

Eco-evo-devo: an emergent integrative discipline of biology

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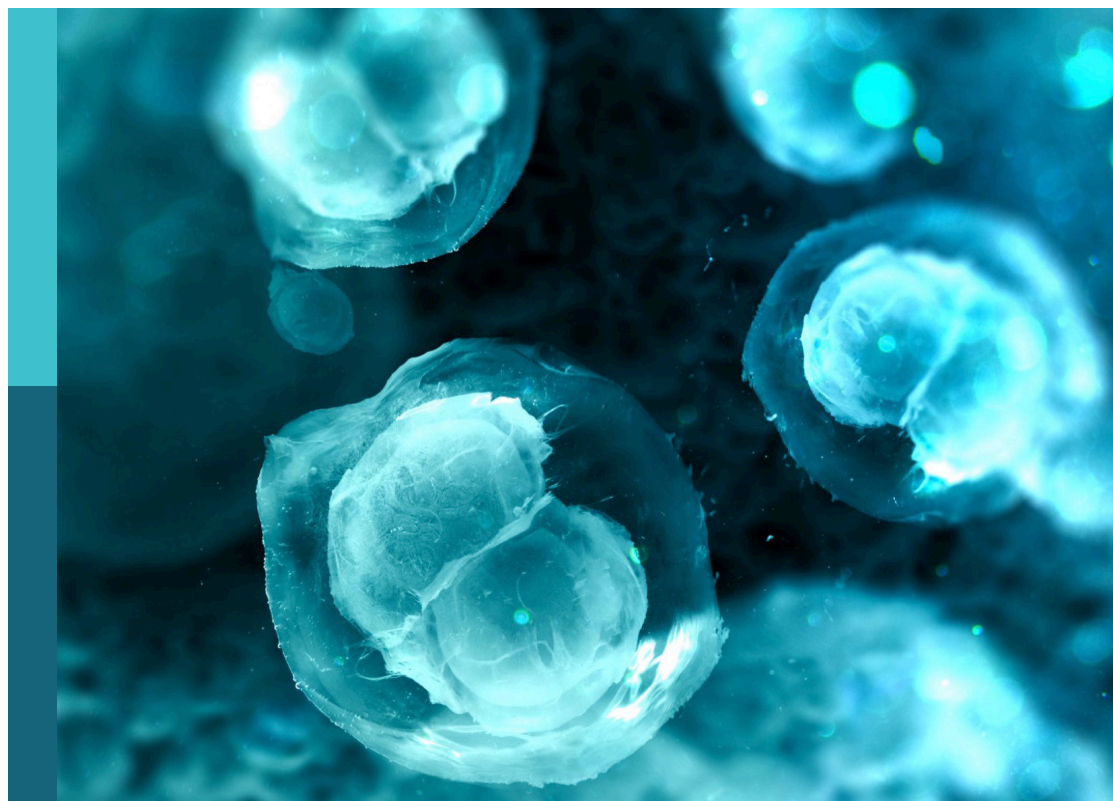
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Eco-evo-devo: an emergent integrative discipline of biology

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Editorial: Eco-evo-devo: an emergent integrative discipline of biology

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evo-devo (evolution and development), developmental and physiological plasticity, holobiont, evolutionary synthesis, theory of biology, developmental bias, life-history, multi-scale causation

Editorial on the Research Topic

Eco-evo-devo: an emergent integrative discipline of biology

Eco-evo-devo (ecological evolutionary developmental biology) has emerged as a highly active field of research, aiming to understand how environmental cues, developmental mechanisms, and evolutionary processes interact to shape phenotypes, morphogenetic patterns, life histories, and biodiversity across multiple scales. Rather than serving as a loose aggregation of diverse research topics and subfields, eco-evo-devo seeks to provide a coherent conceptual framework for exploring causal relationships among developmental, ecological, and evolutionary levels (Figure 1). By doing so, it aspires to be more than the sum of its parts, contributing to the development of a simpler, more elegant, and heuristically powerful biological theory. To illustrate how this integrative approach is taking shape, this Research Topic brings together nine contributions—including empirical studies, conceptual reviews, and opinion pieces, and covering a diverse array of organisms, methodologies and perspectives—that collectively highlight the promise and potential of this emerging discipline.

One central theme is the role of development in shaping plastic phenotypic responses to environmental variation. Beyond the classic reaction-norm-based approaches, which merely establish phenomenological correlations between environmental and phenotypic changes, *eco-evo-devo* aims to provide a causal, mechanistic understanding of how these reaction norms arise during development and evolve over time. Under these premises, Roy et al. present an experimental evolution study in the fruit fly *Drosophila melanogaster*, showing that selection for cold tolerance reduces the plasticity of life-history traits under thermal stress. This finding highlights that, similar to the more widely studied genotype-to-phenotype relationships, development generates complex associations between generative factors - here environmental cues - and phenotypic traits, and that these associations themselves can evolve under sustained environmental selective pressure. Along similar lines, Lofeu et al. demonstrate that the influence of the environment on phenotypic responses

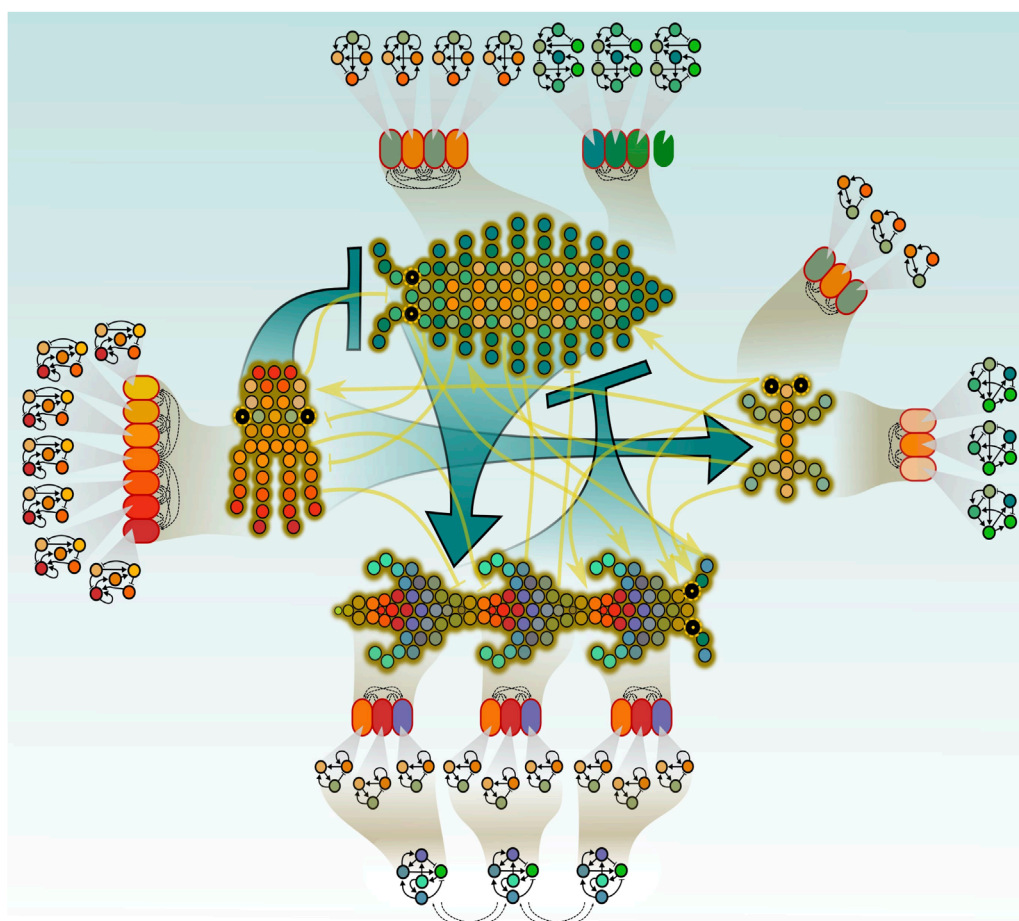


FIGURE 1

This figure provides a conceptual and metaphorical representation of the aim and scope of the Eco-Evo-Devo research field. From the outer layer to the center, nested networks of genetic, cellular, phenotypic, and ecological interactions generate emergent phenomena and bidirectional causal flows across levels. Eco-Evo-Devo offers a framework to explore this multilevel continuum, revealing hidden regularities, unexpected correlations, and deep organizational principles linking ecology, development, and evolution.

extends to the dynamics of development itself. By examining ontogenetic plasticity in the neotropical fish *Astyanax lacustris*, these authors show how temperature modulates developmental responses to different water flow regimes. Together, these studies not only provide evidence for the crucial instructive role of the environment in shaping development and evolutionary potential, but also suggest that such phenomena occur across distantly related taxa and are likely widespread throughout the tree of life. Thus, by challenging the classic view which privileges genetics as the unique central factor in shaping phenotypic evolution, eco-evo-devo provides a new way to understand and test complex interactions between the environment, ontogeny, and inheritance in the study of diversification.

The importance of symbiosis and inter-kingdom communication in physiology, development and evolution is explored from both empirical and conceptual perspectives. Here, the eco-evo-devo framework sheds light on how interactions between different biological agents can generate new layers of complexity and variation. Although the importance of interactions is well established for genes and cells (whose interaction creates and maintains phenotypes), the eco-evo-devo perspective extends the

interactive principle of biology to novel levels of organization and new causal mechanisms. For example, developmental processes themselves can be shaped by inter-organismal interactions such as symbiosis and inter-kingdom communication—a subject that is explored in this Research Topic both empirically and conceptually. [Mukherjee and Moroz](#) trace the evolution of G-type lysozymes across Metazoa, revealing how these enzymes have been spread by horizontal gene transfer across kingdoms and repeatedly adapted for immune and digestive functions in response to ecological contexts. [Scott Gilbert's](#) opinion article reframes development as a symbiotic process, proposing that organismal identity and morphogenesis are produced through interactions with microbial and environmental partners. These contributions advance the view of organisms as integrated networks of interactions between heterogeneous agents, with eco-evo-devo providing a consistent framework for understanding their mutual development.

Another focus of eco-evo-devo is the role of developmental bias and constraint in directing evolutionary diversification. [Stansfield and Parsons](#) review how biases in developmental systems shape adaptive radiations. Their work indicates that variation is not always

random or isotropic but influenced by the specific architecture of developmental programs. [Singh and Singh](#) explore how gestation length and DNA damage response mechanisms impact life-history traits and correlate with longevity in mammals, emphasizing the developmental foundations of evolutionary transitions in reproductive strategies.

At the intersection of development, morphology and reproductive fitness, two contributions illustrate how developmental processes underpin evolutionary traits. [Pintus et al.](#) investigate how Sertoli cell efficiency and sperm size homogeneity influence reproductive potential in the common eland *Taurotragus oryx*, linking cellular development to fertility and sexual selection. [Knyazeva and Dyachuk](#) review the neural crest's role in gland development across vertebrates, highlighting the conserved developmental modules underlying evolutionary innovation in organogenesis, which shows that even macro-evolutionary trends are shaped by conserved developmental mechanisms. Lastly, [Moroz and Romanova](#) expand the theoretical foundations of eco-evo-devo, advocating for a reconsideration of functional biodiversity from an eco-evo-devo perspective. Drawing from comparative physiology and evolutionary theory, they argue for a synthesis recognizing the centrality of development in shaping organismal function and ecological adaptation.

These nine articles underscore the broad significance of the eco-evo-devo approach. They demonstrate how developmental processes mediate environmental and evolutionary dynamics, how symbioses, inter-kingdom, and ecological interactions contribute to morphogenesis, and how developmental bias and plasticity influence macroevolutionary patterns. They also suggest future directions of research, including mechanistic studies of developmental-environmental interactions, a broader focus on symbiotic development, and integrative modeling across scales and taxa. Furthermore, they offer a conceptual and empirical strategy to challenge long-held views in order to innovate our fundamental ways of thinking about the dynamics and complexity of nature.

Understanding how organisms respond and evolve in relation to their environments is increasingly important as the planet faces ecological change. Eco-evo-devo provides a comprehensive approach for investigating these dynamics, integrating molecular, developmental, ecological, and evolutionary perspectives. The contributions in this Research Topic reflect the current dynamics of the field with the prospect of establishing a foundation for an integrative biology of the 21st century.

Author contributions

MBU: Conceptualization, Visualization, Writing – original draft. RN-F: Conceptualization, Writing – original draft. KK: Conceptualization, Writing – review and editing. JEM-V: Conceptualization, Writing – review and editing. RZ: Conceptualization, Writing – review and editing.

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Neural crest and sons: role of neural crest cells and Schwann cell precursors in development and gland embryogenesis

Aleksandra Knyazeva and Vyacheslav Dyachuk*

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In this review, we consider the multipotency of neural crest cells (NCCs), Schwann cell precursors (SCPs), and their role in embryogenesis base on genetic tracing and knock out model animals and single cell transcriptomic analysis. In particular, we summarize and analyze data on the contribution of NCCs and SCPs to the gland development and functions.

KEYWORDS

neural crest stem cells, pluripotency, cellular hierarchy, Schwann cells, gland development normal

Introduction

Multipotency of neural crest cells and Schwann cell precursors

The neural crest is a population of multipotent migratory cells that are detached from the border of the neural plate during neurulation and differentiate into cells of various organs in the adult organism ([Figure 1; Table 1](#)) ([His, 1868](#)). According to the New Head hypothesis, it was the appearance of the neural crest and epidermal placodes that led to the diversification and widespread distribution of chordates ([Gans and Northcutt, 1983; Martik and Bronner, 2021](#)). Neural crest cells undergo the stage of epithelial–mesenchymal transition and begin migrating to distant areas of the body. The contact of NCCs with growing peripheral nerves, as well as a change in the transcriptional signatures (*Snai1*, *Sox9/10*, *Foxd3*, *Pax3* and others for NCCs; *Sox10*, *Sox2*, *NRG1* for SCP), results in the formation of Schwann cell precursors, whose developmental direction depends on the diameter of the axon along which they migrate.

Depending on the place of their origin and directed migration, the entire population of neural crest cells (NCCs) can be divided into several groups: cranial, trunk, cardiac, and vagal NCCs ([Achilleos and Trainor, 2012](#)).

In mammals, cranial NCCs give rise to the cartilage and bone structures of the jaws and inner ear ([Couly et al., 1998; Freyer et al., 2011](#)). In addition, this cell population gives rise to the tooth's dentin, the bones of the frontonasal process, and the peripheral neurons and glia of the cranial nerves ([Le Lièvre, 1978; Chai et al., 2000; Méndez-Maldonado et al., 2020](#)). The skeletogenic potential of the cranial neural crest has been extensively studied and documented from vertebrates, although cells of the trunk neural crest may also contribute to skeletal components in some animals like the contribution of NCCs to the differentiation of the plastron bones (abdominal carapace bones) of the turtle *Trachemys scripta* ([Cebra-Thomas et al., 2007](#)). The cranial NCCs also differentiate into the Müller glia that play an

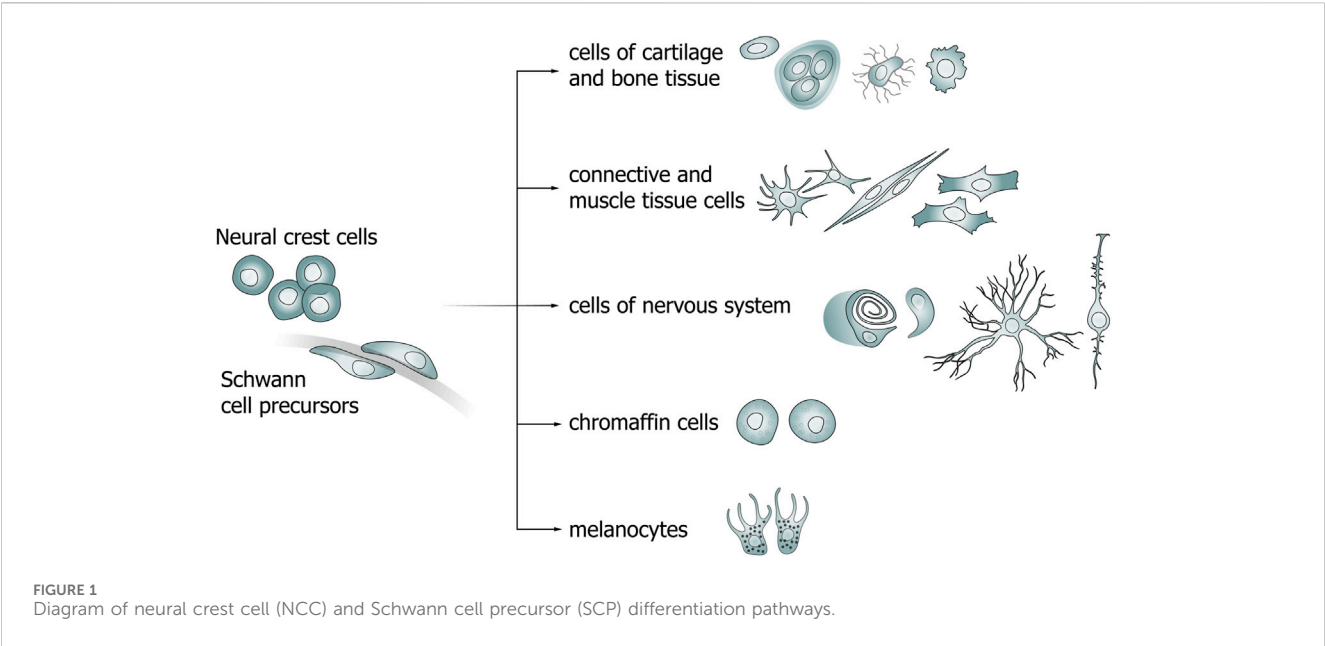


TABLE 1 Cell types derived from neural crest cells and Schwann cell precursors.

NCCs/SCPs descendants	References
Smooth muscle cells	Minoux et al. (2009) , Arima et al. (2012)
Chondrocytes and osteoblasts	Xie et al. (2019)
Melanocytes	Adameyko et al. (2009) , Kaucka et al. (2021)
Pulp cells and odontoblasts	Kaukua et al. (2014)
Sympathetic neurons	Kastriti et al. (2019)
Parasympathetic neurons	Dyachuk et al. (2014)
Sensory neurons	Ito and Sieber-Blum (1991)
Enteric neurons	Nishiyama et al. (2012) , Hutchins et al. (2018)
Promyelinating Schwann cells	Feltri et al. (2016) , Michailov et al. (2004) , Taveggia et al. (2005)
Remak cells	Monk et al. (2015) , Gomez-Sanchez et al. (2017)
Müller glia	Turner and Cepko (1987) , Wetts and Fraser (1988)
Neurons and glia in ovaries	McKey et al. (2019)
Glomus type I cells of carotid bodies	Hockman et al. (2018)
Chromaffin cells of adrenal medulla and organ of Zuckerkandl	Furlan et al. (2017) , Kamenev et al. (2021) , Kastriti et al. (2019)
Prostate neuroendocrine cells	Szczyrba et al. (2017)
Pericytes and all hormone-producing cell lineages of adenohypophysis	Ueharu et al. (2018) , Kato et al. (2021)
Perivascular cells in thymus	Foster et al. (2008)
Connective tissue of different glands	Nakamura (1982) , Suinara et al. (2000) , Müller et al. (2008) , Johansson et al. (2015) , Takahashi et al. (2014) , Jaskoll et al. (2001)

important role in the architecture and proper functioning of the retina ([Turner and Cepko, 1987](#); [Wetts and Fraser, 1988](#)). NCCs in the skull produce a variety of factors that regulate the proper development of brain regions ([Le Douarin et al., 2007](#); [Le Douarin et al., 2012](#)). The post-otic cranial NCCs form the aortopulmonary septum and also differentiate into smooth muscle cells of the third, fourth, and sixth arteries of the pharyngeal arches ([Minoux et al., 2009](#); [Arima et al., 2012](#)). Ablation of the post-otic part

of the cranial neural crest located above somites 1–5 showed that the thymus gland, parathyroid glands, and thyroid glands are underdeveloped or not formed (Bockman and Kirby, 1984). In this regard, the cells of the post-otic part of the cranial neural crest are suggested to play an important role in the morphogenesis not only of the cardiovascular system but also of the endocrine glands (Maeda et al., 2016).

The cardiac NCCs are involved in cardiovascular development in several ways: by remodeling the aortic arch arteries, by dividing the cardiac outflow tract, and by regulating the availability of factors required for normal aortic positioning and functional myocardial maturation in the caudal pharyngeal region (Lee and Saint-Jeannet, 2011). Ablation of the cardiac neural crest resulted in persistent truncus arteriosus (PTA), aplasia or hypoplasia of the thymus, thyroid, and parathyroid glands, and defects in the aortic arch arterial remodeling. Also, the ablation of the cardiac neural crest indirectly led to defects in myocardial function. In studies on quails, a clonal analysis of derivatives showed that cardiac neural crest cells can give rise to melanocytes (Adameyko et al., 2009), smooth muscle, chondrocytes (Xie et al., 2019), connective tissue, and sensory neurons (Ito and Sieber-Blum, 1991).

NCCs migrate to the areas of the forming organs (including the heart) and differentiate into intramural neurons and peripheral glia. Cells from the vagal, trunk, and sacral regions of the neural crest migrate into the intestinal wall, giving rise to enteric neurons and glia from all parts of the intestine. Using frame-by-frame imaging analysis of mouse enteric neural crest cells (ENCCs), Nishiyama et al. (2012) showed that a population of ENCCs moves from the midgut to the hindgut via the mesentery during embryogenesis and that such “transmesenteric” ENCCs make up a major part of the enteric nervous system of the hindgut. This migratory process requires GDNF signaling, and there is evidence suggesting that impaired transmesenteric migration of ENCCs may underlie the pathogenesis of Hirschsprung’s disease (intestinal agangliosis).

The vagal NCCs originating from the neck region colonize the intestine, forming ganglia of the enteric nervous system that control intestinal peristalsis (Hutchins et al., 2018). Chicken trunk NCCs showed the ability to give rise to teeth when implanted into the epithelium of the mouse mandibular arch (Lumsden, 1988). Furthermore, an increase in the level of the transcription factor (TF) microphthalmia (Mitf), which is key TF in melanocyte differentiation, in the trunk NCCs was observed on about day 9.5 of embryonic development, immediately prior to migration. The expression level of the melanogenic enzyme dopachrome tautomerase (Dct) was also found to be elevated in these cells. This enzyme is a marker of melanocytes at all stages, including melanoblasts (MacKenzie et al., 1997; Goding, 2000; Nishimura et al., 2002; Adameyko et al., 2009).

One of the ways by which NCCs differentiate is becoming Schwann cell precursors (SCPs) that also have the ability to differentiate into a wide range of cells (Figure 1) (Furlan and Adameyko, 2018; Kastriti et al., 2022). Primarily, SCPs give rise to myelinating and non-myelinating Schwann cells in the peripheral nervous system (PNS). The transition from NCCs to SCPs is a consequence of the interaction of the former cells with axons (Monk et al., 2015). The molecular mechanisms that direct a portion of the SCPs population to peripheral nerves and the causes of selection of

specific nerve types are not fully understood. The transition from SCPs to immature SCs (imSCs) is accompanied by an important mechanistic step involving the selection of axons for myelination (radial sorting) of imSCs which, in turn, differentiate into promyelinating SCs (Feltri et al., 2016). The remaining imSCs are connected to axons of smaller diameters and differentiate into non-myelinating SCs (Remak cells) (Monk et al., 2015; Gomez-Sanchez et al., 2017). The choice of imSCs to differentiate into myelinating or non-myelinating SCs is determined by the expression levels of neuregulin 1 (NRG1). While lower levels of NRG1 released by a relatively smaller axon lead to the maturation of imSCs into non-myelinating SCs, higher levels lead to the development of myelinating SCs (Michailov et al., 2004; Taveggia et al., 2005; Gomez-Sanchez et al., 2017).

In addition, SCPs are capable of transforming into neurons of internal organs (e.g., intramural neurons of the intestine). It is known that SCPs and Schwann cells originating from the cranial region of the neural crest, forming the dental mesenchyme, take part in the development, growth, and regeneration of teeth. Moreover, SCPs, like NCCs, contribute to the formation of many parts of the skeleton (e.g., mandible and ribs), as well as nasal and auricular cartilage (Xie et al., 2019).

Evidence confirming the ability of SCPs to specialize into other cell types has been obtained by demonstrating their differentiation into melanocytes of the skin (Adameyko et al., 2009) and into extracutaneous melanocytes of the heart, inner ear, and brain membranes (Kaucka et al., 2021). Quite a large number of studies confirm the contribution of SCPs to the formation of melanocytes in various body structures. For example, after the induction of recombination between stages E9.5 and E10.5 in adult mice of the Plp1^{CreERT2}/R26^{YFP} line, fluorescent signal was detected in the brain membranes, spinal ganglia, spinal cord and brain, and in the supraorbital spaces between the eyeballs. The same team of researchers analyzed internal organs of several fish and amphibian species for the presence of extracutaneous melanocytes and found an association of pigment cells with blood vessels and nerves.

As shown in a study on SCPs *in vitro*, these cells can differentiate into melanocytes if the activity of protein kinase C changes (Hess et al., 1988). There was also a study on NCCs of avian embryos which showed that endothelin 3 *in vitro* promotes the transition of Schwann cells into melanocytes (Dupin et al., 2003). The authors suggested that glial cells and melanocytes share a common precursor. They also stated that glial cells and pigment cells that originated from NCCs are phenotypically unstable *in vitro* and can revert to a bipotential precursor state. Another study on avian NCCs showed that bFGF can promote transdifferentiation of adult Schwann cells into melanocytes (Sherman et al., 1993). It is important to note that the authors used HNK-1 as a marker of Schwann cells with the neural crest origin. Subsequently, it turned out that this molecule cannot be used as a marker of neural crest cells and, therefore, the results obtained require additional verification.

Another example of multipotency of SCPs is their ability to give rise to parasympathetic neurons (Dyachuk et al., 2014). Genetic tracing experiments on transgenic mice, performed by two independent research teams, have convincingly demonstrated that parasympathetic neurons in cranial ganglia, intramural

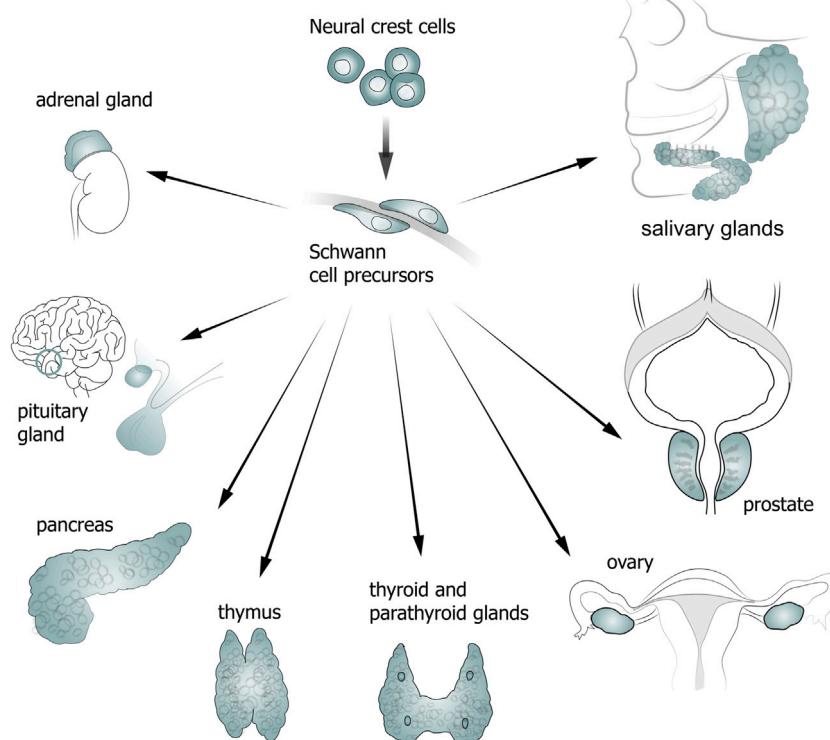


FIGURE 2
Diagram showing contribution of neural crest cells (NCCs) and Schwann cell precursors (SCPs) to gland development.

(interstitial) ganglia of the heart, and sacral parasympathetic ganglia after E12.5 originate from nerve-associated SCPs (Dyachuk et al., 2014; Espinosa-Medina et al., 2014). SCPs are capable of differentiating into enteric neurons during postnatal neurogenesis (Uesaka et al., 2015). Tracing using lipophilic dyes and the Sox10Cre inducible system in a study on *D. rerio* has shown that postembryonic enteric neurons arise from SCPs originating from the neural crest that migrate from the spinal cord to the intestine in this fish (El-Nachef and Bronner, 2020). Furthermore, SCPs can function as a source of mesenchymal cells that produce pulp cells and odontoblasts, as observed in a growing mouse tooth model (Kaukua et al., 2014). In addition to the above-mentioned cell types, neuroendocrine cells of the adrenal medulla (chromaffin cells) have also been shown to originate from the SCPs in mouse embryos and larvae of zebrafish, *D. rerio* (Furlan et al., 2017; Kamenev et al., 2021).

Moreover, it is known that chromaffin cells of the organ of Zuckerkandl and a portion of sympathetic neurons of the posterior paraganglia are largely derived from SCPs (Kastriti et al., 2019). A genetic tracing study has shown that SCPs are involved in the formation of glomus type I cells (primary oxygen-sensitive cells) of carotid bodies (Hockman et al., 2018). Also, a genetic tracing has recently shown that some SCPs are detached from nerve fibers and become mesenchymal cells that further differentiate into chondrocytes and mature osteocytes during the embryonic development in mice. Furthermore, in *D. rerio*, the chondrocyte development is also known to originate from SCPs, indicating that this process is evolutionarily conserved (Xie et al., 2019).

Contribution of neural crest cells and Schwann cell precursors to gland development

NCCs and SCPs play an important role in the development and functioning of various glands (Figure 2). Thus, a chimeric model of chicken/quail embryos has shown that most of the loose connective tissue of salivary glands originates from the neural crest (Nakamura, 1982). In addition, descendants of NCCs are found in the interlobular space and brain matter of the thymus, and also in the connective tissue of parathyroid glands (Sunjara et al., 2000; Müller et al., 2008; Johansson et al., 2015).

NCC descendants in thymus

The thymus has NC-derived mesenchyme which plays a crucial role in the growth and differentiation of glandular epithelium and T cells (Bockman and Kirby, 1984; Foster et al., 2008). In 12-day mouse embryo, NCCs migrated to the thymus region. Ablation of the neural crest in a region between the otic placode and the posterior part of somite 3 prevented the thymus formation or caused its underdevelopment. As the study showed, the number of cells positive to neural crest markers is reduced in thymus tissues, and the size of the forming thymus is directly related to the number of NCCs (Yamazaki et al., 2005).

Removal of perithymic mesenchyme from 12-day mouse embryo lobes blocked most lymphocytes at the CD4⁺CD8⁺ stage of development. However, if the thymus lobes were left intact,

generation of all T-cell subtypes occurred in them *in vitro*. Suniara and co-authors showed that mesenchymal cells directly regulate lymphopoiesis and that a lack of the intrathymic network of NC-derived fibroblasts and their process of extracellular matrix formation in the thymus leads to a decrease in lymphoid tissue development. Furthermore, it is the extracellular matrix that is necessary for integrin and/or cytokine interactions during the thymus development (Suniara et al., 2000).

Yamazaki and co-authors reported that multipotent cells originating from the neural crest are found in the embryonic thymus at stages E14.5 and E15.5, but are absent from the thymus at stage E17.5. The authors concluded that the presence of NC-derived cells in the thymus is important for its early embryonic development (Yamazaki et al., 2005).

In 2008, Foster and co-authors used $Wnt1^{Cre}R26^{eYfp}$ and $Sox10^{Cre}R26^{eYfp}$ transgenic mice to show that NC-derived cells populate the thymus primordium and remain in it during the embryonic development and until 9–10 months of age. In addition, the authors showed that NC-derived cells differentiate into perivascular cells involved in the formation of the blood–thymus barrier (Foster et al., 2008).

In the same year, Müller and co-authors also found NC-derived cells in the adult thymus. NC-derived mesenchyme, according to the authors, may take part in performing specific functions of the blood–thymus barrier in the adult thymus, and are also part of the microenvironment providing maintenance of migrating cells (Müller et al., 2008).

Myoid cells of the thymus are suggested to originate from the neural crest. This hypothesis was advanced by Nakamura and Ayer-Le Lière (1986) whose study, as well as the first studies by Nicole Le Douarin on the neural crest, was based on a hybrid model of chicken embryo, where part of NCCs were replaced by NCCs of quail at the same stage of development. The NC origin of myoid cells in birds was confirmed subsequently, in 2015 (Bódi et al., 2015).

NCC descendants in thyroid and parathyroid glands

Early studies showed that calcitonin-producing thyroid cells originate from NCCs (Le Lievre and Le Douarin, 1970; Le Douarin and Le Lievre, 1970). This seminal study of neural crest cells, where the authors claimed that NCCs carry out invasion of ultimobranchial bodies prior to their fusion with the thyroid gland bud, was performed on chimeric avian embryos.

However, until 2007, there was no evidence whether the same process occurs in mammalian embryonic development. Kameda and co-authors showed that neither ectomesenchymal cells nor neural crest neuronal cells invade the ultimobranchial body primordium at stages E11.5–E12.5 (Kameda et al., 2007).

In 2015, Johansson and co-authors, using a genetic mouse model, confirmed and extended the previous idea that $Wnt1^{+}$ NCCs contribute to the thyroid connective tissue development but are not a source of C cells in the mouse embryonic thyroid. Genetic tracing allowed the authors of this study to conclude that C cells are of endodermal origin (Johansson et al., 2015).

NCC descendants in pituitary gland

The anterior part of the pituitary gland originates from the adenohypophyseal placode (Ueharu et al., 2017). It was long believed that NCCs differentiate into interstitial cells of the adenohypophysis. The presence of interstitial cells (from NCCs) in the developing anterior lobe of the pituitary gland was analyzed using chimeric chicken/quail embryos (Couly and Le Douarin, 1987). Ablation of NCCs using the $Wnt1^{Cre}$ and $P0^{Cre}$ mouse strains showed that these cells are involved in pituitary vascularization, thus, giving rise to pericytes (Davis et al., 2016). The contribution of NCCs to the cerebral vasculature was previously described from mice by several research teams using the $P0$ -Cre and human tissue plasminogen activator (HtPA)-Cre reporter lines (Pietri et al., 2003; Nishiyama et al., 2012).

Ueharu et al. (2018), using genetic tracing in a mouse $P0$ -Cre/EGFP model, showed that NCCs invade the adenohypophysis in several stages (waves). In a study by Kato with co-authors based on a $S100\beta$ /GFP-TG rat model, NCCs populated the pituitary gland in several successive waves (Kato et al., 2021). The first wave was detected in mice at stage E9.5, when the pituitary primordium begins to form with adenohypophyseal placode cells; the second wave of invasion was observed at stage E14.5, when vasculogenesis proceeds from the Atwell's recess. Genetic tracing of NCCs showed that they are capable of differentiating into both pericytes and all hormone-producing cell lineages of the adenohypophysis. According to the results of this study, NCCs contribute to pituitary organogenesis and vasculogenesis together with placode cell derivatives. In addition, the same team of researchers, using a different model, confirmed that SOX10-positive cells found in the pituitary gland are positive to another marker of NCCs, p75NTR. Immunohistochemical staining with antibodies to SOX10 during the pituitary development demonstrated the spatial and temporal pattern of localization of SOX10-positive cells in the posterior, intermediate, and anterior lobes (Ueharu et al., 2018).

NCC descendants in pancreas

As Kirchgessner and co-authors showed on a model of rat intestine and pancreas explants, NCCs migrate to the intestine, and only after that continue their migration to the pancreas, where they give rise to intrapancreatic ganglion neurons (Kirchgessner et al., 1992). It was also reported that NCCs migrating into the pancreas differentiate into sympathetic, parasympathetic, and sensory neurons (Ahren, 2000).

Descendants of NCCs are known to be involved in pancreatic development. Smith (1975) showed that these cells are capable of differentiating into Schwann cells that surround the islets of Langerhans.

Signals from NCCs regulate the mass proliferation and maturation of pancreatic beta cells (Nekrep et al., 2008; Plank et al., 2011). Co-culturing of NCCs and pancreatic islets *in vitro* promoted the regeneration of functional beta cells (Olerud et al., 2009). NCC descendants migrating to the pancreatic region may be important for maintaining beta cell function, development, and maturation. Shimada et al. (2012) reported that NCC descendants maintain beta cell signaling at each stage of the pancreatic development. An earlier study showed that by 15.5 days post-coitum, neural crest-derived neurons were localized close to insulin-expressing clusters of cells and then contacted 98.9% of

beta cell clusters on postnatal day 1 (Plank et al., 2011). However, the results obtained by the team of Shimada with co-authors showed that neural crest derivatives at late stages of embryonic development are closer to alpha cells than to beta cells in the pancreas. According to them, NCC derivatives surround the endocrine cells of the pancreas and influence them through juxtacrine or paracrine regulation (or both) (Shimada et al., 2012).

A number of studies carried out in the 1970s to elucidate the possible embryonic origin of pancreatic endocrine cells from NCCs were largely based on chimeric chicken/quail embryo models. Pictet et al. (1976), who investigated the origin of the islets of Langerhans, cultured rat embryos *in vitro* at early stages of development after removal of the neuroectoderm primordium. Interestingly, the pancreas developed under these conditions, and insulin-producing cells were observed. According to Andrew (1976) who used the chimeric model described above, neuroectodermal cells from the trunk never migrate to the pancreas. Andrew et al. (1986) and Fontaine and Le Douarin (1977), based on the same chimeric model, showed that NCCs migrating to the pancreas originated from the vagal region but did not differentiate into insulin-, glucagon-, somatostatin-, or avian pancreatic polypeptide- (APP) producing cells.

NCC descendants in prostate

In 1999, Aumüller and co-authors found for the first time that human prostate neuroendocrine cells have a neurogenic origin, distinct from that of secretory and basal cells of the gland (Aumüller et al., 1999). Using a Wnt1-Cre/ROSA26-YFP mouse model and abortive human material, Szczyrba and co-authors showed that 64% of mouse prostate neuroendocrine cells originate from NCCs (Szczyrba et al., 2017).

NCC descendants in sex glands

Single NCCs colonize the ovary at around E16.5 and differentiate into neurons and glia that give rise to the entire ovarian neural network. In contrast, NCCs do not infiltrate the testis. Theca cells, derived from the mesenchyme and steroidogenic cells of the ovary, also migrate into the ovary between E17.5 and P5 in a pattern very similar to that of the ovarian innervation development. In the ovary, nerve projections are located in close proximity to the theca cell layer of growing follicles, where they may be involved in stimulating theca or smooth muscle cells during the follicle growth and ovulation (McKey et al., 2019).

NCC descendants in adrenal glands and organ of Zuckerkindl

Chromaffin cells of the adrenal medulla originate from Schwann cell precursors (SCPs) that are descendants of NCCs (Furlan et al., 2017).

SCPs migrate along axons of cholinergic preganglionic neurons in the spinal cord, moving towards the developing sympathoadrenal primordium (Lumb et al., 2018). Upon reaching the rudiment (stages E11.5 and E12.5 in mouse), the SCPs begin to differentiate into chromaffin cells of the adrenal medulla, passing through a

transition state referred to as the “bridge”. It is worth mentioning that most SCPs do not transdifferentiate subsequently but continue the transformation into satellite glial cells located next to neuronal bodies in peripheral ganglia or into Schwann cells covering peripheral nerves. A little later in development, some of the immature, nerve-associated Schwann cells begin to myelinate, while the rest remain dormant on nerves as Remak cells or non-myelinating Schwann cells (Jessen and Mirsky, 2005; Jessen et al., 2015).

A study based on transgenic mice showed that organ of Zuckerkindl cells can also originate from nerve-associated SCPs, similarly to adrenal chromaffin cells (Furlan et al., 2017).

NCC descendants in submandibular salivary glands

Cells originating from NCCs were found in the submandibular salivary glands of adult mice. These cells form a kind of “islets” inside the glands and express various markers characteristic of NCCs such as Sox10 and Ednrb. Furthermore, as was shown on a Wnt1-Cre/R26R lacZ mouse model, the mesenchyme of embryonic submandibular salivary glands originates entirely from the cranial region of the neural crest (Jaskoll et al., 2001; Takahashi et al., 2014).

Conclusion

Neural crest cells (NCCs) are often referred to as the “fourth germinal layer” because of their broad potential to differentiate into a variety of adult cells, ranging from mesenchymal cells to various types of neurons. The emergence of NCCs in the course of evolution has contributed to the generation of a large number of new structures or to the increased complexity of existing ones in chordate organisms. NCCs take an active part in the development of various systems of the body, including the formation of endocrine glands, regulating their proper embryonic development and giving rise to neuroendocrine cells, neurons, and various types of glial cells. Schwann cell precursors, being also multipotent descendants of NCCs, are involved in the gland formation, but many questions as to their role in endocrine embryogenesis remain unaddressed. Despite the ongoing active research in the field of genome evolution and the development of the New Head hypothesis, the origin and evolution of the neural crest and its descendants remains not entirely clear.

Understanding the cellular mechanisms of various organs formation during embryonic development is extremely important for compiling a complete picture of normal embryogenesis, as well as for identifying targets in the treatment of diseases of the adult body and searching for a cellular source for replacement therapy. NCCs and SCPs are among the key participants in the formation of various parts of the nervous and endocrine systems, which makes them an important object for further study.

Author contributions

AK: Data curation, Investigation, Methodology, Visualization, Writing—original draft, Writing—review and

editing, VD: Conceptualization, Funding acquisition, Project administration, Supervision, Writing—original draft, Writing—review and editing.

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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.

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Inter-kingdom communication and the sympoietic way of life

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Organisms are now seen as holobionts, consortia of several species that interact metabolically such that they sustain and scaffold each other's existence and propagation. Sympoiesis, the development of the symbiotic relationships that form holobionts, is critical for our understanding the origins and maintenance of biodiversity. Rather than being the read-out of a single genome, development has been found to be sympoietic, based on multigenomic interactions between zygote-derived cells and symbiotic microbes. These symbiotic and sympoietic interactions are predicated on the ability of cells from different kingdoms of life (e.g., bacteria and animals) to communicate with one another and to have their chemical signals interpreted in a manner that facilitates development. Sympoiesis, the creation of an entity by the interactions of other entities, is commonly seen in embryogenesis (e.g., the creation of lenses and retinas through the interaction of brain and epidermal compartments). In holobiont sympoiesis, interactions between partners of different domains of life interact to form organs and biofilms, wherein each of these domains acts as the environment for the other. If evolution is forged by changes in development, and if symbionts are routinely involved in our development, then changes in sympoiesis can constitute an important factor in evolution.

KEYWORDS

symbiosis, sympoiesis, holobiont, development, evolution, cooperation

1 Introduction: our holobiont heritage

Evolutionary developmental biology is charged with describing and appreciating the mechanisms by which selectable variation is generated, maintained, and propagated. It is the science directly concerned with the origins of the planet's biodiversity. But to do this, it must first have an understanding of what it is that changes. That is to say, if evo-devo is to explain organismal diversity, it must know what an organism actually is. The concept of organism has evolved in a new direction during the past 20 years. Keller (2002) characterized the 20th century as "the century of the gene," but the twenty-first century may become identified as the century of the holobiont relationship. We have become cognizant that multicellular organisms are not (and have never been) biological individuals (Gilbert et al., 2012; McFall-Ngai, 2024). Rather than being the read-out or epiphenomena of genes, our anatomy, physiology, immunity, development, mental health, and evolution are now seen to be performed in concert with other symbiotic organisms. I will argue that appreciating that developing organisms are holobionts (the organism as consortium of symbionts) has important consequences for modeling development, evolution, and the history of life.

"Organisms are holobionts, and life is sympoietic," is a statement that could not have been made from 20th century biology. The term "holobiont" refers to the scientific conclusion that organisms are integrated consortia of a host organism plus numerous

species of other symbiotic organisms (Zilber-Rosenberg and Rosenberg, 2008; Theis et al., 2016)¹. In the adult human body, microbes account for approximately half of our cells (Sender et al., 2016). Moreover, these bacteria, fungi, protists, and archaea are not just travelling in our body and sharing our food. They are critical for our healthy physiology, development, and immunity (Gilbert et al., 2012; McFall-Ngai et al., 2013). Cows, for example, may be herbivores; but there are no genes in their bovine nuclei that encode grass-digesting enzymes. These cellulose-digesting enzymes come from the set of microbes living within the rumen of the cattle's guts (Moraïs and Mizrahi, 2019a). In coral, most of the animal's carbon resources are derived from the photosynthetic reactions of its algal symbionts (Muscattine et al., 1984). Symbiotic fungi extend the roots of plants, allowing them to get water and nutrients more efficiently. These fungal extensions may have been important for permitting plants to adapt to land (Pirozynski and Malloch, 1975). Multicellular organisms exist as multi-species consortia.

Moreover, even “unicellular organisms” are not truly unicellular. Rather, eukaryotic protists (amoebae and diatoms, for instance) are holobionts and are associated with microbial symbionts (Vincent et al., 2018; Kano et al., 2021; Colp and Archibald, 2021). The protist *Mixotricha paradoxa* is largely responsible for synthesizing the lignin-digesting enzymes that permit certain termites to metabolize wood. Only, *Mixotricha* is not a single cell, but a protist whose eukaryotic cell is part of a consortium that includes thousands of bacteria, comprising at least four different species (Margulis and Sagan, 2001). With few (if any) exceptions, animals and plants are holobionts, federated partnerships of numerous species functioning together to generate a healthy organism² (Zilber-Rosenberg et al., 2008; Theis et al., 2016; Roughgarden et al., 2017). An organism is not a monoculture of genetically identical cells.

We now understand, that metabolism is a communal enterprise (Dupré and O'Malley, 2009; Kelty, 2019). In holobionts, the set of symbiotic organisms (including the “host”) participate in each other's metabolisms. In the mealy bug *Planococcus*, the production of the amino acid phenylalanine begins with enzymes encoded by the bug's symbionts, *Tremblaya* bacteria. The metabolites generated by these enzymes then travel into another bacterial species, *Moranella*, which are symbionts within the *Tremblaya* bacteria. The newly made metabolites from *Moranella* then return to the *Tremblaya* bacteria, wherein they are converted into a product that can be metabolized into phenylalanine by

enzymes encoded by the *Planococcus* genome (McCutcheon and von Dohlen, 2011).

In mammals, bacterial products are sent through the circulation of the body, where they sustain digestion, blood circulation, and other physiological functions (McFall-Ngai et al., 2013; Kelty, 2019). In this way, murine gut microbes convert dietary tryptophan into indole, which circulates through the blood and enters the hippocampus. Here, it activates the aryl hydrocarbon receptors of the neural stem cells, converting the receptor into a functional transcription factor that activates the genes responsible for generating neurons. Since these neurons are thought to be critical for memory and learning, the “gut microbes are an evolving, prokaryotic component of the meta-organismal self” (Wei et al., 2021, p. 2). The gut neurons of the esophagus are also beneficiaries of bacterial products. These neurons create the peristaltic waves of muscle contractions that transport food from the throat to the stomach. However, at birth, these neurons are immature. The bacteria produce products (probably short-chain fatty acids) that induce the intestinal cells to synthesize and secrete the hormone serotonin. The serotonin promotes the maturation of the immature neurons and allows efficient peristalsis (De Vadder et al., 2018; Liu et al., 2024).

In 2013, Smith and colleagues coined the term “co-metabolism” to indicate that foods are being metabolized by both host enzymes and microbial enzymes, and that the products of one are often substrates for the other. We have an entangled metabolism. We are not merely organisms; we are biomes, collections of ecosystems (Gilbert, 2019; Suarez and Stencel, 2020). Our development, then, includes both embryogenesis and ecological succession (Blaser and Kirschner, 2007; Gilbert, 2023).

2 Transmission of symbionts

In most all organisms, symbionts are needed to establish or complete normal development. Therefore, the transmission of symbionts from one generation to the next is a crucial feature of their life cycle. Animals receive their first set of microbes in many ways (Funkhauser and Bordenstein, 2013; Theis et al., 2016; Roughgarden et al., 2017). Those organisms using intra-organismal transmission transfer symbionts directly from parent to offspring. Other organisms use intimate neighborhood transmission, wherein a parent provides symbionts as resources at birth or shortly thereafter. In addition, some species utilize a horizontal transmission of symbionts, where the offspring are not given symbionts but inherit a means by which they can select them from the environment. Most organisms probably use combinations of these mechanisms over their lifespan (see Bright and Bugheresi, 2010).

2.1 Intra-organismal transmission

Those organisms using the intra-organismal transmission methods can impart symbionts to their offspring through: 1) vegetative reproduction, 2) oocytes, or 3) embryos. In hydra, budding allows the generative fragments to contain both the nucleated cells and bacterial symbionts (Minten-Lange and Fraune, 2021). In *Drosophila*, for instance, the *Wolbachia* that provide the *Drosophila* fly with immunity against viruses (Hedges et al., 2008; Teixeira et al., 2008) become concentrated in the posterior pole of the embryo, the region that will become the oocytes and the trophocytes

¹ As we will see, Haraway (2016) has pointed out that the host is also in a symbiotic relationship and is therefore another symbiont. It is just larger.

² Some animals have been reported to lack symbiotic partners. While rare, these would not be unexpected. In many instances, horizontal gene transfer has given animals the genes that had made their symbionts essential. Two groups of beetles, for instance, have acquired fungal genes whose protein products digest plant cell walls (Kirsch et al., 2014). In at least one instance of an animal devoid of internal symbionts, external bacteria are critical in propelling the organism from the larval to adult stages of its life cycle (Vijayan et al., 2018; Freckleton et al., 2022).

(“nurse cells”) that feed the oocytes. These bacteria are transported (along the same cytoskeletal pathways as mitochondria, mRNA, and ribosomes) from these gonadal cells into the oocyte, from which they will spread throughout the body (Ferree et al., 2005; Newton et al., 2015). In pea aphids, the obligate symbiont *Buchnera* is transmitted by exocytosis from the mother’s gut into the posterior region of the developing embryo (Koga et al., 2012).

Since bacteria are transmitted to the next-generation only by eggs, several *Wolbachia* species can increase female fecundity (and hence its own propagation) by causing the oocyte precursor cells to undergo extra divisions (Fast et al., 2011; Guo et al., 2020). Moreover, in some insects, *Wolbachia* can even transform male embryos into female embryos, thus continuing its propagation (Ote and Yamamoto, 2020).

The poster-organisms for holobionts, the lichens, emerge from a photosynthetic organism (the “photobiont,” an algae or cyanobacteria) engaging in mutualistic symbiosis with a fungus (often called the “mycobiont” partner). The photosynthesis of the photobionts produce carbohydrates that benefit the fungus, while the photobiont benefits from the fungal hyphae that anchor them to and accumulate moisture and nutrients from the environment. When undergoing asexual reproduction, pieces of existing lichen break off, and all the species travel together. In some species, small groups of algae (and perhaps yeasts) are enveloped in a fungal basket (a soredium) and dispersed. Once the soredium settles, a new lichen can form. Other asexual lichens elongate fungal outgrowths (isidia) that are dispersed to provide a fungus that readily recruits photobionts. Sexual reproduction also occurs in the fungi of most lichen species to form haploid fungal spores. When these disperse and germinate, they initiate a new fungal colony that can recruit photobionts. Remarkably, it is not difficult for fungi and algae to find each other (Hom and Murray, 2014), and this may be an important feature in expanding the domain of living organisms.

