurring color morphs, including xanthic, albino, hypomelanistic, and leucistic, have been recognized in the Texas rat snake (Bechtel and Bechtel, 1985). First, we use transmission electron microscopy (TEM) to show that the leucistic phenotype (Figure 1), an autosomal recessive trait (Bechtel and Bechtel, 1985) in Texas rat snakes, is associated with the lack of both melanophores and xanthophores in the skin. Second, we suggest that the uniform ivory white color in these animals is generated by iridophores and collagen fibers. Third, we perform genomic analyses to identify the causative variant responsible for the leucistic phenotype. Mapping by sequencing is an outstanding approach to determine the genomic interval where a mutation of interest resides (Schneeberger, 2014;



FIGURE 1 | Coloration of wild type **(A)** and leucistic **(B)** Texas rat snakes. A dorsal overview (left), close ups of lateral and ventral views (center), and a head dorsal view are provided. The inset shows the iris coloration.

Ullate-Agote et al., 2014, 2020; Saenko et al., 2015). However, it is a time-consuming protocol that can span over several years for species with seasonal breeding and long generation time. The advent of deep-sequencing technologies has greatly facilitated the genome assembly of non-classical model species, in a fast and affordable manner and with sufficient quality and completeness to annotate complete gene structures. Here, we assembled and annotated a high-quality genome of a leucistic Texas rat snake individual using 10x Genomics linked-reads (Zheng et al., 2016). Then, using a candidate gene approach based on the extensive literature on mutations responsible for leucism, mainly in mammals and birds (Reissmann and Ludwig, 2013; Kinoshita et al., 2014; Woodcock et al., 2017), we identified a single nucleotide deletion in *MITF* that introduces a frameshift and a premature stop codon in the leucistic genome. As this deletion is not present in all the genotyped leucistic animals of independent lineages, we additionally carried out gene expression analyses that revealed a significantly-decreased expression of *MITF* in all leucistic individuals, even in those lacking the above-mentioned single-nucleotide deletion in the coding sequence. We thus suggest that the leucistic phenotype in Texas rat snakes might be associated to mutations affecting the regulation of

MITF, and the single-nucleotide deletion might have appeared subsequently as a secondary consequence of an under- or mis-expressed *MITF*. *MITF* regulates the expression of genes involved in melanocyte migration, survival and differentiation during development (Fock et al., 2019). The absence of xanthophores and melanophores in the leucistic Texas rat snake suggests that in snakes *MITF* might be necessary for the differentiation of neural-crest derived melanophores and xanthophores, but not of iridophores.

MATERIALS AND METHODS

Animals

Texas rat snakes are housed and bred at the LANE animal facility running under the veterinary cantonal permit no. 1008. The individuals were sampled and imaged following Swiss law regulations and under the experimentation permit GE/169/17.

Histology and Transmission Electron Microscopy

Eyes from wild type and leucistic individuals were fixed in PFA 4% and washed in MetOH. Paraffin sections of 7 μ m were

either stained with hematoxylin/eosin or dewaxed with xylene and mounted directly to visualize the pigmentation. Skin pieces of 1 mm² were fixed and sectioned as described in Ullate-Agote et al. (2020). Micrographs were taken with a transmission electron microscope Philips CM100 (Thermo Fisher Scientific) at an acceleration voltage of 80 kV with a TVIPS TemCam-F416 digital camera (TVIPS GmbH). Large montage alignments were performed using Blendmont command-line from the IMOD software (Kremer et al., 1996).

Whole Genome Sequencing

High molecular weight (HMW; >50 Kb) genomic DNA (gDNA) was extracted with the QIAGEN Genomic-tip 20/G kit (10223, QIAGEN) from nucleated erythrocytes of an adult female leucistic individual. The resulting gDNA was used for a 10x Chromium library preparation (Novogene Co., Ltd.) with the Chromium Genome HT Library Kit and Gel Bead Kit v2 (Zheng et al., 2016). We sequenced the resulting barcoded fragments with a HiSeq X instrument, obtaining 522 million 150 bp paired-end reads.

Genome Assembly

We *de novo* assembled all the reads of the 10x Chromium library with Supernova v2.1.1 (Weisenfeld et al., 2017). The total sequencing data corresponded to a 77x raw coverage, above the recommended 56x (Weisenfeld et al., 2017). However, the length-weighted mean of HMW DNA molecule lengths estimated by Supernova is 35.8 Kb, below the ideal 50–100 Kb range. Downstream analyses were based on the pseudo-haplotype Supernova output. We removed highly similar and duplicated scaffolds by running CD-HIT-EST (Li and Godzik, 2006) to cluster sequences with 99% of sequence identity, keeping the longest one in the final assembly (parameters: -g 1 -c 0.99 -s 0.99 -aS 0.99). Finally, we removed mitochondrial DNA scaffolds and looked for contaminants running blastn searches (Camacho et al., 2009) against the closely-related corn snake mtDNA (GenBank accession number: AM236349.1), as well as the “UniVec Build 10,” *Saccharomyces cerevisiae*, *Escherichia coli*, and reptilian ferlaviruses databases with the NCBI VecScreen parameters.

We assessed the gene completeness of the assembly with BUSCO v3.0.2 (Waterhouse et al., 2018) focusing on the 3,950 single-copy orthologous genes of the Tetrapoda set from OrthoDB v9. We also modeled repeat families for the Texas rat snake using RepeatModeler v1.0.11 (Smit, 2008–2019)¹. We masked the assembly with RepeatMasker v4.0.7 (Smit, 2013–2019)² in sensitive search mode with rmbblastn v2.2.27+ in three sequential steps, taking the masked output genome of each run as an input for the following one. First, we considered all the Tetrapoda repeats from the RepeatMasker database (Repbase release 20170127). Second, we looked for classified Texas rat snake repetitive elements coming from RepeatModeler and, finally, we retrieved the repeats with unknown classification.

¹RepeatModeler Open-1.0.11. Available online at: <http://www.repeatmasker.org/RepeatModeler/>.

²RepeatMasker Open-4.0.7. Available online at: <http://www.repeatmasker.org>.

Genome Annotation

We predicted and annotated protein-coding genes in the Texas rat snake genome assembly using the BRAKER v2.1.4 fully automated pipeline (Hoff et al., 2019). Due to the lack of RNA-Seq data, we built a dataset including the non-mitochondrial proteins of five snakes in the NCBI RefSeq database (accessed in November 2019): *Pseudonaja textilis*, *Notechis scutatus*, *Protophops mucrosquamatus*, *Python bivittatus*, and *Thamnophis sirtalis*. After removing identical sequences, we obtained 123,537 proteins. They were aligned to the soft masked Texas rat snake genome using GenomeThreader v1.7.1 (Gremme et al., 2005) with compatible parameters for BRAKER (-gff3out -skipalignmentout). Then, we input the soft-masked genome assembly (adding the-softmasking flag) and the alignments generated with GenomeThreader (using the-protaln option) into BRAKER, with the-prg=gth and-trainFromGth parameters to indicate that the input is GenomeThreader alignments used to train the Augustus gene predictor. This corresponds to the BRAKER annotation pipeline for proteins of short evolutionary distance (Hoff et al., 2019), in which Augustus v3.3.3 (Stanke et al., 2006) is the only gene predictor and it is automatically trained with the protein alignments.

We used the MAKER v3 (Holt and Yandell, 2011) annotation pipeline to calculate the Annotation Edit Distance (AED) quality-control metric (Yandell and Ence, 2012) of the genes

TABLE 1 | List of the nine candidate genes known to be responsible for leucistic phenotypes in vertebrates.

