CONTRIBUTIONS OF BEHAVIOR AND PHYSIOLOGY TO CONSERVATION BIOLOGY

EDITED BY: Susan C. Walls and Caitlin R. Gabor
PUBLISHED IN: Frontiers in Ecology and Evolution

frontiers Research Topics

ISSN 1664-8714
ISBN 978-2-88963-618-1
DOI 10.3389/978-2-88963-618-1

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CONTRIBUTIONS OF BEHAVIOR AND PHYSIOLOGY TO CONSERVATION BIOLOGY

Topic Editors:
Susan C. Walls, United States Geological Survey, United States
Caitlin R. Gabor, Texas State University System, United States

Citation: Walls, S. C., Gabor, C. R., eds. (2020). Contributions of Behavior and Physiology to Conservation Biology. Lausanne: Frontiers Media SA.
doi: 10.3389/978-2-88963-618-1

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frontiers
in Ecology and Evolution

EDITORIAL
published: 21 February 2020
doi: 10.3389/fevo.2020.00033

Editorial: Contributions of Behavior and Physiology to Conservation Biology

Caitlin R. Gabor[1]* and Susan C. Walls[2]

[1] Texas State University, San Marcos, TX, United States, [2] United States Geological Survey, Wetland and Aquatic Research Center, Gainesville, FL, United States

Keywords: behavior, physiology, conservation management, environmental stressors, urbanization

Editorial on the Research Topic

Contributions of Behavior and Physiology to Conservation Biology

Conservation biology is a rapidly evolving discipline, with its synthetic, multidisciplinary framework expanding extensively in recent years. Seemingly disparate disciplines, such as behavior and physiology, are being integrated into this discipline's growing portfolio, resulting in diverse tools that can help develop conservation solutions. Behavior and physiology have traditionally been considered separate fields, yet their integration can provide a more comprehensive approach to developing solutions to conservation and management problems. However, demonstrations are needed of how behavior and physiology—either separately or combined—have contributed to conservation success. Examining species' vulnerabilities to extinction through the lenses of behavior and physiology can provide insight into the mechanisms that drive population declines and extirpations. Our goal is to increase awareness of the benefit of combining behavioral and physiological tools to improve conservation management decisions. Such studies can also help strengthen the basis for evidence-based conservation which, in some cases, has been previously lacking. The diverse studies in our Research Topic illustrate key examples of ways that behavior and physiology can be incorporated into conservation biology. Three main themes emerged from the invited papers with respect to their relevance to conservation: (1) Stress physiology, (2) indicators of health and disease dynamics, (3) and movement ecology. But these themes were also intertwined, thus showing the importance of integrating multiple fields of research to successfully address questions about conservation biology.

Two mini reviews discuss the importance of examining how stress physiology may affect individual fitness and capacity to cope with change which, ultimately, affects the resiliency of populations. Walls and Gabor, in their mini review, promoted combining studies of behavior and physiology to aid in developing conservation strategies for amphibians, which could provide conservation managers with workable solutions to global environmental change. Walls and Gabor also pointed out that studies of behavior are useful to understand how native amphibian species respond to invasive predators. This is supported by Roznik et al. who combined physiology and behavior for a more holistic understanding of the impacts of competition between native and invasive frogs. These authors found that invasive Cuban tree frogs outperformed two native species in jump length and speed, offsetting the costs of dehydration. This work also ties in well to a review

Edited and reviewed by:
Elise Huchard,
UMR5554 Institut des Sciences de l'Evolution de Montpellier (ISEM), France

***Correspondence:**
Caitlin R. Gabor
gabor@txstate.edu

Specialty section:
This article was submitted to Behavioral and Evolutionary Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: 11 January 2020
Accepted: 05 February 2020
Published: 21 February 2020

Citation:
Gabor CR and Walls SC (2020) Editorial: Contributions of Behavior and Physiology to Conservation Biology. Front. Ecol. Evol. 8:33. doi: 10.3389/fevo.2020.00033

by Joly, who indicated the importance of exploring movement ecology (on multiple spatial scales) of invasive species to provide insights for invasive species management. Walls and Gabor also emphasized that more studies on phenological shifts of species in response to recent climate change are needed, as explored by Bókony et al. with birds. These authors found that bird species varied their migratory behaviors in response to contemporary climate change which, in turn, influenced subsequent trends in population size.

Walls and Gabor further promoted the value of examining multiple measures of physiological health to provide a more holistic assessment of an organism's response to environmental change and overall population resilience. Amdekar et al. did just that in their study of lizards. These authors explored multiple markers of health and found no differences in body condition, number of ectoparasites and a measure of immune response between urban and rural populations. However, Amdekar et al. found that urban lizards, compared to rural ones, had lower heterophil to lymphocyte ratios, along with lower testosterone levels—two key measures of physiological health. This research highlights the importance of using multiple physiological measures to explore the health of populations in rapidly changing environments. Last, Walls and Gabor emphasized the contribution that behavioral studies can make to mitigating disease outbreaks. Hettyey et al. provided an example of importance of such studies: these authors suggested that, by providing localized heat sources *in situ*, behavioral temperature preferences, for the heat source, could minimize transmission of the fungal pathogen *Batrachochytrium dendrobatidis*.

In the other mini-review, Narayan et al. focused on multiple non-invasive methods for measuring biomarkers of stress in amphibians, a research gap pointed out by Walls and Gabor. The ability to measure stress non-invasively provides a mechanism for detecting problems in populations before endangerment occurs as it allows one to explore the capacity of a population to cope with a changing environment. The use of such methods allows for studies of imperiled amphibians where destructive measures of physiology are not viable and will aid in understanding the link between population declines and physiological responses to altered environments. Gabor et al. provided an example of the usefulness of non-invasive water-borne hormones to understand mechanisms associated with amphibian population declines. They examined the behavioral and physiological changes associated with exposure to Roundup®, a common pollutant (glyphosate) in aquatic environments that are near agricultural and urban areas. These authors found that exposure to Roundup® may prevent adaptive antipredator responses in larval amphibians but does not affect endogenous corticosterone levels.

Many of the studies in our Research Topic found that examining aspects of movement behavior can help promote conservation outcomes as anthropogenic factors dramatically alter natural ecosystems. For example, McEntire and Maerz, using agent-based models, found that plant climbing behavior in salamanders may help mitigate a species' sensitivity to climate. Bredeweg et al. directly tested movement behavior of metamorphic frogs associated with pond drying and dry habitat conditions. These authors found that larger individuals (from permanent ponds) were more likely to move, and to move further, than smaller individuals, indicating that smaller individuals may not be able to overcome drought conditions by dispersing. As with Bókony et al., there is some indication that organisms are showing adaptive changes in behavior and Bredeweg et al. showed that these behaviors may minimize population declines.

Rapidly changing environments are potentially exposing organisms to unique challenges by generating stressors that affect physiology, behavior, and locomotor efficiency. Understanding how organisms respond to stressors can provide insight into whether populations are able to potentially adapt to these environmental changes. Kern and Langerhans found that fish from urban streams showed higher locomotor efficiency when swimming compared to fish from rural populations, where water velocities are lower during rain events. These authors also found that differences in swimming efficiency in urban populations were the result of genetic evolution, not phenotypic plasticity, and that urban populations were less plastic in their locomotor efficiency than rural populations. These results, together with those of Amdekar et al. and Roznik et al., provide insights into the consequences of urbanization, yet information is still lacking on whether such responses protect these species from population decline or extirpation.

In a more applied setting, Settle et al. explored reproductive behavior of the endangered Ozark hellbender in captivity. These authors found a rapid behavioral change associated with oviposition. This was followed by additional reproductive events in other females that were presumably stimulated by an increase in pheromones/reproductive hormones in the water. Further studies on the physiology of Ozark hellbenders may inform the use of hormones to trigger mating, thus enhancing captive breeding success.

Addressing the biodiversity crisis is inherently complicated because of the multitude of environmental drivers of population declines. Integrating tools and techniques from various disciplines is a more holistic approach in conservation planning and targets this complexity by revealing the mechanisms by which various stressors may be contributing to declines. The articles in this Research Topic provide examples of how studies of behavior and physiology can be integrated into conservation decision-making and strategic planning in a variety of taxa. By highlighting such work, our hope is that others in the conservation community will be motivated to adopt similar approaches, thus allowing a greater understanding of the mechanisms behind population declines and factors that may aid or hinder the future survival of some species.

AUTHOR CONTRIBUTIONS

CG and SW contributed equally to the proposal and editorial work of this Research Topic. Both CG and SW equally contributed to the writing of the Editorial.

ACKNOWLEDGMENTS

We thank all guest editors and reviewers that evaluated the 12 contributions of this Research Topic. Any use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the U.S. Government. This work is ARMI contribution number 726.

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.

frontiers
in Ecology and Evolution

ORIGINAL RESEARCH
published: 07 September 2018
doi: 10.3389/fevo.2018.00128

Measures of Health Provide Insights Into the Coping Strategies of Urban Lizards

Madhura S. Amdekar, Aishwaarya Kakkar† and Maria Thaker*

Centre for Ecological Sciences, Indian Institute of Science, Bengaluru, India

The rapid rate of urbanization worldwide and its consequences for affected species and ecosystems urgently warrants research and action. Whether animals are effectively coping from urbanization is hotly debated, especially since interpretations are based on different measures of animal responses. Here, we take a physiology-centric view of animal coping strategies, and set up predictions for distinguishing urban adaptors and exploiters using multiple markers of health. Our study compares rural and urban populations of a tropical lizard species, the Indian rock agama (*Psammophilus dorsalis*), that lives in a rapidly changing and highly threatened semi-arid ecosystem in Southern India. Contrary to expectations, we did not find any significant differences in body condition, number of ectoparasites, and degree of cell-mediated immune response between lizards from urban and rural areas, despite previously documented differences in aspects of their behavior. However, urban lizards had lower heterophil to lymphocyte ratios, as well as lower testosterone levels compared to rural lizards, which suggest active physiological coping responses. Notably, we find no correlation between the different physiological measures within individuals; strongly reflecting the flexible and independent axes of health responsiveness. Overall, the considerable overlap in responses by urban and rural lizards highlights the importance of using multiple measures of immunity to evaluate health status. These data collectively indicate that *P. dorsalis* is an urban adaptor, and that the tropical urban environment is not associated with harmful consequences for their health.

Keywords: urban, rural, physiology, immune, heterophil, testosterone, Psammophilus, urban adaptor

Edited by:
Caitlin R. Gabor,
Texas State University System,
United States

Reviewed by:
Inger Suzanne Prange,
Ohio University, United States
Richard Anthony Peters,
La Trobe University, Australia

Correspondence:
Maria Thaker
mthaker@iisc.ac.in

†Present Address:
Aishwaarya Kakkar,
Department of Biotechnology, Goa
University, Goa, India

Specialty section:
This article was submitted to
Behavioral and Evolutionary Ecology,
a section of the journal
Frontiers in Ecology and Evolution

Received: *09 May 2018*
Accepted: *09 August 2018*
Published: *07 September 2018*

Citation:
Amdekar MS, Kakkar A and Thaker M
(2018) Measures of Health Provide
Insights Into the Coping Strategies of
Urban Lizards.
Front. Ecol. Evol. 6:128.
doi: 10.3389/fevo.2018.00128

INTRODUCTION

Urban human populations and urban land expansion are increasing at an alarming rate worldwide (Seto et al., 2012; UNDESA, 2014). Rapid urbanization causes modifications to natural ecosystems and introduces unique challenges for its resident flora and fauna. Despite the variation in rate and form around the world, urbanization is characterized by the replacement of natural, often contiguous, habitats by anthropogenic structures, such as roads and buildings (Sol et al., 2013). Presence of these structures causes the loss, fragmentation, and alteration of natural habitats, in addition to introducing pollution and toxicity in the environment (Kylin et al., 2011; Sol et al., 2013). Moreover, urbanization also impacts the biotic components of the ecosystem, which include changes in the composition and intensity of trophic interactions, such as altered predator communities and predation intensities, as well as the composition, distribution and availability of food resources (Sih et al., 2011; Sol et al., 2013). Finally, the altered configuration of suitable habitats in urban areas can affect local population density and competition dynamics of resident wildlife and exposes them to novel disturbances, including humans, domestic animals, and other commensals (Sorace, 2002; Fischer et al., 2012; Sol et al., 2013). All of these abiotic and biotic changes may be perceived as additional stressors for resident wildlife. Consequently, urbanization is expected to negatively impact wildlife, which is why it is not surprising that there is abundant evidence for loss of species richness and diversity in urban areas (Blair, 1996; Blair and Launer, 1997; Germaine and Wakeling, 2001).

Since urbanization is associated with the introduction of novel stressors, animals that manage to live in urban areas are expected to adjust their physiology in response. Numerous studies have therefore tested the prediction that urban animals experience greater stress, and hence have higher circulating glucocorticoid levels, compared to their counterparts in rural areas. Till date there has been mixed evidence for the urban stress hypothesis, with studies reporting increased, decreased, as well as similar glucocorticoid responses in different taxa in urban areas compared to rural areas (French et al., 2008; Bonier, 2012; Scheun et al., 2015; Polich, 2016; Gabor et al., 2018). Hence there is no evidence for a general pattern of how stress physiology of animals is affected by urbanization. The knowledge gap is even greater for physiological processes other than glucocorticoid responses. For instance, the induction of the glucocorticoid stress response can lead to changes in other steroid hormone levels or immune responsiveness (Sapolsky et al., 2000; Davis et al., 2008). Dealing with urban stressors also requires mobilization and reallocation of nutrients to processes involving inflammation or oxidative stress (Isaksson et al., 2017). Thus, acquiring data on multiple physiological markers beyond the glucocorticoid response is imperative. Such information, which can be assessed with minimally-invasive techniques, is critical to determine how animals are perceiving and responding to the potential stress of anthropogenically-altered landscapes.

The impact of urbanization on wildlife is highly variable and many animals persist in urban areas despite the environmental challenges and potential stressors (Ditchkoff et al., 2006; Sol et al., 2013). Urban residents have been defined as either opportunistic "exploiters" that thrive and depend on anthropogenic resources, or as "adapters" that utilize urban environments with relatively similar effectiveness as undisturbed areas (McKinney, 2002; Shochat et al., 2006). Differences in the behavioral ecology of exploiters and adaptors should also correspond to differences in physiological stress and immune responsiveness. For example, in birds, behaviorally-defined adaptor and exploiter species differ in biomarkers of health and nutrition (Isaksson et al., 2017). We extend this distinction between urban exploiter and adaptor species and propose that physiological biomarkers can, in fact, be used to distinguish between these two types of urban residents. Since exploiters are actually benefiting from the anthropogenic conditions, we expect them to have lower stress levels and better health indices in urban areas compared to in undisturbed or rural areas. Alternatively, adaptors should show at least some signatures of physiological coping strategies in urban environments, such as higher stress levels or reduced immunocompetence. At best, given enough time to habituate, adaptor species could show health indices and immune responses that are comparable to those under natural undisturbed conditions. Hence, by comparing physiological indices of urban and undisturbed populations, it is possible to differentiate between adaptor and exploiter strategies. Information on such physiological responses, coupled with knowledge about behavioral changes, can therefore provide a better understanding of whether and how animals are coping with the challenge of urbanization.

Several studies in birds and some in herpetofauna have investigated the health of animals in urban areas using one or two immune parameters (French et al., 2008; Audet et al., 2016; Lazic et al., 2016; Iglesias-Carrasco et al., 2017; Isaksson et al., 2017; but see French et al., 2017). The immune system in vertebrates is a composite system encompassing innate, humoral, and cell-mediated immunity that collectively maintain the health of an organism (Cheng and Lamont, 1988). Measure of a single aspect of the immune system assumes an interconnected response by every component. However, environmental challenges such as urbanization can result in resource limitation, which can force organisms to strategically invest in different physiological and behavioral processes (Norris and Evans, 2000). Trade-offs in resource allocation will therefore arise for example, when investment in one immune component limits investment in other immune components (Norris and Evans, 2000; Matson et al., 2006). Because of differential investment, different components of animal physiology will respond differently to the impact of urbanization; a powerful approach to evaluate the health of an animal would be to assess a combination of immunity measures (Norris and Evans, 2000; Faivre et al., 2003; Adamo, 2004). These measures should include monitoring techniques, which provide information about the health of the individual at the time of sampling, as well as challenge techniques, which measure the response of the immune system after exposure to a novel antigen (Norris and Evans, 2000). Such a combination of parameters would more effectively assess the health status of animals that survive in a potentially challenging urban environment.

India is a rapidly developing nation with one of the highest projected urban land expansion growth rates (Seto et al., 2011). India is also home to an incredible diversity of herpetofauna, with several species found in modified urban environments. In this study, we examined the impact of urbanization on the health of the Indian rock agama *Psammophilus dorsalis*, an agamid lizard species that is found in semi-arid areas of southern peninsular India. This species is also found in human-inhabited areas, such as the city of Bengaluru (Karnataka, India). Here, *P. dorsalis* are restricted to small pockets of suitable areas surrounded by uninhabitable anthropogenic development. Compared to the rural population, lizards from the urban population are habituated to human activity, and they show antipredator behaviors that reflect overall lower reactivity, such as allowing closer approaches before flight (Batabyal et al., 2017). Reduction in antipredator response by urban lizards is not a measure of overall apathy, as these urban lizards are quicker to learn the location of safe refuges than rural lizards (Batabyal, 2017). These behavioral shifts seem to suggest that this species is an urban adaptor. Here we examined whether *P. dorsalis* are urban adaptors based on signatures of physiological coping to the novel environment. By measuring several health parameters in lizards from in and around the city of Bengaluru (Karnataka, India), we explicitly tested the prediction that urban lizards have some biomarkers of compromised health. For both males and females of this species, we employed monitoring techniques, such as the measurement of body condition, ectoparasite infestation, and H:L ratio, as well as a challenge technique to measure cell-mediated immunity (PHA assay). Body condition is a widely accepted index of general health status and reflects lifetime health conditions (Peig and Green, 2009), whereas phytohaemagglutinin (PHA)-induced swelling response is an effective standardized challenge to measure immunocompetence (Kennedy and Nager, 2006). Ectoparasites are known to negatively impact host fitness by decreasing hematocrit, whereas H:L ratio, calculated using leukocyte profiles, is a measure of innate immunity known to be affected by glucocorticoid levels (Dunlap and Mathies, 1993; Davis et al., 2008; Davis and Maney, 2018). Because the hormone testosterone can be immunosuppressive in many vertebrates (Roberts et al., 2004), we also compared plasma testosterone levels in males from both urban and rural areas. These measures from each individual provide us with a multi-variate assay of immunity and health. If *P. dorsalis* is indeed an urban adaptor, we predict that the lizards from urban areas will have at least one of these physiological differences: lower body condition, lower cell-mediated immunity, a greater number of ectoparasites, higher H:L ratio, and lower testosterone levels compared to lizards from rural areas. These physiological responses in a persisting urban population would reflect active measures to cope with the challenging urban environment.

METHODS

Study Areas

The study was conducted during the breeding season for *P. dorsalis* (May-September in 2015 and 2016), since most reptiles exhibit peak stress levels during the breeding period (Romero, 2002). We caught adult male and non-gravid female lizards by noosing from urban and rural areas ($N = 30$ males and 11 females from urban sites, $N = 28$ males and 14 females from rural sites). The urban study area was located within the city of Bengaluru (centroid 13.0422° N, 77.5940° E) while the rural study area was located on the outskirts of Bengaluru, near the Anthargange forest range in Kolar district of Karnataka, India (centroid 13.1243° N, 78.0346° E). These study areas are ∼60 km apart and differ structurally. The urban area consists of semi-developed residential plots, gardens, and construction sites where the lizards are found in small, high-density pockets in the midst of human habitation. Here, they are typically found basking on man-made structures such as cement walls and house ledges. In contrast, the rural area consists of relatively undisturbed boulder fields and rocky outcrops (see Batabyal et al., 2017 for detailed description of the study sites).

Within 4 h of capture, lizards were transported to the laboratory in cloth bags and housed in individual terraria. These terraria (60 × 30 × 25 cm) were equipped with basking lights and rock refuges and were lined with paper towels as substratum. The lizard housing facility allowed for ambient temperature and humidity conditions and natural day/night cycles. Previous research has shown that *P. dorsalis* engage in normal social interactions, such as courtship and aggressive behavior with context specific displays and color changes, in captivity for up to 9 days (Batabyal and Thaker, 2017). All measurements described below were completed within 3 days, during which lizards were consuming live crickets and grasshoppers provided daily (with *ad libitum* water) and showed no signs of distress. All lizards were returned to their site of capture after measurements. Capture and experimental protocols have been approved by the Institutional Animal Ethics Committee at the Indian Institute of Science (CAF/Ethics/394/2014).

Body Condition

All lizards were weighed and their snout-vent length (SVL) was measured in order to calculate body condition, using the scaled mass index (SMI) method:

$$\mathrm{SMI} = M_i \left(\frac{L_o}{L_i}\right)^b$$

Where M_i and L_i are the mass and the SVL of the individual i respectively, L_o is the mean value of SVL for the sample population and b is the slope of the standardized major axis regression of log transformed mass on log transformed SVL (Peig and Green, 2009). The SMI was calculated separately for urban and rural males and females, respectively.

Number of Ectoparasites

Number of ectoparasites present on each lizard was counted manually. Ectoparasites were morphologically distinguishable as either orange-red colored chigger mites (family Trombiculidae) or dark red colored mites, *Pterygosoma foliosetis* Jack (B. Mallik personal communication; Jack, 1961). The chigger mites were typically found on the neck of the lizards while *Pterygosoma* mites were found on the folds of the limbs under the scales.

Cell-Mediated Immunity

Cell-mediated immunity of each lizard was measured using the phytohaemagglutinin (PHA) assay. PHA is a lectin protein which, when injected sub-dermally, causes a complex reaction involving local inflammation and proliferation of lymphocytes, as well as mobilization of heterophils and macrophages (Kennedy and Nager, 2006). The resulting swelling from the PHA injection is therefore a response that involves components of both innate and adaptive immunity, wherein the size of swelling is an indication of the strength of the immune response (Kennedy and Nager, 2006). For the assay, the thickness of the palm on the left and right front limbs of lizards was first measured using digital calipers. Following this, one of the palms was injected with 50 mg of PHA (Sigma, L-8754) dissolved in 10 ml phosphate buffer saline (PBS) such that the total volume injected was 3 μl of this solution per gram of lizard mass (Belliure et al., 2004). The other limb was injected with same volume of PBS. The position of PHA injection in the left or right palm was randomized. Lizards were released in their individual terraria after injections and the thickness of swelling was measured 18 h later. This time-line was determined after a pilot study where maximum swelling in response to PHA injection was found to be at 18 h post injection ($N = 5$ lizards tested; **Supplementary Figure S1**). Response to PHA was calculated as the change in thickness of the PHA-injected hand (difference between thickness 18 h after injection and thickness before injection) minus the change in thickness of PBS-injected hand.

H:L Ratio

After measuring the PHA-induced response, a small blood sample (30–60 μl) was obtained from each lizard through its retro-orbital sinus using a heparinized microcapillary tube. Immediately upon collection, a single drop of blood was used to prepare blood smears on microscopic slides. These slides were fixed for 3 min in absolute methanol and then stained with Giemsa stain. Slides were examined under a compound microscope under oil immersion at 100X to determine the heterophil to lymphocyte (H:L) ratio. To achieve this, white blood cells (WBCs) were counted until a total of 100 WBCs were reached. These were segregated into different cell types (basophils, eosinophils, monocytes, heterophils etc.) based on morphological characteristics, and only the number of heterophils and lymphocytes was used to calculate the H:L ratio (Fokidis et al., 2008; Stacy et al., 2011; Campbell, 2015). Each slide was counted twice in random order under blind conditions by the same person (AK) and the mean value of H:L ratio was used.

Hormone Assay

The rest of the blood sample was centrifuged and the plasma was stored at −20°C until hormone assays were conducted. Plasma levels of testosterone for males only were measured using Enzyme-Immuno Assay kits from Arbor Assays (K032-H1), optimized for the species. Because females are smaller than males, we obtained a lower volume of blood for females compared to males and thus did not analyse testosterone levels for females. Each sample was run in duplicate with a plasma volume of 4 μl and a dilution ratio of 1:140. Some of the samples were subsequently re-run at dilution ratios 1:100 and 1:50 in order to detect the lower levels of testosterone that were not detectable at 1:140 dilution ratio. The intra-assay coefficient of variation was 0.06–8.7% and the inter-assay coefficient of variation was 13% ($n = 4$ kits), based on a single lab standard of testosterone placed in each kit.

Statistical Analysis

Assuming independent axes of responses, we first fitted separate linear models to test whether body condition, cell-mediated immune response, and H:L ratio differed in lizards as a function of site (urban or rural) and sex (male or female). Each of the models had site as the explanatory variable, with sex and an interaction between site and sex as covariates. Since the cell-mediated immune response was found to be highly correlated with body length (SVL), we divided that value by SVL of each individual and used this size-dependent transformed variable for the general linear model. We similarly SVL-corrected the number of ectoparasites per individual and square root transformed that value to meet assumptions of normality. The H:L ratio was log-transformed to meet assumptions of normality. For both H:L ratio and number of ectoparasites, we graphically show the non-transformed values for ease of interpretation. All the models were checked for normality of residuals. Testosterone values of males from the two study areas were compared with a Wilcox test. Testosterone data from one rural male was excluded from this analysis as it was considerably higher (876 ng/ml) than the typical range observed in this species (this study; Batabyal and Thaker, in review), and thus could have been due to measurement error.

We also tested for correlations between each of the health measures within individuals, i.e., body condition, cell-mediated immune response, number of ectoparasites, H:L ratio, and plasma testosterone (for males only). Finally, we performed a principal component analysis (PCA) using three variables (SVL-corrected cell-mediated immune response, SVL-corrected number of *Pterygosoma* mites, and H:L ratio) to examine overall health differences between sites and sexes. All the data used in this study are provided in **Data Sheet 1** (Supplementary Material). All the analysis was performed in R version 3.2.3 (R Development Core Team, 2016).

To ensure that the results of the statistical tests above were not being driven by a few outliers in the data, we also re-ran the statistical analyses after removing outliers in H:L ratio ($N = 2$) and number of *Pterygosoma* mites ($N = 4$), which were two standard deviations from the mean. Results from the conservative data set remained qualitatively unchanged on the exclusion of these data, and therefore we present results from the complete dataset.

RESULTS

We found no significant interaction between site and sex for any of the health parameters, and thus report only the main effect comparisons below.

Body Condition

Consistent with reported sexual dimorphism, males (SVL range 101–150 mm, body mass range 40–100 g) of this species are larger than females (SVL range 77–111 mm, body mass range 16–36 g). We found that males also had significantly greater body condition than females [$F_{(1,79)} = 319.11$, $p < 0.0001$, $N = 58$ males, 25 females, **Figure 1A**], with no significant difference between urban and rural lizards [$F_{(1,79)} = 1.75$, $p = 0.18$, $N = 42$ rural, 41 urban, **Figure 1A**].

Ectoparasites

Rural lizards were found to have both chigger mites as well as the *Pterygosoma* mites, while only the *Pterygosoma* mites were present on the skin of urban lizards (exception: 1 urban male and 1 urban female had both types of mites). Therefore, we used only the number of *Pterygosoma* mites in our linear model to determine differences between sites and sexes. The number of *Pterygosoma* mites on a lizard was positively correlated with its SVL (Spearman's rank correlation rho 0.36, $p < 0.001$). After correcting for SVL, we found that males had significantly higher number of *Pterygosoma* mites compared to females [$F_{(1,79)} = 6.79$, $p = 0.01$, $N = 58$ males, 25 females, **Figure 1B**]. There was no significant difference in the number of *Pterygosoma* mites between urban and rural lizards [$F_{(1,79)} = 3.85$, $p = 0.05$, $N = 42$ rural lizards, 41 urban lizards, **Figure 1B**]. The number of red chigger mites present on rural lizards was not significantly different between males and females (Wilcoxon rank sum test $W = 228$, $p = 0.39$, mean ± 1 se = 136.8 ±22.19, $N = 28$ males, 14 females).

Cell-Mediated Immunity

Cell-mediated immune response of lizards was positively correlated with SVL (Pearson's correlation coefficient 0.409, $p < 0.001$), and after correcting for SVL, we found no significant difference between males and females [$F_{(1,79)} = 3.607$, $p = 0.06$, $N = 58$ males, 25 females, **Figure 1C**], or between lizards from urban and rural sites [$F_{(1,79)} = 2.082$, $p = 0.15$, $N = 42$ rural lizards, 41 urban lizards, **Figure 1C**].

H:L Ratio

We found that urban lizards had lower H:L ratio compared to rural lizards [$F_{(1,64)} = 14.41$, $p < 0.001$, $N = 37$ urban lizards, 31 rural lizards, **Figure 1D**]. There was no significant difference in the H:L ratio between males and females [$F_{(1,64)} = 0.0001$, $p = 0.99$, $N = 48$ males, 20 females, **Figure 1D**].

Testosterone

Urban males had significantly lower testosterone levels (63.39 ± 21.20 ng/ml) compared to rural males (89.58 ± 21.57 ng/ml, Wilcoxon rank sum test $W = 384$, $p = 0.009$, $N = 25$ rural males, 22 urban males, **Figure 2**).

Correlations and PCA of Health Parameters

Overall, only body condition and SVL-corrected cell-mediated immune response was correlated, such that lizards with high

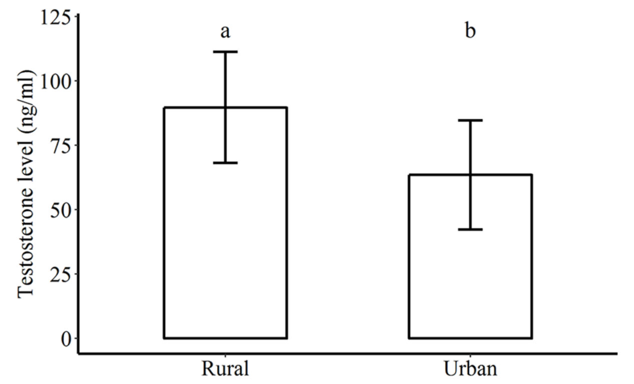

FIGURE 2 | Testosterone levels (mean ± se) of male lizards measured from urban and rural sites. Letters above the bars denote significant difference at $p < 0.05$.

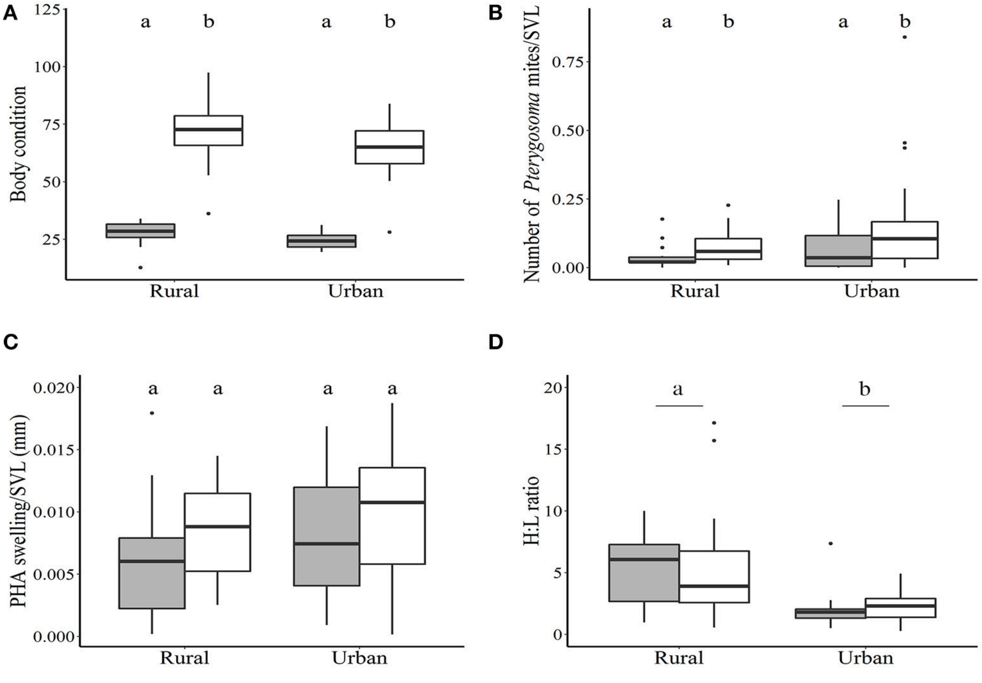

FIGURE 1 | Health parameters of lizards captured from urban and rural areas. **(A)** body condition, **(B)** SVL-corrected number of *Pterygosoma* mites, **(C)** SVL-corrected PHA swelling (in mm), and **(D)** Heterophil:Lymphocyte ratio. Box plots display median, quartiles, 5th and 95th percentile and extreme values. White and gray bars represent males and females, respectively. Significant differences at $p < 0.05$ are denoted by lower case letters above the box plots.

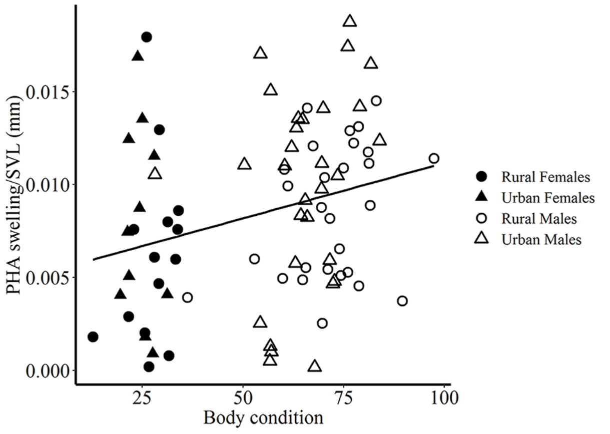

FIGURE 3 | Positive relationship between body condition and size-corrected immune response to PHA (in mm). Circles denote rural lizards and triangles denote urban lizards. Filled and open shapes indicate female and male lizards respectively.

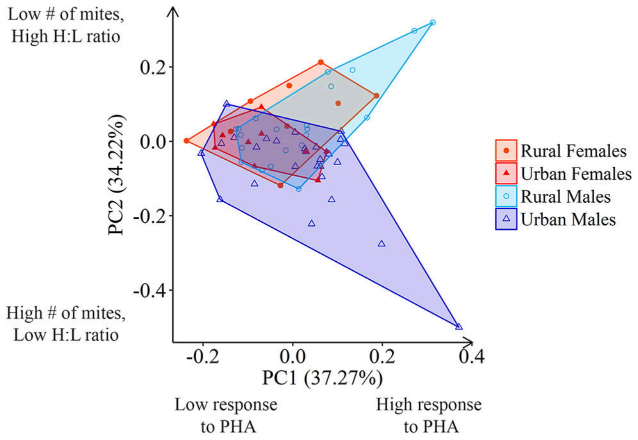

FIGURE 4 | Principle Component Analysis shows almost complete overlap of health parameters (SVL-corrected response to PHA and number of *Pterygosoma* mites, and H:L ratio) between males and females from urban and rural areas.

body condition had a greater swelling in response to PHA (Pearson's correlation coefficient 0.273, $p = 0.012$, **Figure 3**).

A PCA using three health parameters (H:L ratio, SVL-corrected number of *Pterygosoma* mites, and SVL-corrected response to PHA) generated three principal components. The first principal component (PC1) accounted for 37.27% of the variation, while the second principal component (PC2) accounted for an additional 34.22% of the variation. Response to PHA (positive loading of 0.742) was explained by PC1 while number of ectoparasites (negative loading of -0.734) and H:L ratio (positive loading of 0.678) loaded on the PC2 axis. When the PC scores were plotted against each other, urban females ($N = 10$) and rural females ($N = 10$) showed almost complete overlap, while urban males ($N = 27$) and rural males ($N = 21$) showed greater variation in their immune responses (**Figure 4**).

DISCUSSION

Urbanization can affect the physiological processes of animals. Both increases as well as decreases in various immune functions have been reported for many taxa living in urban areas, but we still lack sufficient information for lizards (see review in Bradley and Altizer, 2007). Most of our knowledge about the impact of urbanization on physiology has also been generated from studies in temperate regions of the world, with considerable focus on birds, which are highly mobile organisms (e.g., Bonier et al., 2007; Fokidis et al., 2008; French et al., 2008, 2018; Partecke et al., 2010; Giraudeau and McGraw, 2014; Meillère et al., 2015; Audet et al., 2016; Lazic et al., 2016; Iglesias-Carrasco et al., 2017; Isaksson et al., 2017). However, organisms such as herpetofauna are less mobile and by virtue of being range restricted, are likely to be strongly affected by urbanization. In addition, tropics are at the global center of rapid urban expansion while simultaneously being rich in endemic herpetofauna. To our knowledge, this study is the first to examine the impact of urbanization on biomarkers of health in a tropical agamid lizard species. Of the measures of health and physiological responses in this study, we found that H:L ratio and testosterone were significantly lower for lizards in urban areas compared to those from rural areas. In addition, except for a positive association between body condition and cell-mediated immune response, none of the other health parameters were correlated with each other. Taken together, these results emphasize the importance of considering several measures of immunity and suggest that urbanization has altered the innate physiology of the Indian rock agama such that they are effectively coping as urban adaptors.

Urban and rural lizards differed in some, but not all physiological parameters. Urban lizards had a lower H:L ratio compared to rural lizards, a result in agreement from another study in tree lizards (*Urosaurus ornatus*) in USA (French et al., 2008). Evidence from other experiments with *P. dorsalis* support the urban stress hypothesis, as urban males have higher corticosterone levels than rural males under various control and social conditions (Batabyal and Thaker, in review). Stress is known to affect leukocyte profiles such that increased glucocorticoid levels lead to an increase in circulating heterophils and a decrease in circulating lymphocytes, thereby increasing the overall H:L ratio (Davis et al., 2008). Another factor that can affect leukocyte numbers is the presence of infection but we have detected no infection by typical blood endoparasites in our study system (Jalal, Thaker and Ishtiaq, unpublished data). We are reluctant to conclude that lower H:L ratio in urban lizards compared to rural lizards indicates lower levels of overall stress in urban areas compared to rural areas (Davis and Maney, 2018). We do think that lower H:L ratio in urban lizards is a strong indication that lizards in the city are actively coping with urban stressors.

Male lizards from urban areas also had lower testosterone levels compared to those from rural areas. These levels are comparable to testosterone levels exhibited by males under control conditions in captivity, in the absence of any social interactions (Batabyal and Thaker, in review). Hence, they reflect baseline levels of the hormone required by these lizards to perform activities such as courting, fighting or maintenance of territories during the breeding season. In urban sites, *P. dorsalis* reside in small environmentally suitable pockets in high density and frequently encounter mates as well as competitors (Amdekar and Thaker, unpublished data). The "challenge hypothesis" postulates that males present in high density populations should exhibit higher levels of plasma testosterone compared to males in lower density populations because the former are expected to engage in more encounters with females as well as competitive interactions with males (Ball and Wingfield, 1987). Although support for this hypothesis has been found in many species (Hirschenhauser and Oliveira, 2006), we find the opposite pattern in *P. dorsalis*. Lower levels of testosterone found in males from urban areas may reflect a strategy to avoid engaging in frequent social interactions, such as fighting with other males throughout the breeding season, since such frequent agonistic interactions can have high physiological and physical costs (Wingfield et al., 2001). Sexual signals (color and social behavior) in males of this species are also less elaborate in urban areas compared to rural areas (Batabyal and Thaker, 2017; Batabyal and Thaker, in review). Many studies in lizards have found that sexual signaling is strongly mediated by testosterone (Salvador et al., 1996; Cox et al., 2008) which also supports the pattern we find here; urban lizards have lower testosterone and lower sexual signaling.