2.2 Intimate neighborhood transmission

2.2.1 Coprophagy

One of the most efficient ways of transmitting symbionts from parent to offspring is by coprophagy—the ingestion of feces (Linaje et al., 2004; Kovacs et al., 2006). Many insects lay eggs in or on their feces, which the larval offspring consume upon hatching (Blum et al., 2013; Jahnke et al., 2021). This is especially important for herbivores, since the feces that the larvae eat are comprised mostly of cellulose. Most insects do not have the genes that synthesize cellulose-digesting enzymes, and such fecal pellets lack most essential amino acids. These enzymes and amino acids are produced by the microbes. In many dung beetles, the mother rolls mammalian dung into a ball, buries the ball, and lays an early embryo atop it. But between the dung ball and the embryo she excretes a *pedestal*, a pellet of her own feces that contains the microbial symbionts that can digest the plant-containing dung dropped by the herbivores (Ledón-Rettig et al., 2018; Rohner and Moczek, 2024).

The squash bug *Anasa tristis* requires the bacterial symbiont *Caballeronia* for normal growth and development (Acavedo et al., 2024). While the squash bug is born without these symbionts, it acquires them as immature newly hatched nymphs. The nymphs can accurately smell the presence of *Caballeronia*-containing feces left by adult squash beetles. Moreover, using fluorescently-tagged microbes,

it was shown that this preference was specifically for the *Caballeronia* bacteria and not for a species-specific component of the feces.

Coprophagy can take many forms. In some species of termites, workers feed newly hatched juveniles the feces of adults (a process that has been given the wonderful name, proctodeal trophallaxis) (Brune and Dietrich, 2015). Koalas use a special adaptation of coprophagy (Osawa et al., 1993) wherein the mother feeds her infant a mixture of milk and feces, which she smears on their faces. The microbes are able to colonize the gut, permitting the newly weaned joey to digest eucalyptus leaves. Some species of caecilians, a legless amphibian group, transmit their symbiotic bacteria through their skin. The females tend their newborns, and the newborns then eat their mother’s particularly fatty skin. The young amphibians thereby acquire nutrition and a set of symbiotic microbes on their skin and in their gut (Kouete et al., 2023).

2.2.2 Vaginal delivery

In humans and most other mammals, a starter set of microbes colonizes our bodies as the fetus passes through the birth canal. During the third trimester of pregnancy, particular species of microbes are selected in the female reproductive tract and anus. Many of these are spore-forming bacteria that can withstand the different conditions they will encounter (Browne et al., 2016), and this property facilitates transmission. These bacteria appear to help a pregnant woman adapt to the physiological stresses of carrying a fetus and also establish the conditions that enable other bacteria to find niches in the intestines (Koren et al., 2012). In humans, there is a remarkable “crosstalk between our human “first genome” and microbial “second genome.” Wacklin et al., 2011; Zhernakova et al., 2024), and expression of the *ABO* (blood group) and *FUT2* (secretor) genes allows the gut epithelium to select for different bacterial groups (Rausch et al., 2017; Rühlemann et al., 2021).

In addition, mammalian milk provides microbes and a microbe-promoting diet from their mothers. Milk provides an additional route for microbes to travel from parent to offspring (Jin et al., 2011; Jost et al., 2013; Addis et al., 2016). Human milk contains about 10⁵ bacteria per ml, and it comprises hundreds of species. The DNA of bacterial strains isolated from mother’s milk appears very similar to the DNA found in the offspring’s gut (Milani et al., 2015). In addition to providing bacteria, mother’s milk contains complex oligosaccharides that support the growth of the major group of these bacteria, *Bifidobacterium*, but which are not digestible by the infant or by other bacteria (Sela et al., 2011). *Bifidobacterium* contains genes encoding the enzymes that digest these oligosaccharides (Garrido et al., 2016 (Zivkovic et al., 2011; de Muinck and Trosvik, 2018);³. Suggesting that symbiotic bacteria and their human host co-evolved. The gut microbiome will continue

³ *Sympoiesis* is a concept in developmental biology referring to developmental inputs from several sources. We are not merely the products of the zygotic cells; we are created “together” with the help of other species. This has been confused with the notion of *symbiogenesis*, which is a concept in evolutionary biology meaning that evolutionary events such as speciation and the formation of the first cells arose through the acquisition of new genomes (rather than by the mutation of existing ones).

to expand as the baby becomes part of a complex environment (Kort et al., 2014; Milani et al., 2017; Stewart et al., 2018).

2.3 Horizontal acquisition

Last, symbionts can be acquired from the environment. This probably happens in most animals, but it is critical for those animals whose development requires these organisms that are not supplied by their parents. These animals inherit the ability to recognize and respond to particular environmental microbes, which become affordances⁴ for normal development. This “horizontal” or “environmental” recruitment of symbionts also allows juvenile animals to use microbes from the environment to create diverse phenotypes that depend on the symbiont.

The Hawaiian bobtail squid, *Euprymna scolopes*, is the best studied organism for the horizontal transmission of symbionts. A 2-inch long swimmer of shallow Hawaiian waters off Hawaii, this nocturnal squid preys on shrimp. However, predatory fish can readily see the squid if the moon casts the squid’s shadow on the sea floor. *Euprymna scolopes*, has solved this problem by developing a ventral light that can shine on the seafloor and hide its shadow from potential predators. Making this light organ requires the help of the symbiotic bacterium *Vibrio fischeri*. Indeed, it is the bacteria that glow. It has to collect these bacteria, and the bacteria have to build the light organ. Other species of bacteria in the sea water attempt to stick to the squid, but the squid repels these bacteria species with toxic mucus, acid, and nitric oxide (Troll et al., 2010; Wang et al., 2010; Schwartzman et al., 2019). *Vibrio fischeri* survive these chemicals and induce gene expression changes in the squid epithelial cells, allowing the *V. fischeri* to aggregate (Altura et al., 2013).

Once aggregated, the bacteria migrate inward through a gradient of chitobiose made by the epithelial cells (Mandel et al., 2012; Kremer et al., 2013). Inside the crypts, the bacteria’s bioluminescence is stimulated through a quorum-sensing feedback loop. At critical densities, the bacteria produce two molecules that induce transcription of their own *lux* genes, which are the genes responsible for luminescence (Visick et al., 2000; Millikan and Ruby, 2001). Thus, the light organ is a product of inter-kingdom communication.

2.4 Community ecology as part of holobiont development

While embryonic development and organismal succession are usually studied in the separate fields of developmental biology and ecology, respectively, they are united in the holobiont. If we take seriously the idea that organisms are both individuals and collections of ecological communities, then we have to realize

that animals are simultaneously the products of embryological development and ecological succession (Gilbert, 2023). Indeed, Skillings (2016) maintains that “most holobionts share more affinities with communities than they do with organisms.”

Ley and colleagues (2007, p. 3) note, “When a new human being emerges from its mother, a new island pops up in microbial space.” And if we are islands popping up in microbial space, then the colonization and succession patterns of island biogeography prevail. We should therefore look at mammalian development in terms of dispersal, local diversification, environmental selection, and ecological drift (Costello et al., 2012). Our various microbiomes have ecological succession (Gonzales et al., 2011; Bordenstein and Theis, 2015; Ratsika et al., 2022), resulting from a combobulation of several factors (Milani et al., 2017), including the bacteria available for colonization (Wampach et al., 2018; Shao et al., 2019), the diet that preferentially enables the proliferation of certain microbes (Carmody et al., 2015; Rothschild et al., 2018), and the genotype of the organisms that provide the environment for the microbes⁵ (Goodrich et al., 2014; Brooks et al., 2016; Kurilshikov et al., 2021; Zhernakova et al., 2024).

Breast-fed and formula-fed macaque monkeys, for instance, generate different communities of gut microbiota, and the microbiota of such breast-fed monkeys are more capable of producing the lymphocytes responsible for eliminating opportunistic pathogens (Ardeshir et al., 2014). Here, diet produces two alternative equilibrium states with different capacities. In other cases, different populations of microbes act to perform similar functions (Doolittle and Inkpen, 2018). Different herds of cattle have different symbiotic populations of microbes in their rumen. Although each of these microbial communities allow sympoiesis and nutritional symbiosis, they can have different phenotypes. Some of these communities, for instance, produce higher level of methane gas than do others (Moraïs and Mizrahi, 2019). Some microbes are generalists and will colonize numerous species, whereas other microbes are specialists that recognize species-specific genetic markers on particular hosts (Theis et al., 2016; Lim and Bordenstein, 2020).

3 Sympoiesis

3.1 Generalized sympoiesis

The example of *Euprymna/Vibrio* brings us to sympoiesis, the mutualistic partnerships to generate a phenotype, a “making-with” process whereby development occurs as a team activity. Indeed, as important as symbiosis is to the adult organism, such symbioses are not just between mutually consenting adults. Symbiosis occurs during development to *generate* the adult. Sympoiesis refers to the observation that multicellular eukaryotic organisms use

⁴ When the organism evolves such that something in its environment can now be utilized, the newly perceived item is sometimes called an “affordance,” since the environment *affords* a different opportunity for exploitation (Walsh, 2015).

⁵ This could be the cell surfaces of the “host” or the community of other microbes that support the membership of a bacterium within a biofilm. Blaser and Kirshner (2007) propose that cross-signaling relationships between humans and their microbes constitute evolutionary stable strategies for holobiont development.

symbiotic microbes to co-construct organs. It is a critique of autopoiesis, the idea that organisms are self-generating (Dempster, 1998; Haraway, 2016; Clarke and Gilbert, 2022).

Sympoiesis should be familiar to embryologists. When the bulge from the brain meets the surface ectoderm of the head, it tells the ectoderm that it will become lens. Reciprocally, as the lens begins to form, it tell the brain bulge cells that they will become retina. In this way, the eye will be formed. The lens and retina co-constructed each other. Neither existed previously. The dozen cell types of the kidney are made by the interactions of the ureteric bud and the mesonephrogenic mesoderm, neither of which would have made any structure on its own (see Barresi and Gilbert, 2023). Similarly, *Vibrio* bacteria will activate the *Euprymna* squid genes needed to form a functional light organ (McFall-Ngai, 2021), and the squid will tell the bacterial genes to produce light (Dunn et al., 2006). The functional light organ did not exist before the bacteria and the squid cells made it together.

Animal (and plant) development is predicated on such sympoieses. The nerves that promote peristalsis and hearing, the lymphocytes that constitute the immune system, the intestinal capillaries that take nutrients to the body, and even portions of the mammalian brain are generated or matured by microbial symbionts (Steppenbach et al., 2002; see Gilbert and Epel, 2015). Indeed, the cow rumen is constructed in response to signals from the bacteria whose progeny will dwell within it (Sander et al., 1959; Baldwin and Conner, 2017). *Wolbachia* bacteria are responsible for the proper orientation of the second mitotic division of the nematode *Brugia malayi* (Landman et al., 2014), and they are needed for the formation of ovaries in the *Asobara* wasp (Dedeine et al., 2001). Almost all animals are co-created through interactions between their zygotically derived cells and their environmentally derived microbes³.

Gut microbes were crucial for orchestrating gut formation in mammals. The mammalian gut becomes colonized by bacteria as the fetuses pass through the mother's birth canal. Stappenbeck and colleagues (2002) demonstrated that in the absence of these normal intestinal bacteria, the capillaries lining the villi of the small intestine fail to develop complete vascular networks. Microarray analyses of mouse intestinal cells show that the normally occurring gut bacteria upregulates the transcription of numerous mouse intestinal genes, including those encoding colipase (which is important in nutrient absorption), angiogenin-4 (which promotes the formation of blood vessels) and Sprr2a (a small, proline-rich protein that is thought to fortify matrices that line the intestine). In other words, products of the bacterial cells can induce gene expression in the mammalian intestinal cells (Hooper and Gordon, 2001). Certain gut microbes, mainly strains of *Bacteroides*, are critical for activating the transcription of the gene encoding angiogenin-4 in the Paneth cells of the intestine.

The mRNA for angiogenin-4 is translated into the angiogenin-4 protein, which is then secreted and induces the mesodermal cells lining the intestine to organize into capillaries. But *Bacteroides* bacteria and angiogenin-4 have other properties, as well. First, angiogenin-4 acts through Wnt and Notch to expand the population of Lgr5⁺ intestinal stem cells. At higher concentrations it causes apoptosis of these cells, thereby becoming a regulator of intestinal homeostasis (Abo et al., 2023). Second, Ang4 is toxic to *Listeria*, *Candida* and

Salmonella, potential competitors of *Bacteroides*. Administration of Ang4 not only increased beneficial bacteria such as *Lactobacillus*, *Akkermansia*, *Dubosiella*, and *Adlercreutzia*, but also decreased certain pathogenic bacteria, including *Alistipes* and *Enterohabdu*, indicating that angiogenin-4 “regulates the shape of gut microbiota composition” (Sultana et al., 2022). Angiogenin-4 is also toxic to *Bacteroides thetaiotaomicron*, preventing it from dominating the bacterial population. Third, *Bacteroides thetaiotaomicron* secretes a DNase to form bile-dependent biofilms. Just as bacteria induce gut cells, the gut induces new properties in the microbes. “Physiological concentrations of bile extract induce the formation of biofilm in almost all tested *B. thetaiotaomicron* strains” (Béchon et al., 2022). Therefore, the microbes help establish and regulate the gut microenvironment. Moreover, it is the holobiont community--the zygotic-derived cells and the bacteria--that work together to protect it from invasions of other external bacteria (Chiu et al., 2017).

Zebrafish guts also have a vast assortment of microbes (see Jemielita et al., 2014), and these microbial symbionts activate the Wnt paracrine signaling pathway to initiate cell division in the intestinal stem cells (Rawls et al., 2004). Without this bacterially induced stem cell division, the zebrafish have thinner intestines, with a deficiency of enteroendocrine and goblet cells (Bates et al., 2006). The region of the gut that forms the zebrafish pancreas and its insulin-secreting beta-cells is also induced by bacteria. In zebrafish, the proliferation of insulin-secreting pancreatic beta cells depends on a protein generated by a relatively rare microbe, *Aeromonas* (Hill et al., 2016; 2022). This bacterial species contains a gene, *BefA*, whose secreted protein product stimulates the proliferation of beta cells in the zebrafish from their fetal levels to their adult levels. Zebrafish lacking *Aeromonas* (or whose *Aeromonas* bacteria lack the *BefA* gene) have a small number of beta cells and a diabetes-like syndrome. This number can be expanded to normal levels, however, by supplying the fish with the protein synthesized by the wild-type *BefA* gene. Moreover, providing additional *BefA* protein to diabetic mice alleviated their diabetic symptoms by increasing the number of beta cells (Wang et al., 2022).

Indeed, insulin signaling may be a multi-species effort throughout the animal kingdom. *Drosophila* larvae eating the rotten fruit upon which their egg was laid acquire about 20 species of bacteria and yeasts in their guts. Some of these microbes produce compounds that stimulate insulin synthesis. The insulin regulates the speed of developmental as well as the body size, energy metabolism, and intestinal stem cell activity of the larvae (Douglas, 2011; Shin et al., 2011; Storelli et al., 2011).

3.2 The sympoiesis of the rumen ecosystem

One of the most important of the sympoietic events has been the generation of the rumen in cattle. The cow's rumen is the example *par excellence* of an ecosystem residing within an organism. As a recent review (Mizrahi and Jami, 2021) succinctly notes, “Ruminants, more than any other mammalian group, also represents the epitome of mammalian-microbe symbiosis, as they rely completely on microbial fermentation to sustain their lives.” A cow is an herbivore that is

dependent upon digesting grass. But it cannot digest grass alone. The genome of the domestic cattle, *Bos taurus*, makes no enzymes capable of digesting cellulose or other plant wall compounds. The digestion of plant materials in both these organisms is accomplished by symbiotic microbes that resides within their guts. Cattle can only survive as holobionts. They are rumnants, as they have a specialized region of their gut, the rumen, that houses these symbiotic microbes. A rumen can be simultaneously viewed as an organ, a complex ecosystem, and “a large anaerobic fermentation chamber where plant-degrading rumen microbiota (bacteria, protozoa, archaea, and fungi) ferment otherwise non-digestible plant-based foodstuffs into primarily the volatile fatty acids acetate, propionate, and butyrate” (Daniels and Yohe, 2014).

The microbial composition of the rumen changes in a way that resembles ecological succession. The first colonizers of the rumen (i.e., the bacteria acquired in the passage through the birth canal) are aerobic bacteria. However, these bacteria utilize the oxygen in the ruminal region over the next 2 days, so this volume becomes populated by anaerobic bacteria that can survive without oxygen (Jami et al., 2013). The microbial ecosystem of the rumen from then on depends on the starter set of microbes it receives from its mother, the microbes it receives from its environment, the diet of the animal, and the genome of the animal. Different breeds of cow select for different microbes, and different locations allow different microbes to colonize the gut. As Taxis and colleagues (2015) have shown, “the players may change but the game remains.” The functions are what’s important, not the species that perform them. As Doolittle and Booth (2017) have noted, “it’s the song not the singer” that’s critical.

Still, there does exist a core community of microbial species, some of which are found in 100% of cows surveyed. These are the “keystones” of rumen ecosystem. Two of them are *Ruminococcus flavifaciens* and *Fibrobacter succinogenes*, bacteria capable of digesting cellulose into soluble sugars. Another keystone genus, *Prevotella*, appears important for converting products to short-chain fatty acids (SCFA) such as propionate and butyrate (Wallace et al., 2019; Furman et al., 2020). So in addition to “It is the Song Not the Singer,” there is also “Nobody Does It Better.”⁶

But in addition to this nutritional *symbiosis* in the adult cow, there is also *sympoiesis* between the microbes and the bovine cells to *generate* the rumen. The calf is not born with an anatomically or physiologically mature rumen. The gut gets colonized by bacteria, but in the distal esophagus and proximal stomach, these bacteria are not given food to metabolize (Warner, 1956; Giesecke et al., 1979; Daniels and Yohe, 2014; Malmuthuge et al., 2019). Rather, the newborn calf is fed milk, and this milk passes through a duct, the esophageal groove, that bypasses the rumen. When the calf is weaned and feeds on grass or grain, the groove disappears, the plant material enters the proximal stomach, and the bacteria digest and ferment these plant cell wall carbohydrates into short chained fatty acids. Butyric acid induces new gene expression patterns in this region of the gut, and the immature rumen is converted into a functional rumen (Baldwin and Conner, 2017; Liu et al., 2024). Calves that retain a milk-only diet do not

mature their rumen, and adding butyric acid to an unweaned calf’s diet will cause the rumen to develop prematurely (Sander et al., 1959; Baldwin and Conner, 2017). In other words, as the bacteria proliferate by metabolizing the grasses and grains, they produce the short chain fatty acids that cause the rumen to differentiate and grow.

It appears that *Prevotella*, *Bacteroides*, and *Ruminococcus* bacteria work together to initiate new transcription in the rumen (Malmuthuge et al., 2019). They create SCFA from cellulose, and the SCFA activates the genes for numerous transcription factors that promote the growth and differentiation of rumen tissue become activated. This new activation of gene expression appears to be accomplished by acetylating specific nucleosomes. When butyrate is given to cultured rumen cells in the laboratory, the levels of nucleosome modification correspond to the levels of butyrate, and there is a correlation between the levels of butyrate and the developmental maturity of the rumen (Deng et al., 2023; Kang et al., 2023). Thus, the bacteria help construct the rumen, which will house them and allow the survival of the host organism. Here we see both symbiosis in the adult and sympoiesis in the juvenile (Chiu and Gilbert, 2020). What is critical is understanding that the cow is a holobiont that develops both as an organism (the product of zygotic cells) and as the ecosystem (of the acquired cells).

3.3 Sympoiesis of cognitive systems

The development of mammalian immune and nervous systems, the major cognitive interfaces that allow us to interact with our environments (Tauber, 2013) develop sympoietically. Germ-free mice have serious neurological and immunological deficiencies. Symbiotic gut microbes are required for generating the B-cells and T-cells of the gut-associated lymphoid tissue (Rhee et al., 2004), and the immune systems of such mice have fewer lymphocytes, less active intestinal macrophages, lower cytokine production, and lower titers of serum immunoglobulin (Dobber et al., 1992; Kieper et al., 2005; Mazmanian et al., 2005; Ratsika et al., 2022). Therefore, as mentioned in our earlier discussion of angiogenin-4, immunity is a holobiont property, made through sympoiesis. It is not merely a function of the host (Gilbert and Tauber, 2016; Pradeu, 2019; Schneider, 2021)⁷.

The mammalian brain also develops sympoietically (Cryan et al., 2019; Morais et al., 2021; Nagpal and Cryan, 2021). Compared to conventionally bred mice, germ-free mice have higher titers of serotonin, while maintaining lower levels of the mRNAs for transcription factor Egr1 and the paracrine factor BDNF in the relevant portions of their brains (Diaz et al., 2011; Clarke and Gilbert, 2022). These changes correlate with behavioral differences between the germ-free and conventionally raised mice. Diaz Heijtz and colleagues (2011, p 3047) concluded that

⁶ This was also seen in the above-mentioned studies analyzing the induction of angiogenin-4 by bacteria. Several bacteria were able to induce angiogenin-4 gene expression; but *Bacteroides thetaiotamicron* did it better than the other species.

⁷ Gilbert and Tauber (2016) have proposed that the boundary of the holobiont is the immune system, a system created through the interactions of host and symbionts. This system defines “self” in a dynamic manner, changing constantly as new are encountered. “Whatever these interactions are between microbe and host, the holobiont is being continuously constructed.”

“during evolution, the colonization of gut microbiota has become integrated into the programming of brain development, affecting motor control and anxiety-like behavior.”

Although mammalian embryos may develop within an aseptic amnion (Kennedy et al., 2023), about 30% of the metabolome coursing through the maternal bloodstream of a pregnant mouse emerges directly or indirectly from symbiotic microbes (Nicholson et al., 2012; McFall-Ngai et al., 2013). Therefore, microbes in the maternal gut can create compounds that circulate throughout the body, enter into the fetal circulation, and affect the developing fetus. When the gut microbes of a pregnant mouse digest and ferment plant fibers, they produce short-chain fatty acids (such as those seen to direct the development of the cattle rumen) that enter the maternal bloodstream and enter the organs of her developing embryos. In the embryo, these bacterial products activate particular genes in the pancreas, nervous system, and intestines, generating proteins that mature the sympathetic neurons and provide life-long obesity-resisting metabolic phenotypes to the offspring (Kimura et al., 2020). Other soluble digestion products made by the maternal gut microbiome (e.g., hippurate and trimethyl-5-aminovalerate) stimulate the maturation of auditory neurons in the fetal mouse brain (Vuong et al., 2020). If these latter metabolites were not generated by the pregnant maternal microbiome, her adult progeny have hearing deficiencies.

If the microbes of mammalian guts appear to be crucial for stimulating “basic neurogenerative processes such as the formation of the blood-brain barrier, myelination, neurogenesis, and microglia maturation” (Sharon et al., 2016, p. 915), then could such microbes also be critical for normal mental functioning? There is now evidence that the microbiome may also be stimulating mammalian social behaviors (Stilling et al., 2018; Sherwin et al., 2019). Germ-free mice have a suite of abnormal behaviors, including excessive time spent in repetitive self-grooming, social avoidance, and very little time spent in social investigation. Desbonnet and colleagues (2014) remarked that these traits appeared to be similar to those of autistic children. Moreover, some of these behavioral traits can be normalized by providing the germ-free mice with gut bacteria early in post-natal life. There are many pathways to accomplish this. Wu and colleagues (2021) have discovered that these “socializing” bacteria (especially *Enterococcus faecalis*) can act, in part, by suppressing corticosterone release from the paraventricular region of the brain. This may make socializing with other mice less stressful. The asocial behavior of germ-free mice may also be due to the deficiency of oxytocin-releasing signals from the vagus nerve. This behavior which can be reversed by providing the germ-free mice with *Lactobacillus reuteri* or with microbes from normal mice or even from neurotypical humans (but not with microbes from some autistic patients; Sgritta et al., 2019; Sharon et al., 2019; Bravo et al., 2011)⁸.

Therefore, the microbiome appears to be critical for normal animal development. In mammals, the gut, pancreas, immune system, and brain form from the interactions of microbial cells with zygote-derived cells. Animals develop sympoietically, in partnership at every level. Indeed, if bacteria help make us into healthy and social animals, both the bacteria and the mammal benefit. Indeed, perhaps we animals are the bacteria’s way of making more environments for our bacteria’s descendants (Gilbert, 2018; 2021).

3.4 Reconfiguring biology sympoietically

In contributing to physical and behavioral development, it should not be surprising that microbes are critical to the completion of life-cycles. This can readily be seen in marine invertebrates, where the metamorphosis from larva to adult is often mediated by microbes. In the life cycle of the sponge *Amphimedon*, for instance, symbiotic bacteria are needed to supply the arginine that is utilized to synthesize the nitric oxide cue for settlement of the larvae and their metamorphosis into the adult sponge (Song et al., 2021). Similarly, lipopolysaccharide from the biofilm-forming bacterium *Cellulophaga lytica* is needed for the settlement and subsequent metamorphic events of *Hydroides elegans*, the common tube worm (Freckelton et al., 2022). Recent studies (Freckelton et al., 2024) strongly suggest that the bacteria may be forming a prepattern that would become the template for the biogeographic distribution of benthic marine organisms.

Sympoiesis must be foregrounded as a crucial parameter for the conception of life. First, sympoiesis means the end of our thinking of ourselves and other organisms as monogenomic, self-contained, self-making individuals (Demster, 1998; Gilbert et al., 2015; Haraway, 2016). As Donna Haraway noted, we literally “become one with others.” We receive 22,000 genes from the zygote; we receive over eight million different genes from our symbionts (Funkhauser and Bordenstein, 2013).

If organisms are to be perceived as individuals, we are individual teams. And just like teams, organisms are formed through a mixture of cooperative and competitive processes. One competes to become part of a cooperating team, and “making the team” can be intensely competitive, whether it be for the Chicago Bears or for an ursine holobiont. We saw this in our discussion of *Euprymna* and *Vibrio*. Moreover, like a team, each player forms part of the environment for the other players. The co-symbionts mutually scaffold and provide affordances for each other’s existence (Clark and Gilbert 2022; Chiu and Gilbert, 2020). The symbionts in the rumen allow the existence and propagation of cows; the cows allow the existence and propagation of their ruminal bacteria. There is no “host” in the symbiotic relationship (Haraway, 2016). Animals constitute the environment of bacteria, as bacteria constitute the environment of the animals (Formosinho et al., 2022.)

This has important consequences for evolution. As in sports teams, what gets selected in evolution may be the “team” of organisms, not the individual players (Roughgarden et al., 2017; Osmanovic et al., 2018; Roughgarden, 2020). Evolutionary speciation often occurs, as Lynn Margulis and Dorian Sagan (2002) announced, through “acquiring genomes.” On the microevolutionary level, new combinations of symbionts have given rise to new phenotypes. Whether a pea aphid is green or red, thermotolerant or thermolabile, immune or susceptible to parasitoid wasp infection,

⁸ Although human autism spectrum anomalies probably have many causes, pilot studies have shown that replacing the gut bacteria of severely autistic children with those of neurotypical children can dramatically enhance the sociability of many patients (Kang et al., 2019; Puricelli et al., 2022). A recent international study (Morton et al., 2023) claims to find a set of bacteria and bacterial products in autistic patients that correlates with human brain gene expression changes and pro-inflammatory cytokine profiles.

depends on the bacteria associated sympositically with the developing aphid body (Dunbar et al., 2007; Oliver et al., 2009; Tsushida et al., 2010). Similarly, new phenotypes can arise from new combinations of symbionts. The acquisition of new fungal symbionts has enabled the relatively benign red turpentine beetle of the Oregon and Washington states to become a major killer of Chinese pine trees (Sun et al., 2013; Taerum et al., 2013). The acquisition of pesticide-resistant bacteria has allowed the *Riptortus* bean bug to acquire the resistance to insecticides (Kikuchi et al., 2012; Kim et al., 2016; Lee et al., 2019). And, of course, the acquisition of symbionts allowed animals to digest plants.

These modules can be tightly connected (as in *Buchnera*'s being an obligate symbiont of pea aphids; Monnin et al., 2020; Bennett and Moran, 2015) or loosely tied to an animal's development (as in the above-mentioned cases for a pea aphid's color, thermotolerance, and parasitoid resistance). In loosely coupled symbioses, the short-lived symbionts can provide adaptation to changing environments (as in metamorphosis). In tightly coupled symbioses (as in *Buchnera* and pea aphids), the symbiont's contribution becomes canalized into the normal developmental trajectory (Bennett and Moran, 2015). As Žukauskaitė (2020) points out, "sympoietic systems carry different bits of information in their components . . . This makes sympoietic systems more flexible and adaptive, in the sense that they can easily adapt to changing environments, and also create something new, produce new forms of organization (in this regard they are allopoietic)." Thus, symbionts add a new modular dimension to evolution. We have shown here that symbionts are critical for normal development. If, as evolutionary developmental biologists insist, evolution is made possible by changes in development, then changes in symbionts can cause changes in evolution.

3.4.1 The warp and woof of biodiversity

Sympoiesis may be the defining characteristic of biodiversity both temporally (as studied by evolutionary biology) and spatially (as studied by ecology). First, in the temporal dimension, major evolutionary innovations may have emerged through new sympoietic associations. This may include multicellularity (Dayel et al., 2011), meiosis (Woznica et al., 2017), herbivory (Gilbert, 2020), and the mammalian uterus (Emera and Wagner, 2012). Each appears to have arisen through new combinations of animals and symbiotic microbes (Gilbert, 2019). Even the animal nervous system may have evolved partly out of a need to orchestrate the complex interactions between animals and their associated micro-organisms (Augustin et al., 2017; Klimovich and Bosch, 2018).

Sympoiesis also seems to be critical in forming the great network that hold the planet's biodiversity together. The root nodules on legumes, which provide fixed nitrogen into the soil, are neither organs of the plant nor the bacteria. They are made from both participants, and they result from an intimate communication between the plant root and the rhizobium bacteria involving flavonoids and nodulation factors. Similar factors are required to form the symbiosis between the plant roots and the fungi that produce the mycorrhizae. The tidal and coral ecosystems are similarly sustained by sympoiesis. The worldwide coastal tidal zone, is sustained by a quadruple symbiosis involving seagrass, nitrogen-fixing bacteria, clams, and the sulfide-oxidizing bacteria living inside the clam's gills (Cardini et al., 2022). For the formation of coral reefs, mutualistic unicellular algae (dinoflagellates of the family

Symbiodinaceae) enter into and reside in the gastric cells of the corals, where they transport up to 95% of their photosynthetically produced carbon compounds to their hosts (Muscattine et al., 1984). The photosynthesis by the algal symbionts fuels coral growth and the calcification that creates the reef. Most coral-algae symbioses are established horizontally, with the larval or post-settlement stage acquiring the algae from the environment. It appears that these early stages of development can acquire different species of algae, and that the interactions eventually favor a single species (del Gomez-Cabrera et al., 2008; Matsuda et al., 2022). Therefore, sympoiesis is crucial in creating the major ecosystems of the planet.

When we talk about life on earth, we are talking about holobionts. Therefore, when we discuss development, we must discuss the development of holobionts. This entails studying the ways that microbes signal developmental phenomena to occur. It also involves studying how these interactions allow the persistence of microbial communities. And when we look at evo-devo, we are studying holobiont evo-devo. The study of life is the study of holobionts and the development of relationships between organisms.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

Author contributions

SG: Conceptualization, Writing—original draft, Writing—review and editing.

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Horn size is linked to Sertoli cell efficiency and sperm size homogeneity during sexual development in common eland (*Taurotragus oryx*)

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Background: In polygynous species, the development of secondary sexual characters is usually decisive for male reproductive success. However, our understanding about the links between the growth of these traits and reproductive efficiency is still elusive. Most research efforts in this topic have been also focused on adult males, although the development of some secondary sexual characters, like bovid horns, typically starts after birth, continues during the puberty and in some species, such as the common eland, slows or even stops during adulthood. In this study, we investigated the relationships between horn size and testicular function during sexual development in common elands using a comprehensive approach that considers both spermatogenic and sperm parameters.

Methods: Twenty-two non-sexually mature common elands were used for the present study. Horn size, body mass, testes mass, and gonadosomatic index were assessed. Spermatogenic activity was determined by cytological and histological analyses. Sperm concentration, morphology, morphometry, and intramale variation in sperm size were evaluated on epididymal sperm samples. Cluster analysis was performed to explore the influence of age on relationships between horn size and reproductive function.

Results: We found that bigger horns are associated with increased Sertoli cell efficiency and reduced intramale variation in sperm size. Both parameters were not related to one another while they have shown to be associated with enhanced sperm quality in ungulates. Moreover, horn size was positively linked to the testis mass, sperm concentration, and testicular investment in the seminiferous epithelium. Spiral length and basal circumference were the horn traits most strongly correlated with spermatogenic and sperm parameters as well as those responsible for the sexual dimorphism in this species. Cluster analysis rendered two groups: the first one including males ≤ 30 months old, while the second one those > 30 months old. Horn development and reproductive function were still correlated within age groups, with the strongest relationship found between horn size and sperm size homogeneity in males > 30 months old.

Conclusion: Taken together, our results indicate that horn size can be regarded as a good index of male reproductive potential during sexual development and provide insights into the role of secondary sexual characters in sexual selection dynamics.

KEYWORDS

male fitness, resource allocation, secondary sexual characters, sexual selection, sperm morphometry, spermatogenic efficiency

1 Introduction

In sexually dimorphic and polygynous species, male secondary sexual traits are regarded as the hallmark of sexual selection as their expression may influence mating success rendering males more successful in male-male contests (e.g., weapons like tusks, antlers, and horns) and/or more attractive to females (e.g., ornaments like plumage/fur color and manes) (Snook et al., 2013; Simmons et al., 2017). The question on whether the investment in secondary sexual traits growth might predict male fertility has been a matter of a vibrant debate over the last decades. Several studies have explored the relationship between male secondary sexual characters and sperm traits in a variety of taxa (e.g., crustaceans: Paschoal and Zara, 2022; insects: Rogers et al., 2008; fish: Kekäläinen et al., 2014; amphibians: Doyle, 2011; birds: Navara et al., 2012; mammals: Malo et al., 2005a), often finding mixed or weak empirical evidence (Mautz et al., 2013). An aspect to be considered is that no study has so far explored the relationship between spermatogenic function and the morphology of secondary sexual characters, inasmuch testis size and sperm quality are commonly regarded as the main determinant of sperm production and competitiveness, respectively. To fill this gap of knowledge, Ramm and Schärer (2014) advocate a more comprehensive approach that, over testis size, also considers the testicular architecture, spermatogenic cell organization and their hierarchical relationships. Testis mass is indeed a vague measure of male investment in the seminiferous epithelium as it also includes connective tissue, smooth muscle, nerves, blood and lymphatic vessels with the fluids herein. Another aspect that has been attracting the attention of evolutionary studies entails the role of intramale variation in sperm size, which has been mostly explored in a small number of mammalian species (i.e., rodents: Šandera et al., 2013; Varea-Sánchez et al., 2014). To date, only one study has investigated the implication of intramale variation in sperm size in an ungulate species, the red deer (Ros-Santaella et al., 2015). In this study, a reduced coefficient of variation (CV) in sperm size was associated with greater testes mass, sperm velocity and normal morphology, but it is still unknown whether the homogeneity in the sperm size is associated with the development of secondary sexual characters. Additionally, most research efforts exploring the relationships between pre- and post-copulatory traits in mammals have been so far focused on sexually mature males (Ferrandiz-Rovira et al., 2014; Dines et al., 2015), even though the development of secondary sexual characters in many species (e.g., some bovids) starts early in the male lifetime and sometimes even do not appreciably grow during adulthood (Geist, 1966).

In several bovids and cervids, horns and antlers are one of the most diverse and elaborate male secondary sexual characters. In addition to their role as sexual traits, horns and antlers play a major role as weapons against predators and body temperature regulators (Picard et al., 1999; Bro-Jørgensen, 2007). Previous studies have shown that horn/antler size and shape in male ungulates are associated with fighting behavior (Lundrigan, 1996; Caro, 2003), major histocompatibility complex (MHC) traits (Ditchkoff et al., 2001), parasite abundance (Ezenwa and Jolles, 2008), testis size (Malo et al., 2005a), sperm motility and velocity (Malo et al., 2005b; Santiago-Moreno et al., 2007), and reproductive success (Kruuk et al., 2002; Preston et al., 2003; Willisch et al., 2015). In a comparative study in ungulates, Ferrandiz-Rovira et al. (2014) found that longer weapons were significantly associated with shorter sperm cells, but there was no association between weapons length and testes mass (but see also Lüpold et al., 2015). In addition to their size, also fluctuating asymmetry in bilateral paired organs, like horns, can be used as an index of developmental stability (Benítez et al., 2020). In ungulates, fluctuating asymmetry in male secondary sexual traits has been related to poor ejaculate quality, at least in captive populations (Roldan et al., 1998). Surprisingly, the links between horn size/asymmetry and testicular function are still largely unknown in ungulates.

The aim of this study was to explore the relationships between horn morphology (i.e., size and asymmetry) and reproductive competence during the sexual development of male common eland (*Taurotragus oryx*). The common eland is one of the largest antelope species native to the southern and eastern Africa. This non-territorial and sexually dimorphic species is currently classified as “least concern” by the IUCN, with a stable population of ~100,000 individuals (IUCN, 2024). The age of sexual maturity is estimated to be around 2.5 years in females and 4 years in males (Pappas, 2002). Elands can reproduce at any time of the year, although in wild conditions breeding and calving season peak might occur during the rainy season (Pappas, 2002; Pennington, 2009). Both sexes have spiral horns, which are shorter, thicker, and have tighter and more pronounced spirals in males than in females (Pappas, 2002). Final horn length is achieved relatively early during male development and do not appreciable grow after reaching sexual maturity (Jeffery and Hanks, 1981). For this reason, sexual development can represent a crucial phase of the male lifetime in which a link between primary and secondary characters is established. The understanding of the role played by horn morphology during the male sexual development can reveal early important traits of individual fitness that might be determinant for reproductive success during adulthood.

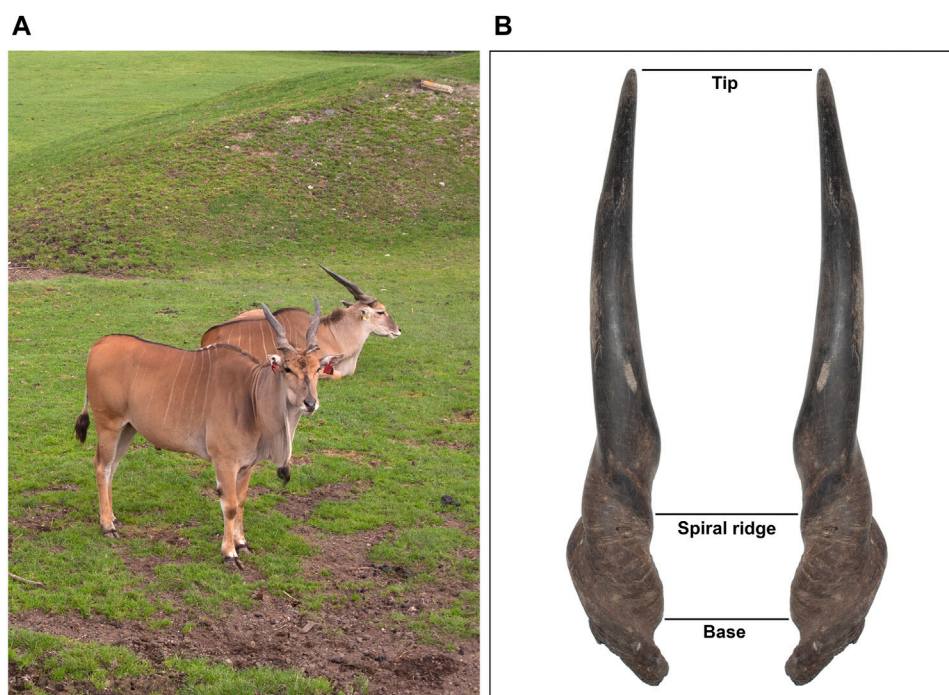


FIGURE 1
Post-pubertal males and horn measurements in common eland. **(A)** Post-pubertal male common elands. **(B)** Specular image that representatively shows the points of reference for horn size measurements: horn length (from the front base and straight up to the tip), spiral length (from the front base following the spiral ridge to the point where the latter is not pronounced and then straight up to the tip), and basal circumference.

2 Methods

2.1 Animal management and experimental design

From September 2013 to December 2017, 22 male common elands (age: 15–44 months old; [Figure 1A](#)) were slaughtered at the farm of the Czech University of Life Sciences Prague (Lány, Czech Republic). The farm is accredited as research facility according to European and Czech laws for ethical use of animals in research (permits no. 58176/2013-MZE-17214 and no. 63479/2016-MZE-17214). Slaughter of males is part of farm regular production and management to reduce the number of animals due to overwintering capacity. All individuals were born in captivity, individually identified by ear tags since birth, and bred under the same environmental conditions. They represented the seventh captive generation after their import from East Africa from 1969 to 1972 ([Vágner, 1974](#)). Genetic diversity of the herd has been maintained through exchanges of individuals from zoological gardens. All eland males were group-housed in straw bedded pens in a barn together with the rest of the herd of maximum of 50 animals, which was separated usually into two groups based on reproductive state of adult females and time of year. All animals were fed with mixed diets *ad libitum* consisted of corn silage (60%), lucerne haylage (30%), meadow hay (7%), and barley straw (3%). This mixture contained 16.6% of crude protein and 16.2% of crude fiber. From April to November, they had access to 2.5 ha of paddock with grass to enhance contact between animals and received up to 1 kg/individual/day of barley grain. The health of animals was

randomly checked by veterinarian inspection and monitored on yearly basis. The animals were slaughtered, exsanguinated, eviscerated at the farm, and transported to the abattoir of the Institute of Animal Science in Prague for further processing (see more in [Bartoň et al., 2014](#)). All slaughter process was carried out under the supervision of a state veterinarian according to EU and national legislation and conditions for farm animals (slaughter permits no. SVS/WS22/2012-KVSS and no. SVS/2015/077267-S). Testes (within the scrotum) were removed directly after the slaughter, stored in sealed plastic bags, labelled according to individual identification code, and transported at room temperature to the laboratory for further processing. Testicular and sperm samples were collected and processed within approximately 2 h after the death of the animals.

2.2 Biometrics and blood testosterone levels

Body mass was determined using tensometric scale (EC 2000, True-Test Limited, Auckland, New Zealand) to the nearest 0.5 kg. Horn size was determined by flexible tape measure to the nearest 0.5 cm. Horn size measurements were determined as it follows: horn length (from the front base and straight up to the tip), spiral length (from the front base following the spiral ridge to the point where it is not pronounced and then straight up to the tip), and basal circumference ([Figure 1B](#)). All horn measurements were taken by the same trained observer (RK) and averaged from the left and right sides. Horn asymmetry was calculated as the signed difference between right and left sides of each trait ([Palmer, 1994](#)). Horn

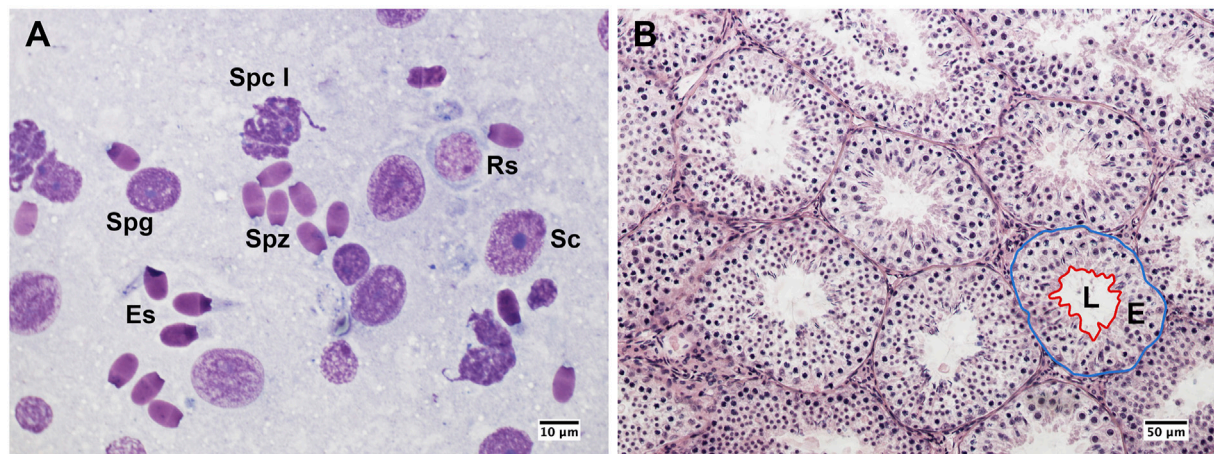


FIGURE 2
Testicular cytology and histology from post-pubertal common eland. **(A)** Germ cells and Sertoli cells from a cytological smear. Sc: Sertoli cell; Spg: spermatogonium; Spc I: Primary spermatocyte; Rs: round spermatid; Es: elongated spermatid; Spz: spermatozoon. **(B)** Histological section of seminiferous tubules that shows a representative example of seminiferous tubule measurements: the blue line indicates the area of the seminiferous tubule, whereas the red line indicates the epithelial (E) and luminal (L) areas.

size was not adjusted for body size as scaling relationship between organs typically occurs during ontogenetic growth (Vea and Shingleton, 2021). In comparative biology, recent studies have also questioned traditional methods for body-size adjustment as they i) do not adequately separate the effects of body size from those of other biological and ecological factors on a specific phenotypic trait (Glazier, 2022) and ii) can spuriously change the sign of regression coefficients compared to the original values, which could lead to inferential biases in biological studies (Rogell et al., 2019). Testosterone levels were assessed from blood samples collected from the jugular veins and carotid arteries into ethylenediaminetetraacetic acid vials. At the laboratory, the samples were immediately centrifuged at $3,500 \times g$ for 20 min at 4°C. After that, blood plasma was transferred into a new vial and stored at -80°C till analyses. Testosterone levels were assessed in duplicate by an enzyme immunoassay with a double-antibody technique and expressed as ng/mL (Roelants et al., 2002). Because of logistic issues, blood testosterone levels were determined only during the first phase of the study ($N = 13$).

2.3 Testes mass and spermatogenic function

Testes mass was recorded to the nearest 0.1 g using an electronic balance (EK-600G, LTD, Japan). The gonadosomatic index (GSI) was calculated as the relative proportion of testes mass to the body mass. Cytological samples were collected from each testis using the fine needle aspiration technique, which has proven to be a reliable method for the assessment of testicular function in ungulates both under physiological and pathological conditions (Pintus et al., 2014; Pintus et al., 2015a). Testicular smears were stained with Hemacolor (Merck, Darmstadt, Germany) and evaluated under a $\times 100$ objective using bright-field microscopy (Nikon Eclipse E600, Nikon, Tokyo, Japan). Assessment of germ cell proportions and spermatogenic indices were determined on at least 200 Sertoli and spermatogenic cells per testis (Figure 2A).

Then, testicular indices were assessed as follows: i) the Sertoli cell index (SEI), which is the percentage of Sertoli cells per total germ cells and estimates the spermatogenic activity; ii) the spermatozoa-Sertoli cell index (SSEI), which is the number of spermatozoa per Sertoli cell; iii) the meiotic index (MI), which is the ratio of round spermatids to primary spermatocytes and estimates the germ cell loss during meiosis; iv) the ratio of elongated spermatids to round spermatids (ES/RS), which estimates the germ cell loss during the post-meiotic phase; v) the ratio of elongated spermatids to total germ cells (ES/GC), which estimates the overall germ cell loss during spermatogenesis; vi) the ratio of round spermatids to Sertoli cells (RS/SC), vii) the ratio of elongated spermatids to Sertoli cells (ES/SC), which both estimate the Sertoli cell function; and viii) the ratio of total germ cells to Sertoli cells (GC/SC) that estimates the Sertoli cell workload capacity (Ros-Santaella et al., 2019). Samples for testicular histology were collected and processed as previously described (Pintus et al., 2015a). Briefly, a fragment of approximately 1 cm³ was isolated from the equatorial region of each testis, fixed in modified Davidson's solution (30% formaldehyde, 15% ethanol, 5% glacial acetic) for 24–48 h, then stored in 70% ethanol until analysis. Then, the samples were embedded in paraffin, cut into 4 µm sections, and stained with hematoxylin and eosin. To evaluate the morphology of the seminiferous tubules, 25 roundish cross-sections of the seminiferous tubules were photographed per each testis using a high-resolution camera (Digital Sight DSFi1, Nikon, Tokyo, Japan) under a $\times 20$ objective. Then, the area of the seminiferous tubule, lumen, and epithelium were assessed using ImageJ software (National Institutes of Health, Bethesda, MD, United States) (Figure 2B). The proportion of the seminiferous epithelium to the total tubular area was then calculated. Values from cytological and histological analyses were averaged from the left and right testes. Because of suboptimal sample quality or reduced smear cellularity, testicular cytology and histology were not performed in one and two males, respectively. All measurements were taken by the same trained observer (EP).

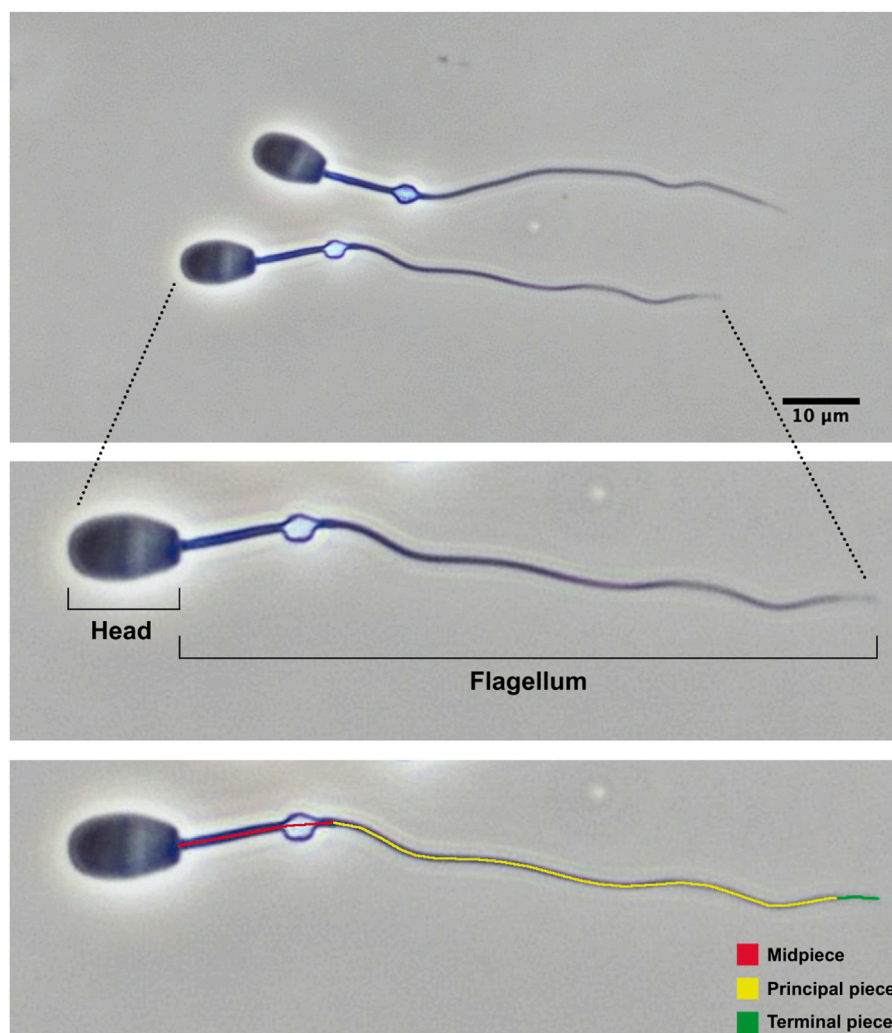


FIGURE 3
Epididymal spermatozoa from post-pubertal common eland and their main structures.