Gene name	Gene description	Corn snake	Texas rat snake
EDN3	Endothelin 3 (X4 in corn snakes)	XM_034433122.1 573 nt/190 aa	573 nt/190 aa
EDNRB	Endothelin Receptor Type B	XM_034417959.1 1,302 nt/433 aa	1,302 nt/433 aa
EDNRB2	Endothelin Receptor Type B2 (in birds) Endothelin Receptor Type B-like (in snakes)	XM_034424043.1 1335 nt/444 aa	1,335 nt/444 aa
KIT	KIT Proto-Oncogene, Receptor Tyrosine Kinase	XM_034411440.1 2,880 nt/959 aa	2,859 nt/952 aa
KITLG	KIT Ligand	XM_034403862.1 852 nt/283 aa	852 nt/283 aa
MITF	Melanocyte Inducing Transcription Factor (X2 in corn snake)	XM_034440585.1 1,725 nt/574 aa	1,725 nt/574 aa
PAX3	Paired Box 3 (X1 in corn snake)	XM_034439420.1 1,455 nt/484 aa	1,455 nt/484 aa
SNAI2	Snail Family Transcriptional Repressor 2	XM_034429739.1 807 nt/268 aa	807 nt/268 aa
SOX10	SRY-Box Transcription Factor 10	XM_034402787.1 1,386 nt/461 aa	1,386 nt/461 aa

They are mainly involved in neural crest-derived cells migration, differentiation and survival during development. We provide the GenBank reference for each corn snake transcript (considering the longest isoform, or the one stated) with the CDS and protein length, and the corresponding manually-curated transcript and protein length for the Texas rat snake. In bold, the mutated gene in leucistic Texas rat snakes.

predicted by BRAKER as explained in (Campbell et al., 2014). AED measures the concordance between the annotated gene models and the supporting evidence data, with a value of one corresponding to lack of support. We input the BRAKER gene annotation into the MAKER pipeline in GFF format, together with the RefSeq snake protein database as evidence and the complex repetitive elements information from RepeatMasker, masking simple repeats during the MAKER run. Then, we ran InterProScan-5.39-77.0 (Jones et al., 2014) to look for Pfam protein domains. The final gene set includes the BRAKER gene models that are supported by evidence ($AED < 1$) or have a Pfam protein domain identified. Finally, we performed blastp searches against UniProtKB/SwissProt and Squamata proteins in RefSeq to annotate the genes (both accessed on 26.11.2019). A protein was considered complete if it covered at least 95% of its best blastp hit.

Candidate Gene Approach

We built a list of nine candidate genes responsible for leucistic phenotypes in other species which are mainly involved in the migration, survival, and differentiation of neural crest-derived melanocytes during development (Table 1). We obtained from the NCBI RefSeq database the protein sequences of these genes for the corn snake *Pantherophis guttatus* and the eastern brown snake *Pseudonaja textilis* (accessed in July 2020). We aligned them to the leucistic Texas rat snake genome assembly using exonerate v2.2.0 (Slater and Birney, 2005) in protein2genome mode to retrieve any disruptive modifications

in their coding sequence, i.e., frameshifts, premature stop codons, or modification of intron-exon boundaries.

Variant Confirmation

We extracted genomic DNA from animal sheds using the DNeasy Blood & Tissue Kit (Qiagen 69504) and genotyped them for the point mutation identified by deep-sequencing. We extracted mRNA with Direct-zol RNA Miniprep kit (Zymo Research R2050) from dorsal skin and prepared complementary DNA with PrimeScriptTM Reverse Transcriptase (Takara Bio 2680A). We then amplified and sequenced the *MITF* transcript in leucistic and wild type individuals. All primers are provided in Supplementary Table 1.

MITF Transcript Quantification

We obtained 50 μ l of whole blood and mixed it with 1 ml DNA/RNA Shield (Zymo Research) before storing at -80°C . We extracted RNA with the Quick-RNATM Whole Blood kit (Zymo Research). Reverse transcription starting with 500 ng of RNA was performed with the PrimeScriptTM RT Reagent Kit with gDNA Eraser (Takara Bio) which includes a step for the elimination of any gDNA contamination. We used the PowerUp SYBR Green Master Mix (Thermo Fisher Scientific) for the quantitative RT-PCR run on a QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific). All samples were run in triplicates. We verified the PCR efficiency for the amplification of *MITF* and *TUBB*, the reference gene, as described in Pfaffl (2001). Biogazelle qbasePLUS 3.2 software was used to analyze the results and

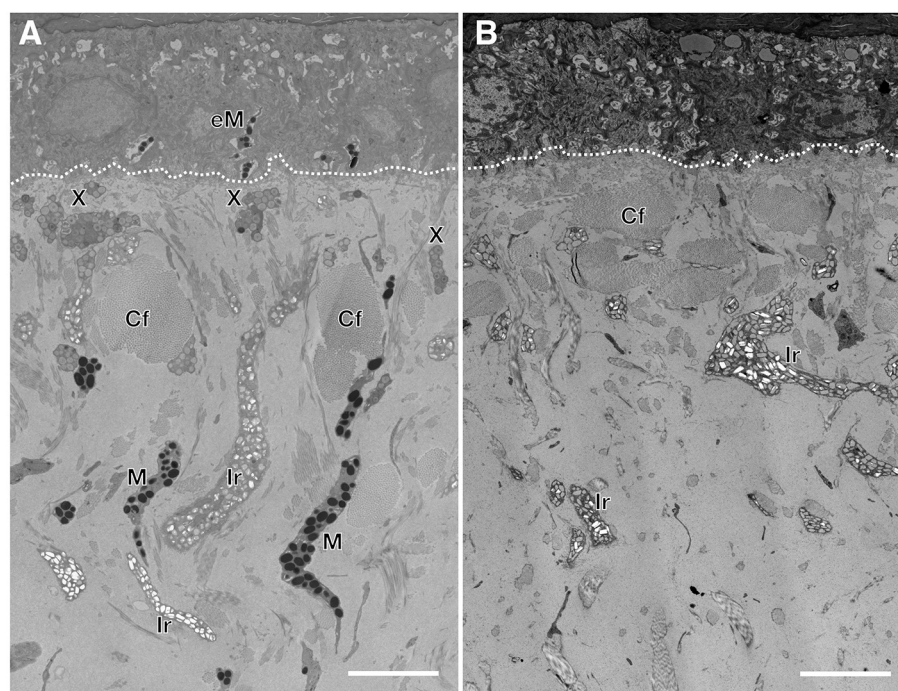


FIGURE 2 | Semi-thin sections of wild type (A) and leucistic (B) Texas rat snake dorsal skin. In the wild type, xanthophores (X) are situated closer to the basal membrane (dashed line) whereas the iridophores (Ir) and the melanophores (M) are spread in the loose dermis among collagen fibers (Cf). In the leucistic skin, only iridophores and collagen fibers are observed. Epidermal melanophores (eM) are observed only in the wild type. Scale bar: 5 μm .

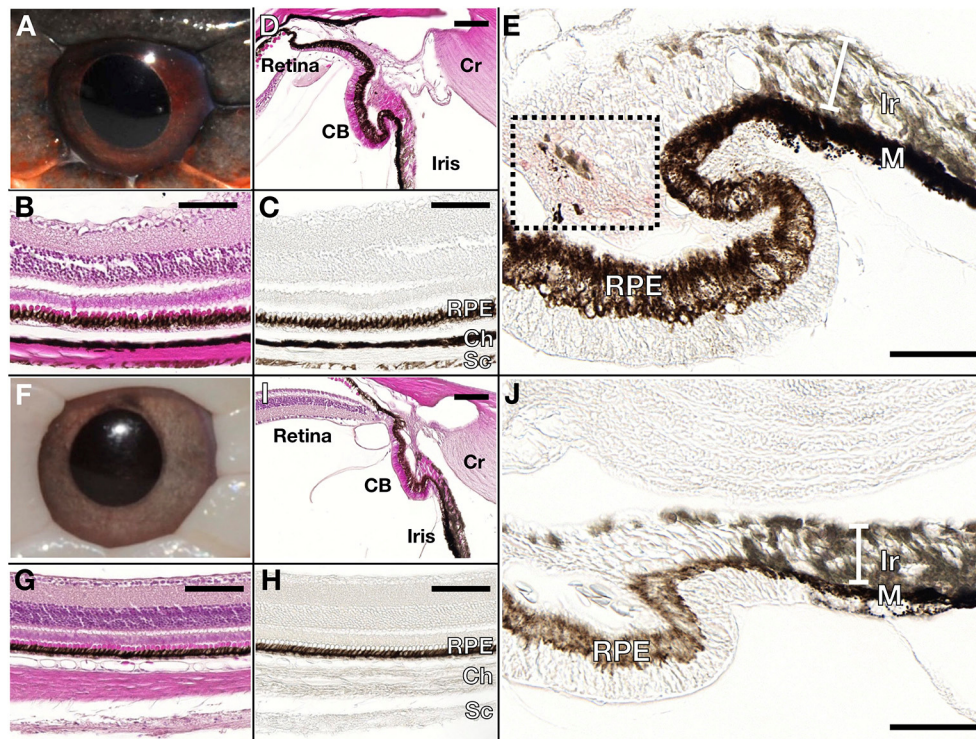


FIGURE 3 | Wild type (A–E) and leucistic (F–J) eye anatomy. Hematoxylin/Eosin stained (B,G) and unstained (C,H) paraffin sections through the retina show that pigmentation is present in the Retinal Pigmented Epithelium (RPE) of both wild type and leucistic snakes, whereas only the wild type choroid (Ch) and sclera (Sc) contain melanophores. (D,I) The RPE of the retina extends in the ciliary body (CB) and in the posterior part of the iris. Red pigmentation (area in black box) is visible in the CB of the wild type (E) but not in the leucistic snake (J), whereas iridophores (Ir) are found in the anterior part of the iris in both phenotypes (E,J). M: melanophores, Cr: cornea. Scale bar: 100 μ m.

perform statistical analyses (Biogazelle, Zwijnaarde, Belgium - www.qbaseplus.com).