Contrary to our expectations, we found no effect of urbanization on PHA-induced immune responses and number of ectoparasites on lizards. Studies with other lizards have found mixed results, with some species showing no difference in cell-mediated immune response between urban and natural habitats, while others reporting reductions in immunocompetence in response to human disturbance (Amo et al., 2006; Iglesias-Carrasco et al., 2016; French et al., 2017). Poor body condition in lizards from disturbed areas compared to those from natural areas has been documented for *Anolis antonii* and *Psammodroumus algirus*, but we did not find differences in body condition between urban and rural lizards in our study (Amo et al., 2007; Gallego-Carmona et al., 2016). Species that are able to exploit food resources, despite the altered conditions in urban areas, would match or even fare better than their rural counterparts. Although we currently do not have information about prey resource distribution across the urban-rural gradient, we have found that dietary overlap in *P. dorsalis* from urban and rural areas is high, thereby showing no restriction to acquiring resources (Balakrishna et al., 2016).

We did not find sex-based differences in any of the health parameters except for the expected difference in body condition due to sexual dimorphism and the number of *Pterygosoma* mites. In general in vertebrates, males are predicted to suffer from reduced immunocompetence and higher parasite infection during the peak breeding season because of the immunosuppressive effects of the hormone testosterone, as well as through their aggressive and territorial behavior (Zuk and McKean, 1996). Accordingly, studies in many different taxa have reported lower immune response in males as compared to females (Saad, 1989; Moreno et al., 2001; Tschirren et al., 2003). We found no evidence for immunosuppression in males, and the lack of sex differences for most of the health parameters measured in our study may be due to similar exposure and susceptibility of both the sexes to immune challenges in urban and rural environments.

Our results show lack of either positive or negative correlations between the different health parameters at the individual level. This suggests that there is neither a trade-off nor a correlated response between the multiple health parameters, and that these parameters act as independent axes of health. Evidence for trade-offs between different immune components has been found in some birds in other environmental contexts (Ardia, 2007). For example, in house sparrows (*Passer domesticus*), individuals that have a greater humoral immune response also have a lower cell-mediated immune response (Buchanan et al., 2003). Other studies in birds, but not lizards, have found varying degrees of correlation between other sets of physiological measures such as immune function and basal metabolic rate, or antioxidative measures (e.g., Cohen and McGraw, 2009; Buehler et al., 2012). We also did not find any correlation between testosterone levels in males and any of the immune parameters measured, a result inconsistent with the proposed immunosuppressive nature of testosterone (Roberts et al., 2004). Lack of trade-offs between physiological components is possible if environments have sufficient resources, such that individuals are not forced to trade-off across multiple processes. In *P. dorsalis*, dietary overlap between urban and rural lizards is high (Balakrishna et al., 2016), suggesting sufficient resources in these environments. Independent health axes would also enable these lizards to independently adjust each of these components in response to a challenge, especially if the environment is such that different selection pressures affect different parts of physiology. Further experiments in free-living vertebrates that manipulate one immune component and examine the impact on other components, or directly manipulate access to resources will shed more light on the trade-offs possible between immune components. Such trade-offs are more likely between immune components that are innate vs. induced, as they signify investment in current health state and the ability to combat future infections.

Urbanization creates rapid and dramatic changes in both abiotic and biotic components of the environment. Often, these changes are thought to have immediate negative consequences for animals that utilize these heavily altered landscapes. However, animals differ in their degree of physiological flexibility depending on their ecology and life-history characteristics, as well as the features of the altered environment. We show here that physiological markers can be used to differentiate between adaptor and exploiter species. They are also an effective tool to assess the health status of animals in disturbed areas, a phenomenon that has gained momentum in informing conservation decisions (Wikelski and Cooke, 2006; Busch and Hayward, 2009). Identifying species as adaptors or exploiters can be the first step toward devising better plans for the management of species in disturbed areas. As opposed to blanket strategies for the conservation of urban residents, information about how animals are coping would allow for more targeted approaches, especially for adaptor species that are exhibiting altered strategies. The natural habitats of *P. dorsalis* are mostly semi-arid savannas of southern India, which are rapidly being converted into agricultural land or urban areas. Protection of *P. dorsalis* would therefore have to include the maintenance of populations in urban areas. The multiple physiological markers of health and the recorded shifts in social and antipredator behaviors of *P. dorsalis* lead us to conclude that this tropical agamid is actively coping in the modified environment. We stress, however, that a single measure of physiological response would have been misleading, as these signatures of coping would have been undetected. Use of multiple physiological tools is essential to generate a more integrative view of whether and how urbanization affects the coping strategies of resident animals.

AUTHOR CONTRIBUTIONS

MA and MT conceived the study; MA and AK performed the experiments; MA carried out statistical analysis; MA and MT wrote the manuscript. All authors have read and approved the final version of the manuscript.

FUNDING

This work was supported by funding from DBT-IISc partnership program, DST-FIST, and the MOEF-CC to MT, as well as a British Herpetological Society Student Grant to MA. AK was supported by the Summer Research Fellowship programme of the Jawaharlal Nehru Centre for Advanced Scientific Research. MHRD supported a research fellowship to MA.

ACKNOWLEDGMENTS

We thank Mihir Joshi and Anuradha Batabyal for help in field and Anuradha Batabyal for advice on statistical analysis.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2018.00128/full#supplementary-material

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Conflict of Interest Statement: 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.

frontiers
in Ecology and Evolution

ORIGINAL RESEARCH
published: 16 October 2018
doi: 10.3389/fevo.2018.00159

Hydric Balance and Locomotor Performance of Native and Invasive Frogs

Elizabeth A. Roznik[1]*, *Camila A. Rodriguez-Barbosa*[2] *and Steve A. Johnson*[2]

[1] *Department of Research and Conservation, Memphis Zoo, Memphis, TN, United States,* [2] *Department of Wildlife Ecology and Conservation, University of Florida, Gainesville, FL, United States*

An animal's physiology should be closely adapted to its environment to optimize organismal function, so physiological traits may be mismatched when animals are introduced to new locations. For introduced amphibians, mismatched traits involving hydric balance or performance across hydric states could have profound impacts on their abundance and distribution, and thus their invasion success. We examined susceptibility to dehydration in invasive Cuban treefrogs (*Osteopilus septentrionalis*) and two sympatric native treefrogs (*Hyla* spp.) in Florida, USA, by measuring rates of evaporative water loss, rates of water uptake, and sensitivity of locomotor performance to dehydration. Of the three species, Cuban treefrogs were the most susceptible to dehydration by experiencing the highest rate of evaporative water loss and lowest rate of water uptake. Green treefrogs (*Hyla cinerea*) were the most resistant to dehydration by losing water the slowest and gaining water the fastest, and squirrel treefrogs (*Hyla squirella*) were intermediate in rates of water loss and uptake. Dehydrating frogs by 20% of their fully hydrated body masses affected the locomotor performance of all species by decreasing maximum jump lengths, decreasing speed, and increasing successive jump lengths. The maximum jump lengths of Cuban treefrogs and green treefrogs were more sensitive to dehydration than those of squirrel treefrogs. Despite this sensitivity, Cuban treefrogs still outperformed both native species in maximum jump length and speed, even when controlling for body size. Consequently, the performance of dehydrated Cuban treefrogs was similar to that of fully hydrated native treefrogs. Our results suggest that Cuban treefrogs are more susceptible to dehydration and its effects than several native competitors; however, they may be able to at least partly compensate for these effects with their large body size, superior locomotor performance, and strong affinity for refugia. Our findings highlight different strategies used by frogs to maintain water balance and suggest that Cuban treefrog densities may be higher in areas with greater moisture availability and habitat complexity, including urban and densely forested areas. Native treefrogs may experience stronger competition and predation from Cuban treefrogs in these areas, which supports anecdotal reports of native frog declines.

Keywords: Cuban treefrog, dehydration, evaporative water loss, *Hyla* sp., introduced species, jumping performance, *Osteopilus septentrionalis*, water uptake

Edited by:
Caitlin R. Gabor,
Texas State University System,
United States

Reviewed by:
Ivan Gomez-Mestre,
Consejo Superior de Investigaciones
Científicas (CSIC), Spain
Inger Suzanne Prange,
Ohio University, United States
James Watling,
John Carroll University, United States

***Correspondence:**
Elizabeth A. Roznik
betsy.roznik@gmail.com

Specialty section:
This article was submitted to
Behavioral and Evolutionary Ecology,
a section of the journal
Frontiers in Ecology and Evolution

Received: *24 June 2018*
Accepted: *24 September 2018*
Published: *16 October 2018*

Citation:
Roznik EA, Rodriguez-Barbosa CA and Johnson SA (2018) Hydric Balance and Locomotor Performance of Native and Invasive Frogs.
Front. Ecol. Evol. 6:159.
doi: 10.3389/fevo.2018.00159

INTRODUCTION

As amphibians made the evolutionary transition from aquatic to terrestrial habitats, many species developed morphological, physiological, and behavioral adaptations that reduced the risk of desiccation, including changes in rates of evaporative water loss and water uptake, increased tolerance to dehydration, and water-conserving behaviors (Toledo and Jared, 1993; Jørgensen, 1997). Although many amphibians have little resistance to water loss and must live in or near water and use behavioral strategies to avoid desiccating conditions, such as nocturnal activity and fossoriality (Wells, 2007), some species have specialized adaptations to reduce and tolerate water loss. For example, some species can tolerate losing up to 50% of their body mass to water loss (Thorson, 1955), and others can substantially decrease evaporative water loss by secreting lipids from specialized glands and spreading them over their bodies (Blaylock et al., 1976; Lillywhite et al., 1997; Barbeau and Lillywhite, 2005), or by forming cocoons from sloughed skin (McClanahan et al., 1976; Withers, 1998). The wide variation in tolerance to dehydration among species is broadly related to their ecological habits, with arboreal species exhibiting high resistance to evaporative water loss, terrestrial species showing moderate resistance, and semi-aquatic species showing low resistance (Thorson, 1955; Wygoda, 1984; Young et al., 2005). Similarly, high rates of water uptake have been reported in species inhabiting arid areas (Bentley et al., 1958; Van Berkum et al., 1982; Tingley et al., 2012).

In addition to maintaining water balance, another challenge that amphibians face in terrestrial environments is maintaining ecological performance while dehydrated. The locomotor performance of amphibians is often sensitive to dehydration, with increasing levels of dehydration causing decreased jump lengths, speed, and endurance (Moore and Gatten, 1989; Rogowitz et al., 1999; Titon et al., 2010; Tingley et al., 2012). Because locomotor performance affects the ability to capture prey and escape from predators, decreased performance due to dehydration can compromise predator escape abilities and foraging efficiency (Preest and Pough, 2003), and consequently, can reduce fitness (Wassersug and Sperry, 1977; Walton, 1988; Miles, 2004; Husak, 2006). The hydric sensitivity of locomotor performance differs among species and is often related to local environmental conditions. Amphibians inhabiting dry areas, which presumably face a greater risk of desiccation than amphibians in wet areas, can often maintain a high level of locomotor performance while dehydrated. For example, dehydrated cane toads (*Rhinella marina*) from an arid area had greater endurance and longer jump lengths than did dehydrated conspecifics from a mesic area (Tingley et al., 2012), and the savanna-dwelling cururu toad (*R. schneideri*) moved longer distances during timed trials when dehydrated than did two congeners found in rainforest (Titon et al., 2010).

An animal's physiology should be closely adapted to its environment to optimize organismal function, so physiological traits may be mismatched when animals are introduced to new locations. For introduced amphibians, mismatched traits involving hydric balance or ecological performance across hydric states could have profound impacts on their abundance and distribution. Environmental conditions that differ from those experienced in their native range could also facilitate adaptations in physiology or behavior, such as those that occurred in introduced cane toads when they invaded an arid region of Australia (Tingley et al., 2012). The Cuban treefrog (*Osteopilus septentrionalis*) is native to the Caribbean and has been introduced to Florida, USA, where it negatively impacts native amphibians by competing with them and preying upon them (Meshaka, 2001; Wyatt and Forys, 2004; Smith, 2005; Knight et al., 2009; Rice et al., 2011). Observational data suggest that Cuban treefrogs may be limited by water availability. For example, Cuban treefrogs are more strongly associated with more urban areas than natural areas (largely due to artificial water sources), and they are more abundant in wetlands and hardwood hammocks than in pine forests (Meshaka, 2001; Johnson, 2007). Furthermore, Cuban treefrogs that were displaced to old-field habitat rapidly moved to nearby hardwood hammock or bottomland forest, which also suggests that they prefer more mesic habitats (McGarrity and Johnson, 2010). The habitat of Cuban treefrogs in their native range has not been described in detail, but observations suggest that they are also associated with urban and forested areas in Cuba, the Cayman Islands, and the Bahamas (Meshaka, 2001).

TABLE 1 | Summary of morphometric measurements for frogs used in this study, including the mean ± SE (range) snout-vent length (SVL), body mass, and surface area (adjusted to account for the water-conserving posture; see methods for details) for each species.

	Cuban treefrogs	Green treefrogs	Squirrel treefrogs
SVL (mm)	37.2 ± 1.1 (28–50)	32.8 ± 0.2 (23–50)	25.9 ± 1.1 (23–32)
Mass (g)	2.6 ± 0.2 (1.1–5.0)	1.9 ± 0.2 (0.6–6.3)	0.9 ± 0.1 (0.6–1.6)
Surface area (cm^2)	11.0 ± 0.5 (7.1–16.3)	9.0 ± 0.6 (5.0–18.6)	6.3 ± 0.2 (5.0–8.5)

We examined susceptibility to dehydration in Cuban treefrogs and two sympatric native treefrogs (*Hyla* spp.) in Florida by comparing rates of evaporative water loss, rates of water uptake, and sensitivity of locomotor performance to dehydration. The goal of our study was to investigate innate differences in these properties among species, rather than size-related differences, so we used frogs of similar body sizes (**Table 1**). Based on apparent preferences for moist microhabitats, as described above, we hypothesized that Cuban treefrogs would be more susceptible to dehydration than the native species by exhibiting higher rates of evaporative water loss, lower rates of water uptake, and locomotor performance that is more compromised by dehydration. Examining these physiological traits will enhance our knowledge of how the Cuban treefrog is affected by water availability, which may influence their habitat use and distribution, and thus their niche overlap with native species.

MATERIALS AND METHODS

Ethics Approval Statement

This study was carried out in accordance with the recommendations of the Institutional Animal Care and Use Committees at the Memphis Zoo and University of Florida. The protocol was approved by the Institutional Animal Care and

Use Committees at the Memphis Zoo (17-110) and University of Florida (201709995).

Frog Collection and Husbandry

We collected 30 Cuban treefrogs (*O. septentrionalis*), 30 green treefrogs (*Hyla cinerea*) and 30 squirrel treefrogs (*Hyla squirella*) from the University of Florida and four private properties in Gainesville, Florida. Frogs were captured by hand or collected from PVC pipe refugia over a three-day period (19–21 October 2017), and the experiments took place within the following 2 weeks. Immediately upon capture, we recorded each frog's snout-vent length (SVL) and body mass in the field (**Table 1**). Frogs were housed individually in ventilated plastic containers (12 cm in diameter, 14 cm in height) with moist paper towels and fed crickets twice per week. At the end of the study, the frogs were maintained in the laboratory for future experiments.

Rates of Evaporative Water Loss and Water Uptake

We measured rates of evaporative water loss and water uptake for all frogs. We first determined the masses of fully hydrated frogs by weighing them after they had been submerged in 3 cm of bottled drinking water for 30 min and their bladders had been emptied by gently pressing their abdomens. Frogs were then dehydrated until 20% of their fully hydrated body masses had been lost by placing them in wind tunnels in front of an electric fan (Titon et al., 2010; Tingley et al., 2012). Wind tunnels were constructed from rectangular plastic containers (11 × 7 × 5 cm) with screened ends. While in the wind tunnels, frogs were weighed every 3 min until they lost 20% of their body mass. We observed the posture of each frog every 3 min during the trial and calculated the proportion of time spent in the water-conserving posture (legs folded under the body and ventral surface pressed to the substrate). All measurements and observations were performed carefully to avoid disturbing the frogs. The goal of our study was to compare physiological differences in water loss rates among three species, rather than differences that were related to body size or behavior. Larger frogs lose water slower than smaller frogs due to a smaller surface-area-to-volume ratio, and frogs in the water-conserving posture lose water slower than frogs in active postures because less surface area is exposed to the air. Therefore, to accurately compare differences in resistance to water loss among species, we controlled for the exposed surface area of each frog due to its body size and posture. We calculated rates of evaporative water loss by dividing the slopes of regressions of body masses vs. time by each frog's surface area. Surface area (cm^2) was calculated using the following equation based on body mass (McClanahan and Baldwin, 1969): surface area $= 9.90\ (\text{body mass})^{0.56}$. We adjusted surface area values by assuming that the water-conserving posture exposes two-thirds of a frog's total surface area (Withers et al., 1982). We tested for effects of species on rates of evaporative water loss (expressed as $\mathrm{mg/cm^2/min}$) using a linear model. We controlled for the time spent in the water-conserving posture by including the residuals from a regression of the proportion of time spent in the water-conserving posture vs. water loss rate as a fixed effect.

After dehydrated frogs were used in locomotor performance trials (as described below), they were immediately rehydrated. To measure rates of water uptake, we placed frogs in plastic containers with 3 cm of bottled drinking water. Every 3 min, frogs were removed from the containers, blotted gently with paper towel to remove excess water, and weighed. These techniques have been used to measure water uptake rates in other studies (Titon et al., 2010; Tingley et al., 2012). Frog body masses were recorded every 3 min until they reached their fully hydrated masses or for a maximum of 30 min. We calculated rates of water uptake by dividing the slopes of regressions of body masses vs. time by each frog's fully hydrated body mass. We used a linear model to test for effects of species on rates of water uptake (expressed as mg/g/min).

We investigated whether rates of evaporative water loss were related to rates of water uptake by using a linear model that controlled for time spent in the water-conserving posture by including the residuals from a regression of the proportion of time spent in the water-conserving posture vs. water loss as a fixed effect. We also tested for potential effects of species and body size (SVL) on water-conserving behavior (proportion of time in the water-conserving posture) during dehydration trials using a linear model. Finally, we examined the natural hydration levels of frogs (calculated by dividing body masses recorded in the field by their fully hydrated body masses determined in the laboratory). We used a linear model to evaluate effects of species and body size (SVL) on natural hydration levels.

Locomotor Performance

We measured the locomotor performance of each frog when it was fully hydrated and dehydrated until 20% of its body mass was lost, as described above. Each frog was tested at each hydric state in a random order, with trials for the same frog occurring at least 48 h apart. Frogs were allowed to jump five times on fabric taped to the floor of a long hallway at 25°C. Most frogs jumped readily, but those that were more reluctant to jump were gently prodded on the urostyle to encourage them to jump. We recorded the time it took each frog to complete five jumps, and we marked the location of each jump using dot stickers. After five jumps were completed, the length of each jump was measured to the nearest 0.5 cm. We used these data to examine the maximum distance jumped, the time to complete five jumps (when controlling for the total distance covered) as a measure of speed, and the change in jumping performance across the five jumps (slope of jump length vs. jump number) as a measure of endurance.

We used two separate linear mixed-effects models to examine whether the maximum jump lengths and the change in jump lengths over time were affected by hydric state, species, SVL, and the interaction between hydric state and species. We used a third linear mixed model to examine whether speed was affected by hydric state, species, SVL, and the interaction between hydric state and species, when controlling for the total distance covered by the five jumps as a fixed effect. For all models, we included individual frog identity as a random effect to account for the fact that each frog was tested at each hydric state and thus the jumping performance data across hydric states were not independent.

All statistical analyses were conducted in R statistical software (version 3.4.3). Linear models were performed in the *stats* package, linear mixed-effects models were performed in the *nlme* package, and all probability values were calculated using the *car* package. We used the *visreg* package to construct partial residual plots to examine the relationships between variables.

RESULTS

Rates of Evaporative Water Loss and Water Uptake

Rates of evaporative water loss ($F = 4.7908$, $DF = 2$, $P = 0.0107$; **Figure 1A**) and water uptake ($F = 14.1540$, $DF = 2$, $P < 0.0001$; **Figure 1B**) differed among species and showed a similar pattern of water balance. Of the three species, the introduced Cuban treefrog was the most susceptible to dehydration by experiencing the highest rate of evaporative water loss and lowest rate of water uptake. The native green treefrog was the most resistant to dehydration by losing water the slowest and gaining water the fastest, and the native squirrel treefrog was intermediate in rates of water loss and gain. Within individual frogs, rates of water loss were negatively related to rates of water uptake ($F = 7.3556$, $DF = 1$, $P = 0.0081$; **Figure 1C**). Water-conserving behaviors differed among species ($F = 4.4239$, $DF = 2$, $P = 0.0148$, **Figure 1D**), but not body sizes ($F = 0.1715$, $DF = 1$, $P = 0.6790$). The amount of time spent in the water-conserving posture was lowest in Cuban treefrogs, highest in green treefrogs, and intermediate in squirrel treefrogs. The average natural hydration level of frogs upon immediate collection from the field was 96% (range: 76–100%) and did not differ among species ($F = 0.1896$, $DF = 2$, $P = 0.8276$) or body sizes ($F = 1.8616$, $DF = 1$, $P = 0.1760$).

Locomotor Performance

The maximum distances jumped by frogs (when controlling for SVL) were affected by hydric state, species, and the interaction between hydric state and species (**Table 2**, **Figure 2A**). Fully hydrated frogs jumped farther than dehydrated frogs in all species, but the magnitude of the effects of dehydration on maximum jump lengths differed by species (**Figure 2A**). Cuban treefrogs and green treefrogs were similarly affected by dehydration, with maximum jump lengths decreasing by an average of 22% (18 cm) and 23% (14 cm), respectively (**Figure 2A**). Both of these species were more sensitive to dehydration than squirrel treefrogs, which experienced a 12% decrease (7 cm), on average (**Figure 2A**). Overall, Cuban treefrogs had longer maximum jump lengths in comparison to the native species, even when controlling for body size. When Cuban treefrogs were dehydrated, their maximum jump lengths were similar to those of native frogs when they were fully hydrated (**Figure 2A**).

Jumping speed was affected by hydric state and species (when controlling for the distance covered and SVL) but there was no evidence for an interaction between these two factors (**Table 2**, **Figure 2B**). Dehydrated frogs took 16% (1 sec) longer, on average, to complete five jumps than fully hydrated frogs (**Figure 2B**). Of the three species, Cuban treefrogs had the fastest speed, green treefrogs had the slowest speed, and squirrel treefrogs had an intermediate speed (**Figure 2B**). The change in jump lengths across the five jumps (slope of jump length vs. jump number) also differed by hydric state, but not by species or by the interaction between these factors, when controlling for SVL (**Table 2**, **Figure 2C**). In all species, the slope for dehydrated frogs was positive, whereas the slope for fully hydrated frogs was zero or negative (**Figure 2C**); this indicates that dehydrated frogs tended to increase their jump lengths over successive jumps, whereas successive jump lengths of hydrated frogs tended to remain the same or decrease.

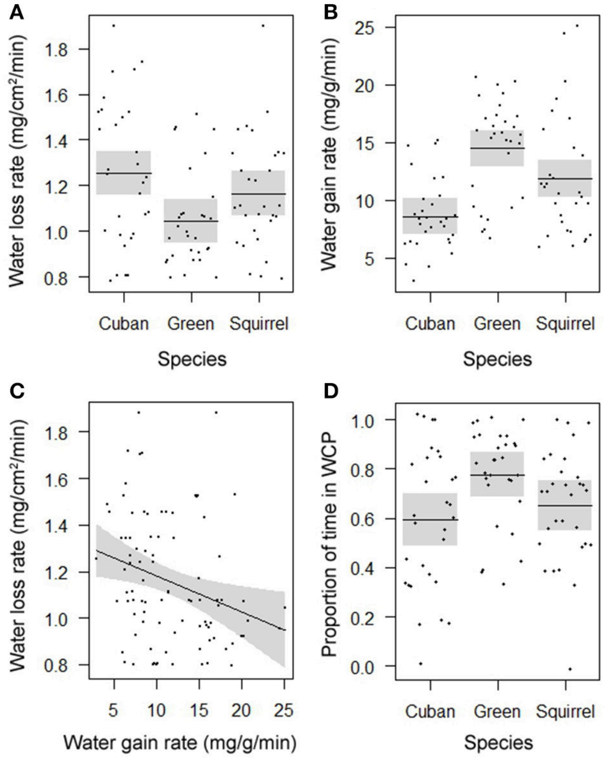

FIGURE 1 | Partial residual plots with lines depicting means (in **A**,**B**,**D**) or regressions (in **C**) and 95% confidence bands (shaded), controlling for all factors in the statistical models that are not shown in the figures, showing the significantly different rates of **(A)** evaporative water loss and **(B)** water uptake for three species, **(C)** the significant correlation between these rates for individual frogs, and **(D)** significant differences in the proportion of time spent in the water-conserving posture (WCP) among three species.

DISCUSSION

The ability of amphibians to maintain hydric balance and locomotor performance across hydric states influences their habitat use, geographic distribution, and evolution (Jørgensen, 1997; Wells, 2007). For introduced amphibians, such as the Cuban treefrog in Florida, these physiological traits may affect their invasion success and impacts on native species resulting from competition and predation. To fully understand hydric balance in amphibians, rates of evaporative water loss and water uptake should both be considered, and our study is the first to do this for introduced Cuban treefrogs and two native competitors in Florida. We found that rates of water loss and water uptake differed among species and were negatively correlated (**Figure 1**). Of the three species, the invasive Cuban treefrog was the most

TABLE 2 | Statistical results from three separate linear models examining the effects of different variables on the locomotor performance of frogs, including maximum jump length, speed, and change in jumping performance across five jumps (slope of jump length vs. jump number).

Predictors	χ^2	DF	*P*
MAXIMUM JUMP			
Hydration	60.0791	1	**<0.0000**
Species	46.4760	2	**<0.0000**
SVL	20.0912	1	**<0.0000**
Hydration × Species	7.9265	2	**0.0190**
SPEED			
Hydration	17.5806	1	**<0.0000**
Species	8.7769	2	**0.0124**
SVL	0.0969	1	0.7556
Distance	0.4115	1	0.5212
Hydration × Species	1.0886	2	0.5802
SLOPE OF PERFORMANCE			
Hydration	16.6373	1	**<0.0001**
Species	0.6622	2	0.7181
SVL	0.5904	1	0.4423
Hydration × Species	2.5475	2	0.2798

Significant probability values are shown in bold typeface.

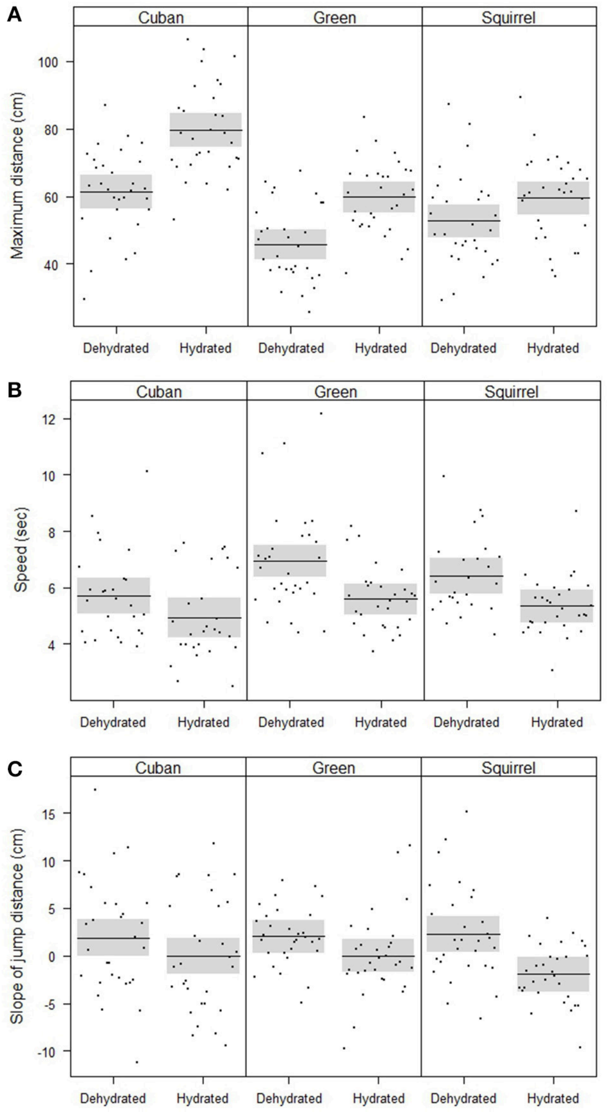

FIGURE 2 | Partial residual plots with means (lines) and 95% confidence bands (shaded), controlling for all factors in the statistical models that are not shown in the figures, showing the locomotor performance of frogs, including **(A)** the significant interaction between species and hydric state on the maximum jump distance, **(B)** significant effects of species and hydric state on speed, and **(C)** the significant effect of hydric state on the change in jump distance across five jumps (slope of jump distance vs. jump number).

susceptible to dehydration by experiencing the highest rate of evaporative water loss and lowest rate of water uptake. The green treefrog was the most resistant to dehydration by losing water the slowest and gaining water the fastest, and the squirrel treefrog was intermediate in rates of water loss and gain. Our water loss findings match those of a study that showed that green treefrogs had the lowest rate of water loss of five arboreal species, including Cuban treefrogs (Wygoda, 1984). However, results from these two studies contradict another study that found that green treefrogs had the highest rate of water loss of six arboreal species, including squirrel treefrogs (Barbeau and Lillywhite, 2005). Native treefrogs in Florida secrete waterproofing lipids from specialized glands and use wiping movements to spread them over their bodies, which reduces evaporative water loss (Barbeau and Lillywhite, 2005). It is not known whether Cuban treefrogs produce lipids or use wiping behaviors, but they have co-ossification of the cranial skin that is thought to reduce evaporative water loss from the head (Seibert et al., 1974). By occupying small refugia and using their casqued heads to seal the entrances (de Andrade and Abe, 1997; Navas et al., 2002), Cuban treefrogs can reduce water loss through a combination of their physiology and behavior. When frogs were placed in front of a fan during dehydration trials, the amount of time spent in the water-conserving posture was lowest in Cuban treefrogs, highest in green treefrogs, and intermediate in squirrel treefrogs. This suggests that species with a higher physiological tolerance to dehydration may rely more on their physiology to withstand dry conditions, whereas species that are less tolerant of dehydration may be more likely to avoid dry conditions by seeking out more favorable microhabitats.

Because Cuban treefrogs had the highest rate of evaporative water loss and lowest rate of water uptake of the species we examined, they may be less tolerant of dry conditions than other frogs of similar sizes. Species that inhabit arid habitats typically lose water more slowly and absorb water more rapidly than species associated with more mesic habitats (Warburg, 1965; Walker and Whitford, 1970; de Andrade and Abe, 1997; Navas et al., 2004; Young et al., 2005). This might explain why Cuban treefrogs are more abundant in wetlands and hardwood hammocks than in pine forests in Florida, and why they are strongly associated with urban areas in their introduced and native ranges (Meshaka, 2001; Johnson, 2007). Urban areas provide greater water availability than many natural areas due to artificial sources of water, such as ornamental ponds and lawn/garden irrigation. When Cuban treefrogs were displaced to old-field habitat in another study, they rapidly moved to nearby hardwood hammock or bottomland forest, which also suggests that they prefer forested areas over more open areas (McGarrity and Johnson, 2010). In addition to providing more moisture, habitats that Cuban treefrogs prefer also provide more potential refugia than non-preferred habitats. Cuban treefrogs' high affinity for refugia (Meshaka, 2001) enables them to invade urban areas and forested areas with high structural complexity and abundant refugia, such as tree hollows. Among the native frogs we examined, we found that squirrel treefrogs lost water more quickly and absorbed water more slowly than green treefrogs. Squirrel treefrogs have been observed using refugia that were more enclosed than refugia used by green treefrogs (Goin, 1958), and squirrel treefrogs also used PVC pipe refugia with a smaller diameter more often than green treefrogs (Zacharow et al., 2003), which may reflect their higher susceptibility to dehydration.

Dehydration affected the locomotor performance of all species by decreasing the maximum jump length, decreasing speed, and increasing (rather than decreasing) jump lengths over time, which is unlikely to indicate an increase in endurance over time and may instead represent a reluctance to move as the trial began(**Figure 2**). These changes in the movements of dehydrated frogs can compromise foraging efficiency and predator escape abilities, and consequently, can reduce fitness (Wassersug and Sperry, 1977; Walton, 1988; Miles, 2004; Husak, 2006). Frogs that jump farther and faster should be more likely to escape from common predators, such as snakes and wading birds. Higher-performing frogs should also be more successful at capturing invertebrate prey (Preest and Pough, 2003). Of the three species we examined, the jumping abilities of Cuban treefrogs and green treefrogs were the most sensitive to dehydration, with maximum jump lengths of dehydrated frogs decreasing by nearly twice as much as those of dehydrated squirrel treefrogs (**Figure 2**). The poorer locomotor abilities of Cuban treefrogs when dehydrated, relative to some native frogs, could contribute to their limited abundance in more open habitats (Meshaka, 2001), in combination with other physiological and behavioral factors.

Overall, we found that Cuban treefrogs were more susceptible to dehydration than similar-sized native treefrogs by losing water more rapidly and absorbing water more slowly. Furthermore, the locomotor performance of Cuban treefrogs was more compromised by dehydration than one of two native species. However, many Cuban treefrogs may be able to compensate for these effects in nature due to their large body sizes and superior jumping abilities. We used frogs of similar sizes in our study to investigate innate differences in water balance and dehydration sensitivity among species, rather than size-related differences. However, adult Cuban treefrogs can grow to be much larger than native treefrogs in Florida (50–100+ mm SVL, depending on sex and locality; Meshaka, 2001; McGarrity and Johnson, 2009), so even though they lost water more rapidly than the native species of the same size, larger frogs should lose water more slowly than smaller frogs due to a smaller surface-area-to-volume ratio. Larger frogs also jumped farther than smaller frogs, but even when controlling for body size, we found that Cuban treefrogs had stronger jumping abilities than the native frogs. Although dehydration had a stronger effect on the jumping performance of Cuban treefrogs than squirrel treefrogs, the maximum jump lengths and speed of dehydrated Cuban treefrogs were similar to those of both native frogs when they were fully hydrated (**Figure 2**). Therefore, the large body sizes and tremendous jumping performance of Cuban treefrogs may allow them to at least partly compensate for the effects of dehydration. However, any decreases in the jumping performance of dehydrated Cuban treefrogs should reduce their competitive advantage and place them on a more level playing field with native species.

In conclusion, we found that Cuban treefrogs were more susceptible to dehydration than two native competitors and their locomotor abilities were more sensitive to dehydration than one of two native species. The relatively poorer water balance of Cuban treefrogs could, in part, explain their lower abundance in more open habitats (Meshaka, 2001) and could affect their survival and activity during periods of drought. Native frogs may experience stronger competition from Cuban treefrogs in more mesic areas, such as urban areas and densely forested areas, which supports anecdotal reports that native frogs have declined in these areas (Meshaka, 2001; Johnson, 2007; Rice et al., 2011). Eliminating or reducing artificial sources of water (e.g., ornamental ponds and lawn/garden irrigation) and potential refugia (e.g., open pipes, pots, and other debris) may help reduce the abundance of Cuban treefrogs in urban areas, which could potentially reduce competition with native treefrogs. Although the geographic distribution of Cuban treefrogs in Florida appears to be limited primarily by low temperatures at the northern edge of their range (Rödder and Weinsheimer, 2009), water availability may also affect their distribution and abundance on a local scale. Selective pressure in dry habitats or during droughts could facilitate adaptations to improve water balance and maintain locomotor performance over a larger range of hydric states, as occurred in invasive cane toads when they invaded an arid region of Australia (Tingley et al., 2012). Our study demonstrates how a physiological mechanism may reduce the spread of a non-native species and its impacts on native species. Studies such as ours illustrate the role that physiology may play in reducing the threat of invasive species and, thus, in conserving natural resources.

DATA AVAILABILITY

The raw data supporting the conclusions of this manuscript will be made available by the authors upon request.

AUTHOR CONTRIBUTIONS

ER and SJ designed the study. All authors collected the animals. ER and CR-B conducted the experiments. ER analyzed the data, prepared the figures, and wrote the first draft of the manuscript. All authors edited the manuscript.

FUNDING

This research was funded by the Memphis Zoo and University of Florida.

ACKNOWLEDGMENTS

We thank Sasha Benitez and Mari Ehlers for assistance with the experiments and frog husbandry, and Andrea Lucky, Rick Owen, and Perran Ross for allowing us to collect frogs from their properties. We are grateful to Brett Scheffers for lending us a balance. This study was conducted under permit LSSC-17-00076 issued by the Florida Fish and Wildlife Conservation Commission.

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Conflict of Interest Statement: 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 handling Editor declared a past collaboration with one of the authors, ER.

frontiers
in Ecology and Evolution

ORIGINAL RESEARCH
published: 12 December 2018
doi: 10.3389/fevo.2018.00205

Quantitative Behavioral Analysis of First Successful Captive Breeding of Endangered Ozark Hellbenders

Rachel A. Settle[1†], Jeffery A. Ettling[2†], Mark D. Wanner[2], Chawna D. Schuette[2], Jeffrey T. Briggler[3] and Alicia Mathis[1*]

[1] Department of Biology, Missouri State University, Springfield, MO, United States, [2] Department of Herpetology, Saint Louis Zoo, St. Louis, MO, United States, [3] Missouri Department of Conservation, Jefferson City, MO, United States

OPEN ACCESS

Edited by:
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Andy Gluesenkamp,
San Antonio Zoo, United States

***Correspondence:**
Alicia Mathis
aliciamathis@missouristate.edu

†Present Address:
Rachel A. Settle,
Florida Fish and Wildlife Conservation
Commission, Naples, FL,
United States
Jeffery A. Ettling,
Sedgwick County Zoo, Wichita, KS,
United States

Specialty section:
This article was submitted to
Behavioral and Evolutionary Ecology,
a section of the journal
Frontiers in Ecology and Evolution

Received: 19 July 2018
Accepted: 14 November 2018
Published: 12 December 2018

Citation:
Settle RA, Ettling JA, Wanner MD,
Schuette CD, Briggler JT and Mathis A
(2018) Quantitative Behavioral
Analysis of First Successful Captive
Breeding of Endangered Ozark
Hellbenders. Front. Ecol. Evol. 6:205.
doi: 10.3389/fevo.2018.00205

Understanding behaviors associated with reproductive events is vital to management of captive breeding programs for threatened and endangered species. The Ozark hellbender (*Cryptobranchus alleganiensis bishopi*) is a federally endangered aquatic salamander with only one successful captive breeding program (the Saint Louis Zoo's Ron Goellner Center for Hellbender Conservation). Although anecdotal observations have been reported for hellbender reproductive behavior from field observations, no quantitative assessments have been made. We quantified hellbender behavior from video-recordings of three successful breeding events at the Saint Louis Zoo that occurred in 2012, including aggressive, sexual, social, and locomotory behaviors. We used transition matrices to organize these data into kinematic diagrams that illustrated behavioral sequences for five time periods: pre-oviposition (2 nights), first oviposition night, inter-oviposition night, second oviposition night, and post-oviposition. General activity and agonistic behaviors increased moderately through the first oviposition night, peaked during inter-oviposition, and declined abruptly following the second oviposition night. Agonistic behavior included bites, charges, chases, and flight. Female-female aggression was common. Surfacing (presumably for accessory air breathing) followed intense activity. Presumed courtship behaviors (tail swishing and circling) occurred at low rates. During oviposition, females remained in the nest box for 1–2+ h. We encourage managers of captive breeding programs to use quantitative behavioral analyses to pin-point critical time periods and conditions for successful reproduction.