2.4 Sperm concentration, morphology, and morphometry

Sperm samples were collected from the epididymal caudae using a sterile surgical blade and fixed into 0.5 mL phosphate-buffered saline solution supplemented with 2% glutaraldehyde. Sperm concentration was determined using a Bürker chamber. The percentage of morphologically normal spermatozoa was determined after evaluating 200 spermatozoa under phase-contrast microscopy ($\times 40$ objective). Sperm morphometry was assessed as previously described (Ros-Santaella et al., 2014; Ros-Santaella et al., 2015). Briefly, sperm pictures were taken with high-resolution camera under phase-contrast microscopy ($\times 40$ objective). The following sperm traits were determined using the ImageJ software: head width, head length, midpiece length, and flagellum length. From these measurements, other morphometric parameters were calculated such as head area, head perimeter, head ellipticity (head length/head width), total sperm length, and principal plus terminal piece length. Twenty-five spermatozoa with normal morphology (i.e., without head or flagellum abnormalities and

without proximal cytoplasmic droplet) were measured per male. For each parameter, the intramale coefficient of variation (CV) was calculated as standard deviation/mean $\times 100$. The main structures of common eland spermatozoa with normal morphology are shown in Figure 3. Sperm morphology and morphometry could not be assessed in five samples because of their low sperm concentration or smear quality. All measurements were taken by the same trained observer (JR-S).

2.5 Statistical analysis

Statistical analyses were performed using the SPSS 20.0 statistical software (IBM Inc., Chicago, IL, United States). The Shapiro-Wilk test was used to check for the normal distribution of data. Data that were not normally distributed were log-transformed. Non-parametric tests were applied to data that were not normally distributed after log transformation. Principal component analysis was used to reduce several closely related variables (i.e., horn size/asymmetry, Sertoli cell efficiency,

TABLE 1 Descriptive statistics of biometrics and testicular parameters in post-pubertal common eland.

	Mean $\pm$ SE	N
<i>Biometrics and blood testosterone levels</i>		
Body mass (kg)	273.25 $\pm$ 16.23	22
Horn length (cm)	55.23 $\pm$ 1.07	22
Horn spiral length (cm)	67.82 $\pm$ 1.50	22
Horn basal circumference (cm)	25.89 $\pm$ 0.29	22
Testes mass (g)	123.43 $\pm$ 7.28	22
GSI (%)	0.05 $\pm$ 0.00	22
Blood testosterone levels (ng/mL)	0.37 $\pm$ 0.06	13
<i>Spermatogenic cells</i>		
Spermatogonia (%)	2.25 $\pm$ 0.25	21
Primary spermatocytes (%)	22.65 $\pm$ 0.83	21
Secondary spermatocytes (%)	0.88 $\pm$ 0.10	21
Round spermatids (%)	37.51 $\pm$ 0.69	21
Elongated spermatids (%)	16.76 $\pm$ 0.67	21
Spermatozoa (SI, %)	19.95 $\pm$ 0.76	21
<i>Spermatogenic indices</i>		
SEI (%)	11.44 $\pm$ 1.96	21
SSEI	2.51 $\pm$ 0.33	21
MI	1.72 $\pm$ 0.08	21
ES/RS	0.46 $\pm$ 0.02	21
ES/GC	0.17 $\pm$ 0.01	21
RS/SC	4.74 $\pm$ 0.68	21
ES/SC	2.05 $\pm$ 0.25	21
GC/SC	12.37 $\pm$ 1.61	21
<i>Testicular architecture</i>		
Area seminiferous tubule (μm^2)	26,520.26 $\pm$ 1,050.76	20
Area seminiferous epithelium (μm^2)	22,033.46 $\pm$ 936.32	20
Area seminiferous lumen (μm^2)	4,486.80 $\pm$ 219.69	20
Proportion of the seminiferous epithelium to the tubular area (%)	83.07 $\pm$ 0.82	20

GSI, gonadosomatic index; SI, spermatogenic index; SEI, Sertoli cell index; SSEI, spermatozoa-Sertoli cell index; MI, meiotic index; ES/RS, ratio of elongated spermatids to round spermatids; ES/GC, ratio of elongated spermatids to germ cells; RS/SC, ratio of round spermatids to Sertoli cells; ES/SC, ratio of elongated spermatids to Sertoli cells; GC/SC, ratio of germ cells to Sertoli cells.

and intramale variation in sperm head size) into a smaller subset that better summarizes the original data. The Bartlett sphericity and Keiser-Meyer-Olkin (KMO) tests for sampling adequacy were applied to test the suitability of data for the principal component analysis. The principal components (PCs) with Bartlett sphericity test's p -value higher than 0.05 and KMO test lower than 0.5 were considered inappropriate (Budaev, 2010). To determine the influence of age on horn and reproductive relationships, two-step cluster analysis was applied using age of males expressed in months as a continuous variable. The number of clusters was automatically determined using the Euclidean distance measure and the Schwarz's

Bayesian criterion. Subsequently, the number of clusters previously obtained was used to set up the K-Means cluster analysis by using the iteration and classification method. Unless otherwise specified, two-tailed Pearson correlations were used when data were normally distributed, otherwise two-tailed Spearman correlations. Correlation analyses were not corrected for multiplicity because, while correction methods decrease the probability of Type I error, they increase the probability of Type II error (Streiner, 2015). Therefore, although the statistical significance was set at $p < 0.05$, p values close to 0.05 must be cautiously considered. Data are shown as the mean $\pm$ SE.

TABLE 2 Descriptive statistics of sperm parameters in post-pubertal common eland.

	Mean ± SE	N
<i>Sperm number and morphology</i>		
Sperm concentration (10 ⁶ /mL)	755.32 ± 100.76	21
Normal sperm (%)	68.79 ± 7.01	17
<i>Sperm size</i>		
Head width (μm)	5.92 ± 0.04	17
Head length (μm)	9.66 ± 0.07	17
Head perimeter (μm)	24.84 ± 0.13	17
Head area (μm ²)	44.95 ± 0.44	17
Head ellipticity	1.63 ± 0.02	17
Midpiece length (μm)	14.52 ± 0.10	17
Principal plus terminal piece length (μm)	47.66 ± 0.38	17
Flagellum length (μm)	62.17 ± 0.42	17
Sperm length (μm)	71.84 ± 0.43	17
<i>Intramale CV in sperm size</i>		
Head width (%)	3.29 ± 0.20	17
Head length (%)	2.85 ± 0.15	17
Head perimeter (%)	2.21 ± 0.10	17
Head area (%)	4.35 ± 0.21	17
Head ellipticity (%)	4.46 ± 0.20	17
Midpiece length (%)	2.68 ± 0.16	17
Principal plus terminal piece length (%)	2.07 ± 0.08	17
Flagellum length (%)	1.57 ± 0.07	17
Sperm length (%)	1.49 ± 0.06	17

CV, coefficient of variation.

3 Results

Descriptive statistics of biometrics, testicular, and sperm parameters of male common elands are shown in [Tables 1, 2](#). On average, horn size was around 55 cm in length and 26 cm at basal circumference, while the spiral length was 68 cm. The signed asymmetry between the right and left sides of each horn trait was: -0.68 ± 0.36 cm for horn length, -0.64 ± 0.46 cm for spiral length, and -0.14 ± 0.19 cm for basal circumference. Except one male in which testicular cytology could not be assessed although spermatozoa were present in the epididymal caudae, the spermatogenesis was complete in all individuals, which indicate that males did already reach puberty. Cytological and histological analyses showed that spermatozoa represent around one-fifth of spermatogenic cell population, with over 80% of the tubular area filled by the seminiferous epithelium. Among spermatogenic cells, the round spermatids were the most abundant, while secondary spermatocytes were the scarcest. On average, over 700×10^6 spermatozoa/mL were collected from the epididymal caudae, with two-thirds of them being morphologically normal. The proportion

of each sperm structure in relation to the total sperm length was: head length, 13.45%; midpiece length, 20.21%; and principal piece plus terminal piece length, 66.34%. Overall, size variation in sperm parameters was below 5%, relatively higher CV values were found in the sperm head than in the flagellum ([Table 2](#)).

3.1 Principal component analysis of horn size and asymmetry

A principal component analysis was performed using the averaged values of the left and right horn measurements to obtain smaller subset of variables that summarizes the horn size. We obtained a single principal component (PC1, horn size) that overall explained 75.89% of the total variance (Keiser-Meyer-Olkin, KMO, measure of sampling adequacy = 0.520; Bartlett's test of sphericity: approx. $\chi^2 = 44.41$, $df = 3$, $p < 0.0001$; [Table 3](#)) and showed normal distribution (Shapiro-Wilk test, $p = 0.872$). Another principal component analysis was performed using the signed difference between the right and left horn measurement to obtain a smaller subset of variables that explain horn asymmetry. We obtained a single principal component (PC1, horn asymmetry) that overall explained 60.29% of the total variance (KMO measure of sampling adequacy = 0.516; Bartlett's test of sphericity: approx. $\chi^2 = 16.30$, $df = 3$, $p = 0.001$; [Table 3](#)). The PC1 of horn asymmetry shows normal distribution (Shapiro-Wilk test, $p = 0.552$) around a mean value close to zero (Skewness = -0.554 ± 0.491 and Kurtosis = 0.496 ± 0.953), which is indicative of fluctuating asymmetry.

3.2 Correlations between horn size and reproductive function

3.2.1 Correlations of horn size with testicular and spermatogenic parameters

We found that horn size was significantly associated with higher testes mass ($r = 0.476$, $p = 0.025$), while it did not correlate neither with the GSI (log-transformed; $r = 0.083$, $p = 0.713$) nor with the blood testosterone levels ($r = 0.176$, $p = 0.565$). Albeit no significant, horn size was positively associated with the body mass (log-transformed; $r = 0.421$, $p = 0.051$). An unexpected finding was that horn size was not related to the proportion of any spermatogenic cell type, including the spermatid index that represents the proportion of spermatozoa over the total germ cells and estimates the sperm production ($r = 0.071$, $p = 0.761$). On the other hand, larger horn size was associated with reduced Sertoli cell index (SEI, log-transformed $r = -0.590$, $p = 0.005$) and increased Sertoli cell function and workload capacity (i.e., spermatozoa-Sertoli cell index, SSEI: $r = 0.531$, $p = 0.013$; ratio of round spermatids to Sertoli cells, RS/SC: $r = 0.540$, $p = 0.012$; ratio of elongated spermatids to Sertoli cells, ES/SC: $\rho = 0.516$, $p = 0.017$; ratio of total germ cells to Sertoli cells, GC/SC: $r = 0.581$, $p = 0.006$, all log-transformed). Among the three descriptors of horn size, spiral length was the one showing the strongest relationship with Sertoli cell function and workload (i.e., spermatozoa-Sertoli cell index, SSEI: $r = 0.603$, $p = 0.004$; ratio of round spermatids to Sertoli cells, RS/SC: $r =$

TABLE 3 Principal component analysis of horn size and asymmetry in post-pubertal common eland.

	PC1	<i>p</i>
<i>Horn size</i>		
Horn length	0.916	<0.001
Spiral length	0.967	<0.001
Basal circumference	0.709	<0.001
Eigenvalue	2.28	
Variance explained (%)	75.89	
<i>Horn asymmetry</i>		
Horn length	0.923	<0.01
Spiral length	0.909	<0.01
Basal circumference	0.361	<0.05
Eigenvalue	1.81	
Variance explained (%)	60.29	

PC, Principal component. Horn asymmetry was calculated as the signed value of the difference between right and left sides of each trait.

0.610, $p = 0.003$; ratio of elongated spermatids to Sertoli cells, ES/SC: $\rho = 0.527$, $p = 0.014$; ratio of total germ cells to Sertoli cells, GC/SC: $r = 0.650$, $p = 0.001$, all log-transformed). To corroborate our findings, we performed a principal component analysis of Sertoli cell indices because of the high correlation among them. We obtained a single principal component (PC1, Sertoli cell efficiency) that explained 96.74% of the total variance (KMO measure of sampling adequacy = 0.803; Bartlett's test of sphericity: approx. $\chi^2 = 268.75$, $df = 10$, $p < 0.0001$; Table 4) and showed normal distribution (Shapiro-Wilk test, $p = 0.055$). Our findings confirm that larger horn size was associated with increased Sertoli cell efficiency ($r = 0.589$, $p = 0.005$, Figure 4).

Horn size was also positively related to the area of the seminiferous tubule and epithelium ($r = 0.462$, $p = 0.040$ and $r = 0.531$, $p = 0.016$, respectively). There was also a significant positive relationship between horn size and the proportion of the tubular area lined by the seminiferous epithelium ($\rho = 0.456$, $p = 0.043$). Among the three descriptors of horn size, basal circumference was the one showing the strongest relationship with the area of seminiferous tubule and epithelium ($r = 0.640$, $p = 0.002$ and $r = 0.629$, $p = 0.003$, respectively). We performed a principal component analysis of testicular architecture, which rendered one principal component that explained 77.13% of the total variance. However, because of the low value of KMO measure of sampling adequacy test (i.e., 0.327), the principal component was rejected.

3.2.2 Correlations of horn size with sperm parameters

Horn size was positively associated with sperm concentration ($r = 0.543$, $p = 0.011$) but it did not correlate with either sperm morphology or size ($p > 0.05$). Albeit not significant, large horn size was associated with a reduced midpiece length ($r = -0.481$, $p = 0.051$). Interestingly, bigger horn size was significantly

TABLE 4 Principal component analysis of Sertoli cell efficiency and intramale variation in sperm head size in post-pubertal common eland.

	PC1	<i>p</i>
<i>Sertoli cell efficiency</i>		
log SEI	-0.997	<0.001
log SSEI	0.968	<0.001
log RS/SC	0.986	<0.001
log ES/SC	0.968	<0.001
log GC/SC	0.998	<0.001
Eigenvalue	4.84	
Variance explained (%)	96.74	
<i>Intramale variation in sperm head size</i>		
log head width CV	0.938	<0.001
Head area CV	0.937	<0.001
Head ellipticity CV	0.901	<0.001
Eigenvalue	2.57	
Variance explained (%)	85.64	

CV, coefficient of variation; SEI, Sertoli cell index; SSEI, spermatozoa-Sertoli cell index; RS/SC, ratio of round spermatids to Sertoli cells; ES/SC, ratio of elongated spermatids to Sertoli cells; GC/SC, ratio of germ cells to Sertoli cells. PC, principal component.

associated with reduced intramale variation in sperm head size (log-head width CV: $r = -0.614$, $p = 0.009$; head area CV: $r = -0.520$, $p = 0.033$) and shape (head ellipticity CV: $r = -0.602$, $p = 0.011$). Among the three descriptors of horn size, basal circumference was the one showing the strongest relationship with intramale CV of sperm head size and shape (i.e., log-head width CV: $r = -0.549$, $p = 0.023$; head area CV: $r = -0.645$, $p = 0.005$; head ellipticity CV: $r = -0.664$, $p = 0.004$). To corroborate our findings, a principal component analysis of sperm head CV parameters was performed. We obtained a single principal component (PC1, intramale variation in sperm head size) that explained 85.64% of the total variance (KMO measure of sampling adequacy = 0.746; Bartlett's test of sphericity: approx. $\chi^2 = 31.275$, $df = 3$, $p < 0.0001$; Table 4) and showed normal distribution (Shapiro-Wilk test, $p = 0.234$). Our findings support that larger horn size is associated with reduced intramale variation in sperm head size and shape ($r = -0.625$, $p = 0.007$, Figure 5).

3.2.3 Correlations between spermatogenic and sperm parameters

To exclude collinearity, we checked for correlation between spermatogenic and sperm parameters that have previously shown to be associated with horn size. We found that increased Sertoli cell efficiency was associated with greater testes size ($r = 0.652$, $p = 0.001$), sperm concentration ($r = 0.475$, $p = 0.034$), and investment in tubular and epithelial areas of the testicular parenchyma ($r = 0.515$, $p = 0.024$ and $r = 0.587$, $p = 0.008$, respectively). Surprisingly, reduced intramale variation in sperm head size was not significantly associated either with testes mass ($r = -0.167$, $p = 0.522$), sperm

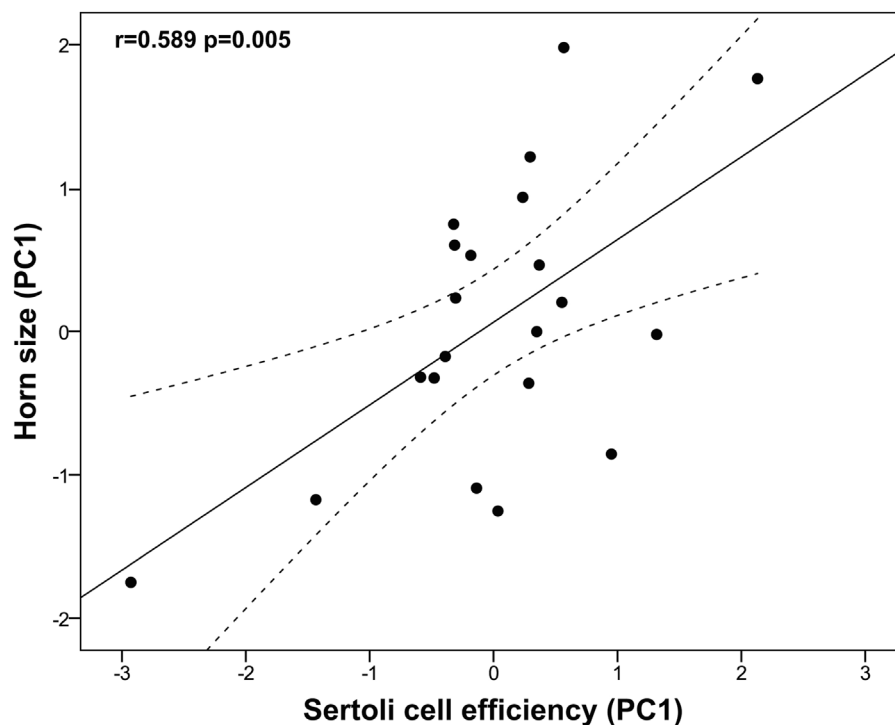


FIGURE 4

Relationship between horn size and Sertoli cell efficiency in post-pubertal common eland. Black line represents linear fit line, while dashed lines indicate 95% confidence intervals. PC: Principal component.

concentration ($r = -0.391$, $p = 0.121$) or Sertoli cell efficiency ($r = -0.248$, $p = 0.338$). However, low intramale variation in sperm head ellipticity was associated with greater sperm concentration ($r = -0.500$, $p = 0.041$) and larger areas of the seminiferous tubule and epithelium ($r = -0.590$, $p = 0.021$ and $r = -0.591$, $p = 0.020$, respectively).

We also checked the correlations between the remaining spermatogenic and sperm parameters (for simplicity, only those with $p < 0.01$ are shown herein). We found that high Sertoli cell efficiency was associated with smaller sperm head (i.e., head width: $r = -0.659$, $p = 0.004$; head area: $r = -0.678$, $p = 0.003$), while high meiotic index was strongly associated with longer sperm length (i.e., principal plus terminal piece length: $r = 0.834$, $p < 0.0001$; flagellum length: $r = 0.822$, $p < 0.0001$; total sperm length: $r = 0.819$, $p < 0.0001$). Sperm concentration was positively associated with tubular and epithelial areas of the testicular parenchyma ($r = 0.756$, $p < 0.001$ and $r = 0.771$, $p < 0.001$, respectively). Moreover, high proportion of normal spermatozoa was associated with increased Sertoli cell efficiency ($\rho = 0.725$, $p < 0.001$) and reduced intramale CV in sperm length (flagellum length: $\rho = -0.635$, $p = 0.006$; total sperm length: $\rho = -0.673$, $p = 0.003$).

3.3 Correlations between horn asymmetry and reproductive function

We found that horn asymmetry (PC1, horn asymmetry) was not significantly correlated neither with biometric, blood testosterone levels, testicular or sperm parameters ($p > 0.05$).

3.4 Effect of age on correlations between horn size and reproductive function

Cluster analysis rendered two groups of similar sample size: the first one includes males ≤ 30 months old ($n = 13$), while the second one those > 30 months old ($n = 9$). Most horn and spermatogenic parameters differed between groups except for horn basal circumference, GSI, blood testosterone levels, percentage of spermatozoa over the total germ cell population, indices of germ cell loss during the post-meiotic phase and whole spermatogenic process (i.e., ES/RS and ES/GC, respectively), and areas of the seminiferous tubule and epithelium ($p > 0.05$, Table 5), to mention but a few. Interestingly, groups did not differ in any epididymal sperm parameter apart from the sperm head width and area that were significantly smaller in elands > 30 months old compared to those ≤ 30 months old ($p < 0.05$, Table 6). New principal component analysis for horn size, Sertoli cell efficiency, and intramale variation in sperm head size were performed for each age group (Supplementary Table S2). Principal component analysis rendered one principal component (PC1) for horn size, Sertoli cell efficiency, and intramale variation in sperm head size in each age group, each of them explaining over 65% of the total variance. The KMO and Bartlett's test of sphericity were > 0.57 and < 0.01 , respectively, for all PCs except for PC1 of horn size in age group ≤ 30 months old (i.e., KMO = 0.376). In this group, correlation analysis showed that, albeit no significant, larger horn size was correlated with increased Sertoli cell efficiency and reduced intramale variation in sperm head size (one-tailed $\rho = 0.455$, $p = 0.069$, Figure 6A and one-tailed $r = -0.468$, $p = 0.086$; Figure 6B, respectively). Although no significant correlation was found with Sertoli

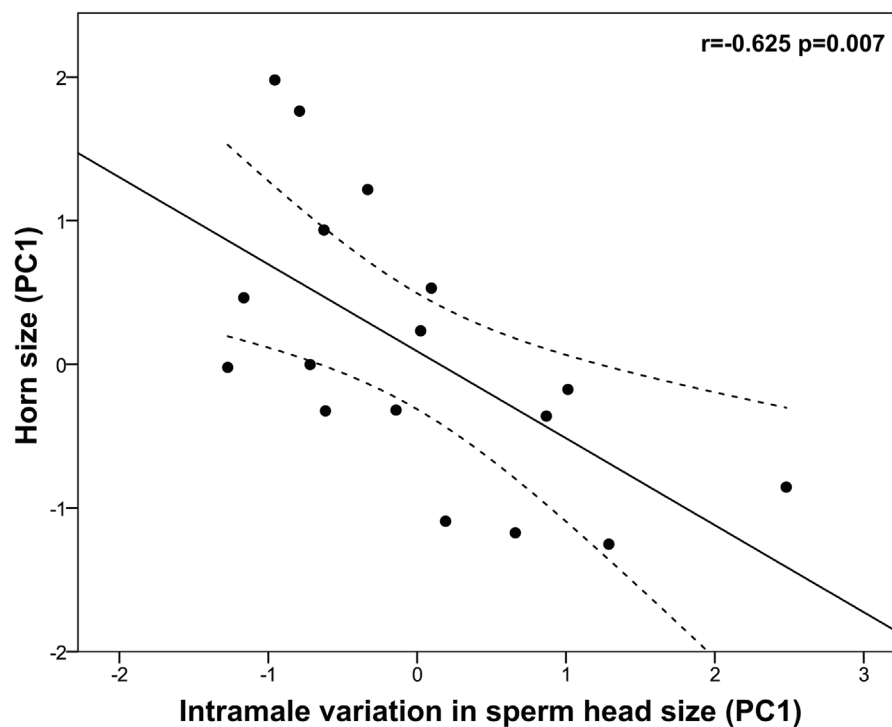


FIGURE 5

Relationship between horn size and sperm head size homogeneity in post-pubertal common eland. Black line represents linear fit line, while dashed lines indicate 95% confidence intervals. PC: Principal component.

cell efficiency in elands >30 months old ($p > 0.05$, Figure 6C), larger horn size was strongly correlated with reduced intramale variation in sperm head size ($r = -0.742$ and $p = 0.028$, Figure 6D) in this age group.

4 Discussion

The present study comprehensively explores the relationships between primary and secondary sexual characters during the sexual development of a polygynous ungulate, the common eland. We provide evidence that large horn size is associated with increased testes mass, spermatogenic activity, Sertoli cell efficiency, sperm concentration and reduced intramale variation in sperm size. It is remarkable to point out that the strength of correlations between horn size and Sertoli cell efficiency and between the former and sperm size homogeneity showed $r > 0.58$ and $p < 0.01$, which provide new insights into the links between primary and secondary sexual characters in a sexually dimorphic mammalian species. After exploring the effect of age using cluster analysis, primary and secondary sexual characters were still correlated within age groups, with the strongest relationship found between horn size and sperm size homogeneity in males approaching sexual maturity. Taken together, our results indicate that, during sexual development, horn size is a good index of reproductive potential in the common eland. It remains however to explore whether increased Sertoli cell efficiency and sperm size homogeneity translate into enhanced male fertility.

Our findings confirm the positive relationship between secondary sexual traits and testis size in ungulates (Malo et al.,

2005b) and suggest that such a relationship is mainly supported by the increased Sertoli cell efficiency. Discovered by Enrico Sertoli in 1865, the Sertoli cell represents the only somatic cell found within the seminiferous tubules. The astonishing ability of Sertoli cells to nurse, protect, and support the development of up to five different types of germ cells at any one time make them one of the most complex cells in the body (O'Donnell et al., 2022). The role of Sertoli cells is crucial during the entire male lifetime: during the embryo and fetal development, Sertoli cells promote the sexual differentiation, while during puberty and sexual maturity they coordinate the spermatogenesis and determine the sperm production. Our findings also indicate that greater Sertoli cell efficiency is associated with higher percentage of normal spermatozoa and smaller sperm head size, possibly because of Sertoli cell's role in phagocytosing abnormal germ cells and residual bodies during spermatogenesis (Oliveira and Alves, 2015). Future studies should be directed towards a deeper understanding of Sertoli cell efficiency using both metabolic and endocrine markers such as lactate production or inhibin levels (Rato et al., 2016; Shah et al., 2021). Taken together, our findings provide further support for the key role of Sertoli cells in determining testis mass, sperm production and quality in ungulates (Pintus et al., 2015b; Ros-Santaella et al., 2019).

Our results show that the investment in secondary sexual characters' growth is not linked to sperm size but rather to the homogeneity in sperm cell dimension. Findings from comparative studies in this taxonomic group have been so far elusive with both negative (Ferrandiz-Rovira et al., 2014) or no (Lüpold et al., 2015) relationships between horn/antler development and sperm size. Under this perspective, our findings highlight the relevance of

TABLE 5 Comparison of biometrics and testicular parameters in ≤ 30 months old and >30 months old post-pubertal common elands.

	≤ 30 months old (Mean $\pm$ SE)	>30 months old (Mean $\pm$ SE)	<i>p</i>
Biometrics			
Body mass (kg)	227.50 $\pm$ 9.44	339.33 $\pm$ 23.93	0.001
Horn length (cm)	53.21 $\pm$ 1.19	58.14 $\pm$ 1.58	0.019
Horn spiral length (cm)	64.56 $\pm$ 1.58	72.53 $\pm$ 2.07	0.006
Horn basal circumference (cm)	25.62 $\pm$ 0.43	26.28 $\pm$ 0.35	0.276
Testes mass (g)	106.09 $\pm$ 7.38	148.47 $\pm$ 9.48	0.002
GSI (%)	0.05 $\pm$ 0.00	0.04 $\pm$ 0.00	0.734
Blood testosterone levels (ng/mL)	0.35 $\pm$ 0.07	0.41 $\pm$ 0.07	0.442
Spermatogenic cells			
Spermatogonia (%)	2.00 $\pm$ 0.36	2.57 $\pm$ 0.30	0.041
Primary spermatocytes (%)	24.13 $\pm$ 0.95	20.68 $\pm$ 1.23	0.027
Secondary spermatocytes (%)	0.82 $\pm$ 0.15	0.95 $\pm$ 0.12	0.553
Round spermatids (%)	37.35 $\pm$ 0.95	37.71 $\pm$ 1.06	0.807
Elongated spermatids (%)	16.39 $\pm$ 1.03	17.27 $\pm$ 0.78	0.529
Spermatozoa (SI, %)	19.30 $\pm$ 1.19	20.83 $\pm$ 0.79	0.332
Spermatogenic indices			
SEI (%)	14.69 $\pm$ 3.11	7.11 $\pm$ 0.84	0.002
SSEI	1.76 $\pm$ 0.23	3.51 $\pm$ 0.57	0.001
MI	1.59 $\pm$ 0.08	1.90 $\pm$ 0.13	0.044
ES/RS	0.45 $\pm$ 0.03	0.47 $\pm$ 0.03	0.705
ES/GC	0.16 $\pm$ 0.01	0.17 $\pm$ 0.01	0.529
RS/SC	3.30 $\pm$ 0.36	6.66 $\pm$ 1.29	0.004
ES/SC	1.45 $\pm$ 0.17	2.85 $\pm$ 0.43	0.001
GC/SC	8.79 $\pm$ 0.89	17.15 $\pm$ 2.94	0.004
Testicular architecture			
Area seminiferous tubule (μm^2)	25,578.69 $\pm$ 1,802.32	27,671.06 $\pm$ 750.57	0.303
Area seminiferous epithelium (μm^2)	20,854.38 $\pm$ 1,574.66	23,474.56 $\pm$ 605.62	0.145
Area seminiferous lumen (μm^2)	4,724.31 $\pm$ 361.58	4,196.50 $\pm$ 189.29	0.253
Proportion of the seminiferous epithelium to the tubular area (%)	81.44 $\pm$ 1.27	85.06 $\pm$ 0.45	0.003

Sample size of ≤ 30 months old eland group is $n = 13$ except for blood testosterone levels ($n = 10$), spermatogenic indices ($n = 12$), and testicular architecture ($n = 11$). Sample size of >30 months old eland group is $n = 9$ except for blood testosterone levels ($n = 3$). GSI, gonadosomatic index; SI, spermatogenic index; SEI, Sertoli cell index; SSEI, spermatozoa-Sertoli cell index; MI, meiotic index; ES/RS, ratio of elongated spermatids to round spermatids; ES/GC, ratio of elongated spermatids to germ cells; RS/SC, ratio of round spermatids to Sertoli cells; ES/SC, ratio of elongated spermatids to Sertoli cells; GC/SC, ratio of germ cells to Sertoli cells.

intraspecific studies as they may provide a better understanding of patterns observed across animal species and reveal the signatures of selection (Kleven et al., 2008). In red deer, for instance, reduced intramale variation in sperm size is associated with enhanced sperm motility and normal sperm morphology (Ros-Santaella et al., 2015), which predict male fertility in this species (Malo et al., 2005a). In agreement with a previous study (Ros-Santaella et al., 2015), our findings also confirm that normal sperm morphology is associated with reduced intramale variation in sperm length. A smaller within-

male CV in sperm size has also been associated with the intensity of sperm competition and testicular investment (Šandera et al., 2013; Ros-Santaella et al., 2015). The lack of significant relationships between intramale variation in sperm size and spermatogenic function in the common elands raise the possibility that other factors, rather than spermatogenesis *per se*, might play a role in the production of a more uniform sperm population. For instance, in the male great tits (*Parus major*), within-male variation in sperm length is associated with a health-related (hematological) trait but

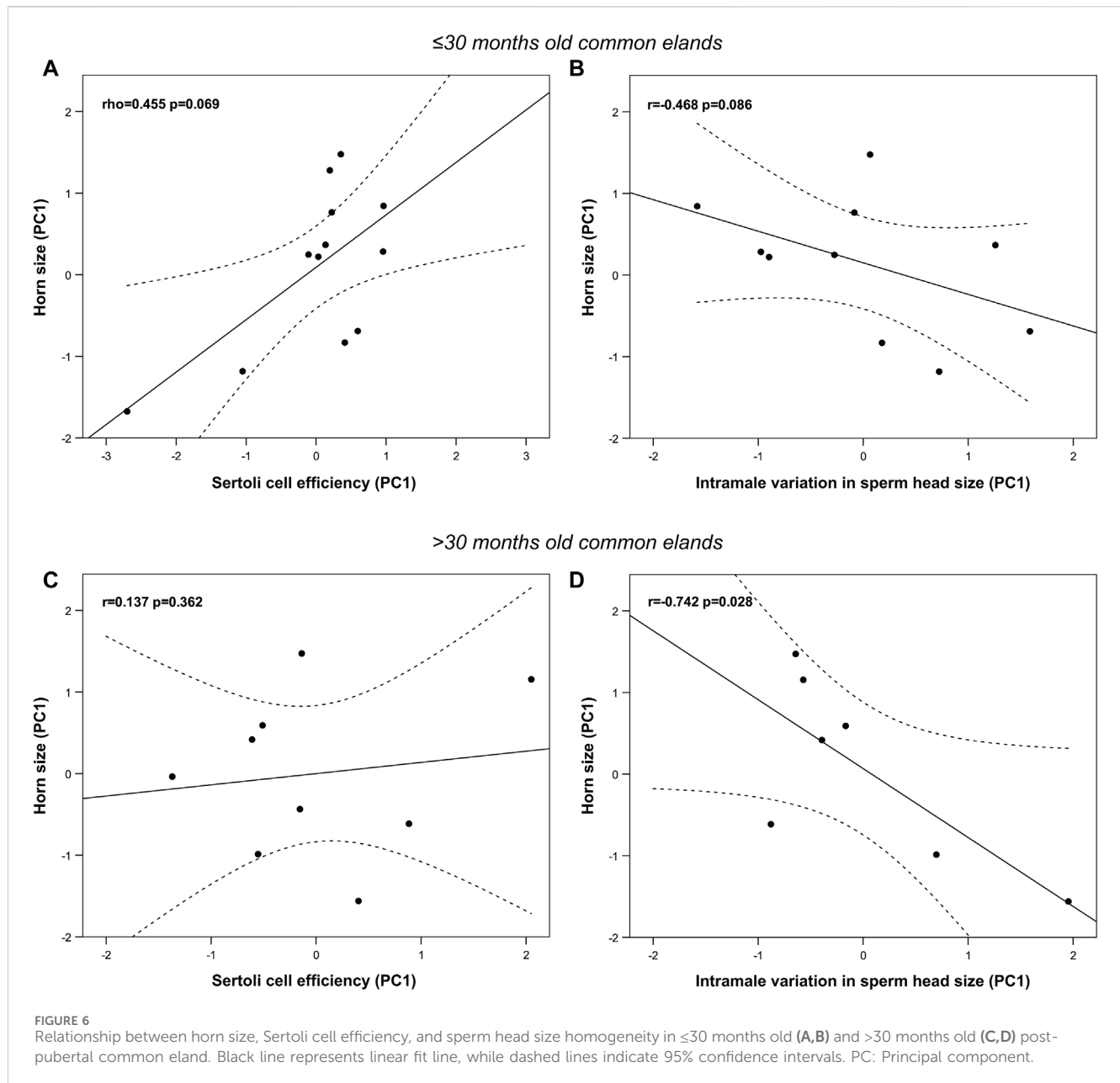
TABLE 6 Comparison of sperm parameters in ≤ 30 months old and > 30 months old post-pubertal common elands.

	≤ 30 months old (Mean $\pm$ SE)	> 30 months old (Mean $\pm$ SE)	<i>p</i>
<i>Sperm number and morphology</i>			
Sperm concentration ($10^6/\text{mL}$)	661.09 $\pm$ 148.02	908.44 $\pm$ 98.48	0.243
Normal sperm (%)	56.70 $\pm$ 10.14	86.07 $\pm$ 3.66	0.055
<i>Sperm size</i>			
Head width (μm)	6.00 $\pm$ 0.05	5.81 $\pm$ 0.04	0.014
Head length (μm)	9.71 $\pm$ 0.09	9.59 $\pm$ 0.11	0.397
Head perimeter (μm)	25.04 $\pm$ 0.14	24.56 $\pm$ 0.21	0.074
Head area (μm^2)	45.77 $\pm$ 0.43	43.78 $\pm$ 0.68	0.020
Head ellipticity	1.62 $\pm$ 0.02	1.65 $\pm$ 0.02	0.318
Midpiece length (μm)	14.57 $\pm$ 0.14	14.44 $\pm$ 0.15	0.545
Principal plus terminal piece length (μm)	47.19 $\pm$ 0.37	48.32 $\pm$ 0.71	0.147
Flagellum length (μm)	61.76 $\pm$ 0.47	62.76 $\pm$ 0.78	0.258
Sperm length (μm)	71.48 $\pm$ 0.50	72.35 $\pm$ 0.77	0.332
<i>Intramale CV in sperm size</i>			
Head width (%)	3.25 $\pm$ 0.18	3.33 $\pm$ 0.44	0.963
Head length (%)	3.01 $\pm$ 0.23	2.63 $\pm$ 0.14	0.223
Head perimeter (%)	2.28 $\pm$ 0.14	2.11 $\pm$ 0.15	0.417
Head area (%)	4.40 $\pm$ 0.20	4.28 $\pm$ 0.45	0.795
Head ellipticity (%)	4.55 $\pm$ 0.24	4.33 $\pm$ 0.35	0.598
Midpiece length (%)	2.89 $\pm$ 0.20	2.37 $\pm$ 0.24	0.112
Principal plus terminal piece length (%)	2.17 $\pm$ 0.09	1.92 $\pm$ 0.13	0.125
Flagellum length (%)	1.65 $\pm$ 0.09	1.47 $\pm$ 0.10	0.216
Sperm length (%)	1.56 $\pm$ 0.09	1.39 $\pm$ 0.06	0.188

Sample size of ≤ 30 months old eland group is $n = 13$ except for sperm morphology and morphometry ($n = 10$). Sample size of > 30 months old eland group is $n = 8$ for sperm concentration and $n = 7$ for sperm morphology and morphometry. CV, coefficient of variation.

not with male ornaments (Svobodová et al., 2018). Beside the cytological and histological assessments provided in this study, analysis of molecular markers and signaling pathways that control germ cell proliferation, differentiation, and apoptosis may contribute to elucidate the mechanisms underlying the relationships between spermatogenic function and sperm traits such as, for instance, the strong positive correlation between the meiotic index and the sperm length found in this study. Another factor that can influence the variation in sperm size is the epididymal environment as sperm morphometry has shown to vary throughout the epididymal regions (Gutiérrez-Reinoso et al., 2016), possibly because of fluid reabsorption, hence increased osmolality. In cats, for instance, reduced within-male variation in sperm head ellipticity is related to higher epididymal mass and sperm concentration (Pintus et al., 2021). In agreement with these findings, we found that low intramale CV in sperm head ellipticity was associated with higher sperm concentration in the epididymal tails of common elands, a finding that may support the role of the epididymal environment in determining a more uniform sperm population.

Overall, the positive relationship between male secondary sexual characters and spermatogenic function found in this study provides support, at least to some extent, to the fertility-linked hypothesis, which predicts that male phenotype covaries with functional fertility (Sheldon, 1994). In contrast, sperm competition games assume a trade-off between pre- and post-copulatory investment (Parker et al., 2013). Across the animal kingdom, evidence has been found to support each or none of these hypotheses (reviewed by Simmons et al., 2017). A complex variety of factors may affect the slope of relationships including genetic variation, mating strategy or environmental conditions. In mammals, for instance, the slope of the relationship between primary and secondary sexual characters can be influenced by the mating strategy, as this trade-off is prominent in species in which weaponry investment is effective in female monopolization (Lüpold et al., 2014). Common elands usually live in large groups (up to 500 animals) that are often composed of mixed-sex herds of one to four adult males and up to more



than 50 females (Pappas, 2002; Bro-Jørgensen et al., 2015). Under such mating system in which males likely fail in female monopolization, it would be more advantageous for males to equally invest their limited resources both in pre- and post-copulatory sexual traits. This would contribute to explain the positive relationship between horn size and testicular function found in this study. Another aspect to consider is that in many sexually dimorphic ungulates, secondary sexual characters like horns or antlers can simultaneously serve both as armaments and ornaments. By contrast, in a comparative study in primate species, Lüpold et al. (2019) found that only those traits that play a role as sexual ornaments trade-off against testes size in contrast to those regarded as weapons. In the light of our findings, future research effort should be directed towards the understanding of other complex signaling traits of male elands such as pelage

ornament (e.g., frontal hairbrush size), dewlap droop, and the peculiar sounds presumably produced by the carpal joints, known as knee-clicks (Bro-Jørgensen and Dabelsteen, 2008; Bro-Jørgensen and Beston, 2015). Another aspect to bear in mind is that, under our experimental conditions, optimal nutritional intake was guaranteed to all animals both in terms of quantity and quality, which may mask any potential trade-off between somatic and testicular investment (Parker, 2016). As previously observed in fallow deer, nutrition has a clear impact on spermatogenic function and sperm morphometry during the sexual development (Ros-Santaella et al., 2019). It remains therefore to be tested whether under restricted access to nutritional resources a positive relationship would be still found between primary and secondary sexual traits' development. It is also important to highlight that our study was performed on a captive population of common eland. On

one hand, this experimental design allowed us to explore the relationship between the development of primary and secondary sexual characters under controlled environmental conditions, but on the other hand, it limits the extrapolation of our findings to natural populations.

The mean horn length found in our study agrees with data reported by previous authors in the adult male common eland (Bro-Jørgensen, 2007; Bro-Jørgensen and Dabelsteen, 2008), which provides support that final horn length is achieved relatively early during the male life and decreases through adulthood because of wearing (Jeffery and Hanks, 1981). Thus, adverse conditions experienced during early development may affect not only the ongoing growth of the individual but also its sexual attractiveness during adulthood (Lindström, 1999). Although age is intrinsically linked to sexual development and underlie the relationships between primary and secondary sexual characters, an unexpected finding of this study was that horn size is not related neither to the spermatogenic index nor to the percentage of sperm cells with normal morphology, which typically increase throughout this phase of male reproductive life (Rajak et al., 2014; Brito, 2021). Nevertheless, both parameters did not differ between common elands in early and late stages of post-pubertal development. Among the traits employed in this study to assess the horn development, it is remarkable to note that the spiral length showed the strongest correlation with the Sertoli cell efficiency. In addition, we also found that horn basal circumference was significantly associated with intramale variation in sperm head size and testicular investment in the seminiferous tubular and epithelial areas. The spiral length and the basal circumference are the horn traits responsible for the sexual dimorphism in common elands as males show horns with more pronounced spirals and greater basal circumference than females (Jeffery and Hanks, 1981; Pappas, 2002). The spiral ridge of the eland horn core has shown different mechanical and chemical properties compared to other parts of this permanent appendage, which may avoid fractures during male-male contests (Cappelli et al., 2018). The spiral of the horn, as bumps and ridges on the horns of various antelopes and gazelles, serve indeed to hold these appendages together during the match, allowing the opponents to develop full strength wrestling engagement (Geist, 1966). In a comparative study across 104 bovid species, Caro et al. (2003) found that, at least in females, twisted horns are associated with wrestling behavior. In the same study, the authors also found that the outward-facing and twisted horns of bovids are also associated with polygynous and large mating groups. Such finding was later supported by Bro-Jørgensen (2007) who found that in polygynous mating system, in which sexual selection is likely to be strong, horn shape is more variable and elaborate than in monogamous species, in which horns tend to be simple and straight. Taken together, male elands with more developed spirals and greater basal circumference may signal not only their fighting ability but also their reproductive competence (i.e., spermatogenic efficiency and sperm size homogeneity). Another non-mutually exclusive hypothesis is that increased horn size may provide a better mechanism for thermoregulation, which may also have beneficial effect on spermatogenesis. Within the thermal neutral zone, the common eland metabolism is estimated to be around 30%

higher than that of Hereford cattle, a bovid species of comparable size (Taylor and Lyman, 1967), although other authors did not find support to this finding (Kotrba et al., 2007). Spermatogenesis requires a cool environment (usually 2°C–6°C below the body core temperature), which is normally guaranteed by the scrotal sac and the complex anatomical structures associated with the latter (i.e., pampiniform plexus, dartos, cremaster, and sweat glands). Because these complex anatomical structures develop with age and influence sperm motility and morphology (Brito et al., 2012), their role is likely more critical in young individuals because of their higher metabolism compared to that of adults. In line with our hypothesis, in the male Alpine ibex (*Capra ibex*), the early development of horns is a suitable predictor of individual reproductive success later in life, whereas the late development of this secondary sexual trait did not seem to significantly relate to it (Willisch et al., 2015). In addition, like other large herbivores adapted to xeric environment (Hetem et al., 2016), common elands can employ adaptive heterothermy by increasing their body temperature in response to high environmental heat load to reduce the water loss (Taylor and Lyman, 1967; but see also Fuller et al., 2004). Because horn size and shape can provide a more efficient mechanism for heat loss (Picard et al., 1994; 1999), and the latter can be beneficial for the spermatogenesis, we can speculate that bigger horn size can provide a more efficient mechanism not only for the body but, indirectly, also for the testicular thermoregulation. On the other hand, no direct link between horn asymmetry and reproductive function was found, probably because of the absence of environmental stressors in our animal population. Using a large dataset, Chirichella et al. (2020) found that environmental stressors (e.g., snow cover duration, population density) experienced in early life influence the symmetrical horn development in the Alpine chamois (*Rupicapra rupicapra*).

In conclusion, greater male investment in secondary sexual characters is related to enhanced spermatogenic function and increased homogeneity in sperm cell dimension. During the sexual development, common elands that display bigger horns show increased testes size, spermatogenic investment, Sertoli cell efficiency and reduced intramale variation in sperm head size and shape. The findings from this study increase our understanding about the role of secondary sexual traits that, beyond sexual selection dynamics, might be involved in the thermoregulatory mechanisms that guarantee the optimal conditions for sperm production.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

Ethics statement

Ethical approval was not required for the study involving animals in accordance with the local legislation and institutional

requirements because the Czech University of Life Sciences' eland farm is accredited as research facility according to European and Czech laws for ethical use of animals in research (permits nos 58176/2013-MZE-17214 and 63479/2016-MZE-17214). All slaughter process was carried out under the supervision of a state veterinarian according to EU and national legislation and conditions for farm animals (slaughter permits nos SVS/WS22/2012-KVSS and SVS/2015/077267-S).

Author contributions

EP: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Supervision, Validation, Writing—original draft. RK: Writing—review and editing, Data curation, Resources. JR-S: Conceptualization, Data curation, Formal Analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Validation, Writing—review and editing.

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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.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcell.2024.1421634/full#supplementary-material>

SUPPLEMENTARY TABLE S1

Principal component analysis of horn size, Sertoli cell efficiency, and intramale variation in sperm head size in ≤30 months old and >30 months old post-pubertal common elands.

SUPPLEMENTARY TABLE S2

Dataset of male post-pubertal common elands.

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Linking DNA damage and senescence to gestation period and lifespan in placental mammals

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The mechanism that synchronizes the timing of parturition remains a mystery. Each mammalian species has a specific duration of gestation that is determined by integrated interactions among the mother, placenta, and fetus. Senescence is primarily driven by DNA damage and is one of the critical factors influencing both parturition and lifespan. In this study, we investigated senescence as a physiological process during pregnancy and observed a gradual physiological increase in senescence in the maternal decidua and placental cells with gestation. This increase in senescence was associated with a gradual physiological increase in DNA damage during gestation. An analysis of the AnAge dataset revealed a positive correlation between the gestation period and maximum lifespan across 740 mammalian species. This finding supports the hypothesis that the rates of DNA damage and senescence may impact both the gestation period and lifespan. We suggest that the relationship between gestation period and lifespan in mammals is mediated by species-specific rates of DNA damage and senescence, necessitating further explorations into their causal roles.

KEYWORDS

placenta, parturition, senescence, lifespan, gestation, genomic instability

1 Introduction

The timing of birth is a major determinant of evolutionary fitness in viviparous species, and there are large variations in the gestational lengths across different mammals. Determination of the gestation period is a complex process involving coordinated interactions among the mother, fetus, and transiently formed organ known as the placenta (Cross, 2005; Burton and Fowden, 2015; Hemberger et al., 2019). Fetuses born preterm (before 37 weeks) have high mortality rates and may develop several neurodevelopmental as well as cardiac abnormalities, whereas fetuses born post-term (after 42 weeks) can pose real threats to the lives of both the mother and fetus (Galal et al., 2012; Singh et al., 2015; Ohuma et al., 2023). Every year, around 13 million babies are born preterm, constituting almost 9.9% of all births globally, which places a significant economic burden on society as most of these preterm babies suffer from long-term disabilities (Ohuma et al., 2023). Despite substantial efforts and extensive research, the molecular mechanisms of parturition (i.e., the action of giving birth to offspring) are not understood fully, even though they are critical determinants of the perinatal outcomes. Fetal organ maturation signals and endocrine signals cannot fully explain the general mechanisms of parturition across all mammals. Changes in the hormonal milieu at

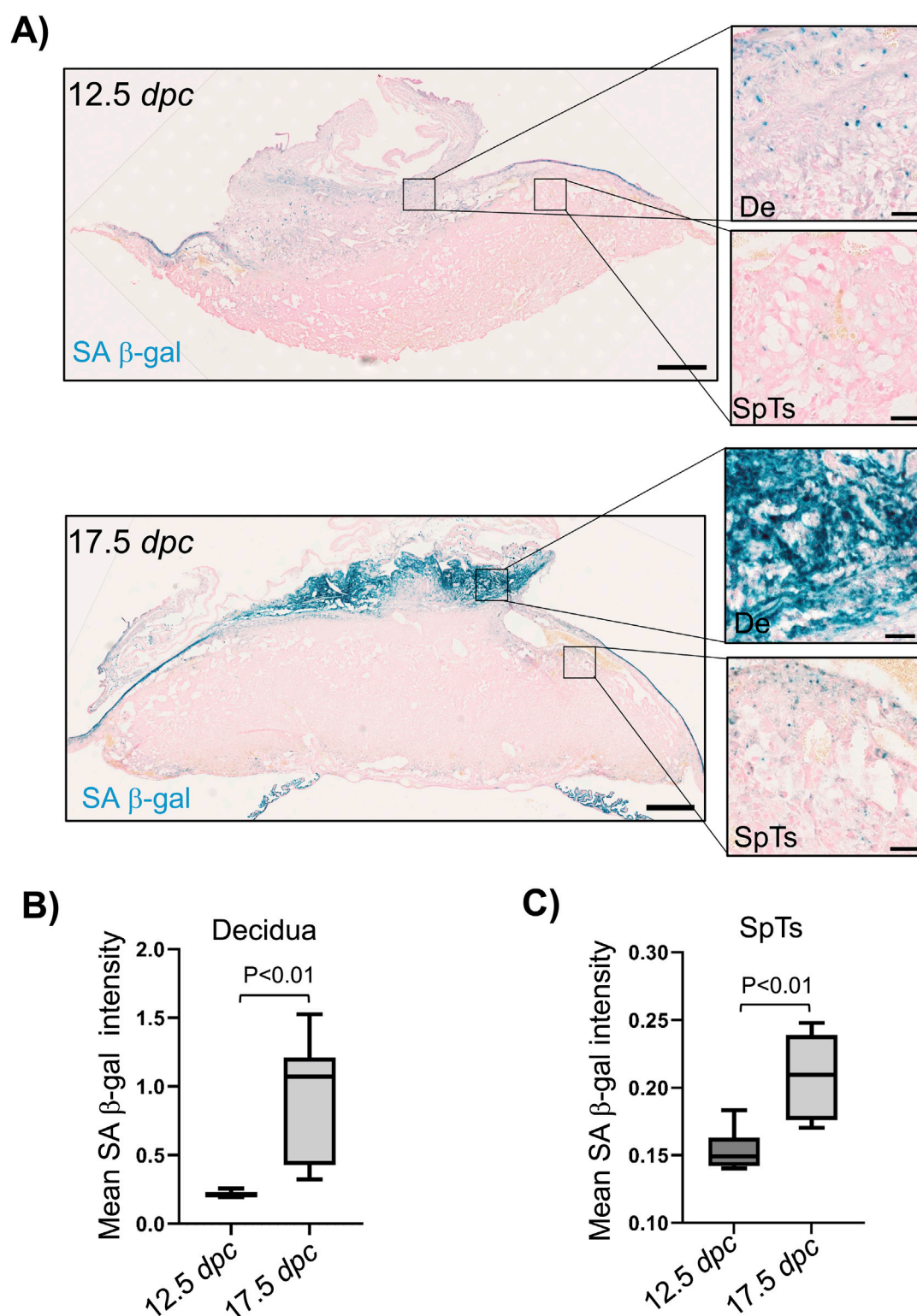


FIGURE 1

Senescence in the maternal decidua and fetal placenta with gestation in mice. **(A)** The maternal decidua and placenta from C57BL/6J inbred strains were fixed at the 12.5 dpc and 17.5 dpc stages of pregnancy, and SA β -gal activity was measured as a marker of senescence after X-Gal staining (dark blue). Hematoxylin was used to counterstain all the nuclei. The decidua (De) and spongiotrophoblasts (SpTs) are shown in the magnified images. **(B)** Quantification of the mean SA β -gal intensities in the maternal decidua at the 12.5 dpc and 17.5 dpc stages of pregnancy ($n = 6$ placentas). The regions of interest (ROIs) were drawn manually in the decidua, and the mean SA β -gal intensities were quantified. **(C)** Quantification of the mean SA β -gal intensity in the placental SpTs at the 12.5 dpc and 17.5 dpc stages of pregnancy ($n = 6$ placentas). The ROIs were drawn manually to mark the SpTs and quantify the mean SA β -gal intensities.

term, such as increased estrogen, oxytocin, and cortisol, along with decreased levels of progesterone can cause COX gene expression and prostaglandin synthesis (Challis et al., 2000). Systemic progesterone withdrawal or addition of oxytocin alone is not found to be solely responsible for inducing parturition in various mammalian species and model organisms (Nishimori et al., 1996; Roizen et al., 2008; Hirota et al., 2010). Interestingly, the administration of exogenous prostaglandins in many of the examined species induced abortion and parturition (Casey and MacDonald, 1988; Gibb, 1998; Pierce et al., 2018). These results suggest that activation of COX and its downstream genes are essential for initiating parturition in many species. Recent studies have suggested the role of senescence in activating COX and inducing sterile inflammation in the maternal decidua, placental, and fetal membranes, which in turn could activate prostaglandin synthesis and parturition (Hirota et al., 2010; Menon et al., 2016; Velicky et al., 2018; Singh et al., 2020). These studies also suggest that premature activation of senescence could cause preterm birth.