RESULTS

Leucistic Texas Rat Snakes Lack Xanthophores and Melanocytes

Wild type Texas rat snakes have black blotches on a red to orange background and a white belly with some traces of yellow (Figure 1A). On the other hand, the dorsal and ventral skin of leucistic individuals is ivory white, their iris coloration is light gray (instead of red as in the wild-type) and the pupil remains black (Figure 1B). TEM analyses indicate that the dorsal skin of wild type individuals contains epidermal melanocytes, dermal xanthophores close to the epidermal basal membrane, as well as iridophores and melanocytes localized deeper in the dermis (Figure 2A and Supplementary Figure 1). No dermal melanophores could be found in the background sections, but we cannot exclude their presence in scarce numbers. The disposition and density of iridophores looks similar in the background and blotch dorsal skin. In comparison to the closely-related corn snake (Ullate-Agote et al., 2020), Texas rat snakes exhibit the following differences in chromatophore composition and distribution: (i) xanthophores are more scarce and can be found deeper in the dermis, instead of forming a continuous

layer just under the basal membrane, (ii) iridophores and melanocytes co-exist, instead of melanocytes being restricted at the bottom of the loose dermis, and (iii) the concentric lamellae of the pterinosomes are less evident and we cannot exclude the presence of carotenoid vesicles in some xanthophores. Indeed, the background coloration of the Texas rat snakes is darker compared to the corn snake, most likely because melanophores are situated higher in the dermis. On the other hand, Texas rat snake leucistic individuals only exhibit iridophores and collagen fibers (Figure 2B) that together produce the ivory white coloration of the animal. Sagittal sections of the Texas rat snake wild type and leucistic eye (Figure 3) both revealed the presence of melanophores in the RPE and the posterior part of the iris, consistent with an optic neuroepithelium origin of these melanophores. In the wild type, melanophores are also present in the sclera and the choroid and we observe red pigmentation in the ciliary body. Iridophores are present in the anterior part of the iris for both wild type and leucistic animals. Therefore, both melanophores and xanthophores of neural crest origin are impacted in the leucistic phenotype, but not the iridophores.

The Leucistic Texas Rat Snake Genome

The karyotype of the Texas rat snake consists of 18 pairs of chromosomes, of which 10 are micro-chromosomes (Baker et al.,

1972). We sequenced a 10x Chromium library and obtained 522 million paired-end reads from an adult leucistic female individual, female being the heterogametic sex, and we assembled them using the Supernova software (Weisenfeld et al., 2017). After removing redundant and contaminant sequences, the final rat snake genome assembly (GenBank accession number GCA_012654085.1) is 1.69 Gb long, consists of 36,795 scaffolds >1 Kb and presents only 3.6% of gaps. The N50 is 14.5 Mb with an L50 of 29 sequences. We thus obtained a highly contiguous genome in a single step, presenting similar statistics to other high-quality snake genomes, such as *Pantherophis guttatus*, *Notechis scutatus*, and *Pseudonaja textilis* (Table 2).

We assessed gene completeness using the 3,950 Tetrapoda single-copy gene set of BUSCO v3.0.2: we retrieved 3,481 full genes (88.1%), 270 partial genes (6.9%), and only 199 (5.04%) were missing. These values indicate high levels of completeness similar to other Squamata genomes (Supplementary Figure 2). The RepeatModeler library for the Texas rat snake consists of 1,496 repeat families, of which 687 are classified. Repetitive elements cover 45.18% of the genome and their diversity resembles that of the closely-related corn snake [(Ullate-Agote et al., 2020); Supplementary Figure 3 and Supplementary Table 2]. The two species have an estimated evolutionary divergence time of 13 Mya (Kumar et al., 2017); hence, it is not surprising that the repeat content is highly conserved.

Using the BRAKER pipeline and snake proteins from RefSeq, we predicted 24,107 genes with a total of 39,255 transcript isoforms. We only kept the gene models that were either supported by protein evidence data [i.e., with an Annotation Edit Distance lower than one or that had a Pfam domain (Supplementary Figure 4A)]. The annotation of 38,323 proteins is supported by other Squamata or UniProtKB/SwissProt proteins, with 21,435 being complete (Supplementary Figure 4B). Thus, most annotated genes are complete, which is crucial for the retrieval of variants affecting protein-coding sequences, as we attempt in this study. We provide a genome browser of the Texas rat snake genome to visualize the assembly and annotation (https://www.reptilomics.org/jbrowse/Pantherophis_obsoletus).

MITF Could Be Associated With the Leucistic Phenotype in a Captive-Bred Family

The leucistic phenotype of the Texas rat snake is caused by a recessive allele, so the assembled genome of a leucistic individual should harbor the causative variant in a homozygous state. The persistent coloration of the retinal pigment epithelium in leucistic animals (Figure 1B) rules out genes related to pigment synthesis or the biogenesis of color-producing organelles. Thus, we focused on genes involved in the migration, survival and differentiation of neural crest-derived chromatophores during development, i.e., genes whose variants can cause similar pigmentation phenotypes, like white skin and colored eyes, in other vertebrate species (Table 1). In mice, mutations in *Edn3* and its receptor Endothelin receptor type B (*Ednrb*) affect

TABLE 2 | Statistics of the final Texas rat snake genome assembly compared to other published snake genomes.

Species	Assembly length in Gb (% of gaps)	Number of sequences	N50 (Kb)	L50
<i>Pantherophis obsoletus lindheimeri</i> (this article)	1.69 (3.3%)	36,795	14,520	29
<i>Pantherophis guttatus</i>	1.71 (4.7%)	34,268	16,790	24
<i>Boa constrictor</i>	1.44 (3.9%)	19,927	4,505	90
<i>Python bivittatus</i>	1.44 (3.5%)	39,113	213.97	1,939
<i>Deinagkistrodon acutus</i>	1.51 (5.9%)	162,571	2,075	199
<i>Pseudonaja textilis</i>	1.59 (2.5%)	28,550	14,686	31
<i>Notechis scutatus</i>	1.67 (4.8%)	52,414	5,997	66
<i>Crotalus viridis</i>	1.34 (6.2%)	7,043	103,291	3

the migration of neural crest-derived melanocytes and enteric ganglion neurons precursors from E10.5 to E12.5, resulting in both leucism and an aganglionic megacolon (Lee et al., 2003). The Endothelin receptor type B2 gene (*EDNRB2*), absent in mammals, is a paralog of *EDNRB*. In birds, it is known to interact with *EDN3* and mutations in *EDNRB2* have been associated with leucistic plumage in chicken and the Japanese quail (Miwa et al., 2007; Kinoshita et al., 2014). *MITF* is the key transcription factor for melanocyte differentiation and survival and regulates the transcription of melanocyte-specific genes, such as *TYR* and *TYRP1* (Steingrimsson et al., 2004). *PAX3* and *SOX10* transcription factors are activators of *MITF* transcription, binding a proximal region of its promoter (Bondurand et al., 2000). Moreover, *SOX10* is expressed early in pre-migratory neural crest cells and then during migration, influencing the fate specification of certain neural crest derivatives, such as melanocytes (Kelsh, 2006). The Snail Family Transcriptional Repressor 2 (*SNAIL2*) gene is involved in the epithelial-to-mesenchymal transition and it is expressed in migrating neural crest cells, impairing melanoblast migration and survival when absent (Cobaleda et al., 2007). Mutations in any of the genes listed above, besides *EDNRB2* which is absent in mammals, can cause different types of Waardenburg syndrome in humans (Pingault et al., 2010). Finally, the *KIT* receptor and its ligand (*KITLG*) are involved in melanogenesis, gametogenesis and hematopoiesis (Besmer et al., 1993; Fleischman, 1993): in mice, mutations in these genes cause leucism and sterility and, in some cases, are lethal.