Keywords: reproduction, kinematic analysis, captive breeding, endangered species, hellbender

INTRODUCTION

Captive breeding and subsequent reintroduction can be an important tool in conservation of declining populations (Griffiths and Pavajeau, 2008), particularly when the reason for the decline is unclear or unresolved. Zoos, aquariums, and other ex situ breeding facilities frequently do an excellent job of developing ethograms (lists and descriptions of behavior), which can be helpful in design and implementation of captive breeding programs (e.g., Stanton et al., 2015). Quantitative studies of behavior of animals in captivity are less common (Maple and Segura, 2015), but these detailed analyses can lead to improved captive breeding success. For example, due to expense, space, and availability of reproductive adults, captive breeding efforts often are made only between assigned pairs of males and females, but quantitative behavioral studies showed that mating can be enhanced when females are allowed to choose their mating partners (Martin-Wintle et al., 2015; Hartnett et al., 2018). Behavioral studies have also helped to define receptivity periods for species in captivity (e.g., duck-billed platypus *Ornithorhynchus anatinus*, Hawkins and Battaglia, 2009; collared peccary, *Pecari tajacu*, da Silva et al., 2016), which helps program directors to better target breeding efforts.

Historically, captive breeding efforts have focused on large, charismatic species, particularly mammals (Leader-Williams and Dublin, 2000). As species are added to threatened and endangered lists at an unprecedented rate, captive breeding efforts are expanding to include many nontraditional species, including fishes, amphibians and invertebrates, which have added benefits of often requiring less space, having higher birth rates and being easier to reintroduce than larger fauna (Keulartz, 2015). Amphibians, in particular, have received increasing attention (Griffiths and Pavajeau, 2008; Harding et al., 2015; Murphy and Gratwicke, 2017) due to the rapid widespread severity of their population declines (41% of amphibian species listed as threatened with extinction by the IUCN: https://www.iucn.org/theme/species/our-work/amphibians). However, studies of behavior related to captive breeding of amphibians are not as well developed as for taxa with a longer history of ex situ breeding efforts.

About half of salamanders (Amphibia: Urodela) are considered by the IUCN to be threatened or extinct. One family of particular conservation concern is the Cryptobranchidae, which contains the world's largest extant salamanders and which is represented by only two genera, *Andrias* in Asia and *Cryptobranchus* in the United States. All species of these fully aquatic salamanders are threatened or endangered (Browne et al., 2014). Generally, captive breeding efforts have been more successful for *Andrias* (Kuwabara et al., 1989) than for *Cryptobranchus,* which has had only one known successful breeding program (the Ron Goellner Center for Hellbender Conservation at the Saint Louis Zoo: Ettling et al., 2013).

Two subspecies are currently recognized within the genus *Cryptobranchus*, the Eastern (*Cryptobranchus alleganiensis alleganiensis*) and Ozark (*C. a. bishopi*) hellbenders, although both are paraphyletic (Crowhurst et al., 2011; Tonione et al., 2011). The Ozark subspecies is listed as federally endangered in the United States (USFWS, 2011), and the Eastern hellbender (*Cryptobranchus alleganiensis alleganiensis*) is currently petitioned to be listed as threatened or endangered under the Endangered Species Act (USFWS, https://ecos.fws.gov/ecp0/profile/speciesProfile?spcode=D043). A Population and Viability Assessment indicated a high probability of extinction within 75 years without significant intervention, including captive propagation (Briggler et al., 2007; Ettling et al., 2017).

Hellbenders are exceptionally long-lived for amphibians, with a lifespan of over 50 years (Nickerson and Mays, 1973). These large salamanders are habitat specialists, requiring clear, cool, fast-flowing water with rocky substrates (Nickerson and Mays, 1973). During a short breeding season (several weeks), males aggressively defend spawning sites under rocks or within bedrock, court females, and guard eggs after spawning. The cause(s) for the decline have not been specifically identified, although numerous factors have been suggested, including river sedimentation/siltation and changes in electrical conductivity due to deforestation, pollution from run-off, increased predation from introduced or reintroduced species, amphibian chytrid fungus infections, and over-collection (Nickerson and Briggler, 2007; Briggler et al., 2008; Gall and Mathis, 2011; Nickerson et al., 2017; Pitt et al., 2017).

As part of a strategy to combat the decline of hellbenders, captive rearing efforts were initiated at the Saint Louis Zoo's (SLZ) Ron Goellner Center for Hellbender Conservation (RGCHC) and the Missouri Department of Conservation's (MDC) Shepherd of the Hills Fish Hatchery in Branson, Missouri. Both programs have successfully hatched eggs collected from naturally-occurring nests and reared larvae for release in the wild (Briggler, 2007; Briggler et al., 2011; Crowhurst et al., 2011; Bodinof et al., 2012).

Captive breeding of hellbenders proved to be more difficult. In 2011, a conservation milestone was reached when the RGCHC, in collaboration with MDC, reported the first successful breeding of Ozark hellbenders in captivity (Ettling et al., 2013). The SLZ continued to successfully breed Ozark hellbenders each year between 2011 and 2016 (Briggler, 2007; Briggler et al., 2011; Ettling et al., 2017).

The success of the captive breeding program at RGCHC appears to be largely attributable to use of artificial breeding streams that closely mimic natural conditions, including temperature, photoperiod, precipitation, water quality, and prey availability (Ettling et al., 2013). Adjusting the ionic composition (total dissolved solids) and the introduction of artificial nest boxes were likely major contributing factors to the success of fertilized clutches (Ettling et al., 2013). At the time of the first successful breeding events, an indoor artificial stream was outfitted with a four-camera surveillance system that recorded hellbender activity around the clock.

In this study, we provide an analysis of the video recordings of the behavior of the hellbenders during the three successful sequential oviposition events of 2012, culminating in kinematic diagrams of sequences of behavior that occurred before, during, between, and immediately after the successful reproductive events. Although there have been numerous anecdotal descriptions of reproductive events in the wild (Smith, 1907; Huheey and Stupka, 1967; Floyd and Unger, 2016), there has not been a systematic ethological analysis of the steps involved in courtship and mating. Herein, we (a) describe the behaviors that we observed during pre-oviposition, oviposition, inter-oviposition and post-oviposition periods, (b) quantify the frequency of each behavior during each period, and (c) use transition matrices to describe sequences of behavior. These observations will help to identify social interactions and other behaviors that contributed to successful captive breeding and help to identify behaviors that signal that reproduction is imminent and that signal transitions between sequential breeding periods.

METHODS

Broodstock and Artificial Stream

The successful breeding events occurred at the RGCHC in an indoor artificial stream (9.7 m × 1.7 m × 0.6 m; **Figure 1**) containing five male and three female adult Ozark hellbenders.

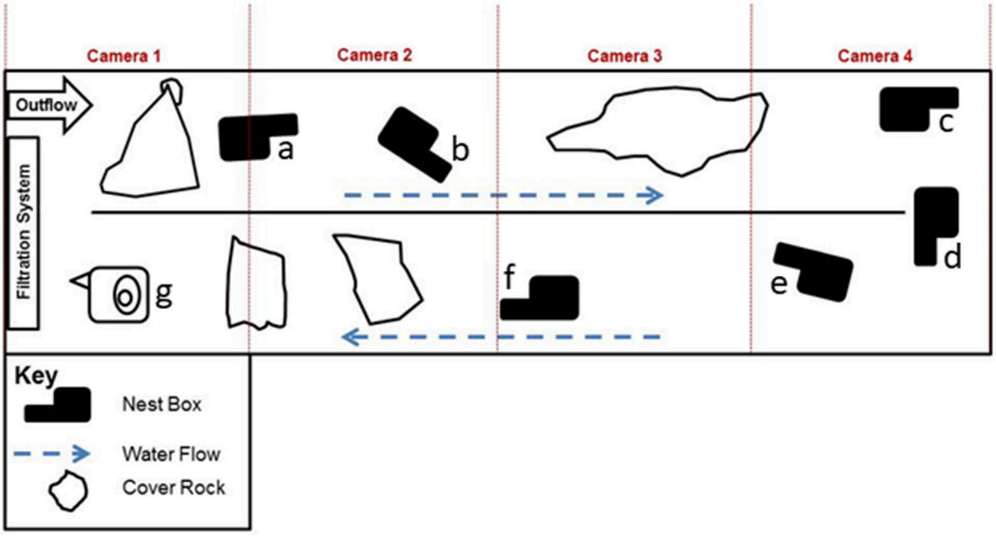

FIGURE 1 | Diagram of the artificial indoor stream (9.7 m × 1.7 m × 0.6 m), including nest box **(a–g)** and cover rock locations, at the Saint Louis Zoo Herpetarium. For boot-shaped nest boxes **(a–f)**, the neck of the boot is the entrance chamber and the foot of the boot is the nest chamber. Nest box **(g)** is of an older, non-boot, design and was not used during any oviposition events.

Broodstock (**Table 1**), collected from the North Fork of the White River, Ozark County, Missouri, were added to the indoor artificial stream at the time of collection and were kept in the stream until after the successful breeding events reported in this study. The excess number of males vs. females was used to provide increased opportunities for mate selection by females. For details about quarantine and husbandry protocols, see Ettling et al. (2013). All males had the typical donut cloacal swelling that indicates reproductive condition. The range of male sizes (**Table 1**) was chosen to maximize the probability of healthy sperm by including a range of individuals from small/young (near lower-end of sexual maturity) to large/old (near high end of size range) (e.g., Peterson et al., 1983). Females all exhibited abdominal swellings consistent with egg production.

The stream was a closed recirculating system with water flow in a circular direction at 227 L/min at an average depth of 0.3 m. Mechanical and biological filtration together with ultraviolet sterilization helped to maintain water quality, and reconstituted reverse osmosis water was used for water changes. Year-round light:dark cycles, water temperature, water quality and precipitation events were selected to mimic values that occurred in natural habitats in the river of origin. A chiller was used to manually adjust temperatures each day to match data recorded by data loggers in the river of origin; annual temperatures ranged from 4.4 to 22.2°C. Total dissolved solids were also kept similar to natural river water at 175–300 mg/L because related characteristics, such as salinity and osmolality, can influence sperm motility in some aquatic species (Alavi and Cosson, 2006; Bonislawska et al., 2015). Data for other measures of water quality (pH, nitrates, nitrites, ammonia, phosphates, dissolved oxygen) are provided by Ettling et al. (2013). A manual sprinkler system plus adjustment of water levels was used to mimic natural precipitation, and photoperiods were adjusted daily via an automatic timer. The floor of the artificial stream was covered with river gravel (10.2–15.2 cm), and a variety of large (approximately 0.2–0.7 m) sandstone and moss-covered rocks were scattered over the gravel. Crayfish (*Orconectes* spp., *Procambarus* spp.), darters (*Etheostoma* spp.), sculpins (*Cottus bairdi*, *Cottus carolinae*), and shiners (*Notropis* spp.) were collected from various streams near the St. Louis, MO, area and introduced into the stream as a source of natural forage.

Artificial nest boxes ($n = 7$) were positioned in the stream (**Figure 1**) with the open end of the entrance tunnel of the boxes facing downstream of water flow. As described in Briggler and Ackerson (2012), nest boxes were constructed with a chicken-wire base frame covered with hardware cloth and a concrete/sand mixture. All but one of the nest boxes were a modified "boot" design, with an entrance tunnel ("leg" of the boot; ∼27 tunnel length × 7.3 entrance height × 10 entrance width cm) connected to a nesting chamber ("foot" of the boot, ∼ 39 × 31 cm). An opening with a removable lid was made on the surface of the nesting chamber so that eggs deposited inside the chamber could be monitored periodically with minimal disturbance. The seventh box (**Figure 1**, g) was an older non-"boot" design and was not used during any of the oviposition events.

A four-camera (**Figure 1**) infrared video recording system positioned directly above the stream monitored the hellbenders between 20:00 and 08:00 h daily because hellbenders are primarily nocturnal (Noeske and Nickerson, 1979; Coatney, 1982). Video recordings were archived to computer hard drives at the RGCHC.

Behavioral Sequence Data Collection and Analysis

Our analyses are based on video data collected from 21 to 26 September 2012, during which time three oviposition events occurred (Ettling et al., 2013). We quantified the behavior of

TABLE 1 | Collection and demographic data for hellbender broodstock.

Collection date	Sex	Snout-Vent Length (cm)	Mass (g)
2004	F	32.0	713
2004	M	25.5	444
2004	M	34.0	596*
2005	F	33.0	907
2005	F	36.0	995
2007	M	30.0	569
2007	M	33.0	815
2011	M	29.5	552

**Missing both hind limbs.*

TABLE 2 | Ethogram of behaviors recorded during video observations.

Behavior	Description
AGONISTIC	
Bite	One hellbender bites or snaps at another
Charge	A hellbender swims toward another at a noticeably increased swimming speed
Chase	One hellbender follows another
Flee	An individual quickly swims away from another (flight)
SOLITARY/LOCOMOTORY	
Surface	Hellbender contacts surface with any part of body
Swim	Wave-like movements of the tail propel the body forward, and limbs are not in contact with the substrate
Walk	Hellbender moves forward while limbs are in contact with substrate
SEXUAL	
Oviposition	Female deposits eggs
Circle	Hellbender swims in tight circle near another who may or may not perform circling at the same time
Tail Swish	Male swishes tail laterally while stationary
SOCIAL	
Approach	One hellbender moves to within 0.5 m of another without changing swimming speed
Nose-to-nose	Individuals touch or nearly touch their noses while stationary

the hellbenders on the night before the first oviposition to illustrate “pre-oviposition behavior”; qualitatively, the behavior on this night was similar to the behavior on the preceding three nights (personal observations). The first oviposition night (two oviposition events) occurred on 22 September 2012 and the second oviposition night (one oviposition event) occurred on 24 September 2012. The night between the two oviposition events (23 September 2012) was categorized as “Inter-oviposition” behavior. Post-oviposition behavior was quantified for 2 days following the last oviposition event.

Each night’s videos (4 videos × 12 h) were viewed in their entirety using Milestone XProtect® Smart Client 2013 R2 – Player v. 8.1b. The hellbender keepers at the zoo developed a list of behaviors that they observed during their daily surveys, and this list formed the basis of the ethogram (list of species-specific behavior describing the elements and putative function of each behavior) (**Table 2**) we used in this study. Behaviors were categorized as “agonistic,” “solitary/locomotory,” “sexual,” or “social.” We recorded every occurrence of any of the defined behaviors, the location of the behavior (camera number, nest box number, etc.), and, when possible, the sex of the individual. An individual’s sex was identified based on physical features unique to that individual, and these features were not always visible on the video; we estimate that we were unable to identify the individuals, and, thus, their sex, for about 10% of observations.

We defined a behavioral sequence as beginning when one or multiple individuals performed any of the defined behaviors (**Table 2**) and ending when the hellbender(s) was/were inactive for a period of 5 min, began a new defined behavior, or when the individual(s) entered a next box or other cover object (i.e., natural rock). We calculated transitional probabilities (the probability that one behavioral pattern follows another) through the use of transition matrices (Martin and Bateson, 2007), which were calculated for all individuals combined. The columns and rows of the matrix consisted of all behavioral patterns, and the numbers in each cell were the percentage of times that the first behavioral pattern (rows) was followed by the second behavioral pattern (columns). We illustrated the transition probabilities using kinematic graphs (flow diagrams) (Lehner, 1996). Separate transitional matrices and kinematic diagrams were made for the periods of pre-oviposition, first oviposition night, inter-oviposition, second oviposition night, and post-oviposition (2 nights).

Consider the following two examples of sequence scenarios. The first example is one sequence comprised of four sequential behaviors: Hellbender A (1) Walked out of a nest box onto a rock. He (2) Approached and (3) Bit Hellbender B, while Hellbender B (4) Fled. The second example is comprised of two sequences, with the first consisting of one behavior only and the second consisting of four behaviors: Hellbender A (1) Walked, rested for 5 or more min, (1) Walked, (2) Approached Hellbender B, (3) Bit Hellbender B and caused Hellbender B to (4) Flee.

Transitional sequences between any two specific behaviors did not occur with sufficient frequencies for statistical analysis. However, we increased sample sizes by combining behaviors into functional categories so that we could address two questions. First, does Approach lead to a higher proportion of interactive behaviors (e.g., combined Bite, Flee, Swim, Oviposition, additional Approaches) than non-interactive behaviors (walk)? Second, does Surfacing follow a greater proportion of high-activity behaviors (e.g., combined Swim, Chase, Flee) than low-activity behaviors (e.g., Walk). These two comparisons were made via two-tailed Binomial tests (Minitab, v. 16). Note that each event was treated as a unique data point even though the same individual hellbender may have initiated multiple events.

RESULTS

In our artificial streams, individuals commonly shared cover objects, including nest boxes, prior to the breeding season, but exclusive residency occurred as the breeding period approached (Ettling et al., 2013). The reproductively successful males in our study had become established in their nest boxes before we began our observations. However, all males did not engage in nest box defense, and nest boxes were sometimes occupied by single females. Hellbenders were generally most active during 01:00–07:00 h, and oviposition occurred between 02:00 and 07:00 h, with females remaining in the nest box with the male for 65 min to over 2 h. For the results below, the transitional matrices used to construct kinematic diagrams are in **Supplementary Material**.

Pre-oviposition Night

On the night before oviposition (21 September), most behaviors were Solitary/locomotory (**Figure 2A**). Almost all (97%) of these locomotory movements were Walking, with the rest being Swimming (**Figure 2A**). Only one agonistic sequence was recorded during the pre-oviposition period. This sequence was initiated by a hellbender Approaching another hellbender and Biting it, resulting in the bitten hellbender Fleeing. No sexual behaviors (Tail swish, Circle) were observed during the quantified pre-oviposition period (21 September 2012) or during our observations of the videos for 18–20 September 2012, which are not included in **Figure 2A**.

First Oviposition Night

During the first oviposition night (22 September), the overall level of activity was higher and the diversity of behavior increased to include all behavioral categories (agonistic, solitary/locomotory, reproductive and social) (**Figure 2B**). Solitary/locomotory behavior continued to be the most frequently performed behavior, but, in comparison to the previous night, the frequency of Walking decreased by about 50%, from 103 to 54 instances, and Swimming behavior increased by a factor of 8 (from 3 to 25 instances). Agonistic behavior also increased in frequency, with Biting occurring five times, Fleeing occurring three times, and the first occurrences of Chasing. Surfacing behavior was also observed for the first time during this event (four times).

Some patterns in behavioral sequences were apparent (**Figure 2B**). Although Bites sometimes (1/5) led to Circling behavior, Circling did not lead directly to escalated agonistic or sexual interactions, but only to more circling (1/5) or locomotory behaviors (4/5). Flight resulted only from either Bites (2/3) or Chases (1/3). Approach led to swimming (1/8) or the intense social interactions of Biting (3/8), Nose-to-nose (2/8), and Oviposition (2/8) (Interactive vs. Noninteractive, $Z = 1.76$, $P = 0.078$). Surfacing events only followed the high-activity behaviors of Swimming (2/4) and Chasing (2/4).

The two oviposition behaviors during the first oviposition night occurred as follows. After two females Approached a nest box **(Figure 1)**, nest box e containing a male, one female Bit the other, and the bitten hellbender Fled away from the nest box while being chased. The female that initiated the bite then slowly entered the nest box (∼02:00 h). She stayed inside the nest box for approximately 120 min and exited without any indication of coercion by the male. After approximately 90 min, the second female Approached and entered the nest box (∼06:00 h) and stayed inside the nest box until the video stopped recording (08:00 h). The male did not leave the nest box after oviposition occurred.

Inter-Oviposition

During the Inter-oviposition period (23 September), locomotory behavior occurred at the highest frequencies of the entire data collection period. Walking initiated behavioral sequences 330 times, and Swimming initiated sequences 110 times (**Figure 3A**). In addition, an increased number of interactions between hellbenders were observed. Approach ($n = 30$) almost always led to interactive events (20 Bites, 5 Flees, 2 additional Approaches; interactive vs. noninteractive: $Z = 4.20$, $P < 0.001$) (**Figure 3A**). Agonistic behaviors also occurred at the highest frequencies during the Inter-oviposition period: 20 Bites, 23 Flees, 23 Chases, and 2 Charges. Charges always led to Walking and Biting always led to Fleeing. Female-female aggression occurred in 55% of the aggressive acts, with male-male (30%) and female-male (15%) aggression explaining the remainder. Tail swishing ($n = 1$) and Circling ($n = 6$) were the only sexual behaviors to occur, and both of these behaviors led only to locomotory behaviors. As in the previous night, Surfacing followed high-activity behaviors (Flee, Swim) or other Surfacing (high-activity vs. low activity: $Z = 2.12$, $P = 0.034$). During this period, the male defending the nest box with eggs briefly emerged a few times, but generally did not engage in aggressive acts.

Second Oviposition Night

Although locomotory behaviors were not as frequent during the second oviposition night (24 September) as during the Inter-oviposition period, locomotory behavior was still moderately frequent and initiated behavioral sequences at a higher rate than on the first oviposition night (Walk, increase of 257%; Swim, increase of 72%) (**Figure 3B**). Agonistic sequences occurred, but at a lower frequency than the previous night and similar to that during the first oviposition night. Behavioral transition sequences showed some similar patterns as observed during the first oviposition night. With one exception, Surfacing events only followed the high-activity behaviors of Fleeing and Chasing (High-activity vs. Low-activity: $Z = 2.41$, $P = 0.020$). Flight continued to result only from Bites or Chases. The most intense interactions of Biting and Oviposition followed from Approach behavior (Interactive vs. Noninteractive: $Z = 1.76$; 0.042). In general, sexual behavioral transitions were less complex than those occurring in the first oviposition night; neither Circling nor Nose-to-nose behaviors were observed during this oviposition event.

The oviposition activity during the second oviposition night was less complex than in the first oviposition night. The remaining non-spent female approached a separate nest box (**Figure 1**, nest box a) that was occupied by a different male than the male that fertilized both clutches on the first oviposition night. After Approach, the sequence of behavior by the female was: Walk, Walk, Walk, Swim, Surface, Walk, Swim, Swim, Walk, Walk, Walk, Approach, Walk, Approach, Oviposition (06:00 h). The female stayed within the nest box for approximately 65 min and slowly exited after Oviposition. The male remained within the nest box, and so we could not observe his behavior.

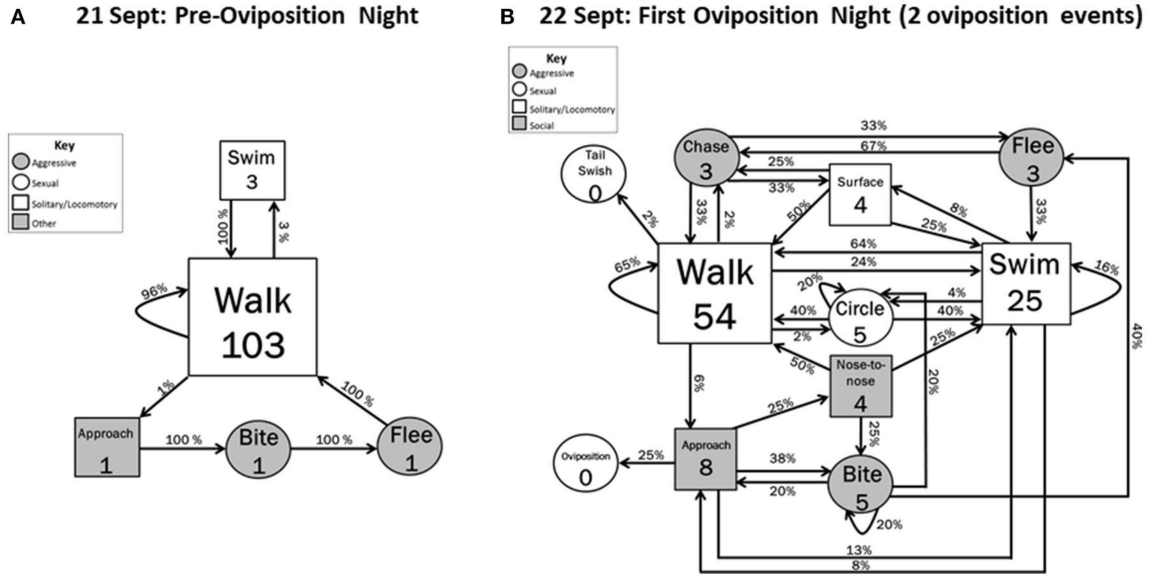

FIGURE 2 | Behavioral transitions during **(A)** the night before the oviposition events occurred and **(B)** the first night of oviposition when 2 oviposition events occurred. Frequency of initiating behavioral actions (inside boxes) and % time initiated action was followed (arrow) by other actions are indicated. Zeros indicate that the behavior occurred but did not initiate a sequence within the 5 min designated time frame. Shape sizes are indicators of the relative frequency in which the initiating behavior occurred (e.g., larger boxes indicate the behavior occurred more often than smaller boxes).

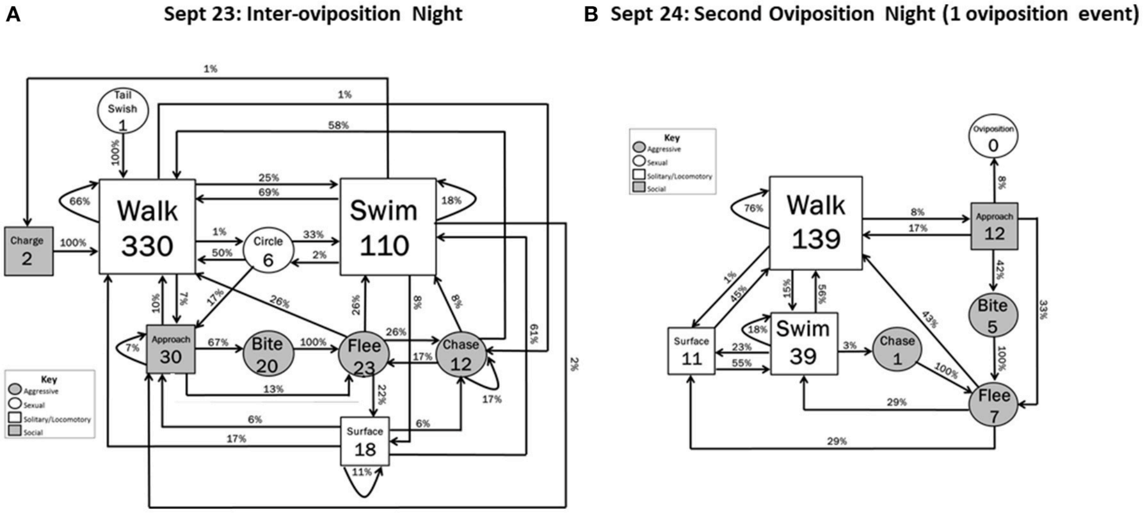

FIGURE 3 | Behavioral transitions during **(A)** the night between the two oviposition and **(B)** the second oviposition night when 1 oviposition event occurred. Frequency of initiating behavioral actions (inside boxes) and % time initiated action was followed (arrow) by other actions are indicated. Zeros indicate that the behavior occurred but did not initiate a sequence within the 5 min designated time frame. Shape sizes are indicators of the relative frequency in which the initiating behavior occurred (e.g., larger boxes indicate the behavior occurred more often than smaller boxes).

Post-oviposition

The post-oviposition period began on 25 September 2012 and ended on 26 September 2012. This period was characterized by an abrupt decrease in frequency of all behaviors, with only Solitary/locomotory behavior exhibited. On the first night post-oviposition, Walking was the only behavior exhibited, and it occurred only four times (**Figure 4A**). To determine whether this very low level of activity continued, we also quantified behavior on the second night post-oviposition; 14 instances of Walking and one of Swimming occurred (**Figure 4B**).

Summary Comparisons Across Nights

Figure 5 shows a clear pattern of behavioral changes across the six nights of the study. Frequencies of all behavioral categories were relatively low during pre-oviposition. Although the total number of behavioral events remained relatively low on the first

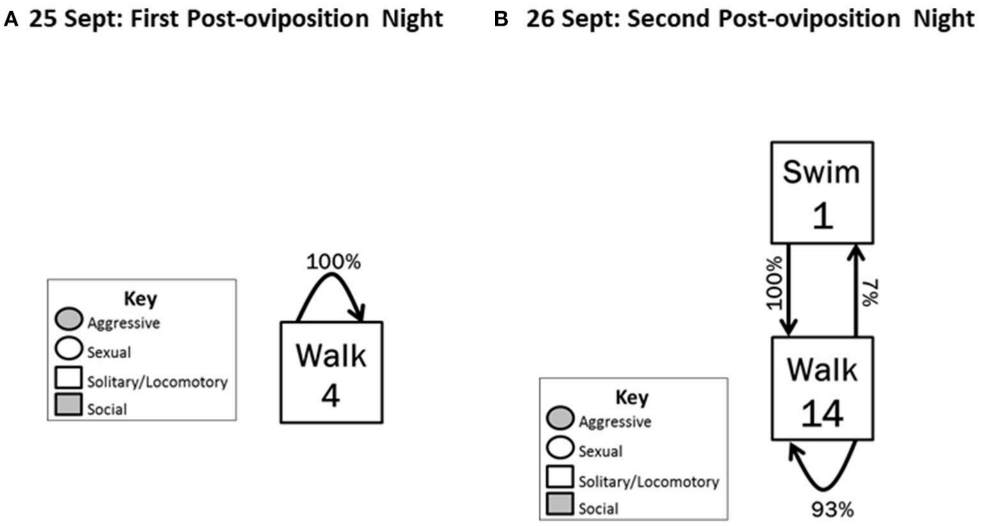

FIGURE 4 | Behavioral transitions during **(A)** the first night post-oviposition and **(B)** the second night post-oviposition. Locomotory behaviors were followed (arrows) by only other locomotory behaviors. Frequency of initiating behavioral actions (inside box) and the % time the initiated action was followed (arrow) by another action are indicated.

oviposition night, there was a shift to include more agonistic and sexual/social behaviors. By far, the highest level of all activity was on the inter-oviposition night, with an approximately 5-fold increase in all categories of behavior from the previous night. On the second oviposition night, the frequency of behavioral events decreased to only about 2× that of the first oviposition night. On the two post-oviposition nights, the frequency of behavior dropped abruptly to below that of the pre-oviposition night, and the only behavior that occurred was solitary/locomotory (mostly walking).

DISCUSSION

Descriptions of the reproductive behavior of hellbenders is limited both in natural habitats due to their secretive nature, and in captivity, where the first successful reproductive event occurred relatively recently (Ettling et al., 2013). This study provides the first quantitative ethological analysis of the behavior of hellbenders immediately prior to, during and after an oviposition event. These data, which were collected from video recordings of the captive reproductive events reported in Ettling et al.'s (2013) study, help to fill in the details of sequences of behavior previously reported in anecdotal field observations. Studies of captive breeding events for threatened and endangered species, including our study, often suffer from low sample sizes due to availability of reproductive individuals and appropriate-sized of enclosures (Snyder et al., 1996). In our study, the minimal information on individual variation of the behaviors due to low sample size limits the strengths of the inferences that can be drawn. However, behavior surrounding our three observed reproductive events were generally consistent, and there were several similarities with some anecdotal observations from nature.

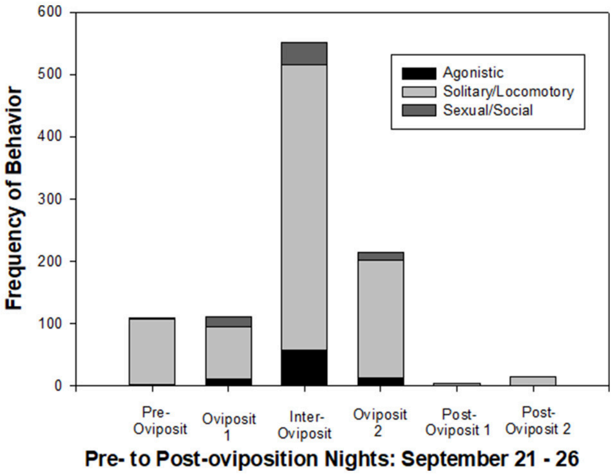

FIGURE 5 | Summary of frequencies of behavioral transitions across the six nights of the study, which included one night each, including pre-oviposition, oviposition night 1 (2 breeding events), inter-oviposition, oviposition night 2 (1 breeding event), and two nights post-oviposition. Agonistic behaviors were Bites, Charges, Chases and Flight. Solitary/Locomotory behaviors were Walking, Swimming, and Surfacing. Sexual and Social behaviors were Ovipositions, Circling, Tail Swishes, Approach, and Nose-to-Nose.

As reported in numerous previous studies (Smith, 1907; Bishop, 1941; Peterson, 1988), males began defending nesting sites prior to the oviposition period. In nature, males typically defend a "den" site consisting of a depression located under a flat cover rock, or within crevices or holes in the bedrock (Bishop, 1941; Pfingsten and Downs, 1989). Although flat rocks were available, the hellbenders in our study defended only the boot-shaped nest boxes. The same type of nest boxes have been successfully used for oviposition by hellbenders in the wild, with success likely due primarily to the ease of defensibility provided by the single, narrow neck opening and the spacious chamber for eggs (Briggler and Ackerson, 2012).

Our findings are consistent with other studies that suggest that aggression increases during the breeding period (Smith, 1907; Peterson, 1988; Foster et al., 2009). Although our data span a limited period (after initial establishment of den sites by males), the increase in aggression we observed was abrupt, with increases in both number and types of overt acts. The night before the first oviposition included only three agonistic acts (one each of approach, bite and flee), whereas the night of the first oviposition included 38 acts of six overt behaviors (chase, flee, nose-to-nose, approach, circle, bite). Locomotory activity also became more intense, with swimming (as opposed to walking) comprising only 3% of pre-oviposition locomotory movements, increasing to 28% on the night of oviposition. It is not known whether the observed increase in aggression and movement intensity is as abrupt in the field or whether the very low level of aggression and movement intensity on the night before oviposition is typical of a more extended pre-oviposition period under natural conditions. In any case, we recommend that managers of captive breeding facilities carefully monitor hellbenders for increased aggression and swimming activity as a possible indicator of imminent oviposition. Continued high levels of activity, including aggression, after one oviposition event, could indicate that additional oviposition events are forthcoming.

Most previously-reported anecdotal field observations of aggression and the apparent territorial spacing of males in the field suggest that aggression has three primary functions: male-to-male competition for breeding sites (Alexander, 1927; Hillis and Bellis, 1971; Nickerson and Mays, 1973), (2) male attempts to coerce females to enter or leave their nest sites or (3) male attempts to protect their eggs from oophagy (Smith, 1907). However, the aggressive acts that we observed in the artificial stream were mostly (55%) female-female, with females apparently competing to occupy the oviposition sites. Female-female aggression associated with reproduction may be more common than previously thought; relatively few overt aggressive acts have been observed in the field, and the contestants are rarely definitively identified with respect to sex (e.g., Nickerson and Mays, 1973). Alternatively, female-female aggression could be a result of the specific conditions/densities within the artificial stream, which could be tested with artificial streams with varying sizes and densities if sufficient numbers of adults in breeding condition were available. The consequences of aggression may be severe. After this breeding period, both males and females in our study had severe lacerations on the limbs, bite marks along the lateral folds, and even lost limbs (Ettling et al., 2013).

It is possible that the dramatic reduction of population sizes of Ozark hellbenders in recent decades (e.g., Wheeler et al., 2003) has also resulted in alterations in the frequency or intensity of aggressive behavior in natural habitats. For example, limitation of available receptive females or fertile males (see Unger and Mathis, 2013) may have resulted in more intense male-male or female-female competition. Alternatively, lower population densities may have led to an overall reduction in aggressive encounters in the wild. The latter seems unlikely since fresh wounds, in at least some cases resembling conspecific bite marks, have been reported in post-decline (∼ early 1980's: Wheeler et al., 2003) populations (Pfingsten, 1990; Wheeler et al., 2003; Miller and Miller, 2005; Williams and Groves, 2014).

The kinematic analysis also allows for inferences about whether there are consistent transitions from one behavior to the next. Although variability of transitions was high, some general patterns were apparent from the data. Not surprisingly, Flight was typically the result of being bitten or chased. Both Swimming and Chasing appear to be energetically costly because they were frequently followed by surfacing behavior, presumably for accessory air breathing. Strenuous activity can lead to respiratory and metabolic acidosis in hellbenders (Boutilier et al., 1980); although hellbenders rely primarily on cutaneous respiration (Guimond and Hutchison, 1973), lung-based respiration may be important for maintenance of sufficient blood oxygen levels during stressful periods. Although we did not measure levels of stress hormones, we hypothesize that corticosterone may increase during reproductive events to mobilize energy for high activity levels, as has been reported for some other salamanders (Reedy et al., 2014). Overall, hellbenders have very low plasma corticosterone levels, but levels rise during periods of acute stress (restraint), and, at least during the early breeding season, males have higher corticosterone levels than females (Hopkins and DuRant, 2011).

Circling behavior, which occurred 11 times, has been reported during courtship in a taxonomically wide range of salamanders (e.g., Plethodontidae: Cupp, 1971; Salamandridae: Bruni and Romano, 2011), frequently leading to oviposition; however, in our observations, circling consistently led only to locomotory behavior or more circling. Approach typically led to physical interactions (bite, nose-to-nose, and oviposition). Tail Swishing by the male was observed on only two occasions and so may not play as strong a role as the tail undulations that are a part of courtship of some other salamander taxa (Houck and Arnold, 2003).

Our set-up had an excess of males to allow females opportunities for mate choice. However, no particular feature stands out as a basis for success. The two successful males were intermediate in size (SVL), and the male that fertilized the third clutch was missing both hind limbs (**Table 1**; see Nickerson et al., 2011 for discussion of recent increases in hellbender abnormalities). Oxygen concentration (see Settle et al., 2018) or other features of the nest box might also be important, but the successful nest boxes were at opposite sides of the artificial stream (**Figure 1**, nest boxes a and e), suggesting general nest box location was not a critical factor.

Two females in our study laid eggs in the same nest box, with fertilization by the same male, and clutches of multiple females in the same nest has also been reported for hellbenders in nature (Nickerson and Mays, 1973). Spawning of several females in one nest site also occurs in the other species in this family, the Asian giant salamanders (*Andrias* sp.: Browne et al., 2014), but the function of this behavior is not known. Generally, such spawning decisions by females could result either from preferred characteristics of the nest site or preferred characteristics of the male (e.g., Refsnider and Janzen, 2010). In any case, we recommend that managers provide females with multiple nest sites and multiple males during the spawning season [see also details in (Ettling et al., 2013)]. In addition to mate choice opportunities, multiple individuals could provide increased concentrations of potential pheromones or reproductive hormones that are released into the water. For example, in lampreys, *Petromyzon marinus*, odors from mature males facilitate sexual maturation for both sexes, attract females, and are important for nest construction and gamete release (review in Buchinger et al., 2015).

CONCLUSIONS

The use of quantitative behavioral data to predict timing of potential reproduction should be useful in captive breeding programs for a wide range of species. In addition, understanding the sequence of events that lead to copulation/oviposition can help managers to pinpoint the point at which failure occurs so that problems can be more effectively addressed. For example, detailed behavioral observations of Giant Pandas at a breeding center near Wolong, China, led to the conclusion that copulation failure was due to lack of motivation by the male (Zhang et al., 2004). Even though mounting successfully occurred, unsuccessful males frequently had improper mounting positions, low persistence, and low penetration success. Mitigation efforts could then be focused on steps to increase the motivation of the male.