DNA damage is the main driver of senescence and plays a central role in regulating its processes (Schumacher et al., 2021). Both DNA damage and senescence are critical determinants of species aging, and species with robust DNA damage repair mechanisms tend to have extended lifespans and slower rates of senescence (Maynard et al., 2015; Yousefzadeh et al., 2021). This begs the question of why different mammals have different lifespans and large variations in their gestation periods. Moreover, is there a relationship between lifespan and gestation period? As both lifespan and gestation period are regulated by senescence, we hypothesize that mammals with robust DNA damage repair mechanisms would have both longer lifespans and longer gestation periods. In the present work, we examine and discuss the above fundamental questions from this perspective. We also systematically analyze senescence and its main driver (DNA damage) for different gestational stages in mouse pregnancy. Using the AnAge dataset, we examine the relationships between gestation period and lifespan in mammals. We propose that DNA damage and senescence may be the key drivers regulating gestation period and lifespan in placental mammals.

2 Physiological increases in senescence and DNA damage in murine placenta

Senescence is typically estimated by measuring the activity of senescence-associated β -galactosidase (SA- β -gal) using X-Gal (a hallmark of senescence) as the substrate (Dimri et al., 1995; Hirota et al., 2010). The number of placental cells is highest at 12.5 days post-coitum (dpc) in mouse pregnancy (Eaton et al., 2020), suggesting that there is minimal cell division after 12.5 dpc and that the senescence cells do not divide. Thus, we decided to analyze senescence at this time point as well as at a later time point, such as 17.5 dpc. We focused on the decidual cells derived from the mother's endometrium and spongiotrophoblasts (SpTs) of the fetal placenta owing to their distinct morphological features at different stages of pregnancy (Rossant and Cross, 2001). We observed some senescence cells in the placenta and decidua at 12.5 dpc (Figure 1A). As the pregnancy progresses, there is a significant increase in the number of senescence cells (Figure 1A). At 17.5 dpc, there was an almost 4.4-

fold increase in the decidual mean SA- β -gal activity measured through X-Gal staining compared to the decidua at 12.5 dpc (Figures 1A, B). In contrast, the increase in mean SA- β -gal activity in the SpTs was only 1.3-fold at 17.5 dpc compared to that at 12.5 dpc (Figures 1A, C). These results suggest that the major increase in senescence during pregnancy is primarily through the contribution of the maternal decidua and to a lesser extent from the fetal placental cells, which are consistent with previously published results (Bonney et al., 2016).

Because persistent DNA damage is a major driver of senescence, we analyzed the DNA damage at the early stages of pregnancy in mice using γ H2A.X immunostaining as the marker of DNA damage (Rodier et al., 2009; Singh et al., 2020). We observed a gradual increase (3.5-fold) in the mean γ H2A.X intensity in the maternal decidua from 9.5 dpc to 12.5 dpc (Supplementary Figures S1A, B). Similarly, the placental SpTs showed a gradual increase (3.8-fold) in the mean γ H2A.X intensity from 9.5 dpc to 12.5 dpc (Supplementary Figures S1A, C). Both the maternal decidua and placental SpTs showed maximum and persistent DNA damage at 12.5 dpc since no further increase was observed at 17.5 dpc (Supplementary Figures S1B, C). Because the placenta is a polyploid organ and an increase in ploidy may elevate DNA damage during the replication process, we also analyzed the ploidy (nuclear size) in the maternal decidua and placental SpTs at different stages of mouse pregnancy (Singh et al., 2020, 2023). Interestingly, there was no change in the nuclear size in the maternal decidua from 9.5 dpc to 17.5 dpc (Supplementary Figures S2A, B). However, we observed a 1.6-fold increase in the nuclear size of placental SpTs from 9.5 dpc to 12.5 dpc (Supplementary Figures S2A, C); furthermore, there was a slight reduction (0.8-fold) in the size of the SpTs from 12.5 dpc to 17.5 dpc, possibly due to reduction in the overall placental volume at the end of pregnancy, as reported previously (Eaton et al., 2020). Numerous studies have previously shown that increased senescence in the maternal decidua and placental cells can cause preterm birth and fetal growth restriction (Hirota et al., 2010; Perez-Garcia and Turco, 2020; Singh et al., 2020, 2023). Overall, these results indicate that persistent DNA damage during gestation induces senescence in the maternal decidua as well as fetal placental cells and that this senescence is not related to the ploidy of the cells. Based on these results, we propose that these gradual physiological increases in DNA damage and senescence during gestation may be critical drivers of parturition in mammals and that changes in these processes can affect the perinatal outcomes.

3 Correlation between the gestation period and lifespan in different inbred mouse strains

DNA damage and senescence are critical factors in the aging of any organism, and it is now widely accepted that the number of senescence cells increases with age (López-Otín et al., 2013; Schumacher et al., 2021). Somatic mutations accumulate in aged humans and model organisms to promote senescence (Moskalev et al., 2013). Each species has a definite lifespan, and species with longer lifespans have robust DNA repair processes, suggesting lower rates of senescence (MacRae et al., 2015). We propose that the physiological rates of DNA damage and senescence during placental

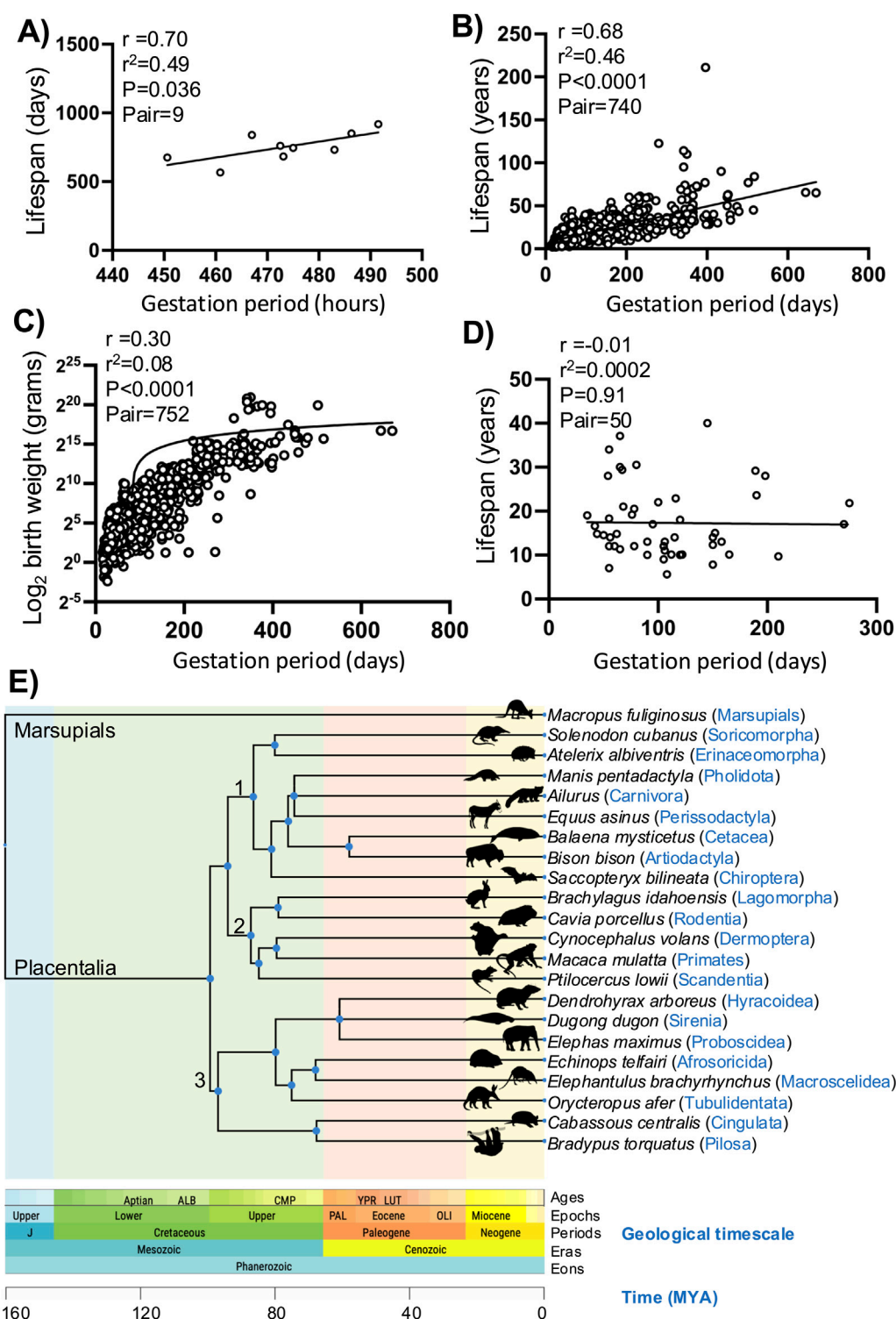


FIGURE 2

Correlation plots showing the relationships between gestation period, lifespan, and body weight. (A) Correlation plot between the gestation periods and lifespans of different inbred strains of mice. The solid line represents the regression curve, and each circle represents a mouse strain. (B) Correlation plot between the gestation periods and lifespans for different placental mammals. The solid line represents the regression curve, and each circle represents a single mammalian species. (C) Correlation plot between the gestation periods and birth weights for different placental mammals. The solid line shows the regression curve, and each circle represents a single mammalian species. (D) Correlation plot between the gestation periods and lifespans in the mammalian order *Chiroptera* (bats). The solid line represents the regression curve, and each circle represents a single bat species. (E) Time-tree of the placental mammals. A single representative species was selected from each of the 21 mammalian orders, and MEGA11 (TIMETREE5) was used to plot the evolutionary relationships. A marsupial species was used to illustrate the evolution of the placental mammals; the shaded colors indicate the geological timescale periods, and the distinct clusters are marked as 1, 2, and 3. The images representing each of the mammalian orders were obtained from PhyloPic (<http://phylopic.org>).

development may reflect the normal aging process in placental mammals. To test this hypothesis, we first analyzed the link between gestation period and lifespan across different inbred strains of mice. Murray et al. (2010) published the gestation periods of 15 inbred strains of mice (AKR/J, CAST/EiJ, KK/H1J, BTBR-T+tf/J, NOD.B10-H2b, DBA/2J, A/J, NZW/LacJ, BALB/cBy, FVB/NJ, C3H/HeJ, 129S1/SvImJ, PWD/PhJ, C57BL/6J, and WSB/EiJ) and showed that strain genetics is a major determinant of the gestational period. We investigated the lifespans of these strains using another dataset published by Yuan et al. (2009). The correlations between gestation period and lifespan for all 15 mouse strains were positive but not significant. However, some of these inbred strains are reported by the Jackson Laboratory to have certain limitations, such as AKR/J that develops lymphoma, KK/H1J that develops diabetes, BTBR-T+tf/J that lacks a corpus callosum, NOD.B10-H2b that shows immune dysfunction, A/J that has a high incidence of lung adenomas, and C57BL/6J that shows seasonal gestational variations, which could affect our analysis; hence, these were excluded from the analysis (Delahunty et al., 2009). By analyzing the remaining nine mouse strains, we observed positive correlations between gestation period and lifespan, with $r^2 = 0.49$ and $p = 0.036$ (Figure 2A). These results suggest that mice with longer gestation periods tend to have extended lifespans.

4 Correlation between the gestation period and lifespan in mammals

We aimed to analyze the relationships between gestation period and lifespan across all placental mammals. Therefore, we downloaded the aging data from the AnAge resource (Human Ageing Genomic Resources) (De Magalhães et al., 2007; Tacutu et al., 2013). Most of the lifespan records in this dataset are obtained in captivity and tend to be slightly longer compared to species in the wild. The lifespan data for the mammalian order *Chiroptera* (bats) were primarily derived from banding studies, and those for the order *Cetacea* were mostly obtained using indirect methods (De Magalhães et al., 2007). We obtained data on the gestation period, birth weight, and lifespan for more than 740 placental mammals across all 21 mammalian orders. We analyzed the correlations between gestation period and lifespan in these data and observed a strong significant positive correlation, with $r^2 = 0.46$ and $p < 0.0001$ (Figure 2B). When we analyzed the correlation between gestation period and birth weight, we found a moderate association, with $r^2 = 0.08$ and $p < 0.0001$ (Figure 2C).

We also categorized all placental mammals according to their order. Using MEGA11 (TIMETREE5), we analyzed the evolutionary relationships among all 21 mammalian orders (Figure 2E; Supplementary Figure S3) (Tamura et al., 2021; Kumar et al., 2022; Li et al., 2022) and observed three distinct clusters: 1) *Soricomorpha*, *Erinaceomorpha*, *Pholidota*, *Carnivora*, *Perissodactyla*, *Cetacea*, *Artiodactyla*, and *Chiroptera*; 2) *Lagomorpha*, *Rodentia*, *Dermoptera*, *Primates*, and *Scandentia*; 3) *Hyracoidea*, *Sirenia*, *Proboscidea*, *Afrosoricida*, *Macroscelidea*, *Tubulidentata*, *Cingulata*, and *Pilosa*. We performed correlation analyses between the gestation period and lifespan in all these mammalian orders with more than ten genera in the dataset

(Carter and Mess, 2008; Carter, 2012). We found that most orders had positive associations between the gestation period and lifespan, except for *Chiroptera* (bats) (Figure 2D; Supplementary Table S1). Closely related orders such as those in cluster 1 (*Artiodactyla*, *Carnivora*, and *Soricomorpha*) and cluster 2 (*Lagomorpha*, *Rodentia*, and *Primates*) showed strong positive correlations between the gestation period and lifespan (Figure 2E; Supplementary Figure S3; Supplementary Table S1). Interestingly, *Chiroptera* (bats) exhibited that an increase in the gestational period did not increase the lifespan, making them an outlier among all mammalian orders. This finding contrasts with previous observations that bats have higher life expectancies relative to mammals of similar body size (Ricklefs, 2010). Our results suggest that bats should have even longer lifespans given their gestation period. These results indicate that the correlation between gestation period and lifespan is significant. Since both the gestation period and lifespan are regulated by DNA damage and senescence, we propose that the causal roles of these factors in regulating the gestation period and lifespan should be explored.

5 Discussion

It is generally believed that traits associated with placental development exhibit antagonistic pleiotropy to support early development but have deleterious effects later in life. Our results suggest that DNA damage and senescence are physiological processes during placental development but have detrimental effects later that contribute to aging. We observed positive correlations between the gestation period and lifespan in placental mammals. Fetal organ maturation and endocrine signals alone could not explain the general mechanism of parturition in all placental mammals. From this perspective, we propose that DNA damage and senescence provide evolutionarily consistent mechanisms to regulate the gestation period and lifespan in mammals. Although our results are correlative and require further validation in several mammalian species, they provide valuable insights into the aging processes of mammals.

Large cohort studies have shown that around 78% of the babies born at or before 27 weeks of gestation develop at least one chronic health condition compared to 37% for full-term babies (Dance, 2020). There are indications of high mortality rates in preterm babies due to these chronic health conditions, suggesting a decreased lifespan (Dance, 2020). These findings indicate a possible relationship between gestation period and lifespan in humans. Black women in the United States have preterm birth rates that are twice that of Caucasians (Ekwo and Moawad, 1998). Similarly, preterm births are very high in certain populations of Sub-Saharan African countries, such as Nigeria and Kenya, as well as tribal populations in India (Shah et al., 2021; Ohuma et al., 2023). Several factors may contribute to preterm births in these populations, including low income, high infection rates, and potentially genetic causes. There are also indications that these populations have lower life expectancies, but there is a lack of systematic analysis on preterm babies and their health conditions. Understanding genetic factors may thus help improve the quality of life for these populations.

In humans, among the natural conceptions for which the ovulation dates are known, there may be a 37-day variation in the gestation period (Jukic et al., 2013). It is unclear why there is such a large variation in the gestation period in humans. The decidua genotype is always maternal, whereas the placenta and fetal membrane consist of both paternal and maternal genotypes. Our results suggest that the major contributor to senescence is the maternal decidua, but there may also be some contributions from the placenta and fetal membrane. In each pregnancy, the decidual genotype is consistent for a mother but there are large variations in the placental and fetal membrane genotypes because of random segregation of genes during gamete formation. Thus, the gestation length is determined by integrated interactions among the maternal, fetal, and placental genotypes. Given the extensive diversity of human population, such variations in gestational length are expected.

Despite having robust DNA damage repair mechanisms, it is unclear why bats exhibit longer gestation periods but still have shorter lifespans. One possibility is that bats are reservoirs of several viruses, which may contribute to their reduced lifespans (Gorbunova et al., 2020). Given the high load of viruses in bats, they have evolved very strong DNA damage repair pathways (Wang et al., 2011; Huang et al., 2016, 2019); this could be why bats have slower rates of DNA damage and senescence in the maternal decidua and fetal placenta despite their longer gestation periods. It has been shown that the entire PYHIN gene family, including DNA sensors AIM2 and IFI16, are missing in bats along with dampened cytosolic DNA sensors, such as STING-dependent interferon activation (Xie et al., 2018; Gorbunova et al., 2020). This may be another reason for the reduced sterile inflammation and senescence observed in the placentas of bats, despite their longer gestation periods.

Many biologists believe that the rate of aging has a genetic basis; it has also been shown in different strains of mice that the gestation period is genetically determined (Murray et al., 2010). In this report, we propose that DNA damage and senescence could be the most likely links between the gestation period and lifespan in placental mammals. Bats have evolved robust DNA damage repair processes as well as suppressed DNA sensing mechanisms to dampen inflammation and coexist with viruses. We provide some possible explanations for their unusually long gestation period but require further experimentation to test the DNA damage and senescence in bat placentas at different time points during gestation. Overall, our results suggest that the rates of DNA damage and senescence may be determinants of the gestation period and that perturbations in these processes could lead to preterm births or fetal growth restrictions. We propose that there are critical thresholds for the rates of DNA damage and senescence and that accelerating or decelerating these processes will affect the parturition process. Understanding the molecular regulators of placental DNA damage and senescence will thus aid in elucidating the molecular etiologies of preterm births and parturition.

Data availability statement

The original contributions presented in the study are included in the article/[Supplementary Material](#); further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was approved by the Stowers Institute for Medical Research, Kansas City, MO, United States. The study was conducted in accordance with all local legislations and institutional requirements. This study was approved by the institutional ethical committee of ICMR-NIRTH, Jabalpur (NIRTH/IEC/202302/09 dated 16/05/2023).

Author contributions

VPS: conceptualization, data curation, funding acquisition, methodology, validation, visualization, writing—original draft, and writing—review and editing. PS: supervision, writing—review and editing, and funding acquisition.

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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.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcell.2024.1480695/full#supplementary-material>

SUPPLEMENTARY FIGURE S1

DNA damage in the maternal decidua and fetal placenta with gestation in mice. (A) The maternal decidua and placenta from C57BL/6J inbred strains were fixed at the 9.5 dpc, 12.5 dpc, and 17.5 dpc stages of pregnancy, and γ H2A.X immunostaining was performed to identify the damaged DNA sites using a chromogenic substrate (dark brown). Hematoxylin was used to counterstain all nuclei. The decidua (De) and spongiotrophoblasts (SpTs) are shown in the magnified images. SpTs from the 9.5 dpc placenta are highlighted with rectangles, given their small numbers. (B) Quantification of the mean γ H2A.X intensities in the maternal decidua at the 9.5 dpc, 12.5 dpc, and 17.5 dpc stages of pregnancy ($n = 3-6$ placentas). The regions of interest (ROIs) were drawn manually in the decidua, and the mean γ H2A.X intensities were quantified. (C) Quantification of the mean γ H2A.X intensities in placental SpTs at the 9.5 dpc, 12.5 dpc, and 17.5 dpc stages of pregnancy ($n = 3-6$ placentas). The ROIs were drawn manually to mark the SpTs and quantify the mean γ H2A.X intensities.

SUPPLEMENTARY FIGURE S2

Nuclear areas in the maternal decidua and fetal placenta with gestation in mice. (A) The maternal decidua and placenta from C57BL/6J inbred strains were fixed at the 9.5 dpc, 12.5 dpc, and 17.5 dpc stages of pregnancy, and Feulgen staining was performed to quantify the nuclear areas (light violet). The decidua (De) and spongiotrophoblasts (SpTs) are shown in the magnified images. SpTs from the 9.5 dpc placenta are highlighted with rectangles, given their small numbers. (B) Quantification of the integrated Feulgen densities in the maternal decidua at the 9.5 dpc, 12.5 dpc, and 17.5 dpc stages of pregnancy ($n = 3-6$ placentas). The regions of interest (ROIs) were drawn manually in the decidua, and the integrated Feulgen densities (mean intensity multiplied by area) were quantified. (C) Quantification of the integrated Feulgen densities in placental SpTs at the 9.5 dpc, 12.5 dpc, and 17.5 dpc stages of pregnancy ($n = 3-6$ placentas). The ROIs were drawn manually to mark the SpTs and quantify the integrated Feulgen densities.

SUPPLEMENTARY FIGURE S3

Time-tree of placental mammals. At least one representative species was selected from each mammalian family (Supplementary Table S2), and MEGA11 (TIMETREE5) was used to plot the evolutionary relationships. The shaded colors indicate geological time periods, and distinct clusters are marked as 1, 2, and 3. The images representing each of the mammalian orders were sourced from PhyloPic (<http://phylopic.org>).

SUPPLEMENTARY TABLE S1

Correlation analysis between the gestation period and lifespan for different mammalian orders. Taxonomic orders with a minimum of 10 genera are included in this table.

SUPPLEMENTARY TABLE S2

Dataset downloaded from AnAge. Sheet 1 contains the entire dataset, sheet 2 lists all placental mammalian species, and sheet 3 includes all placental mammals according to their order.

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Developmental bias as a cause and consequence of adaptive radiation and divergence

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Efforts to reconcile development and evolution have demonstrated that development is biased, with phenotypic variation being more readily produced in certain directions. However, how this “developmental bias” can influence micro- and macroevolution is poorly understood. In this review, we demonstrate that defining features of adaptive radiations suggest a role for developmental bias in driving adaptive divergence. These features are i) common ancestry of developmental systems; ii) rapid evolution along evolutionary “lines of least resistance;” iii) the subsequent repeated and parallel evolution of ecotypes; and iv) evolutionary change “led” by biased phenotypic plasticity upon exposure to novel environments. Drawing on empirical and theoretical data, we highlight the reciprocal relationship between development and selection as a key driver of evolutionary change, with development biasing what variation is exposed to selection, and selection acting to mold these biases to align with the adaptive landscape. Our central thesis is that developmental biases are both the causes and consequences of adaptive radiation and divergence. We argue throughout that incorporating development and developmental bias into our thinking can help to explain the exaggerated rate and scale of evolutionary processes that characterize adaptive radiations, and that this can be best achieved by using an eco-evo-devo framework incorporating evolutionary biology, development, and ecology. Such a research program would demonstrate that development is not merely a force that imposes constraints on evolution, but rather directs and is directed by evolutionary forces. We round out this review by highlighting key gaps in our understanding and suggest further research programs that can help to resolve these issues.

KEYWORDS

phenotypic plasticity, plasticity-led evolution, parallel evolution, evolvability, eco-evo-devo, extended evolutionary synthesis, ecotype evolution, quantitative genetics

1 Introduction

The neo-Darwinian view of evolution commonly proposes that random genetic mutations lead to random phenotypic variation, which is then sorted by natural selection (Gregory, 2009). Through this view selection and genetic allele frequency changes have been at the centre of evolutionary enquiry over the past decades, from the emergence of evolutionary ecology (Endler, 1986) toward the incorporation of genetics (Mousseau et al., 2000), and ultimately genomic data. However, decades of molecular, developmental, and theoretical findings have shown that this view is limited and understates the role of development in driving evolutionary change (Laland et al., 2015; Muller, 2007).

TABLE 1 Properties of adaptive radiations that can be better understood by integrating developmental bias into our thinking.

Property of adaptive radiations	Conventional perspective (neglecting development)	Incorporating developmental bias
Rapid evolution	Adaptive evolution occurs through random mutations that are unguided in their phenotypic consequences. Additional role for relaxed selection in a novel environment	Phenotypic consequences of mutations and environmental inputs are non-random (biased), and evolution can be accelerated if such biases align with axes favored by selection. Biases can evolve, and this may be facilitated by the release of competition
Parallel evolution	Similar selection regimes lead to similar phenotypic outcomes	In order to evolve in parallel, lineages must have similar patterns of bias, or be able to diverge along similar trajectories. These biases can evolve. If a lineage encounters an environment an ancestor has already adapted to, evolution may be accelerated if “current” biases reflect past selection
Radiating lineages have a “competitive advantage”	Certain lineages radiate because they claim a competitive advantage. Often, such successful colonisers are described as “generalists”	Lineages that gain a competitive advantage may do so if biases align with selection or evolve to do so before competitors. “Generalists” may show flexibility in these biases
Facilitated by ecological release	Adaptive radiations begin when a lineage invades a new environment. This “ecological release” from competition allows diversification into previously uninhabited niches	Shifts in developmental biases may break constraints. This “developmental release” would allow invasion into novel regions of morphospace. Thus, adaptive divergence and radiation may not require environmental changes
Repeated emergence of “ecotypes”	Radiations are characterised by the repeated emergence of “ecotypes” (or “ecomorphs”), characterised by the co-occurrence of several ecologically-relevant traits. Ecotypes emerge due to selection on this “complex” of traits	Ecotype emergence is driven by patterns of covariation that link such traits, and thus represents different points on a “line of least resistance.” Once these patterns of covariation evolve once within a lineage, future divergence along this axis will be facilitated and accelerated
Radiating lineages are characterised and facilitated by common ancestry	Genetic similarity will increase the likelihood of parallel evolution	Common ancestry may correspond to more similar patterns of developmental bias, thus increasing the likelihood of parallel evolution
Adaptive divergence is often seeded through phenotypic plasticity	Plasticity-led evolution can accelerate adaptation and ensure phenotype-environment correlations	Responses to environmental and genetic perturbations are similarly biased by a shared developmental system. Closely-related lineages may show similarly-biased plastic responses, driving parallelism. Plasticity-led evolution alters the environment-phenotype and genotype-phenotype maps, increasing the likelihood of similar plastic responses in the future

Phenotypic variation is the target of selection and arises through a range of developmental processes. Far from providing a blank slate for selection to act on, it is now understood that phenotypic development is “biased,” in that developmental systems respond to genetic and environmental perturbations in non-random ways (Uller et al., 2018). Indeed, it has been demonstrated that both phenotypic patterns of mutational variation (Braendle et al., 2010; Houle et al., 2017) and trajectories in multivariate phenotypic space are biased in nature (McGlothlin et al., 2018; Rohner et al., 2022; Schluter, 1996). This suggests that developmental processes impose structure upon phenotypic variation, including biases that may or may not align with patterns of macroevolutionary divergence (Houle et al., 2017; McGlothlin et al., 2018; Rhoda et al., 2023). However, how these biases shape, and are shaped, by evolutionary change is poorly understood, and requires a synthesis of ideas about how adaptation unfolds, and how phenotypic variation arises.

Investigating adaptive radiations may facilitate such a synthesis, as they can address both generative developmental processes and the sorting processes of selection. The predominant view in adaptive radiation research – ecological speciation – has focused more upon the contribution of selection. Adaptive radiations are characterised by the explosive diversification and speciation within a lineage, often triggered by the colonisation of a novel environment (Schluter, 2000). Such radiations have produced much of earth’s diversity (Wilson, 1999), including some of the most well-characterised study

systems in evolutionary biology, such as African Rift Lake cichlids (Santos and Salzburger, 2012), Galapagos finches (Grant and Grant, 2002), and Caribbean anoles (Losos, 2011). However, whilst a growing body of work has investigated the role of development in adaptive radiations (e.g., Abzhanov et al., 2004; Maan and Sefc, 2013), the links between developmental bias and adaptive radiations have been neglected. This is despite many of the defining features of adaptive radiations suggesting a role for developmental bias. Therefore, we contend that radiating lineages could be used to reconcile developmental bias with adaptive evolution.

In this review, we will argue that many classic characteristics of adaptive radiations imply that developmental biases are both causes and consequences of these evolutionary patterns (Table 1). Specifically, these classic features are i) common ancestry within a radiating lineage; ii) rapid evolution along evolutionary “lines of least resistance,” often manifesting in parallel evolution; iii) the subsequent repeated and parallel evolution of ecotypes; and more recently the idea that iv) evolutionary change is “led” by biased phenotypic plasticity upon exposure to novel environments (Levis and Pfennig, 2019). In the process, we hope to demonstrate that adaptive radiations provide unprecedented opportunities to study developmental biases, and to integrate adaptive evolution and development. Finally, we suggest areas of research that will be required to fully appreciate the role that developmental biases play in adaptive radiations and divergence.

2 A primer on developmental bias

Darwin was the first to observe and recognise both the discontinuities of variation and the importance of understanding its underlying mechanisms, but lacking knowledge of genetics and development he was unable to provide such explanations (Darwin, 1859; Gliboff, 2023). Since his time, a coherent understanding of genetics preceded knowledge of development, leading to a gene-centric view of evolution referred to as the “modern synthesis,” which remains central today (Laland et al., 2014a). In this highly quantitative view of evolution, any potential biases in the distribution of mutational effects are overpowered by selection (Fisher, 1930; Yampolsky and Stoltzfus, 2001), thus relegating development to the role of an uninteresting and undirected process that produces the substrate for selection to “sculpt” evolution to its liking (Hendrikse et al., 2007). Findings from molecular biology, genetics and paleontology led to a renewed interest in development at the tail end of the 20th century, led by researchers such as Stephen Jay Gould (Gould, 1977; Gould, 1989; Gould and Lewontin, 1979; Alberch 1980; Alberch 1989), and John Maynard-Smith (Maynard-Smith et al., 1985) amongst others. This research program sought to understand “rules of development,” which were typically viewed as constraining forces that influenced evolution only by impeding selection (Gould, 1989), with Maynard-Smith et al. (1985) recommending “extreme caution in claiming that such constraints are responsible for evolutionary trends.” It was not until the turn of the millennium when researchers began to understand these developmental “rules” as forces that could accelerate or improve the efficacy of evolution (Arthur, 2001; Schluter, 1996). Thus, the need to integrate evolution and development, and “extend” the modern synthesis (Pigliucci, 2007), was recognized.

The “extended evolutionary synthesis” (EES) focuses on bridging the explanatory gap between genotype and phenotype, by understanding how developmental processes can explain evolutionary trends (Laland et al., 2015; Pigliucci, 2007). In this framework, embryos are not simply a collection of genes, but are developmental systems capable of guiding their own ontogenetic trajectory (Laland et al., 2014b; Walsh, 2015). Furthermore, evidence from models of tooth development (Kavanagh et al., 2007) and pattern formation (Turing, 1952; Yamaguchi et al., 2007) demonstrate that adaptive variation can be produced by interactions between morphogenic elements, often in dynamic and reciprocal fashion, that suggest levels of developmental causation occurring above the level of genes (Müller and Newman, 2003). The overarching conclusion of this research program so far is that evolvability – the ability to produce adaptive and heritable phenotypic variation (Kirschner and Gerhart, 1998) – is itself capable of evolving (Pigliucci, 2008; Wagner and Altenberg, 1996). In other words, developmental architectures evolve to become “biased” towards adaptive regions of phenotypic space (Uller et al., 2018).

3 Characteristics of adaptive radiations that imply role for biases

3.1 Common ancestry

The fact that members of an adaptive radiation necessarily share a recent common ancestor (Schluter, 2000) allows us to study how

the combined effects of selection and development determine the generation and persistence of phenotypic variation within a radiating lineage. Note that adaptive radiations do not simply reflect the sorting of ancestral variation into new forms, but rather are characterised by the production of novel and exaggerated variants that far exceeds that observed in the ancestor. However, common ancestry means that members of a radiation share a common ancestral developmental system making it more likely that the responses of lineages to various environmental and genetic inputs are biased along similar developmental trajectories (Parsons et al., 2020; Schluter, 1996) (Figure 2A). This ultimately ties to the themes we discuss next.

3.2 Rapid evolution along lines of least resistance

Another defining feature of adaptive radiations are their greatly accelerated rates of diversification and speciation (Gillespie et al., 2020; Schluter, 2000). As a famous example, over 500 species of cichlids have emerged within Lake Victoria from common ancestry in approximately 10,000 years (Galis and Metz, 1998), involving accelerated rates of speciation (Henning and Meyer, 2014) and the emergence of diverse morphologies (Witte et al., 2008), colour patterns (Maan and Sefc, 2013), and life-histories (Ribbink, 1990) (Figure 1A). In search of a general mechanism for such rapid rates of evolution, Schluter (1996) used a quantitative genetics approach to demonstrate that phenotypic evolution in threespine stickleback was in closer alignment to $\mathbf{g}_{\max}$ – the multivariate direction of greatest additive genetic variance within a population – than would be expected by chance. This phenomenon has since been confirmed by a large body of literature (Bégin and Roff, 2004; Blows and Higgie, 2003; Brattström et al., 2020; Marroig and Cheverud, 2005; McGlothlin et al., 2018; McGuigan et al., 2005; Rhoda et al., 2023; Figure 1B; Renaud et al., 2006; Rohner and Berger, 2023; Walter et al., 2018), although counter-examples also exist (Badyaev and Hill, 2000; Merilä and Björklund, 1999). Quantitative-genetic models (Lande, 1979) then suggest that evolutionary trajectories do not necessarily follow the direction of fastest ascent in the fitness landscape (referred to as the “selection gradient”), but rather are biased towards $\mathbf{g}_{\max}$, which Schluter calls the “genetic line of least resistance.” While misalignment between selective and developmental axes can constrain adaptive evolution and reduce fitness gains, strong alignment can accelerate it (Figure 2B). For example, Marroig and Cheverud (2005) showed that evolutionary rates decreased by a factor of 3–4 when selection and $\mathbf{g}_{\max}$ (i.e., development) are not aligned. However, while evidence suggests that evolution tends to occur along lines of least resistance initially, without understanding the stability of the structure of multivariate phenotypic variation over generations the predictive power of such models is limited.

Indeed, while the quantitative genetics program has yielded many important evolutionary insights, the $\mathbf{G}$ -matrix – a matrix of trait variances and covariances from which $\mathbf{g}_{\max}$ is derived – carries predictive power only from one generation to the next (Pigliucci, 2006), as it is itself subject to drift and selection. Thus, how such a research program can bridge micro- and macroevolution is a persistent issue (Arnold et al., 2001). For

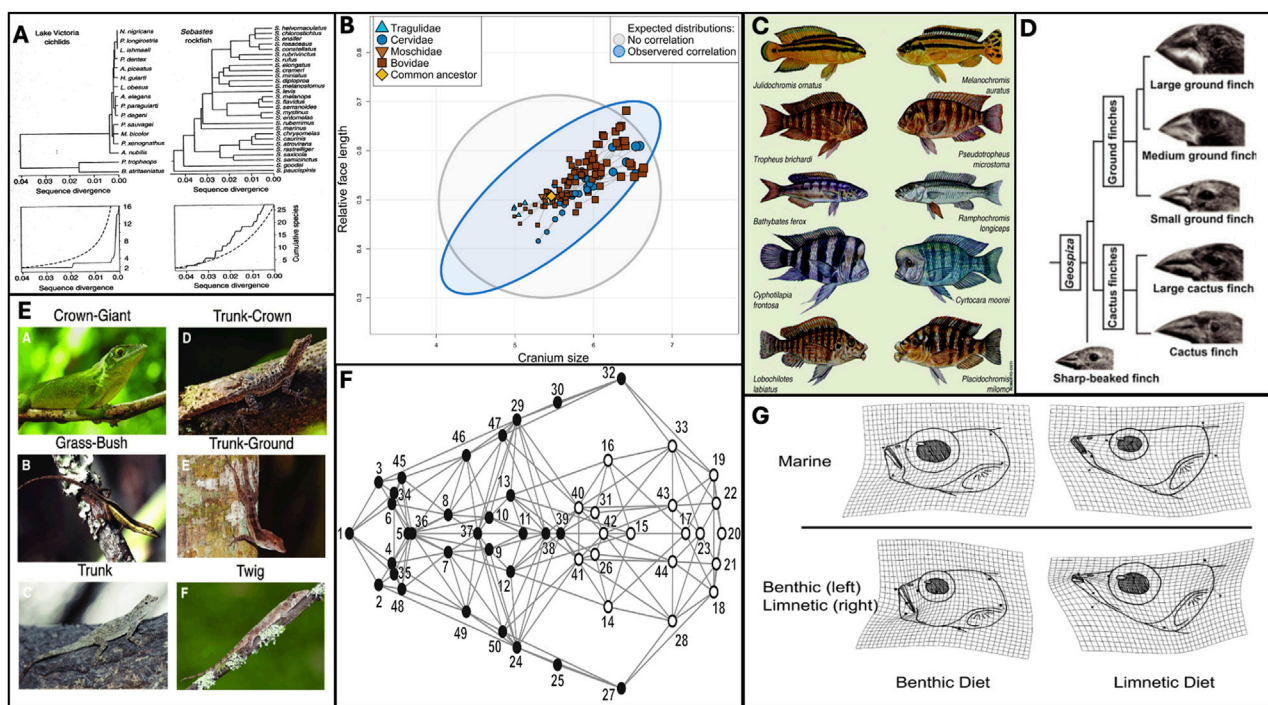


FIGURE 1

Defining features of adaptive radiations suggest a role for developmental bias. (A) If lines of least resistance align with axes of selection, evolution can be greatly accelerated. Cichlid speciation (left) and sequence divergence is rapid, surpassing neutral expectations, and evolutionary rates in a non-radiating family, *Sebastes* rockfish. Figure from Schluter (2000) p.15. (B) Evolution tends to follow "lines of least resistance." Rhoda et al. (2023) found that ruminant cranial evolution follows a line of least resistance driven by an allometric relationship between face length and cranium size. Thus, variation is restricted to a narrower region of phenotypic space (blue) than would be expected under the null hypothesis of isotropic variation (grey). (C) Similar patterns of developmental bias within a lineage can contribute to parallel evolution. Many similar species have emerged independently in three African Rift Lakes – Malawi, Victoria and Tanganyika. Diagram from Stiasny and Meyer (1999) shows fish from lake Tanganyika on the left, and similar varieties from Lake Malawi on the right. (D) Adaptive radiation is characterised by ecotypic divergence, driven by altered patterns of covariation. Galapagos finches represent a textbook example, due to divergence in skull and beak morphology in response to different feeding niches. Figure from Mallarino et al. (2011). (E) The *Anolis* adaptive radiation is characterised by the repeated emergence of six ecotypes, inhabiting different microhabitats. Figure from Huie et al. (2021). (F) Adaptive radiation are often facilitated by "key innovations," seeded through altered patterns of covariation that bias evolution towards certain regions of phenotypic space. Drake and Klingenberg (2010) demonstrated that the canine skull is composed of two modules, mandibular (black) and cranial (white), and associated this developmental lability with the diversity observed within domestic dog breeds, which eclipses that of the entire carnivora family. (G) Adaptive divergence is often seeded through phenotypic plasticity, which is biased in nature. Wund et al. (2008) demonstrated that specialised benthic and limnetic feeding morphologies (bottom), characterised by patterns of covariation, could be recapitulated plastically in the marine ancestor (top). Such "plasticity-led evolution" is a characteristic feature of adaptive radiations.

example, Boell (2013) found that the G -matrix was unique for each generation, while Björklund et al. (2013) and Doroszuk et al. (2008) found fluctuations in the G -matrix over relatively short time frames. Schluter (1996), based on the models of Lande (1979) and Via and Lande (1985), predicted that his model carried predictive power only for the initial stages of divergence, and that selection should overpower developmental constraints given enough time. Nevertheless, (Schluter, 1996) showed that stickleback evolution had followed the line of least resistance for at least 4 million years, as far back as the data allowed inference. Similarly, (Renaud et al., 2006) observed that evolution in rodents aligned with g_{max} for 6.5 million years, before eventually diverging, and evidence from *Anolis* (McGlothlin et al., 2018), *Sepsidae* flies (Rohner and Berger, 2023), *Drosophila* (Houle et al., 2017), and *Onthophagus* dung beetles (Rohner et al., 2022) show alignment between lines of least resistance and macroevolutionary patterns seemingly persisting over tens of millions of years. Thus, in spite of the ephemeral nature of the G -matrix, a meta-analysis by Arnold et al. (2008) found that several measures, and particularly the

orientation of g_{max} were broadly similar in 74% of studies comparing experimental treatments, sexes, populations, and species. These results suggest that g_{max} and thus the underlying developmental processes – may play a role in dictating macroevolutionary patterns.

While Houle et al. (2017) argue that the alignment between lines of least resistance and macroevolution is suggestive of evolutionary constraint, it could also be argued that the G -matrix and g_{max} are subject to selection and are therefore capable of evolving. Theoretical models suggest that pleiotropy (Jones et al., 2003; Jones et al., 2007) and epistasis (Jones et al., 2014) – the processes determining patterns of covariation – can be molded by selection so that the distribution of mutations becomes aligned with the fitness landscape. The explanation that the G -matrix and lines of least resistance are evolvable is preferred by Rohner and Berger (2023), McGlothlin et al. (2018), and Schluter (1996). Indeed, Schluter (1996) remarks that the G -matrix provides explanatory power for patterns of variation that are demonstrably the result of natural selection, suggesting alignment between selective and development

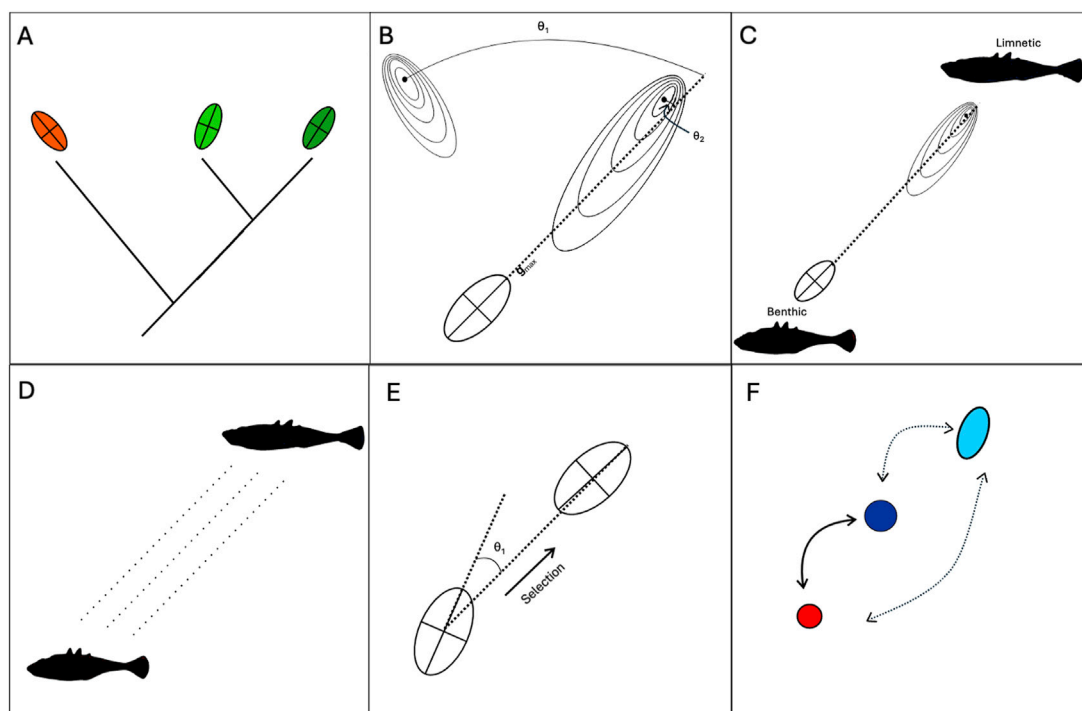


FIGURE 2

Bias as a cause and consequence of adaptive radiations and divergence. (A) Common ancestry may lead to similarly-biased developmental systems.

As a result, parallel evolution may be more likely within a lineage, or one lineages may be able to evolve in the face of ecological “opportunity” while another lineage cannot. (B) Evolution tends to occur along “lines of least resistance,” captured by $G_{\max}$ – the axis along which the most additive genetic variation is produced. If $G_{\max}$ aligns with the fitness landscape (θ is small), then adaptive evolution may be accelerated (e.g., θ_1). Conversely, if $G_{\max}$ for a population or lineage is not aligned with the fitness landscape (θ is large, e.g., θ_2) adaptation may be slowed or constrained entirely. Hence, biases can be viewed as permissive and constraining forces. (C) Lines of least resistance typically correlate with multiple ecotypes. For example, Schluter’s (1996) line of least resistance for threespine stickleback contained slender, shallow-bodied limnetic forms at one end and deep-bodied limnetic forms at the other end. (D) Parallel evolution of ecotypes can occur through a combination of parallel selection pressures and parallel patterns of bias. Benthic–limnetic divergence has occurred numerous times independently in sticklebacks, as has the emergence of similar divergent phenotypes in response to different environmental gradients. (E) Adaptive divergence results in altered patterns of developmental bias. Thus, biases can be viewed as consequences, as well as causes, of adaptive divergence and radiation. Future evolution can be accelerated if it occurs along axes previously favoured by selection. (F) Rapid evolution can occur via biased phenotypic plasticity. “Learning” can occur in gene regulatory networks, allowing rapid plastic switching between ecotypic forms that can become refined over time (hard arrows). Developmental noise induced by novel environmental cues will further be directed along axes previously favoured by selection (dashed arrows).

explanations. To support this, the meta-analysis of Arnold et al. (2008) found that, although G -matrices and $g_{\max}$ were fairly stable across published studies, divergence was observed in species under divergent selection (Cano et al., 2004; Doroszuk et al., 2008; Eroukhanoff and Svensson, 2011; Fong, 1989; Jernigan et al., 1994; Johansson et al., 2011; Roff et al., 2004; Shirk and Hamrick, 2014; Land et al., 1999), further suggesting malleability of these matrices. Direct evidence comes from Walter et al. (2018), who found G -matrix divergence between wildflower ecotypes evolving in different microhabitats, suggesting developmental divergence driven by diverging selection regimes. When taken together, these data suggest that G -matrices, and thus the underlying developmental processes, impose structure on the phenotypic variation made available to selection, and these biases evolve and subsequently influence macroevolution. Understanding both the stability and evolvability of these biases is required to fully understand their role in adaptive radiations.

A persistent criticism of the modern synthesis is that it fails to consider developmental processes, and thus can explain what happens to phenotypic variation that is generated but does not

provide insights into how this variation originates. A similar criticism can be made of quantitative genetics, which typically uses modelling approaches that are naïve to underlying development processes (Hansen and Houle, 2008). However, a growing number of interdisciplinary researchers are now successfully integrating development and quantitative genetics to help bridge the gap between micro- and macroevolution and improve the predictive powers of such models. Recently, Mongle et al. (2022) and Machado et al. (2023) both utilised a study system with well understood developmental “rules” – the inhibitory cascade model (ICM) that describes mammalian molar development – to study evolution within a “biologically-informed” morphospace. Predictable interactions between inhibitory and activating factors maintain a consistent ratio of molar sizes and create a discontinuous morphospace of patterns that can be produced by development, thus limiting the trajectories that evolution can take. Indeed, such a morphospace was able to unite micro- and macroevolutionary patterns, leading Machado et al. (2023) to suggest that observed variation is the result of stabilising selection that is “enforced” by development. Thus, the existing literature suggests that evolution

tends to occur along axes with high additive genetic variation, but integration of these quantitative genetic metrics with developmental knowledge will further improve their predictive power.

3.3 Parallel and repeated evolution of ecotypes

The lines of least resistance as described by Schluter (1996) often describe axes of ecotypic divergence. “Ecotypes” represent body plans defined by a set of ecologically relevant traits that tend to covary together. Radiating lineages are thought to evolve distinct ecotypes in response to different environments that are encountered during colonisation (Schluter, 2000). Theory suggests that correlated selection acting on a set of traits and their respective covariances should stabilize the **G**-matrix so that the line of least resistance aligns with a ridge on the fitness landscape (Jones et al., 2004). This ridge may straddle two or more fitness peaks that represent ecotypes, thus allowing accelerated transitions between body plans sharing patterns of trait covariance that have previously been favoured by selection. For example, (Schluter, 1996) line of least resistance for sticklebacks had “limnetic” morphologies at one end, possessing smaller and more slender bodies than “benthic” counterparts at the other end of the axis (Figure 2C). Similarly, Caribbean anoles have repeatedly evolved into a limited set of ecotypes, each of which corresponds to a different microhabitat (Huie et al., 2021; Losos, 2011). These ecotypes are primarily differentiated by size and limb proportions, which corresponds to the line of least resistance observed by McGlothlin et al. (2018). Freedom to vary along ecologically relevant axes of covariation can facilitate rapid evolution. For example, the capacity to rapidly produce well-adapted benthic and limnetic trophic morphologies has been strongly linked to the prodigious evolution displayed by cichlids (Albertson and Kocher, 2006). The propensity for the above groups to recapitulate these ecotypic forms through plasticity in lab settings (Calsbeek et al., 2007; Parsons et al., 2016) further suggests a prodigious capacity to vary along these axes in the face of genetic or environmental inputs into development. In all, ecotypic divergence driven by developmental lability along an ecologically determined set of trait covariations is a prominent feature of adaptive radiations and may contribute to accelerated rates of diversification.