We aligned the closely-related corn snake and the eastern brown snake proteins of the nine candidate genes to the leucistic genome and discovered a single nucleotide deletion in the coding sequence (CDS) of *MITF*. Genotyping of wild type and leucistic Texas rat snakes in our colony (Figure 4A and Supplementary Table 3) confirmed that indeed a cytosine is missing in exon 6 at position 825 of the coding sequence in all leucistic individuals, introducing a frameshift and a premature stop codon downstream (Figure 4B). As a result, the leucistic *MITF* protein is only 286 amino acids long, instead of 574, with the last 12 aa being different from the ones in the wild type (Figure 4C). The sequences of the other candidate genes

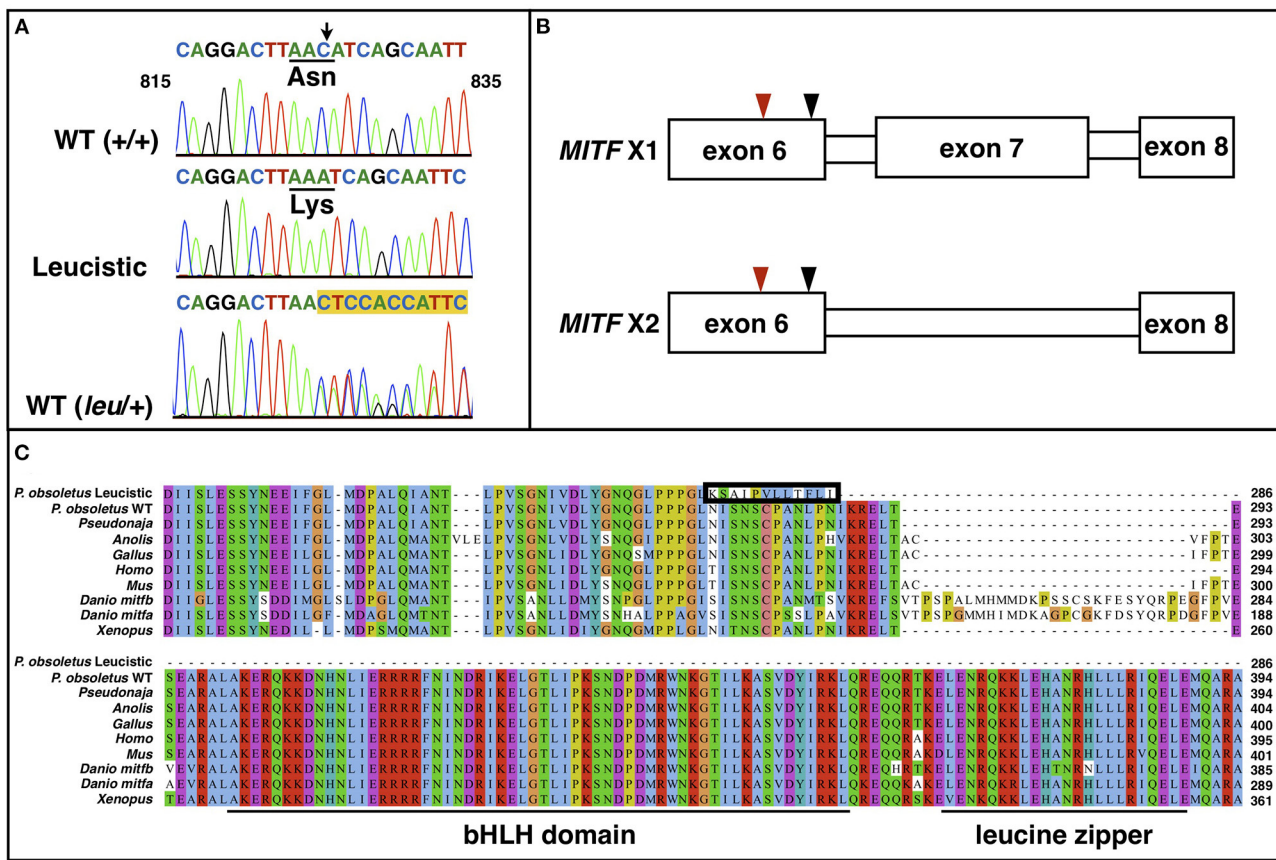


FIGURE 4 | (A) Chromatogram sequences of *MITF* between positions 815 and 835 of a WT (+/+), a leucistic and an heterozygous WT snake. The black arrow points to the deleted cytosine. The amino acid change is shown. Highlighted in yellow are the double peaks in the heterozygous individual. (B) Representation of the two *MITF* isoforms, which only differ by the presence/absence of exon 7. The red arrowhead points to the location of the cytosine deletion, while the black one indicates the location of the premature stop codon. (C) Multiple sequence alignment of *MITF* proteins from different vertebrate lineages along with the wild type (isoform X2 in 4B) and leucistic *Pantherophis obsoletus* *MITF*. The 12 amino acids that are modified in the leucistic *MITF* after the frameshift and before the premature stop codon are included in the black box. The basic helix-loop-helix and the leucine zipper domain are underlined.

do not present any obvious disruptions of the gene structure (Supplementary Text).

There are six annotated *MITF* isoforms for the corn snake. We confirmed the presence of two of them—isoform 2 (XM_034440585.1), which is one of the longest, and isoform 5 (XM_034440588.1), where exon 7 is missing—in the wild-type Texas rat snake by sequencing complementary DNA of multiple animals. As the premature stop codon in *MITF* of leucistic snakes is located in exon 6, we expect only one truncated protein to be produced in these animals (Figure 4B). The truncated protein misses the bHLH-Zip domain, which includes a DNA binding region, the helix-loop-helix domain and the leucine-zipper domain, with the latter two being involved in homo- and heterodimerization (Tachibana, 2000; Steingrimsson et al., 2004). These domains are highly conserved among all vertebrates (Figure 4C), suggesting that the mutated *MITF* protein is probably non-functional. A similar mutation has been identified in mice [in two different genetic backgrounds, *mi^{di}* and *mi^{ce}* (West et al., 1985; Zimring et al., 1996)]: a C to T transition at position 916 bp that introduces a

stop codon after amino acid 262. These mutants feature a white coat and reduced iris pigmentation. Depending on the genetic background, it has been reported that females have reduced reproduction.

MITF Expression Levels in Leucistic Individuals

As all the leucistic individuals of our colony originate from the same four related founders (Supplementary Table 3; individuals OBS01, 02, 05, and 06), we investigated if the mutation of the *MITF* CDS described above is present in other lineages of leucistic Texas rat snakes. Indeed, as discussed in (Bechtel and Bechtel, 1985), leucistic individuals exist in the wild and they have been independently introduced to the pet trade in different occasions. We thus purchased four additional leucistic snakes from three different sources and lineages (Supplementary Table 3; individuals OBS42–45). One of the animals had red pupils (OBS42; Supplementary Figure 5A) and we thus assume that it is a double mutant: amelanistic and leucistic. When we genotyped these new individuals for the