For hellbenders, in combination with husbandry details described by Ettling et al. (2013), close monitoring of hellbender behavior during the breeding season can provide clues to the imminent onset of oviposition. The most striking result was the rapid on-set of behavioral changes. We recommend that the behavior of hellbenders in captive breeding programs be monitored closely each night during the breeding season. An increase in surfacing events is easy for even staff with minimal training to detect. Closer observation should reveal increased aggression and other social interactions as well as a substantial increase in the proportion of locomotory events involving swimming as opposed to walking. Such observations allow managers to detect newly deposited eggs early and to intervene if aggression levels are high enough to endanger the lives of the adults. Although the presence of a guarding male undoubtedly increases survival of eggs in natural habitats, we recommend removal of the eggs from the nest for rearing; at the RGCHC, we remove the eggs 14 days after oviposition. Separate rearing allows for the elimination of potential predation, including by the guarding male, and allows for close control of water quality, maintenance of high levels of oxygenation, and removal of eggs that become infected with disease.

Surprisingly, much of the observed aggression in this study of captive individuals was among females, so it is important that females are provided with multiple males and multiple nest sites. Even so, two females in this study spawned in the same nest with the same male. A relatively large space is required for captive-breeding of this species, and having more than one gravid female per breeding stream increases the probability of at least one successful mating event. Moreover, it is not known whether female-female social interactions are important to maintaining normal behavior. However, managers should be aware of the potential cost of the high level of aggression (female-female, male-male, male-female) during reproductive activities, and carefully examine individuals for injuries post-reproduction.

ETHICS STATEMENT

Handling and maintenance associated with captive rearing were performed in entirety by the staff and interns of the Saint Louis Zoo according to Association of Zoos and Aquariums accredited institutional protocol.

AUTHOR CONTRIBUTIONS

RS and AM conceived and conducted the quantification and analyses of data from the video recordings. JB made field collections of the adult broodstock. JE, MW, CS, and JB developed housing and maintenance procedures, cared for study animals, and provided videos. CS contributed to observations that formed the basis of the ethogram.

ACKNOWLEDGMENTS

Support was provided by the Ron Goellner Center for Hellbender Conservation and the Saint Louis Zoo, the Missouri Department of Conservation, and the Graduate College and Biology Department at Missouri State University. Protocols were approved by the Saint Louis Zoo's Institutional Animal Care and Use Committee. Ruthie Carter worked with CS on development of the initial ethogram and provided training on identification of behaviors and use of software for this project. We thank the late Ron Goellner for designing and building the runway, and Ron and his wife, Karen Goellner, for their work in initiating the captive-breeding program for hellbenders at the Saint Louis Zoo. We appreciate the help of the many Zoo staff who assisted with captive-breeding efforts over the years. The manuscript was considerably improved by the thoughtful comments of three reviewers.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2018.00205/full#supplementary-material

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Conflict of Interest Statement: 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.

frontiers
in Ecology and Evolution

ORIGINAL RESEARCH
published: 07 January 2019
doi: 10.3389/fevo.2018.00229

Urbanization Alters Swimming Performance of a Stream Fish

Elizabeth M. A. Kern*† and R. Brian Langerhans

Department of Biological Sciences and W. M. Keck Center for Behavioral Biology, North Carolina State University, Raleigh, NC, United States

Human activities cause many changes in wildlife populations, including phenotypic shifts that represent adaptations to new conditions and influence population dynamics, diversity, and persistence. Although many examples of phenotypic adjustment to anthropogenic disturbance exist, we rarely know the extent to which these changes result from genetic evolution or phenotypic plasticity. Furthermore, our understanding of how whole-organism performance changes as a result of habitat alteration is very limited. We tested how urbanization, an important type of global disturbance, influences fish swimming performance in urban streams. Because urban streams have higher water velocities during rain events than rural streams, we tested for increased steady-swimming performance in fish from urbanized watersheds. Across ten populations of wild-caught Creek Chub (*Semotilus atromaculatus*), we found that fish from streams in more urbanized areas exhibited a longer propulsive wavelength and a lower tailbeat frequency, expending lower levels of hydromechanical power during steady swimming. In a laboratory experiment, we reared individuals collected as fry from four populations with different urbanization histories, and found evidence for genetically based differences in swimming kinematics. Laboratory-reared fish derived from urbanized populations exhibited higher locomotor efficiency, matching predictions for adaptation to urban environments. We exposed laboratory-reared Creek Chub from urban and rural populations to artificially increased water velocity for 4 months and observed that only some populations exhibited plasticity. Urban populations may have lost maladaptive, velocity-induced plasticity in locomotor efficiency that is still present in rural populations. Evolutionary change in freshwater species may represent a widespread yet unrecognized consequence of anthropogenic activity.

Keywords: biomechanics, contemporary evolution, Creek Chub, land use, phenotypic plasticity, *Semotilus atromaculatus,* urbanization, whole-organism performance

Edited by:
Susan C. Walls,
United States Geological Survey,
United States

Reviewed by:
Sean Lema,
California Polytechnic State University,
United States
Michael J. Pauers,
Milwaukee Public Museum,
United States
Jay A. Nelson,
Towson University, United States

****Correspondence:***
Elizabeth M. A. Kern
ekern@ewha.ac.kr

†Present Address:
Elizabeth M. A. Kern,
Department of Life Science, Ewha
Womans University, Seoul, South
Korea

Specialty section:
This article was submitted to
Behavioral and Evolutionary Ecology,
a section of the journal
Frontiers in Ecology and Evolution

Received: *23 May 2018*
Accepted: *07 December 2018*
Published: *07 January 2019*

Citation:
Kern EMA and Langerhans RB (2019)
Urbanization Alters Swimming
Performance of a Stream Fish.
Front. Ecol. Evol. 6:229.
doi: 10.3389/fevo.2018.00229

INTRODUCTION

Human impacts on the environment present one of the greatest challenges for conservation worldwide (Young et al., 2005; Worm et al., 2009; Barbier, 2011). Urbanization in particular is a pervasive, rapidly expanding type of impact that causes dramatic changes in natural ecosystems (Marzluff, 2001; McKinney, 2008). These changes can lead to altered selection and phenotypic divergence in traits such as life history, endocrine traits, birdsong, and seed dispersal, all of which are known to evolve rapidly in urban settings (Partecke and Gwinner, 2007; Cheptou et al., 2008; Luther and Baptista, 2010; Bonier, 2012; Miranda et al., 2013; Alberti et al., 2017). Understanding and predicting trait changes can provide a better understanding of evolution and inform decisions for conservation and sustainable urban development (Carroll et al., 2014; Smith et al., 2014). However, there are substantial gaps in our knowledge: we rarely know the extent to which phenotypic responses are genetic or plastic, or how certain types of traits like whole-organism performance respond to anthropogenic disturbance. Also, although we know of some evolutionary responses to urbanization in terrestrial species, we know practically nothing about how stream species respond evolutionarily to the environmental changes caused by urbanization (Kern and Langerhans, 2018). This is surprising since much attention has been devoted to urban stream degradation (the "urban stream syndrome") and other effects of human development on aquatic ecosystems (Paul and Meyer, 2001; Wenger et al., 2009; Knouft and Chu, 2015). It is becoming clear that our knowledge of human-induced *ecological* impacts in streams has far outpaced our needed understanding of *evolutionary* impacts.

Urbanization strongly affects freshwater streams because their topographic position makes them especially vulnerable to changes in land use (Walsh et al., 2005). One of the most salient changes to urban streams is their rapid rise in discharge following rain events. Impervious land cover like roads and buildings decreases infiltration and hastens the transport of water to streams such that urbanized streams experience faster water velocities during rainfall and more frequent high-discharge events (Poff et al., 2006; Jacobson, 2011). These greater peak flows and more frequent floods have been well documented in urban streams (Dunne, 1978; Shaw, 1994; McCuen, 1998).

This widespread urban hydrologic pattern provides the opportunity to test fundamental evolutionary questions. Studies of fish inhabiting undisturbed systems have uncovered adaptive differences in swimming performance among sites with different current velocities (Nelson et al., 2003; Langerhans, 2008), and it seems reasonable to predict that fish persisting in urban streams might similarly respond to human-altered hydrologic regimes (Nelson et al., 2008). In particular, fish in urban streams might exhibit greater "steady-swimming" performance (i.e., more energetically efficient cruising at constant speeds) than rural fish owing to the demands of human-caused hydrological changes. We tested this hypothesis by measuring differences in swimming performance among fish populations from streams that vary in degree of urbanization.

Swimming ability is an example of whole-organism performance (i.e., the performance of an ecologically relevant task such as running, biting, or jumping), and represents a key component of fitness (Arnold, 1983; Irschick et al., 2008; Langerhans, 2009a). Documented cases of evolution in complex phenotypes—like whole-organism performance—in response to human activity are exceedingly rare (Merilä and Hendry, 2014; Donihue and Lambert, 2015), and phenotypic responses to urbanization are often not tested for a genetic basis (Alberti et al., 2017). However, testing for a genetic basis can be valuable because the genetic vs. plastic basis of trait differentiation may help determine species persistence. Many responses to human impacts seem more plastic than genetic, which on the one hand may constrain species' ability to handle future conditions, since plasticity has a theoretical limit (Hendry et al., 2008). On the other hand, plasticity might allow species to persist long enough to evolve additional adaptations to modified environments (Pigliucci, 2001).

To test the effects of urbanization on steady-swimming abilities of stream fish, we conducted swim-tunnel experiments with a cyprinid minnow, Creek Chub (*Semotilus atromaculatus*), from streams across a range of urbanization levels. Creek Chub commonly inhabit rural and urban streams, and because of their short generation time (1–2 years to sexual maturity) they could have evolved measurable responses to urbanization over the past 20–120 years. We measured four kinematic swimming variables (tailbeat frequency, tailbeat amplitude, rostral amplitude, and propulsive wavelength) and an estimate of overall locomotor efficiency to assess differences among fish from historically urban, recently urbanized, and rural habitats. These kinematic variables are associated with swimming efficiency and allow us to test specific predictions about phenotypic divergence (McHenry et al., 1995). Based on biomechanical principles, urban fish that experience faster flowing water should benefit from stiffer bodies (longer propulsive wavelengths), reduced tailbeat frequency, and possibly reduced rostral and tailbeat amplitudes (McHenry et al., 1995; Langerhans, 2009b).

To test whether differences observed in the wild reflect plasticity, genetic differentiation, or both, we performed the same swimming measurements on laboratory-reared individuals from a subset of populations. Testing fish raised in a laboratory also reduces the likelihood that unmeasured environmental factors in urban streams (such as pollution) are responsible for any observed differences in swimming performance.

MATERIALS AND METHODS

Wild-Caught Fish

For trials of wild-caught fish, we collected 64 adult Creek Chub from ten streams in the Piedmont region of North Carolina, USA (**Table 1**). This particular region has well-documented differences in flow regime between urban and rural streams; previous work has demonstrated that urban streams in our study area are flashier than nearby rural streams (Brown et al., 2009). Using maps and aerial images, we specifically selected study sites to span variation in urbanization across this region. To avoid confounding factors such as habitat size, we further selected similarly sized streams (first and second order). To provide a quantitative measurement of urbanization at each site, we measured the percent of developed land cover within a one-mile radius of each collection site using the 2011 National Land Cover Database and QGIS 2.10. Developed land cover highly correlates with impervious surface cover (Jennings et al., 2004). Estimated developed land cover included all four subcategories in the National Land Cover Database, which comprises constructed materials (e.g., roads, parking lots, businesses, apartments, homes) and open spaces associated with urban development (e.g., lawns, golf courses, urban-associated vegetation). We also examined maps of each watershed, to ensure for example that major upstream development was absent above rural sites. Visual inspection of satellite and aerial images across time (**Figure S1**),

TABLE 1 | Collection site information for Creek Chub examined in this study.

Site name	Urbanization status	Developed land cover (%)	Lat. N	Long. W	N
Poplar Creek	Rural	24.8	35.7893	−78.4718	17
Hodges Creek	Rural	22.5	35.8548	−78.4984	2
Little White Oak Creek	Rural	8.3	35.6669	−78.9210	2
Rocky Branch	Rural	3.3	35.6484	−79.1582	6 (13)
Beaver Creek	Recently urbanized	79.0	35.7426	−78.8819	(29)
Richland Creek	Recently urbanized	51.1	35.9960	−78.5109	3 (9)
Middle Creek	Recently urbanized	40.1	35.7082	−78.8342	8
Lower Barton Creek	Recently urbanized	33.2	35.9322	−78.6735	10
Mine Creek	Historically urban	87.9	35.8767	−78.6457	5 (5)
House Creek	Historically urban	85.8	35.8120	−78.6916	6
Bolin Creek	Historically urban	75.2	35.9239	−79.0502	5

Number of individuals tested (N) is given for both wild-caught fish and laboratory-reared fish (the latter in parentheses).

combined with our quantitative measurements of land use, placed each of our sites into one of three "urbanization status" categories which were meant to capture each site's history of urban development (**Figure 1**). First, historically urban sites ($n = 3$) had high levels of urbanization (defined as here as $>$ 70% developed land cover) and showed very little change in urban development across aerial photographs from the 1980s and satellite images from the 2010s. Second, recently urbanized sites ($n = 3$) had moderate to high levels of urbanization (30–80% developed land cover) and showed a substantial increase in developed land cover since the 1980s (**Figure S1**). Finally, rural sites ($n = 4$) had a continuously low proportion of developed land cover from the 1980s to the present ($<$ 30% developed land cover). Since phenotypic change may lag behind environmental change (especially for genetic evolution rather than phenotypic plasticity), using these three categories—historically urban, recently urbanized, and rural—could prove more useful than a dichotomous urban/rural designation. The three categories capture the bulk of variation in urbanization in this geographic region, which includes an "old" New World city (Raleigh, North Carolina, founded in 1792), rapidly accelerating localized urban growth, and considerable rural area.

We collected Creek Chub during October 2013 to November 2014 using dip nets and a backpack electroshocker, and transported them in coolers to animal care facilities at North Carolina State University, where they were gradually introduced over several hours to 21°C water temperatures. We allowed each fish to acclimate to laboratory housing for at least three days prior to experimentation. This acclimation period may or may not completely remove stress effects, but previous work indicates that stress does not significantly impact fish swimming performance (Gregory and Wood, 1999; Lankford et al., 2005). Fish were housed in 76-liter aquaria (no flow-through) at 21°C and fed dry flakes daily *ad libitum.* For swimming trials, fish were moved with as brief air exposure as possible using a bucket and aquarium net into a clear Plexiglas tunnel measuring 15 × 15 × 50 cm. Once each fish recovered from the move (came to rest or began exploring the tunnel), water velocity in the tunnel was increased to 1.4 m/s. This velocity was designed to mimic challenging conditions during stormwater runoff in an urbanized area, yet still remain within the range of the steady-swimming gait (also known as cruising) for this species. Tunnel water velocity was more than twice the speed of average baseflow conditions (0.51 m/s) at our field sites.

Water flow inside the tunnel was powered by an electric sump pump (Leader Provort 540a; Ladson, SC, USA). Water was pumped into the tunnel through a honeycomb baffle of short drinking straws, which helped approximate laminar flow. To control for slight variations in water speed, we used a digital water velocity meter (Global Water Flow Probe) to measure the velocity at the specific location within the tunnel where the fish swam during each trial (mean ± standard error, 1.43 m/s ± 0.01). We also recorded water temperature after each trial for potential use as a covariate, in case small variations in temperature (e.g., from the running pump motor) might affect swimming performance (20.93°C ± 0.14). In rare cases where fish did not respond to the artificial current by swimming against it, they were returned to aquaria and retested on a different day. In our dataset these individuals ($n = 3$) represent $<$5% of the total number of individuals.

Swimming trials were recorded with a high-speed digital video camera (IDT N4-S1). A mirror propped underneath the tunnel at 45° allowed us to simultaneously capture ventral and lateral views of the fish as it swam at constant speed, maintaining position in the tunnel. From the video sequences (set to 400 fps with a few exceptions at 200 fps) we used tpsDig (Rohlf, 2013) to collect spatial coordinates of the fish's snout, body, and tail positions in order to measure four kinematic variables: tailbeat frequency, tailbeat amplitude, rostral amplitude, and propulsive wavelength (McHenry et al., 1995). Tailbeat frequency is measured in beats per second, with one beat representing a full cycle from one side of the body to the opposite side and back to the original location. Tailbeat amplitude is half the distance between the maximum left and right excursions of the most distal tip of the caudal fin during one complete tailbeat. Similarly, rostral amplitude is half the distance of the maximum left and right excursions of the tip of the snout. We calculated propulsive wavelength by doubling the propulsive half-wavelength, which is the distance between

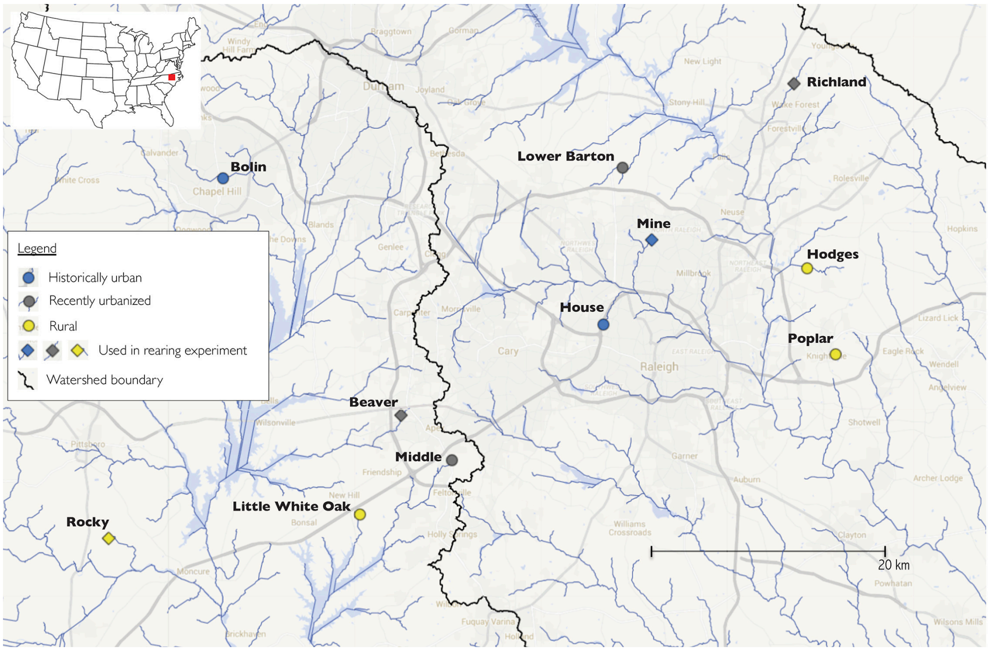

FIGURE 1 | Map of study area in North Carolina, USA.

TABLE 2 | Trait loadings and variance explained by the first two principal components for the PCA of swimming kinematic data in wild-caught Creek Chub (loadings ≥ |0.60| in bold text).

Kinematic variable	PC 1	PC 2
Tailbeat frequency	**-0.74**	−0.31
Rostral amplitude	0.22	**0.90**
Tailbeat amplitude	**0.74**	−0.36
Propulsive wavelength	**0.83**	−0.19
Variance explained:	45.54%	27.03%

upper and lower crests of the sinusoidal wave created between the midbody and midtail region of a fish in motion (Webb et al., 1984). We measured each variable three times in each video and used the means for final analysis. Because kinematic variables may be correlated with one another, we performed principal components analysis (PCA) on the correlation matrix of the four kinematic variables to reduce data dimensionality. We retained PCs with eigenvalues ≥ 1 for analysis of kinematic variation, resulting in retention of the first two PCs, explaining 72.6% of the variance (**Table 2**).

To provide a summary of the magnitude of thrust production, we estimated total hydromechanical power using Lighthill's elongated-body theory (Wu, 1971; Lighthill, 1975; Videler, 1993). We calculated power (P) as $f^2H^2B^2(1–U/c)$, where f, tailbeat frequency; H, tailbeat amplitude; B, caudal fin depth; U, swimming speed, and c, wave speed (propulsive wavelength times tailbeat frequency). This parameter can be thought of as the total lateral force produced by the tail to overcome drag forces and maintain constant-velocity swimming. Holding swimming speed constant, a lower P indicates greater overall locomotor efficiency (i.e., less power consumed to swim at a given speed, analogous to miles per gallon).

We performed two sets of analyses to test for effects of urbanization on steady-swimming performance in wild-caught Creek Chub: one examining variation across categories of urbanization, and one utilizing estimates of continuous variation in degree of urbanization. First, we tested for kinematic and power differences between the three habitat types using a general linear mixed model (using restricted maximum likelihood) for each of the three response variables (PC 1, PC 2, and hydromechanical power) with body lengths per second as a covariate, habitat as a fixed effect, and population as a random effect. We included body lengths per second as a covariate to control for both variation in body size (**Table S1**) and any slight variation in water velocity—i.e., statistically adjust response variables to allow comparison among groups at a constant relative swimming speed. Body lengths per second is a standard metric for measuring and comparing fish swimming speed (Bainbridge, 1958). It was measured by recording fish swimming at a known speed in the tunnel (i.e., swimming "in place" against a constant current of known velocity) and dividing water velocity by body length (standard length). Thus, swimming speed was highly correlated with body length ($r = -0.92$, $P < 0.0001$), and all results are qualitatively no different than if we use body size as a covariate instead of swimming speed. We initially included water temperature as a covariate but excluded it due to non-significance. Second, we calculated population means ($n = 10$) for all three response variables (least-squares means, controlling for body lengths per second) and conducted Spearman rank correlations between swimming performance means and site-specific estimates of urban development land cover. The latter analyses took advantage of continuous variation in present-day urbanization levels to test whether populations surrounded by more urban land cover tend to exhibit greater steady-swimming performance. The amount of urban land cover was quantified by measuring the percentage of developed land cover in the 2.6 km (1 mi) radius around each site, using QGIS and the 2011 National Land Cover Database (Homer et al., 2015).

Because multiple comparisons (e.g., multiple tests on the same set of fish) can increase Type I error rates, we used QValue (Storey, 2003) to control the false discovery rate at 5% using the bootstrap procedure for estimating the probability of a true null hypothesis (Benjamini and Hochberg, 1995). The false discovery rate (FDR) is the proportion of significant tests that are in fact null. We accepted tests in this study as significant only when both the *p*-value of the test and its *q*-value (determined by the QValue program) were ≤ 0.05. Because all observed *p*-values ≤ 0.05 in the present study remained significant at the FDR of 5%, we straightforwardly interpret *p*-values ≤ 0.05 as being significant. Due to modest sample sizes in several populations, we avoid making strong conclusions regarding the absence of effects based on non-significant findings. Moreover, all results reported in this study are qualitatively unchanged using permutation tests in place of our parametric *F* tests (using the permuco R package; Kherad-Pajouh and Renaud, 2010, 2015).

Laboratory-Raised Fish

To test whether differences between populations persisted in fish reared in a common laboratory environment (which would suggest a genetic basis), we conducted swimming trials of laboratory-raised Creek Chub from four populations. We used dip nets and aquarium nets to catch late postlarval to very early stage juvenile Creek Chub (1.5–3.0 cm standard length; Buynak and Mohr, 1979) from four stream sites during the hatching and emergence period (June–July 2014; Washburn, 1948; **Table 1**). Sample sites were selected to cover the entire urbanization gradient. Fish were transported in 19-liter buckets to facilities at North Carolina State University and each population was randomly separated into two treatment groups—high velocity and low velocity (descriptions below). Fish were divided among eight ten-liter tanks according to population and treatment (2–15 Creek Chub per tank; **Table S3**). All tanks were part of a single recirculating system (Aquatic Habitats; Apopka, FL), which allowed populations and treatment groups to experience identical water conditions. Fish were fed brine shrimp and dry flakes twice daily and kept on a 12:12 h light-dark cycle. Water conditions were maintained at 0.5 ppt salinity and 25°C.

After 2 weeks of rearing all fish in common conditions to allow acclimation to the laboratory-rearing environment, we initiated water velocity treatments designed to roughly mimic flow conditions of urban and rural stream. High-velocity treatment groups received 60 min of intensified current once per week for 16 weeks to simulate increased velocity in urban streams during rainfall events. Current was generated by fully opening the two tank nozzles for each tank (two separate nozzles provide water to each tank within the recirculating system), which flushed tanks at a constant rate of 4.2 L/min (compared to 0.7 L/min at other times and throughout the low-velocity treatment). This created elevated, non-uniform water velocity within the tanks (~0.9–5.5 cm/s) and forced fish to swim against a moderate current to maintain position (~1–3.5 body lengths per second). Low-velocity treatment groups never received any water velocity increases, and experienced non-uniform water velocity ~0.1–0.9 cm/s within the tanks at all times throughout the experiment.

TABLE 3 | Trait loadings and variance explained by the first two principal components for the PCA for swimming kinematic data in laboratory-raised Creek Chub (loadings ≥ |0.60| in bold text).

Kinematic variable	PC 1	PC 2
Tailbeat frequency	**-0.60**	0.29
Rostral amplitude	**0.62**	**-0.63**
Tailbeat amplitude	**0.79**	0.20
Propulsive wavelength	**0.67**	**0.66**
Variance explained:	46.53%	22.99%

After 18 weeks of laboratory rearing (16 weeks of treatment and 2 weeks of initial acclimation time), fish were recorded swimming in a Plexiglas swim tunnel measuring 5 × 6 × 21 cm against a current of 0.27 m/s. Water temperature was held constant at 25°C. Dimensions and velocity differ from that used for the wild-caught fish above owing to the differences in body size between adult wild-caught fish and juvenile laboratory-raised fish (if we had tested juveniles at speeds of 1.4 m/s they would not have been able to swim against the current). As before, we used high-speed video and an angled mirror to capture a lateral and ventral view of the fish swimming, and we used the same methods as above to measure the same four kinematic variables and hydromechanical power.

We again performed a PCA on the four kinematic variables as described above, and retained the first two PCs for analysis, explaining 70% of the variance (**Table 3**). We fit a general linear model to examine kinematic and hydromechanical power variation across treatments, populations, and the interaction of treatment and population, with body lengths per second as a covariate to control for possible effects of body size. Our inclusion of body lengths per second in the model was in essence equivalent to including body size because all fish were tested at a common velocity, and thus this procedure appropriately adjusts for size effects. We further tested for non-linear effects of body size (squared term), but no non-linear effects were observed ($p > 0.91$). Moreover, we confirmed that body size broadly overlapped among populations within this smaller subset of populations (**Table S2**), permitting the proper statistical test and adjustment for body size. The treatment term tests for water velocity–induced phenotypic plasticity. The population term tests for environment-independent differences between populations, which would imply genetically based variation in swimming performance, though parental effects cannot be ruled out. The interaction between treatment and population tests whether velocity-induced plasticity differs between populations. For each term we calculated η^2 (an estimate of effect size) to

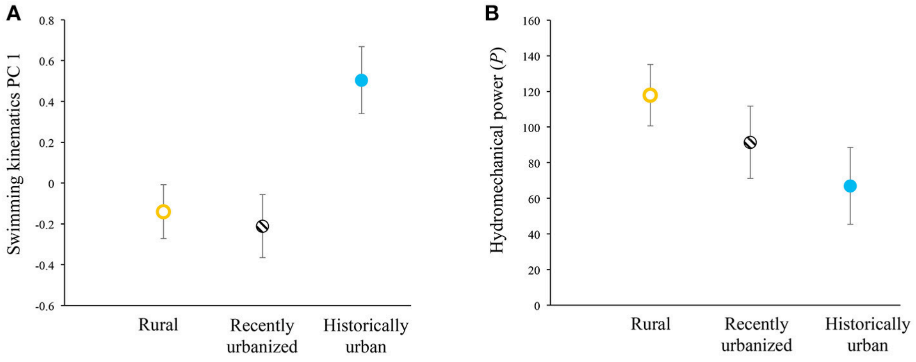

FIGURE 2 | Variation in **(A)** swimming kinematics PC 1 scores and **(B)** hydromechanical power across the three habitat types for ten populations of wild-caught Creek Chub. Higher PC 1 scores reflect longer propulsive wavelengths (i.e., stiffer bodies), reduced tailbeat frequencies, and greater tailbeat amplitudes (**Table 2**), controlling for relative swimming speed (body lengths per second). Least-squares means ± 1 SE depicted.

directly assess the relative importance of the terms (Langerhans and DeWitt, 2004). We used Tukey's honestly significant difference test to interpret significant categorical model terms (only groups with $p \leq 0.05$ treated as significantly different).

Animal Use Statement

This study was approved by the Institutional Animal Care and Use Committee at North Carolina State University.

RESULTS

Wild-Caught Fish

In our tests of swimming-performance differences among the three categories of urbanization status, we found that only one of the three response variables showed significant differences. PC 1 scores of steady-swimming kinematics differed between habitats ($F_{(2,59)} = 6.24$, $p = 0.0035$) and negatively co-varied with body lengths per second ($F_{(1,59)} = 135.36$, $p < 0.0001$). Fish from historically urban populations exhibited significantly higher PC 1 scores than other populations, when statistically controlling for body lengths per second (**Figure 2A**). Higher PC 1 scores in historically urban fish indicated longer propulsive wavelengths (i.e., stiffer bodies), reduced tailbeat frequencies, and greater tailbeat amplitudes. For PC 2, the covariate was non-significant ($F_{(1,59)} = 0.14$, $p = 0.7126$) and habitat type was marginally non-significant ($F_{(2,59)} = 2.79$, $p = 0.0694$), suggesting a trend where rural fish had lower PC 2 scores than recently urbanized fish, with historically urban fish intermediate. Hydromechanical power negatively scaled with body lengths per second ($F_{(1,59)} = 14.22$, $p = 0.0004$). Hydromechanical power did not differ significantly with habitat types ($F_{(2,59)} = 1.80$, $p = 0.1746$), even though the trends did match *a priori* predictions, with rural fish tending to require more hydromechanical power to swim at a given swimming speed compared to historically urban fish, and with recently urbanized fish intermediate between the two (**Figure 2B**).

Spearman rank correlation tests revealed that Creek Chub from sites with greater urban development land cover had significantly higher swimming kinematic PC 1 scores ($\rho = 0.69$, $p = 0.0289$) and lower hydromechanical power ($\rho = -0.67$, $p = 0.0330$) (**Figure 3**); no relationship was observed for swimming kinematic PC 2 ($\rho = 0.35$, $p = 0.3282$). Thus,

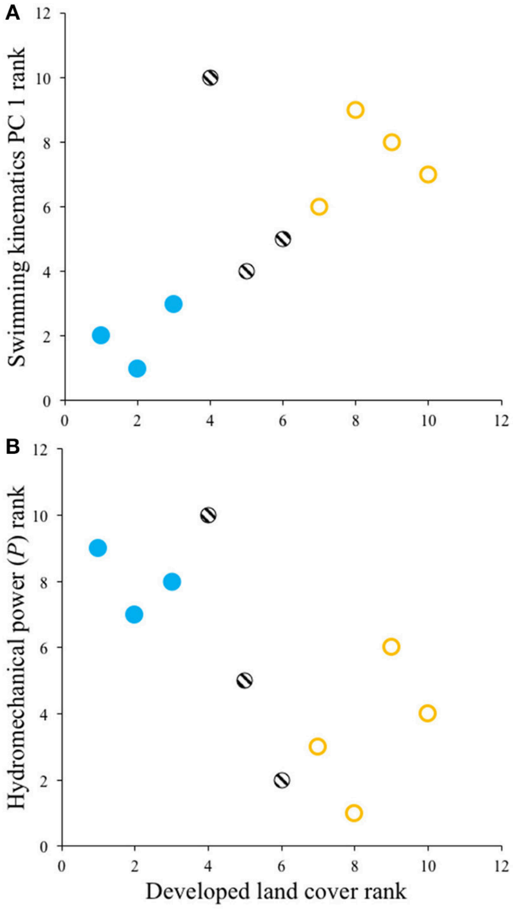

FIGURE 3 | Spearman rank correlations showing that Creek Chub from streams with greater urban development had **(A)** higher swimming kinematics PC 1 scores and **(B)** lower hydromechanical power. Symbols follow **Figure 2**.

during steady swimming, Creek Chub populations in more urban areas tended to exhibit longer propulsive wavelengths, reduced tailbeat frequencies, greater tailbeat amplitudes, and employ lower overall hydromechanical power to swim at a given speed.

Laboratory-Raised Fish

We uncovered evidence suggesting genetically based differences between populations in swimming performance (all three variables) and for population-specific variation in water velocity–induced plasticity (two variables), but found no evidence of shared patterns of velocity-induced plasticity across populations (**Table 4**).

For the laboratory-raised Creek Chub, PC 1 scores were negatively correlated with body lengths per second and were significantly different between populations. For one population (Beaver Creek), PC 1 scores varied between velocity treatments (**Figure 4A**). A rural population, Rocky Branch, tended to exhibit higher PC 1 scores than other populations regardless of treatment. Plasticity was only evident for a recently urbanized population (Beaver Creek), with significantly lower PC 1 scores in the high-velocity treatment than in the low-velocity treatment (**Figure 4A**).

PC 2 also exhibited a negative relationship with body lengths per second and significantly differed among populations, but showed no significant velocity-induced plasticity (**Figure 4B**). Again, the rural site (Rocky Branch) exhibited the highest PC 2 scores.

Hydromechanical power was negatively associated with body lengths per second, significantly differed among populations, and showed velocity-induced plasticity within one population (**Figure 4C**). Rocky Branch exhibited the highest overall power, indicating that the rural population tended to use a greater amount of hydromechanical power to swim at the same speed as fish from urbanized areas. Rocky Branch was also the only population that exhibited significant velocity-induced plasticity in hydromechanical power: higher power was produced by fish raised in the high-velocity treatment.

DISCUSSION

The ecological and evolutionary importance of whole-organism performance is undisputed, yet we know little about how human activities affect it (Irschick et al., 2008) or whether its response to human impacts more likely reflects genetic evolution or phenotypic plasticity (Hendry et al., 2008). Here we found that the steady-swimming performance of Creek Chub (wild and laboratory-raised) differs with stream urbanization, and that although some aspects of performance in some populations show velocity-induced plasticity, most of the differences between populations appear to reflect genetic differentiation, although controlled breeding experiments would be necessary to confirm this. Wild-caught Creek Chub from rural streams expended more hydromechanical power during steady swimming than urban fish swimming at the same speed, and patterns in laboratory-raised fish suggest a genetic basis to these differences as a rural population still showed lower steady-swimming efficiency than populations from urbanized streams. Our results demonstrate one way in which humans can impact the evolution of phenotypic traits and suggest that urbanization may be leaving a noticeable mark on whole-organism performance evolution in some stream species. The underlying biological causes of altered swimming performance were not measured here, but presumably, evolved differences in physiological and morphological attributes provide key sources: in fact, past work in Creek Chub has shown that body shape changes subsequent to urbanization (Kern and Langerhans, 2018), and the morphology and physiology of various stream fish also differ between impacted and unimpacted streams (Blevins et al., 2013; Gaulke et al., 2015; King et al., 2016; Pease et al., 2018).

For wild-caught Creek Chub, the results partially agreed with our predictions for how fish should adapt to higher peak water velocities in urbanized watersheds. First, Creek Chub from historically urban sites and from sites with greater overall developed land cover had longer propulsive wavelengths than fish from rural or recently urbanized sites. Longer propulsive wavelengths indicate greater body stiffness in fish (Blight, 1977; Long et al., 1996) and should increase steady-swimming efficiency (McHenry et al., 1995; Langerhans, 2009b); thus, the changes we observed could be adaptive. Second, wild-caught urban fish had lower tailbeat frequencies than rural fish, which should also indicate increased efficiency since tailbeat frequency often provides a useful surrogate for oxygen consumption (Herskin and Steffensen, 1998; Steinhausen et al., 2005). On the other hand, urban fish did not have significantly lower tailbeat amplitudes as might have been expected (since decreased amplitude is one way to reduce mechanical work). Altogether, the kinematics results indicate that Creek Chub swim differently in streams with different urbanization levels, and that in urbanized environments their kinematics indicate greater locomotor efficiency. Indeed, wild-caught Creek Chub from streams in more urbanized areas actually did exhibit greater swimming efficiency, as shown by their lower hydromechanical power during steady swimming. This suggests that Creek Chub populations from more urbanized areas incur lower metabolic costs while swimming steadily at the same speed as their more rural counterparts.

Do these differences in swimming performance arise via evolutionary change or phenotypic plasticity? By raising multiple populations in a common environment, we found evidence for a genetic basis to variation in swimming kinematics. We uncovered significant among-population variation in all three variables tested, mainly reflecting differentiation between the rural population and the three more urbanized populations. These patterns are consistent with our findings in wild-caught adults; fish derived from urbanized environments exhibited greater hydromechanical efficiency than fish derived from a rural environment, even after they had been raised in common conditions. This suggests adaptive evolution, in which fish responded to urbanization by evolving greater locomotor efficiency, which is probably an advantage when coping with the strong currents that frequently occur in urban streams after rain events.

While our results support genetic differentiation, parental effects on swimming performance cannot be ruled out because

TABLE 4 | Results of general linear models examining steady-swimming kinematic variation in laboratory-raised Creek Chub.

Source	d.f.	PC 1			PC 2			Power		
		F	p	η^2	F	p	η^2	F	p	η^2
Body lengths per sec	1,47	5.1	**0.0286**	**5.29**	4.99	**0.0304**	**5.25**	8.73	**0.0049**	**8.66**
Population	3,47	5.11	**0.0039**	**15.87**	5.47	**0.0026**	**17.26**	4.53	**0.0072**	**13.48**
Treatment	1,47	0.66	0.4209	0.68	0.01	0.9408	0.01	2.71	0.1067	2.68
Population × Treatment	3,47	5.12	**0.0038**	**15.93**	1.75	0.1693	5.53	3.11	**0.0352**	**9.25**

Statistically significant effects are in bold.

practical constraints (i.e., time to maturity in this species) precluded rearing multiple generations in the laboratory. However, in fish species the existence of maternal effects is commonly manifest in life-history traits such as offspring size (Reznick et al., 1996; Heath et al., 1999; Green, 2008), while our findings involve whole-organism performance independent of body size; and even maternal effects can facilitate contemporary evolution (Räsänen and Kruuk, 2007). Furthermore, prior work has often found that fish locomotor performance has a genetic basis (Nicoletto, 1995; Garenc et al., 1998; Ghalambor et al., 2004; Langerhans, 2009a; Johnson et al., 2010). Another possible alternative explanation is that natural selection or environmental effects on steady-swimming performance could have already occurred in the field prior to collection of laboratory-reared fish. To address this, as much as possible we minimized the amount of time for selection or environmental induction to have occurred by collecting very small fish soon after hatching within each population. We then raised all fish in the laboratory for a total of 18 weeks, which includes an especially formative period of development: their entire first summer. However, more prolonged laboratory rearing might produce more plasticity (Kelley et al., 2017).

Although laboratory-raised Creek Chub from urbanized populations showed some hallmarks of greater locomotor efficiency as predicted, such as lower tailbeat amplitudes and lower hydromechanical power, other among-population differences in laboratory-raised fish kinematics (as seen in PC 1) did not conform to predicted patterns even though kinematics in wild-caught fish did. It is unclear why kinematics in wild-caught and laboratory-raised Creek Chub were not more similar, but swimming efficiency in living animals is a complex activity influenced by many factors, including ontogeny, muscle fiber composition, physiological adaptations, body and fin morphology, and possibly genetic constraints on kinematics (Ghalambor et al., 2004; Lailvaux et al., 2010; Walker, 2010; Dalziel and Schulte, 2012), any one of which could be responsible for wild and laboratory-raised differences. Alternatively, the differences between wild and laboratory-raised fish could be due to countergradient evolution, in which genetic differences (along an environmental gradient) compensate for the impact of the environment on phenotype, thus minimizing phenotypic differences (Conover and Schultz, 1995).