Ecotypic divergence, and subsequent adaptive radiations, have been observed on numerous occasions to be driven by “key innovations” – such as varied beak morphologies in Galapagos finches (Grant and Grant, 2002) – that typically involve altered patterns of covariation between traits. Modularity – the semi-independence of traits and/or developmental programs (Schlosser and Wagner, 2004) – has been shown to be a driver of numerous adaptive radiation events by providing the “degrees of freedom” required to explore morphospace. For example, the developmental decoupling of the oral and pharyngeal jaws in cichlids has long been linked to trophic diversification by allowing these semi-independent structures to evolve distinct functions (Liem, 1973; Parsons et al., 2012 - but see Conith and Albertson, 2021). Similarly, division of the canine skull into cranial and mandibular modules has been implicated as a factor in the diversification of dogs under domestication, the diversity of which eclipses the entire *carnivora*

family (Figure 1F) (Drake and Klingenberg, 2010; Wilson et al., 2021). Modularity has been implied in the diversification of apes (Parins-Fukuchi, 2020) and carnivorous mammals (Law et al., 2022) amongst other radiating lineages, further implying a link between trait independence and evolutionary rates. Conversely, the coupling of traits - integration - has similarly been linked to rapid exploration of morphospace by allowing correlated trait complexes to evolve together, requiring minimal regulatory changes (Gerhart and Kirschner, 2007). For example, “domestication syndrome” describes rapid evolution due to the shared developmental origin of cell populations in neural crest cells (NCCs) (Wilkins et al., 2014). Artificial selection during domestication is predicted to lead to a reduced contribution of NCCs to developmental structures, leading to fewer cartilage, pigment and neuronal cells, and thus concomitant changes in linked traits (Lesch and Fitch, 2024; Trut, 1999; Wilkins, 2020). While the involvement of NCCs as an explanation for domestication syndrome has been controversial (see Gleeson and Wilson, 2023; Wright et al., 2020) evidence from “de-domestication” (Flynn, 2022) and the evolution of aggression (Feiner et al., 2024) suggest that the traits that contribute to domestication syndrome can easily covary in the other direction, demonstrating how integration at the developmental level allows rapid evolution along axes of covariation.

The repeated evolution of ecotypes at the level of species, assemblages, and taxa, further implicates a role for developmental biases (Figures 1C–E). For example, independent invasions of freshwater habitats by marine sticklebacks have led to the repeated parallel evolution along a marine-freshwater axis (Roberts Kingman et al., 2021) (Figure 2D). These marine and freshwater ecotypes bear anecdotal resemblance to benthic and limnetic ecotypes as described by Schluter (1996), as well as populations that have diverged along lake-stream (Ravinet et al., 2013), geothermal-ambient (Pilakouta et al., 2023), and mud-lava (Kristjánsson et al., 2002) axes, suggesting that shared patterns of covariation have led to adaptive divergence being channeled along the same or similar phenotypic axes. Benthic-limnetic divergence has been observed in a wide range of fish clades such as cichlids (Hulsey et al., 2013), labrids (Larouche et al., 2023), and several postglacial fishes (Parsons and Robinson, 2007; Skúlason et al., 2019), suggestive of a taxa-level bias that has shaped, and perhaps been shaped by selection to align with this ecological axis, thus potentially accelerating evolution. This ecotypic divergence is well characterised in the African Rift Lake cichlid radiations, in which parallel patterns of trophic diversification have been identified (Cooper et al., 2010; Hulsey et al., 2013). These patterns have been shown to be underpinned by parallel patterns of craniofacial modularity (Parsons et al., 2012), suggesting an interactive role between selection and developmental bias in driving adaptive radiations.

However, whilst these replicated radiations present some of the most well-characterised evolutionary events, such patterns are observed less frequently than theory would suggest (Losos, 2010), with E.O. Wilson (1999) observing that “many clades fail to radiate although seemingly in the presence of ecological opportunity.” In these cases, the absence of an expected pattern is significant, and suggests that natural selection plays an important, but incomplete role as only a sorting mechanism in adaptive radiations. Explanations of parallel evolution must therefore look beyond

parallel selection pressures and consider that the variation exposed to selection is produced by developmental systems that are biased and may be pre-disposed to producing variation in directions favoured by previous selection. Such biases may be particularly relevant when investigating populations that share common ancestry, as this may pre-dispose development along similar trajectories. A driving role for developmental processes is substantiated by the observation that parallel evolution is infrequently mirrored at the genetic level (Barghi et al., 2019; Conte et al., 2012; Poore et al., 2023), and can be achieved by mutating alternative genes in the same pathway (Steiner et al., 2009). Furthermore, replicated radiations tend to occur within the same clade (Losos, 2010), with repeated benthic-limnetic divergence across fish clades being an exception. Thus, phylogenetically related, but geographically distant, populations can follow similar evolutionary trajectories (e.g., threespine stickleback - Roberts Kingman et al., 2021), while other clades co-existing with radiating lineages fail to radiate themselves (e.g., Galapagos Mockingbirds - Arbogast et al., 2006; Losos, 2010). Hence, what have long been labelled as “contingencies” (Blount et al., 2018; Gould, 1977) or “phylogenetic constraint” (McKittrick, 1993) may reflect divergence in developmental systems, which may preclude or facilitate divergence along certain trajectories. For example, finches and honeycreepers have radiated on both the Galapagos islands and mainland South America while mockingbirds and thrushes, while present in both regions, have failed to radiate (Arbogast et al., 2006). Navalón et al. (2020) demonstrated links between clade divergence and altered patterns of integration between the beak and cranium, suggesting that these evolutionary patterns can be linked, at least in part, to intrinsic factors. Similarly, some butterfly taxa such as the *Mycalesina* subtribe have diversified into over 250 species originating from numerous radiation events spanning multiple continents (Brakefield, 2010; Kodandaramaiah et al., 2010), while most other butterfly clades are relatively species-poor. An alternative explanation is that radiating clades possess a competitive advantage, but this could also be interpreted as a developmental explanation, with niche expansion being facilitated by a labile developmental system. Looking at initial stages of colonisation, we should also ask if this evolvability was present in colonisers, or if it rapidly evolved in response to ecological opportunity. Thus, by understanding why some clades radiate while others fail to do so, we can understand how developmental factors co-exist and interact with other factors influencing evolutionary patterns.

3.4 Biased plasticity as a driver of adaptive divergence

Perhaps the defining quality of adaptive radiations is rapid evolution upon exposure to new environments. New environments should induce a plastic response, and there is now plentiful evidence that adaptive radiations are in some cases seeded by phenotypic plasticity – the capacity of a developmental system to produce phenotypic variation in response to environmental cues (Levis and Pfennig, 2019; West-Eberhard, 2003). Plasticity-induced trait variation can help populations persist in novel environments (“the Baldwin effect.” Baldwin, 1896; Simpson, 1953) and can then become refined (“accommodated”) and eventually “assimilated”

whereby an environmental cue is no longer a requirement of its development (Waddington, 1953; West-Eberhard, 2003). Models of plasticity-led evolution, such as the flexible stem hypothesis (West-Eberhard, 2003), state that extant patterns of phenotypic diversity are partly determined by the plastic capacities of ancestral (“stem”) lineages (Parsons et al., 2016; Wund et al., 2008). The capacity of ancestral populations to produce derived ecotypes when reared in novel environments (Parsons et al., 2016; Rohner et al., 2022; Wund et al., 2008; Figure 1G) further suggests a role for plasticity-led evolution in adaptive radiations (Levis and Pfennig, 2019; Pfennig et al., 2010).

Although plastic responses to novel environments have long been considered as a “noisy” variable in evolution (Murren et al., 2015), increasing evidence suggests that plastic “noise” is indeed biased. West-Eberhard (2003) proposed that genetic and environmental cues can be viewed interchangeably, by both providing sources of information into the same developmental system. Indeed, a recent meta-analysis by Noble et al. (2019) demonstrated that noise induced by genetic and environmental perturbations was biased along the same axis, while Rohner et al. (2022) found that genetic and environmental interventions biased *Onthophagus* beetle horn development in similar ways. The production of phenocopies – phenotypic forms that can be replicated in the lab through non-genetic manipulations – suggests that genetic and environmental inputs are processed by the same developmental system, with its inherent biases (Alberch and Gale, 1985; Parsons et al., 2014; Wund et al., 2008). If closely related species show similar patterns of developmental bias, we may expect similar plastic responses upon exposure to the same environmental cue (s). Under such a scenario, plasticity-led evolution would impose a phylogenetic signal due to developmental biases, potentially explaining why replicated radiations tend to occur within lineages (Wilson, 1999). Thus, developmental biases may act to homogenise adaptive plastic responses in sister taxa, perhaps explaining patterns of benthic-limnetic divergence within and across fish clades (Larouche et al., 2023; Parsons and Robinson, 2007). The observation that ancestral plasticity tends to align with patterns of phenotypic variation (Radersma et al., 2020) further suggests a role for biased plasticity in determining the trajectories taken during adaptive divergence.

Understanding the role of developmental bias in driving phenotypic plasticity may ameliorate some of the oft-cited costs of plasticity. It is thought that plasticity must carry costs, otherwise organisms should be perfectly plastic and able to match any fitness optima (DeWitt et al., 1998), although empirical attempts to demonstrate such costs have rarely succeeded (Murren et al., 2015; Van Buskirk and Steiner, 2009). A commonly cited cost of plasticity is the risk of mismatches between organism and environment, or more broadly the production of phenotypes upon exposure to a novel environment that have not been “vetted” by selection (Murren et al., 2015). However, as we posit that plastic responses are produced by a biased developmental system, we would argue that plastic noise is far from random (e.g., Noble et al., 2019). Rather, we may expect that plastic responses will be closely aligned with past selection, thus falling closer to the phenotypes of parents and ancestors than would be expected by chance. If plasticity-led evolution is driven by extreme

(“transgressive”) phenotypes, such leaps in morphospace may be aligned with such axes, producing “hopeful monsters” with underlying logic (Figure 2F) (Alberch, 1989). A view of plasticity that considers developmental trajectories rather than development of explicit phenotypes may also explain why plastic capabilities do not degrade over thousands or millions of years when the “machinery” associated with a trait lays dormant. Evolution may favour flexibility along a multivariate axis defined by a pattern of covariation, accelerating and refining plastic responses when a cue is re-encountered. Furthermore, a growing body of evidence suggests that the genetic architecture of traits is environment-dependent, with loci underpinning trait variation differing under experimental treatments (Bao et al., 2018; Conith et al., 2018; Ishikawa et al., 2017; Mathews et al., 2008; Messmer et al., 2009; Navon et al., 2021; Parsons et al., 2016; Snoek et al., 2017; Yadav et al., 2016; Zhang et al., 2010; Zogbaum et al., 2021). “Canalising” plasticity to produce discrete variants that have been refined by selection mitigates much of the stochasticity associated with plastic responses. In addition, modularisation of the environment-specific gene expression networks underlying plasticity can avoid disruptive pleiotropy and epistasis, thus, in the context of the specialist-generalist dichotomy pervasive in the plasticity literature, allow an organism to evolve specialisations for multiple sets of environmental conditions without encountering tradeoffs (Snell-Rood et al., 2010). For example, many *Mycalesina* butterfly species show polyphenisms, in which either a “wet season form” or a “dry season form” will develop in response to environmental conditions (Halali et al., 2024). These forms are primarily characterised by finely detailed (Prudic et al., 2015) and adaptive wing patterns (Brakefield and Frankino, 2009), but also show correlated changes in behaviour, physiology, and life-history. A developmental architecture that situationally shields developmental programs from selection may also relax selection, allowing the accumulation of cryptic genetic variation that enables the production of transgressive phenotypes (Snell-Rood et al., 2010). Thus, biased properties of development may ameliorate some of the costs and limitations attributed to phenotypic plasticity.

3.5 Bias as a cause and consequence of adaptive radiation

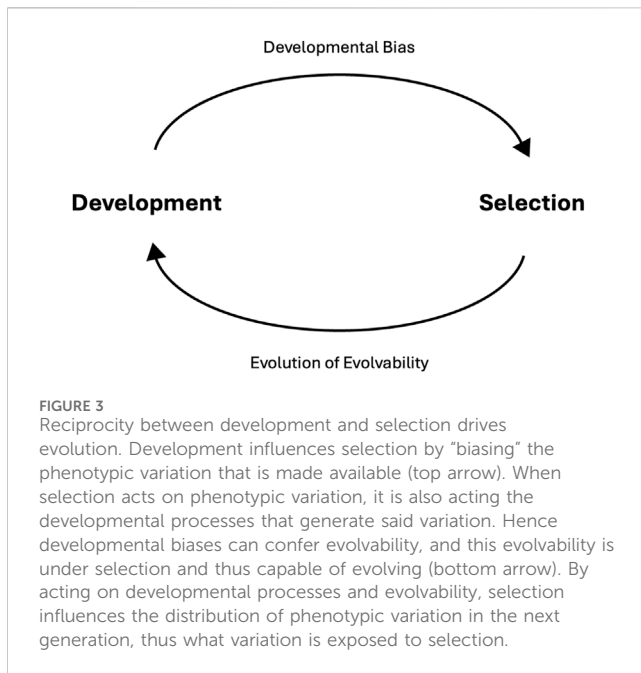
Central to the assertion that developmental bias is a cause and consequence of adaptive radiations are the observed mathematical equivalencies between the evolution of gene regulatory networks (GRNs) and learning in neural networks (Watson and Szathmáry, 2016). GRNs have been shown to demonstrate “Hebbian learning,” informally known as “fire together wire together” (Pavlicev et al., 2011; Watson and Szathmáry, 2016). Epistatic interactions between regulatory genes that confer a selective advantage will be strengthened while those that are deleterious will be weakened, leading the epistatic landscape to act as a logbook for prior selection. Hebbian learning allows GRNs to “generalise,” learning which interactions should and should not be conserved, and thus causing evolution to be guided to adaptive regions of morphospace containing both previously adaptive solutions and structurally similar novel phenotypes (Kouvaris et al., 2017; Parter et al., 2008). In extreme cases, adaptive solutions can be memorised and recalled if conditions demand. For example, (Rajakumar et al.,

2012) demonstrated that several species of ant within the genus *Pheidole* were able to produce supersoldier castes when induced in the lab, suggesting this developmental program had been “memorised” for 45–60 million years despite not being used. Incorporating learning theory into evolution therefore means appreciating that developmental responses will be informed by past experience. Indeed, Brun-Usan et al. (2021) demonstrated that changes in the environment-phenotype map will lead to changes in the genotype-phenotype map, and vice-versa, thus aligning genetic and plastic responses along previously adaptive trajectories (Figure 2E). The utilisation of past information can also offer an explanation for the rapidity of evolution under adaptive radiations. Many of these radiations involve the repeated colonisation of similar habitats, for example, caused by fluctuating water levels in African Rift Lakes (Nevado et al., 2013), exposure to new freshwater habitats through deglaciation (Skúlason et al., 2019), or colonisation of emerging islands in an archipelago (Freed et al., 1987). Learning theory suggests that prior evolution along an environmental gradient could lead to flexibility along such axes in derived lineages. In such cases, radiating species may not need to mount an evolutionary response “from scratch,” instead riding the coattails of their ancestors. Work on regulatory circuits supports this theory, demonstrating that exposure to fluctuating selection, between environments that share structural regularities, led populations to regions of morphospace in which all solutions or adaptive “peaks” were readily accessible, in the best models through a single regulatory change (Kashtan and Alon, 2005; Parter et al., 2008). We therefore suggest that biased development can represent both causes and consequences of adaptive radiations by i) allowing “recall” of “memorised” adaptive solutions previously vetted by selection and ii) by channelling noise along previously fruitful axes.

We therefore argue that empirical and theoretical data point to the conclusion that developmental bias is both a cause and consequence of adaptive radiation and divergence. Rather than viewing development and selection as separate and/or conflicting processes, we recognize the inherent reciprocity present here that drives evolution (Figure 3). Development determines what variation is made available for selection, while selection acts both on variation and the developmental system that has produced it. Hence, future evolution will be driven by variation produced by a biased developmental system that is the product of selection. Thus, the role of development in evolution should be expanded from just being a driver of evolutionary change, to acknowledging that development, and its biases, are also the consequence of evolution. As Jones et al. (2004) say, “The response of a population to selection is a consequence of selection.”

4 Discussion

In this review we have described evolutionary patterns – parallel evolution, rapid diversification and phenotypic plasticity – that have for decades been attributed to random mutations and the guiding hand of selection. We therefore outline a set of questions that aim to demonstrate that developmental bias can explain the accelerated and exaggerated patterns of evolution that characterise adaptive radiations. While we have provided findings from several distinct



fields that support these conclusions, the lack of explicit links between developmental bias and adaptive radiations leaves a number of key questions unanswered.

4.1 Macroevolutionary consequences of developmental bias

While developmental bias has been empirically demonstrated at the microevolutionary level (Braendle et al., 2010; McGlothlin et al., 2018; Rohner et al., 2022), its macroevolutionary consequences have only been tentatively suggested (e.g., Houle et al., 2017), as in this review. This represents a major gap in our understanding, as without knowledge of the stability of these biases we can only hypothesize about their role in evolutionary change. One way to bridge this gap would be to use gene-editing, RNAi, or mutagenic substances to induce mutations across a radiating lineage in the lab and observe how development is perturbed. If mutations, especially in different genes, bias developmental noise along axes corresponding to macroevolutionary patterns within said lineage, this would provide strong support that such biases are channeling long-term evolutionary change. For example, Rohner et al. (2022) used RNAi to disrupt distinct signalling pathways in the dung beetle *Onthophagus taurus* and observed that i) independent mutations produced similar phenotypic consequences and ii) these patterns aligned with phylogenetic trends observed across the *Onthophagus* lineage. Deploying and expanding this approach in different radiating lineages would provide a clearer picture of how biases influence evolution over longer timeframes.

4.2 Biased plasticity and adaptive divergence

Phenotypic plasticity undoubtedly can play a driving role in adaptive radiations, but whether plastic responses are a consequence

of developmental bias is still unclear. Thus, demonstrating biased plastic responses will only strengthen links between bias and adaptive radiations. These links can be tested by exposing a radiating species to an environmental cue and observing if the developmental responses mirror macroevolutionary patterns. Such findings would provide support for the flexible stem model of plasticity-led evolution, as has been done in numerous lineages (McGlothlin et al., 2018; Parsons et al., 2016; Wund et al., 2008). Whether genetic and environmental perturbations lead the production of variation in similar phenotypic directions has seldom been investigated (but see Rohner et al., 2022), but would lend support to theories tying developmental system properties to macroevolutionary patterns. Furthermore, whether different environmental variables induce similar developmental responses has not, to our knowledge, yet been investigated. The threespine stickleback may be an excellent system in which to test this, as they show similar patterns of divergence in response to different environmental gradients (Roberts Kingman et al., 2021), with plasticity known to be central to such responses (Pilakouta et al., 2023; Skúlason et al., 2019; Wund et al., 2008). If the observed ecotypic divergence is indeed mediated through shared patterns of trait covariation, if and how these covariation structures respond to external cues would give further insight into mechanisms controlling divergence (Navon et al., 2021).

4.3 Bias as consequence and cause of adaptation

Our central thesis in this review has been that developmental bias can influence, and be influenced by, evolutionary history to shape patterns of adaptive divergence. While we have provided examples from the literature that support this claim of reciprocity, explicit testing of these hypotheses is required. Comparisons of radiating and non-radiating lineages should be further utilised to determine the role of development. If evolvability with respect to key evolutionary innovations could be demonstrated in radiating lineages, but not in non-radiating ones, this would suggest links between evolvability and evolutionary patterns. These fundamental questions can also be studied by using lineages that have evolved in parallel, or alternatively, lineages we might expect to have diverged in this way but have “failed” to do so. If parallel evolution can be linked to similarly biased developmental systems, or if non-parallel evolution can be attributed to a lack of evolvability, this would implicate a role for bias in facilitating and/or preventing parallel evolution and adaptive divergence. Conversely, comparing populations with known evolutionary histories may inform us of how past evolutionary shifts have influenced evolvability, and thus may influence future divergence. Experimental evolution, in which model organisms are exposed to an artificial selection regime, may allow links between selection and developmental variability to be solidified. Understanding how developmental factors can influence the predictability of evolution could then be utilised to predict how species respond to pressing ecological threats such as climate change (Campbell et al., 2017; Feiner et al., 2021), invasive species (Cordeschi et al., 2022), and increasing urbanisation (Thompson et al., 2022).

4.4 Bias and speciation

With the greatly accelerated speciation rates seen in adaptive radiations (Schluter, 2000), how this phenomenon could be influenced by developmental bias is unknown and seldom considered. If populations are diverging towards different ends of the same ecological axes (e.g., ecotype formation), then this initial process could be accelerated through developmental bias. This would especially be true if divergence is seeded by biased phenotypic plasticity, and/or utilises developmental axes used in the lineage's evolutionary history (Parsons et al., 2020). However, if and how developmental biases drive reproductive isolation, perhaps through reduced hybrid fitness, is unknown. Understanding the role of biases in driving speciation is an essential step in understanding how such biases drive adaptive radiations.

4.5 Bias and selection

While selection and development have been largely considered as antagonistic forces, with the former permitting evolution and the latter providing limits (Maynard-Smith et al., 1985), we have drawn on evidence from learning theory to demonstrate that this dichotomy is unhelpful (Kouvaris et al., 2017; Pavlicev et al., 2011). Instead, theoretical models suggest that development is influenced by past selection (Pavlicev et al., 2011; Watson and Szathmáry, 2016), and that the phenotypic variation that selection is given is influenced by developmental parameters (Machado et al., 2023; Mongle et al., 2022). However, if and how developmental biases interact with selection, and how this can influence broader evolutionary patterns, has rarely been studied outside of computational systems (but see Walter et al., 2018). Thus, how malleable biases are in the face of selection, and how stable such biases are over evolutionary time, are important questions pertaining to the evolutionary consequences of developmental biases.

5 Conclusion

In this paper we have outlined a prominent role for developmental bias as a driver of many of the hallmark features of adaptive radiations. In the process, we have cited studies on many evolutionary topics, such as parallel evolution and phenotypic plasticity, that we argue are studying developmental bias, although this term may not be used directly. We may then suggest that a failure to invoke developmental explanations results not from a lack of empirical evidence, but from under-

appreciating the role of development in adaptive evolution. By discussing developmental biases in the context of adaptive radiations, and their concomitantly exaggerated patterns of evolution, we hope to exemplify that biases do not merely constrain adaptive evolution but can also facilitate it. These astounding evolutionary events should also arm us with data and discoveries that can allow a proper understanding of development's role in evolution.

Author contributions

CS: Writing–review and editing, Writing–original draft, Conceptualization. KP: Writing–review and editing, Supervision, Conceptualization.

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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.

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Functional evolution and functional biodiversity: 150 years of *déjà vu* or new physiology of evolution?

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Introduction

Research at the intersections of different scientific fields is the primary catalyst to solicit novel disciplines and expand the frontiers of knowledge. Quantum physics in chemistry and biology, artificial intelligence, nanoscience, and advances in genomic revolution with cost-efficient sequencing conceptually and radically changed scientific paradigms with profound public and economic impacts. The current Frontiers' research topic further stresses the need for integrative studies, advocating *Eco-evo-devo* as an emerging discipline of biology by expanding "traditional evo-devo" to ecology.

On a deeper level, this is a recurring and persistent *déjà vu* in the history of biology ever since Darwin-Wallace papers (Darwin and Wallace, 1858), when it was confirmed, and it is still impossible to understand biological phenomena without deciphering evolutionary mechanisms at *all* levels in *changing ecosystems* (using modern terminology). Arguably, this "first rigorous formulation of the concept of evolution, made just over a century ago, was the most useful one we have ever had" (Carneiro, 1972). In his masterpieces, Darwin bridged geology, ecology, biogeography, biodiversity, different types of selection, development, psychology and behaviors (Darwin, 1872; Darwin, 1880). Today, the unification of disciplines for mechanistic and systemic understanding of evolutionary processes reflects the still ongoing scientific and societal challenges under an accelerating information tsunami. Here, we emphasize two aspects of current challenges demanding integrative approaches.

The first problematics is the bottleneck in education, when the evolutionary training and biosystematics courses, which deal with the most fundamental concepts in biology, have quietly lost their place of eminence within the biomedical curriculum—"outcompeted" by escalating specialization and the increasingly technical nature of many disciplines (Moroz, 2010).

The second problematics is an equally critical bottleneck in our understanding of functional evolution (Figure 1) as hierarchical architectures of *real-time* physiological and metabolic/biochemical *interactions* underlying mechanisms of adaptations that can explain the biodiversity dynamics on the changing planet. The incorporation of a physiological web of life into "genomic" evolution will better forecast biota's resilience to environmental stressors. That is functional biodiversity—the strategy to *marry physiology at all levels of organizations (from genomes to behaviors) with classical biodiversity to understand micro- and macro-evolution*. We stress the more integrative term of *functional evolution* in

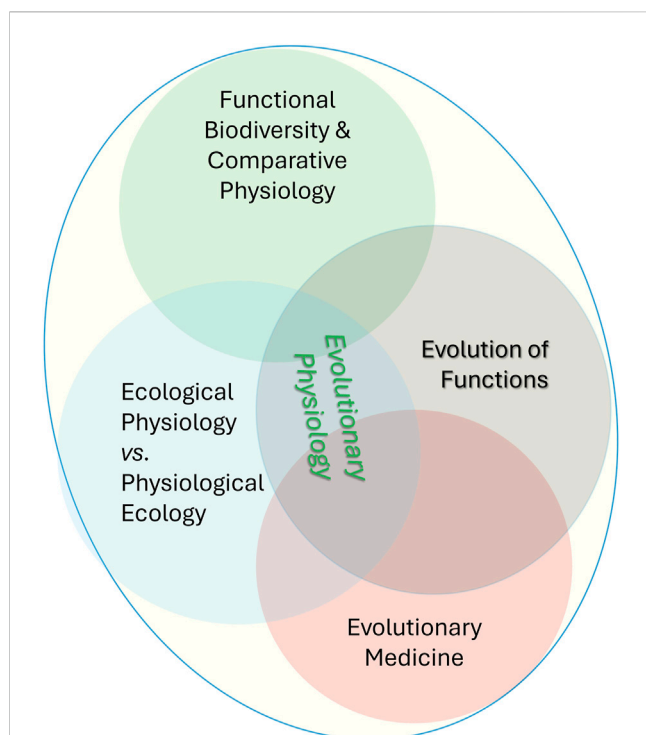


FIGURE 1
Functional Evolution and its subdisciplines were also united as classical Evolutionary Physiology. Functional evolution highlights the importance of understanding how all biological *functions* evolve, from the molecular level to the whole organism, and how these changes contribute to species adaptation, survival, and diversification. Under this framework, the emerging, conceptually similar term **Physiology of Evolution** further illuminates the importance of physiological approaches to understanding evolution. Notable, these terms and research strategy do not revisit the concept of evolution; we emphasize the urgent need for paying more attention to real-time physiological aspects of various biological traits, focusing on integrative mechanisms such as neuronal, hormonal, and immune functional interactions at the top of the organismal and behavioral hierarchies.

changing ecosystems rather than the evolution of particular functions, that is, the multilevel reconstruction of the history of individual traits from proteins to behaviors, also known as evolutionary physiology. In these definitions, numerous details of the evolution of diverse functions in cells, tissue, and organs' systems have to be united as components of functional evolution (Figure 1). In the broadest sense, we should not differentiate the terms "functional evolution" from "evolution" as such. We emphasize that the current evolution studies need significantly more physiological approaches rather than over-dominance of gene-centric approaches by continuing to focus on genes and genomes and then "jump" to organismal phenotypes without real-time physiological studies. Thus, modern *physiology of evolution* is a richer and broader framework, where a web of functions is understood at different levels, as opposed to the gross (organismal) definition of function in changing ecosystems.

Competition and selection occur at all levels of physiological integration, requiring more research on traits critical to decision-making, including learning and memory, homeostatic mechanisms, adaptive metabolism, and immunity (Figure 2). We especially point out the necessity of understanding organisms as highly dynamic hierarchical systems subjected to selection that work on semi-

autonomous integrated systems with emergent properties at each level of increased complexity. Such architectures can be viewed as the collective intelligence of heterogeneous cell populations both in simpler and complex metazoans (from placozoans to cuttlefishes) with social behaviors.

Functional evolution also embraces the phenomena of physiological selection (Romanes and Moulton, 1886) and the so-called Baldwin effect (Baldwin, 1896c; Morgan, 1896; Osborn, 1896; Baldwin, 1897; Simpson, 1953; Newman, 2002; Sznajder et al., 2012). Here, the ability to adaptability accelerates evolution by forming, and even making, novel internal and environmental adaptive spaces, therefore giving extra time for natural selection to act; and favoring a broader range of physiological, epigenetic, stereotyped, and learned behavioral outcomes as feedback mechanisms and platforms for functional evolution.

What is remarkable—these critical integrative questions and directions have been recurrently emerging over and over again for 150 years, sometimes with changes in the scope and meaning of terms. Thus, it would be informative to refer to the history of terms briefly, and then summarize past and modern frameworks.

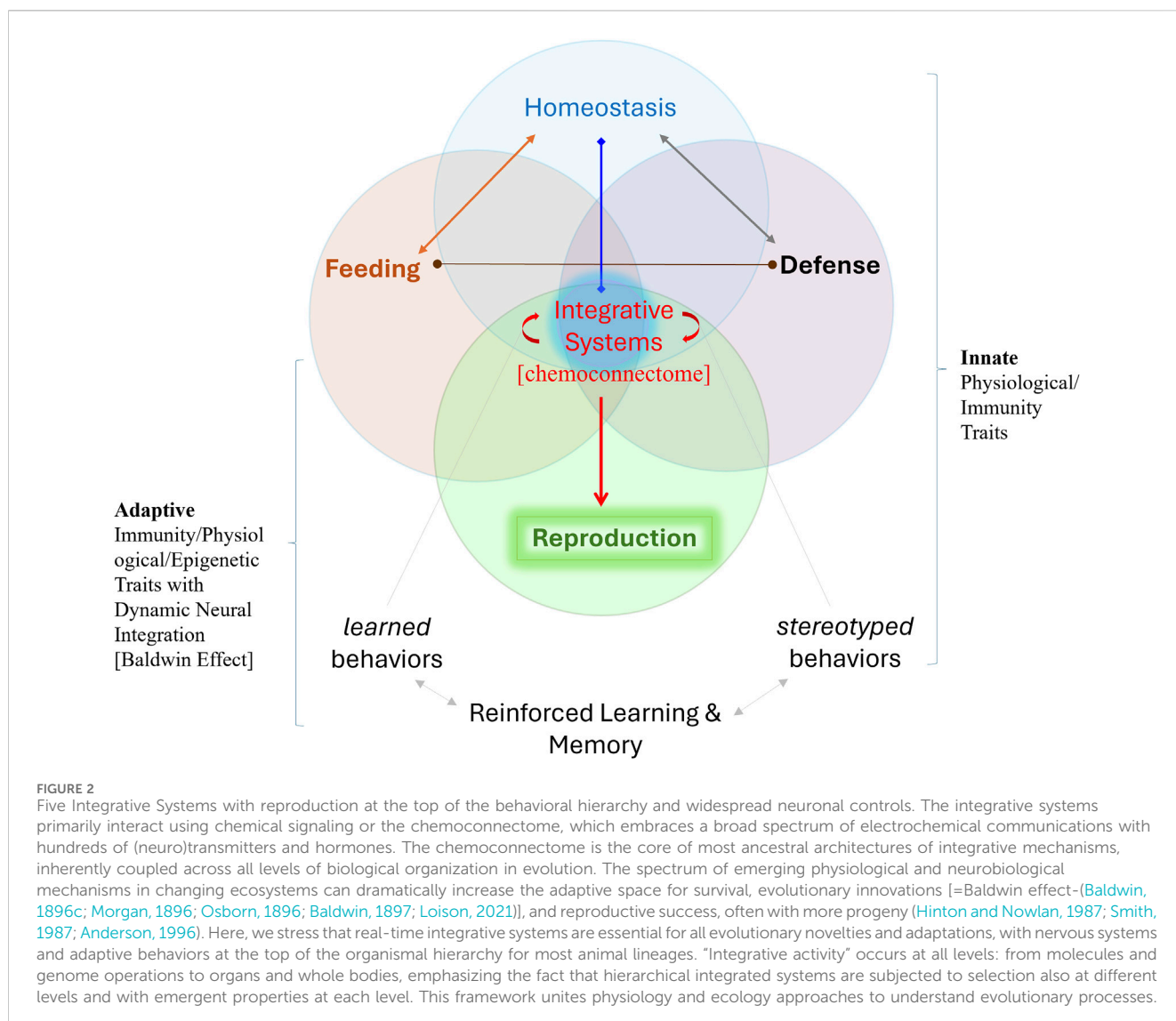
What is physiology?

Physiology is somewhat a missing elephant in a palace of evolutionary biology today and evo-devo in particular. The term physiology is derived from the ancient Greek φύσις (*phúsis* - "nature, origin") and -λογία (*-logía* - "study of") (Fletcher, 1835; Gontier, 2024) and traced back to 1628 - the classical work of William Harvey on blood circulation with a remarkable integrative conceptual framework (Harvey, 1628; Whitteridge, 1964; Whitteridge, 1978). The American Physiological Society defines physiology as the *science of life* (<https://www.physoc.org/explore-physiology/what-is-physiology/>), and its primarily based on dynamic cellular-molecular interactions. In this sense, the subject of physiology is the *living* systems (i.e., cells and organisms with self-autonomous respective integrative functions) vs. studies of *non-living* systems, which include the deciphering of gene sequences, or locations of their expression, subcellular molecular ensembles (and even gene regulatory networks as an array of regulatory components vs. *functional* networks within defined cell and organismal homeostasis identity and behaviors).

For all definitions, the key to the strategy of physiology is the *integration* and *real-time* interactions of organismal multifunctional networks to obtain energy, survive, and reproduce (Figure 2). In this sense, Darwin and Wallace were comparative integrative physiologists and ecologists who worked to decipher the origin of species. In fact, Darwin was a member of the Physiological Society from the very beginning of its foundation in 1876 (Sharpey-Schafer, 1927), collaborating with physiologists on mechanisms of selection (Romanes and Moulton, 1886), see details in (Noble and Noble, 2023a).

From classical evolution to functional evolution

Literally, the word evolution means "*unrolled*," which refers to ancient books that were rolled on wooden rods (McCabe, 1921),



gradually transforming to Bonnet's unfolding of the human embryo from a perfectly formed "homunculus," and eventually to the concept of "descent with modification," theories and facts (Mayr, 2001) illuminating causes, tempo, and mechanisms of the 3.5+ billion-year life history on our planet with 10–100 million of extant species, including 33 animal phyla, changing global ecosystems.

As noted by (Carneiro, 1972), because of the original doctrine of *preformations* (e.g., reading of an already written text in rolled books or unfolding of a tiny preexisting homunculus), Lamarck did not use the term evolution in his famous book (Lamarck, 1809), focusing on the role of physiology in the development of emerging properties. Interestingly, Darwin also did not use the term evolution throughout the text of the first five editions of his "Origins of Species." Darwin concluded, however, his masterpiece with the word "evolved." Spencer's evolution terminology preempted biology (Spencer, 1863) with the famous "survival of the fittest" at the *complex interplay with the environment*.

Soon, evolutionary embryology concepts led to impactful theories of animal origins, known as *Gastrea* (Haeckel, 1874) and

Phagocytella (Metschnikoff, 1886). Thus, integrative and comparative biology at the end of the XIX century can already be viewed as *eco-evo-devo* in modern terms. Furthermore, Metschnikoff's phagocytella concept included the unification of *cell physiology* and *immunity* (phagocytosis) to explain system mechanisms of the germ layers' formation as predecessors and factors for functional evolution in general and the origins of developmental processes (e.g., gastrulation) in particular. These views were conceptually different from past preformation theories by employing dynamic physiological processes (rather than static morphology) as subjects of selection and adaptation.

Questions of "how" and "why"

The evolutionary focus shifted early to real-time physiology, where mechanistic questions "how" ("what for") something works were coupled with the questions of "why" this system works in such a particular way (or "how come" - (Mayr, 1961)) as a result of its genealogy and long planetary history. Why do we observe certain

types of cellular or systemic organizations in ctenophores, placozoans, cnidarians, molluscs, arthropods, or chordates instead of others? These “*why*” questions target both primary causes and exaptations (Gould and Vrba, 1982) to reconstruct the extant functional biodiversity in ecosystems.

Why, for example, are individual neurons so different from each other within a given species and across phyla? One possible answer is the functional demands within a given neural circuit and behavior. Another is that each distinct neuronal population forming complex neural nets or brains has a different evolutionary history, and, as a result, neurons carry the heavy molecular burdens of primordial integrative systems within their complex evolutionary past—*neurons are also different because they have different genealogies*. In other words, neurons might independently evolve from distinct types of secretory cells with different secretory products and functional interactions. These multilevel interactions could be preserved over many million years, therefore explaining the astonishing diversity of signal molecules in the brains and complex dynamics of extant chemoconnectomes (Figure 2) as a result of their deep ancestry (Moroz, 2014; 2021). Such distinctive cellular ancestries and integrative mechanisms might either limit or facilitate future evolutionary opportunities to adapt to changeable environments. In other words, past evolutionary history might provide constraints for the emergence of novel behaviors within a given time or resistance to stress, disease, or injury. Remarkable examples of extensive parallel evolution of physiological functions (compared to morphology) exist within all animal phyla, enabling forecasts for resilience (or not) to anthropogenic or climate changes. Yet, new experimental designs are needed to reveal and explain the origins or loss of biological complexity, and past or ongoing extinctions of species and ecosystems.

As stressed by one of the reviewers: “Understanding the “*physiological functional web of life*” in modern terms means gaining better purchase on *how, why, when, and with whom* eukaryotic cells choose to partner for their physiological mechanisms that propel evolutionary solutions to cellular problems.” Holozoan animal ancestors evolved in Ediacaran ecosystems dominated by prokaryotes. And, as in today’s ecosystems, microbiome complexity continues to shape, drive, and even dramatically change paths of evolutionary trajectories via cross-kingdom signaling (Heyland and Moroz, 2005) and horizontal gene transfer (HGT) with omnipresent viromes, further contributing to animal innovations via transposon-derived transcription factors (Mukherjee and Moroz, 2023), for example. By itself, it fully justifies the efforts to preserve the entire biodiversity from microbes (Averill et al., 2022) to all eukaryotes as primary preconditions of Planetary Health (Bertram et al., 2024).

Past and modern frameworks for evolutionary physiology

Even Aristotle noted that living organisms are causes of themselves (Okasha, 2024), which, in modern terms and our perception, refers to the physiology. As a separate discipline, evolutionary physiology was not established for nearly a century since Darwin (Orbeli, 1941; 1961; Natchin and Chernigovskaya,

1997; Svidersky, 2002; Natchin, 2009; Natchin, 2017). In part, this situation was due to the complexity of processes and emerging systemic properties of multicellular interactions across phyla. The deficit of studies on the evolution of functions was clearly recognized early in the XX century (Lucas, 1909). The term “*evolutionary physiology*” was coined by A.N. Severtzov in 1914 to complement what was already established by that time as evolutionary morphology and development (Severtzov, 1914; Natchin, 2010). Physiology was then and remains today the most integrative approach for functional evolution, inherently focused on real-time interactions of myriads of molecular and cellular processes leading to organismal homeostasis with stereotyped and learned behaviors.

The original framework of evolutionary physiology and functional evolution (Figure 1) included the natural integration of (a) comparative and ecological physiology coupled with neural controls, (b) ontogenesis, (c) clinical studies, including stress, recoveries (or not) from numerous pathologies, and (d) the development of unique experimental methodology (Orbeli, 1941; 1961). If the first two approaches were traditional from the very beginning of evolutionary thoughts, the remaining two were entirely novel in the 1940s–1960s and still not yet well established to enable sufficient cross-links across fields. Leon Orbeli developed an earlier strategy for evolutionary physiology (Figure 1), specifically with interpretations of medical pathologies in evolutionary terms as well as insights into the evolution of ion transport and homeostasis (Orbeli, 1941; 1961; Ginetzinsky, 1996). 60 years ago, in the USSR, under the leadership of Orbeli formed the laboratory, and then the Institute of Evolutionary Physiology and Biochemistry (1956); the Journal of Evolutionary Biochemistry and Physiology was established in 1965. A new wave of reviews occurred 30 years later (in the 1990–2000s), calling for a renaissance in the field with more focus on molecular mechanisms (Garland and Carter, 1994; Feder et al., 2000; Garland et al., 2016; Galván et al., 2022).

Today, with many thousands of genomes sequenced, evolutionary approaches in biomedicine have gained momentum with successful stories that explain specific adaptations to various pathogens, preconditions, preventive diagnostics for Mendelian diseases, and forecast of outcomes as parts of personalized medicine. Most pathologies might recruit or be constrained by ancestral gene/cellular regulatory programs and signaling networks under stressful disease-driven tissue microenvironments. For example, particular modern human adaptations or constraints were acquired from the Neanderthal ancestry and beyond, and most of them are deeply embedded in the dynamics of metabolic architectures (Zeberg et al., 2024), rather than specific genes for cognitive capabilities. All these events were affected by countless environmental factors over thousands of generations, with even deeper evolutionary ancestral innovations due to the modularity of multi-domain protein assemblies (McCoy and Fire, 2024; Moroz, 2024).

We highlight the recent strong statements by Denis Noble: (i) *Physiology restores purpose to evolutionary biology* (Noble and Noble, 2023a); (ii) *Genes are not the blueprint for life* (Noble, 2024). In Noble’s words: “The genome is not a code, blueprint or set of instructions. It is a tool orchestrated by the system” (Noble and Noble, 2023b). First, because the vast majority of genes do not have a single “pre-set” function that can be determined from their DNA

sequence with perfect examples of multi-modal roles of ion channels in control of cells and organismal behaviors (Noble, 2021) due to the compensatory redundancy of regulatory systems; second, there are emerging, often unpredictable, properties of complex living systems, which can not be deduced from properties of individual components. Here, we emphasize that only real-time physiology in natural habitats (*in situ*) can experimentally unroll the integrative logics of life as we know it, perhaps under the unified theme to proactively “explore” the environment to “learn” to “eat” to reproduce.

Admittedly, we do not know how deep in time we can trace the origins of organismal integrative systems (e.g., neural, hormonal, and immune - (Moroz, 2021)) or mechanisms of genome-scale integration in any particular cell type genealogical lineage. Indeed, cancer is a disease of the genome operation within specific cellular and organismal contexts. Integrative properties of 3D genome operation with thousands of co-expressed genes in each cell are largely unknown and often referred to as a ‘genomic dark matter’ with undetermined redundancy. With trillions of individual cells in the human body, with more than 10,000 differentially expressed genes in each cell type, the task of uncovering such complexity seems to be impossible. Traditional knockout approaches or gains of particular functions are often not sufficient, due to compensatory mechanisms and redundancy of molecular and system signaling and with limited success of a small number of so-called model organisms representing a few specialized animal lineages out of 30+ phyla and 100+ classes of extant metazoans (Moroz, 2018). As a result, a broader concept of *reference species* has been introduced (Striedter et al., 2014), stressing the importance of studying diverse taxa from multiple ecological niches across all phyla.

We are confident that the complementary comparative ecophysiological strategy is needed and possible; it can be and should be executed in the nearest time by proactively learning from experiments performed by Mother Nature over 3.5 billion years of biological evolution with the advent of floating field laboratories (Moroz, 2015). Pioneering work led by Knut Schmidt-Nielsen (Schmidt-Nielsen, 1997) and George Somero (Somero and Hochachka, 2002; Somero et al., 2017; Somero, 2022) in *ecophysiology* of system, cellular and biochemical adaptations validate both feasibility and conceptual breakthroughs in these integrative strategies.

Today, **4D+ (space and time) single-cell multi-omics and real-time imaging of cellular dynamics** can be efficiently integrated with the ecophysiology of life cycles, including the identification of functional networks from heterogeneous cell populations and signal molecules with deciphering events of convergent evolution and recruitments of homologous cell lineages, therefore **transforming the animal tree of life into the cell type trees and physiological functional web of life**. However, novel technologies are required for real-time imaging and quantification of molecular and cellular dynamics to probe the interplay between eukaryotes, bacteria, archaea, and viromes in changing ecosystems.

Conclusion and future directions

“Physiology became completely excluded from evolutionary biology and, in many countries, evolutionary biology was no

longer taught within physiology and medical courses in universities. Nor has physiology been taught in Evolutionary Biology courses” (Noble, 2023). Denis Noble calls it “a profound mistake,” associated with a gene-/genome-centric view of evolution. Although ideas of evolutionary physiology were introduced in the XX century, they apparently lost their influence on modern evolutionary theories.

We argue that eco-evo-devo can’t “live and survive” without real-time physiology. *In situ*, comparative real-time physiological studies in natural habitats (not in the lab cages) are urgently needed for functional biodiversity and physiology of evolution at all levels of biological organization, from cells to behaviors. We must focus on currently neglected so-called “minor” phyla such as Placozoa and Ctenophora, Dicyemida and Orthonectida, and 20+ others (Nielsen, 2012; Moroz, 2018), which, by their relative simplicity and phylogenetic position, represent crucial reference species to integrate hundreds and even thousands of individually traced signaling pathways from cells to behaviors.

What are physiological processes that cause and drive “functional” evolution? Here, we view the *behavior as the pacemaker of evolution* (Mayr, 2001), critical for most ecological adaptations and stress resilience on the changing planet. As indicated by one of the reviewers: “*learned behaviors characterize all of evolution from its beginnings*,” placing behavior before genes.

We know little about three fundamentals.

- (1) What make and integrate complex hierarchies of stereotyped and learned behaviors at the cellular-molecular level?
- (2) How do these behaviors *affect* genes, neuron-specific genomic changes, and functional connectivity across cell types and species?
- (3) How do behaviors, elementary and complex cognitions, or learned “intelligence” of multicellular aggregates (Levin, 2023) trigger subsequent genetic flow in populations, potentially reinforcing adaptive behavioral patterns?

The recognition of these physiological processes in evolution, known as the Baldwin factor (Baldwin, 1896c; Morgan, 1896; Osborn, 1896; Baldwin, 1897; Simpson, 1953), has grown with the evidence of its accelerated importance in natural selection (Hinton and Nowlan, 1987; Smith, 1987), adaptability, with enigmatic origins of complex innate behaviors or instincts (Baldwin, 1896b; Baldwin, 1896a; Dennett, 2003; Bateson, 2004; Pigliucci, 2005; Crispo, 2007; Badyaev and Uller, 2009; Sznajder et al., 2012; Loison, 2021).

Nearly all behaviors can be modified by learning. Phylogeny of learning is traced to the dawn of animal evolution as a memory of injury (Walters and Moroz, 2009) mediated by a conservative toolkit of small signal molecules and secretory peptides (= chemoconnectomics (Moroz et al., 2021; Moroz and Romanova, 2023) that expand cellular dynamics and phenotypic plasticity at all levels. Organismal behavioral learning can dramatically increase survival; it occurs in somatic cells (not germ cells or gametes) and then affects genes as tools (Hinton and Nowlan, 1987; Smith, 1987). Pioneering neuroplasticity studies on numerically simpler neural systems of *Aplysia* and kin revealed rapid epigenetic changes by co-opting DNA and RNA methylation machinery and piwi genes as toolkits (Day and Sweatt, 2010; Rajasethupathy et al., 2012; Kandel

et al., 2014; Pearce et al., 2017; Bedecarrats et al., 2018; Carney, 2018; Yang et al., 2018; Kim, 2019; Huang et al., 2023). Thus, behaviors, learning, and memory give time and space for evolutionary playgrounds.

The quest for innate or learned integrative mechanisms also inherently reopens the discussion and physicochemical definition of “agency” and “purpose” in evolution (Mayr, 1961; Corning et al., 2023; Noble and Noble, 2023a,b). How would any such Darwinian “agency” (Levin, 2023) integrate cell-cell dynamics and propel physiological interactions and adaptability in general? The modern framework of the biological agency, as a self-autonomous organism (Okasha, 2024) with individuality and apparent “goal-directness,” is also a testable hypothesis with a focus on experimental deciphering emerging properties (from cells to ecosystems) that are not directly forecasting from a gene-centric approach. Here, “organism-as-agent” heuristic experimental motivation can be viewed as not that cell ensembles or organisms “consciously aim to maximize inclusive fitness in their social interactions, but rather they behave as if they do” (Okasha, 2024). It would require an understanding of still elusive integrative logics of life, hopefully over the next century, which is an optimistic forecast.

All species continue to evolve together with their symbionts and parasites in land and ocean ecosystems. Rephrasing Peter Medawar, we conclude the alternative to thinking in *evolutionary physiological* terms is not to think at all. In the conceptual sense, “physiology” means “Logic of Life” (Noble, 2023). The sooner the *physiology of evolution* and *functional biodiversity* are inherent and required parts of every biomedical student’s curriculum, the greater progress we can expect from a new generation of scientists in the clinic, the laboratory, and in natural ecosystems. Perhaps we need to include evolution and biodiversity, Darwinian ‘agency’ in the curriculum not only in medical and all biomedical training (evolutionary medicine) but also as a crash course(s) (introductory lectures, principles) in the curriculum of chemists, physicists, bioengineers, and mathematicians/computer/AI scientists worldwide for the

Planetary Health and interdisciplinary Frontiers across scientific fields and politics.

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LM: Conceptualization, Funding acquisition, Investigation, Project administration, Resources, Writing–original draft, Writing–review and editing. DR: Data curation, Validation, Writing–review and editing.

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Evolution of g-type lysozymes in metazoa: insights into immunity and digestive adaptations

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Exploring the evolutionary dynamics of lysozymes is critical for advancing our knowledge of adaptations in immune and digestive systems. Here, we characterize the distribution of a unique class of lysozymes known as g-type, which hydrolyze key components of bacterial cell walls. Notably, ctenophores, and choanoflagellates (the sister group of Metazoa), lack g-type lysozymes. We reveal a mosaic distribution of these genes, particularly within lophotrochozoans/spiralian, suggesting the horizontal gene transfer events from predatory myxobacteria played a role in their acquisition, enabling specialized dietary and defensive adaptations. We further identify two major groups of g-type lysozymes based on their widespread distribution in gastropods. Despite their sequence diversity, these lysozymes maintain conserved structural integrity that is crucial for enzymatic activity, underscoring independent evolutionary pathways where g-type lysozymes have developed functionalities typically associated with different lysozyme types in other species. Specifically, using *Aplysia californica* as a reference species, we identified three distinct g-type lysozyme genes: two are expressed in organs linked to both feeding and defense, and the third exhibits broader distribution, likely associated with immune functions. These findings advance our understanding of the evolutionary dynamics shaping the recruitment and mosaic functional diversification of these enzymes across metazoans, offering new insights into ecological physiology and physiological evolution as emerging fields.

KEYWORDS

ctenophores, *Aplysia californica*, porifera, spiralia, placozoa, innate immunity, gene retention, horizontal gene transfer

1 Introduction

Lysozymes, first discovered by Alexander Fleming in 1922, are key antimicrobial enzymes within the innate immune system, widely known for their role in hydrolyzing the 1,4-beta-linkages between N-acetylmuramic acid (NAM) and N-acetyl-D-glucosamine (NAG), which are critical components of bacterial cell walls. This enzymatic activity is ubiquitous across various biological systems and tissues, including mammalian blood, tears, sweat, and milk, as well as in the egg whites of birds (Fleming, 1922; Rupley, 1967). Beyond their enzymatic role, lysozymes also exhibit potent antibacterial properties, as even their proteolytic fragments demonstrate antimicrobial effects (Ibrahim, Matsuzaki and Aoki, 2001).

Lysozymes are classified into several distinct types, each with evolutionary variations. These include the c-type (chicken-type), g-type (goose-type), i-type (invertebrate-type), and the less common ch-type (*Chalaropsis*-type). Additionally, lysozymes are found in bacteriophages (phage-type), bacteria (bacterial-type), and plants (plant-type), each contributing to breaking down bacterial cell walls and immune defense in different organisms. Despite differences in their tissue and organismal origins, and minimal sequence similarity, lysozymes exhibit conserved protein crystal structures, indicating convergent evolution across eukaryotic kingdoms from animals to plants and even prokaryotes (Beintema and Terwisscha van Scheltinga, 1996; Callewaert and Michiels, 2010; Fischetti, 2005; Grutter, Weaver and Matthews, 1983; Holtje, 1996; Monzingo, Marcotte, Hart and Robertus, 1996; Weaver et al., 1984).