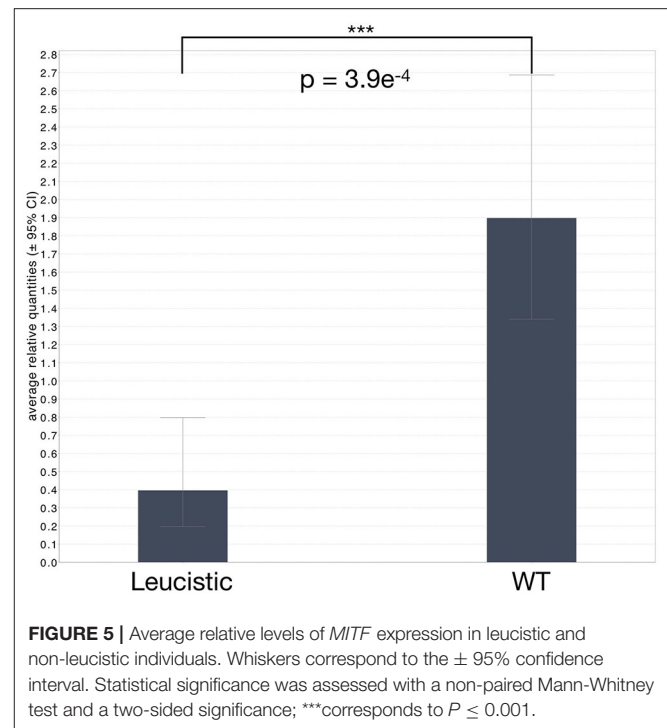
single-nucleotide deletion in *MITF*, we found that only one of them was homozygous for it (OBS42). The other three were either heterozygous (siblings OBS44 and 45) or homozygous for the wild type allele (OBS43). Both *MITF* isoforms were present in these four individuals without any other modification of the transcript, besides the presence in some cases of the single-nucleotide deletion as seen in their gDNA. Based on this genotypic heterogeneity, one could argue that the single-nucleotide deletion introducing a stop codon in the *MITF* protein cannot be solely responsible for the leucistic phenotype, as it is absent in some leucistic individuals (OBS43), or it is present on only one allele in others (OBS44 and 45).

To assess whether *MITF* is involved in the determination of the leucistic phenotype, we performed gene expression analyses. Red blood cells in reptiles are nucleated and on average 25,000 proteins are transcribed in the blood, making it a valid starting material for gene expression analyses when minimally invasive sampling is required (Waits et al., 2020). We thus performed our analyses with this type of samples. It was not possible to collect more adequate target tissues (namely, embryonic skin) because the leucistic individuals in our colony produce a very small number of eggs, compared to their heterozygous and wild-type counterparts.

We extracted RNA from the blood of all living individuals in our colony, as well as from the four new animals, and performed quantitative RT-PCR on all samples (Figure 5 and Supplementary Figures 6, 7). The results clearly show that *MITF* expression is significantly reduced in all leucistic individuals ($p = 3.9 \times 10^{-4}$; non-paired Mann-Whitney test with two-sided significance), independently of the presence or absence of the single-nucleotide deletion in the coding sequence. We thus hypothesize that a regulatory mutation could down-regulate *MITF* expression in all leucistic snakes and a more recent single-nucleotide deletion might have appeared in another lineage. Indeed, a down-regulated expression or mis-expression of *MITF* could facilitate the accumulation of mutations in the coding part of the gene. The advantage of using blood as starting material for these analyses is that the expression levels detected are independent of the number of chromatophores present in the skin. Indeed, in leucistic individuals, a reduced *MITF* expression in the skin could also be due to the absence of melanophores and xanthophores.

DISCUSSION

Vertebrate pigmentation has been extensively studied, mainly focusing on human related syndromes and mouse mutants in mammals, the zebrafish in teleosts and *Xenopus* in amphibians [for example Jackson (1997), Baxter and Pavan (2013), Mitros et al. (2019), Patterson and Parichy (2019)]. Mammals have limited interest for studying the development of chromatophores as they exhibit only melanophores. Fishes and amphibians go through metamorphosis and the origin of the embryonic and adult chromatophores is not the same for the different types. Squamates are important new models for understanding chromatophore development because they display remarkable



coloration patterns that are produced by different combinations of the three chromatophore types: melanophores, xanthophores, and iridophores. Although color patterns can evolve over time, as is the case of the ocellated lizard [*Timon lepidus*; (Manukyan et al., 2017)], squamates do not undergo metamorphosis such that the time evolution of color patterns must be associated to spatial reorganization of existing cell lineages or the redistribution of their pigmented organelles (Szydlowski et al., 2016). Here, we focus on the Texas rat snake whose pigmentation pattern, produced by the three types of chromatophores, does not change after hatching. Whole-genome sequencing and assembly, in combination with a candidate-gene approach, suggest that reduced *MITF* expression could be associated with leucism in this species. Additional expression analyses focusing on the developmental stage at which the cell fate of the neural-crest cells is determined would be necessary to provide additional support to our observations. Obviously, one cannot exclude the possibility that additional and independent mutations in *MITF* exist in other leucistic animals of the species, as is the case of the numerous *MITF* mutations that have been identified in humans and which all cause the Waardenburg syndrome type 2 and the Tietz syndrome (Grill et al., 2013). Both syndromes are characterized by generalized hypopigmentation and hearing loss.

In humans, mutations in *MITF* cause the Waardenburg syndrome type 2A (Pingault et al., 2010), an autosomal-dominant condition where depigmented skin patches are associated with sensorineural deafness and sometimes *Heterochromia Iridis* (the two eyes presenting different colors). Some *Mitf* variants in mice cause, in addition to the Waardenburg syndrome traits, abnormalities in eye development, such as microphthalmia. Zebrafish is a model species for coloration with similar

chromatophore types as in snakes. The zebrafish *mitfa* mutants lack melanophores, while they exhibit an excess of iridophores and a reduction in xanthophores (Lister et al., 1999). Despite being expressed in the RPE of wild type individuals, *mitfa* mutations appear to have no effect on the RPE pigmentation or development (Lister et al., 1999). Indeed, following the whole genome duplication at the origin of the teleost lineage, zebrafish have acquired a functional paralog gene, named *mitfb*. The latter is not expressed in neural crest cells but only in the RPE, compensating for the lack of *mitfa* expression in this structure in *mitfa* mutants (Lister et al., 2001).

One major developmental difference between snakes and zebrafish is that the latter undergo metamorphosis. As a result, the early larval and adult patterns in zebrafish are distinct; the former is generated directly from the neural crest cells, whereas the latter mainly originates from multipotent pigment progenitors located in the dorsal root ganglia (Singh et al., 2016). However, extensive support exists on the dual origin of adult xanthophores. Most larval xanthophores lose their pigmentation during metamorphosis and become cryptic, but they re-acquire pigmentation later in development and participate to the adult pattern (McMenamin et al., 2014; Saunders et al., 2019). Some of the adult xanthophores also originate from the multipotent pigment progenitors, as do most melanophores and iridophores.

Single-cell RNA sequencing analyses performed during the post-embryonic zebrafish development show that *mitfa* is expressed in pigment cell progenitors, melanophores and xanthophores, but not in iridophores (Saunders et al., 2019). Among xanthophores, the expression is higher in the actively differentiating population [xanthophores 2 in Saunders et al. (2019)] rather than in the cryptic one [xanthophores 1 in Saunders et al. (2019)]. This observation coupled with the reduced yellow coloration observed in *mitfa* zebrafish mutants suggests that the differentiation of xanthophores originating from the multipotent pigment cell progenitors is more impacted by the lack of *mitfa* than the state of the cryptic xanthophores, whose fate has already been defined during early larval development. Thus, the xanthophores that persist in *mitfa* mutants might be the early-larval xanthophores, containing pteridines. Although, the carotenoid content of *mitfa* mutants was not quantified, it was shown that their xanthophores maintain their pteridine pigmentation as adults (Lister et al., 1999), consistent with the presence of early-larva xanthophores.

The common origin of chromatophores from a multipotent precursor has been suggested long ago (Bagnara et al., 1979). Detailed lineage marker experiments propose the presence of a melanophore/iridophore bi-potent precursor in zebrafish (Curran et al., 2010; Petratos et al., 2018), where the expression of *mitfa* inhibits iridophore fate and promotes the melanophore differentiation. The co-expression of melanoblast and xanthoblasts markers, as well as the increase of melanophores in the absence of xanthophores (Minchin and Hughes, 2008) suggests that there might exist a melanophore/xanthophore bi-potent precursor as well. A common melanophore/xanthophore lineage has also been suggested with clonal experiments during

the fin regeneration of the zebrafish (Tu and Johnson, 2011). Our observations in the leucistic Texas rat snake substantiate the latter suggestion. Reduced expression of *MITF*, coupled in some individuals with a truncated *MITF* protein production, could result in the absence of melanophores and xanthophores, whereas the iridophore lineage remains intact. Indeed, the density of iridophores seems to be the same between wild type and leucistic individuals, whereas zebrafish *mitfa* mutants lacking melanophores have an increased number of iridophores (Lister et al., 1999). Our results corroborate the possible involvement of *MITF* in the differentiation of melanophores and xanthophores in a non-metamorphic species, that does not bear a paralogue of this gene. We did not observe an increase of iridophores in the leucistic samples, so we cannot deduce how *MITF* expression could impact iridophore fate. Melanophores persist in the RPE of leucistic snakes (Figure 3), so we can assume that their fate determination is probably independent of *MITF*, as is the case in other leucistic species (Bharti et al., 2006). On the other hand, in leucistic animals, both xanthophores and melanophores are absent from the anterior part of the iris whereas iridophores persist (Figure 3), as is the case in the skin. In conclusion, neural-crest derived melanophores and xanthophores are absent and only iridophores remain.