The timescale of the evolutionary divergence we document here is within the range of previously documented, human-induced contemporary evolution (Hendry et al., 2008). Fish can evolve substantial divergence in fewer than 13 generations (Hendry et al., 2000), and given the 50-year or longer history of urbanization at many of our urban sites, and the 1–2 year generation time for Creek Chub, the divergence we observed is not unprecedented despite the common perception of evolution as a much slower process.

Our laboratory rearing experiment included a test for velocity-induced plasticity, which is not uncommon in fish (e.g., Broughton and Goldspink, 1978). In response, some Creek Chub populations showed plasticity in some performance variables. However, the overall effect of treatment for all populations taken together was not significant for any performance variable, and the effect size of the population term was as large as or larger than the effect of the interaction term in all cases. The modest sample sizes in the laboratory-reared populations lead us to interpret these non-significant results with caution; plasticity may be present in some populations at a magnitude too small for detection in this study. It is also conceivable that a stronger experimental treatment (faster or more frequent high-velocity exposure) would have elicited plastic responses from more populations or in more kinematic variables, though the high-velocity treatment should have been adequate since it produced a more than 6-fold increase in water velocity, caused elevated swimming activity, and approximated the frequency and duration of summer storms in the region for 4 months of postlarval rearing, which is a substantial growing period for these fish.

The fact that some laboratory-reared populations did respond in some kinematic variables to the velocity treatment could reflect adaptive plasticity. For instance, in the recently urbanized Beaver Creek population, fish in the high-velocity treatment displayed lower tailbeat amplitudes, which should indicate enhanced locomotor efficiency. However, other aspects of observed plasticity appear to counter adaptive hypotheses. For example, in the rural Rocky Branch population, fish in the high-velocity treatment showed higher hydromechanical power, which could indicate reduced locomotor efficiency under challenging flood conditions (rarer in rural streams). In light of this result, it is possible that urbanized populations have evolved means of diminishing the negative consequences of increased water velocities, thus maintaining similar locomotor efficiencies regardless of flow regime—that is, urban populations could have evolved changes in unmeasured traits (including adaptive plasticity in those traits) which eliminated the maladaptive velocity-induced plasticity historically present in locomotor efficiency. Fish might also be responding to changes in urban

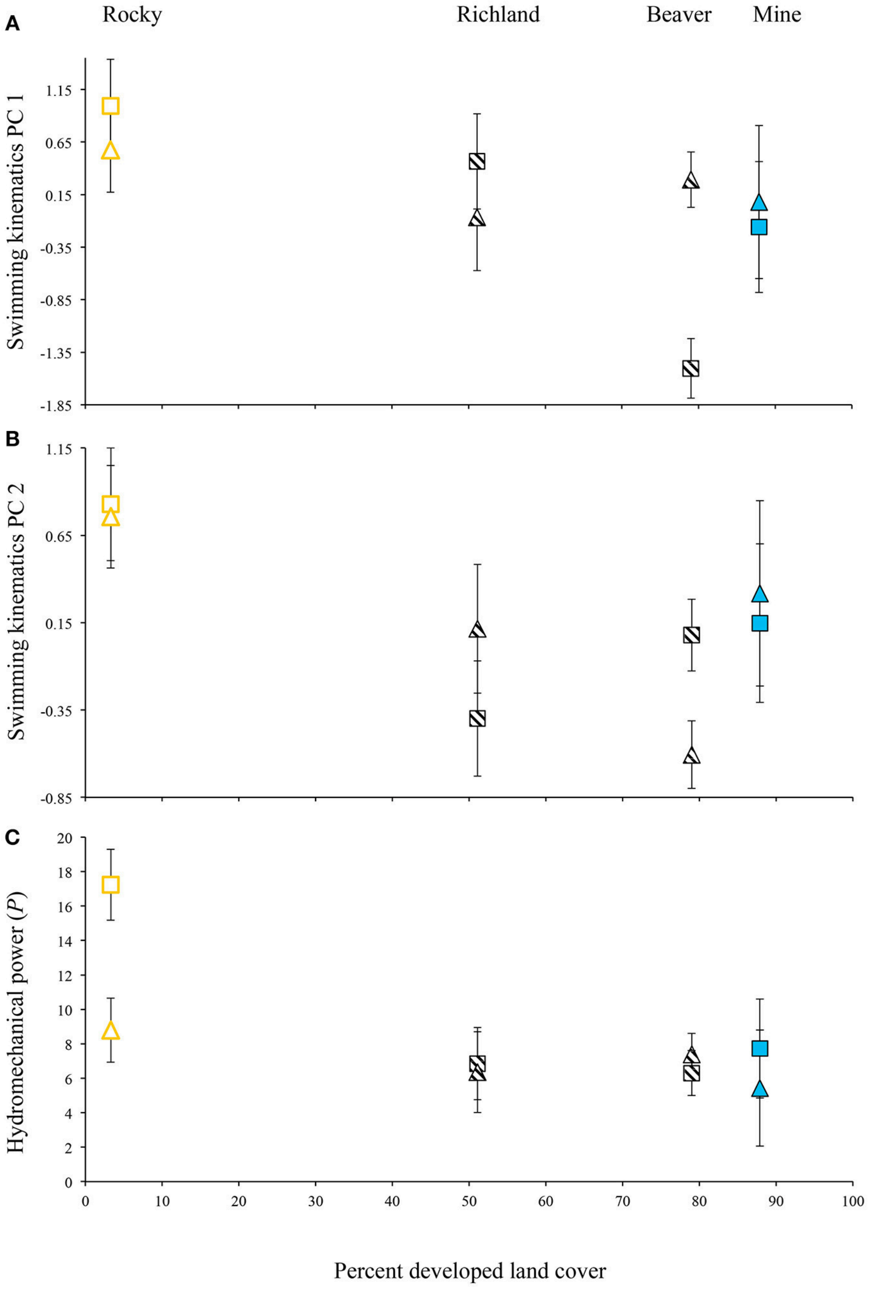

FIGURE 4 | Variation in **(A)** swimming kinematics PC 1 scores, **(B)** swimming kinematics PC 2 scores, and **(C)** hydromechanical power for four Creek Chub populations raised in the laboratory under two velocity treatments, controlling for relative swimming speed (body lengths per second). Triangles, low-velocity treatment; squares, high-velocity treatment; open symbols, rural; hatched symbols, recently urbanized; solid symbols, historically urban. Least-squares means ± 1 SE depicted.

stream morphology (fewer riffles, less woody debris) that reduce spatial variation in water velocity. In general, to better understand how urbanization leads to changes in phenotypic plasticity, as may often occur in the wild (Lande, 2009, 2015; Crispo et al., 2010), future work will need to investigate velocity-induced plasticity in multiple traits that affect steady-swimming abilities.

In conclusion, we found that Creek Chub exhibit greater steady-swimming efficiency in urbanized streams, as predicted, suggesting urbanization could be an unappreciated yet major driver of evolution in aquatic organisms. Urbanization causes many changes in addition to water velocity: it can alter thermal regimes and structural composition of streams, introduce pollutants, and greatly reduce species diversity (Mcdonald et al., 2008; Brown et al., 2009), any of which could have also played a role in the present study, but we now know that alterations in flow regimes have major evolutionary consequences for fish

(Langerhans, 2008; Haas et al., 2010; Franssen, 2011). This has at least two conservation ramifications: first, it suggests that altered flow regimes in urban streams are a strong driver of biological changes, implying that slowing down stormwater discharge may be an important part of mitigating the urban stream syndrome. Second, it indicates that for some species, urban populations may be genetically divergent from rural populations, which in turn has implications for how these populations are managed. However, whether adaptive responses to urban conditions could protect some species from population decline or extirpation is still unknown (Kark et al., 2007; Schiffers et al., 2012).

DATA AVAILABILITY STATEMENT

Data is available upon request: the raw data supporting the conclusions of this manuscript will be made available by the authors, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

ACKNOWLEDGMENTS

We gratefully thank Jonathan Otten and Natalia Zbonack for their contributions to fish photography and video analysis; Benjamin Pluer, Chris Schalk, and Carmen Montaña for their aid in the field; and Erik Archer for assistance with live fish care.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2018.00229/full#supplementary-material

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Conflict of Interest Statement: 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.

frontiers
in Ecology and Evolution

ORIGINAL RESEARCH
published: 08 February 2019
doi: 10.3389/fevo.2019.00022

Integrating Ecophysiological and Agent-Based Models to Simulate How Behavior Moderates Salamander Sensitivity to Climate

Kira D. McEntire[1,2]* *and John C. Maerz*[1]

[1] *Warnell School of Forestry and Natural Resources, University of Georgia, Athens, GA, United States,* [2] *Department of Biology, Trinity University, San Antonio, TX, United States*

Developing rigorous ecological models is a fundamental goal of conservation biologists seeking to forecast biotic responses to climate change. A limitation of many models is they are amechanistic and lack integration of behavior, which is fundamental to animal biology. We integrated biophysical and agent-based models (ABM) to examine how behavior could affect the sensitivity of Plethodontid salamander activity time to climate. Specifically, our model used a temperature differential to stimulate plant climbing, a widely observed behavior among salamanders, which would allow salamanders to reduce body temperatures and associated dehydration rates. Consistent with expectations, predicted activity time was positively correlated with precipitation. The model showed that climbing plants increased activity time in drier conditions, particularly for smaller salamanders. The predicted importance of climbing behavior, a form of behavioral plasticity, was highly sensitive to assumptions about the threshold of water loss an individual was willing to tolerate. Because activity time is associated with fitness, increased activity time as a consequence of climbing behavior could moderate salamander sensitivity to shifts in weather patterns. Our results demonstrate the potential and importance of integrating behaviors into ecophysiological models when evaluating a species' potential sensitivity to climate.

Keywords: individual-based models, amphibian, conservation, ecology, management

Edited by:
Caitlin R. Gabor,
Texas State University System,
United States

Reviewed by:
Brittany Ousterhout,
Lewis and Clark Community College,
United States
Vincent R. Farallo,
Virginia Tech, United States

****Correspondence:***
Kira D. McEntire
kmcentir@trinity.edu

Specialty section:
This article was submitted to
Behavioral and Evolutionary Ecology,
a section of the journal
Frontiers in Ecology and Evolution

Received: *14 November 2018*
Accepted: *22 January 2019*
Published: *08 February 2019*

Citation:
McEntire KD and Maerz JC (2019)
Integrating Ecophysiological and
Agent-Based Models to Simulate How
Behavior Moderates Salamander
Sensitivity to Climate.
Front. Ecol. Evol. 7:22.
doi: 10.3389/fevo.2019.00022

INTRODUCTION

Models are important tools for understanding ecological systems and predicting how those systems may change across spatial and temporal gradients. The development of rigorous distribution, population, and performance models has reemerged as a major focus of conservation biologists attempting to forecast population responses to environmental change (Guisan et al., 2006; Araújo and New, 2007; Jackson et al., 2009; Urban et al., 2016). Due to relative ease of data accessibility, distribution models based on correlations between species presence data and environmental attributes are commonly used to make predictions about how species distributions may change under future environmental conditions (Pearson and Dawson, 2003; Araújo et al., 2005; Buckley et al., 2010). Criticisms of these bioclimatic models include the assumption that key mechanisms are captured within the correlations, missing other key parameters such as dispersal, migration, or biotic interactions, and concerns about extrapolation to future novel environments (Pearson and Dawson, 2003; Hampe et al., 2004; Araújo et al., 2005; Soberon and Peterson, 2005; Peterson et al., 2015). Mechanistic models address the assumptions outlined above and are potentially more robust than correlative bioclimatic models. For example, ecophysiological models are a type of mechanistic model that use mathematical models and fundamental principles of physics to predict how an organism's performance (e.g., metabolic rate, energy acquisition, activity, survival) varies in response to environmental variation (e.g., Peterman and Gade, 2017). Assuming that variation in individual performance correlates with spatial or temporal variation in abundance, ecophysiological models can be particularly useful for modeling species responses to environmental change (Kearney and Porter, 2009). The development of mechanistic distribution models remains inaccessible for many species because key relationships between organisms and environmental factors are poorly understood (Kearney and Porter, 2009; Buckley et al., 2010). Even for well-studied species, mechanistic models may not be robust for predicting changes in species distribution or abundance if those models do not account for other key processes such as physiological plasticity, biotic interactions, or compensatory behaviors (Buckley et al., 2010).

Behavior is a key process that—when missing—may limit the predictive capacity of ecophysiological models (Beever et al., 2017; Riddell et al., 2018). Behavior permits flexibility and is the proximate means by which most animals—particularly ectotherms—remain relatively homeostatic despite environmental heterogeneity (i.e., Hertz and Huey, 1981; Bauwens et al., 1996; Belasen et al., 2016; Muñoz and Losos, 2018). Importantly, behaviors interact with an animals' physiology to determine performance. For example, animals engage in thermoregulatory behaviors in response to extreme temperatures such as seeking warmer microhabitats when conditions are cool, and cooler microhabitats when conditions are hot. These compensatory behaviors allow animals to occupy a wider range of climates at higher abundances than might be predicted by physiological tolerances alone (Sears et al., 2011; Riddell et al., 2018). Of course, while behaviors may allow animals to occupy a wider range of climates, there are likely performance costs associated with behavioral compensation. For example, choosing to bask in the sun to increase motor function consequently requires increased caloric intake to compensate for a higher metabolic rate. Identifying how behaviors interact with physiological processes to allow animals to occupy a broader range of environments while potentially constraining performance within some environments is particularly relevant to understanding how animals may respond to future novel environments. Novel environments provide additional complexity for predicative models (Williams and Jackson, 2007), and using mechanistic models that address behavioral and physiological plasticity can yield more realistic projections about species performances in those novel environments. A few recent studies have integrated behavioral plasticity into predictive, bioclimatic models in an effort to provide more realistic projections of species' distributions and performance under current and future climates (Gifford and Kozak, 2012; Sears and Angilletta, 2015; Peterman and Gade, 2017; Riddell et al., 2018).

Individual or agent-based models (hereafter, ABMs) provide a useful platform for integration of behavior into ecophysiological models. This use of ABMs was first applied to animal systems to model recruitment in fish populations and has been subsequently used for a variety of animal species, primarily focused on movement or migration behavior (summarized in DeAngelis and Grimm, 2014). Sears and Angilletta (2015) and Sears et al. (2011) integrated behavior and physiology into an ABM to model how local thermal heterogeneity might affect lizard performance. Those studies highlighted the importance of behavioral plasticity and local environmental heterogeneity to determine lizard energetics. Beyond these examples, ABMs remain an underutilized tool in ecological research specifically seeking to understand how behavior may act as a mechanism influencing animal responses to environmental change. That is not to suggest the relationship has not been considered in other contexts or using other methods.

We integrated ecophysiological models into an agent-based modeling framework to explore the influence of two behaviors—surfacing and plant climbing—on the sensitivity of Plethodontid salamander activity to variation in air temperature, soil temperature, and relative humidity. Because Plethodontid salamanders are lungless, they are dependent on moist skin for gas exchange and vulnerable to water loss. Consequently, Plethodontid activity and performance are strongly linked to moisture and limiting water loss (Feder, 1983). The Southern Appalachian Mountains are a global hotspot for Plethodontid diversity, and within the topographically complex region, species occupy landscapes with steep natural gradients in rainfall and temperature. The region is expected to experience increased temperature and more variable precipitation over the coming century, prompting a growing number of efforts to forecast how plethodontids will respond to future climate scenarios (Milanovich et al., 2010; Gifford and Kozak, 2012; Riddell et al., 2018). We simulated weather and environmental conditions based on field measurements and estimated the effects of the threshold of water loss on surfacing activity and climbing behavior on nightly and seasonal activity times. Plethodontid salamanders are known to retreat from the surface to below ground refugia to avoid water loss, though this comes at the expense of foraging time (Fraser, 1976). Other recent models of salamander activity or energetics have included surfacing and retreating behaviors (Gifford and Kozak, 2012; Peterman et al., 2013; Caruso et al., 2014; Peterman and Gade, 2017; Riddell et al., 2018), but no prior models have considered other compensatory behaviors like plant climbing. When active above ground, plethodontids will routinely move between the ground and climbing on vegetation, which may allow animals to alter dehydration rates by altering their body temperature (McEntire, 2016). We also included multiple size classes of animal in our model, which has only been included in one prior model (Riddell et al., 2018).

MATERIALS AND METHODS

Model Overview

The model estimated activity time using salamander dehydration rates. Although increased surface activity could make salamanders more vulnerable to predators, surface activity is directly correlated with foraging activity (Jaeger, 1972, 1980; Fraser, 1976), which serves a as a good proxy for fitness (Adolph and Porter, 1993). Correlations between higher predicted activity time and higher estimated density in the field (Peterman and Semlitsch, 2013, 2014), supports activity time as a good proxy for fitness despite any altered predation risk. We based our model on prior models of salamander activity and dehydration rates (Feder, 1983; Gifford and Kozak, 2012; Caruso et al., 2014; Peterman and Gade, 2017). As an additional compensatory behavior, we modeled plant climbing effects on activity, through its relationship to thermal-dehydration regulation. We varied the probability of rainfall to examine how surfacing and climbing behavior affected the sensitivity of salamander activity time to variation in precipitation patterns.

Model Landscape Development

Detailed methods and explanations of model structure are presented in the form of an "ODD" (Overview, Design concepts, and Details) protocol (Grimm et al., 2006, 2010) in the **Supplementary Materials**. We used NetLogo (Version 6.0.2, U. Wilensky, 1999) to simulate salamander activity on a 50 × 50 tile landscape, with each tile representing one square meter of forest habitat. Daily probability of rainfall ranged from 0.3 to 0.9 and was consistent for all cells in the landscape to create a gradient from relatively wet to dry climates among simulations. The simulations ran 20 times for each set of parameters and a single simulation lasted for one active season (April through October) with alternating day and night time steps (428 total steps). "Night" time steps varied in absolute length in terms of minutes, dependent on the monthly hours of darkness (see below), and dehydration rate was calculated on this minute-by-minute scale. The code is available from the authors upon request.

We simulated weather events, including rainfall events and nightly temperature, once every "24 h" (two time steps) for the entire virtual landscape, where all cells maintained the same values. Rainfall events occurred based on a probability ranging from 0.3 to 0.9 (in 0.05 increments). These events were assumed to be large enough to saturate the ground surface, but rainfall amounts were not directly modeled. Because rainfall events were used to determine relative humidity, the amount of rainfall would not be as important as frequency of events in this simulation. During rainfall events the relative humidity was set at 100%. Relative humidity is the result of a large number of interacting factors including cloud cover, temperature, air pressure, and rainfall events. For simplicity, when not raining, the humidity decreased by 10% each day it did not rain. Though a simplification of how relative humidity fluctuates, it created variability over time within the virtual environment, and comparable values to those observed during field studies (Howard, 2018). The model simulated seasonal changes in air temperature by generating temperatures from a random-normal distribution using the monthly average and standard deviation for nightly temperature data recorded at the Coweeta Hydrologic Laboratory at 5 stations spread over an area of 25 square kilometers from 2013 to 2014 (Miniat et al., 2017). Soil temperature was set to the average monthly soil temperature at 5 cm (Miniat et al., 2017). We used temperature data collected during this time period, because it was all that was available at the time of model development. Directly modeling vapor pressure deficit rather than calculating it may have been more efficient, but it is more challenging to model over time as it depends on relative humidity which is also difficult to simulate. Although a simplification, because air temperatures are typically measured at a height above the plants, plant temperatures are generally intermediate between measured soil and air temperatures (Geiger, 1965); therefore, we set vegetation surface temperature to an average between the soil and air temperature. Hours of daylight were set for each month to the monthly average for the latitude of the Coweeta Hydrologic Laboratory (35° 3′35.70″N, http://aa.usno.navy.mil/data/docs/Dur_OneYear.php).

Agent Simulation

The agents were modeled as salamanders in the genus *Plethodon*, specifically the *Plethodon jordani* species complex. We included three size classes of individuals: hatchlings [snout-vent-length (SVL) <32 mm], juveniles (SVL 32–42 mm), and adults (SVL 43–72 mm). We simulated 1,000 agents of each size class for a total of 3,000 agents randomly distributed across the uniform landscape, and we did not include any density-dependent affects.

Resistance to water loss is a critical value for estimating activity time using biophysical models. Some studies found amphibians' skin acts as a free water surface (Spotila and Berman, 1976), meaning their resistance value is very close to zero. However, other studies have found species-specific differences and variable resistance values based on environmental conditions with values greater than zero (Littleford et al., 1947; Cohen, 1952; Ray, 1958; Spotila, 1972; Riddell and Sears, 2015; Riddell et al., 2017). We used data for *P. jordani* reported in Spotila (1972) to create a function of predicted resistance to water loss based on soil temperature Equation (1). We used soil temperature as the primary temperature because salamanders regularly experience soil temperatures for extended periods compared to brief episodic climbing events. Therefore, we expect salamanders to be physiologically acclimated to ambient soil temperatures. There is little data on acclimation rates of salamander physiological processes, but available studies suggest processes such as water loss rate are unlikely to occur within the time frame of a single night of foraging (e.g., Riddell et al., 2017).

$$R = 0.425T_S + 0.8136 \qquad (1)$$

Where R is the resistance to water loss in seconds per centimeter and T_s is the soil temperature in Celsius. While recent physiological models suggested the importance of including calculations of boundary layer resistance as part of the estimate of salamander's resistance to water loss (Riddell et al., 2017), we used literature values for skin resistance based on field-recorded water loss, which effectively included the boundary layer resistance in the value of skin resistance to water loss. Riddell et al. (2018) recently demonstrated seasonal plasticity in skin resistance, suggesting the monthly changes in water loss resistance in our model were appropriate.

We assumed body temperature was equivalent to the surface the salamander was sitting on. This deviates from previous studies that estimated operative body temperature using mathematical equations (Gifford and Kozak, 2012; Peterman and Gade, 2017). Body temperatures measured in the field were equivalent to the temperature of the substrate upon which they were found (McEntire, 2018) and were on average 2.38 ± 1.04°C cooler than the air temperature (McEntire, 2018). These relationships, along with previous studies using the temperature of the substrate beneath the salamander as the body temperature (Feder and Lynch, 1982), suggest it was reasonable to assume modeled soil temperature as the agents' initial body temperature. If a salamander remained on the ground, they maintained the soil temperature as their body temperature; when a salamander climbed on vegetation, they adopted the temperature of the plant as their body temperature. Salamanders on the ground maintained consistent conditions; we did not include the possible differences in microclimate above or below leaf litter. We assumed consistent relative humidity and still (no wind) conditions for both agents on the ground and climbing.

When air temperatures were cooler than soil temperatures, agents had a 0.50 probability of climbing once they were surface active. This value was not chosen to represent natural climbing rates, which remain unknown. By setting a fixed probability of climbing, this resulted in ∼50% of the agents climbing on any given evening, which allowed us to compare differences in activity time between those agents that climbed and those that did not. We did not allow salamanders to climb when soil temperatures were cooler than air temperatures because we assumed that this would create conditions where dehydration was always faster for climbing animals. This was not to imply that real animals would not climb under such conditions in the field (see McEntire, 2018).

Model Process

We constrained surface activity based on field observations and other published studies to times when: (1) the relative humidity was above 45% and (2) the animal's water deficit was $<4\%$ (Feder and Londos, 1984). Although salamanders sometimes retreated before reaching 4% water loss (the 3% water loss threshold simulations), this threshold offered the possibility of losing water while in the soil or failing to fully rehydrate. Simulated body temperatures did not fall out of the range of active temperatures used in previous studies (Peterman and Gade, 2017; Riddell et al., 2018), so this restriction was not included. The salamanders had one opportunity to surface at the beginning of the night and remained active until they reached their water loss threshold. Nightly foraging time in minutes was calculated based on dehydration rate Equation (2)

$$EWL = \frac{\rho_{sal} - \rho_{air}}{R} \tag{2}$$

where EWL is evaporative water loss (g cm^{-2} sec^{-1}); R is the resistance value of the salamander to dehydration (sec cm^{-1}); ρ_{sal} is the vapor pressure density at the surface of the salamander and is assumed to be equal to the saturation vapor pressure density (Tracy, 1976); ρ_{air} is the vapor pressure density of the air given the relatively humidity. We estimated these values based on standard equations related to temperature (**Supplementary Materials**). We multiplied the evaporative water loss rate by an agent's surface area (cm^2) to estimate grams of water lost per minute. The agents remained active until they lost 3–10% of their body mass, which is the threshold range observed in dehydration studies and used in other models (Ray, 1958; Feder and Londos, 1984; Gifford and Kozak, 2012; Caruso et al., 2014; Peterman and Gade, 2017; Riddell et al., 2018). For each simulation, the percentage of water lost was held constant and consistent among all individuals, but we compared 3, 5, 7, and 10% water loss thresholds because previous models suggested high sensitivity to this value (Peterman and Gade, 2017).

When simulated salamanders were inactive, either during the day, at night when not surfacing, or after retreating for the night, they rehydrated on a minute-by-minute basis as determined by soil temperature and soil moisture Equation (3) (Spotila et al., 1992):

$$r = (\varphi_{sal} - \varphi_{soil}) \times K \tag{3}$$

Where rehydration is measured in grams per cm^2 per minute, φ_{sal} is the water potential of the salamander in pascals, φ_{soil} is the soil moisture tension in pascals, and K is the hydraulic conductance, set at 0.00000013 g cm^{-2} min^{-1} pa^{-1} as calculated for Leopard frogs, *Rana pipiens*, (Tracy, 1976). The water flux of the salamanders was estimated using the following Equation (4) established for Leopard frogs (Tracy, 1976).

$$\varphi_{sal} = \left(-284.802\theta^3 + 773.427\theta^2 - 703.223\theta + 0.0214809\right) \times 100 \tag{4}$$

Where θ is the hydration level of the salamander (1—percent of mass lost to water loss). These parameters are currently unavailable for salamanders; however, the leopard frog values produce reasonable rehydration rates reflective of those found in previous studies with salamanders (Cohen, 1952; Spight, 1967a,b; Spotila, 1972; Feder, 1983). Soil moisture was modeled simply and remained well above the minimum water potential for salamanders to rehydrate (−2 atm, Spight, 1967a,b; Spotila, 1972) as modeling soil moisture dynamics went beyond the scope of this study.

Agents recorded their nightly foraging time in minutes, which allowed for comparison of nightly activity with simulated environmental factors. At each "night" time step, we recorded the average nightly activity time of each size class. When climbing occurred, we divided the agents into "climbers" and "non-climbers," then recorded the average nightly time for each size class in both categories.

Sensitivity Analysis

We conducted a sensitivity analyses for the assigned threshold for water loss. We did not conduct sensitivity analyses on other

TABLE 1 | Simulated abiotic factors, and biophysical parameters and functions used in the model.

Parameter name	Parameter value	Source
Hour	9.5–13	http://aa.usno.navy.mil/data/docs/Dur_OneYear.php
Air temperature	Random normal draw from:	Average nightly temperatures recorded between 2013 and 2014 at the Coweeta LTER (± 1 SD) (Miniat et al., 2017)
April	11.89 ± 1.85	
May	15.00 ± 1.83	
June	18.57 ± 0.70	
July	19.07 ± 0.95	
August	19.15 ± 1.01	
September	17.36 ± 1.94	
October	12.07 ± 3.00	
Soil temperature		Average nightly temperatures recorded between 2013 and 2014 at the Coweeta LTER (Miniat et al., 2017)
April	11.64	
May	14.17	
June	17.44	
July	18.61	
August	18.62	
September	17.95	
October	14.13	
Probability of climbing	0 or 0.5	For model evaluation
Rainfall probability	0.3–0.9 at 0.05 increments	For model evaluation
Water loss threshold	0.03, 0.05, 0.07, 0.10	Peterman and Gade, 2017
Resistance to water loss	$0.425 * T_{soil} + 0.8136$	Derived from Spotila (1972)
Snout-vent length (mm)	$32.8 * \text{mass}^{-0.34}$	Howard, 2018
Surface area	$8..42 * \text{mass}^{0.694}$	Whitford and Hutchison, 1967
Hydraulic conductance	1.3×10^{-7}	Tracy, 1976

Additional details can be found in ***Supplementary Materials****.*

model values that have been validated by previous studies or were based on the best available published values (**Table 1**; Whitford and Hutchison, 1967; Spotila, 1972; Tracy, 1976; Peterman and Gade, 2017). There were 52 possible simulations combining possible rainfall probabilities and thresholds for water loss.

Data Analysis

We limited comparison of nightly activity between climbing and non-climbing salamanders to simulated nights when air temperature was cooler than soil temperature. We subtracted the recorded average activity time of non-climbing agents from the time for climbing agents to determine any difference in nightly activity time associated with the behavior. We visually examined the effects of weather, body size class, and threshold for water loss on differences in nightly activity time between climbing and not climbing salamanders. We used general additive models with smoothing to make patterns easier to visualize. To evaluate the effects of climbing on sensitivity of salamanders to "climate," we summed the nightly activity times for each size class and rainfall probability per simulation across the rainfall probability gradient. We assumed animals were active every night of the simulation, so the absolute values do not reflect true estimates of seasonal activity for salamanders.

RESULTS

As part of the model structure, climbing only occurred on nights when simulated air temperature was lower than soil temperature. This resulted in ∼85 nights when climbing could occur out of 214 nights per active season simulation. The maximum potential difference in nightly activity time between climbing and non-climbing animals ranged from 6.1 to 7.0 h. There were a few instances when climbing resulted in lower activity times compared to remaining on the ground, with those differences not exceeding 22 min. During rainy nights with only slight differences between soil and air temperature, there was little to no difference in activity time between climbing and non-climbing salamanders (**Figure 1**). As the temperature difference between the ground and air increased, the benefits of climbing increased for all size classes of salamander and all thresholds of water loss (**Figure 1**). Hatchlings were the most responsive to very small temperature differences regardless of water loss threshold or time since last rainfall (**Figure 1C**). Importantly, as conditions dried [days since rain increased], the advantages of climbing manifest with a smaller temperature difference between the ground and air, though days since rain also constrained the realized activity time because of the negative relationship to relative humidity. The general patterns of increased activity among climbing animals with increasing temperature difference did not vary with the threshold of water loss; however, the benefits of climbing manifest at a lower temperature difference as the threshold for water loss declined (**Figure 1**). The unimodal patterns of differences in activity time as a function of temperature differences during rainy nights reflected other constraining conditions. Specifically, in our observed weather data used to simulate weather in the model, we observed air temperatures ∼5+ °C cooler than soil temperatures when cold fronts moved in, creating near freezing air temperatures while soils remained relatively warm (**Figure 2**). These cold conditions constrained salamander activity independent of factors affecting dehydration rates. Across our rainfall probability gradient, total seasonal activity time increased with increasing probability of rainfall (wetter climate), and the benefit of climbing was consistent across the rainfall probability gradient (**Figure 3**). Overall, climbing had the largest benefit for hatchlings across the rainfall probability gradient (**Figure 3**). The total seasonal activity time was sensitive to the water loss threshold, with increasing activity time in relation to increased thresholds (**Figure 3**).

DISCUSSION

Inclusion of climbing behavior increased activity time of salamanders under the specified conditions and acted as a compensatory behavior when interacting with both time since last rain and differences in air and soil temperatures. As a compensatory behavior, climbing may decrease the predicted sensitivity of salamanders to climate change compared to models

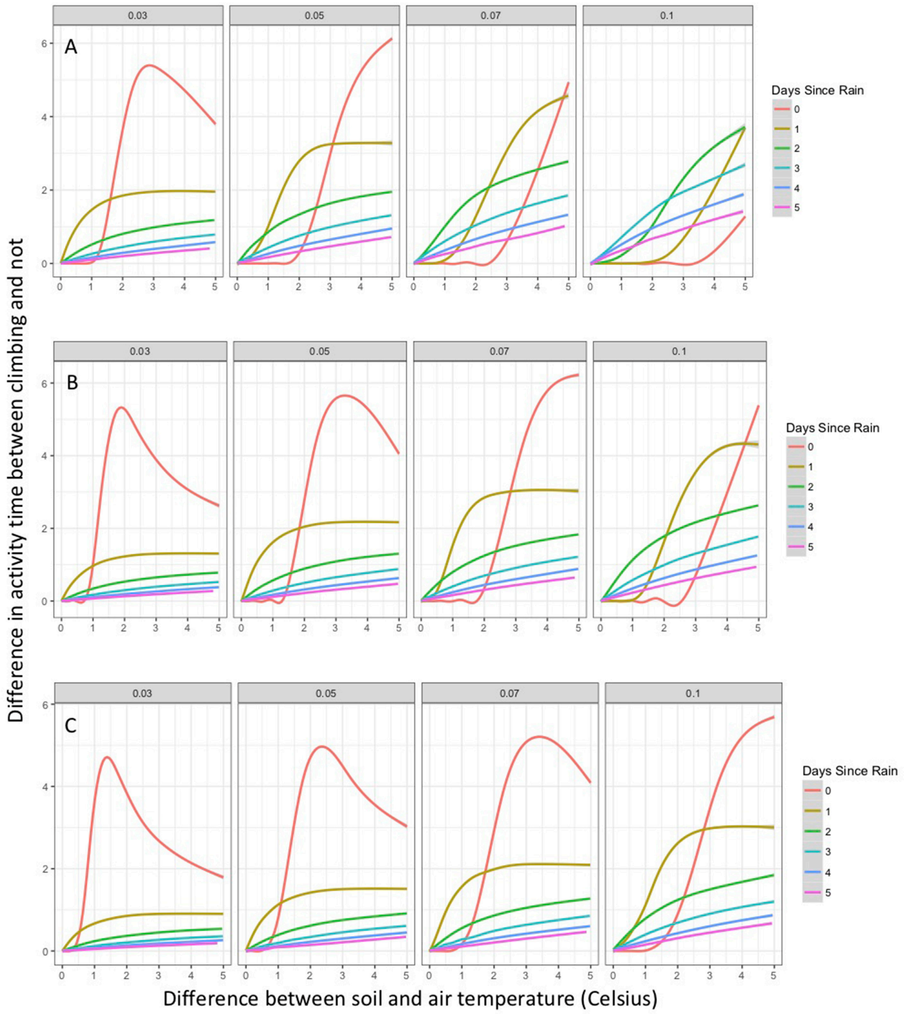

FIGURE 1 | Percent change in activity time of climbing compared to non-climbing salamanders as a function of the difference between air and ground temperature (limited to most common values), days since last rain, water loss threshold, and body size class. Shown is the generalized additive model of the percent increase in mean activity time for non-climbing salamanders compared to the mean activity time of climbing salamanders on the same simulated night. Positive value indicates greater activity time among climbing salamanders. The gray area around each line represents a 95% confidence interval. **(A)** adult salamanders, **(B)** juvenile salamanders, and **(C)** hatchling salamanders.

that do not including climbing. This is broadly consistent with other recent models that show the inclusion of behavioral and physiological plasticity reduces the predicted impact of climate warming on the performance and distribution of salamanders (Riddell et al., 2018) and other taxa (Sears et al., 2011; Sears and Angilletta, 2015). In laboratory studies, *Plethodon cinereus* and *P. metcalfi* have demonstrated physiological plasticity in response to temperature (Riddell and Sears, 2015; Novarro et al., 2018). An interesting outcome of our model results is the demonstration that the addition of climbing behavior creates a mechanism by which salamanders that occupy areas with lower rainfall probabilities can realize comparable amounts of activity time to salamanders that occupy areas with a higher rainfall probability. We caution that these inferences do not account for any costs that may be associated with climbing such as increased predation pressure or altered temperature-dependent metabolic rate. In addition, variation in rainfall likely affects productivity and food availability independent of activity time, and we are assuming that equivalent activity time translates to equivalent food intake rates regardless of whether the salamander is climbing or not. This assumption has not been validated in the field. Jaeger (1978) found that eastern red-backed salamanders (*P. cinereus*) had larger, higher-quality prey in their stomachs compared to animals found on the ground; and Mitchell and Taylor (1986) reported arboreal invertebrates in the diets of red-legged salamanders (*P. shermani*), suggesting climbing salamanders can access specific prey. However, a more recent study of *P. shermani* found similar diets and volume of prey between individuals collected on plants and those collected on the ground (Lewis et al., 2014), and Roberts and Liebgold (2008) estimated that potential prey of *P. cinereus* were actually less abundant on plants compared to the ground. Therefore, it remains to be determined whether salamanders that climb are able to translate increased activity time to equal or greater intake of prey or some other fitness benefit.

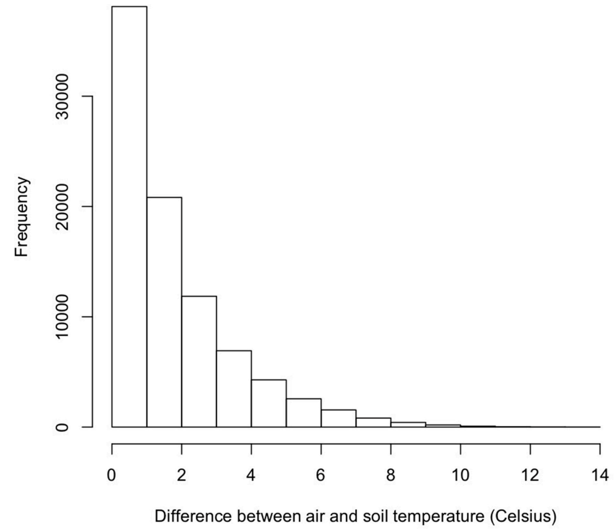

FIGURE 2 | Histogram of differences between simulated air and soil temperature.

Assuming that climbing is adaptive, particularly in areas with less frequent rainfall, our model results would suggest a shallower relationship between rainfall gradients and salamander abundance than would be predicted by models that exclude climbing or other plastic traits. Because salamander activity is tied to rainfall, this may suggest climbing offers a mechanism for salamanders to increase their activity time in drier areas. Our predictions are consistent with other studies that find hatchling salamanders should be most sensitive to drier conditions (created by either declining precipitation or increased temperature, Riddell et al., 2018). We currently lack empirical data that can be used to test this prediction. Our model predictions are generally consistent with two studies that demonstrate both reduced spatial abundance of salamanders in relation to mean annual precipitation (Howard, 2018) or soil moisture (Peterman and Semlitsch, 2013). In both studies, the largest contributor to declining abundance was the decline in hatchling or juvenile abundance, which is also consistent with model predictions. Howard (2018) estimated salamander abundance and population structure across the Coweeta basin, which served as the context for our simulation model and was the source of our weather conditions in simulations. Her results demonstrate a rather shallow decline in abundance over a large range of mean annual precipitation until condition become very dry. Qualitatively, the shape of this relationships is similar to our modeled estimates of activity time across the rainfall probability gradient.