In vertebrates, lysozymes are key components of the innate immune system, serving as a frontline defense against invading pathogens. These enzymes are typically associated with immune cells such as monocytes, macrophages, and neutrophils (Ragland and Criss, 2017; Rosowski, 2020). Neutrophils, in particular, are produced in the bone marrow and circulate in the bloodstream, mobilizing quickly during infections (Burgener and Schroder, 2020). These neutrophils are major phagocytic cells across vertebrates, including fish, amphibians, reptiles, birds, and mammals. During immune responses, neutrophils perform key functions such as phagocytosis, apoptosis, and degranulation, and can form neutrophil extracellular traps (NETs) to capture and neutralize pathogens (Brinkmann et al., 2004; Fingerhut, Dolz and de Buhr, 2020).

While vertebrate lysozymes are well-studied, little is known about highly diverse invertebrate groups, many of which rely entirely on innate immunity due to their lack of adaptive immune systems. In invertebrates, lysozymes serve both defense and digestive functions, particularly in filter-feeding species such as bivalves. These enzymes help protect against pathogens and can digest bacteria filtered through their gills. This dual role of lysozymes highlights a significant evolutionary adaptation in invertebrates, where digestive and immune functions have co-evolved together. Specifically, arrays of i-type lysozymes play critical roles in these processes, contrasting with the c-type lysozymes' recruitment in vertebrate ruminants, where they facilitate similar digestive functions (Itoh et al., 2010; Itoh and Takahashi, 2009; Matsumoto et al., 2006).

The understanding of g-type lysozymes in invertebrates remains limited. These lysozymes were originally discovered in the egg whites of the *Anser anser* (Emden goose) and have since been identified in other chordates as well as various molluscs, such as scallops and mussels. This widespread presence across different phyla suggests that g-type lysozymes perform essential functions in both ecological and evolutionary contexts. Their functions range from aiding in digestion to providing defense against pathogens, highlighting their versatility and significance in the immune responses of these organisms (He et al., 2012; Hikima et al., 2001; Nilsen et al., 2003; Wang et al., 2012; Zhang et al., 2012; Zhao et al., 2007; Zou et al., 2005).

Lateral Gene Transfer (LGT) or Horizontal Gene Transfer (HGT) plays a significant role in biological innovations, shaping the evolutionary trajectories by cross-kingdom signaling and transfer novel genetic material across species. Recent studies have

highlighted the importance of HGT in enabling organisms to acquire adaptive traits such as enhanced metabolism, versatile immunity, and reproductive functions (Li et al., 2022; Liu et al., 2023). The identification of g-type lysozymes in metazoans, which exhibit strong sequence similarities to bacterial lysozymes, particularly those of predatory myxobacteria, suggests that HGT may have contributed in their acquisition. This process may explain the mosaic distribution of g-type lysozymes across taxa, as observed in our study.

Here, we conducted an extensive screening of g-type lysozymes across metazoans, uncovering a complex and intermittent distribution pattern, particularly within the lophotrochozoan/spiralian superclade. Our findings suggest that this distribution may be driven by lateral gene transfer (LGT) events from bacteria. We also identified two major g-type lysozyme groups in gastropods, using *Aplysia californica* as the reference species. Notably, our analysis suggests that *A. californica* and kin lacks both c-type and i-type lysozymes, indicating its reliance on three unique g-type lysozymes. Of these, two are highly expressed in the hepatopancreas, a key digestive organ, aligning with findings from other mollusks. The third g-type lysozyme shows variable expression across organs associated with both feeding and immune defense, highlighting the gene's functional diversity.

These targeted expression patterns point to evolutionary adaptation through convergent evolution and lateral gene transfer to meet specialized dietary and defense challenges and needs. Additionally, we observed high sequence similarity between metazoan g-type lysozymes and those in myxobacteria, suggesting horizontal gene transfer from bacteria to animals. These findings provide insights into the evolutionary dynamics of lysozymes, shedding light on their mosaic functional diversification across metazoans.

2 Results

2.1 Mosaic distribution and evolutionary insights of g-type lysozyme genes across metazoans

Two copies of g-type lysozyme (Lyg1 and Lyg2) have been identified in all extant mammals in a study that analyzed 250 species, with the exception of cetaceans and sirenians, which have lost both copies. Additionally, a potential loss of these genes was observed in the tarsier (Zhang et al., 2021). Similarly, genome sequences of two bony fish species (gar and tilapia) revealed no g-type lysozyme genes, while other teleost fish species possessed single or multiple g-type lysozyme genes (Irwin, 2014). Interestingly, g-type lysozyme genes have been found in various bird species, though not universally, as some species, like ducks, may carry pseudogenes (Irwin, 2014). Considering the wealth of data available on vertebrates, we chose to shift our focus beyond vertebrates to explore the presence of g-type lysozyme genes in other lineages.

To further explore the evolutionary presence of g-type lysozyme genes, we performed an extensive screening for the presence of genes encoded g-type lysozymes across the sequenced genomes from representatives of all five basal metazoan lineages: Ctenophora, Porifera, Placozoa, Cnidaria, and Bilateria, viewing ctenophores

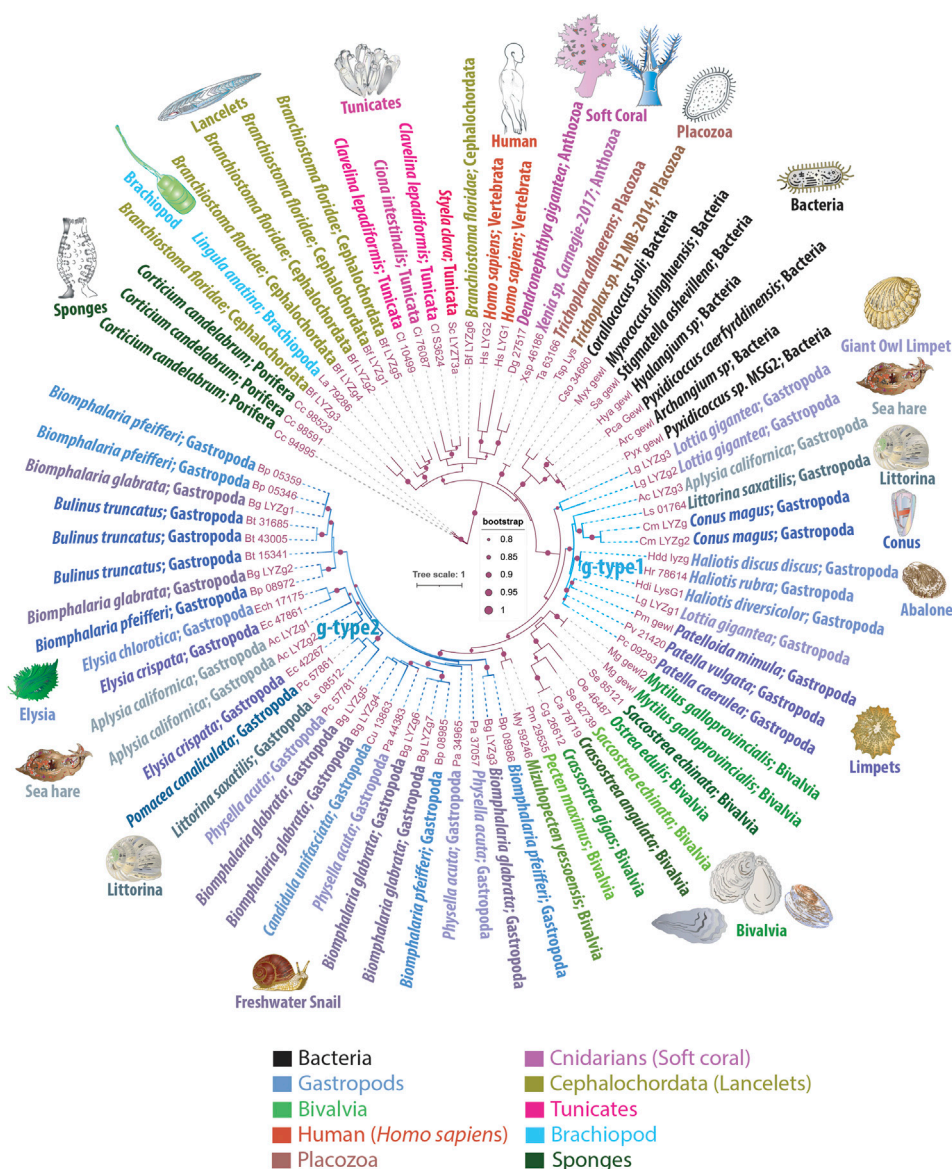


FIGURE 1

Phylogenetic Tree Illustrating the Mosaic Distribution of g-Type Lysozyme Genes Across Metazoans. This phylogenetic tree depicts the evolutionary mosaic distribution of g-type lysozyme genes across animal taxa, with gene names displayed alongside their respective genus and species name and the taxonomic group they belong to. Bootstrap values below 80% have been excluded for clarity. Lysozymes from predatory myxobacteria, highlighted in black, exhibit strong sequence similarities with metazoan g-type lysozyme genes, suggesting potential horizontal gene transfer events. The tree distinguishes two groups of lysozymes: g-type1, representing more ancestral genes found in basal species of gastropods, and g-type2, which appears to have arisen from more recent gene duplication events and is primarily found in the more recently evolved *Euthyneura* group, including species like *Elysia* spp., *Biomphalaria* spp., *Physella acuta*, and the coenogastropod *Littorina saxatilis*.

as the sister lineage to the rest of Metazoa (Moroz et al., 2014; Ryan et al., 2013; Schultz et al., 2023; Whelan et al., 2015; Whelan et al., 2017). We expanded our investigation to include choanoflagellates and other holozoans, as well as prokaryotes, encompassing single-cell eukaryotes, protists, and fungi. In this survey, by screening five genomes (*Pleurobrachia bachei*, *Hormiphora californensis*, *Bolinopsis microptera*, *Mnemiopsis leidyi*, and *Beroe ovata*, see (Moroz et al., 2014; Ryan et al., 2013; Schultz et al., 2023; Vargas et al., 2024) and dozens of transcriptomes (Moroz et al., 2014; Whelan et al., 2017), we were not able to identify recognizable g-type lysozymes in ctenophores. In the homoscleromorph poriferan

Corticium candelabrum we found three g-type related lysozymes, which we used as an outgroup, and no similar genes in the other three major groups of sponges (demosponges, glass, and calcareous sponges). The genomes of two haplotypes of *Trichoplax* (Placozoa) contain just one g-type lysozyme gene (Figure 1). Importantly, the genomes of both *Trichoplax* and *C. candelabrum* lacked other lysozyme types, such as c-type or i-type. We also discovered a single g-type lysozyme gene in the soft coral species *Xenia* sp. and *Dendronephthya gigantea* (Figure 1). These genes were not found in other cnidarian classes, such as hydrozoans and anthozoans. Notably, that a single g-type gene identified in brachiopods is

nested within chordates. *Branchiosoma floridae* (lancelet, Cephalochordata) has six g-type lysozyme genes, one of the largest species-/genus-specific radiation events across metazoans.

This sporadic presence and distribution patterns strongly suggest that basal metazoan lineages acquired the g-type lysozymes via LGT events. We observed phyletic relationships between animal g-type lysozyme genes and several bacterial genes (Figure 1), illustrating the robust branching of prokaryotic genes within animal g-type lysozyme genes.

Notably, the sequenced genomes of five unicellular holozoans, sisters to Metazoa (*Monosiga brevicollis*, *Capsaspora owczarzewski*, *Pigoraptor chileana*, *Pigoraptor vietnamica*, and *Ministeria vibrans*), do not contain g-type lysozyme genes. However, these holozoan genomes contain molecularly distinct, unclassified lysozyme genes that do not belong to the traditionally recognized types, such as i-type or c-type lysozymes (KM and LLM, manuscript in preparation). Similarly, our screening of fungal genomes also did not yield any g-type lysozyme genes. Moreover, phylogenetic analyses revealed significant sequence homology between animal g-type lysozyme genes and those found in several bacterial species, as depicted in Figure 1. These bacterial homologs, belonging to myxobacteria, include representatives of the genera *Corallococcus*, *Myxococcus*, *Pyxidicoccus*, *Archangium*, *Stigmatella*, and *Hyalangium*. These myxobacteria are well-known for their predatory behaviors, utilizing complex mechanisms to hunt and consume other microorganisms, including bacteria, fungi, and algae (Contreras-Moreno et al., 2024). This phylogeny implies horizontal gene transfer between these predatory bacteria and metazoans, underlining the adaptive significance of g-type lysozymes for innate immunity and defense, and early possible metazoan diversification.

The molluscs, and gastropods, in particular, revealed the extensive acquisition and lineage-specific radiation of g-type lysozymes. The graser, freshwater snail *Biomphalaria glabrata* has seven g-type genes and five lysozyme-encoded genes present in closely related species of *Biomphalaria pfeifferi*. Most other gastropod species have 2–4 copies of g-type lysozyme genes per genome (Figure 1). One or two copies of g-type lysozymes were found in the sequenced bivalves' genomes. In contrast, cephalopods, *Octopus bimaculoides*, *Octopus vulgaris*, *Octopus sinensis*, and *Amphioctopus fangsiao*, each possess one or two i-type lysozyme genes, but none had g-type lysozymes. We did not identify any g-type lysozymes in the sequenced annelid, nemertines or phoronid genomes.

In summary, the distribution patterns of g-type lysozyme genes suggest two possible evolutionary scenarios: either the g-type lysozyme gene was initially present in the common ancestor of all metazoans and subsequently underwent widespread losses throughout ~530 million years of subsequent evolution, or certain species sporadically acquired it within some metazoan lineages through domestication or horizontal gene transfer. The mosaic distribution and the rare occurrence of g-type lysozyme genes rather support the latter scenario. This indicates a selective evolutionary process characterized by intermittent gene retention, loss, and horizontal gene transfer within this particular family. This varied pattern of distribution across metazoans not only challenges the conventional view of a uniform presence across

animal taxa but also enhances our understanding of the intricate evolutionary dynamics that have shaped the existing diversity of g-type lysozyme presence in the animal kingdom.

2.2 *Aplysia californica* encodes phylogenetically distinct g-type lysozyme genes

The sea slug *A. californica* is a powerful reference species and model for understanding cellular basis of behaviors and neuroplasticity (Kandel, 1979; Kandel, 2001; Moroz, 2011). As with many invertebrates, *A. californica* lacks c-type or i-type lysozymes. Figure 1 highlights the presence of three g-type lysozyme genes in *A. californica*, designated as LYZg1, LYZg2, and LYZg3. Notably, LYZg3 forms a unique, possible ancestral subgroup and lacks close orthologs within the Euthyneura group, which includes genera such as *Elysia*, *Biomphalaria*, and *Physella acuta* (Brenzinger et al., 2021). Instead, LYZg3's closest orthologs appear in the more distantly related Caenogastropoda clade, particularly within the genomes of *Littorina saxatilis* and *Conus magus*. The phylogenetic relationships of this gene reach even further, to distant relatives within the Patellogastropoda, such as *Lottia gigantea* and *Haliotis* spp. (Schoch et al., 2020). Due to its unique evolutionary path and distinct phylogenetic position, we have classified the more ancestral LYZg3 under a new category, termed g-type1 lysozyme genes (Figure 1).

The evolutionary analysis suggests that the acquisition of LYZg3 by *A. californica* predates the more recent gene duplication events responsible for LYZg1 and LYZg2. These newer genes show closer evolutionary relationships within the Euthyneura group, with species like *Elysia chlorotica* and *Elysia crispa* each harboring two copies of similar lysozyme genes. Conversely, within the Caenogastropoda, notably in the genome of *L. saxatilis*, only a single copy of this gene type is present. This finding has led us to categorize LYZg1 and LYZg2 as g-type2 lysozyme genes, thus distinguishing them from the older g-type1 group (Figure 1).

Our analysis of the g-type lysozyme genes in *A. californica* reveals the presence of two phylogenetically distinct gene groups: g-type1 and g-type2. A detailed sequence identity analysis showed that g-type1 genes have an average sequence identity of 68.48%, with values ranging from 44.49% to 100%, indicating higher conservation. In contrast, g-type2 genes demonstrated greater variability, with an average sequence identity of 58.28% and a range of 45.69%–100%. These results suggest that g-type1 lysozymes may perform more conserved and essential functions. In contrast, the g-type2 lysozymes are likely subject to adaptive pressures, possibly reflecting diverse roles in immune response or digestive functions.

In addition to the sequence identity analysis, we observed that g-type1 lysozyme genes are exclusively present in the studied marine gastropods, whereas g-type2 lysozymes are found in both marine and freshwater species. This distribution suggests that g-type1 lysozymes may be optimized for the relatively stable environmental conditions of marine ecosystems, while the broader range of g-type2 lysozymes indicates that these genes may have evolved to handle the more variable and diverse environmental pressures encountered in freshwater habitats.

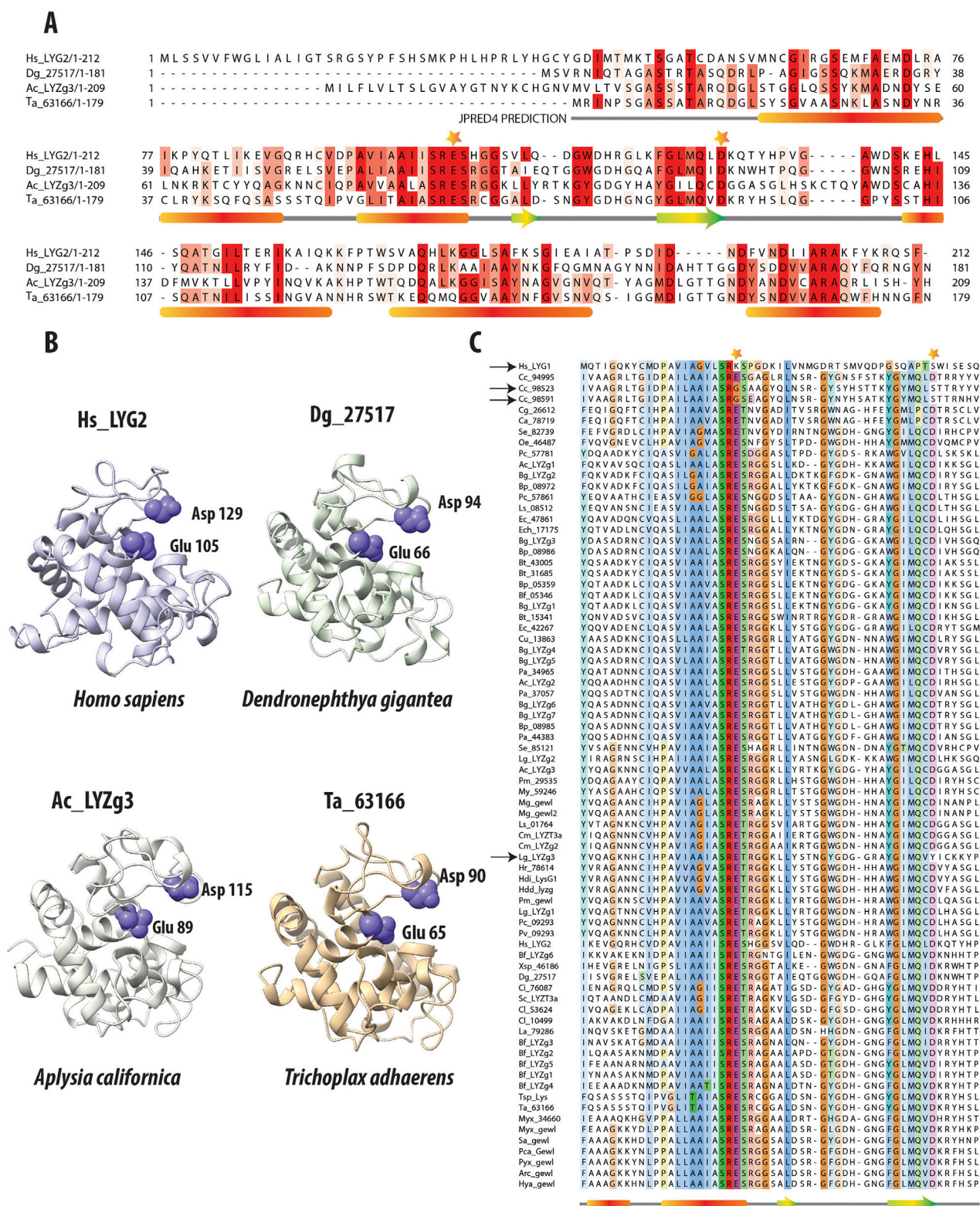


FIGURE 2

Comparative Analysis of g-type Lysozyme Genes Across Diverse Species. (A) Multiple Sequence Alignment of g-type lysozyme genes from representative species, including humans, the soft coral *Dendronephthya gigantea*, one of the g-type lysozyme genes from the *Aplysia californica* genome, and a g-type lysozyme gene from the placozoan *Trichoplax adhaerens*. The alignment, enhanced by the secondary structure predictions from Jpred (Drozdetskiy et al., 2015), reveals alpha-helices marked by red bars and beta-sheets denoted by green and yellow arrows. These structural elements are essential for the enzyme's lytic function, highlighting the evolutionary conservation despite sequence variability. Catalytic residues crucial to enzymatic activity are highlighted with stars above the alignment, emphasizing their conserved placement across species. (B) The 3D structural models of these proteins, generated by AlphaFold2 (Jumper et al., 2021), confirm the structural similarities in the regions noted in the sequence alignment. Their

(Continued)

FIGURE 2 (Continued)

lysozymes also exhibit highly similar structural folds. The preservation is especially pronounced in the catalytic residues, Glutamic and Aspartic acids, which are crucial for the enzyme's function (Malcolm et al., 1989). The consistent arrangement of these residues across representatives of different taxa underscores the structural fidelity of these proteins. It reflects evolutionary pressures to preserve key functional aspects of lysozymes crucial for antimicrobial defense. (C) Multiple sequence alignment of g-type lysozymes from a variety of species, focusing on the catalytic residues (Glutamic and Aspartic acids); these residues are marked with stars. Notably, deviations such as the non-conservation of these residues in the human Hs_LYG1 gene and two genes from *Corticium candelabrum* are indicated with black arrows. Additionally, a unique substitution in the gene Lg_LYZg3, where Tyrosine replaces Aspartic acid, is also highlighted, capturing the evolutionary dynamics of these residues.

3 Structural conservation of g-type lysozymes across metazoans

Figure 2A presents a multiple sequence alignment (MSA) of g-type lysozyme genes, including one from humans (LYG2), Anthozoan *D. gigantea*, the more ancestral *A. californica* gene (LYZg3), as well as the *Trichoplax* g-type lysozyme. Accompanying this alignment, secondary structure predictions obtained from the JPREDD4 server underscore the preservation of key structural elements across these diverse taxa (Drozdetskiy et al., 2015). Notably, the catalytic residues (aspartic acid and glutamic acid) are highlighted with stars on the MSA, illustrating their surprising conservation across broad phylogenetic distances. These catalytic residues are also conserved across all lysozyme types, including c-type and i-type lysozymes (Kuwano et al., 2013; Taylor et al., 2019).

Further, 3D structural analyses were performed using AlphaFold2 (Jumper et al., 2021) (Figure 2B). Predictions from AlphaFold2 demonstrated that the g-type lysozyme genes across a broad evolutionary spectrum fold into a structural motif observed in other lysozyme types, such as c-type or i-type (Kuwano et al., 2013). Despite lacking sequence similarity with these types, g-type lysozymes adopt similar structural conformations. Thus, the 3D structures predicted by AlphaFold2 reveal a remarkable consistency in the structural architecture of these enzymes across metazoans, again highlighting the deep evolutionary conservation of essential structural features critical for antimicrobial defense.

In order to validate the structural models of the g-type lysozyme genes across metazoans, we employed AlphaFold for structure prediction. The predicted accuracy, represented by the pLDDT (predicted Local Distance Difference Test) and pTM (predicted Template Modeling score), was calculated for each lysozyme model. As summarized in Table 1, the highest-ranked models for each gene showed consistent and high accuracy, with pLDDT values ranging from 87.4 to 97.8 and pTM values from 0.815 to 0.940, indicating reliable predictions. These results further illuminate the evolutionary preservation of g-type lysozyme architectures.

In addition to the predicted accuracy, we compared the Root Mean Square Deviation (RMSD) between different model pairs for each gene, as shown in Table 2. The RMSD values across model comparisons ranged from 0.100 to 0.183, indicating close structural similarity between different AlphaFold predictions for each gene. Furthermore, the structural alignments between the AlphaFold2-predicted models and experimentally determined structures yielded the following RMSD values: 0.717 Å for Hs_LYG2, 0.566 Å for Dg_27517, and 0.503 Å for Ta_63166 (all aligned with PDB ID: 4G9S). Additionally, the alignment between the AlphaFold2-predicted model for Ac_LYZg3 and the experimentally determined structure (PDB ID: 4G9S) produced an RMSD of 0.644 Å,

demonstrating a high degree of similarity between the predicted and experimentally derived conformations.

4 Conservation of catalytic residues in g-type lysozymes across metazoans

Figure 2C presents a multiple sequence alignment of g-type lysozymes from various representative species, focusing on the amino acids surrounding the critical catalytic residues, glutamic and aspartic acids. These essential residues are highlighted with stars above the alignment to underscore their importance in the enzyme's catalytic mechanism. Previous research has demonstrated that site-directed mutagenesis of aspartic acid (D52N) and glutamic acid (E35Q) in the chicken egg white lysozyme significantly impairs enzymatic activity. The mutant enzyme D52N retains approximately 5% of the wild-type lytic activity against *Micrococcus luteus* cell walls, whereas E35Q exhibits virtually no measurable activity ($0.1\% \pm 0.1\%$) (Malcolm et al., 1989). These findings affirm the pivotal role these residues play in the enzyme's catalytic function.

Interestingly, we observed variations in the conservation of these residues across different species. In the human *Hs_LYG1* gene, both glutamic acid and aspartic acid are not conserved, as indicated by the black arrow next to the gene name. *Hs_LYG1* is highly expressed in the kidney, which plays a key role in detoxification and filtration, with lower expression levels noted in the liver and testes. A similar pattern of non-conservation is found in two of the three g-type lysozyme genes from the poriferan *C. candelabrum* (genes *Cc_98523* and *Cc_98591*), which also lack conservation of these catalytic residues, marked by black arrows next to the gene names. In contrast, these catalytic residues are generally conserved in most other g-type lysozymes across other taxa. An exception to this trend is the gene *Lg_LYZg3*, where the aspartic acid (D) is replaced by tyrosine (Y), again indicated by a black arrow. While the specific effects of these mutations on enzyme activity have not yet been experimentally validated, it is hypothesized that these changes may result in reduced or altered enzymatic function, as seen with the chicken lysozyme mutants (D52N and E35Q).

In summary, the broad preservation of these catalytic residues highlights their evolutionary significance in maintaining enzymatic functions, with a few notable exceptions. These exceptions likely represent lineage-specific adaptations that have altered the canonical amino acids involved in the catalytic mechanism.

4.1 Tissue-specific expression patterns of *Aplysia californica* g-type lysozyme genes

Using RNA-seq data, we characterized the tissue-specific expression profiles of g-type lysozyme genes (LYZg1, LYZg2,

TABLE 1 AlphaFold Model Prediction Accuracy for Selected g-type Lysozyme Genes.

Gene	Model	pLDDT	pTM	Recycles	Top ranked
Hs_LYG2	alphafold2_ptm_model_2_seed_000	87.4	0.815	3	Yes
Dg_27517	alphafold2_ptm_model_3_seed_000	97.0	0.937	3	Yes
Ac_LYZg3	alphafold2_ptm_model_3_seed_000	95.2	0.896	3	Yes
Ta_63166	alphafold2_ptm_model_5_seed_000	97.8	0.940	3	Yes

TABLE 2 Comparison of Root Mean Square Deviation (RMSD) Between Models for g-Type Lysozyme Genes.

Gene	Model 1 vs. Model 2	Model 1 vs. Model 3	Model 2 vs. Model 3	RMSD exp vs. predicted
Hs_LYG2	0.183	0.177	0.151	0.717
Dg_27517	0.163	0.131	0.124	0.566
Ac_LYZg3	0.130	0.148	0.138	0.644
Ta_63166	0.100	0.133	0.124	0.503

LYZg3) with distinct expression patterns for each gene, which suggests the division of labor and specialized roles of different isoforms in physiology and development. LYZg1 and LYZg3 are associated with the digestive system and functions, whereas LYZg2 is more broadly distributed and might participate in innate immunity.

Specifically, the LYZg1 gene in *A. californica* has the highest expression levels in the radula region (with a value 1061 TPM, Transcripts Per Million). The radula is equipped with chitinous radular teeth for the mechanical breakdown of food (Scheel et al., 2020). Additionally, digestive organs (esophagus and stomach) also show a significant expression (TPM of 91, Figure 3A and Supplementary Material). LYZg3 was predominantly expressed in the hepatopancreas - the major digestive gland (1610 TPM), suggesting a specialized role in food processing. During development, both LYZg1 and LYZg2 exhibited low levels of expression (Figure 3C). This gene also showed moderate expression levels in some abdominal neurons and possible associated glia, implying immune-like functions (Figure 3E). At very low levels, LYZg1 is expressed in neural tissues (7–27 TPM), which might be associated with either non-functional ‘leakage’ of promoters in polyploid cells or some other unknown functions (Figure 3E, Supplementary Material).

In contrast, LYZg2 showed more varied expression patterns across different tissues (e.g., the hermaphroditic glands [41TPM], chemosensory regions of mouth and rhinophores [17 TPM], penis muscle [26 TPM], skin [86 TPM], and foot [28 TPM] – see Figure 3B). During development, the expression of LYZg2 was noted on the 9th day [33 TPM] and significantly increased during metamorphosis, peaking in post-metamorphic stages [3339–3436] TPM (Figure 3D and Supplementary Material), suggesting its involvement in the broad spectrum of functions, during developmental and tissue differentiation, likely innate immunity. *Aplysia californica* is a simultaneous hermaphrodite with biphasic life cycle and evident metamorphosis. The expression data for all tissues, ganglia, and developmental stages are provided in Supplementary Material.

5 Discussion

In various organisms, lysozymes play dual roles: they protect against microbial invasions and facilitate essential digestive processes (Dobson et al., 1984; Itoh and Takahashi, 2007). This study reveals a highly mosaic distribution of g-type lysozyme genes across metazoan phyla, with the apparent absence of these enzymes in ctenophores and most sponge classes (except the homoscleromorph *C. candelabrum*). The potential absence of g-type lysozymes in basal groups like ctenophores and sponges may represent either their primary absence in the common metazoan ancestor or early divergence of lysozyme subtypes and gene loss in many lineages, as opposed to lateral gene transfer in bilaterians, for example, illuminated in insects (Li et al., 2022; Liu et al., 2023).

Notably, the demosponge *Suberites domuncula*, possesses a different lysozyme subtype (i-type) and employs it for immune defense and digestive processes (Wiens et al., 2005). Sponges, being bacterial feeders, utilize specialized cells, choanocytes, for a filter mechanism similar to unicellular and colonial choanoflagellates. Both sponges and choanoflagellates share a critical structural feature for feeding—a collar: an apical cilium that generates water currents to draw bacteria surrounded by microvilli, which capture bacteria for intracellular digestion by phagocytosis (Mah et al., 2014). Although we did not identify a g-type lysozyme in the sequenced choanoflagellate genomes, we discovered geneologically unrelated families of genes encoding ch-type (*Chalaropsis*-type) and other unconventional lysozyme-like proteins and their potential involvement in digestive and defensive functions.

A broader look at the distribution of g-type lysozymes across bilaterians further suggests the same two alternative hypotheses: first, these genes may be conserved across the Tree of Life, playing fundamental roles across bacteria and animals, which we think is unlikely due to the absence of these enzymes in many other eukaryotic lineages. Alternatively, the observed distribution in Metazoa could be explained by lateral gene transfer (LGT) followed by differential gene loss in some lineages, too.

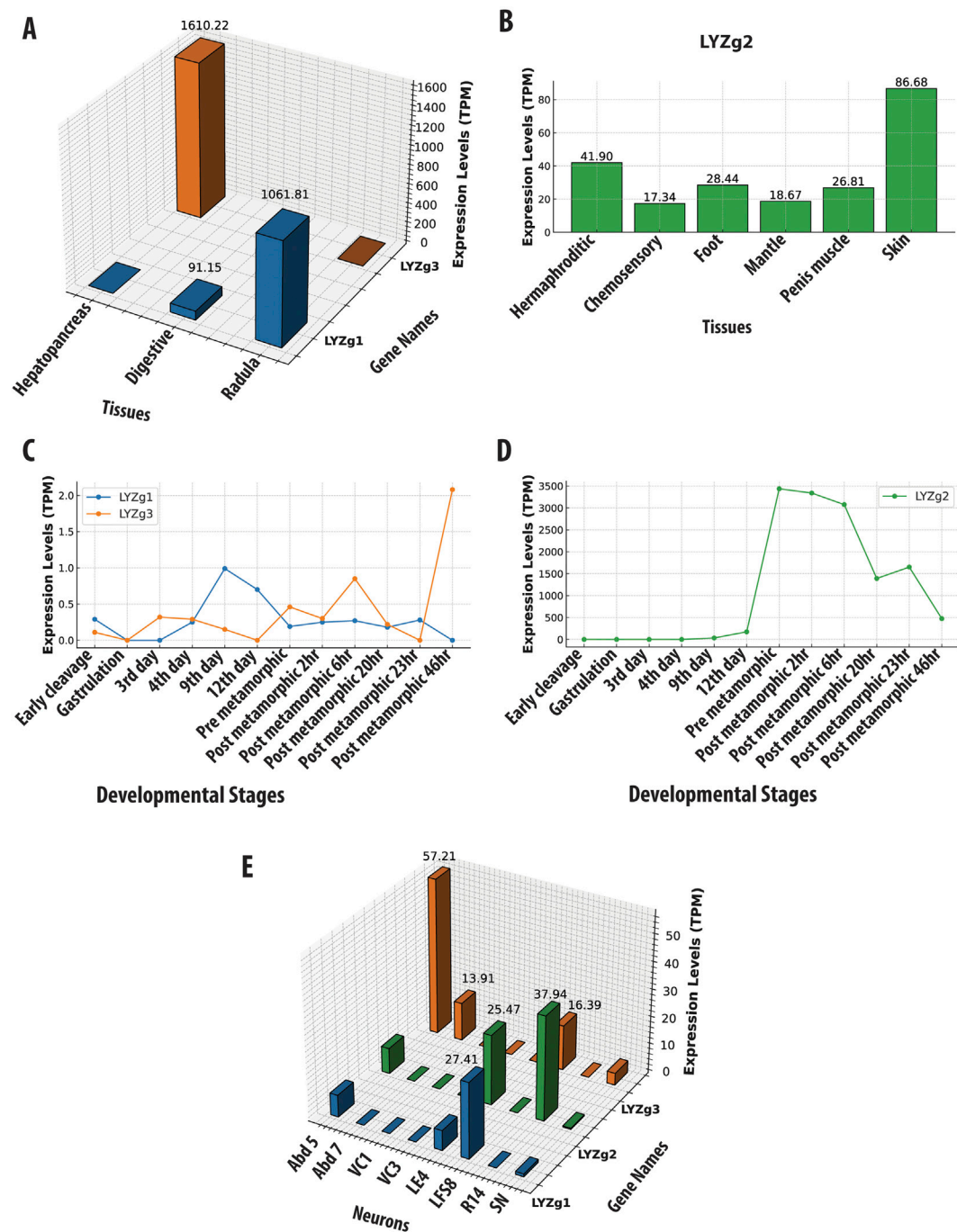


FIGURE 3

Gene Expression Profiles of *Aplysia californica* g-Type Lysozyme Genes Across Various Contexts. This figure illustrates the expression profiles of g-type lysozyme genes from *Aplysia californica* across multiple biological contexts. (A) Tissue-specific expression patterns of LYZg1 and LYZg3, normalized to Transcripts Per Million (TPM). (B) Tissue-specific expression patterns of LYZg2, highlighting its activity in immune-related tissues. (C) Developmental stage-specific expression patterns of LYZg1 and LYZg3, showing notable peaks during early post-metamorphic stages. (D) Expression levels of LYZg2 across developmental stages, indicating significant increases during post-metamorphic transitions. (E) Expression profiles of LYZg1, LYZg2, and LYZg3 in individual neurons, emphasizing their roles in neural function. All expression data are normalized to TPM. (See [Supplementary Material](#) for additional details).

Additional studies exploring the phylogenetic relationships and functional similarities between bacterial and metazoan lysozymes will be necessary to pinpoint whether the pattern is a result of gene conservation or LGT events.

Placozoans, the simplest metazoans with the primary absence of neurons and muscles, have developed extracellular digestion without a mouth and gut (Romanova and Moroz, 2024). G-type lysozyme gene in *Trichoplax*, with no evidence of other lysozyme types, might

likely contribute to both immunity and digestion as in soft coral species like *Dendronephthya* and *Xenia* sp., with possible functional specialization for both diet and defense. While specific physiological reports on lysozymes in anthozoans are lacking, lysozyme-like activity has been observed in the mucus of *Actinia equina* (Stabili et al., 2015). Furthermore, our findings suggest that cnidarians, including *Nematostella vectensis*, may possess non-conventional types of lysozyme (unpublished data). The hemichordate *Saccoglossus kowalevskii* appears to encode genealogically different i-type and other non-conventional lysozyme as examples of convergent evolutionary adaptation, likely for both defensive and digestive roles.

Lysozymes broadly exhibit multifunctionality in marine bivalves, including scallops, clams, oysters, and mussels. These enzymes are utilized to degrade bacteria ensnared within the mucus of their gills, a process vital for nutrient uptake and enhancing digestive efficacy (Van Herreweghe and Michiels, 2012). Similarly, terrestrial invertebrates such as worms and flies, which feed on decomposing organic matter, depend on other families of lysozymes for both defensive measures against potential pathogens and the efficient extraction of nutrients from their microbial food sources. In flies, specifically in *Drosophila melanogaster*, lysozymes are part of the immune response system and are known to target bacterial peptidoglycans, as well as perform digestive roles in the midgut (Hultmark, 1996). Other arthropods, such as crustaceans, also utilize lysozymes for immune defense, breaking down bacterial cell walls to protect against infections (Bachali et al., 2002). In nematodes like *Caenorhabditis elegans*, lysozyme genes are expressed in the intestine, playing roles in pathogen defense and digestion (Boehnisch et al., 2011). Genealogically different i-type lysozymes also perform digestive roles akin to the function of c-type lysozymes in the foreguts of ruminants, highlighting a striking example of convergent evolution spanning diverse biological groups (Bachali et al., 2002; Olsen et al., 2003).

The observed high sequence similarity between g-type lysozymes in animals and those found in myxobacteria such as *Corallococcus*, *Myxococcus*, *Pyxidicoccus*, *Archangium*, *Stigmatella*, and *Hyalangium* presents a compelling case of both molecular convergence and horizontal gene transfer across diverse domains of life. This striking homology not only suggests that similar selective pressures may have independently shaped these enzymes in vastly different organisms but also highlights the essential functional role of lysozymes in both predation and immune defense.

In myxobacteria, these lysozyme-like proteins are likely integral to their predatory mechanisms, facilitating the breakdown of bacterial cell walls, similar to the role of g-type lysozymes in the immune systems of animals. These conserved enzymatic functions across evolutionary distances underscore the adaptive significance of lysozymes and may reflect ancient evolutionary connections (LGT) that have preserved these critical activities. This finding contributes to our broader understanding of how essential functions can be recruited or arise independently across phyla, thus offering new insights into the evolutionary dynamics of molecular function and adaptation.

The phylogenetic analysis of g-type lysozyme genes in *Aplysia californica* illuminate functional divergence and suggests distinct evolutionary pressures resulting in division of labor between

digestive and likely immune functions. The characteristic distribution of g-type1 and g-type2 lysozymes across marine and freshwater gastropods further supports functional divergence associated with different habitats. G-type1 lysozymes are exclusively found in marine gastropods in more stable marine environments. In contrast, g-type2 lysozymes, present in both marine and freshwater species, likely reflect phenotypic plasticity to cope with fluctuating salinity, temperature, and microbial diversity in freshwater habitats. This observation highlights shaping the evolutionary trajectories of differential recruitments of these lysozyme families, closely tied to the ecological niches. The positioning of bivalve g-type lysozyme genes between the two gastropod groups (g-type1 and g-type2) in the phylogenetic tree could reflect divergence from a common ancestral lineage shared by gastropods and bivalves or LGT events or both. This finding underscores the complexity of lysozyme evolution in molluscs.

In this context, *A. californica* further demonstrates the molecular division of labor for three g-type lysozymes, showing distinct expression patterns across cells and tissues. Similarly, among scallops and abalone, g-type lysozymes are primarily located in organs vital for nutrient processing and environmental pathogen management, such as the hepatopancreas and mantle (Bathige et al., 2013). In *A. californica*, LYZg1 and LYZg3 are predominantly associated with components of the digestive system, also implying that *A. californica* leverages these enzymes to decompose bacterial cells, thus pursuing an evolutionary path distinct from that of mammals (Mackie, 2002). On the other hand, the pronounced expression of LYZg2 in the skin, mantle, foot, chemosensory organs, and reproductive organs likely represents *A. californica*-specific adaptation, with a highly reduced shell, boosting its defenses against pathogens.

In summary, our findings highlight the multifunctional and evolutionary significance of lysozymes across Metazoa. The roles of these enzymes extend beyond immune defense, showcasing their involvement in critical digestive processes, and are shaped by lineage-specific adaptations. The observed mosaic distribution of g-type lysozymes and their varying conservation patterns point to complex evolutionary trajectories influenced by ecological and physiological pressures.

6 Conclusions

1. In this study, we explored the evolutionary diversification and functional roles of g-type lysozymes across a broad range of metazoans, revealing a mosaic pattern in their distribution. Certain phyla, such as ctenophores and most sponge classes, lack these enzymes entirely, while others, like *C. candelabrum* and *Trichoplax*, retain or acquire g-type lysozymes that play critical roles in both immune defense and digestion. Additionally, the identification of non-conventional lysozyme-like proteins in choanoflagellates suggests that digestive and defensive functions may have evolved in parallel in these organisms despite the absence of g-type lysozymes.
2. We also uncovered significant homology between g-type lysozymes in animals and myxobacteria, indicating a possible case of molecular convergence or horizontal gene transfer. This observation highlights shared evolutionary pressures across vastly different organisms, suggesting that lysozymes may have been

recruited independently throughout evolution for similar functional roles in predation and immune defense.

- Finally, the distinct expression patterns of g-type lysozymes in various tissues of *A. californica* emphasize their specialized roles. LYZg1 and LYZg3 are primarily involved in digestion, while LYZg2 plays adaptive roles in immune defense. These tissue-specific patterns reflect the evolutionary pressures that shaped lysozyme diversification and demonstrate the molecular division of labor in different metazoan lineages. There is clear divergence between g-type1 and g-type2 lysozymes in *Aplysia*. G-type1 lysozymes exhibit strong sequence conservation, likely due to their essential roles in immune defense and physiological processes. In contrast, the greater sequence variability in g-type2 lysozymes suggests adaptive evolution in response to environmental challenges, such as fluctuating microbial communities in different habitats. This functional diversification, together with the diversity of these enzymes in marine and freshwater species, highlights the role of sequence variation in shaping molluscan physiology, where g-type lysozymes serve distinct functions.

This study provides new insights into the evolutionary dynamics of g-type lysozymes and their recruitment for diverse functional roles across metazoans. Future studies, including experimental validation of lysozyme activity and further genomic analysis, will be crucial to understanding the full scope of their contributions to both immune defense and digestion.

7 Methods

7.1 Sequence retrieval and database preparation

We established both local and online sequence retrieval systems. Locally, we configured the NCBI standalone BLAST tool on a UNIX-based platform, accessible via the NCBI BLAST Download. We generated a dedicated BLAST database using the `makeblastdb` utility, incorporating genomic data across species. Additionally, we conducted searches within the Neurobase transcriptomic dataset (<https://neurobase.rc.ufl.edu/>) for lysozyme genes against Ctenophore transcriptomes, which we assembled using the Trinity assembler (Grabherr et al., 2011). The genomic and transcriptomic datasets or assemblies used for the standalone BLAST searches are listed in a table provided as [Supplementary Material](#). This database enabled targeted searches for lysozyme genes using TBLASTn, with e-value thresholds set between 10^{-5} and 10^{-10} to ensure the identification of potential homologs with high specificity. In instances where genomic regions lacked annotated gene models, we extracted sequences surrounding predicted coding regions and subjected them to hidden Markov model analysis using FGENESH+, thereby enhancing our ability to predict gene structures accurately. We stored all the genomic information in our locally created FileMaker Pro database (<http://claris.com>).

We broadened our search for lysozyme genes, utilizing species-specific online platforms such as the Ensembl genome browser. Furthermore, we employed the NCBI BLAST server to retrieve as well as cross-examine the lysozyme gene data from a wide array of

sources, including metazoans, premetazoans, single-celled eukaryotes, protists, fungi, and prokaryotic origins. By maintaining the default e-value cutoffs, we achieved a comprehensive retrieval of homologous sequences, thereby considerably expanding our dataset and enriching the comparative dimension of our study. This dataset underwent rigorous manual validation and reverse BLAST searches to ensure accurate identification of lysozyme genes. Following this, we conducted multiple sequence alignments and phylogenetic reconstructions to categorize the sequences within their appropriate lysozyme groups. The summary table of all species from which g-type lysozyme sequences were retrieved is provided in [Supplementary Material](#). This includes the NCBI GenBank Accession numbers, LOC numbers where available, along with their corresponding protein and cDNA sequences.

7.2 Protein domain identification

To elucidate the structural and functional elements of the protein sequences under study, we employed a multifaceted approach for comprehensive protein domain identification. We utilized three major databases known for their robust domain detection capabilities: the Pfam database (version 34.0), the SMART database (Simple Modular Architecture Research Tool), and the NCBI's Conserved Domain Database (CDD). Each protein sequence was processed and submitted to these search tools, which utilize hidden Markov models (HMMs) to detect known protein domains within the queried sequences. This approach allows for the identification of conserved domains that are critical to understanding the functional capabilities and evolutionary history of the proteins. The search parameters across all platforms were harmonized by setting an E-value cutoff of 0.01 to minimize the inclusion of potentially spurious matches, thereby enhancing the specificity of the domain identification process. Identified domains from Pfam, SMART, and NCBI CDD were cross-referenced to confirm domain predictions (Edgar, 2004; Letunic and Bork, 2018; Marchler-Bauer et al., 2011).

7.3 Protein multiple-domain alignment

Protein sequences were aligned using the MUSCLE (Multiple Sequence Comparison by Log-Expectation) algorithm, a method recognized for its high quality and rapid alignment capabilities. We applied MUSCLE either through the European Bioinformatics Institute (EBI) online interface or directly *via* a command-line utility on a UNIX system. This meticulous alignment process is critical for maintaining the precision necessary for our subsequent evolutionary and functional analyses. The reliability and effectiveness of MUSCLE in generating accurate alignments make it a preferred choice in bioinformatics studies (Edgar, 2004). The multiple sequence alignment file is provided as part of the [Supplementary Material](#) (see [Supplementary Material](#)) for reproduction and further analysis.

7.4 Phylogenetic analysis

We constructed phylogenetic trees using PhyML v3.0, a tool renowned for its efficiency and accuracy in tree estimation. To determine the most appropriate evolutionary model, we used ProtTest to evaluate models based on the Akaike Information Criterion (AIC). This model was applied with adjustments for rate heterogeneity and other relevant evolutionary parameters to best fit our data. Tree topology was refined employing both Nearest Neighbor Interchange (NNI) and Subtree Pruning and Regrafting (SPR) moves to optimize the tree structure. Clade support was rigorously assessed *via* the SH-like approximate likelihood ratio test, which provided a robust statistical framework for evaluating phylogenetic reliability and accuracy. This comprehensive approach ensures that our phylogenetic interpretations are both scientifically robust and reliable, facilitating detailed evolutionary insights (Anisimova et al., 2011; Guindon et al., 2010; Guindon and Gascuel, 2003). The multiple sequence alignment file and the phylogenetic tree in Newick format, along with detailed statistics, including the final model (WAG), are provided in [Supplementary Material](#) for reproducibility.

7.5 Visualization and post-processing of phylogenetic trees

Phylogenetic trees were initially visualized using iTOL (Interactive Tree Of Life), an online tool that allows for the comprehensive display and annotation of phylogenetic and other hierarchical trees. To improve visual clarity and enhance publication quality, the resulting diagrams were further refined using Adobe Illustrator. This post-processing step enabled us to fine-tune graphic details, adjust color schemes, and add text annotations, thus enhancing the overall readability and aesthetic appeal of the tree presentations. These refinements were crucial for effectively conveying complex evolutionary relationships among the studied lysozyme genes, facilitating a clearer understanding and interpretation by the scientific community. This approach ensured that our visual representations were not only scientifically accurate but also visually compelling.

7.6 Prediction of protein secondary and tertiary structures

Protein secondary structures were predicted using the JPRED4 online server, which is highly regarded for its accuracy and reliability in secondary structure prediction (Drozdetskiy et al., 2015). We uploaded the Multiple Sequence Alignment (MSA) file, saved in MSF format from the ClustalX MSA viewer, directly to JPRED4. This step facilitated the automated prediction of alpha-helices, beta-sheets, and coil regions within the protein sequences, providing a foundational understanding of the structural elements.

For the prediction of protein tertiary structures, we employed the AlphaFold2.ipynb notebook on the AlphaFold2 server hosted

on Google Colab (ColabFold v1.5.5). This platform leverages MMseqs2 for sequence searching, significantly enhancing the accuracy and reliability of the protein folding predictions (Jumper et al., 2021; Mirdita et al., 2022). AlphaFold2's deep learning-based approach allows for the generation of highly accurate 3D models of protein structures based on the amino acid sequences provided.

7.7 *Aplysia californica* cDNA library preparation and sequencing

A comprehensive cDNA library for *Aplysia californica* was constructed by the Moroz laboratory as a part of the *A. californica* genome/transcriptome project, using an Illumina kit, specifically designed for high-fidelity and efficient cDNA synthesis (Moroz and Kohn, 2010). The transcriptomes were sequenced using an Illumina sequencing platform. The sequencing was optimized to achieve high coverage and depth, enhancing the reliability of gene expression data. Links to the *A. californica* tissue, individual neurons, and developmental stage datasets, created in our lab and available for online access from the NCBI SRA database, are provided in [Supplementary Tables 1, 3](#).

7.8 Data processing and expression analysis

The initial step involved using Trimomatic for trimming adapter sequences and removing low-quality bases, which is critical for maintaining the integrity of subsequent analyses. Following this preprocessing, the cleaned reads were aligned to the *Aplysia californica* genome using the STAR aligner, a tool renowned for its efficiency and accuracy in mapping large numbers of RNA-seq reads (Dobin et al., 2013).

After alignment, gene expression levels were quantified using EdgeR, an established bioinformatics tool for differential expression analysis (Robinson et al., 2010). In this phase, we focused on calculating Transcripts Per Million (TPM), a normalization method that facilitates accurate comparison of gene expression levels across different samples by accounting for both the depth of sequencing and the gene length.

7.9 Visualization of gene expression data

For the gene expression data from our *A. californica* study, we used bar and line graphs generated in the R programming environment with the ggplot2 package. The data were log-transformed to better visualize differences in gene expression levels across tissues; however, the graphs are labeled with the actual TPM (Transcripts Per Million) values to ensure accuracy in reporting. Bar graphs were used to compare gene expression across tissues, with each bar representing the average expression in TPM, and 3D bar diagrams were employed for more complex visualizations. Line graphs were used to display temporal changes in gene expression across developmental stages or under varying experimental conditions.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repository and accession number(s) can be found in the article/[Supplementary Material](#).