Knowledge is building up on the development of Squamate pigmentation (McLean et al., 2017; Andrade et al., 2019; Kuriyama et al., 2020; Ullate-Agote et al., 2020), clearly showing that there are similarities with the zebrafish in terms of gene regulation and expression. Thus, it is now important to understand how different chromatophores are specified and migrate in these species. Using known neural crest and chromatophore markers established in the zebrafish, it is conceivable to study the spatial and temporal distribution of these cells during development. These analyses would improve our understanding of the role of *MITF* in chromatophore differentiation in squamates. It would also allow to visualize the routes along which chromatophores migrate to populate the skin and generate the remarkable diversity of colors and color patterns in snakes and lizards.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://www.ncbi.nlm.nih.gov/assembly/GCA_012654085.1.

ETHICS STATEMENT

The animal study was reviewed and approved by the Geneva Cantonal Veterinary Authorities.

AUTHOR CONTRIBUTIONS

AT conceived and directed the study and organized all wet lab experiments. AT and AU-A designed the study, analyzed all data,

and wrote the manuscript. All authors read and approved the final manuscript.

FUNDING

Funding was provided by the University of Geneva and the Georges et Antoine Claraz Foundation. AU-A received an iGE3 Ph.D. salary award.

ACKNOWLEDGMENTS

We would like to thank Ingrid Burgelin, Carine Langrez, and Valerie Haechler for technical assistance, as well as Adrien Debry and Florent Montange for maintaining and imaging the animals.

REFERENCES

- Andrade, P., Pinho, C., Perez, Id. L. G., Afonso, S., Brejcha, J., Rubin, C. J., et al. (2019). Regulatory changes in pterin and carotenoid genes underlie balanced color polymorphisms in the wall lizard. *Proc. Natl. Acad. Sci. U. S. A.* 116, 5633–5642. doi: 10.1073/pnas.1820320116
- Anistoroaei, R., Krogh, A. K., and Christensen, K. (2013). A frameshift mutation in the *LYST* gene is responsible for the Aleutian color and the associated Chediak-Higashi syndrome in American mink. *Anim. Genet.* 44, 178–183. doi: 10.1111/j.1365-2052.2012.02391.x
- Bagnara, J. T., Matsumoto, J., Ferris, W., Frost, S. K., Turner, W. A., Jr., Tchen, T. T., et al. (1979). Common origin of pigment cells. *Science* 203, 410–415. doi: 10.1126/science.760198
- Baker, R. J., Bull, J. J., and Mengden, G. A. (1972). Karyotypic studies of 38 species of North-American snakes. *Copeia* 257:1442486. doi: 10.2307/1442486
- Barbosa, M. D., Nguyen, Q. A., Tchernev, V. T., Ashley, J. A., Dettler, J. C., Blaydes, S. M., et al. (1996). Identification of the homologous beige and Chediak-Higashi syndrome genes. *Nature* 382, 262–265. doi: 10.1038/382262a0
- Baxter, L. L., and Pavan, W. J. (2013). The etiology and molecular genetics of human pigmentation disorders. *Wiley Interdiscip. Rev. Dev. Biol.* 2, 379–392. doi: 10.1002/wdev.72
- Baynash, A. G., Hosoda, K., Giaid, A., Richardson, J. A., Emoto, N., Hammer, R. E., et al. (1994). Interaction of endothelin-3 with endothelin-B receptor is essential for development of epidermal melanocytes and enteric neurons. *Cell* 79, 1277–1285. doi: 10.1016/0092-8674(94)90018-3
- Bechtel, H. B. (1991). Inherited color defects. Comparison between humans and snakes. *Int. J. Dermatol.* 30, 243–246. doi: 10.1111/j.1365-4362.1991.tb04628.x
- Bechtel, H. B., and Bechtel, E. (1985). Genetics of color mutations in the snake, *Elaphe obsoleta*. *J. Heredity* 76, 7–11. doi: 10.1093/oxfordjournals.jhered.a110026
- Besmer, P., Manova, K., Duttlinger, R., Huang, E. J., Packer, A., Gyssler, C., et al. (1993). The kit-ligand (steel factor) and its receptor c-kit/W: pleiotropic roles in gametogenesis and melanogenesis. *Development*. Supplement, 125–137. doi: 10.1538/expanim1992.9.1
- Bharti, K., Nguyen, M. T., Skuntz, S., Bertuzzi, S., and Arnheiter, H. (2006). The other pigment cell: specification and development of the pigmented epithelium of the vertebrate eye. *Pigment. Cell Res.* 19, 380–394. doi: 10.1111/j.1600-0749.2006.00318.x
- Bondurand, N., Pingault, V., Goerich, D. E., Lemort, N., Sock, E., Le Caignec, C., et al. (2000). Interaction among *SOX10*, *PAX3* and *MITF*, three genes altered in Waardenburg syndrome. *Hum. Mol. Genet.* 9, 1907–1917. doi: 10.1093/hmg/9.13.1907
- Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K., et al. (2009). BLAST+: architecture and applications. *BMC Bioinform.* 10:421. doi: 10.1186/1471-2105-10-421
- Campbell, M. S., Holt, C., Moore, B., and Yandell, M. (2014). Genome annotation and curation using MAKER and MAKER-P. *Curr. Protoc. Bioinform.* 48, 4–39. doi: 10.1002/0471250953.bi0411s48
- Jean Deraspe and Rodrigue Peraldi participated in the TEM sample processing and imaging. The TEM sample processing and imaging were performed at the Electron Microscopy Facility, University of Lausanne (Switzerland). The computations were run on the Baobab cluster (<https://baobabmaster.unige.ch/iface/>) of the University of Geneva (Switzerland). Michel Milinkovitch commented on an earlier version of the manuscript.
- ## SUPPLEMENTARY MATERIAL
- The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fevo.2021.583136/full#supplementary-material>
- Chang, C. M., Coville, J. L., Coquerelle, G., Gourichon, D., Oulmouden, A., and Tixier-Boichard, M. (2006). Complete association between a retroviral insertion in the tyrosinase gene and the recessive white mutation in chickens. *BMC Genom.* 7:19. doi: 10.1186/1471-2164-7-19
- Cobaleda, C., Perez-Caro, M., Vicente-Duenas, C., and Sanchez-Garcia, I. (2007). Function of the zinc-finger transcription factor *SNAIL2* in cancer and development. *Ann. Rev. Genet.* 41, 41–61. doi: 10.1146/annurev.genet.41.110306.130146
- Curran, K., Lister, J. A., Kunkel, G. R., Prendergast, A., Parichy, D. M., and Raible, D. W. (2010). Interplay between *Foxd3* and *Mitf* regulates cell fate plasticity in the zebrafish neural crest. *Dev. Biol.* 344, 107–118. doi: 10.1016/j.ydbio.2010.04.023
- Fleischman, R. A. (1993). From white spots to stem cells: the role of the Kit receptor in mammalian development. *Trends Genet.* 9, 285–290. doi: 10.1016/0168-9525(93)90015-A
- Fock, V., Gudmundsson, S. R., Gunnlaugsson, H. O., Stefansson, J. A., Ionasz, V., Schepsky, A., et al. (2019). Subcellular localization and stability of MITF are modulated by the bHLH-Zip domain. *Pigment. Cell Melanoma Res.* 32, 41–54. doi: 10.1111/pcmr.12721
- Fuhrmann, S., Zou, C., and Levine, E. M. (2014). Retinal pigment epithelium development, plasticity, and tissue homeostasis. *Exp. Eye Res.* 123, 141–150. doi: 10.1016/j.exer.2013.09.003
- Gremme, G., Brendel, V., Sparks, M. E., and Kurtz, S. (2005). Engineering a software tool for gene structure prediction in higher organisms. *Inform. Softw. Tech.* 47, 965–978. doi: 10.1016/j.infsof.2005.09.005
- Grill, C., Bergsteinsdottir, K., Ogmundsdottir, M. H., Pogenberg, V., Schepsky, A., Wilmanns, M., et al. (2013). MITF mutations associated with pigment deficiency syndromes and melanoma have different effects on protein function. *Hum. Mol. Genet.* 22, 4357–4367. doi: 10.1093/hmg/ddt285
- Hart, J. C., and Miller, C. T. (2017). Sequence-based mapping and genome editing reveal mutations in stickleback *Hps5* cause oculocutaneous albinism and the casper phenotype. *G3* 7, 3123–3131. doi: 10.1534/g3.117.1125
- Hoff, K. J., Lomsadze, A., Borodovsky, M., and Stanke, M. (2019). Whole-genome annotation with BRAKER. *Methods Mol. Biol.* 1962, 65–95. doi: 10.1007/978-1-4939-9173-0_5
- Holt, C., and Yandell, M. (2011). MAKER2: an annotation pipeline and genome-database management tool for second-generation genome projects. *BMC Bioinform.* 12:491. doi: 10.1186/1471-2105-12-491
- Iwanishi, S., Zaitou, S., Shibata, H., and Nitasaka, E. (2018). An albino mutant of the Japanese rat snake (*Elaphe climacophora*) carries a nonsense mutation in the tyrosinase gene. *Genes Genet. Syst.* 93, 163–167. doi: 10.1266/ggs.18-00021
- Jackson, I. J. (1997). Homologous pigmentation mutations in human, mouse and other model organisms. *Hum. Mol. Genet.* 6, 1613–1624. doi: 10.1093/hmg/6.10.1613
- Jones, P., Binns, D., Chang, H. Y., Fraser, M., Li, W. Z., McAnulla, C., et al. (2014). InterProScan 5: genome-scale protein function classification. *Bioinformatics* 30, 1236–1240. doi: 10.1093/bioinformatics/btu031