One powerful use of models is to examine and estimate latent processes that cannot be observed. It is currently not

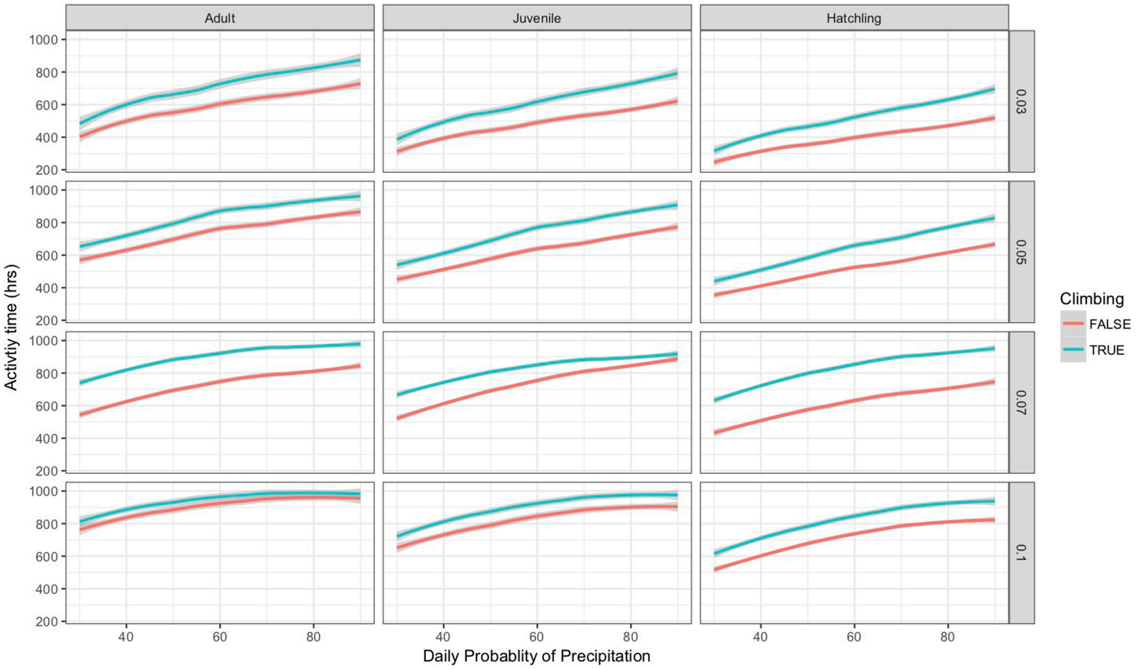

FIGURE 3 | Estimated mean total seasonal activity time, restricted to nights when climbing occurred, for climbing and non-climbing adult, juvenile, and hatchling salamanders as a function of the probability of precipitation and water loss threshold in the model simulation. The lines were smoothed using a logarithmic linear model fit to the data, gray bands surrounding each line indicate a 95% confidence interval. Each water-loss threshold is shown individually as indicated by the far right axis.

practical (or likely even possible) to directly measure salamander activity time in the field, yet it is widely accepted as a critical currency in salamander fitness. One emergent pattern in our model was that the benefits of climbing increase as time since rain decreases (decreased relative humidity). This contradicts earlier characterizations of this behavior. Jaeger (1978) hypothesized that dehydration during plant climbing would restrict the behavior to rainy nights. Our model results, across a range of weather conditions and thresholds for water loss, suggest the benefits of climbing to activity time increase with time since rain and are instead likely to be determined by differences in temperature between the ground and air rather than rain events. In fact, at higher water loss thresholds, which have been demonstrated for our model *Plethodon* species (Riddell et al., 2018), our model predicts little to no advantage in terms of activity time for climbing during rainy nights unless extreme temperature differences occur. Although the model is constrained by the coded rules, this interaction between temperature and relative humidity (a function of days since rain) was not directly coded and was an emergent result. Whether this predicted relationship is true requires field validation.

There are some important limitations to our model in terms of predicting the advantages of climbing or when we expect to see increases of climbing in the field. First, as mentioned previously, our model does not incorporate potential costs that may be associated with climbing. Second, in addition to regulating water loss, salamanders may be selecting among thermal microhabitats to optimize metabolic rate and may be balancing increases in activity time with metabolic efficiency (Riddell et al., 2018). Though our model can integrate metabolic rates and efficiencies, we did not evaluate that parameter in these simulations. In our model, we strictly evaluate the benefits of climbing on activity time as a function of water loss, because any potential costs are unknown. Our results suggest that—generally—the benefits of climbing increase as the air becomes progressively cooler compared to the ground. In reality, weather data indicate that this occurs most frequently during cooler seasons when the ground is warmer but the air is particularly cold. Under these conditions, it is reasonable that a salamander would remain on the ground where temperatures are more metabolically optimal even though their dehydration rate would be greater and their activity time shorter. Ultimately, field studies that can directly or indirectly relate activity time to behavior as a function of weather are needed to validate our and other models and the importance of behaviors in determining salamander fitness and demography.

Another outstanding issue in ecophysiological models of salamanders and other amphibians is the threshold for evaporative water loss before an animal will cease activity and retreat into moist refugia. An early laboratory study on *Desmognathus ochrophaeus* suggested salamanders will "give up" activity at much lower levels of water loss than they can tolerate before becoming impaired, and that water loss thresholds may be plastic depending on the dehydration potential of the environment (Feder and Londos, 1984). A model by Peterman and Gade (2017) estimated similar high sensitivity to the threshold for water loss for *Plethodon*. However, field measures of *Plethodon metcalfi* water deficits recorded by Riddell et al. (2018), suggested the species may tolerate higher water loss thresholds than has been demonstrated in the lab or through other models. The sensitivity of our and other model estimates to an assumed water loss threshold (e.g., Gifford and Kozak, 2012; Peterman and Gade, 2017; Riddell et al., 2018) illustrates the need to better understand this parameter, particularly for models that will be used in climate change projections. Moreover, differences in water loss resistance and threshold water loss tolerances among size classes and species would aid in understanding behavioral differences in the field and comparing the relative sensitivity of species to spatial and temporal climate change. For example, our model predicts that size classes or species with reduced water loss resistance or lower water loss thresholds or greater surface area to volume ratios would realize a greater benefit from climbing at a lower temperature differential. We would expect these size classes or species to show a greater propensity to climb compared to larger size classes or species with greater skin resistance to water loss.

A final limitation of our model, and all models to date, was our inability to deal with natural variation in soil moisture and soil tension. Our model does include some variability in soil moisture, but we were unable to provide additional insight about this poorly understood relationship. During model development, when soil moisture dropped below −0.2 atm, the majority of animals would "die" from an inability to rehydrate in the soil. However, Spotila (1972) and Spight (1967a) demonstrated that *Plethodon* could rehydrate up to a soil moisture of −2 atm. Salamanders are also known to create a "wetting front" to slow water loss in dry soils (Spotila, 1972), but the mechanism and the conditions under which this front is formed along with the hydraulic conductance or water flux rates for salamanders remains undescribed. This indicates that current rehydration models for salamanders [and other amphibians] are incomplete. This also illustrates that while salamanders spend a large portion of their time underground or in retreats, all current models of salamander interactions with climate have focused only on their time above ground (i.e., Walls, 2009; Milanovich et al., 2010; Gifford and Kozak, 2012; Ficetola et al., 2016; Peterman and Gade, 2017; Riddell et al., 2018). Until our knowledge gap about salamander below ground relationships to soil conditions is filled, our models of these organisms will remain incomplete and prone to error. This illustrates a wider problem with the development of mechanistic models. Plethodontid salamanders are a group that are well-studied and for which there is a relatively rich physiological literature, and yet our current knowledge is still insufficient to construct a full working physiological model of how these animals interact with their environment (Gifford, 2016). Knowledge of most other animal species is far more deficient, which will limit the ability to develop and apply mechanistic models to understanding the performance of those species in current or future novel environments.

Because of the heterogeneous nature of most environments, most animals use behaviors to compensate for less suitable conditions that might otherwise limit their performance and ability to occupy a site. Behavioral and physiological plasticity and acclimation are one of the most proximate phenotypic responses of animals to environmental change, and compensatory behaviors and plasticity in physiology act as key mechanisms for acclimatization to seasonal climates or short-term changes in weather, food availability, predation (Muñoz et al., 2016; Beever et al., 2017). These compensatory traits, therefore, shape the potential sensitivity of animals to longer-term environmental changes (Beever et al., 2017). After field validation, by including multiple behaviors and physiological processes into mechanistic models, we can make more robust predictions about how organisms might respond to future novel environments. Although recent models are incomplete, the demonstration by several recent studies that the integration of behavioral and physiological plasticity dramatically reduces the projected impacts of climate change on some species is heartening and should have important ramifications for how we think about threats and conservation actions.

DATA AVAILABILITY STATEMENT

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

AUTHOR CONTRIBUTIONS

JM contributed to idea generation, designing sensitivity analyses, and writing. KM was primarily responsible for model development and contributed to idea generation and writing.

ACKNOWLEDGMENTS

We thank Dr. Jeffrey Hepinstall-Cymerman, Heather Abernathy, Jenny Asper, Jillian Howard, and Elizabeth Hunter for assistance with model development. This research was supported by two National Science Foundation awards from the Long Term Ecological Research Program to the Coweeta LTER Program at the University of Georgia (DEB-0823293 and DEB-1637522). Additional support was provided by the Daniel B. Warnell School of Forestry and Natural Resources at the University of Georgia and the Graduate School at the University of Georgia. Precipitation data was provided by the Coweeta LTER and the USDA Forest Service. We thank Dr. Chelcy Miniat for logistical support and for permission to use Forest Service data when appropriate.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2019.00022/full#supplementary-material

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Conflict of Interest Statement: 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.

frontiers in Ecology and Evolution

ORIGINAL RESEARCH
published: 02 April 2019
doi: 10.3389/fevo.2019.00089

Changing Migratory Behaviors and Climatic Responsiveness in Birds

Veronika Bókony[1]*, *Zoltán Barta*[2] *and Zsolt Végvári*[3]

[1] Lendület Evolutionary Ecology Research Group, Centre for Agricultural Research, Plant Protection Institute, Hungarian Academy of Sciences, Budapest, Hungary, [2] MTA-DE Behavioral Ecology Research Group, Department of Evolutionary Zoology, University of Debrecen, Debrecen, Hungary, [3] Department of Conservation Zoology, University of Debrecen-Hortobágy National Park Directorate, Debrecen, Hungary

Change of avian migratory behavior is one of the best-studied phenomena presumably associated with contemporary climate change, yet to what degree these behavioral changes represent responses to climate warming is still controversial. We investigated interspecific variation in migratory behavior over three decades at a Central-European site, testing whether the type and extent of behavioral change are predicted by species' responsiveness to short-term variation in large-scale climatic indices. We found that species with earlier arrivals at the breeding grounds after winters with higher North-Atlantic Oscillation indices were more likely to overwinter at the study site. This behavior was more frequent in the second half than in the first half of the study, although the extent of this change was not predicted quantitatively by short-term climatic responsiveness. Overwintering was more prevalent in short-distance migrants with more complex diets and larger population sizes. Furthermore, species arriving earlier after summers with higher Sahel rainfall indices increasingly advanced their first arrival date, whereas species that do not molt in the pre-breeding season increased their frequency of overwintering in more recent years. Our results demonstrate that interspecific variation in short-term climatic responsiveness predicts long-term changes in migratory behaviors, supporting that the latter are responses to climate change. Furthermore, the type of response (advancing arrivals or overwintering near the breeding grounds) depends on life history. Finally, we found that overwintering behavior during the study period predicted subsequent trends in population size, suggesting that information on temporal changes in migratory strategy may help conservation planning and risk assessment.

Keywords: migration phenology, overwinter residency, population trends, Hortobágy, waterbirds

Edited by:
Susan C. Walls,
United States Geological Survey,
United States

Reviewed by:
Jose A. Masero,
University of Extremadura, Spain
Piotr Tryjanowski,
Poznan University of Life Sciences,
Poland

Correspondence:
Veronika Bókony
bokony.veronika@agrar.mta.hu

Specialty section:
This article was submitted to Behavioral and Evolutionary Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: *30 September 2018*
Accepted: *08 March 2019*
Published: *02 April 2019*

Citation:
Bókony V, Barta Z and Végvári Z (2019) Changing Migratory Behaviors and Climatic Responsiveness in Birds. Front. Ecol. Evol. 7:89. doi: 10.3389/fevo.2019.00089

INTRODUCTION

In parallel with global climatic changes, various alterations of physiology, distribution, and phenology in a wide range of species have been documented and interpreted as biological responses to climatic changes (Hughes, 2000; Walther et al., 2002; Parmesan and Yohe, 2003; Parmesan, 2006; Poloczanska et al., 2013; Thackeray et al., 2016). An interesting aspect of these alterations is that they show large variation among species (Visser and Both, 2005; Møller et al., 2008; Végvári et al., 2010). Understanding the causes and consequences of this interspecific variation is crucial for several reasons. For example, research on the effects of climate change contributes to a better understanding of the ecological and evolutionary significance of environmentally induced phenotypic change. Studying how these changes occur can shed light on the relative roles of phenotypic plasticity and genetic differentiation in adaptation (Van Buskirk et al., 2012; Urban et al., 2014). Furthermore, climate-driven changes can be used to inform species-specific conservation planning, as phenotypic changes over recent decades have been shown to be an important predictor of population declines in a number of species of key conservation importance (Møller et al., 2008; Végvári and Barta, 2016).

Notably, however, not all the phenotypic changes over recent time are necessarily driven by climate change. For example, while increasing temperatures are predicted to bias the sex ratios of reptiles with temperature-dependent sex determination (Mitchell and Janzen, 2010), historical trends in turtle sex ratios were found to be explained instead by the growing density of roads which increases female mortality (Gibbs and Steen, 2005). Similarly, the recent loss of migratory behavior documented in several bird species as an apparent response to warmer winters (Pearce-Higgins and Green, 2014) has been suggested to be a part of an adaptation to increasing urbanization, with residency being facilitated by overwinter availability of anthropogenic food (Møller et al., 2014). Although disentangling the causes of long-term phenotypic changes is challenging, evidence is needed to ascertain whether such changes actually represent responses to climate change. If they do, then these responses remain the most readily usable indices of climatic responsiveness, enabling comparisons across a large number of species for scrutinizing drivers and constraints of climate-driven adaptations (Rubolini et al., 2007; Végvári et al., 2010; Bókony et al., 2017; Usui et al., 2017).

Changes in migratory behaviors, especially in birds, are one of the best-studied phenomena in relation to contemporary climate change (La Sorte and Jetz, 2010; Knudsen et al., 2011; Scridel et al., 2018). The timing of migratory movements has been shifting in several taxa in parallel with climate warming, such that the migrants arrive at the breeding grounds of the northern hemisphere earlier in spring and may depart from there later in autumn (Hüppop and Hüppop, 2003; Marra et al., 2005; Gordo, 2007; Usui et al., 2017). It has been shown that birds have decreased the distances they migrate (Visser et al., 2009) and changed their distribution and abundance at wintering sites (Maclean et al., 2008). In several species, loss of migration has also been documented, such that a part or all of a population started to overwinter at the breeding sites (Pearce-Higgins and Green, 2014). The degree to which different species alter their migration has been shown to be related to a number of selective forces and constraints. For instance, interspecific comparisons support that the advancement of spring arrivals is constrained in species with long migration distances, specialized diets, and extensive pre-breeding molts (Rubolini et al., 2007; Végvári et al., 2010). In contrast, arrival is accelerated in species with greater fecundity (Møller et al., 2008; Végvári et al., 2010) and in some taxa with strong sexual selection (Spottiswoode and Møller, 2004; Rubolini et al., 2005; Spottiswoode et al., 2006). These findings suggest that ecology and life history influence the species' responsiveness to climate change. However, these ecological and life-history traits may also facilitate or constrain the species' adaptation to other contemporary environmental changes including habitat urbanization, which is also thought to influence migratory behaviors (Møller et al., 2014).

In comparative studies involving a large number of species, a typical difficulty is that the responses to climatic processes have to be quantified in a way that is similar across species. Detailed study of individual species can identify specific weather variables, time windows, and geographical areas with the strongest effects on migratory and wintering behaviors (Haest et al., 2018a,b), but this kind of information is not available for all species, and it is not trivial to make comparisons on the basis of data that vary from species to species. As a solution, local weather conditions can be approximated by using climatic indices measured at large spatial scales (Sandvik and Erikstad, 2008), such as the North Atlantic Oscillation (NAO) and the Sahel Rainfall Index (SRI). In many species, arrival dates are correlated with the values of NAO that describes fluctuations in the difference of atmospheric pressure at sea-level between the Icelandic low and the Azores high, indicating the strength and direction of westerly winds and thereby the climate fluctuations in the North Atlantic region and in the surrounding humid areas (Bradley et al., 1999; Forchhammer et al., 2002; Cotton, 2003; Hüppop and Hüppop, 2003; MacMynowski and Root, 2007). Positive NAO phases are accompanied by warmer winters in Europe with more precipitation in northern areas and less precipitation in central and southern areas, although the strength of these correlations is not homogenous across the continent (Cleary et al., 2017). Sahel Rainfall Index, a widely used metric of precipitation anomalies in the African Sahel zone between June and October (Dai et al., 2004), seems to also affect the spring arrival dates of long-distance migrants in southern Europe (Gordo et al., 2005; Gordo and Sanz, 2006; Saino et al., 2007). Trans-Saharan migrants can be directly affected by SRI, whereas species wintering in more northern areas might be influenced indirectly due to the climatic connectivity between the Mediterranean and the Sahel regions (Rowell, 2003; Park et al., 2016). Thus, both NAO and SRI can be used as proxies of weather conditions encountered by wintering and migrating birds, so species' responses to year-to-year variability in NAO and SRI can be considered as measures of responsiveness to weather and, thereby, to climate change. Indeed, bird species have been shown to vary greatly in how much their migration timing and winter distribution are explained by NAO (Haest et al., 2018a; Pavón-Jordán et al., 2018) or by weather variables at wintering and stopover sites (Van Buskirk et al., 2012; Haest et al., 2018b). Although some of this interspecific variation may be due to methodological issues (Haest et al., 2018a), biological differences such as habitat type (Pavón-Jordán et al., 2018) and migration distance (Van Buskirk et al., 2012) may also cause different species to vary in weather responsiveness. It remains unclear whether these differences explain why not all species have adjusted their migratory behaviors to the same extent over time as climate has been warming (Van Buskirk et al., 2012; Haest et al., 2018a,b; Pavón-Jordán et al., 2018).

Using this approach, we hypothesized that species which are more readily tracking inter-annual variation in weather are more likely to respond to long-term changes in climate such as a systematic increase of mean temperatures over time. Assuming that the species that are more strongly influenced in the short term—i.e., by year-to-year variability in NAO and/or SRI—have greater climatic responsiveness, one would also expect that these species show the largest changes in migratory behavior in response to long-term climatic changes. Therefore, we predicted that short-term responsiveness to climatic indices (weather responsiveness) should correlate with the extent of long-term changes in migratory behaviors if the latter are driven by climate change. To test this prediction, we examined variation in change in migratory behavior over ca. 30 years among 107 bird species. We quantified two aspects of long-term behavioral change over time: temporal shift of spring arrivals and the frequency of overwintering at the breeding site. We then quantified short-term climatic responsiveness as the slope of linear regressions of arrival date against SRI and NAO. We tested whether these two measures of short-term climatic responsiveness are correlated across species with (i) a long-term shift in arrival date over time and (ii) temporal change in the likelihood of overwintering. Such significant correlations would strongly suggest that these behavioral changes are responses to climate change. We also investigated whether the advancing spring arrivals and more frequent overwintering at breeding sites, observed at the population level, are predicted by ecological and life-history traits that are likely to influence a species' ability and/or need to adjust to climate change (Végvári et al., 2010). Finally, we examined whether the subsequent trends in population size were explained by the changes in migratory behaviors to infer their potential adaptiveness and conservation importance.

METHODS

Data Collection

We obtained the arrival data of migratory birds in Hortobágy National Park (HNP, Eastern Hungary, N47° 30′, E21°10′) covering 80,200 hectares. The area is the most extensive continuous alkaline grassland in Europe consisting of steppe areas interspersed with alkali marshes. Out of this area, 27,000 hectares are designated as Ramsar sites, being a stopover site of key importance for migratory and breeding waterbirds in Central Europe (Ecsedi, 2004). Our database was compiled from the field records of Gábor Kovács collected between 1969 and 2007 during his regular field trips as a ranger in the south-western part of the HNP. His surveys followed standardized census routes, covering a representative set of natural habitats in the studied areas avoiding settlements but not excluding farmlands between 1st January and 31st May of each year. This dataset contained data on the first observation date (FOD) of each species in multiple years (**Supplementary Table 1**). All data were collected by the same person following the same protocol, thus sampling effort was highly standardized and constant over the years. During the study period, 42,720 h were spent in the field (mean ± SD: 219.1 ± 27.7 h per month) and a total of 154,000 km were covered (789.7 ± 107.7 km per month), demonstrating the high effort invested into the detection of birds present in that area.

We examined the species that met the following criteria: (1) data available for at least 17 years, which is more than half of the period between 1975 and 2006 (we excluded the years 1969–1974 because the sampling effort was lower in these first years and 2007 because it was an extreme year, see below) and (2) the length of continuous time span with missing data at the start of the study period not longer than 3 years, so the earliest year with FOD not later than 1978. In total, 108 species met these criteria, with 17–32 (median: 30) years of available FOD data per species (**Supplementary Table 1**). Note that the missing data mean that the species was not observed in that particular year; this was due to certain species occurring less frequently in HNP. Nevertheless, our criteria ensured that the time series were comparable across species, because even if a species was not observed in some years, the gaps were relatively evenly distributed across the study period (**Supplementary Table 1**). We disregarded the data from 2007 because the autumn and winter preceding the spring of 2007 were extremely warm, accompanied by anomalous phenologies (Luterbacher et al., 2007). As this extremely warm year happened to be the last year for which we had the data, including it would have biased our estimates, resulting in spuriously steep changes of migratory behavior over time. Omitting this extreme year makes our inference conservative. As first observations can be influenced by factors affecting detectability, we conducted several analyses to check the effects of such factors (see **Supplementary Methods and Results**); based on these results, we consider our data to be unbiased for the purposes of the current study.

We defined individuals observed before 15 January in a given year as overwinterers, whereas we treated all FODs recorded after 15 January as first arrival dates (FAD). Therefore, when overwintering individuals of a species were detected in a year, we have no data on FAD for that year because the arriving migrants and overwintering individuals cannot be distinguished in our dataset (**Supplementary Table 1**). We do not know whether the observations of overwintering individuals were due to shortening of migration distance (a northward shift of wintering grounds) or complete loss of migration in these birds. Nevertheless, both mechanisms result in the occurrence of overwinterers at the population level. Variation in these occurrences across years and species is worth studying because they represent a different response than the earlier arrivals, i.e., overwinterers are affected by local weather at the wintering grounds, whereas the migrating birds may make behavioral decisions in response to climatic variables at larger scales.

Our dataset includes 41 passerines and 67 non-passerines, predominantly waterfowl and waders (**Supplementary Table 1**). For these species, we previously collected data on life-history traits (Végvári et al., 2010). Here, we included only four variables that we found to be important predictors of FAD advancement (Végvári et al., 2010). We estimated migration distance from the summer to winter distribution midpoint values of longitudes and latitudes following previous studies (Imboden and Imboden, 1972; Svensson and Hedenström, 1999). Using the data of Snow and Perrins (1998), we categorized pre-breeding molt as absent or present, annual fecundity (the average number of broods raised consecutively per season) as single-brooded or multi-brooded, and we scored diet complexity by recording whether the species' diet included plant materials, insects, other invertebrates, fish, amphibians, reptiles, birds, and mammals, and counting the number of food types (range: 1–7). In addition, we collected the data on each species' population size as the mean of minimum and maximum estimated numbers of mature individuals in Europe, which were obtained from the International Union for the Conservation of Nature (IUCN) Red List (IUCN, 2017), and we categorized the trends observed in population size in HNP as stable, decreasing, or increasing. The latter was based on expert opinion compiled by more than 50 local experts (Ecsedi, 2004; also verified by ZV) and agrees well with numeric estimates of population trends where those exist (Végvári and Barta, 2016).

We obtained monthly data on the NAO index from the NOAA National Weather Service's Climate Prediction Center (http://www.cpc.ncep.noaa.gov/products/precip/CWlink/pna/nao_index.html) and on the SRI from the Joint Institute for the Study of the Atmosphere and Ocean (http://jisao.washington.edu/data/sahel/). For each year, we calculated the average winter values (December to March) of NAO and the average rainy season values (June to October) of SRI, which are two commonly used variables in the studies of climatic responsiveness (Ottersen et al., 2001; Gordo and Sanz, 2006).

We collected data on the species' population size trends in Hungary between 2000 and 2017 from the database of the Hungarian Common Bird Monitoring Scheme (Szép et al., 2012). This database provided the slope of estimated linear change in population index (population size as percentage of the 1999 census) and a categorization of each species as significantly increasing, significantly decreasing, stabile, or uncertain. These data were available for 44 out of our 108 species (we did not count or use data that were categorized as uncertain). In spite of the partial overlap in time, the two datasets are not redundant, as there was no significant association between the 1969–2006 HNP trends and the 2000–2017 Hungarian trends (see **Supplementary Methods and Results**).

Statistical Analyses

For each species, we estimated the relationships of FAD separately with NAO and SRI by fitting linear regressions of yearly FAD data on the given climatic index of the same year (NAO) or the previous year (SRI). We used the slope of each of these relationships as measures of short-term climatic responsiveness (hereafter "NAO responsiveness slope" and "SRI responsiveness slope") to quantify how closely the species' migratory behavior tracks year-to-year variation in large-scale climatic indices (see **Figure 1** for graphical examples). Negative slopes indicate earlier arrival in years with higher values of NAO (warmer, wetter winters in Europe and warmer, drier winters in HNP) and SRI (more rainfall in the Sahel zone). Notably, neither NAO ($r = -0.05$, $p = 0.795$, $N = 32$ years) nor SRI ($r = 0.23$, $p = 0.204$, $N = 32$ years) values changed linearly over time during the study years (**Figure 1**), and there was no correlation between the yearly values of NAO and SRI ($r = 0.15$, $p = 0.415$, $N = 32$ years). This means that any systematic changes in migratory behaviors over time during the study period cannot be attributed to the systematic changes in NAO or SRI. Therefore, the effects of "NAO responsiveness slope" and "SRI responsiveness slope" on temporal changes are not mere by-products of a common effect of time. Note that this does not necessarily mean that birds experienced no climate change during the study period.

In the first phylogenetic analysis, we investigated whether overwintering occurred in more than 1 year. For each species, we counted the number of years in which overwintering individuals were observed in HNP. Species that never overwintered in HNP during the study period ($N = 80$) or overwintered in only 1 year ($N = 5$) were categorized as "migrants" ($N = 85$), whereas species that overwintered in more than 1 year were categorized as "overwinterers" ($N = 22$). This latter group includes species that were frequently observed overwintering in HNP already at the beginning of the study (e.g., water rail *Rallus aquaticus,* mallard *Anas platyrhynchos,* common blackbird *Turdus merula*) as well as species that started to overwinter regularly in the second half of the study period (e.g., graylay goose *Anser anser,* gray heron *Ardea cinerea,* common starling *Sturnus vulgaris*). To examine whether short-term climatic responsiveness and life history predicted to which group each species belonged, we used phylogenetic logistic regression (Ives and Garland, 2010) in which the dependent variable was the type of migratory strategy (overwinterers vs. migrants).

We then investigated predictors of temporal changes in migratory behavior, analyzing overwinterers and migrants separately. In the second phylogenetic analysis, we focused on overwinterers and examined whether short-term climatic responsiveness and life history predicted the magnitude by which these species increased their overwintering frequency. We used a robust approach in which we calculated the change between the first half (1975–1990) and the second half (1991–2006) of our study period in the proportion of years in which a given species was observed overwintering in HNP. Larger differences indicated greater increases in overwintering frequency over time. We used phylogenetic generalized least squares (PGLS) models (Freckleton et al., 2002) to test which climatic-responsiveness and life-history variables predicted the change in overwintering frequency.

In the third phylogenetic analysis, focused on migrants, we analyzed whether short-term climatic responsiveness and life history predicted the degree to which these species advanced their spring arrival dates over time. To express the strength of this response, we fit a linear regression on FAD as a function of time (years) for each species and calculated the slope of the regression line (hereafter "FAD slope over time"). More negative values of FAD slope indicate more rapid advancements of spring arrival during the study period (see **Figure 1** for graphical examples). Note that the years in which the species overwintered in HNP were excluded from this calculation (1 year in 5 species each). We used PGLS models to test which climatic-responsiveness and life-history variables predicted the FAD slope over time.

For each dependent variable we built a full model that contained all of the 4 life-history and 2 climatic-responsiveness predictors. In addition, we took into account that the first observation dates may depend on population size (Tryjanowski and Sparks, 2001; Miller-Rushing et al., 2008). In the model of

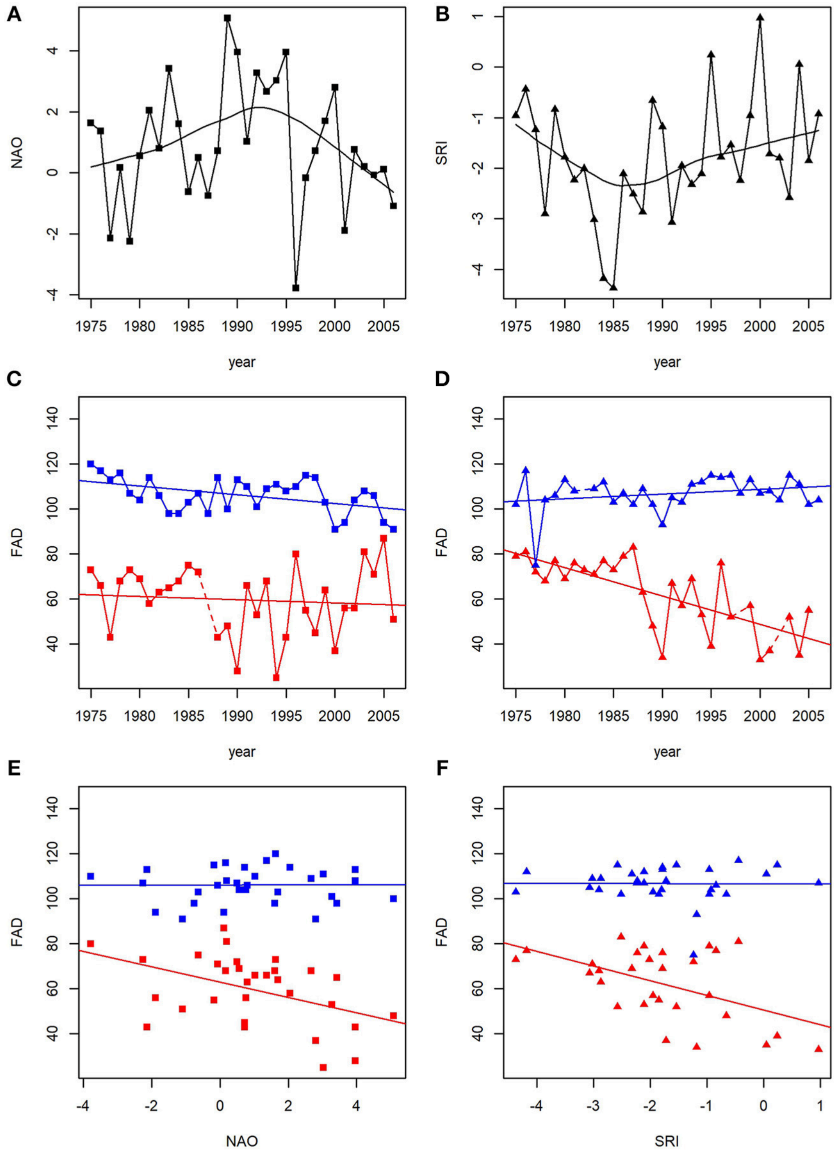

FIGURE 1 | Variation over time in **(A)** average winter (December to March) values of NAO, **(B)** average rainy-season (June to October) values of SRI, and **(C–F)** first arrival dates (FAD; number of days since 1 January). Examples are shown for FAD as function of year **(C–D)**, NAO **(E)**, and SRI **(F)** in four species as follows: red squares, strong NAO responsiveness (gadwall *Anas strepera*); blue squares, weak NAO responsiveness (black tern *Chlidonias niger*); red triangles, strong SRI responsiveness (Eurasian marsh harrier *Circus aeruginosus*); blue triangles, weak SRI responsiveness (Eurasian reed warbler *Acrocephalus scirpaceus*). LOESS curves are fit for NAO and SRI **(A,B)** to illustrate their non-linear changes during the study period. For FAD **(C–F)**, linear regressions are fit to illustrate slopes. To illustrate year-to-year variation **(A–D)**, consecutive data points are connected by solid lines; dashed lines indicate gaps in the time series.

migratory strategy, we included population size as a covariate because overwinterers may be more likely detected if their numbers are large. In the models of overwinterers and migrants, we included population trend as a fixed factor (stable, decreasing, or increasing population in HNP), because temporal changes in population sizes may cause changes in the first observation dates even if the birds' behavior does not change (Miller-Rushing et al., 2008). To ensure adequate distribution of residuals, we used the square root of migration distance and the base-10 logarithm of population size. All numeric predictor variables were z-transformed (mean-centered and divided by standard deviation), so that the model parameter estimates can be compared among predictors and interpreted as effect-size estimates, expressing the change in the dependent variable in response to a change corresponding to one SD in the predictor. As we had low sample size for overwinterers ($N = 22$), the number of estimated parameters was relatively high in the full models (9 in the first analysis and 10 in the second analysis). Therefore, we conducted model reduction to ensure that no important effect was missed due to over-parameterization. We reduced each full model stepwise, omitting the predictor with the largest *p*-value in each step, until only significant ($p < 0.05$) effects remained. To ensure that full and reduced models were comparable, all models for each dependent variable were run with the same dataset. We omitted one species (common cuckoo *Cuculus canorus*) for which the annual number of broods is missing because it is a brood parasite; all sample sizes presented in the "Statistical analyses" section excluded this species.

We tested whether the changes in migratory behavior observed in HNP between 1969 and 2006 predicted trends in population size in Hungary between 2000 and 2017 using PGLS models. We compared the slope of the Hungarian population trend between overwinterers ($N = 10$) and migrants ($N = 34$). Within migrants, we tested whether the subsequent population trends were correlated with FAD slope over time (we allowed for different variances among species with increasing, stabile, and decreasing trends). We did not conduct a similar analysis for the degree of change in overwintering frequency as our sample size for overwinterers was too small. In both models, we controlled for overall population size by adding the IUCN estimate of European population size as a covariate.

In all phylogenetic models, we used the phylogeny compiled for our earlier study (Végvári et al., 2010) and estimated the level of phylogenetic correlation (α in phylogenetic logistic regression and λ in PGLS) by the maximum likelihood method (Freckleton et al., 2002; Ives and Garland, 2010). All analyses were conducted in R 3.4.1 (R Core Team, 2018), using the packages "phylolm" (Ho and Ané, 2014) for phylogenetic logistic regression and "caper" (Orme et al., 2013) for PGLS analyses. Our data are available in **Supplementary Data 1, 2**.

RESULTS

Species that overwintered in more than 1 year differed in several traits from the other species (**Table 1**). Overwinterers had shorter migration distances (**Figure 2A**), more complex diets (**Figure 2B**), larger population sizes (**Figure 2C**), and, according to the reduced model, steeper (more negative) NAO responsiveness slopes (**Figure 2D**). The two groups of species did not differ significantly in SRI responsiveness slope, pre-breeding molt, or annual fecundity (**Table 1**).

In 18 of 22 species that overwintered more than once, the proportion of years with overwintering individuals was almost 3 times larger in the second half of the study (range: 0.125 −1, mean ± SE: 0.49 ± 0.07) than in the first half (range: 0–0.6, mean ± SE: 0.15 ± 0.04). Overwintering frequency increased significantly from the first to the second half period (paired *t*-test: $t_{21} = 4.39$, $p < 0.001$), by ca. 4 years on average. The difference in overwintering frequency over time was not significantly related to any of the predictor variables in the full model (**Table 2**). The reduced model showed that the largest increases of overwintering frequency were shown by species that do not molt in the pre-breeding season (**Table 2**, **Figure 3**).

In migrants, the greatest advancements in arrival timing (most negative FAD slope values) were associated with the most negative SRI responsiveness slopes (**Table 3**, **Figure 4**). Furthermore, the full model indicated that species with pre-breeding molt had advanced their FAD to a greater extent than species with no pre-breeding molt (**Table 3**, **Figure 4**), although this effect was not retained in the reduced model ($p = 0.054$ before exclusion). None of the other predictors had a significant effect on FAD advancement (**Table 3**).

Of 10 species that were overwinterers in HNP between 1969 and 2006, 8 had significantly increasing population trends in Hungary between 2000 and 2017 and only 2 were decreasing. All but one of these overwinterers had increased their overwintering frequency by 11–95% in HNP during the study period. In contrast, for 34 migrants, subsequent population trends were increasing only in 6 (17%), whereas 19 (54%) were decreasing. The slope of Hungarian population trend was significantly higher for overwinterers (mean ± SE: 3.60 ± 1.29) than for migrants (mean ± SE: −1.22 ± 0.70; PGLS: $t_{41} = 3.29$, $p = 0.002$; overall population size: slope ± SE = −0.27 ± 0.62, $p = 0.668$). However, for migrants, FAD slope over time did not correlate

TABLE 1 | Phylogenetic logistic regression models predicting whether overwintering at the study site was detected in the species in more than 1 year during the study ($N = 107$ species).

	Full model		Reduced model	
Model parameters	**b ± SE**	***p***	**b ± SE**	***p***
Intercept	−1.228 ± 0.681	0.071	−1.678 ± 0.349	<0.001
NAO responsiveness slope*	−0.506 ± 0.332	0.128	−0.603 ± 0.305	0.048
SRI responsiveness slope*	0.029 ± 0.297	0.923		
Migration distance	−1.067 ± 0.400	0.008	−0.981 ± 0.363	0.007
Pre-breeding molt (present)	−0.544 ± 0.636	0.392		
Diet complexity	1.025 ± 0.346	0.003	1.051 ± 0.334	0.002
Fecundity (multi-brooded)	−0.870 ± 0.797	0.275		
Population size	1.100 ± 0.446	0.014	0.987 ± 0.366	0.007

The dependent variable was a binary factor coding whether or not overwintering occurred in more than 1 year between 1975 and 2006. All numeric predictor variables were z-transformed. The parameter estimates are on log-odds scale. The estimate of phylogenetic signal (i.e., –log α) was −1.2 in the full model and −1.3 in the reduced model.

**More negative values represent stronger responses; i.e., greater advancement of FAD with increasing NAO or SRI values.*

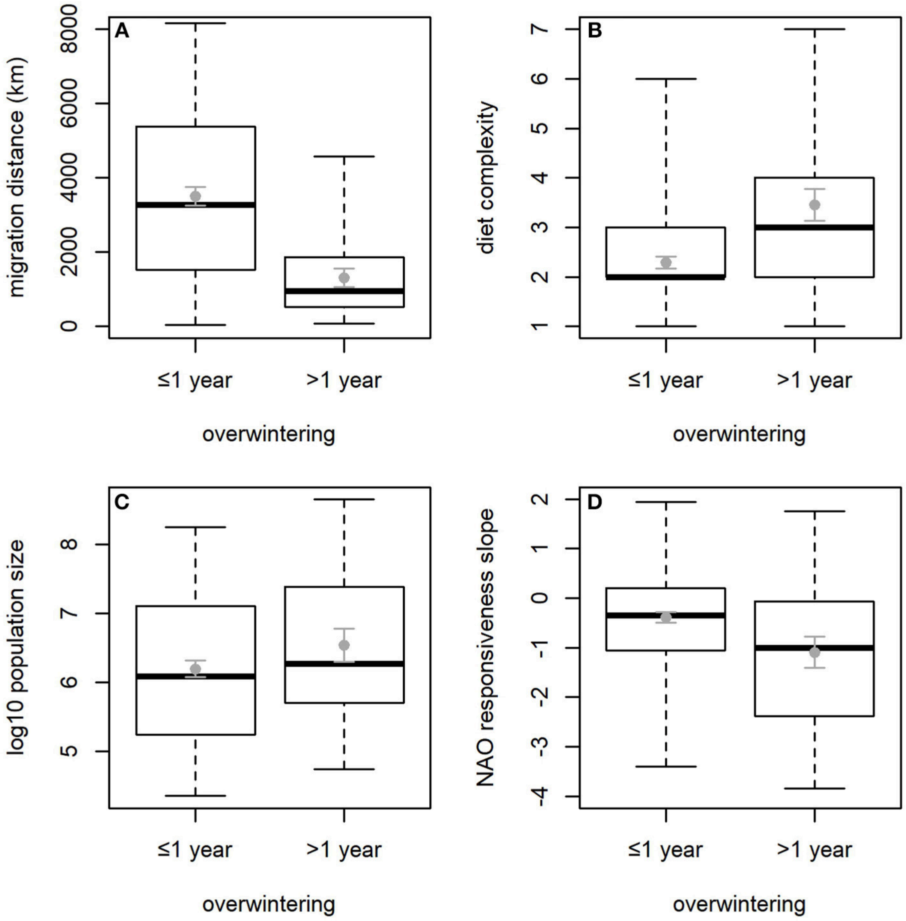

FIGURE 2 | Comparison of **(A)** migration distance, **(B)** diet complexity, **(C)** population size, and **(D)** NAO responsiveness between species that were ($N = 22$) or were not ($N = 85$) observed overwintering at the study site in more than 1 year from 1975 to 2006. Box plots show the median (thick middle line), interquartile range (box), and data range (whiskers); the gray error bars depict the mean ± standard error. NAO responsiveness slope has more negative values if the species had earlier arrivals after high-NAO winters.

significantly with the subsequent population trends (PGLS: slope $\pm$ SE $= -0.31 \pm 1.70$, $p = 0.855$; overall population size: slope $\pm$ SE $= 0.93 \pm 0.49$, $p = 0.067$).