Ethics statement

The manuscript presents research on animals that do not require ethical approval for their study.

Author contributions

KM: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Resources, Software, Validation, Visualization, Writing—original draft, Writing—review and editing. LM: Conceptualization, Funding acquisition, Investigation, Project administration, Resources, Supervision, Writing—original draft, Writing—review and editing.

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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.

The author(s) declared that they were an editorial board member of *Frontiers*, at the time of submission. This had no impact on the peer review process and the final decision.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcell.2024.1487920/full#supplementary-material>

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Reduction in thermal plasticity of life history traits in response to cold selection: an experimental evolution study using *Drosophila melanogaster*

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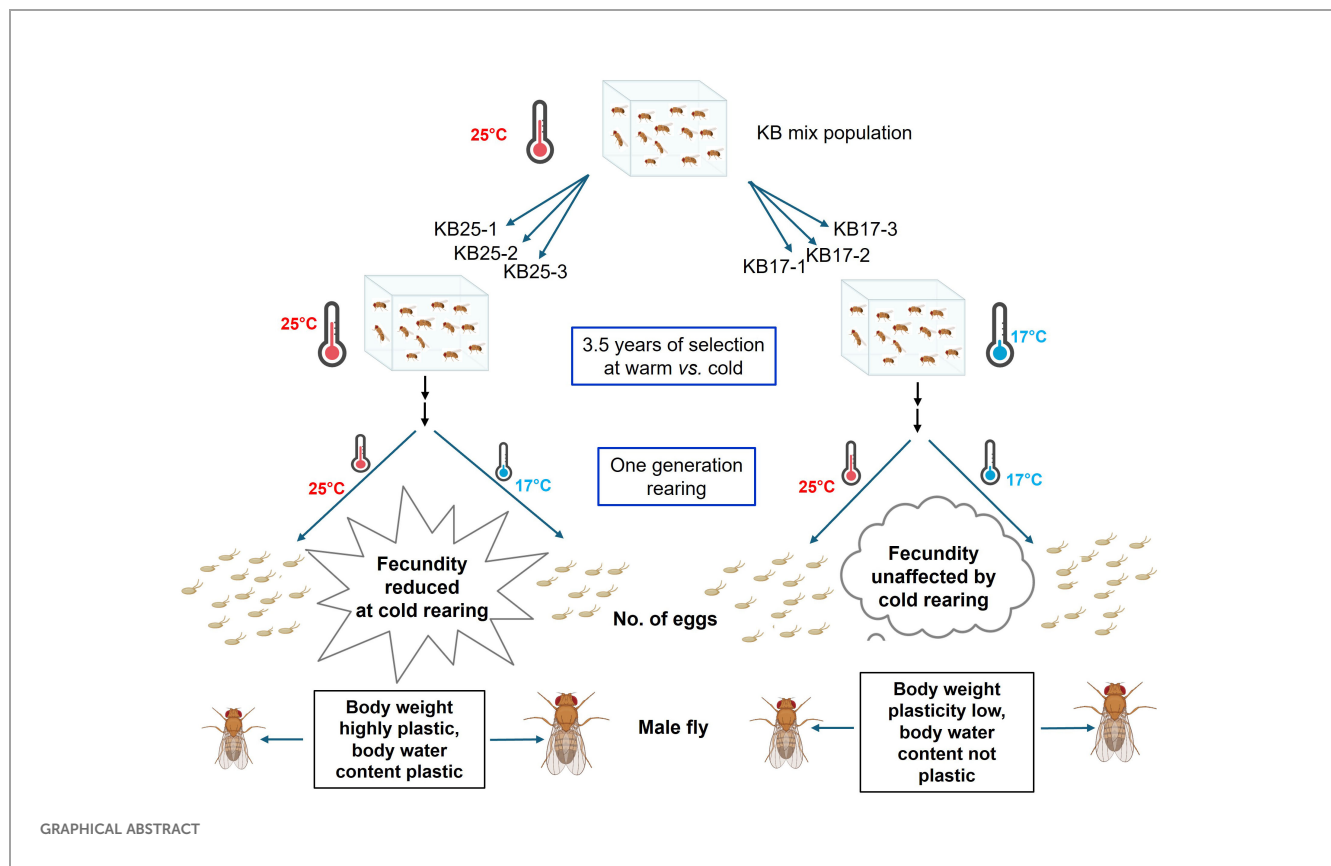
Introduction: How selection influences phenotypic plasticity is an important question in evolutionary biology. We report an experimental evolution study that examined how prolonged selection at cold vs. warm temperature impacts the thermal plasticity of traits like reproductive output, body size, and body water content in *Drosophila melanogaster*.

Methods: We conducted the study on two sets of large, outbred fly populations: one maintained at the standard fly rearing temperature, i.e., 25°C, and another selected at cold temperature, i.e., 17°C, for 3.5 years. Both selection lines were derived from the same ancestral population.

Results and discussion: We found that while 25°C selected females lay significantly fewer eggs in cold compared to warm or optimal rearing temperature of 25°C, the 17°C selected females have consistent or canalized fecundity levels across warm and cold conditions. Sustained fecundity levels across cold and warm conditions are potential marks of adaptation to a broader thermal range. While phenotypic plasticity may aid in adaptation to new environments, for traits such as fecundity, consistent levels across environments, that is, low plasticity, may be more adaptive. We also found that male flies from cold vs. warm selection regimes differ in their thermal plasticity. Plasticity of dry weight and body water content was reduced in the cold-selected males, indicating the evolution of canalized levels for these traits too. While canalized fecundity levels across temperatures can potentially help in thermal adaptation, the significance of reduced plasticity of male body size and water content needs to be investigated in the future.

KEYWORDS

thermal plasticity, thermal adaptation, *Drosophila*, experimental evolution, fecundity, body size, canalization, body water



Introduction

Temperature induces both plastic and evolutionary changes in organisms. Short-term or within-generation effect of temperature on a trait, that is, thermal plasticity, is observed in a wide variety of biological traits. Thermal plasticity is often viewed as an inevitable effect of temperature on different biological parameters like growth, metabolism, and physiology (Van Der Have and De Jong, 1996; Angilletta and Dunham, 2003; Zuo et al., 2012; Ghosh et al., 2013). Nonetheless, there is ample empirical evidence that suggests thermal plasticity could be adaptive, and different species harbor genetic variation controlling how a trait is influenced by temperature. Therefore, thermal plasticity can be shaped by selection (Nettle and Bateson, 2015; van Heerwaarden and Sgrò, 2017; McDonald et al., 2018; Lafuente et al., 2018; MacLean et al., 2019).

Whether selection can alter the level of plasticity for a trait is an interesting question in evolutionary biology. The impact of selection on plasticity is context-dependent (Leonard and Lancaster, 2020; Barley et al., 2021; Carbonell et al., 2021; Schaum et al., 2022). How selection changes the level of plasticity of a trait in a given population would depend on various factors, such as a) if the population is inhabiting a stable or unpredictable environment, b) if plasticity for the trait incurs any energetic cost in its bearers, c) if there is genetic variation for the extent of phenotypic plasticity in the trait and whether or not there are genetic constraints influencing plasticity of the trait, and d) whether high or low plasticity is favored for that given trait.

Direct selection for high or low plasticity may alter the plasticity of a trait. Moreover, selection for a trait *per se*—and not its plasticity—can also lead to evolved changes in the plasticity of the trait as a by-product of selection. For empirical investigation of the effect of selection on plasticity, temperature can be a suitable factor as it engenders both proximate and ultimate changes, reflecting thermal plasticity and thermal evolution. Ectothermic organisms experience a greater impact of temperature because they do not have internal thermoregulation the way endotherms do. The ectotherm *Drosophila* is particularly well-suited for studies focusing on temperature-induced plasticity and evolution, because of its a) amenability to studies manipulating temperature in the laboratory and (b) wide geographic range across latitudes and continents covering thermally diverse regions (James et al., 1997; Trotta et al., 2006; Mayekar et al., 2023). In *Drosophila*, cold developmental temperature leads to slow growth and metamorphosis, which translates into the emergence of bigger flies, demonstrating the temperature size rule (Atkinson, 1994; Kingsolver and Huey, 2008; Ghosh et al., 2013). Cold temperature during the adult stage usually leads to prolonged lifespan but reduced reproductive output or fecundity (Partridge et al., 1995; Moñón et al., 2020). The plastic effects of cold temperature on growth, body size, and fecundity are mostly consistent across studies. However, the evolutionary impact of temperature may vary from population to population, depending upon the genetic composition and evolutionary history of the concerned population and also upon the specific thermal range considered for the study. Studies exploring the effect of temperature in *Drosophila* have often explored thermal

selection response, clinal adaptation, or thermal plasticity, but it is rare to find research focusing on how thermal selection shapes the thermal plasticity of life history traits.

Studies performed on fly populations collected from different latitudes have added important information about thermal adaptation, but clinal adaptation may be influenced by factors other than temperature, like humidity, photoperiod, and altitude. Therefore, some of the evolved changes may have been shaped by factors other than temperature. In contrast, the experimental evolution approach enables one to study evolution in a carefully controlled laboratory setup, under the influence of clearly defined selection pressures (Garland and Rose, 2009; Kawecki et al., 2012; Lenski, 2017; Prasad and Joshi, 2003; Schlötterer, 2023). While experimental evolution studies may not mimic multidimensional and complex selection pressures experienced by populations in a natural environment, they nonetheless hold considerable merit in identifying evolutionary responses to specific selection pressures. Consequently, tracking the evolution of populations subjected to specific thermal regimes under regulated laboratory conditions for many generations can potentially help us understand the role temperature plays in shaping adaptive evolution.

While experimental evolution has been used by a number of research groups to study thermal evolution in *Drosophila* (James and Partridge, 1995; Partridge et al., 1995; Santos et al., 2006; Schlötterer, 2023), there is more than one reason that warrants renewed efforts to study thermal selection using experimental evolution. For example, some laboratory thermal selection studies were conducted for a very short duration (Fragata et al., 2014; Tobler et al., 2014), while others employed a small population size, making it difficult to draw meaningful conclusions at the evolutionary level (Cavicchi et al., 1995). One long-term thermal selection study conducted by Linda Partridge's group performed extensive characterization of life history, growth, and size traits (James and Partridge, 1995; Partridge et al., 1995; Gilchrist et al., 1997). However, this study did not control for rearing densities that can potentially interfere with thermal selection, as warm temperature leads to crowding that, in turn, leads to evolutionary changes caused by high densities and not warm temperature per se [see Santos et al. (2004) for a discussion]. We used a well-replicated selection design for studying the laboratory thermal evolution of *Drosophila*, focusing majorly on how the plasticity of various traits evolves with thermal selection.

The thermal range of *D. melanogaster* ranges from ~11°C to ~32°C (Trotta et al., 2006; Klepsatel et al., 2019). While 25°C is optimal for the growth and survival of this species and can be considered warm, 17°C is cold, which extends the developmental time twofold compared to that at 25°C, produces bigger flies (Ghosh et al., 2013; McDonald et al., 2018), and suppresses fecundity (Mołón et al., 2020; Partridge et al., 1995). In this study, we investigated how some life history traits of the fly and the plasticity thereof change as a result of evolving at 17°C vs. 25°C for many generations. Reproductive output is a direct measure of the Darwinian fitness of an organism, and in *Drosophila*, it can be measured as the number of eggs laid and/or as the offspring produced by the flies. We used fly fecundity, i.e., the number of eggs laid per female, as a measure of reproductive output or fitness of flies. Except for a few studies (Nunney and Cheung, 1997;

Novoseltsev et al., 2005), fecundity is often measured as reproductive output during the early life of flies (Fragata et al., 2014; Klepsatel et al., 2019; Santos et al., 2020). However, we chose to measure eggs laid by individual females every day, up to 22 days of adult life, covering a large part of their lifespan, in order to get a clearer picture about the distribution of egg output across age. Another important life history trait in *Drosophila* is body size. Body size is positively correlated with a) female fecundity and b) male mating success in *Drosophila* (Pitnick, 1991; reviewed in Prasad and Joshi, 2003; Flatt, 2020) and shows plastic and evolutionary changes to rearing temperatures. Therefore, we investigated the evolved and plastic changes in the body size of flies caused by temperature. Different researchers have examined the variability in wing area, thorax length, and body weight to measure body size variation in flies. In this study, we measured body weight variation, similar to various earlier studies focusing on the life history traits in *Drosophila* (James et al., 1997; reviewed in Cavicchi et al., 1995; Watanabe and Riddle, 2021). However, there are some concerns that the body weight of a fly can vary considerably depending upon its age, activity, and in the case of females its egg, laying status (before and after peak fecundity). Taking these factors into consideration, we measured the weights of only freshly eclosed unmated flies, within 4 h of emergence from pupae, keeping age, activity, and reproductive status consistent.

Materials and methods

We employed a 2 × 2 full factorial design, in which flies from the two thermal selection regimes were reared and assayed at two treatment temperatures (17°C and 25°C). This design allowed us to investigate both a) the evolutionary effect of cold vs. warm selection and b) the plastic effect of cold and warm temperatures on traits like fecundity and body size of flies. While we examined the egg production of flies for fecundity, the wet and dry body masses of flies were assayed for body size. This also allowed us to quantify the relative content of water with respect to the body mass of the flies. The main effect of selection temperature revealed the *evolutionary change* in fecundity and body size traits, and a comparison of the trait values across treatment temperatures for a given selection regime allowed us to evaluate the extent of *plasticity* of the traits. The study design thus allowed us to find out whether selection in warm vs. cold has changed the extent of plasticity of the traits under study.

Study populations and selection regimes

We used six large, outbred (see Garland and Rose, 2009) laboratory populations of *D. melanogaster*: three populations allowed to evolve at 17°C (KIIT Base populations 17 or KB17 1–3) and three populations maintained at 25°C (KIIT Base populations 25 or KB25 1–3). Both KB17 and KB25 populations are descendants of MB (melanogaster Base) populations, whose ancestry has been described in detail in Sheeba et al. (1998) and Sarangi et al. (2016). MBs were maintained on cornmeal medium at 25°C on a 21-day discrete generation cycle, under constant light and high humidity in the laboratory of Amitabh Joshi for

over 200 generations (Sheeba et al., 1998). Five replicate populations of MB were mixed and maintained for ~2 years, and two thermal selection lineages were initiated from the mixed population, namely, KB17 (KIIT Baseline 17) 1–3 and KB25 (KIIT Baseline 25) 1–3. KB17 populations were allowed to evolve at 17°C, on a discrete generation cycle of 24 days. KB25 populations were maintained at 25°C, on a 14-day discrete generation cycle. Both sets of populations were maintained on cornmeal medium, 24 h light, and high humidity. At the time of this study, both lineages had evolved for a little over 3.5 years at their respective temperatures. *Drosophila melanogaster* takes 17–20 days to develop from egg to adult at 17°C, whereas it takes 8–10 days to develop at 25°C. Thus, the generation time is much longer at 17°C. Except for temperature and generation time, the same protocol was followed for maintaining both sets of populations. For each replicate population, flies were reared in 25 vials containing food, and the larval density was controlled at ~70 per vial. Upon eclosion, flies from all 25 vials were transferred to plexiglass cages containing food smeared with supplementary live yeast-acetic acid paste. On the 14th day after the previous generation egg collection in KB25 populations and the 24th day after the previous egg collection in KB17 populations, eggs were collected for the next generation. Thus, egg collection was done on the 4th–6th day of adult age for KB25 and the 4th–7th day of adult age for KB17 populations. A day prior to the egg collection, each population cage was provided with a Petri dish filled with fly food. Flies were then allowed to oviposit on it. After 12 to 16 h, the food plate was taken out and placed under a microscope, and the food was cut into small pieces, each containing approximately 60–80 eggs. Each piece was placed inside a vial containing 6 mL of food. Twenty-five such vials were used for each replicate population. These vials were then incubated at a specific temperature (25°C or 17°C), and upon eclosion, all flies were transferred to a population cage for the next generation and the cycle was repeated.

Generation of flies for the experiments

Body weight and fecundity of both 25°C and 17°C selection lines were assayed at two treatment temperatures, namely, a) 25°C and b) 17°C. For comparing the selection lines at a) 25°C, flies from both 17°C and 25°C selection lines were reared at a common temperature of 25°C for one generation, and these flies were referred to as standardized flies. The progeny of standardized flies was reared and assayed at 25°C. This way both selection lines were standardized, and any non-genetic parental effect of divergent temperature on the progeny was eliminated, and only selection response or evolved changes between the two thermal selection lines could be identified (Rose, 1984). Similarly, to compare the selection response at 17°C, both 17°C and 25°C selected populations were standardized at 17°C for one generation, and their progeny was subsequently reared and assayed at 17°C. The larval density was controlled at ~70 per vial for the generation of flies for all our experiments.

All assays reported here were conducted between 56 and 63 generations of selection for KB17 populations. The KB25s, the predecessors of the MB flies and their ancestors, all had been maintained at the optimal rearing temperature of 25°C for decades. Hence, the number of generations is somewhat irrelevant

for the KB25s, and they should rather be considered as control baseline or populations that had been adapted to 25°C for very long.

Fecundity assay

This assay was conducted after 56 generations of selection of the KB17 populations. Twenty vials of eggs were collected from all three replicates of KB17, over an oviposition window of 13–14 h at 17°C, and the vials were transferred to 25°C. From this step onward, the entire assay was conducted at 25°C. Upon eclosion, these flies were transferred to cages and were referred to as the standardized flies (for 25°C assay temperature). These flies were then provided with food and excess yeast-acetic acid paste for 3 days, and their eggs were collected in vials over a small oviposition window of 3 h. The flies growing from these eggs are referred to as assay flies. After 8 days of egg collection, flies started eclosing, and those eclosed in the first 4 h were discarded. After this point, every 4 h, freshly eclosed flies were transferred from the rearing vials to empty vials and subjected to chill coma for ~30 min by placing the vials in ice. After being immobilized by chill coma, the male and female flies were separated and then transferred to vials containing food. By this time, the flies recovered from the chill coma. This process was repeated every 4 h, till the next day, and males and females were thus collected in separate storage vials. Several such storage vials were maintained, each containing 30–40 flies. Once flies were collected in sufficient numbers covering the complete middle part of the eclosion time distribution, virgin collection was stopped. Similar to very early flies, very late eclosed flies too were excluded from the assay. Given the KB25 populations were already maintained at 25°C, no standardization was needed for them. Eggs were collected from them over an oviposition window of 3 h and incubated at 25°C. Once eclosed, these were our assay flies. A similar protocol was followed for virgin separation in these flies. Once the virgin collection was completed for both selection lines, one male and one female randomly drawn from the storage vials were introduced into one fecundity assay vial containing 2 mL of food. Fifteen such vials were set up for the fecundity assay for each selection regime and replicate population. Upon completion of oviposition for 24 h, each fly pair was transferred to a fresh food vial to allow for the next day's egg laying, and the previous day's vial was taken under a microscope and the eggs laid in it were counted. This cycle was repeated for 22 days. The assay was conducted at 25°C. If a male from a pair died, it was replaced with an unmated male of the same age. If a female died, it was not replaced, and data from the same vial were included in the analysis up to the death of the female. Similarly, for the treatment temperature of 17°C, eggs were collected directly from the generation 56 flies of the 17°C selection lines, and assay flies were generated at 17°C. Simultaneously, 25°C selected populations were standardized for a generation at 17°C, and the fecundity assay was performed using their offspring, also maintained at 17°C. KB17 flies were reared similarly at 17°C, and after virgin separation, a similar assay setup was done for them. Total fecundity was analyzed cumulatively over a period of 22 days using a 2 selection × 2 treatment temperature full factorial design. The total number of eggs laid by the female was averaged across 15 vials and used for the analysis. The fecundity data were also analyzed separately to investigate the age-specific pattern of egg production. For this, the weekly total fecundity was calculated for

weeks 1, 2, and 3, and an ANOVA was performed that included age (week) as a variable. The data for day 22 were not included in the analysis.

Weight assays

The dry and wet weights of the flies from all replicates of KB25 and KB17 populations were assayed after 63 generations of cold selection. The assays were conducted at both 25°C and 17°C, and the weight of the progenies of standardized flies was assayed. The assay flies were collected within 4 h of eclosion, stored immediately in microcentrifuge tubes (MCTs), and freeze-killed by keeping the flies at −20°C for 40–45 min. Wet weight was assayed for males and females separately. For each combination of selection regime, treatment temperature, replicate population, and sex, eight MCTs were set up with five flies in each. After freeze-killing, these flies were taken out of the freezer and weighed immediately. Five flies were weighed at a time using a Sartorius Quintix 35 ($d = 0.01$ mg) balance, and the average weight per fly was calculated for each MCT. After measuring the wet weight of the flies, the dry weight of the same flies was also assayed. For this, after collection of the wet weight data, the flies were returned to their respective MCTs and placed in a hot air oven set at 70°C. They were kept at 70°C for 36 h, thus being dehydrated completely, and then taken out and weighed again.

Relative water content of the flies

The water content of the flies was calculated by measuring the water lost during dehydration in the hot air oven. The total dry weight of five flies stored in each MCT was subtracted from the wet weight of the same flies to obtain the water content (in mg) of flies in each MCT. The relative water content was calculated by dividing the water content by the wet weight of the flies in the respective MCT (in percent).

Statistical analysis

Mixed model analyses of variance (ANOVA) were performed for all the assays. Replicate population means were used for testing the significance of fixed factors and their interactions in all the analyses. *Post-hoc* comparisons were done using Tukey's honest significant difference (HSD) test. Data analyses were carried out on JMP Pro 17. To analyze the fecundity data, 22 days' total fecundity per female was averaged across vials to obtain replicate population means. ANOVA was performed on replicate population means. Selection and treatment temperature were treated as fixed factors, and replicate population was treated as a random factor nested within selection and treatment temperature. We also analyzed the week-wise fecundity data (weeks 1, 2, and 3) to investigate the effects of selection and treatment temperature on early fecundity, mid-stage fecundity, and late fecundity till 21 days of adult life. A three-way ANOVA was conducted with selection, treatment, and age (week post-eclosion) as fixed factors, with the random factor population replicate nested within all three of these.

For both wet weight and dry weight measurements, the mean weight per fly from each MCT was averaged across eight MCTs to obtain the replicate population mean. ANOVA was performed on replicate population means of individual fly weight, with selection, treatment temperature, and sex being treated as fixed factors. The replicate population was treated as a random factor nested within all three fixed factors. Similar analyses were performed separately for wet weight and dry weight data. The relative water content data were obtained in percentage and therefore were subjected to arcsine-square root transformation to meet the normality assumption of ANOVA. Population means were used for testing the significance of fixed factors such as selection, treatment temperature, sex, and their interactions.

Results

Total fecundity

After evolving at 17°C for 56 generations, KB17 populations did not show any significant difference in total fecundity from KB25 populations ($p = 0.1672$) (Supplementary Tables 1; 4A, B), but treatment temperature had a significant main effect on fecundity ($p = 0.0001$) as fecundity was significantly less at 17°C treatment compared to 25°C. However, the interaction of selection temperature and treatment temperature was significant ($p = 0.0356$), and *post-hoc* comparisons showed that KB25 populations exhibit a significant drop in fecundity from 25°C to 17°C treatment temperature ($p < 0.05$), but KB17 populations did not show any significant change between 25°C and 17°C treatment (Figure 1).

Age-specific fecundity

Separate analysis of the week-wise fecundity data showed significant effects of treatment temperature, age (week), and interaction of treatment temperature and age ($p < 0.0001$, for all three effects) (Supplementary Tables 2; 4A, B). The 17°C treatment reduced the overall fecundity compared to 25°C (Figure 2). Pooled over treatments and selection regimes, *post-hoc* comparisons showed that fecundity was significantly higher during the first week compared to weeks 2 and 3 ($p < 0.05$). However, the interaction of treatment temperature and age showed that fecundity declined significantly across weeks only in the 25°C treatment. In contrast, fecundity did not differ significantly across weeks in the 17°C treatment. The main effect of selection and its interactions was not significant for the week-wise fecundity data (Figure 2).

Wet weight

After 63 generations of selection at 17°C, the wet weight of the flies was strongly affected by selection temperature, treatment temperature, and sex (Figures 3A, B). Overall, the flies of KB17 populations were significantly heavier than their counterparts from KB25 ($p = 0.0093$), and the flies reared at 17°C had significantly greater wet weight than those reared at 25°C ($p < 0.0001$) (Supplementary Table 5). Females had

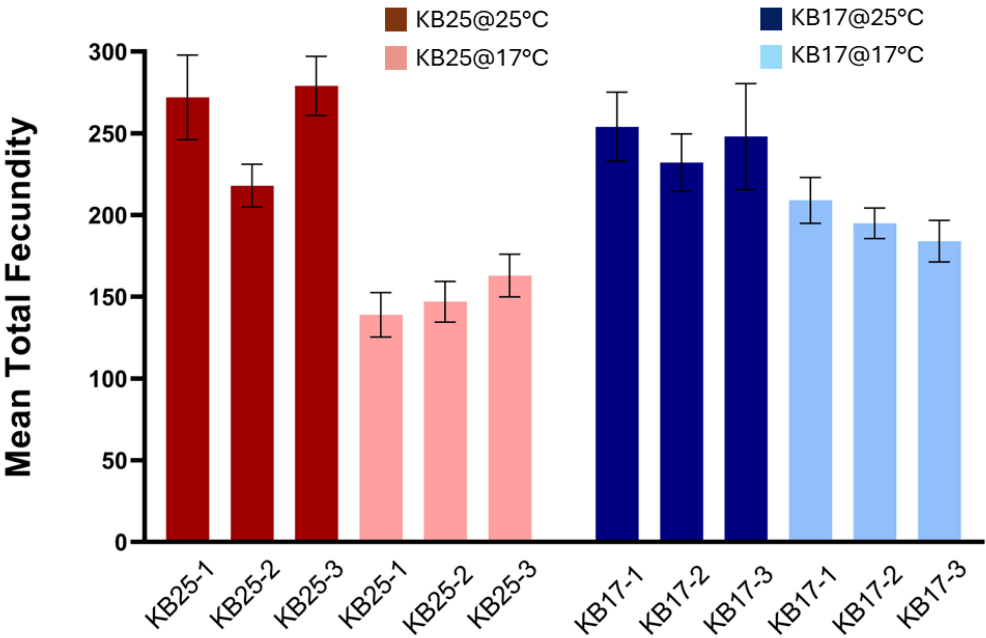


FIGURE 1
Mean total egg production per female in KB25 and KB17 replicate populations, assayed at 25°C and 17°C. The error bars represent standard error across replicate vials.

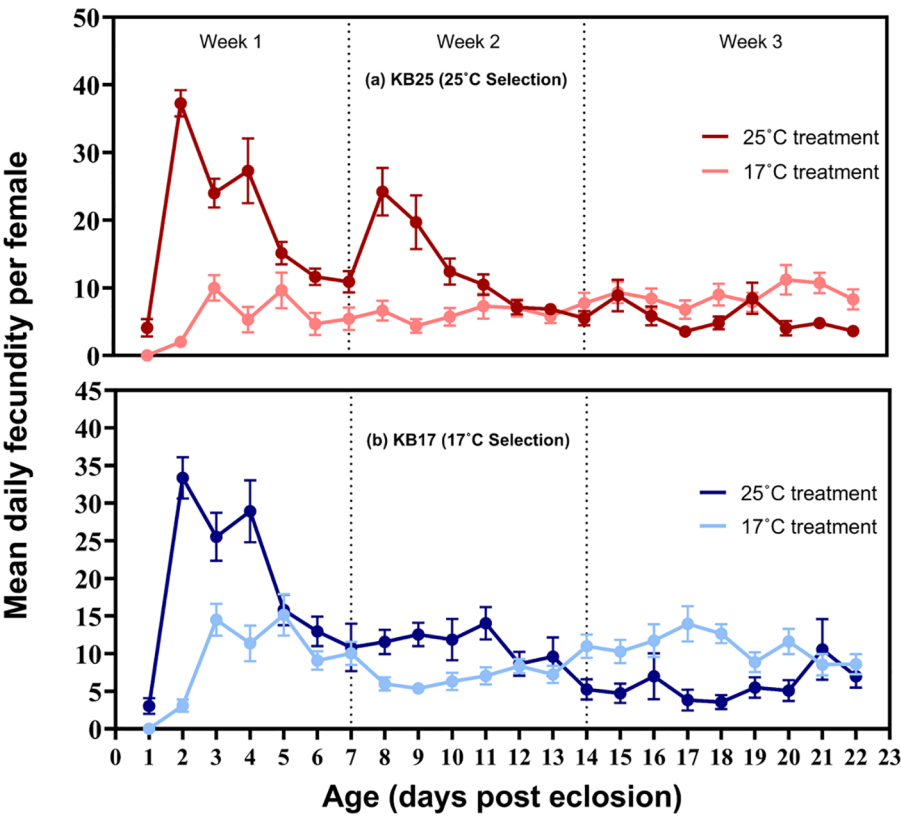


FIGURE 2
Mean daily egg production per female of (A) KB25 and (B) KB17 populations, assayed at 17°C and 25°C. The error bars represent standard error across replicate populations.

significantly greater wet weight than males ($p < 0.0001$) (Figures 3A, B). Fixed factor interactions were not significant (Supplementary Table 3).

Dry weight

The dry weight of the flies was strongly affected by treatment temperature and sex, but not selection temperature (Supplementary

Tables 3, 5). Flies reared at 17°C had significantly greater dry weight than those reared at 25°C ($p < 0.0001$) (Figures 3C, D). Females were significantly heavier than males across conditions ($p < 0.0001$). Two-way interaction between treatment and sex ($p < 0.0001$) and three-way interaction among selection, treatment, and sex were significant ($p = 0.025$) (Supplementary Table 3). Both males and females of the KB25 population and females of the KB17 population had 44% to 47% greater dry weight at 17°C than at 25°C. However,

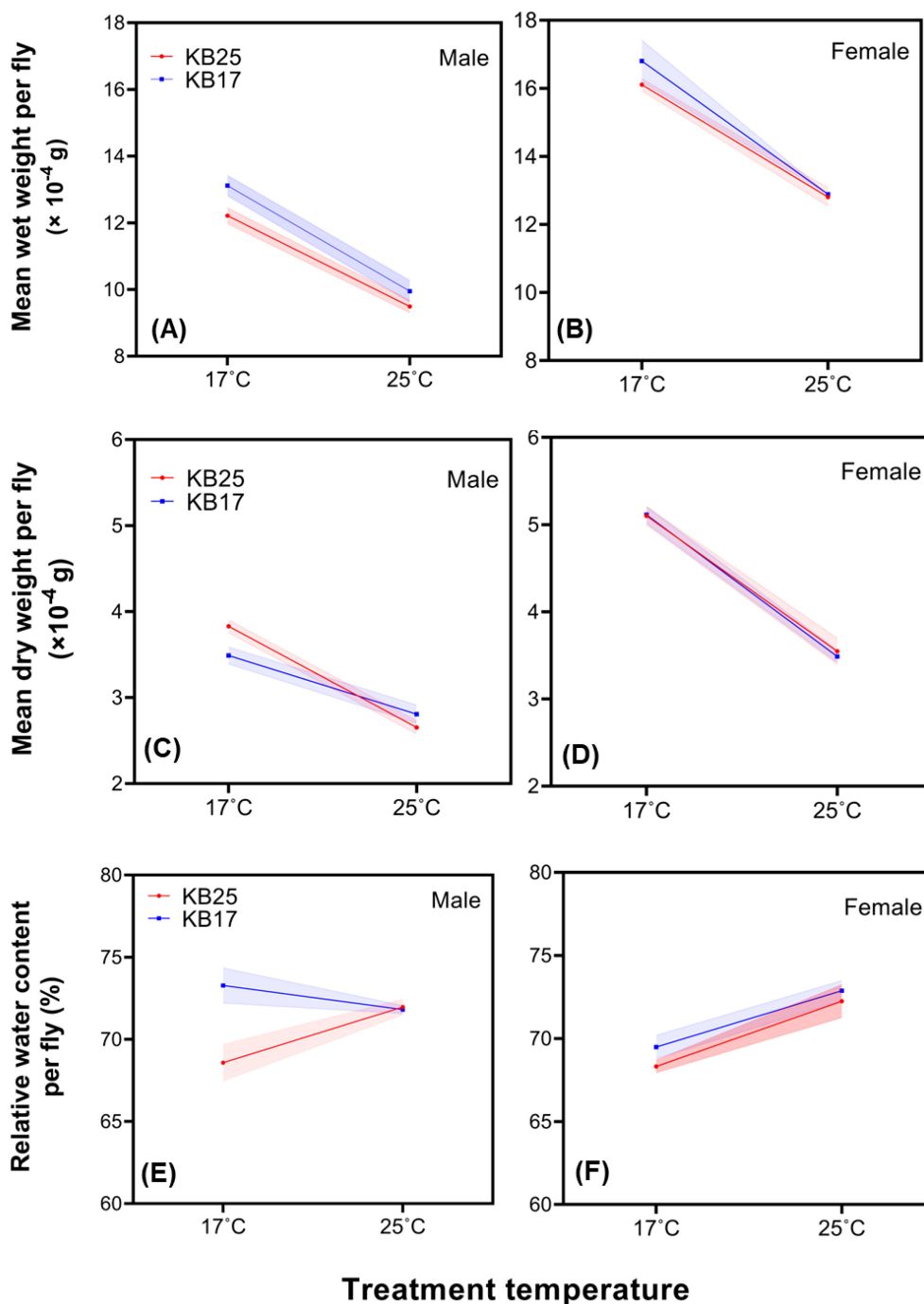


FIGURE 3

Mean wet weight of individual (A) male and (B) female fly at eclosion, mean dry of individual (C) male and (D) female fly at eclosion, and mean relative water content per (E) male and (F) female fly at eclosion, in KB25 and KB17 populations, reared at 25°C and 17°C. The error bars represent standard error of the three replicate population means.

KB17 population males had only 24% increase in dry weight in the same thermal range (Figures 3C, D).

Relative water content of the flies

For water content per unit wet weight of flies, the main effects of selection temperature and treatment temperature were significant ($p = 0.0031$ and $p = 0.0001$, respectively) (Supplementary Tables 3, 5; Figures 3E, F). The two-way interactions between selection temperature and treatment temperature and between sex and treatment temperature were significant ($p = 0.0083$, for both interactions) and so was the three-way interaction among selection, treatment temperature, and sex ($p = 0.0303$). KB25 males and females had significantly less water content (68%–69%) when reared at 17°C compared to 25°C (71%–72%) ($p < 0.05$). Similarly, KB17 females had significantly less water content (69%) when reared at 17°C compared to 25°C (73%) ($p < 0.05$), but KB17 males did not show a significant change in relative water content when reared at 17°C vs. 25°C (72%–73%) (Figures 3E, F). Consequently, the relative water content of KB17 and KB25 males was not significantly different at 25°C, but it was significantly higher in KB17 males than KB25 males at 17°C ($p < 0.05$).

Discussion

Evolution of fecundity

Cold temperature reduced the pooled fecundity of the two selection regimes, but the interaction of selection and treatment temperature revealed that the impact of cold treatment on the fecundity of warm- and cold-evolved flies was different. The control groups, i.e., the warm selection lines, suffered a significant decline in fecundity when assayed at cold temperature, in comparison to their fecundity in warmer conditions. In contrast, the cold selection lines did not suffer any significant reduction in fecundity at cold treatment temperature compared to the warm treatment. For *D. melanogaster* rearing, 25°C is the laboratory optimum, and the findings from our control populations show that 17°C represses their fecundity, as observed in numerous previous studies (Nunney and Cheung, 1997; Klepsatel et al., 2019; Molón et al., 2020). However, the cold-selected populations seem to have evolved the ability to withstand cold, such that cold temperature does not cause a significant reduction in their egg production. This clearly indicates that the cold-evolved flies have adapted to cold as a result of selection. The sustained fecundity of the cold-adapted flies transitioning from warm to cold environments, an attribute not observed in the warm-evolved controls, hints at an evolved ability to neutralize the cold's suppressive effect. There could be various possibilities, such as a) cold perception that potentially modulates egg production might have diverged in the two selection lines or cold tolerance might have improved in cold selected flies, and/or b) cold selection might have led to bigger flies such that an increased size buffers against the repressive effect of cold on fecundity, as body size and fecundity are positively correlated in flies (Pitnick, 1991;

Lefranc and Bundgaard, 2000; Flatt, 2020). There was a main effect of selection temperature on the wet weight of flies in our study, indicating that KB17 flies had evolved a higher wet weight compared to KB25. This could be one of the factors contributing to an increased fecundity of cold-evolved flies, while there could be possible alternative or additional mechanisms accounting for their thermally canalized fecundity. We also evaluated the progeny survivorship in a later generation (gen 78) and found no significant effect of selection, treatment temperature, or their interaction on the trait (Chattopadhyay et al., unpubl. data). This indicates that the different effects of cold temperature on the reproductive output of warm- and cold-selected populations were primarily exerted through an effect of cold on egg production and not through progeny survivorship.

Comparison of different thermal selection studies

Interestingly, one study conducted by the research group of Mauro Santos involved the selection of *Drosophila subobscura* populations at 13°C, 18°C, and 22°C for over 4 years. In this study, warm-adapted populations showed greater net fitness at all three test temperatures, while cold-adapted populations had low fitness in the warm environment (Santos, 2007). On the other hand, in a thermal selection study under laboratory conditions conducted by Linda Partridge's research group, cold-adapted populations of *D. melanogaster* lived longer and laid more eggs compared to warm-adapted flies, at their maintenance temperature, i.e., 18°C (Partridge et al., 1995). Similarly, the warm-adapted flies (25°C) lived longer and laid more eggs than warm-adapted flies when tested at 25°C. Thus, while our study suggests “colder is better,” Santos' work suggests “warmer is better,” and Partridge's findings indicate the existence of a trade-off between adaptation to cold vs. warm environments. The contrasting findings from the different studies suggest that thermal adaptation may manifest diverse evolutionary patterns as it can vary across populations and species.

Age-specific fecundity pattern

Apart from total fecundity, our study revealed a noticeable impact of treatment temperature on the age-specific pattern of fecundity. The distribution of fecundity along the age axis in wild-caught *Drosophila* and those maintained in the laboratory tends to be positively skewed. A triangular shape of the lifetime fecundity distribution characterized by an early peak is a typical feature of iteroparous insects (Dixon and Agarwala, 2002). This peak has been shown to be triggered by the onset of mating in flies (Modak, 2009). In our study, the typical early-life spike in fecundity was observed at 25°C treatment, which also coincides with the age of egg collection in our fly populations. Surprisingly, we did not observe such a spike at the 17°C treatment for either of the selection lines, which suggests that the early-life fecundity spike may not be canalized across conditions and can be absent in colder environments. When flies were reared and assayed at 25°C, fecundity was the highest during the first week, after which it

showed a gradual decline during weeks 2 and 3. At 17°C, in contrast, the pooled fecundity did not differ across 3 weeks, and the data rather show a somewhat moderate steady fecundity level at 17°C throughout the duration of the assay, which is different from the pattern observed at 25°C. These differences between the 25°C and 17°C treatments can be suitably explained by the lifespan of the flies at the said temperatures. Cold temperature increases the lifespan in flies, and a trade-off between lifespan and reproductive output is well-documented in evolutionary biology (reviewed in Prasad and Joshi, 2003). In conjunction with the existence of this trade-off, flies appear to maintain a relatively steady yet reduced egg production throughout their entire lifespan at cold temperature. In contrast, at warm temperature, flies lay a maximum number of eggs early in life which subsequently dwindles to a lesser egg output for the remaining part of their lifespan. The strategy at cold temperature seems to be conserving resources, facilitating a prolonged lifespan and an extended period of egg production. The energy needs to support a longer lifespan perhaps do not allow flies to exhaust too much resource early in life. The age-specific fecundity pattern, therefore, may correspond to the energy need of the flies to live and reproduce during their respective lifespans at warm vs. cold conditions. It was observed that this temperature-dependent age-wise fecundity pattern remained unaffected by selection. However, it is important to note that in our cold selection regime, neither a long lifespan nor a consistent egg output throughout the lifespan was relevant to fitness, because similar to KB25, eggs for the next generation were collected from KB17 populations on the 4th–7th day of adult life. It is possible that a somewhat flat pattern of fecundity as a plastic response to cold environmental temperature is hardwired in flies. Whether continued cold selection in which a) only early-life fecundity is favored and b) a longer lifespan is not relevant to fitness can alter this pattern can be potentially investigated in these populations in the future. It is important to note that some flies from the KB25-1 and KB25-3 populations approximately days 8–9 laid a large number of eggs that led to a smaller second peak in the daily fecundity pattern (Figure 2). In the MB populations and their ancestor JB, eggs were collected after 10–12 days of eclosion, which led to the evolution of a subsidiary peak at that age (Prasad and Joshi, 2003). We speculate that the small second peak observed in the KB25 populations could be a result of their evolutionary history. However, it was not found in KB17 populations.

Evolution and plasticity of body size and relative water content

Cold temperature of 17°C led to greater wet weight of flies both at plastic and evolutionary levels. Dry weight on the other hand was influenced only by the treatment temperature implying the plastic influence of cold temperature, but it remained unaffected by cold selection. Females being heavier than males under all conditions is expected and hence does not require much discussion. As revealed by the interactions of selection temperature, treatment temperature, and sex, KB17 males showed significantly less thermal plasticity of dry weight compared to KB17 females and KB25 flies. While cold

vs. warm selection did not alter wet weight plasticity, this indicates that cold selection however lowered the dry weight plasticity, albeit only in male flies. At present, it is not very clear why cold selection led to reduced plasticity of the dry weight, albeit only in the male flies. In future generations, investigating how body weight plasticity evolves further may reveal some more details about this trend. Male *Drosophila* take a longer time to develop, yet they are smaller and lighter than females. Flies become sexually mature and start mating within a few hours of eclosion, and it is suggested that the reproductive maturation of males takes more time than that of females, accounting for the longer development time of the former (reviewed in Prasad and Joshi, 2003). How the development time difference of the two sexes fares across the two selection regimes and treatments would be worth investigating, in order to gain more insight about the reduced size plasticity observed only in cold-evolved males.

Apart from the weights, the trend observed for relative water content was interesting. KB25 flies as well as KB17 females had a plastic reduction in relative body water content when developing in cold temperature. Some earlier studies suggest that a reduced body water content may be a sign of cold tolerance in insects in freezing temperatures as it may help to reduce the damage caused by water crystal formation (Worland, 1996). However, it is not clear why flies would have a lower water content at a tolerable cold temperature like 17°C than at a warmer temperature of 25°C. Given that both KB25 flies and KB17 females showed a small but significant reduction in relative water content when developing at 17°C, this could be common in *Drosophila*, but that KB17 males instead evolved the same body water percentage across 25°C and 17°C is surprising. Whether or not these flies have evolved a different metabolism, retaining more water at cold temperatures compared to the females of their population and the ancestral flies from which they have evolved, would be worth investigating in the future. Our work thus indicates that the cold-evolved males have diverged from the warm-evolved populations both in terms of thermal plasticity of dry weight and relative body water content.

To sum up, we show that while cold temperature suppresses the reproductive output of *D. melanogaster*, prolonged cold selection can lead to improved egg production in cold temperature, showing clear evidence of adaptation to the cold environment. The fecundity of the cold-evolved flies also remained unaffected under warm conditions. Therefore, compared to warm-adapted populations that suffer a significant decrease in egg production from warm to cold conditions, cold-adapted populations evolved fecundity levels that were less thermally plastic or more canalized. We speculate that the evolution of canalized or more consistent fecundity levels across environmental conditions can potentially aid in range expansion of a species. While for some traits phenotypic plasticity may aid in adaptation, for others, canalization might be more adaptive. In addition to fecundity, we found that male flies of cold-selected populations evolved reduced plasticity of dry body weight and relative body water content. Future studies exploring the connections between weight, body composition, temperature, and metabolism can potentially help us understand the significance of the evolution of low plasticity in these traits.

Data availability statement

The original contributions presented in the study are included in the article/[Supplementary Material](#). Further inquiries can be directed to the corresponding author.

Ethics statement

The manuscript presents research on animals that do not require ethical approval for their study.

Author contributions

RR: Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – review & editing. AC: Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – review & editing. SS: Investigation, Methodology, Writing – review & editing. AM: Investigation, Methodology, Writing – review & editing. PB: Investigation, Methodology, Writing – review & editing. SG: Investigation, Methodology, Writing – review & editing, Conceptualization, Data curation, Formal analysis, Funding acquisition, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft.

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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.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fevo.2024.1452948/full#supplementary-material>

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Water temperature modulates multidimensional plastic responses to water flow during the ontogeny of a neotropical fish (*Astyanax lacustris*, characiformes)

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Introduction: Plastic phenotypes result from multidimensional developmental systems responding to distinct yet simultaneous environmental signals, which may differently affect the magnitude and directions of plastic responses. Concomitant environmental signals during development may result in dominant, synergistic, or even antagonistic phenotypic effects, so that a given condition may amplify or minimize plastic responses to other environmental stimuli. Knowledge on how external information shapes complex plastic phenotypes is essential to predict potential evolutionary trajectories driven by developmental plasticity.

Methods: Here, we manipulate water temperature to evaluate its effects on the well-described phenotypic accommodation of fish growth in the presence of water flow, using the neotropical species *Astyanax lacustris*. We include larval and juvenile ontogenetic stages to examine the interaction between these two environmental signals in plastic responses related to body size and shape, skeleton ossification and gene expression, using *bmp4* as a proxy for ossification pathways.

Results and discussion: Our results demonstrate that water temperature plays a crucial role determining the expression of plastic variation at all dimensions, and effects of water flow were restricted to specific thermal regimes. Combination of high temperature and water flow has a major effect on body shape and unveils unique phenotypic patterns, supporting the prediction that high temperatures can amplify plastic responses to external signals. Specifically, fish raised in the presence of water flow at warmer environments grew faster and ossified earlier, and this condition increased *bmp4* expression levels especially at later developmental stages. Such plastic phenotypes likely involve a functional relationship with swimming performance in running-water environments. Our findings highlight the importance of studying developmental plasticity in complex environments using a multidimensional approach, especially considering increments in water temperatures due to

accelerated climate changes that likely impact the fish developmental potential to mitigate environmental changes through plastic responses.

KEYWORDS

BMP4, body shape, body size, ossification, phenotype, plasticity

Introduction

Developmental systems integrate responses to genetic and/or environmental inputs in several dimensions to produce well-suited organisms that are able to survive and reproduce in specific ecological settings (West-Eberhard, 2005; Badyaev, 2009; Duclos et al., 2019). These dimensions interact among each other, and occur both along vertical (e.g., molecules-cells-tissue-organ) and horizontal (i.e., communication among different trajectories and systems within each vertical level) axes (see Duclos et al., 2019). External environmental signals may affect each of these developmental dimensions, shaping the phenotypic outcomes in the ontogenetic landscape along with genes (Duclos et al., 2019). This phenomenon is termed developmental plasticity and designates changes that are frequently permanent in anatomical, physiological and behavioral phenotypes induced by the external environment (West-Eberhard, 2005; Bonini-Campos et al., 2019; Pfennig, 2021; Lofeu et al., 2021; Lofeu et al., 2024). Although developmental plasticity often produces irreversible changes or at least biases subsequent ontogenetic trajectories (West-Eberhard, 2005; Forsman, 2015; Ledón-Rettig and Ragsdale, 2021), literature also recognizes other types of phenotypic plasticity involving responses that may be reversible (Forsman, 2015). Adaptive developmental plasticity allows organisms to handle environmental perturbations by producing functional phenotypes (phenotypic accommodation, see West-Eberhard (2005), which enables population persistence in changing environments (Diamond et al., 2021) and also facilitates adaptive evolution (Hagen, 2008; Hendry, 2016; Uller et al., 2020). Moreover, new complex phenotypes may be revealed by developmental plasticity, which often results in a quick burst of phenotypic diversification (Pfennig and McGee, 2010; Lofeu et al., 2021; Levis and Pfennig, 2021).

Natural environments concomitantly encompass several signals, which may sometimes act in opposite directions in developmental plastic responses (e.g., Kasumovic, 2013; Chevin and Lande, 2015; Groothuis and Taborsky, 2015; Reyes Corral and Aguirre, 2019; Mohanasundaram and Pandey, 2022; Lock et al., 2024). In contrast, some environmental signals seem to amplify the plastic responses to a given stimulus (e.g., Kleiven, et al., 1992; Langerhans et al., 2007; Kasumovic, 2013; Chevin and Lande, 2015; Groothuis and Taborsky, 2015; Lock et al., 2024). Identification of interaction effects among environmental signals presume experimental designs that combine different conditions, although more often studies manipulate one stimulus at a time. For example, in aquatic organisms such as fishes, both temperature and water flow influence development at several dimensions, including gene expression, growth, differentiation and behavior (temperature: Sfakianakis et al., 2004; Witten and Hall, 2015; Riera-Heredia et al., 2018; Shuai et al., 2018; Reyes Corral and Aguirre, 2019; Han et al., 2020; Fey and Greszkiewicz, 2021; Kourkouta et al., 2021; water-flow; Grünbaum et al., 2007; Langerhans, Chapman and

DeWitt, 2007; Fischer-Rousseau, Chu and Cloutier, 2010; Fiaz et al., 2012; Witten and Hall, 2015; Kelley et al., 2017; Shuai et al., 2018; Reyes Corral and Aguirre, 2019; Kourkouta et al., 2021). Effects of water flow on fish development are particularly well understood (Langerhans, 2008), although it remains unknown if temperature modulates some of these effects. Water flow often accelerates growth, muscle development (Langerhans, 2008), and chondrogenesis and osteogenesis (Grünbaum et al., 2007; Cloutier et al., 2010; Fiaz et al., 2012), and also induces more streamlined body shapes (Grünbaum et al., 2007; Langerhans, 2008; Reyes Corral and Aguirre, 2019). These plastic responses likely enhance swimming performance in current aquatic environments (Grünbaum et al., 2007; Fiaz et al., 2012), and therefore can be interpreted as phenotypic accommodation (West-Eberhard, 2005). Plastic responses to temperature are also well-known during fish development, and thermal regimes may influence both growth rates and ossification sequences of specific bone sets (see Mabee et al., 2000). Given that changes in water flow might occur in rivers experiencing different thermal regimes (Shuai et al., 2018), temperature effects may overlap and interact with water flow during fish development in three possible outcomes: i) both signals act together, and their interaction induces new responses not observed by only one signal acting individually; ii) one signal has a dominant effect over the other and explains most of the expressed variation; iii) one signal reduces the magnitude of effects of the other. For example, effects of temperature and water flow would be opposite if low temperatures decelerates ossification processes (see Cordova-de la Cruz et al., 2022) while water flow accelerates them (see Fiaz et al., 2012). This complex pattern in the expressed phenotypic variation results from the multidimensional nature of developmental processes along the vertical and horizontal axes described by Duclos et al. (2019). The present study innovates by using an integrative approach in which we evaluate how thermal regimes modulate plastic responses to water flow in several phenotypic traits known to be influenced by both variables. As aforementioned, studies addressing developmental plasticity usually investigate isolated effects of such environmental signals, and here we aim to fill this gap by understanding how effects of one signal (temperature) can influence developmental plastic responses to another signal (water flow).