- Kelsh, R. N. (2006). Sorting out Sox10 functions in neural crest development. *Bioessays* 28, 788–798. doi: 10.1002/bies.20445
- Kinoshita, K., Akiyama, T., Mizutani, M., Shinomiya, A., Ishikawa, A., Younis, H. H., et al. (2014). Endothelin receptor B2 (EDNRB2) is responsible for the tyrosinase-independent recessive white [mo(w)] and mottled (mo) plumage phenotypes in the chicken. *PLoS ONE* 9:e86361. doi: 10.1371/journal.pone.0086361
- Kremer, J. R., Mastronarde, D. N., and McIntosh, J. R. (1996). Computer visualization of three-dimensional image data using IMOD. *J. Struct. Biol.* 116, 71–76. doi: 10.1006/jsbi.1996.0013
- Kumar, S., Stecher, G., Suleski, M., and Hedges, S. B. (2017). TimeTree: a resource for timelines, timetrees, and divergence times. *Mol. Biol. Evol.* 34, 1812–1819. doi: 10.1093/molbev/msx116
- Kuriyama, T., Misawa, H., Miyaji, K., Sugimoto, M., and Hasegawa, M. (2013). Pigment cell mechanisms underlying dorsal color-pattern polymorphism in the Japanese four-lined snake. *J. Morphol.* 274, 1353–1364. doi: 10.1002/jmor.20182
- Kuriyama, T., Miyaji, K., Sugimoto, M., and Hasegawa, M. (2006). Ultrastructure of the dermal chromatophores in a lizard (Scincidae: *Plestiodon latiscutatus*) with conspicuous body and tail coloration. *Zool. Sci.* 23, 793–799. doi: 10.2108/zsj.23.793
- Kuriyama, T., Murakami, A., Brandley, M., and Hasegawa, M. (2020). Blue, black, and stripes: evolution and development of color production and pattern formation in lizards and snakes. *Front. Ecol. Evol.* 8:232. doi: 10.3389/fevo.2020.00232
- Lee, H. O., Levorse, J. M., and Shin, M. K. (2003). The endothelin receptor-B is required for the migration of neural crest-derived melanocyte and enteric neuron precursors. *Dev. Biol.* 259, 162–175. doi: 10.1016/S0012-1606(03)00160-X
- Li, W., and Godzik, A. (2006). Cd-hit: a fast program for clustering and comparing large sets of protein or nucleotide sequences. *Bioinformatics* 22, 1658–1659. doi: 10.1093/bioinformatics/btl158
- Lister, J. A., Close, J., and Raible, D. W. (2001). Duplicate mitf genes in zebrafish: complementary expression and conservation of melanogenic potential. *Dev. Biol.* 237, 333–344. doi: 10.1006/dbio.2001.0379
- Lister, J. A., Robertson, C. P., Lepage, T., Johnson, S. L., and Raible, D. W. (1999). nacre encodes a zebrafish microphthalmia-related protein that regulates neural-crest-derived pigment cell fate. *Development* 126, 3757–3767.
- Manukyan, L., Montandon, S. A., Fofonjka, A., Smirnov, S., and Milinkovitch, M. C. (2017). A living mesoscopic cellular automaton made of skin scales. *Nature* 544, 173–179. doi: 10.1038/nature22031
- McLean, C. A., Lutz, A., Rankin, K. J., Stuart-Fox, D., and Moussalli, A. (2017). Revealing the biochemical and genetic basis of color variation in a polymorphic lizard. *Mol. Biol. Evol.* 34, 1924–1935. doi: 10.1093/molbev/msx136
- McMenamin, S. K., Bain, E. J., McCann, A. E., Patterson, L. B., Eom, D. S., Waller, Z. P., et al. (2014). Thyroid hormone-dependent adult pigment cell lineage and pattern in zebrafish. *Science* 345, 1358–1361. doi: 10.1126/science.1256251
- Minchin, J. E., and Hughes, S. M. (2008). Sequential actions of Pax3 and Pax7 drive xanthophore development in zebrafish neural crest. *Dev. Biol.* 317, 508–522. doi: 10.1016/j.ydbio.2008.02.058
- Mitros, T., Lyons, J. B., Session, A. M., Jenkins, J., Shu, S., Kwon, T., et al. (2019). A chromosome-scale genome assembly and dense genetic map for *Xenopus tropicalis*. *Dev. Biol.* 452, 8–20. doi: 10.1016/j.ydbio.2019.03.015
- Miwa, M., Inoue-Murayama, M., Aoki, H., Kunisada, T., Hiragaki, T., Mizutani, M., et al. (2007). Endothelin receptor B2 (EDNRB2) is associated with the panda plumage colour mutation in Japanese quail. *Anim. Genet.* 38, 103–108. doi: 10.1111/j.1365-2052.2007.01568.x
- Montoliu, L., Gronskov, K., Wei, A. H., Martinez-Garcia, M., Fernandez, A., Arveiler, B., et al. (2014). Increasing the complexity: new genes and new types of albinism. *Pigm. Cell Melanoma R.* 27:12167. doi: 10.1111/pcmr.12167
- Nakayama, T., Nakajima, K., Cox, A., Fisher, M., Howell, M., Fish, M. B., et al. (2017). No privacy, a *Xenopus tropicalis* mutant, is a model of human Hermansky-Pudlak Syndrome and allows visualization of internal organogenesis during tadpole development. *Dev. Biol.* 426, 472–486. doi: 10.1016/j.ydbio.2016.08.020
- Patterson, L. B., and Parichy, D. M. (2019). Zebrafish pigment pattern formation: insights into the development and evolution of adult form. *Ann. Rev. Genet.* 53, 505–530. doi: 10.1146/annurev-genet-112618-043741
- Petratou, K., Subkhankulova, T., Lister, J. A., Rocco, A., Schwetlick, H., and Kelsh, R. N. (2018). A systems biology approach uncovers the core gene regulatory network governing iridophore fate choice from the neural crest. *PLoS Genet.* 14:e1007402. doi: 10.1371/journal.pgen.1007402
- Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucl. Acids Res.* 29:e45. doi: 10.1093/nar/29.9.e45
- Pingault, V., Ente, D., Dastot-Le Moal, F., Goossens, M., Marlin, S., and Bondurand, N. (2010). Review and update of mutations causing Waardenburg syndrome. *Hum. Mutat.* 31, 391–406. doi: 10.1002/humu.21211
- Reissmann, M., and Ludwig, A. (2013). Pleiotropic effects of coat colour-associated mutations in humans, mice and other mammals. *Semin. Cell Dev. Biol.* 24, 576–586. doi: 10.1016/j.semcdb.2013.03.014
- Saenko, S. V., Lamichhaney, S., Martinez Barrio, A., Rafati, N., Andersson, L., and Milinkovitch, M. C. (2015). Amelanism in the corn snake is associated with the insertion of an LTR-retrotransposon in the OCA2 gene. *Sci. Rep.* 5:17118. doi: 10.1038/srep17118
- Saenko, S. V., Teyssier, J., van der Marel, D., and Milinkovitch, M. C. (2013). Precise colocalization of interacting structural and pigmentary elements generates extensive color pattern variation in *Phelsuma* lizards. *BMC Biol.* 11:105. doi: 10.1186/1741-7007-11-105
- Saunders, L. M., Mishra, A. K., Aman, A. J., Lewis, V. M., Toomey, M. B., Packer, J. S., et al. (2019). Thyroid hormone regulates distinct paths to maturation in pigment cell lineages. *Elife* 8:40. doi: 10.7554/eLife.45181.040
- Schneeberger, K. (2014). Using next-generation sequencing to isolate mutant genes from forward genetic screens. *Nat. Rev. Genet.* 15, 662–676. doi: 10.1038/nrg3745
- Singh, A. P., Dinwiddie, A., Mahalwar, P., Schach, U., Linker, C., Irion, U., et al. (2016). Pigment cell progenitors in zebrafish remain multipotent through metamorphosis. *Dev. Cell.* 38, 316–330. doi: 10.1016/j.devcel.2016.06.020
- Slater, G. S., and Birney, E. (2005). Automated generation of heuristics for biological sequence comparison. *BMC Bioinform.* 6:31. doi: 10.1186/1471-2105-6-31
- Stanke, M., Schoffmann, O., Morgenstern, B., and Waack, S. (2006). Gene prediction in eukaryotes with a generalized hidden Markov model that uses hints from external sources. *BMC Bioinform.* 7:62. doi: 10.1186/1471-2105-7-62
- Steingrimsson, E., Copeland, N. G., and Jenkins, N. A. (2004). Melanocytes and the microphthalmia transcription factor network. *Ann. Rev. Genet.* 38, 365–411. doi: 10.1146/annurev.genet.38.072902.092717
- Szydlowski, P., Madej, J. P., and Mazurkiewicz-Kania, M. (2016). Ultrastructure and distribution of chromatophores in the skin of the leopard gecko (*Eublepharis macularius*). *Acta Zool.* 97, 370–375. doi: 10.1111/azo.12132
- Tachibana, M. (2000). MITF: a stream flowing for pigment cells. *Pigm. Cell Res.* 13, 230–240. doi: 10.1034/j.1600-0749.2000.130404.x
- Teyssier, J., Saenko, S. V., van der Marel, D., and Milinkovitch, M. C. (2015). Photonic crystals cause active colour change in chameleons. *Nat. Commun.* 6:6368. doi: 10.1038/ncomms7368
- Tu, S., and Johnson, S. L. (2011). Fate restriction in the growing and regenerating zebrafish fin. *Dev. Cell* 20, 725–732. doi: 10.1016/j.devcel.2011.04.013
- Ullate-Agote, A., Burgelin, I., Debry, A., Langrez, C., Montange, F., Peraldi, R., et al. (2020). Genome mapping of a LYST mutation in corn snakes indicates that vertebrate chromatophore vesicles are lysosome-related organelles. *Proc. Natl. Acad. Sci. U. S. A.* 2020:117. doi: 10.1073/pnas.2003724117
- Ullate-Agote, A., Milinkovitch, M. C., and Tzika, A. C. (2014). The genome sequence of the corn snake (*Pantherophis guttatus*), a valuable resource for EvoDevo studies in squamates. *Int. J. Dev. Biol.* 58, 881–888. doi: 10.1387/ijdb.150060at
- Waits, D. S., Simpson, D. Y., Sparkman, A. M., Bronikowski, A. M., and Schwartz, T. S. (2020). The utility of reptile blood transcriptomes in molecular ecology. *Mol. Ecol. Resour.* 20, 308–317. doi: 10.1111/1755-0998.13110
- Wasmeier, C., Hume, A. N., Bolasco, G., and Seabra, M. C. (2008). Melanosomes at a glance. *J. Cell Sci.* 121, 3995–3999. doi: 10.1242/jcs.040667
- Waterhouse, R. M., Seppey, M., Simao, F. A., Manni, M., Ioannidis, P., Kliuchnikov, G., et al. (2018). BUSCO applications from quality assessments to gene prediction and phylogenomics. *Mol. Biol. Evol.* 35, 543–548. doi: 10.1093/molbev/msx319