DISCUSSION

We demonstrated that the behavioral changes of migratory birds comprised shifts to earlier arrival (FAD) as well as an increased frequency of overwintering in HNP over the recent decades. Our main finding is that certain aspects of these long-term behavioral changes were predicted by short-term climatic responsiveness. Firstly, the 22 species in which overwintering occurred and typically increased in parallel with the contemporary climatic changes were those that matched their arrival dates better to year-to-year fluctuations of NAO values compared with the 85 species that remained migratory in HNP. This result indirectly suggests that NAO responsiveness could play a role in the increasing incidence of overwintering. Secondly, species whose arrival dates more closely tracked the year-to-year variation in SRI had the steepest FAD slopes over time. Altogether, these results support the hypothesis that earlier arrivals and recent overwinterings at or near the breeding grounds may be responses to contemporary climatic changes. We also found that the migratory species differed from overwinterers in several key characteristics of life history and ecology, whereas other characteristics were important in predicting the magnitude of behavioral changes over the years. Thus, our study corroborates the earlier findings (Spottiswoode and Møller, 2004; Rubolini et al., 2005; Spottiswoode et al., 2006; Møller et al., 2008; Végvári et al., 2010), suggesting that the type and extent of phenotypic changes by which different species respond to climate change can depend on various aspects of their biology.

Although changes in arrival times are intensively researched, relatively little is known of the factors that explain variation across species in the tendency to change wintering grounds (Pearce-Higgins and Green, 2014). We found that species that were observed overwintering in HNP during the study period

TABLE 2 | Phylogenetic generalized least squares models predicting the change in the frequency of overwintering at the study site (*N* = 22 species).

	Full model		Reduced model	
Model parameters	**b ± SE**	***p***	**b ± SE**	***p***
Intercept	0.19 ± 0.224	0.412	0.383 ± 0.072	<0.001
NAO responsiveness slope*	0.086 ± 0.090	0.359		
SRI responsiveness slope*	−0.057 ± 0.091	0.542		
Migration distance	−0.038 ± 0.082	0.653		
Pre-breeding molt (present)	−0.222 ± 0.175	0.225	−0.266 ± 0.107	0.022
Diet complexity	−0.011 ± 0.062	0.858		
Fecundity (multi-brooded)	0.062 ± 0.184	0.744		
Population trend (decreasing)	0.252 ± 0.184	0.193		
Population trend (increasing)	0.165 ± 0.179	0.375		

The dependent variable was the difference in the proportion of years with overwintering individuals between the second and first half of the study period. All numeric predictor variables were z-transformed. The estimate of phylogenetic signal (λ) was zero in both models.

**More negative values represent stronger responses; i.e., greater advancement of FAD with increasing NAO or SRI values.*

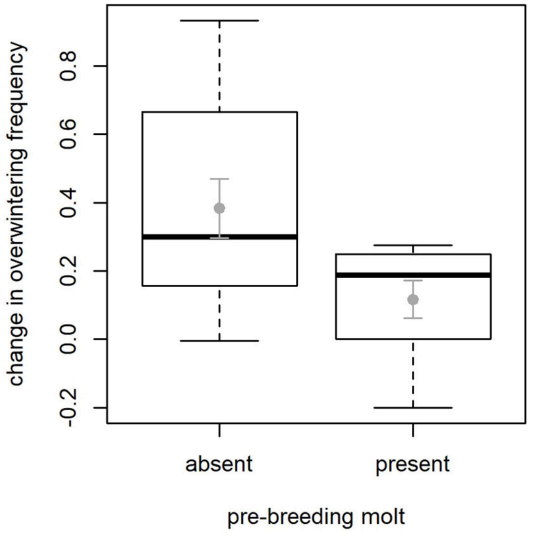

FIGURE 3 | Change from 1975–1990 to 1991–2006 in proportion of years in which a species was observed overwintering at the study site, in species with (*N* = 10) and without (*N* = 12) pre-breeding molt. See **Figure 2** for explanation of boxplots and error bars.

were those with strong responsiveness to NAO, short migration distances, and more complex diets. These characteristics probably make the birds less constrained in switching to wintering at more northern latitudes, appearing as overwinterers in study sites like ours, for the following reasons. Superior ability to track the changes in NAO, which affects the winter weather in Europe, may indicate that these birds are able to detect signs of mild winter before they leave the breeding grounds or migrate further south. Furthermore, waterbirds that arrive earlier after winters with higher NAO values (when the region of HNP receives less precipitation) may be more tolerant of dry conditions, which might make them more capable of overwintering near the breeding grounds instead of southern wetlands. Shorter migration distances mean that less energy is expended to reach the wintering grounds. This further encourages forgoing migration, because if winter unexpectedly becomes harsh, then the birds can easily leave. More general diets should facilitate survival at more northern latitudes during winter. Thus, these three traits are likely to promote behavioral plasticity in migratory strategies. Interestingly, a study on blackbirds found that loss of migration was facilitated by increasingly urbanized life style (Møller et al., 2014), but the switches to overwintering we report here are unlikely to be driven by urbanization because the majority of birds we investigated do not breed in areas with significant human population (Ecsedi, 2004), and overwintering species were not over-represented near human settlements (see **Supplementary Methods and Results**). Nevertheless, our result that species with larger population sizes were more likely to overwinter in HNP indicates that population size affects the probability of detecting overwintering individuals, as has been reported for first arrivals (Tryjanowski and Sparks, 2001; Miller-Rushing et al., 2008). Therefore, it will be important to validate our findings further with more detailed data for each species—controlling for fine-scale variation in the size of overwintering and migrating populations—and to tease apart the biological and methodological factors that may be contributing to interspecific differences (Tryjanowski et al., 2005).

We found that the magnitude of change in migratory behaviors was predicted by different traits from those that predicted the type of migratory strategy (overwinterers vs. migrants). Both the slope of FAD advancement and the change of overwintering frequency were unrelated to NAO responsiveness, but FAD slope correlated with the species' responsiveness to SRI. Thus, species that typically arrive earlier in years with high SRI (after more rainy summers and falls in the Sahel zone) have been arriving increasingly earlier over the recent decades in HNP. This relationship was robust as it persisted despite controlling for migration distance, which was a potentially confounding variable. Previous studies indicated that short-distance migrants were more likely to advance their FAD (Butler, 2003; Thorup et al., 2007; Møller et al., 2008; Végvári et al., 2010).

TABLE 3 | Phylogenetic generalized least squares models predicting the change in FAD over time in species that did not overwinter at the study site in more than 1 year ($N = 85$ species).

	Full model		Reduced model	
Model parameters	**b ± SE**	***p***	**b ± SE**	***p***
Intercept	−0.005 ± 0.075	0.943	−0.127 ± 0.039	0.001
NAO responsiveness slope*	−0.047 ± 0.032	0.152		
SRI responsiveness slope*	0.124 ± 0.032	<0.001	0.123 ± 0.030	<0.001
Migration distance	−0.004 ± 0.032	0.904		
Pre-breeding molt (present)	−0.152 ± 0.070	0.033		
Diet complexity	−0.009 ± 0.032	0.771		
Fecundity (multi-brooded)	−0.009 ± 0.067	0.896		
Population trend (decreasing)	0.083 ± 0.094	0.378		
Population trend (increasing)	−0.084 ± 0.070	0.235		

The dependent variable expresses the average number of days FAD has changed per year, with more negative values representing stronger responses; i.e., greater advancement of FAD over time. All numeric predictor variables were z-transformed. The estimate of phylogenetic signal (λ) was zero in both models.

**More negative values represent stronger responses; i.e., greater advancement of FAD with increasing NAO or SRI values.*

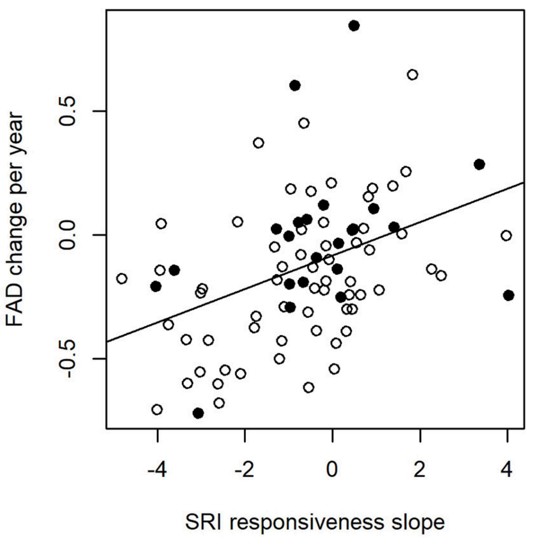

FIGURE 4 | FAD slope over time in relation to the slope of SRI responsiveness and pre-breeding molt. The regression line was fit from a PGLS model containing SRI responsiveness as single explanatory variable. More negative slopes indicate earlier arrivals in more recent years (y axis) and after wetter rainy seasons in the Sahel zone (x axis). Empty and filled circles, respectively, represent species in which pre-breeding molt is present ($N = 63$) or absent ($N = 22$); note that species with pre-breeding molt tend to cluster in the lower part of the figure.

In contrast, long-distance migrants may be affected more by Sahel climate than short-distance migrants that typically do not cross the Sahara. Our finding that SRI responsiveness predicted FAD advancement regardless of migration distance might be explained by the effect of Mediterranean sea surface temperatures on Sahel rainfall variability: in years when the Mediterranean is warmer, the Sahel tends to be wetter (Rowell, 2003; Park et al., 2016). Thus, short-distance migrants may appear to respond to SRI because they are responding to climatic conditions at their Mediterranean wintering grounds, which are correlated with Sahelian conditions experienced by the long-distance migrants. Our finding that NAO responsiveness did not predict the slope of FAD advancement might be due to the fact that the predictive value of NAO on winter weather is rather heterogeneous along the Baltic-Hungarian flyway (Cleary et al., 2017).

The other predictors we investigated had relatively little effect on temporal changes in migratory and wintering behaviors. Pre-breeding molt appeared important in some, but not all analyses, and had opposite effects in overwinterers and migrants. These statistically weak effects may represent biologically meaningful relationships, as the presence of pre-breeding molt might indicate strong sexual selection (Tökölyi et al., 2008) which, in turn, may facilitate FAD advancement in migrants (Spottiswoode and Møller, 2004; Rubolini et al., 2005; Spottiswoode et al., 2006). In contrast, the absence of a costly pre-breeding molt may facilitate spending the winter at or near the breeding grounds. The relatively small sample size of overwintering species prevents us from drawing strong conclusions. However, we urge further studies on the ecological and life-history predictors of changes in wintering behaviors.

Responses to climate change may be crucial for population persistence because failure to advance phenology can lead to temporal mismatches among trophic levels (Thackeray et al., 2010) with potentially detrimental consequences for fitness and demography (Sanz et al., 2003; Miller-Rushing et al., 2010; Franks et al., 2018). Such a mismatch might have contributed to population declines observed in migratory birds that had not advanced their migration timing (Møller et al., 2008; Végvári and Barta, 2016). Although we did not find a similar relationship between population trends and FAD slope in the present study, we found that population sizes were more likely to increase over the last 17 years in species that had been observed overwintering in HNP during our study period. Because overwintering species in our study tended to increase their overwintering frequency over time, this strategy might be an especially adaptive response to climate change. Thus, we propose that monitoring changes in migratory strategy may provide useful information for risk assessment and conservation planning. This may be a fruitful direction to explore because the appearance of locally new overwintering species or their increases in abundance might be monitored more easily and efficiently—for example, by involving citizen science and amateur ringing efforts—than subtle changes in migration timing. Furthermore, changes in populations' wintering distributions and the shortening or loss of migration can alter patterns of gene flow and metapopulation connectivity, which may then affect the degree of responses to local selection pressures and also population trends (Webster et al., 2002; Pulido and Berthold, 2010). Thus, we need more detailed studies on the possible adaptive value of shortened or lost migration and its mechanistic links to individual fitness and population persistence.

Taken together, our results suggest that long-term changes of avian migration occurring in parallel with contemporary climate change, including the advancement of spring arrivals and an increasing frequency of overwintering at or near the breeding grounds, may be explained by a species' ability to track short-term variability in large-scale climatic factors. This implies that changes in migratory behavior over time can provide an appropriate measure of climatic responsiveness for comparing adaptation to climate change across species. Our findings indicate that these adaptations take the form of different behavioral changes (i.e., overwintering vs. earlier arrivals) depending on the ecology and life history of the species, and the type of change may contribute to predict the population trends. Thus, a sound understanding of climatic effects on diverse behavioral responses will be important for informing conservation management of migratory birds.

DATA AVAILABILITY

All data used in this study are available in the Supplementary Material of this paper.

AUTHOR CONTRIBUTIONS

VB and ZV designed the study. ZV assembled the data. VB performed the analyses. VB, ZV, and ZB contributed significantly to writing the manuscript and gave final approval of the version to be published.

FUNDING

We were supported by the National Research, Development and Innovation Office (NKFIH) of Hungary (K115402 to VB, K112527 to ZB).

ACKNOWLEDGMENTS

VB was supported by the János Bolyai Scholarship of the Hungarian Academy of Sciences.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fevo.2019.00089/full#supplementary-material

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Conflict of Interest Statement: 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.

frontiers in Ecology and Evolution

ORIGINAL RESEARCH
published: 02 April 2019
doi: 10.3389/fevo.2019.00097

Starting on the Right Foot: Carryover Effects of Larval Hydroperiod and Terrain Moisture on Post-metamorphic Frog Movement Behavior

Evan M. Bredeweg[1]*, Jenny Urbina[1], Anita T. Morzillo[2] and Tiffany S. Garcia[1]

[1] Department of Fisheries and Wildlife, Oregon State University, Corvallis, OR, United States, [2] Department of Natural Resources and the Environment, University of Connecticut, Storrs, CT, United States

Changing patterns of precipitation and drought will dramatically influence the distribution and persistence of lentic habitats. Pond-breeding amphibians can often respond to changes in habitat by plastically shifting behavioral and developmental trait response. However, fitness tradeoffs inherent in life history strategies can carry over to impact development, behavior, and fitness in later life stages. In this experiment, we investigated carryover effects of hydroperiod permanence on the movement behavior of newly-metamorphosed juvenile Northern Red-legged Frogs (*Rana aurora*). Frogs were raised through metamorphosis in mesocosms under either permanent or ephemeral hydroperiod conditions. After metamorphosis, individuals were removed from the mesocosms, measured, uniquely tagged with elastomer, and moved to holding terrariums. Movement behavior was quantified under two terrain conditions: a physiologically-taxing, dry runway treatment, or a control, moist runway treatment. Individuals were given 30 min to move down the 1 × 20m enclosed structure before distance was measured. We applied a hurdle model to examine two distinct components of movement behavior: (1) the probability of moving away from the start location, and (2) movement distance. We found that hydroperiod condition had an indirect carryover effect on movement via the relationship between individual size and the propensity to move. Individuals from ephemeral mesocosm conditions metamorphosed at a smaller size but showed increased growth rates as compared to individuals from permanent hydroperiod conditions. Individual snout-vent length and runway condition (moist or dry) were significant predictors of both aspects of movement behavior. Larger individuals were more likely to move down the runway and able to move a farther distance than smaller individuals. In addition to the influence of size, dry runway conditions reduced the probability of individuals moving from the start location, but increased the distance traveled relative to the moist runway. The demonstrated cumulative impact of stressors suggests the importance of addressing direct, indirect, and carryover effects of stressors throughout ontogeny.

Keywords: plasticity, movement behavior, amphibian, latent effects, life history, ontogeny

Edited by:
Caitlin R. Gabor,
Texas State University System,
United States

Reviewed by:
Elizabeth A. Roznik,
Memphis Zoo, United States
Ivan Gomez-Mestre,
Estación Biológica de Doñana (EBD),
Spain

Correspondence:
Evan M. Bredeweg
evan.bredeweg@oregonstate.edu

Specialty section:
This article was submitted to
Behavioral and Evolutionary Ecology,
a section of the journal
Frontiers in Ecology and Evolution

Received: *30 November 2018*
Accepted: *11 March 2019*
Published: *02 April 2019*

Citation:
Bredeweg EM, Urbina J, Morzillo AT and Garcia TS (2019) Starting on the Right Foot: Carryover Effects of Larval Hydroperiod and Terrain Moisture on Post-metamorphic Frog Movement Behavior. Front. Ecol. Evol. 7:97.
doi: 10.3389/fevo.2019.00097

INTRODUCTION

Climate change is projected to influence many aspects of the environment, including temperature and storm intensity (Hughes, 2000). Critical to lentic organisms, climate change also alters precipitation patterns, which are key in the predictability and variability of water cycling and storage (Williams et al., 2015; Smith et al., 2018). For animals such as aquatic insects and amphibians, with complex life histories that require movement between aquatic and terrestrial habitats, this variation can determine the success of individual development and survival (Robson et al., 2011; Suislepp et al., 2011; Cayuela et al., 2016).

Life history theory suggests that a dynamic yet predictable environment would select for a flexible life history strategy, such as life-cycle staging or phenotypic plasticity (Meyers and Bull, 2002; Roff, 2002). Developmental plasticity has allowed some amphibian species with complex life histories to adjust trait response, such as development rate, antipredator behavior, and metamorphosis timing in response to a variety of aquatic environmental stressors (desiccation—Denver et al., 1998; competition and predation—Relyea, 2004; multiple predators—Vonesh and Warkentin, 2006; temperature—Thurman and Garcia, 2017). However, plasticity has physiological costs and trade-offs that can constrain the degree of response both immediately and across ontogeny (Relyea, 2002). The true price of plasticity may only be understood when considering the entire life cycle.

Carryover effects, also termed latent effects, have been increasingly apparent in biological systems (Pechenik, 2006; O'connor et al., 2014). Carryover effects of larval plasticity may be important in determining response to changing environmental conditions (Johansson et al., 2010). Early life stages can be more susceptible to shifting environmental conditions than adults, an effect that has been observed in diverse taxa from reptiles to butterflies (Radchuk et al., 2013; Levy et al., 2015). Several carryover effects to larval stress in anurans have been observed: for example, drying conditions experienced by developing Túngara frog tadpoles reduced leg length and jumping performance (Charbonnier and Vonesh, 2015), and high larval densities of Red-eyed treefrogs resulted in smaller juveniles with reduced livers and fat bodies (Bouchard et al., 2016).

To date, assessment of potential impacts of climate change on amphibians have focused on adult stages, with little attention to other sensitive life history stages or across ontogeny (Levy et al., 2015; Lancaster et al., 2017). Dramatic shifts in environmental conditions that are stressors during early life stages may fundamentally change the ability of individuals to respond to stress later in development. The physiological changes and energy investment in larval trait response can represent an opportunity cost that limits the ability to respond to future stressors (Weinig and Delph, 2001; Amburgey et al., 2016). As such, investments in plastic responses at the larval, aquatic stage have the potential to carry over to influence the developmental trajectory and behavior of juvenile frogs, and limit capacity to manage stress in terrestrial life stages.

We studied the combined influence of aquatic and terrestrial environmental condition across a life history transition in the Northern Red-legged Frog (*Rana aurora*) to examine the interaction of direct and carryover effects on juvenile movement behavior. We chose movement behavior as our response variable because of its importance in a range of ecological aspects, such as foraging, predator avoidance, refuge discovery, and population connectivity (Osbourn et al., 2014; Schwalm et al., 2016; Székely et al., 2017). We selected pond permanence as a larval stressor because of the extensive documentation of amphibian plasticity in response to drying conditions (Johansson et al., 2010; Charbonnier and Vonesh, 2015; Thurman and Garcia, 2017). *Rana aurora* utilizes both ephemeral and permanent lentic waters for breeding habitat, with ephemeral ponds being important for larval success (Adams, 2000; Hayes et al., 2008). After metamorphosis, we tested for carryover effects on juvenile movement behavior under two physiologically distinct conditions: a moist, low-stress terrain or a dry, high-stress terrain. Based on past research (see above), we hypothesized that individuals who (plastically) responded to drying (ephemeral) hydroperiods would be less suited to movement under stressful conditions. A trade-off between structural growth and energy stores (Morey and Reznick, 2004) suggests that larval development has inherent resource limitations. The increased density and limitations of ephemeral conditions may tax this trade-off and change the physiology of juvenile frogs and their subsequent movement behavior.

Amphibians are species that are dependent on water throughout their life cycle. Yet, to our knowledge, impacts of 2 critical aspects of amphibian habitat affected by changing precipitation—drying ponds and dry terrain—have not been directly assessed for amphibian species across metamorphosis. As climate change influences precipitation patterns and the availability of moisture in the environment, the mechanisms by which amphibians modify their development, manage the physiological stress, and respond behaviorally to these changing conditions will be important for continued survival of those species (Mazerolle and Desrochers, 2005; Watling and Braga, 2015).

METHODS

Animal Collection and Rearing

On 31 January 2015, we collected eight recently laid *R. aurora* egg masses from three separate populations in the Willamette Valley, Oregon. Egg masses were immediately transported to Oregon State University and placed in de-chlorinated water in individual 75 L glass aquaria. Individuals were reared in the laboratory through hatching until Gosner developmental stage 25 (Gosner, 1960) and fed a mixture of ground fish flakes and rabbit chow *ad libidum*. Water changes were performed every 10 days. On 3 March 2015, tadpoles were combined into a common pool and randomly assigned to a mesocosm tub. Each mesocosm was populated with 50 randomly selected larvae, with 10 replicates per hydroperiod treatment (permanent or ephemeral). Mesocosms were held outdoors at the Lewis Brown Horticultural Farm (44.551346, −123.215831) under a mixed oak canopy. Mesocosms (120 L HDPE stock tanks) were prepared with 100 grams of dried and autoclaved oak leaves and 5 grams of rabbit chow in 100 liters of well-water. Each mesocosm tub was then inoculated with algae and microorganism communities 2 weeks prior to the introduction of tadpoles using 4 L of water from an onsite source-mesocosm. Mesocosm tubs were then fitted with mesh screen lids to prevent predation or introduction of other species.

Two weeks after the introduction of *R. aurora* larvae, water levels in the ephemeral treatment mesocosms were drawn down by 4 liters every week until water levels reached a total 30 liters. At this point, water levels were maintained for the remainder of the experiment. Permanent hydroperiod treatments were maintained at 100-liter levels with additional well-water every week as needed. As individuals neared metamorphosis, two mesh floats were added to mesocosms to allow individuals to emerge. Individuals were removed from the mesocosms at Gosner developmental stage 45 (Gosner, 1960), measured (snout-vent length, total length, and mass), and marked with a unique color tag. Color tags consisted of 3 subcutaneous injections of visible implant elastomer (Northwest Marine Technology, Inc.) in the belly, right leg, and left leg (Govindarajulu et al., 2006; Swanson et al., 2013). Individuals were then transferred to outdoor terrariums adjacent to the mesocosm array. Treatment groupings were maintained throughout this process. Terrariums (120 L HDPE tubs) contained moistened coconut husk bedding, well-water pools, and dried grass and leaves for refuge. Individuals in terrariums were fed an excess of wingless fruit flies (*Drosophila melanogaster*) 3 times a week. Emerging individuals were collected starting on 25 June and continued until we collected at least 200 individuals which occurred on 14 September. Logistical seasonal weather constraints of our runway assays prevented the inclusion of individuals that metamorphosed after our collection window, which does limit our inference to those early emerging individuals. This is an important consideration as emergence date has shown to change the size and dispersal of individuals (Chelgren et al., 2006).

Behavioral Assay- Runway Dispersal

Four 1 × 20 m runways were constructed at the Lewis Brown Horticultural Farm. Each runway was constructed from a single piece of 7-millimeter white plastic sheet draped across parallel wires suspended 75 centimeters above the perimeter of the runways. This plastic sheeting prevented individuals from escaping the runways and maintained moisture conditions within assays. The runway substrate consisted of smoothed and compacted topsoil to a depth of 7 cm. The 4 runways were arranged in pairs and placed on level ground under the dappled shade of several surrounding trees on an angle from northeast to southwest. Runways were paired, with each pair containing a dry and wet treatment runway.

Immediately prior to each assay, all runways were prepared according to their assigned moisture treatment. Wet condition runways had the entire length of substrate moistened with well-water to the point of near saturation to minimize standing pooled water. Dry condition runways had no added water except for the release point. In each runway, a release point was constructed by placing a 15 cm disk of moist sphagnum moss in the northeast end of the runs. The immediate area around this release point was gently moistened in both the wet and dry conditions to provide a place of hydrologic refuge. Runways were cleaned of detritus prior to each assay and the release point moss was replaced each day.

Individuals from a single terrarium were ordered by age and alternately assigned to runway condition to provide equal representation across ages and terrariums. Each animal was measured (snout-vent length, mass, etc.) and then allowed to acclimate within the runway under a clay pot cover in the center of the release point. Individuals were anointed with a small application of high visibility fluorescent powder (ECO Aurora Pink® Pigment, DayGlow Color Corp.) on their back. After a 5-min acclimation period, the clay pot was remotely removed, and the animal was given 30 min to freely move in the runway. Runway assays occurred during daylight-hours; average start time was at 13:25 h (SD = 1:45 h) and ranged from 08:25 to 15:40h. At the end of the assay, animals were located, recaptured, and returned to their terrarium. In between each assay, the moss disk at the release point was rinsed and immediate area remoistened. Wet runways were also remoistened as needed in between each assay. Each animal was run through the assay twice with at least 14 days between each run. Animals assigned to a runway condition were maintained in the same runway condition for their second assay. At the end of the second runway assay, individuals were humanely euthanized with a solution of Tricaine Methanesulfonate (Leary et al., 2013).

Statistical Analysis

We examined the relationship between larval hydroperiod and frog body size using two models to assess the responses of individual length at two time points: once upon metamorphic emergence from the mesocosms, and once at the time of the runway assay (juvenile growth). To assess potential carryover effects of hydroperiod conditions on frog size, we fit a linear mixed-effects regression model of metamorphic emergence snout-vent length (SVL) based on mesocosm hydroperiod, day of emergence, and a random effect of mesocosm tub. To assess carry over on juvenile growth, we built a second linear mixed-effects model that examined SVL growth after metamorphic emergence and before the first the runway assay. For this model, we fit data from the first assays with predictor variables of mesocosm hydroperiod, day of emergence, days since emergence, and a random effect of enclosure. We also included the total number of animals per enclosure (container density) as a predictor variable. The measure of density provided a continuous classification of terrarium density outside of the random effect of enclosure but was not unique for each individual. Despite feeding an excess of fruit flies, the density of animals in a terrarium could have had other effects on growth (Altwegg, 2003; Harper and Semlitsch, 2007).

We tested two distinct movement aspects of each individual: the decision to attempt a movement, and how far to travel during a movement event. A hurdle regression model was used to assess these two aspects of an individual response (Falke et al., 2013). Individuals that were within 20 cm from the start location (a

15 cm disk of moss) were not considered to have moved since they were near the moss refuge of the release point; this created a binomial response variable of a movement attempt. This analysis simultaneously fits a binomial model for the decision to leave or stay in the start location with a zero-truncated count regression model of distance for individuals that move down the runway. The models were fit using the "glmmTMB" library in R, which allowed for inclusion of random effects of experimental enclosure (Brooks et al., 2017). Only animals that completed both trials were included in the final analysis.

As this experiment ranged across a metamorphic life history transition, there were a suite of individual measurements that could be included as covariates in this modeling framework. To reduce the likelihood of an over fit model, predictor covariates were grouped into three sets: environmental conditions (day of assay, time of assay, ambient temperature), individual characteristics (SVL, body condition, days since emergence), and individual traits at metamorphic emergence (emergence SVL, emergence body condition, day of emergence). Humidity is likely an important aspect of the environmental conditions in shaping movement (Chan-McLeod, 2003), but is integrally determined by temperature which had a finer resolution measurement and was used in place of humidity. Correlation between covariates within each group were assessed to check for multicollinearity. In addition to our experimental treatments (hydroperiod, runway condition, run number, random effect of enclosure), these covariate groups were used to develop four candidate models (**Table 1**). Body condition was calculated using the scaled mass index proposed by Peig and Green (2009), which incorporates allometric scaling of body size to better represent an individual's energy stores. Predictor covariates were standardized by subtracting the mean and dividing by their standard deviations to make them more directly comparable in the model and subsequently back transformed onto their original scales for interpretation. These four candidate models were then compared using AIC (Symonds and Moussalli, 2011). The best fit model (lowest AIC) was based on individual characteristics including SVL, body condition, and days since emergence. We assessed the presence of interaction with the best fitting model and adding interactions with and between all experimental factors and covariates. The AIC values from all interaction models showed no significant improvement (ΔAIC > 2) in model fit over the additive model. In the interest of parsimony, we used the additive individual characteristic model for interpretation (Aho et al., 2014). All statistical analyses were performed in R (version 3.5.0) using RStudio (version 1.1.447, RStudio Team, 2016).

TABLE 1 | Candidate model sets with included variables.

Model	Variables	ΔAIC
BASE	Hydroperiod + runway condition + run# + (Random effect of enclosure)	26.3
Environmental condition	*BASE* + experimental day + time of assay + ambient temp during assay.	30.1
Individual characteristics	*BASE* + SVL + body condition + days since emergence	0
Emergence characteristics	*BASE* + emergence SVL + emergence body condition + emergence day	12.4

All candidate models used runway movement distance as their response and included the random effect of enclosure and experimental treatment factors of larval condition, runway treatment, and runway assay number. The addition of each group of covariate variables were then compared against the base model using AIC.

RESULTS

We tested 204 animals that emerged within our experimental window and successfully completed both runway assays. These animals emerged from 18 of our mesocosms (9 Ephemeral and 9 Permanent) with an average emergence of 11.3 (SD = 4.8) animals per mesocosm. Two of the mesocosms did not have any emergence within our experimental window (1 Ephemeral and 1 Permanent). The 82 individuals from Ephemeral mesocosms were subsequently divided evenly into Wet and Dry runway treatments. Permanent mesocosms had 122 individuals emerge, resulting in 63 and 59, respectively in Wet and Dry runway treatments.

Our analysis on size at emergence found that both day of emergence ($z = -5.16$, $p < 0.001$) and hydroperiod ($z = 2.98$, $p = 0.003$) had a significant effect on emergence SVL. Snout-vent length at emergence decreased by 0.12 mm for each additional week (95% CI = 0.08 to 0.17), meaning that individuals emerging earlier were relatively larger than later-emerging individuals. Individuals from permanent hydroperiods had 0.66 mm longer SVL (95% CI = 0.23 to 1.10) than individuals from ephemeral hydroperiods (**Figure 1A**). In addition to this effect of hydroperiod on size at emergence, we also detected a significant difference in juvenile growth rates based on larval hydroperiod. Juvenile SVL was significantly affected by day of emergence ($z = -5.09$, $p < 0.001$), days since emergence ($z = 2.65$, $p = 0.008$), and hydroperiod ($z = -2.84$, $p = 0.005$). Individuals from ephemeral hydroperiods emerged at a smaller size relative to their counterparts from permanent hydroperiods but had faster growth post-emergence. Enclosure density did not have a significant effect on SVL growth ($z = -0.78$, $p = 0.437$). Like the above analysis, day of emergence had a negative effect on SVL growth after emergence with 0.19 mm less growth for each additional week (95% CI = 0.12 to 0.27). Increased time elapsed since emergence resulted in increased SVL growth, with an additional 0.14 mm for each week post-emergence (95% CI = 0.04 to 0.24). Further, we found the effect of mesocosm hydroperiod was opposite of the previous analysis, with individuals from permanent hydroperiods increasing SVL 0.61 mm less than ephemeral conditions (95% CI = 0.19 to 1.04) after emergence (**Figure 1B**).

In our analysis of individual movement behavior, the best fit model included individual characteristics at the time of the runway assay, including SVL, body condition, and days since emergence, as covariates with experimental factors of larval hydroperiod, runway condition, and runway assay number (**Table 1**). The hurdle model allows the movement of each treatment group to be separated into the decision to leave the release point and the subsequent decision of how far to move.

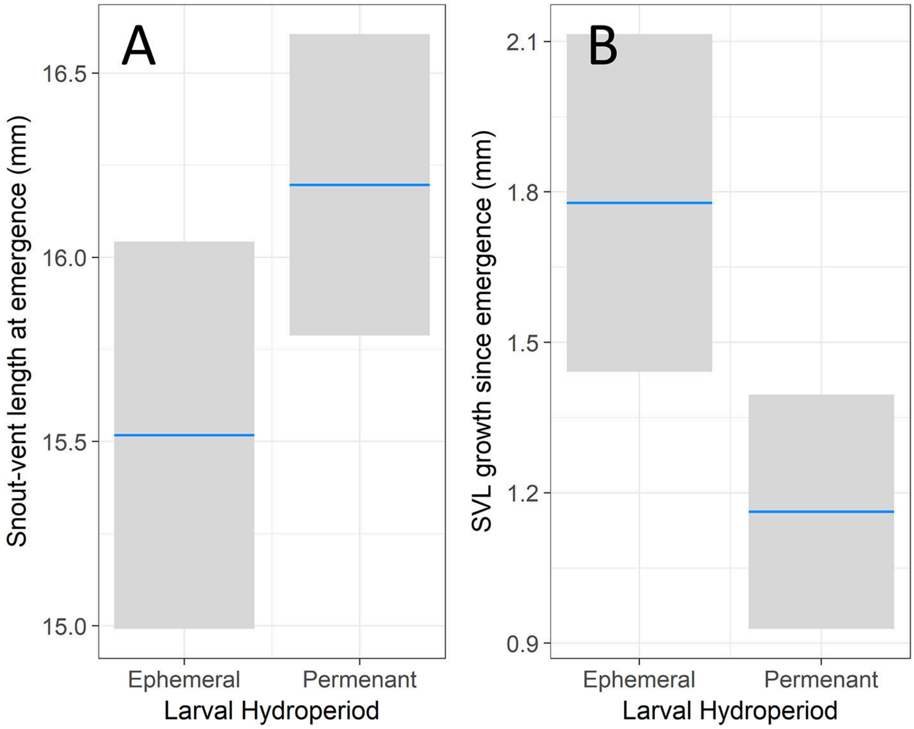

FIGURE 1 | The effect of hydroperiod on snout-vent length. The left **(A)** shows the effect of hydroperiod on emergence SVL after controlling for the emergence day. The right **(B)** shows the effect of hydroperiod on juvenile SVL growth during the first assay after controlling for emergence day, days since emergence, enclosure density. The blue bar represents the modeled response of an average individual with the 95% confidence interval in the shaded bar.

These two aspects of the model were fit with identical predictors but have distinct results. In order to distinguish between these two aspects of the behavior, they are referred to as the movement hurdle (decision to move, zero-inflated model component) and the movement distance (distance undertaken after movement decision, conditional model component).

From our model, two variables were significant to both the movement hurdle and movement distance: runway condition and SVL. Runway condition was a significant predictor of a frog crossing the movement hurdle ($z = -4.16$, $p < 0.001$). An individual in dry runway conditions is 60% less likely to move than in wet runway conditions (95% CI = 38 to 74%). After controlling the other variables, the probability an average individual moves away from the start position was 0.24 (±0.05 SE) in a dry runway compared to 0.44 (±0.06 SE) in a wet runway. Snout-vent length significantly influenced an individual's willingness to cross the movement hurdle ($z = -2.86$, $p = 0.004$). With each millimeter increase in SVL at the time of the assay, the odds of crossing the movement hurdle and leaving the start position increased by 16% (95% CI = 0.05 to 25%). The probability of crossing the movement hurdle increased in wet runway condition and as SVL increased (**Figure 2**). However, after crossing the movement hurdle, the impact of runway condition changed. Runway condition still significantly influenced the distance an individual moved down the runway ($z = -3.01$, $p = 0.003$), but now wet runway condition reduced the distance moved by 36% (95% CI = 14% to 52%) compared to the dry runway condition. As in the movement hurdle, SVL was a significant predictor of movement distance ($z = 2.94$, $p = 0.003$). An increase of 1 mm SVL increased the movement distance by 12% (95% CI = 4 to 21%). Movement distance was highest for large individuals in dry runway conditions (**Figure 3**).

Neither body condition nor time since emergence (age) were significant in our model for movement hurdle (body condition: $z = 0.20$, $p = 0.840$; age: $z = -0.28$, $p = 0.773$) and movement distance (body condition: $z = 0.81$, $p = 0.416$; age: $z = 0.40$, $p = 0.688$). Repeatability of behavior by an individual across assays was not strongly correlated ($R^2 = 0.0283$ distance, $R^2 = 0.0193$ hurdle). Snout-vent length and runway condition were the only important factors in both the ability and behavior around movement, yet hydroperiod was not significant in either the movement hurdle ($z = 1.25$, $p = 0.212$) or movement distance ($z = 0.23$, $p = 0.816$). The opposite effect of ephemeral conditions with smaller size at emergence and subsequent increased growth rates likely reduced the significance of hydroperiod in the behavioral assay because of the spectrum of ages included (Range = 2–83 days after emergence, mean = 35 ± 19 days SD).

DISCUSSION

The ability of an amphibian species to respond plastically to changing hydroperiods in seasonally-variable habitats is an essential life history adaptation, particularly in the face of

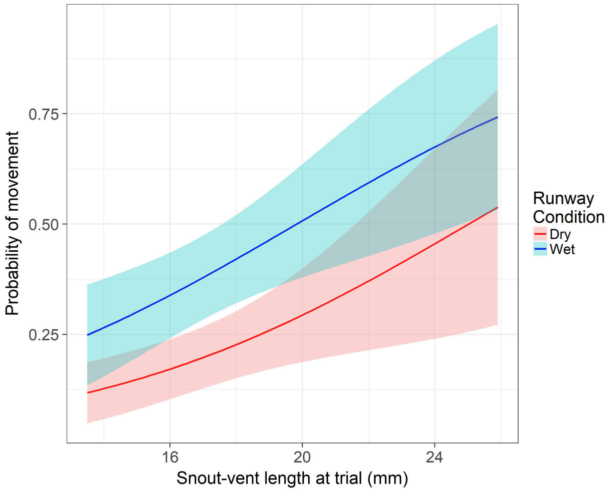

FIGURE 2 | The impact of snout-vent length and runway condition on probability of movement across the movement hurdle. Shaded areas represent the 95% confidence interval. Response is modeled off an individual from the permanent hydroperiod in their first runway assay with average body condition and time since metamorphosis.