The patterns of plastic responses to specific environmental signals have been described for several fish species (Machado-Schiaffino et al., 2014; Kelley et al., 2017; Härer et al., 2017; O'Dea et al., 2019; Gilbert et al., 2023), but most studies evaluated environmental effects separately, while in nature we expect many signals simultaneously affecting different developmental dimensions. For example, combinations of environmental signals produce different head morphotypes in *M. macrocephalus* fish (Lofeu et al., 2021), and plastic phenotypes encompass developmental changes in several dimensions (Lofeu et al., 2024). In that fish species, some environmental signals seem to have stronger effects in the plastic variation revealed (Lofeu et al., 2021; Lofeu et al., 2024),

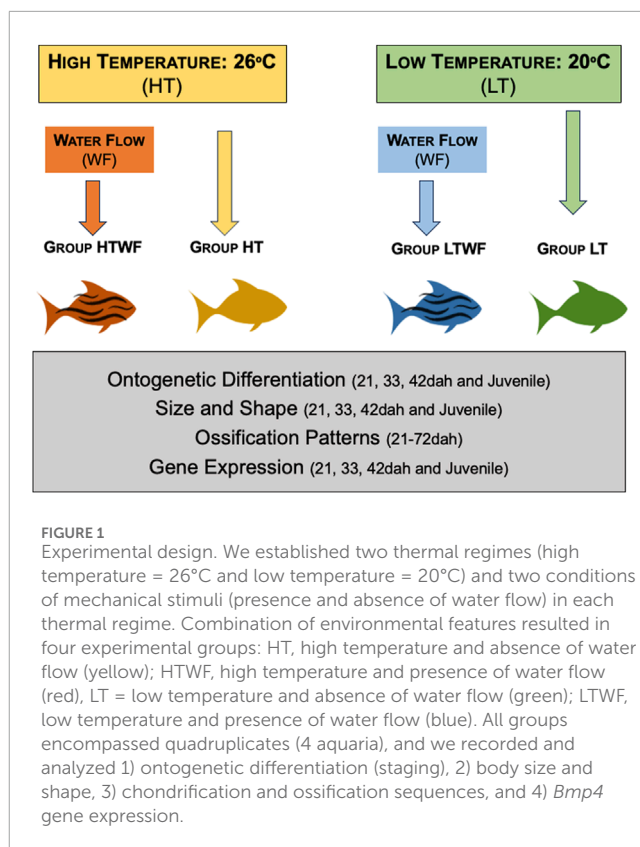
and we can expect that such effects may also vary along ontogeny. The present study aims to provide a multidimensional perspective about interaction effects of environmental signals on developmental plasticity. Specifically, we investigate the effect of water temperature on plastic responses induced by the presence of water flow, focusing on body size and shape, skeleton ossification, and gene expression during larval and juvenile development of the neotropical fish *Astyanax lacustris* (Characiformes). Several characteristics turn this species an ideal biological system for studies addressing developmental plasticity: these fishes are common for aquarium hobby, as they are small and easy to maintain in captivity; they grow fast and quickly reach sexual maturity, and eggs can be obtained through the year. In addition, previous studies described adaptations to flowing water environments in this species and reported phenotypic plasticity in other species of *Astyanax* (see Costa-Pereira et al., 2016; de França et al., 2024; Reyes Corral and Aguirre, 2019). We manipulated the presence of water flow at two thermal regimes (high temperature = 26°C; low temperature = 20°C) to establish four experimental developmental environments, and quantified plastic responses at different dimensions: 1) growth and differentiation, 2) ossification, 3) gene expression (Figure 1). For this later dimension, we used *bmp4* as a proxy (i.e., an indicator) for ossification pathways, based on the extensive literature describing participation of this gene in processes of bone differentiation (e.g., Ahi, 2016; Li et al., 2021; Wu et al., 2024) and also responses to mechanical stress (e.g., Ikegame et al., 2016; Jang et al., 2016; Dayawansa et al., 2022; Lofeu et al., 2024). Different expression levels of *bmp4* have been identified in plastic responses of fish raised in different developmental environments (Lofeu et al., 2024), which turns this gene an ideal candidate to be a proxy of cellular processes modulating ossification rates in specific environmental conditions. We hypothesize that plastic responses to water flow will be magnified at warmer thermal regimes (see O'Dea et al., 2019). Understanding how water temperature modulates plastic responses to mechanical stimulus is particularly relevant considering accelerated effects of climate changes warming up the aquatic environments (Czernecki and Ptak, 2018).

Methods

In this study, we aimed to investigate temperature effects in plastic responses triggered by water flow, evaluating multiple interconnected levels: ontogenetic differentiation (staging), growth and size, body shape, ossification rates and gene expression (*bmp4*). As described in introduction, these levels express well-known plastic responses to water flow and also are affected by temperature. Despite being interconnected, we analyze each level as a separated unity, and applied to each dimension the most appropriate methods, considering also specific methodological restrictions for each set of traits.

Animals and experimental design

We established four experimental developmental conditions that combined presence/absence of water flow (0.10 m/s for 6 h every day) with two thermal regimes (high temperature = 26°C and low temperature = 20°C): 1) no water flow at high temperature (HT); 2) presence of water flow at high temperature (HTWF); 3) no water



flow at low temperature (LT); and 4) presence of water flow at low temperature (LTWF). The temperatures used correspond to extremes in the thermal range tolerated by this species (Santos et al., 2016; dos Santos et al., 2020), and an interval of six degrees Celsius is sufficient to modify developmental rates of most biological levels investigated here (Sfakianakis et al., 2004; Sfakianakis et al., 2011; Reyes Corral and Aguirre, 2019; Han et al., 2020; Clarkson et al., 2021; Kourkouta et al., 2021). Each experimental condition was assembled in quadruplicate (see detailed description in Supplementary Methods and Supplementary Table S1). We used the species *Astyanax lacustris* as study system. Larvae were acquired from the National Center of Research and Conservation of Continental fishes - CEPTA/ICMBio, Pirassununga, São Paulo, Brazil (Coordinates 21°56'02.8"S 47°22'21.4"W°), a governmental institution established as a genetic bank for conservation of Brazilian fish species. We randomly sampled 1200 larvae that hatched from 600.000 eggs resulting from a mating matrix of 64 females and 128 males (hatching rate of approximately 70%), using a reproductive design that ensured genetic variation similar to wild populations of *A. lacustris*. Twelve days-old larvae (=12 days after hatching, hereafter referred to as 12 dah) were sampled, transported to the lab at University of São Paulo in Ribeirão Preto, São Paulo, Brazil, and acclimatized for 5 days in the same water from the CEPTA at 23°C and constant aeration. We chose to use 12 dah larvae because this stage is the subsequent window after the critical first days of eclosion characterized by high mortality in this species and the individuals were still in the middle of the preflexion stage, which precedes the developmental window analyzed here. After acclimation time, larvae were distributed in quadruplicates

(i.e., four tanks) in each of the four experimental developmental conditions (75 fish per tank, four tanks for each condition, total of 16 tanks). To equally comprise ontogeny in all experimental conditions, we standardized sampling events to occur every 3 days along the entire larval development period up to the juvenile stage. Specifically, at each 3-day interval we randomly collected six specimens from each aquarium (see [Supplementary Table S1](#)), in a total of 24 individuals for each experimental condition in each sampling event. We opted to use time in days to standardize sampling events and establish comparison points because this approach avoids bias from metrics like size and ontogenetic stage, which are among the response traits we focus in this study. In addition, strict boundaries among stages are not always clearly demarcated by phenotypic traits in *A. lacustris*, especially within the flexion stage (see [Supplementary Methods](#)). By using this approach, we could evaluate which traits differed among experimental groups after fish were maintained for 9 (21dah), 21 (33dah), 30 (42dah) or 60 (72dah) under specific environmental conditions (see further details). Immediately after sampling, we euthanized the animals using an overdose of lidocaine anesthetic solution (1 g/L of water). Two specimens from each aquarium (total of 8 specimens for each experimental group) were stored in RNAlater (Sigma-Aldrich R0901 – Missouri, EUA), and the remaining individuals were fixed in 10% formaldehyde and stored in ethanol 70% for morphological and osteological analyses. The experiments were continued until individuals in all treatments reached the juvenile stage, which happened 60 days after we distributed fish in different environmental conditions. Therefore, we established comparisons based on days after hatching along the experiments, but also compared plastic responses among fish that were at the same stage in the end of the experiments (juveniles). A detailed description is provided in the [Supplementary Methods](#). All procedures were approved by the Ethics Committee for the use of Animals of the Faculty of Philosophy, Sciences and Letters of University of São Paulo in Ribeirão Preto (Protocol #2018.5.310.59.6).

We photographed the specimens in right lateral view by positioning each individual in a petri dish using a brush, using scale and a digital image capture system by a LEICA DMC6200 camera coupled to a LEICA M205 FA stereomicroscope. Pictures were used for ontogenetic staging and also linear and morphometric analyses. Then, we chose three specific sampling time points to better analyze the influence of experimental conditions on ontogenetic differentiation, growth and body shape - 21, 33 and 42 days after hatching, which respectively represented early, middle and late time points of the experiment (~10 days of difference between each interval) - and defined the juvenile stage as a final point of our analyses (approximately 51dah, depending on the experimental group).

Ontogenetic staging

To evaluate possible effects of developmental conditions on ontogenetic differentiation (i.e., the rate different ontogenetic stages are established), we staged the specimens sampled from each experimental group following [Santos et al. \(2016\)](#), which describes phenotypic characteristics that separate ontogenetic stages for embryonic, larval and juvenile development in *A. lacustris*.

Based on ontogenetic descriptions from [Santos et al. \(2016\)](#), we identified three developmental stages in our sampling: flexion larvae, postflexion larvae, and juvenile. The flexion larvae stage can be subdivided into “early” and “late” according to larvae traits (see [Supplementary Methods](#)).

Body size and shape

We investigated environmentally-induced variation on growth rate and size using linear and geometrics morphometry, and the last was also used to evaluate shape variation (morphospace analysis). For each collection time point (21, 33 and 42dah) we used 16 individuals, except for the juvenile stage, in which we used 64 individuals by group because this was the end of the experiment. Therefore, in addition to the comparisons based on days after hatching along the experiments, we also compared plastic responses among fish that were at the same stage in the end of the experiments (juveniles). Linear measurements of total Body Length (BL) were obtained using ImageJ software version 1.54a ([Supplementary Figure S1A](#)). To test for group differences in the average size during ontogeny, we log10 transformed the body length values and performed ANOVA corrected by a Bonferroni post-hoc test.

The same photographed specimens were used for geometric morphometrics analysis. As aforementioned, we based our comparisons on days after hatching, instead of ontogenetic stages. Given that high temperatures accelerate growth ([Mabee et al., 2000](#); see also our results), prior to our shape analyses we performed linear regressions between body height and body length, in order to evaluate if temperature during development affected allometric relationships (see [Supplementary Methods](#)). Slopes of allometric relationships did not differ between fish from high and low temperatures raised in the absence of water flow, and slopes were also not different between thermal regimes in fish raised in the presence of water flow (see [Supplementary Methods](#)), so we applied morphometric geometrics removing size effects using the pooled-within group approach described in [Klingenberg \(2016\)](#), which is widely applied in studies comparing different species, experimental groups and sex ([Sidlauskas et al., 2011](#); [Reyes Corrol and Aguirre, 2019](#); [DeLorenzo et al., 2023](#); [Hetzel and Forsythe, 2023](#); [Pan et al., 2025](#)). We used the software TPSdig version 3.2 ([Rohlf, 2005](#)) to position 14 landmarks over homologous points in the larval stages and 17 landmarks in the juvenile stage (anatomic points where landmarks were placed are described in the [Supplementary Figures S1B,S1C](#)). We then exported landmark data to the MorphoJ software ([Klingenberg, 2011](#)) and performed Procrustes superimposition (“full Procrustes fit”) to remove position, scale (size) and rotation effects, remaining only the shape information that was our trait of interest (see [Klingenberg, 2010](#); [Webster and Sheets, 2010](#)). To further remove any remaining allometric effect, we regressed Superimposition-adjusted Procrustes against centroid size, and used the Procrustes residuals for all shape analyses. We implemented these two steps to have confidence that all possible confounding effects of body size were eliminated and only shape information was accessed in the morphospace analysis. A Canonical Variate Analysis was performed to evaluate the morphospace as a function of developmental

conditions during ontogenetic windows; using this analysis, we can observe how groups are distributed in the morphospace and which environmental factor explains most of the variation. To test for statistical differences among groups in the morphospace distribution, we performed a one-way ANOVA followed by Bonferroni post-hoc test using the CV scores generated from the Canonical Variate Analysis. We also ran a multivariate regression of Procrustes coordinates (body shape) as a function of centroid size (body size) to evaluate growth rates among experimental groups. These statistical analyses and graphic results were performed in the Prism Software (Swift, 1997). Finally, to test if average shape differs among experimental groups after developing for 61 days in distinct environments and represents new morphotypes, we used the Procrustes coordinates in a Permutational Multivariate Analysis of Variance using Distance Matrices (NPERMANOVA) followed by a Bonferroni *post hoc* test (see Anderson, 2014; Lofeu et al., 2021); this analysis was implemented in the Past Software (Hammer et al., 2001). Similar approaches have been used in previous studies addressing plastic responses in fish to environmental signals manipulated in the lab (Bonini-Campos et al., 2019; Lofeu et al., 2021; Lofeu et al., 2024).

Ossification patterns of skeletal elements

We evaluated ossification patterns of post-cranium skeletal elements focusing on the time of ossification and the element identity. Specifically, three specimens from each sampling event (three-days intervals along the entire experiment, total of 18 sampling events, see [Supplementary Table S1](#)) in each experimental group were stained with Alcian Blue and Alizarin Red, which colors cartilaginous structures in blue and bone structures in red respectively (protocol adapted from Taylor (1985); see [Supplementary Methods](#)). Using a digital image capture system in a LEICA DMC6200 camera coupled to a LEICA M205 FA stereomicroscope, we determined the emergence and ossification status of post-cranium skeletal elements as follows: if the element was blue-stained it was classified as cartilaginous, if it was red-stained it was classified as completely ossified. We focused on emergence, chondrification and ossification of the following bone structures: pectoral, caudal, anal, dorsal and pelvic fins, and Weber Apparatus, Vertebral and Supraneural Centers ([Supplementary Figure S2](#)).

Gene expression: *bmp4*

To measure expression levels of *bmp4* (Bone Morphogenetic Protein 4), we preserved fresh samples of post-cranium and caudal fin skeleton in RNAlater (Sigma-Aldrich R0901; Missouri, EUA) and stored at -80°C . We evaluated *bmp4* expression in two ontogenetic windows: 21 and 33dah, respectively representing initial and middle points of the experiments. Larvae at these stages are very small and have low amount of tissue, so we established a pool of two individuals as a unity biological sample. We used three biological samples (each one corresponding to a pool of two individuals) from each experimental group at each ontogenetic stage. Each biological sample was divided into technical triplicates,

establishing a total of 9 samples for each group at each ontogenetic stage. The bone structures corresponding to the post-cranium were dissected, mixed, and stored in Trizol (TRI Reagent® RNA Isolation Reagent Sigma Aldrich) for RNA preservation and tissue digestion. We used individuals with similar size and adjusted tissue volume to avoid effects of different volumes between samples. Total RNA was extracted following Trizol RNA-extraction protocol from Invitrogen (T9424; Massachusetts, EUA), and quantified using a Spectrophotometer (NanoVue Plus® Biochrom). The extracted RNA was treated with Invitrogen® Dnase I Kit to remove eventual remaining DNA. Finally, we synthesized cDNA from RNA aliquots (Reverse Transcription) using the Invitrogen® SuperScript® III Kit (Invitrogen 18080051; Massachusetts, EUA).

Expression of *Bmp4* was quantified using real-time PCR (qPCR) and GAPDH as endogenous control. We used primers for *bmp4* and GAPDH from the literature (Casadei et al., 2011; Gross et al., 2016). The qPCR assays were performed using SYBR Green PCR Master Mix (Applied Biosystems 4309155; Massachusetts, EUA) to the final reaction volume of 10 μL and the following cycle conditions: 2 min at 50°C , 10 min at 95°C , followed by 40 cycles of 15 s at 95°C and 1 min at 60°C . All trials were performed in the AriaMx® from Agilent Technologies®.

We analyzed qPCR results using the delta $\Delta\Delta\text{CT}$ method, based on Comparative Cycle Thresholding (qCT). Prior to the analyses, we performed an Efficiency (E) test for qPCR experiments, and obtained the following values: *Bmp4* – E = 0.962 (96.2%), Slope = -3.400 ; GAPDH–E = 0.981 (98.1%), Slope = -3.282 . Data were normalized by log10 transformation. We then calculated the average values of technical triplicates for each biological sample, using 3 biological samples for each experimental group. A one-way ANOVA with Bonferroni post-hoc test was performed to test for differences in quantitative expression among experimental groups. Changes on *bmp4* expression levels as a function of days after hatching was tested using a linear regression (expression levels as response trait and days after hatching as the independent variable). All these analyses were performed in Prism software (Swift, 1997).

Results

Temperature modulates the effects of water flow on growth and differentiation

The presence of water flow accelerated ontogenetic differentiation within each thermal regime (low or high temperatures), but the major factor affecting ontogenetic differentiation was temperature ([Figure 2A](#)). At high temperature (HT and HTWF groups) fish already exhibited traits of late flexion larvae 21dah ([Supplementary Methods](#)), in addition to better-differentiated eyes, more pigmented body, better developed fins and head when compared to individuals raised at low temperatures, which developed slower regardless of water flow and still resembled early flexion larvae at this sampling event ([Figure 2A](#); see also [Supplementary Methods](#)). The same pattern was observed at 33dah: LT and LTWF fish exhibited phenotypic traits of previous flexion substages when compared to HT and HTWF ([Figure 2A](#); see also [Supplementary Methods](#)). At 42dah, fish raised at high temperature already exhibited all the features

of the postflexion stage: swimming bladder, notochord no longer visible, spread body pigmentation, scales appearing along the body, advanced development of pectoral fins and fin rays, among others (Figure 2A; see also [Supplementary Methods](#)). In this condition (i.e., high temperature) it was possible to observe fully-developed juveniles at 51dah, while fish from low temperatures did not reach the juvenile stage until later than 57dah in the LTWF group and later than 60dah in the LT group. Indeed, some individuals raised at low temperatures even did not show phenotypes from late flexion larvae yet at 51dah.

We also observed larger allometric differences between thermal regimes when comparing fish raised in developmental environments with constant water flow, especially because these animals were significantly bigger along larval development when compared to those developed in the absence of water flow (Figure 2B; [Supplementary Table S2](#); [Supplementary Figure S3](#)). This observation suggests additive effects on growth between water flow and high temperature, as fish grew faster in warmer environments and water flow also stimulated this process. As a result of this interaction, we identified two contrasts clearly established at 42dah (Figure 2B; [Supplementary Table S3](#); [Supplementary Figure S3](#)), which remained during the juvenile stage: 1) fish raised at high temperature (HT and HTWF) significantly larger than those from low temperature (LT and LTWF); 2) fish from water flow conditions (HTWF and LTWF) significantly larger than those raised in the absence of water flow (HT and LT). The multivariate regression of Procrustes coordinates by centroid size confirmed that temperature influenced growth rate, and fish raised at high temperature grew faster than those from low temperature conditions (Figure 2C; [Supplementary Table S4](#)).

Temperature enhances shape responses to water flow and reveals a new morphotype

The Canonical Variate analysis revealed complex changes in body shape integrating water flow and temperature in two Principal axes (CV 1 and CV 2): in the Canonical Variate 1 (CV1), we detected a major effect of temperature explaining 45% of variation in body shape at 21dah, 71.20% of variation at 33dah, 78.66% at 42dah, and 67.22% at the juvenile stage (Figure 3A; [Supplementary Figures S4A,C,E](#); [Supplementary Table S5](#)). In the Canonical Variate 2 (CV2), we detected a major effect of water flow in group positioning (positive and negative values in Figure 3B; [Supplementary Figures S4B,D,F](#); see also [Supplementary Table S5](#)). The CV2 explained 36.54% of shape variation at 21dah, 17.39% at 33dah, 12.25% at 42dah, and 22.28% at the juvenile stage. Interestingly, variation explained by water flow decreased in flexion stages of larval development, from 36.54% at 21dah to 12.25% at 42dah, but then increased back to 22.28% at the juvenile stage, which suggests that effects of water flow on body shape increase during the ontogeny of *A. lacustris*.

Temperature effects on body shape were mostly related to body height (i.e., more robust and shorter bodies that are dorsal-ventrally enlarged) in fish raised at high temperatures (HT and HTWF, Figure 3A), while we observed thinner and more elongated bodies that were dorsal-ventrally flattened in fish raised at low temperatures (LT and LTWF, Figure 3A). We also observed that

fish from the in HT and HTWF groups were characterized by wider heads that were shortened in the front and had an upturned mouth. At the juvenile stage, fins were positioned closer to the operculum in fish from the LT than in HT groups, suggesting differences in ventral width induced by temperature (Figure 3A). The Canonical Variate 2 was mostly related to water flow and expressed patterns in body shape directly related to swimming function in flow environments (Grünbaum et al., 2007; Langerhans, 2008; Reyes Corral and Aguirre, 2019) that seem exclusive to fish raised in the presence of water flow (HTWF and LTWF conditions). These patterns were already noticeable at 33dah in fish raised from these groups (HTWF and LTWF): a streamlined body, wider dorsal fins, overall more robust and straightforward caudal peduncle, and an upturned rostrum ([Supplementary Figures S4D,F](#)). These patterns were even more prominent at the juvenile stage, when fish from the HTWF and LTWF conditions exhibited the typical shape observed in water-current environments: a hydrodynamic streamlined body that is thinner and more fusiform when compared to fish raised in the absence of water flow (HT and LT conditions, see the negative axis at Figure 3B). Moreover, we observed that fish from the HTWF and LTWF groups exhibited a fin shape that is commonly induced as a function of swimming in flow environments, expressing a phenotype characterized by adipose and dorsal fins that are wider (negative axis in Figure 3B). Finally, our results suggest that high temperature promotes shape diversification by enhancing plastic responses to water flow: in high temperatures, fish from the HTWF condition differ from those from HT concerning average shape, while in low temperatures, the presence of water flow is not sufficient to differentiate fish from the LT and LTWF groups regarding average shape (Table 1; Figure 3C). Therefore, interaction between high temperature and presence of water flow induces expression of a new body morphotype that was not observed in environments without water flow (HT group). Comparing shape differences among all groups, it is evident that complex environmental interactions amplified the divergence among fish morphotypes (Table 1; Figure 3C).

Emergence and ossification patterns of post-cranium skeletal elements are affected by temperature and water flow

Overall, chondrification and ossification of most skeletal elements was anticipated in fish raised in higher temperature when compared to those from lower temperature conditions. Interaction of water flow with high temperature (HTWF) resulted in the fastest rates of osteological development, suggesting an additive effect (Figure 4). Low temperatures decelerated ossification rates, but the presence of water flow accelerated ossification of specific elements in this condition, suggesting possible interactions in opposite directions when temperatures are low thermal. Sequence and ossification patterns differed among bone complexes, as further detailed (see also Figure 4; [Supplementary Figures S5,S6](#)).

- i) Pectoral fin (Figure 4A): the first element to chondrify and ossify in the pectoral fin was the cleithrum (cle), which was completely ossified in all experimental groups at 21dah. The pectoral girdle (ptg), which connects the scapula and coracoid,

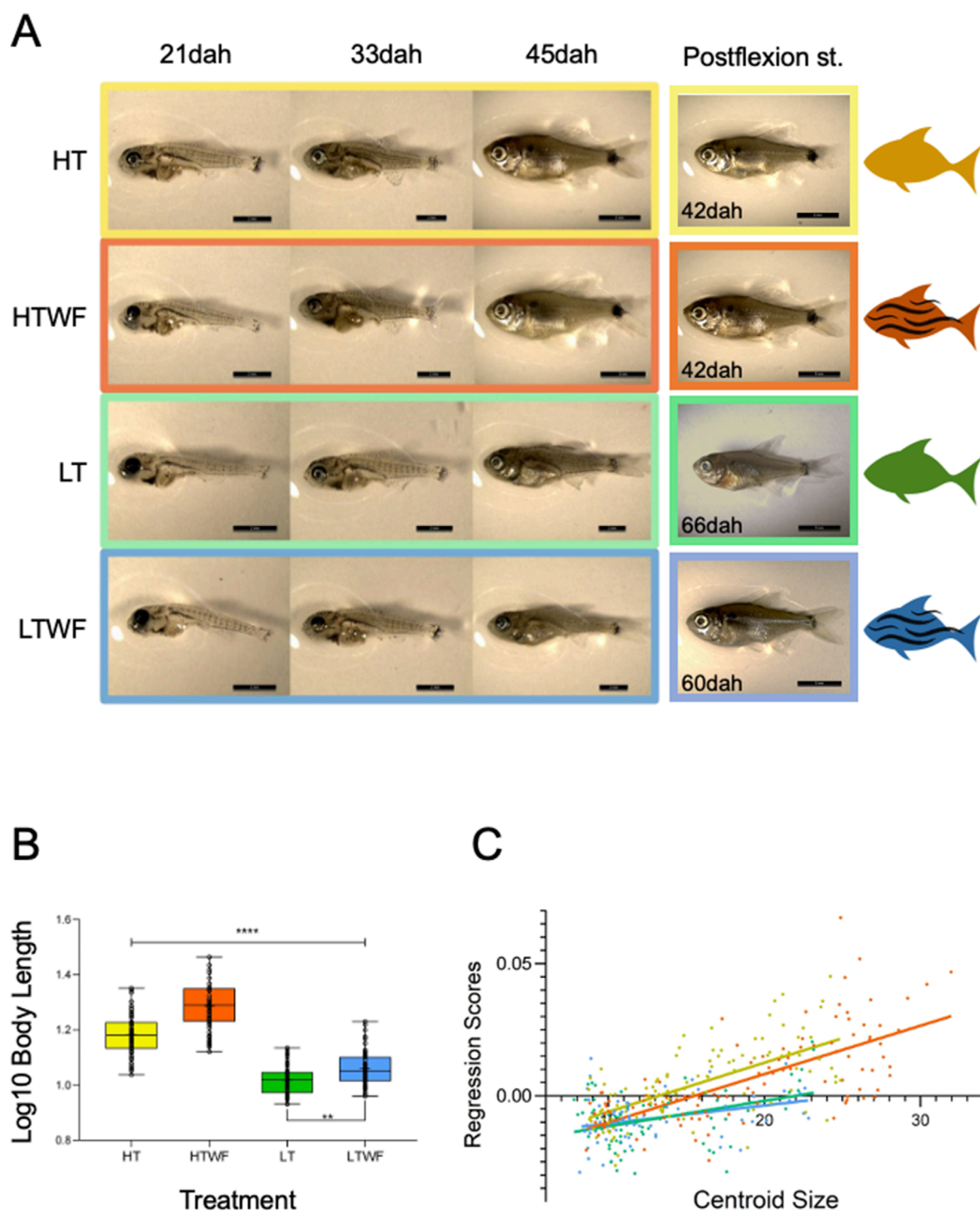


Fig. 2

FIGURE 2

Temperature and water flow affect ontogenetic differentiation and growth. (A) Fish phenotypes sampled at 21, 33 and 42dah in each experimental group, and fish phenotypes at the postflexion stage (HT and HTWF fish reached this stage at 42dah, LT reached this stage at 66dah, LTWF reached this stage at 60dah). (B) Group differences in Body Length (log10 transformed values, p -values HT-HTWF/HT-LT/HT-LTWF/HTWF-LT/HTWF-LTWF (****) < 0.0001 , LT-LTWF (**) = 0.007). (C) Multivariate regression of Procrustes coordinates by Centroid Size (HT: Rsquared = 0.421, $F = 77.25$, p -value < 0.0001 . HTWF: Rsquared = 0.488, $F = 101.10$, p -value < 0.0001 . LT: Rsquared = 0.164, $F = 13.99$, p -value < 0.0004 . LTWF: Rsquared = 0.113, $F = 9.436$, p -value < 0.003). Color codes according to experimental groups: yellow = HT, red = HTWF, green = LT, blue = LTWF.

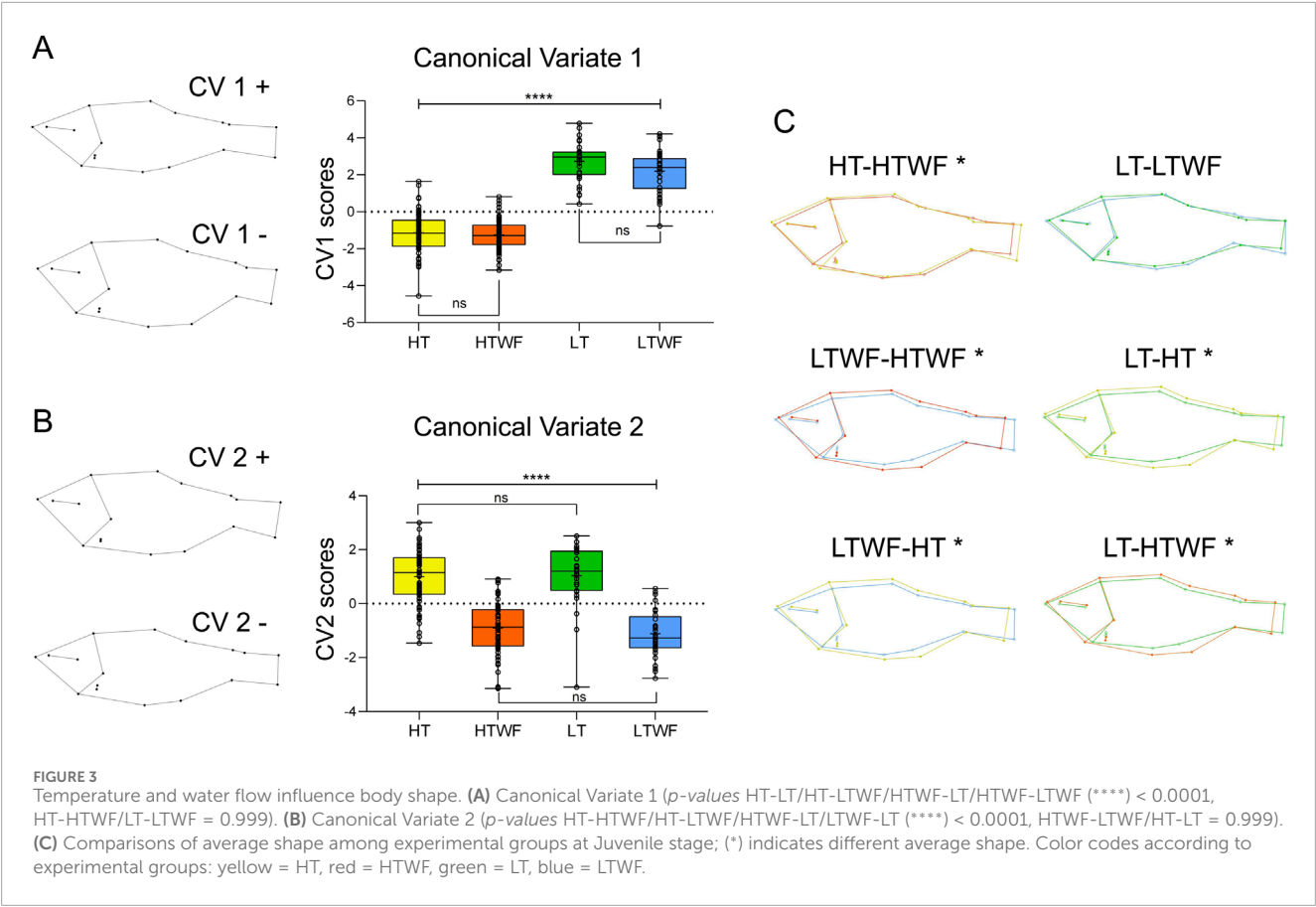


TABLE 1 NPERMANOVA results testing average shape difference between experimental groups at juvenile stage.

NPERMANOVA				
Permutations	Total sum of squares	Within-group sum of squares	<i>F</i>	<i>p</i> -value
N = 9,999	0.4022	0.3295	13.89	0.0001
Pairwise comparisons: bonferroni-corrected <i>P</i> -values				
	HT	HTWF	LT	LTWF
HT	-	0.0006	0.0006	0.0006
HTWF	0.0006	-	0.0006	0.0006
LT	0.0006	0.0006	-	0.2532
LTWF	0.0006	0.0006	0.2532	-

HT, high temperature, HTWF, high temperature + water flow, LT, low temperature, LTWF, low temperature + water flow. Significant values ($p < 0.05$) highlighted in bold.

began to chondrify before 21dah, and was completely ossified by 30dah in fish raised in the HTWF condition, and by 36dah in fish from the HT group. In fish that developed at low temperature (LT and LTWF groups), the pectoral girdle did not ossify before 72dah. The pectoral fin rays (rfpt) appeared by 33dah in fish raised at high temperature, and at 65dah in those developed at low temperature. In low temperature, these structures ossified earlier in the group developed in the

presence of water flow (LTWF). The distal pectoral fin radials (rdpt) ossified by 48dah at high temperature, but did not ossify before 72dah at low temperature. Unexpectedly, this structure began to ossify earlier in fish from the group HT than in those from HTWF.

ii) Caudal fin (Figure 4B): The main rays (rm) were the first structure to ossify in the caudal fin. The ural and pre-ural centers (cpu1) ossified first in groups submitted to water flow

in both thermal regimes. The same pattern was observed for the dorsal procurent rays (dpcr), which ossified 10 days earlier in HTWF than in HT group, while the difference in the low temperature groups (LTWF and LT) was approximately 3 days between them. Development of the neural arches of pre-ural centers 2 and 3 (napu23) did not differ between fish raised in the presence or absence of water flow, but these structures ossified near 25dah in fish from high temperature treatments and only by 33dah in those raised at low temperature. We observed a clear interaction between temperature and water flow in the hemal arches of pre-ural centers 2 and 3 (hapu23), which ossified by 25dah in fish from the HTWF group and by 33dah in those from the HT, while in fish from low temperature environments they did not ossify before 72dah. The hypurals (hyp 1-6), epurals (ep) and paripurals (pp) ossified around 50dah in fish raised at high temperature, and did not ossify until 72dah in those from low temperature conditions. Ventral procurent rays (vpcr) ossified by 25dah and 30dah at high and low temperatures, respectively. Intramuscular (intra) ossified 30 days earlier at high temperature when compared to individuals maintained at low temperature conditions.

- iii) Dorsal and anal fins (Figure 4C): dorsal and anal fins showed similar patterns of chondrification and ossification. Dorsal (dr) and anal (ar) rays were the first elements to ossify in both structures, both at high and low temperature conditions. Subsequent ossification was observed in the proximal dorsal and anal radials (pdr and par). We observed a clear influence of water flow in the development of these structures in the low temperature groups, and ossification occurred only in fish from the LTWF group. Distal dorsal and anal rays (ddr and dar) chondrified earlier in fish raised in the presence of water flow (LTWF), but only ossified in the HTWF group. Temperature had an effect also in the dorsal fin endpiece (dfep), which chondrified 20 days earlier in fish from high temperature conditions compared to those from low temperature conditions. In the LTWF group this chondrification was earlier than LT group.
- iv) Pelvic fins (Supplementary Figure S5): pelvic fin was the last structure to start ossification. Pelvic fin rays (plfr) ossified by 30dah in fish developed at high temperature conditions, which contrasts with the pattern observed in fish from the LTWF (40dah) and LT (63dah) groups. The pelvic girdle (plg) ossified by 30dah in groups from high temperature conditions. We identified a clear effect of water flow in the low temperature groups: chondrification started by 36dah, and ossification by 65dah in LTWF groups, while in LT groups these started by 45dah and 70dah respectively. Pelvic fin radials (plfrd) ossified by 50dah in high temperature conditions, and did not ossify before 72dah in low temperature conditions. Water flow groups chondrified earlier for both temperatures.
- v) Weber Apparatus, Vertebral and Supraneural Centers (Supplementary Figure S6): Most of the structures of the Weber Apparatus and vertebral centers were already ossified by 21dah in fish from high temperature conditions: vertebral centers 1, 2, 3 and 4 (vc14), vertebral centers after post-Weber apparatus (vcpw), and neural (napw) and hemal (hapw) arches. The Supraneural 3 (sn3), post-Weber apparatus supraneurals (snpw) and Parapophyses (parp) were already chondrified by

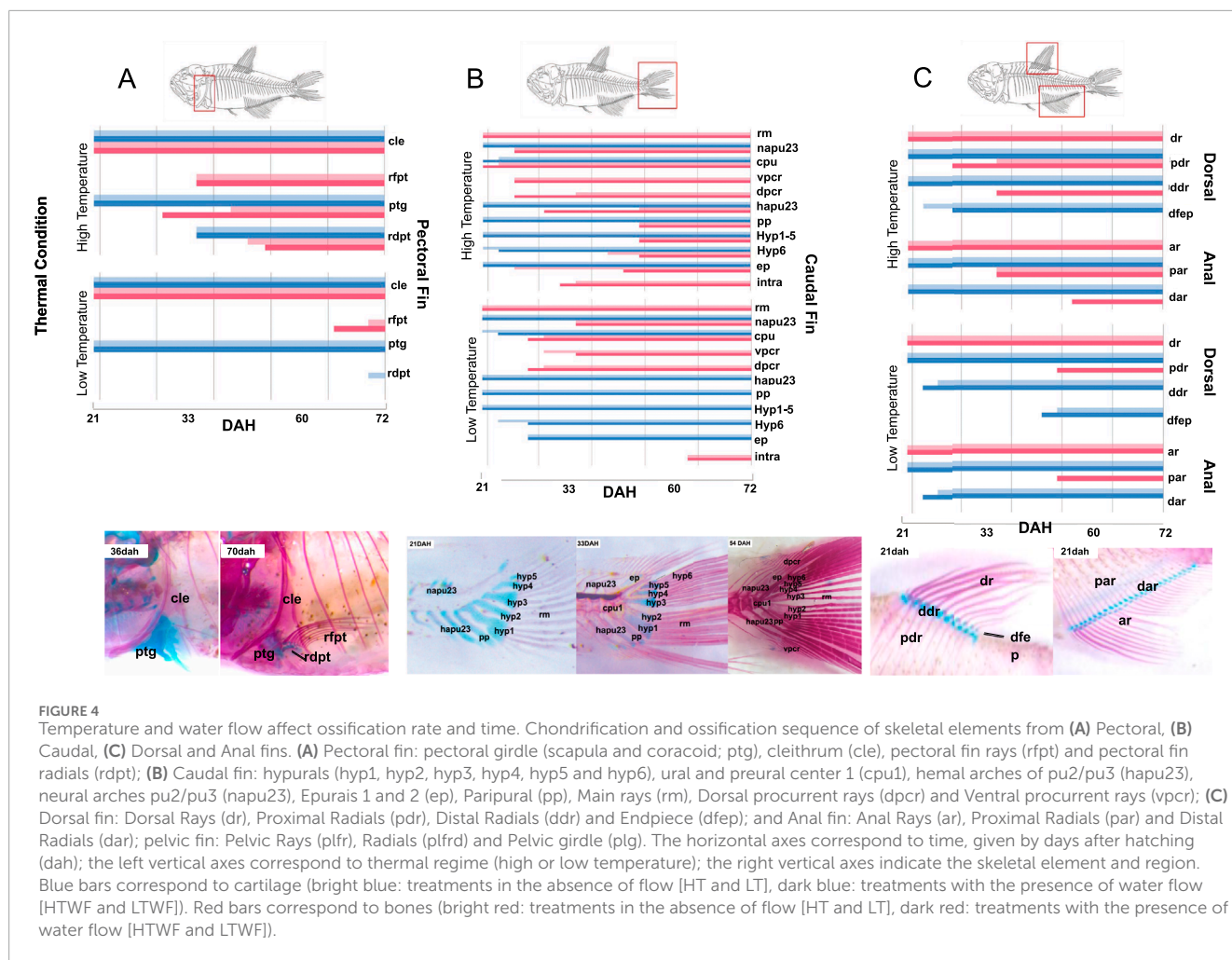
21dah in fish from the high temperature conditions. Contrary to the pattern observed to the fins, most structures of Weber Complex ossified earlier in fish from HT than those from the HTWF group, while the pattern was inverse in LT and LTWF groups. Neural arcs of the Weber apparatus (na34) ossified by 25dah in high temperature and 36dah in low temperature. Supraneural 3 (sn3) ossified by 33dah in fish from the HT condition, 50dah at HTWF, 63dah at LTWF, and by 72dah at LT condition. Paripurals (parp) ossified 30 days earlier in groups from high temperature conditions than those from low temperature. Post Weber appliance (snpw) supraneurals ossified by 50dah at high temperature and by 72dah at low temperature.

Water flow affects *Bmp4* expression levels during skeletal ontogeny

At 21dah, we identified temperature as a major factor affecting *bmp4* expression in analyzed samples, with higher expression values measured in groups developed at higher temperature (HT and HTWF) when compared to those from lower temperature conditions (LT and LTWF). However, significant differences were only identified between HT and LT and LTWF groups (Supplementary Figure S7; Supplementary Table S6). At 33dah, we also identified effects of water flow in *bmp4* expression levels, especially in the HTWF group, which exhibited higher expression values than all other groups (Supplementary Figure S7; Supplementary Table S6). Expression levels of *bmp4* increased with time (days after hatching) both in fish from HTWF and LTWF conditions, but not in the HT and LT groups, suggesting effects of water flow on *bmp4* expression during larval ontogeny (Figure 5; for specific data for each condition, see also Supplementary Figure S8; Supplementary Table S7).

Discussion

Developmental plastic responses often produce complex highly-integrated phenotypes (Westneat et al., 2019) and encompass multidimensional processes in which different structures at several biological levels interact to determine the ontogenetic landscapes (Duclos et al., 2019). In each dimension, a myriad of environmental signals may concomitantly determine and modulate trajectories in landscapes (Duclos et al., 2019). Moreover, distinct environmental signals may differ in the strength of their effects on development, and may interact in an additive, synergistical or even antagonist way. Understanding how distinct yet simultaneous environmental signals shape plastic responses is essential to predict how developmental plasticity may affect trajectories of phenotypic evolution in specific lineages. This is especially relevant in the current scenario of global warming and accelerated climate changes. In the present study, we manipulated water temperature to evaluate multidimensional plastic responses to water flow during larval and juvenile ontogeny of the fish *Astyanax lacustris*. Our results provide evidence of interaction effects between temperature and water flow in growth, ossification and gene expression in this tropical fish.



Thermal regimes impact several levels of ontogenetic development, and effects of water flow are modulated by temperature

When studying developmental plasticity in complex environments, a very relevant question is whether a given variable has a major effect over the others (Westneat et al., 2019), and if there is a driver determining the magnitude and direction of plastic responses to other variables (see Lofeu et al., 2021). In the system used in the present study, manipulation of two environmental variables simultaneously suggests that effects of temperature on fish development seem more prominent than those of water flow in most of the processes evaluated. Specifically, we identified two remarkably distinct phenotypic groups as a function of thermal regimes, especially regarding body size and external differentiation during ontogeny (early flexion, flexion, postflexion larvae, and juvenile stages). Development at higher temperature resulted in larger, well-developed and differentiated fish, which contrasts with the smaller and less-differentiated fish observed in colder thermal regimes. Despite reports of accelerated bone development in fish raised in the presence of water flow (see Cloutier et al., 2010; Fiaz et al., 2012), we observed that temperature is actually a major effect that overlaps mechanical stimulus and defines ossification time

of skeletal elements in *Astyanax lacustris*, decelerating ossification at colder environments. Given that mechanical stress by differential exercise seems to induce robust ossification in fish (Fiaz et al., 2012; Lofeu et al., 2024), we interpret that cold environments may decelerate bone ossification regardless the presence of water flow. Nonetheless, phenotypic effects of water flow were still noticeable in the groups developed in the presence of water flow, as this factor accelerated chondrification and ossification of specific elements related to swimming. At warmer thermal regimes, water flow has an additive effect to high temperature, maximizing growth and ossification (see Grünbaum et al., 2007; Cloutier et al., 2010; Fiaz et al., 2012).

Temperature and water flow elicit expression of shape plasticity - higher temperature reveals a "water-flow plastic morphotype"

We identified that thermal regimes explain most of the variation in body shape during all ontogenetic stages we studied in *A. lacustris*, and water flow seems to interact together with temperature producing unique morphotypes. Rather than only

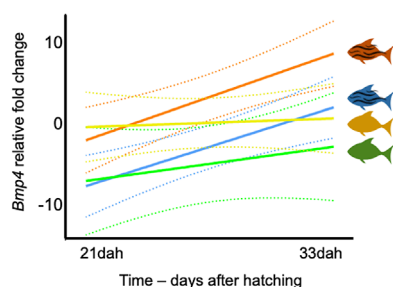


FIGURE 5
Expression of *bmp4* is mostly affected by temperature. Regression of *bmp4* expression levels in two sampling events: 21dah and 33dah. HT: $R^2 = 0.056$, $F = 0.2382$, $p\text{-value} = 0.651$. HTWF: $R^2 = 0.870$, $F = 26.94$, $p\text{-value} = 0.006$. LT: $R^2 = 0.279$, $F = 1.554$, $p\text{-value} = 0.280$. LTWF: $R^2 = 0.863$, $F = 25.29$, $p\text{-value} = 0.007$. Color codes according to experimental groups: yellow = HT, red = HTWF, green = LT, blue = LTWF. Relative fold changes in the vertical axis indicate how much the *bmp4* expression level differs from the calibrator group (HT) after all samples were normalized by the housekeeping gene GAPDH; dashed lines = confidence intervals (95%).

acting additively by enhancing a general phenotypic pattern, such as body size or bone ossification, combination of water flow with high temperature reveals phenotypes that are not induced by one isolated environmental variable. These ‘water-flow plastic morphotypes’ are illustrated by the shape tendencies in the CV2 at 33 days after hatching, which become even more evident in late larval and juvenile stages; these resemble known shape changes related to water flow-associated fish phenotypes (see Grünbaum et al., 2007; Langerhans, 2008; Reyes Corral and Aguirre, 2019). In *A. lacustris*, it is possible to identify four morphotypes, two associated with thermal regimes, and two arising from the interaction between thermal regime and water flow. The warm thermal regime used here apparently enhanced specific plastic responses to water flow in the HTWF group that has not been reported in natural populations, suggesting the potential for variation in *A. lacustris* revealed by specific combinations of environmental factors and may be a source of adaptive plasticity (see Schlichting, 2008; Bonini-Campos et al., 2019; Lofeu et al., 2021). The aquaculture population from which we obtained the hatchlings is maintained in a controlled environment with stable temperature near 23°C and has never been exposed to water flow, and even in nature *A. lacustris* is usually associated to lentic water environments (da Silva et al., 2021). Therefore, the shape morphotypes revealed by our experimental environments in the lab may be interpreted as “novel phenotypes” when compared to the aquaculture system or to natural populations of this species. Although our study did not address eventual differences in oxygen concentration derived from temperature manipulation, we recognize lower concentrations of dissolved O₂ in the water (see Rutjes et al., 2009) might partially explain wider heads in *Aspianax* fish from the high-temperature groups (HT and HTWF). In other fish species, similar phenotypic patterns have been induced by hypoxia derived from high temperatures, and the larger heads observed in fish raised in such conditions may harbor larger gills (Collyder, 2003; Rutjes et al., 2009; Lema et al., 2019). These observations highlight the benefits of gathering as much information as possible about the development environment in future studies

investigating plasticity as an inducer of new phenotypes in ecologically relevant contexts. Our results illustrate how diverse can be the ontogenetic responses to complex ecological contexts, and are also coherent with meta-analyses suggesting lower phenotypic means for morphological traits in fishes from colder water environments (O’Dea et al., 2019). Therefore, warmer environments may enhance phenotypic variation (see O’Dea et al., 2019) and also enable expression of new plastic phenotypes, eventually by magnifying phenotypic responses to other concomitant environmental signals (Westneat et al., 2019; Potts et al., 2021; Lock et al., 2024). The plastic potential discussed here may be a source of adaptive responses to global warming (O’Dea et al., 2019) and should be further investigated in natural populations. Temperature could be an environmental modulator of plastic responses to other environmental factors by facilitating, biasing or even restricting the expression of new plastic phenotypes depending on which factors are interacting with temperature.

Why temperature has a dominant effect on all ontogenetic processes investigated here? And why water flow alone is not able to induce distinct morphotypes at lower temperatures? The answer it is not so clear, but it probably resides on the role of temperature in the cell metabolism and growth (Guderley, 2004; Viadero, 2005; Lema et al., 2019; Fey and Greszkiewicz, 2021), producing overall effects in the organism. Temperature affects efficiency of food intake and conversion (Kinne, 1960) and expression levels of Growth Hormone (GH, see Deane and Woo, 2009). Even knowing that water flow may influence growth dynamics in several tissues, especially cartilage and bones (Fiaz et al., 2012), and also affect body shape (Rutjes et al., 2009; Fischer-Rousseau et al., 2010), responses to flow stimulus may be directly modulated by temperature at metabolic and cellular levels. In fact, temperature affects cartilage and bone development (Blaxter, 1992; Mabee et al., 2000; Hall, 2005; Cubbage and Mabee, 1996; Georgakopoulou et al., 2010; Sfakianakis et al., 2011), and shape emerges as the summatory of different levels (Reyes Corral and Aguirre, 2019), including osteological elements.

Ontogenetic changes induced by water flow suggest developmental responses connecting form to function

We identified a major effect of temperature on all traits investigated here, but water flow also seems to define the appearance of some phenotypic patterns in *A. lacustris* that are known for their functional and adaptive roles related to swimming performance (Fischer-Rousseau et al., 2010; Shuai et al., 2018). Phenotypic patterns observed in fish raised in the presence of water flow involve wider dorsal, adipose and pectoral fins, and longer and streamlined thinner fusiform bodies (Langerhans, 2008; Reyes Corral and Aguirre, 2019), which enhance fish swimming maneuverability in the water, consequently influencing foraging, escape, and predation activities by fish. The presence of water flow accelerates chondrification and ossification in several fish species (see Cloutier et al., 2010; Fiaz et al., 2012), and we observed a similar pattern in *A. lacustris* (see results for HTWF and LTWF groups). The functional bridge between accelerated ossification and water flow if furthermore sustained by increased expression levels of *bmp4* in fish raised in the presence of water

flow, especially at 33 days after hatching (see also Parsons and Albertson, 2009). Together with other members of *bmp* gene family, *bmp4* is involved in several processes related to bone formation, proliferation and remodeling (Ahi, 2016; Li et al., 2021; Wu et al., 2024). Changes along ontogenetic time in *bmp4* expression levels suggest a complex interaction of temperature and water flow modulating *bmp4* expression, especially considering that each factor isolated can affect expression levels of this gene (Han et al., 2020; Clarkson et al., 2021; Li et al., 2021). More interesting, we identified a clear increase of *bmp4* expression levels from 21 to 33 days after hatching in fish from the water flow conditions (HTWF and LTWF). Although here we do not address a direct relationship between genotype and phenotype, empirical studies demonstrated that higher expression levels of *bmp4* enhance chondrogenesis and osteogenesis, also contributing for biomechanical performance of skeletal elements during fish development (Terai et al., 2002; Albertson et al., 2005; Parsons and Albertson, 2009; Li et al., 2021). Mechanical stress and exercise can affect expression levels of *bmp4* (Young and Badyaev, 2007; Ikegame et al., 2016; Jang et al., 2016; Dayawansa et al., 2022; Lofeu et al., 2024), and we interpret changes in *bmp4* expression associated with the presence of water flow in our experiments and the phenotypic patterns induced in *A. lacustris* as indicators of functional plastic responses also at the cellular level. To conclude, our results reiterate the benefits of performing experiments using several relevant environmental signals concomitantly, in order to address the potential of developmental plasticity to induce new phenotypes, an assumption of the ‘Plasticity-led evolution Hypothesis’ (Levis and Pfennig, 2021). Some phenotypes may only be expressed under specific combinations of environmental signals (see also Lofeu et al., 2021; 2024), and plastic responses may constitute a key factor determining extinction or adaptation processes in changing environments.

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.

Ethics statement

The animal study was approved by the Ethics Committee for the use of Animals of the Faculty of Philosophy, Sciences and Letters of University of São Paulo in Ribeirão Preto (Protocol #2018.5.310.59.6). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

LL: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Software, Validation, Visualization, Writing – original draft, Writing – review and editing. BB-C: Data curation, Formal Analysis, Investigation, Methodology, Software, Writing – review and editing. TK: Conceptualization, Data curation,

Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – original draft, Writing – review and editing.

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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.

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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcell.2025.1531162/full#supplementary-material>

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