- Wei, A. H., and Li, W. (2013). HermanskyPudlak syndrome: pigmentary and non-pigmentary defects and their pathogenesis. *Pigm. Cell Melanoma R.* 26:12051. doi: 10.1111/pcmr.12051
- Weisenfeld, N. I., Kumar, V., Shah, P., Church, D. M., and Jaffe, D. B. (2017). Direct determination of diploid genome sequences. *Genome Res.* 27, 757–767. doi: 10.1101/gr.214874.116
- West, J. D., Fisher, G., Loutit, J. F., Marshall, M. J., Nisbet, N. W., and Perry, V. H. (1985). A new allele of microphthalmia induced in the mouse: microphthalmia-defective iris (midi). *Genet. Res.* 46, 309–324. doi: 10.1017/S0016672300022801
- Woodcock, M. R., Vaughn-Wolfe, J., Elias, A., Kump, D. K., Kendall, K. D., Timoshevskaya, N., et al. (2017). Identification of mutant genes and introgressed tiger salamander DNA in the laboratory Axolotl, *Ambystoma mexicanum*. *Sci. Rep.* 7:6. doi: 10.1038/s41598-017-00059-1
- Yandell, M., and Ence, D. (2012). A beginner's guide to eukaryotic genome annotation. *Nat. Rev. Genet.* 13, 329–342. doi: 10.1038/nrg3174
- Zheng, G. X., Lau, B. T., Schnall-Levin, M., Jarosz, M., Bell, J. M., Hindson, C. M., et al. (2016). Haplotyping germline and cancer genomes with high-throughput linked-read sequencing. *Nat. Biotechnol.* 34, 303–311. doi: 10.1038/nbt.3432
- Ziegler, I. (2003). The pteridine pathway in zebrafish: regulation and specification during the determination of neural crest cell-fate. *Pigm. Cell Res.* 16, 172–182. doi: 10.1034/j.1600-0749.2003.00044.x
- Zimring, D. C., Lamoreux, M. L., Millichamp, N. J., and Skow, L. C. (1996). Microphthalmia cloudy-eye [mi(ce)]: a new murine allele. *J. Hered.* 87, 334–338. doi: 10.1093/oxfordjournals.jhered.a023009

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2021 Ullate-Agote and Tzika. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Advantages of publishing in Frontiers



OPEN ACCESS

Articles are free to read
for greatest visibility
and readership



FAST PUBLICATION

Around 90 days
from submission
to decision



HIGH QUALITY PEER-REVIEW

Rigorous, collaborative,
and constructive
peer-review



TRANSPARENT PEER-REVIEW

Editors and reviewers
acknowledged by name
on published articles

Frontiers

Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org

Contact us: frontiersin.org/about/contact



REPRODUCIBILITY OF RESEARCH

Support open data
and methods to enhance
research reproducibility



DIGITAL PUBLISHING

Articles designed
for optimal readership
across devices



FOLLOW US

@frontiersin



IMPACT METRICS

Advanced article metrics
track visibility across
digital media



EXTENSIVE PROMOTION

Marketing
and promotion
of impactful research



LOOP RESEARCH NETWORK

Our network
increases your
article's readership