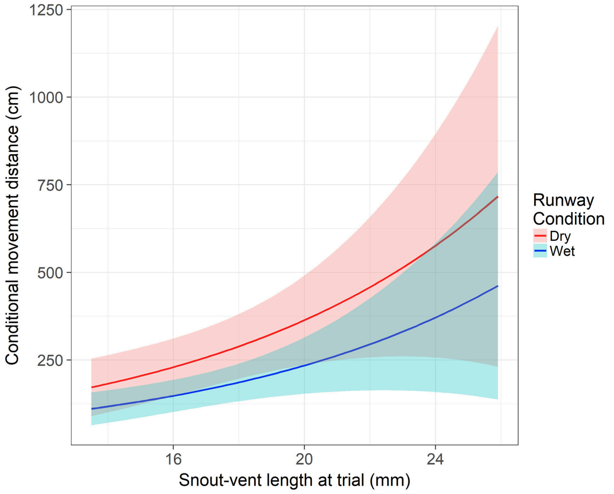

FIGURE 3 | The impact of snout-vent length and runway condition on movement distance after crossing the movement hurdle. Shaded areas represent the 95% confidence interval. Response is modeled off an individual from the permanent hydroperiod in their first runway assay with average body condition and time since metamorphosis.

changing climate. However, if an appropriate larval trait response carries over to fundamentally influence an individual's ability to contend with stressors later in life, this response could be maladaptive. Our objective was to assess the carryover effects of larval hydroperiod permanence onto juvenile growth and movement in low- and high-stress terrestrial conditions. We found that 2 factors, individual *R. aurora* size and terrain condition, were the central predictors of individual movement behavior. The carryover effect of hydroperiod was expressed in both the size at emergence and compensatory growth of individuals soon after metamorphosis. We identified no direct correlation between hydroperiod and movement behavior as hypothesized. However, hydroperiod is directly related to the size of juveniles, which is the main factor determining movement behavior. Thus, larval conditions, including hydroperiod, indirectly influence movement behavior.

Our experiment found that *R. aurora* from permanent hydroperiod conditions metamorphosed at a larger size than individuals reared in ephemeral hydroperiods (**Figure 1**). This pattern is consistent in species that can plastically modify larval developmental rates under stressful conditions (Denver et al., 1998; Morey and Reznick, 2004). Changes in larval duration has been one suggested explanation for the effects of hydroperiod (Morey and Reznick, 2004). We did not observe any difference in larval duration between treatments, which is supported by other observation of reduced size in drying conditions independent of changes in larval duration (Brady and Griffths, 2000; Mogali et al., 2017). However, these smaller sized individuals from ephemeral conditions then demonstrated increased growth rates immediately after emergence, possibly to compensate for suboptimal size at metamorphosis. Compensatory growth of smaller individuals exposed to stressful larval conditions has been observed in other frog species (Boone, 2005; Bouchard et al., 2016). The increased growth rates of the individuals from ephemeral conditions may explain why larval hydroperiod was not significant in our analysis of individual movement. Since our experiment included individuals from a wide emergence window (i.e., emergence dates ranged from 25 June to 24 September), the opposing effect of hydroperiod on emergence size and terrestrial growth created difficulty in deciphering the carryover effect directly. It should also be acknowledged that the relative different in sizes between ephemeral and permanent conditions is only ca. Four percentage after accounting for emergence timing, which match the magnitude of other studies (Semlitsch, 1987; Searcy et al., 2014). What is clear from our analysis is that larval hydroperiod has effects on movement of juvenile *R. aurora* by means of a trade-off in growth between the aquatic and terrestrial environment. This may be common for many species with multiple life history stages to escape drying conditions but requires long-term experiments to uncover fully.

We found body size to be a strong predictor of movement behavior. Size is a critical trait in amphibian biology from physical abilities to individual fitness (Walton, 1988; Van Allen et al., 2010; Earl and Whiteman, 2015), with snout-vent length being a simple measurement that encompassed much of the variation in size. In many amphibian species, larger individual size is often correlated with increased performance (Chelgren et al., 2006; Gomes et al., 2009; Yagi and Green, 2017), as observed in our results (**Figure 3**). Yet increases in snout-vent length were also correlated with increased movement probability (**Figure 2**). This result indicates that an individual's behavior is also influenced by its physical size. One possible explanation for this effect is a change in the perceived cost of a condition relative to body size. Larger individuals can retain moisture more efficiently than small individuals (Levy and Heald, 2016), thereby making movements in dry conditions less risky. Another possibility is inherent differences in behavior that result in differences in size. Individuals with bold personalities can be more aggressive in resource acquisition resulting in differences in body size and these personality traits can also increase predisposition to explore new areas (Kelleher et al., 2018). Whatever the cause of these behavioral differences across a gradient of snout-vent length, this physical trait may be a useful metric for understanding individual movement potential in field contexts.

Terrain moisture is an important aspect of environmental condition that shapes the movement strategies of organisms that are sensitive to dehydration, such as amphibians and invertebrates. With our experimental design, we were able to examine the critical aspects of this behavior: initial decision to move and distance traveled (Martin et al., 2005). The effect of runway condition had predictable effects, such as wet conditions increasing the probability of movement over dry conditions (**Figure 2**). However, dry conditions that can pose significant physiological hazards to young amphibians increased movement distances. Movement may be more effective if individuals quickly traverse the drier terrain to find more hospitable habitat. In contrast, wet terrain conditions are less risky and have the effect of reducing the distance moved (**Figure 3**). Most research has found reduced movement in non-habitat (Eycott et al., 2012) with only occasional evidence for increased movement in inhospitable areas (damselfly—Pither and Taylor, 1998; planthopper—Haynes et al., 2006). However, as indicated by our results, there were several instances of amphibians moving more efficiently over simple and inhospitable habitat (Natterjack toads—Stevens et al., 2004; Northern green frog—Birchfield and Deters, 2005). While there is limited information about the terrestrial movement and habitat of *R. aurora*, there is anecdotal evidence that they are associated with aquatic habitat and moist forests while also moving substantial distances (Haggard, 2000; Hayes et al., 2001; Chan-McLeod, 2003). Amphibians with different habitat requirements or life-history could be expected to respond differently.

Changes in precipitation pattern and droughts likely will also influence the distributions of aquatic habitat and, by extension, amphibian populations and their connectivity (Robson et al., 2011; Jaeger et al., 2014; Cayuela et al., 2016). Insights into carryover and direct effects of stress on juvenile movement behavior provide opportunities to inform management strategies. Beaver canals have been observed to influence the location and emergence of amphibians, making waterway modifications a potential strategy for spatial management of populations (Anderson et al., 2015). Amphibians' likely rely on rare long-distance movements for population connectivity (Semlitsch, 2008). Even small changes in the success of dispersing individuals or distances traversed will likely have great implication for population connectivity (Stevens et al., 2012). Our study informs this pattern by relating hydroperiod permanence with juvenile amphibian body size and growth rates with dispersal probability. The behavioral and developmental trait connections across this life history transition likely influence population connectivity.

This project looked at movement behavior in a simplified setting. However, amphibians have shown to change their movement behaviors based on ground cover and context (Cline and Hunter, 2014, 2016; Osbourn et al., 2014). Accounting for the accumulated impacts of stressors across an individual's development, including terrain complexity, is a key step in providing a complete understanding of these animals. How other stressors, such as terrestrial density, predator presence, or prey availability interact with hydroperiod and terrain conditions could add additional consideration to the movement decisions of these animals (Vonesh and Warkentin, 2006; Rittenhouse et al., 2007; Patrick et al., 2008). Evidence from other amphibian species have also found reduced growth rates in dry conditions creating potential multiplicative effects of environmental conditions, as size was the central determinant of movement (Gomez-Mestre and Tejedo, 2005; Charbonnier et al., 2018). Other research on carry-over effect in amphibians has supported the need for longitudinal studies (Johansson et al., 2010; Charbonnier and Vonesh, 2015; Yagi and Green, 2018). With this broader understanding, we would be better able to combine aquatic structure with terrestrial habitat management to provide for essential habitat components of threatened species (Fellers and Kleeman, 2007; Bartelt and Klaver, 2017).

Amphibian movement has been understudied despite its importance for population connectivity and dispersal of vulnerable species (Pittman et al., 2014; Howell et al., 2018; Zamberletti et al., 2018). Our study details some of the drivers of movement assessed in isolation which can be incorporated into predictions of *in-situ* movement, but there is still need for such knowledge to be explored and validated in field and population studies. There are still important aspects such as directionality and habitat settlement that would further our understanding of movement. In short, assessing movement as a holistic, ontogenetic system, will improve our understanding of this complicated behavior and the myriad factors influencing how an animal moves through life.

DATA AVAILABILITY

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

ETHICS STATEMENT

This work was performed under Oregon State University Institutional Animal Care and Use Committee with Animal Care and Use Protocol 4536 and the Oregon Department of Fisheries and Wildlife Service for Special Use Permit no. 043-15 for animal collections.

AUTHOR CONTRIBUTIONS

EB, TG, and AM conceived the research question. EB and JU collected animals and performed data collection. EB performed the analysis. EB wrote the manuscript with significant assistance from JU, AM, and TG. All authors contributed critically to the drafts and gave final approval for publication.

FUNDING

This work was supported by the National Science Foundation Graduate Research Fellowship under Grant No. 1314109-DGE. The award provided tuition and stipend support with a small amount of research funds.

ACKNOWLEDGMENTS

We would like to thank Hyslop Field Laboratory for their access and use of space. We would also like to thank Jennifer Rowe for statistical consultation, Nicolas Soria and Angie Soken for their assistance with construction and data collection.

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Conflict of Interest Statement: 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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ORIGINAL RESEARCH
published: 11 April 2019
doi: 10.3389/fevo.2019.00114

Roundup™ With Corticosterone Functions as an Infodisruptor to Antipredator Response in Tadpoles

Caitlin R. Gabor, Hannah R. Perkins, Austin T. Heitmann, Zachery R. Forsburg and Andrea S. Aspbury*

Department of Biology, Texas State University, San Marcos, TX, United States

Larval amphibians are frequently exposed to multiple stressors in aquatic environments, so understanding how individual stressors and synergisms of multiple stressors affect amphibians is integral to conservation efforts. Glyphosate, the active ingredient in Roundup^{TM}, is a common pollutant found in aquatic environments. Exposure to glyphosate induces morphological, behavioral, and physiological changes in tadpoles possibly via infodisruption. Urban aquatic environments may have environmentally relevant levels of glyphosate, as well as higher concentrations of exogenous corticosterone (CORT) than rural areas. Elevated CORT levels also affect the morphology and physiology of tadpoles. Dragonfly larvae are common predators of tadpoles, and tadpoles often show elevated CORT and reduced activity in the presence of dragonfly larvae. We tested the hypothesis that combined effects of exogenous CORT and Roundup^{TM} exposure would affect the antipredator behavior, morphology, and stress hormone responses of Gulf coast toad, *Incilius nebulifer,* tadpoles. We exposed tadpoles to one of four treatments: Roundup^{TM}, CORT, Roundup^{TM}+CORT, or control, for 7 days. Tadpoles exposed to CORT or Roundup^{TM}+CORT had elevated CORT release rates. Tadpoles exposed to exogenous CORT had lower tail depth compared to tadpoles in other treatments. Subsequently, we exposed tadpoles to dragonfly diet cues. Tadpoles increased activity after predator cue exposure when they had previously been exposed to Roundup^{TM}+CORT. Taken together, our results suggest that there may be synergistic effects between Roundup^{TM} and exogeneous CORT on organismal behavior but not their physiology or morphology. It appears that glyphosate is an infordisruptor, that prevents tadpoles from demonstrating adaptive antipredator responses, which may contribute to population declines.

Keywords: amphibians, ecotoxicology, glyphosate, conservation physiology, stress, water-borne hormones

Edited by:
Shannon J. McCauley,
University of Toronto Mississauga,
Canada

Reviewed by:
Jessica Hua,
Binghamton University, United States
Travis Eli Wilcoxen,
Millikin University, United States

***Correspondence:**
Caitlin R. Gabor
gabor@txstate.edu

Specialty section:
This article was submitted to
Behavioral and Evolutionary Ecology,
a section of the journal
Frontiers in Ecology and Evolution

***Received:** 31 December 2018*
***Accepted:** 21 March 2019*
***Published:** 11 April 2019*

Citation:
Gabor CR, Perkins HR, Heitmann AT,
Forsburg ZR and Aspbury AS (2019)
Roundup^{TM} With Corticosterone
Functions as an Infodisruptor to
Antipredator Response in Tadpoles.
Front. Ecol. Evol. 7:114.
doi: 10.3389/fevo.2019.00114

INTRODUCTION

Worldwide, amphibian biological diversity is decreasing; 32% of the world's amphibian species are threatened with extinction and 43% of amphibian populations are experiencing declines (Stuart et al., 2004). Environmental pollutants such as herbicides and pesticides not only have a direct effect on amphibian population recruitment, but they also have indirect effects via their immunosuppressive effects on individual organisms (Hayes et al., 2010). Immunosuppression is linked to an increase in glucocorticoid hormones after exposure to chronic stressors. The link between exposure to pollutants and immunosuppression in an organism is likely mediated via the infodisruptive effects of the pollutant that disrupt chemical communication modalities used by the organism. Pollutants also often act as endocrine disruptors that affect hormones and internal communication in organisms (Lurling and Scheffer, 2007). When infodisruptors affect the production of glucocorticoid stress hormones, organisms may show altered immunosuppression in the long-term and altered growth and behavior in both the short- and long-term (McEwen and Wingfield, 2003; Romero, 2004; Denver, 2009; Middlemis Maher et al., 2013). In addition, the breakdown in detection of chemical signals between senders and receivers due to infodisruptors in predator prey systems may increase prey mortality.

Amphibians are exposed to many stressors throughout their life cycle, but frequently early in life aquatic-dependent life stages are particularly susceptible to the effects of exposure to aquatic pollutants (Hayes et al., 2006). During chronic exposure to stressors, the hypothalamic-pituitary interrenal (HPI) axis may become disrupted, and the effects of increased corticosterone (CORT) become immunosuppressive. In frogs and toads, tadpoles show elevated CORT when exposed to pollutants such as atrazine and glyphosate (Burraco and Gomez-Mestre, 2016; McMahon et al., 2017; Gabor et al., 2018), and in the presence of predators (Middlemis Maher et al., 2013; Bennett et al., 2016). In addition, elevated CORT levels in tadpoles induced by exposure to exogenous CORT for 8 days is associated with deeper tails and shorter trunks (Middlemis Maher et al., 2013). Deeper tails may improve the ability of tadpoles to evade predators (Van Buskirk et al., 1997). Tadpoles are exposed to exogenous CORT in their habitat as it is continually released into the water by aquatic vertebrates via urine, feces, and gills (Scott and Ellis, 2007). Tadpoles may uptake the free steroids that are released from the gills (Ellis et al., 2004). Amphibians occupying habitats where they are exposed to more stressors may further release more CORT into the water. For example, the Jollyville Plateau salamander, *Eurycea tonkawae*, releases more CORT and is also exposed to higher exogenous CORT ("background CORT") in urban habitats than in rural habitats (Gabor et al., 2018). This suggests that amphibian habitats in urban areas (that often have higher levels of pollutants such as glyphosate) may also have higher levels of exogenous CORT which may affect the endogenous CORT of the amphibians residing in those waters.

Roundup^{TM} is the most commonly used herbicide in the United States for both agricultural and domestic use (Giesy et al., 2000). Glyphosate, the primary active ingredient in Roundup^{TM}, and the other associated ingredients enter many aquatic ecosystems via runoff, leaching, and mis-use of the product (Annett et al., 2014), thus affecting non-target organisms living in aquatic habitats. Further, repeated applications of Roundup^{TM} contributes to the persistence of glyphosate in aquatic ecosystems (Giesy et al., 2000). Environmentally relevant concentrations range from 0.1 to 2.7 mg a.e./L (Solomon and Thompson, 2003), and acute toxicity of glyphosates for amphibians based on 96-h LC50 estimates range from 1.8 to 3.5 mg a.e./L (Annett et al., 2014). Therefore, amphibians in many aquatic ecosystems are exposed to sub-lethal to lethal concentrations of glyphosate.

Based on results of long-term mesocosm studies we know that tadpoles can exhibit differential responses to exposure to glyphosate. However, it is unclear if there are synergistic effects of exposure to predator cues and glyphosate on tadpole development, morphology, behavior, and physiology. For example, exposure to 2–3 mg a.e./L of glyphosate or to the cues of a dragonfly (*Anax junius*) larvae predator is associated with increased tail depth in two species of *Rana* tadpoles. The increase in tail depth is additive when tadpoles are exposed to both glyphosate and predator cues (Relyea, 2012). In contrast, glyphosate exposure is not associated with morphological changes similar to predator induced changes for tadpoles of *Hyla versicolor* (Relyea, 2018). In addition to its morphology-based interactions with predator cues, glyphosate may interact with behaviors involved in antipredator responses, as environmentally relevant levels of glyphosate interfere with chemical cue detection and processing (e.g., Tierney et al., 2006). Again, there are no clear patterns emergent from the literature on the direction of effects. For example, tadpoles of *Rana sylvatica* exposed to 0.5 mg a.e./L glyphosate do not exhibit a behavioral antipredator response (do not reduce activity) when exposed to predator chemical cues (Moore et al., 2015). The effect of glyphosate on disrupting anti-predator behavior may be concentration dependent; *R. dalmatina* tadpoles exposed to 2 mg a.e./L glyphosate do not differ from control tadpoles in response to predators, but tadpoles exposed to 6.5 mg a.e./L glyphosate exhibited elevated antipredator behavior (reduced activity) (Miko et al., 2017). These results indicate that antipredator response and morphological changes, as a result of glyphosate exposure, vary by exposure level and are species specific in amphibians.

One potential source of variation mediating the relationship between exposure to glyphosate and effects on tadpole morphology and antipredator behavior is the variation in the direction of the stress response. In *Pelobates cultripes* tadpoles whole-body CORT increases with exposure to 1 and 2 mg/L of glyphosate, but CORT decreases with predator exposure (Burraco and Gomez-Mestre, 2016). These results indicate that the interaction of exposure to exogenous CORT and glyphosate may drive additional changes unlike those predicted for each alone. There is a gap in our understanding of the synergistic effects of glyphosate and exogenous CORT on amphibian morphology, antipredator behavior, and physiological stress in amphibians. In this study, we tested the hypothesis that the combined effects of exogenous CORT exposure and Roundup^{TM} exposure would affect antipredator behavior, morphology, and hormonal responses of Gulf coast toad, *Incilius nebulifer,* tadpoles.

MATERIALS AND METHODS

Gulf coast toad (*Incilius nebulifer)* tadpoles were collected from a pond in Prospect Park, San Marcos, TX (29.874°N, 97.963°W) in May 2017 and maintained in the laboratory at Texas State

University. The tadpoles were maintained in 6 L containers with aged tap water and on a 14 h light: 10 h dark cycle at 19°C until Gosner stage 25 (Gosner, 1960). Tadpoles were fed *ad libitum* a mixture of spirulina and fish flakes suspended in an agar medium.

Experiment 1: Stress Response to Glyphosate and CORT Exposure

We haphazardly assigned tadpoles ($N = 120$) that were at or near Gosner stage 25 to one of four experimental treatments that varied in what tadpoles were exposed to for 7 days (12–19 May 2017). Tadpoles were reared in either aged tap water or Roundup^{TM} mixed with aged tap water (final concentration of glyphosate was 0.736 mg a.e./L, analyzed by Mississippi State Chemical Lab). Our target concentration of Roundup^{TM} was 0.5 mg a.e./L based on Moore et al. (2015). The water was also dosed with either 125 nM CORT in 37.5 μL ethanol as the solvent, or only 37.5 μL ethanol. We chose a concentration of glyphosate (from Roundup^{TM}) that represented a low, but environmentally relevant, sub-lethal dosage (Relyea, 2012; Moore et al., 2015) and chose a CORT dosage of 125 nM following Middlemis Maher et al. (2013). The four treatments were: (1) "Control," aged tap water and 37.5 μL ethanol, (2) "Roundup^{TM}," Roundup^{TM} water and 37.5 μL ethanol, (3) "CORT," 125 nM CORT in 37.5 μL ethanol, and (4) "Roundup^{TM} + CORT," Roundup^{TM} water and 125 nM CORT in 37.5 μL ethanol. We replicated each treatment with 5 tanks, each containing 1.5 L of water (either 24 h aged tap water or aged tap water mixed with Roundup^{TM}), that each housed 6 tadpoles. Rearing containers were labeled by code, so the authors were blind to treatments. The volume of ethanol added to each tank was 0.0025% of the total water volume. Prior studies have detected no effects on any measured trait of ethanol at this level so no water control was included (reviewed by Rohr et al., 2013). On the third day, a full water change was completed for all the treatments. Treatments were re-dosed by someone other than the authors. After 7 days of treatment exposure (Day 7), we collected water-borne CORT from 3 of the 6 tadpoles from each of the 5 replicate tanks ($N =$ 15 tadpoles per treatment; except 2 control and 2 CORT tadpoles died in 4 different tanks). To collect water-borne CORT, tadpoles were individually placed into a Nalgene sleeve (with holes on the bottom) fit within 250 ml glass beakers containing 50 ml spring water for 1 h. We then lifted the tadpole from the water using the Nalgene sleeve, leaving the water-borne hormone sample in the glass beaker. We collected water borne-hormones between 0800 and 1400 h to control for circadian variation.

Following water-borne hormone collection, tadpoles were individually photographed from the side in water with a ruler placed below the tadpole, using a digital camera. We used Image J to measure snout vent length (SVL) and tail depth from the photos. After taking photos and mass, we euthanized the tadpoles by overdose of Oragel (active ingredient 1% benzocaine) placed on the ventral side. We preserved the tadpoles in microcentrifuge tubes with 1 ml of 95% ethanol for later Gosner stage determination using a dissecting scope. The water-borne hormone samples were all placed in individual 118 ml HDPE containers and stored at −20°C until hormone extraction (see below). We standardized hormone release rates by dividing by SVL of the given individual (following Gabor et al., 2016). We log transformed CORT release rates but figures present untransformed values. We measured SVL (mm), tail depth (mm), and mass (g) for all tadpoles.

To test the hypothesis that Roundup^{TM} and exogenous CORT affect tadpole endogenous CORT and morphology, we used a linear mixed model with treatment as a fixed effect, and tadpole as a random effect. The response variables were: SVL, tail depth and Ln CORT release rate. All analyses were performed using JMP 14 pro software (SAS Institute, Inc).

Roundup™ Preparation

Roundup^{TM} Ready to Use, Fast Act Technology was purchased from an agricultural supply store in San Marcos, TX USA in 2017. The glyphosate concentration was 20 g acid equivalents (a.e.)/L., so 0.45 ml of Roundup^{TM} base solution was diluted in 18 L of dechlorinated aged tap water to create a stock solution. The glyphosate concentration in the final Roundup^{TM} solution was analyzed from two samples from the stock solution by Mississippi State Chemical Laboratory and determined to be 0.736 mg a.e./L.

Water-Borne Corticosterone Assay

We extracted CORT from the water-borne samples by priming C18 solid phase extraction (SPE) columns (Waters Technology Corporation) with 4 ml of methanol and 4 ml of distilled water (following Gabor et al., 2016). We then pulled the water samples through the column using a vacuum manifold. Samples were eluted with 4 ml of methanol and then the methanol was dried with nitrogen in a 37°C bath. The samples were reconstituted using 5% ethanol, followed by vortexing. We then added 95% enzyme immune-assay (EIA) buffer (from Cayman Chemical Company) up to a volume of 260 μl. All CORT values were multiplied by the resuspension volume. We assayed samples in duplicate using corticosterone EIA plates (No 501320, Cayman Chemical Company) using a spectrophotometer plate reader at 405 nm (BioTek 800XS). Inter-plate variation for four plates was 10.06% and intra-plate variation ranged from 0.22 to 2.91%.

Experiment 2: Antipredator Behavior

On day 7, the remaining tadpoles from the treatments (up to 3 tadpoles in each tank) were placed in 20 clean tanks (the same size as they had been in before) filled with 1.5 L of untreated aged tap water (thus no longer exposed to Roundup^{TM} or CORT). To test for anti-predator behavior, we first collected diet-based chemical cues released by tadpole predators (late instar dragonfly nymphs, genus: *Anax* sp.). The dragonfly nymphs were collected from the same pond as the tadpoles in May 2017 and were kept in individual containers filled with aged tap water and a perch. On day 0 of Experiment 1, we began feeding *Anax* nymphs 2 conspecific tadpoles per day for 7 days. On day 7, *Anax* ($n = 5$) were placed into separate clean beakers with 230 ml water per gram of *Anax* for 24 h. The water samples were then pooled together and mixed. This water-based "diet cue" was frozen at −20°C in 120 ml aliquots (Epp and Gabor, 2008). We defrosted the amount of diet cue needed for the antipredator behavior experiment by letting the aliquot come to room temperature before use.

On days 8, 9, and 10 of the experiment, we set up 20 individual tanks with 1.5 L of aged tap water and with two 60 ml syringes connected to airline tubing with a two-way spout that was placed inside the tank to evenly disperse the water and diet cue. One syringe was filled with aged tap water and the other with the predator diet cue. Individual tadpoles were placed in the tanks and allowed to acclimate for 8 min. We then injected 10 ml of water into the tank at a rate of 1 ml per second and recorded the "pre" exposure activity rate of the tadpole for 8 min using a timer. Activity was defined as any tadpole movement. Then, we injected 10 ml of the diet cue into the tank at the same rate and recorded "post" exposure activity (anti-predator behavior) for 8 min. Syringe tips and tubing were cleaned between trials with 3% hydrogen peroxide and rinsed with deionized water. After the predator cue trial, we collected water-borne CORT samples as outlined previously. Antipredator behavior testing occurred from 0800 to 1200 h with water-borne hormone extraction immediately following predator cue exposure. We also euthanized and collected body measurements from these tadpoles, as outlined in experiment 1.

First, we tested for any significant variation in pre-predator cue exposure activity across the tadpoles from the different treatments using a linear mixed effect model with tank as a random effect and treatment, the day (1, 2, or 3 days after the end of experiment 1) of the behavioral trials, and the treatment X day interaction as the fixed effects. We used a linear mixed effect model on the difference in activity (pre-exposure activity—post-exposure activity) with tank as a random effect, the day of the activity behavior trials, and the treatment X day interaction as the fixed effects. We also used a linear mixed effect model on Ln CORT (post predator cue exposure) release rate with tank as a random effect, the day of the activity behavior trials, and the treatment X day interaction as the fixed effects. All significant treatment effects were further explored with *post-hoc* multiple comparisons. All analyses were performed using JMP 14 pro software (SAS Institute, Inc.).

RESULTS

Experiment 1: Stress Response to Glyphosate and CORT Exposure

After 7 days of exposure to treatments, CORT release rates were significantly higher in the CORT only and the Roundup^{TM} + CORT treated tadpoles than in the control treatment tadpoles [Treatment: $F_{(3,16)} = 41.21$, $p < 0.0001$; **Figure 1a**]. Tadpoles in the CORT only treatment had significantly lower tail depth than the control tadpoles [$F_{(3,16)} = 4.39$; $p = 0.02$; **Figure 2a**]. None of the treatments had significantly different SVL from the control treatment, but tadpoles in the CORT treatment had a significantly smaller SVL than those in the Roundup^{TM} only treatment [Treatment: $F_{(3,16)} = 4.75$; $p = 0.01$; **Figure 2b**).

Experiment 2: Antipredator Behavior

After exposure to the treatments, but before exposure to any predator cues, there was no significant variation in the activity level of the tadpoles (**Table 1**). After predator exposure, there was a significant effect of the treatment on the change in activity of the tadpoles; tadpoles in the Roundup^{TM} + CORT treatment had a significant increase in their activity after predator cue exposure. Tadpoles from all other treatments decreased activity [Treatment: $F_{(3,17)} = 3.36$, $p = 0.043$; **Figure 3**]. CORT release rates did not differ across treatments after exposure to predator diet cues [Treatment: $F_{(3,15)} = 0.34$, $p = 0.80$; **Figure 1b**].

TABLE 1 | Fixed effects tests from LME model on post-treatment, but pre-exposure to predator cue activity (s) of Gulf coast toad, *Incilius nebulifer,* tadpoles.

Source	df	*F*	*p*
Treatment	3	0.705	0.562
Day	2	0.626	0.541
Treatment X Day	6	2.204	0.070

DISCUSSION

Anthropogenic stressors, such as pollutants, contribute to amphibian population declines (Stuart et al., 2004). Because amphibians are often simultaneously exposed to multiple stressors, we evaluated here if multiple stressors can have synergistic effects on tadpole stress response, morphology, and antipredator behavior. We found that Gulf coast toad tadpoles exposed to exogenous CORT and Roundup^{TM} + CORT had significantly higher CORT release rates than those exposed to Roundup^{TM} alone or the control. Our results were consistent with Glennemeier and Denver (2002) who found elevated whole-body CORT in leopard frogs, *Rana pipiens*, after exposure to 125 nM exogenous CORT. Aquatic habitats in urban areas may be more likely to have higher levels of exogenous sources of CORT (background CORT) in the water from other aquatic vertebrates, run-off, terrestrial animals, or sewage line seepage (e.g., Gabor et al., 2018). Because tadpoles can uptake exogenous CORT through their skin, exposure to higher concentrations of exogenous CORT may push tadpoles away from homeostasis and could ultimately result in immunosuppression of the organism. Such indirect effects on organisms as a result of exposure to anthropogenic stressors are associated with greater disease susceptibility (Blaustein et al., 2012), and can ultimately lead to decreases in population recruitment. However, we did not find an infodisruptive effect of a low but environmentally relevant concentration of Roundup^{TM}.

We exposed tadpoles to a very low, concentration of Roundup^{TM} and their CORT release rates did not increase with this exposure. In contrast, (Burraco and Gomez-Mestre, 2016) found that tadpoles exposed to 1 or 2 mg a.e./L glyphosate had increased whole-body CORT after a 10-day exposure period. Future studies will address if higher levels of Roundup^{TM} and/or longer exposure affect CORT release rates in Gulf coast toads.

Despite no effects of exposure to 0.736 mg a.e./L glyphosate for 7 days on Gulf coast toad tadpole CORT release rates, these tadpoles did have significantly greater SVL than CORT exposed tadpoles. Exposure to CORT also affected Gulf coast toad

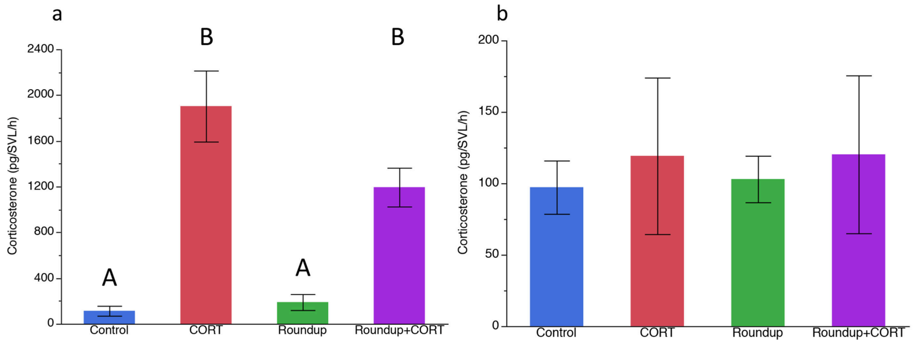

FIGURE 1 | Mean (±SE) corticosterone (pg/SVL/h) by treatment **(a)** after 7 days in the exposure treatments, and **(b)** after predator cue exposure for Gulf coast toad, *Incilius nebulifer*. Different letters indicate significant differences based on Tukey's HSD *post-hoc* test.

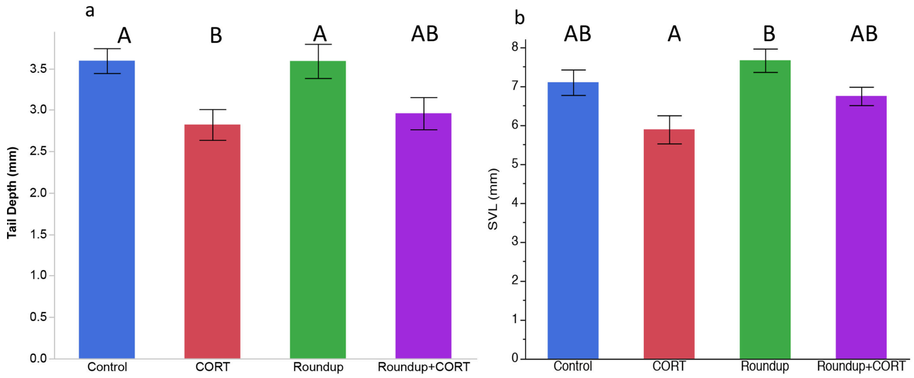

FIGURE 2 | Mean (±SE) **(a)** tail depth and **(b)** snout-vent length (SVL) by treatments on day 7 for Gulf coast toad, *Incilius nebulifer*. Different letters indicate significant differences based on *post-hoc* student's *t*-tests.

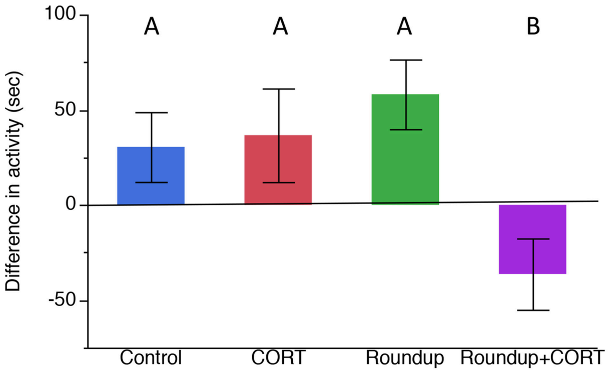

FIGURE 3 | Mean (±SE) difference in activity (time in seconds spent moving) before predator cue exposure and after predator cue exposure across treatments (pre-cue time minus post-cue time). Different letters indicate significant differences based on Tukey's HSD *post-hoc* test.

tadpole tail morphology: tadpoles in the CORT only treatment had significantly lower tail depth than the control tadpoles. Exposure to elevated CORT is associated with tadpoles of *R. sylvatica* and *R. pipiens* developing deeper tails (an antipredator morphology; Hossie et al., 2010; Middlemis Maher et al., 2013). At higher, but still environmentally relevant, concentrations of Roundup^{TM} the antipredator morphology of deeper tails is induced in wood frog and leopard frog tadpoles (Relyea, 2012) but not in gray tree frog tadpoles, *Hyla versicolor* (Relyea 2018). Deeper tails aid in escaping from predators as deeper tails deflect lethal predator attacks toward the tail instead of the body of the tadpole (Van Buskirk et al., 1997). We hypothesize that Gulf coast toad tadpoles do not show antipredator morphology because the tadpoles are noxious to predators (Adams et al., 2011) and thus do not rely on this deflection behavior as an anti-predator strategy.

After predator exposure, tadpoles in the Roundup^{TM} + CORT treatment were more active than they were before exposure to the dragonfly nymph predator cue. Prior to exposure to the predator cue, there were no significant differences in tadpole activity across the treatments, suggesting that the increase in the activity after predator cue exposure of tadpoles exposed to both Roundup^{TM} and CORT was a result of their prior treatment affecting their responsiveness to a predator cue in a seemingly maladaptive way. Tadpoles from all other treatments decreased activity after exposure to a predator cue. Generally, tadpole antipredator behavior consists of freezing in response to sit-and-wait predators like dragonfly nymphs (Middlemis Maher et al., 2013; Moore et al., 2015; Miko et al., 2017). Wood frog, *Rana sylvatic*, tadpoles do not reduce activity when they are exposed to Roundup^{TM} alone and predator diet cues (Moore et al., 2015). For Gulf coast toads, Roundup^{TM} alone did not affect their antipredator behavior but the combination of CORT and Roundup^{TM} did affect their antipredator behavior. Toads could be less sensitive to the effects of Roundup^{TM}, except when they are exposed to stressors. One hypothesis for this is that Bufonid toads synthesize toxic steroids called bufadienolides that are synthesized from the same chemical pathway that produces CORT and the relationship may result in negative feedback that could have mediated a reduction in CORT production (Webb et al., 2008). Bókony et al. (2017) found that longterm exposure of tadpoles, *Bufo bufo*, to 4 mg a.e./L of glyphosate in the lab produced more bufadienolides.

We found that CORT release rates did not increase after exposure to predator diet cues for 10 min as these values were similar to the control values for tadpoles after the first (exposure/no exposure to CORT or Roundup^{TM}) experiment. Amphibian CORT response to predator diet cues is variable and often time-dependent. *Rana sylvatica* tadpoles exhibited an acute (10–20 min post-cue exposure) whole-body CORT increase in response to dragonfly larvae predator cues (Bennett et al., 2016). In some populations of *R. temporaria*, whole-body CORT levels are higher in predator exposed tadpoles at 24 h than in control tadpoles, but this difference is not apparent by 15 days after exposure to predator cues (Dahl et al., 2012). In some cases, there can be initial downregulation of whole-body CORT (within 4 h of non-lethal exposure to predators), but upregulation of CORT after several days (e.g., *R. sylvatica*, Middlemis Maher et al., 2013). One explanation for our result is that CORT had already decreased in the time since the tadpoles were exposed to the initial treatments (1–3 days), and that the short-term predator cue exposure was not enough to elicit a CORT response. CORT may have also been downregulated in the CORT and CORT + Roundup^{TM} treatments after predator exposure, but we are unable to test this hypothesis with or data. However, CORT release rates in tadpoles in the CORT or Roundup^{TM} + CORT treatments dropped to the same levels as the other treatments after exposure to the predator cues. Whether downregulation of CORT is associated with the observed increase of activity in the Roundup^{TM} + CORT treatment is not clear.

Tadpoles exposed to the exogeneous CORT or the Roundup^{TM} + CORT treatments had higher CORT release rates than tadpoles in the control or Roundup^{TM} only treatments prior to predator cue exposure. One interpretation for this result is that tadpoles can uptake exogenous CORT in the environment, which could affect their endogenous CORT levels and behavior. However, it is not known how high the levels of free exogenous CORT need to be in an organism's environment for it to be absorbed and ultimately affect the organism's CORT levels. We should note that in our experiment when we measured water-borne CORT after exposure to exogenous CORT we did not rinse the tadpoles off. In theory a droplet of water from the treatment could have traveled on the tadpole into the water sample and ultimately inflated the CORT release rate values. However, the amount of CORT in a 0.05 mL droplet from a tadpole removed from the 125 nM treated water (∼5.63 pg) would be much lower than the observed difference in CORT release rates between the treatments. In the future it may be better to rinse tadpoles before measuring water-borne CORT after being exposed to exogenous CORT although the additional handling and rinsing could add an additional stressor to the tadpoles.

Our results support the hypothesis that the combined effects of exogenous CORT exposure and environmentally-relevant concentrations of Roundup^{TM} affected antipredator behavior but not morphology or physiology of Gulf coast toads, *I. nebulifer*. Roundup^{TM} alone does not act as an infodisruptor to Gulf coast toads at the tadpole stage, as tadpoles reared in Roundup^{TM} did show decreased activity after predator cue exposure, which is consistent with other studies. However, our results suggest that the synergistic effect of Roundup^{TM} + CORT exposure may act as an infodisruptor in *I. nebulifer* tadpoles as they increased activity after introduction of predator cues. Tadpoles of this species exposed to low levels of Roundup^{TM} could be negatively affected by the synergism of glyphosate and exogenous CORT in urban aquatic environments.

ETHICS STATEMENT

Institutional Animal Care and Use Committee - IACUC201563714.

AUTHOR CONTRIBUTIONS

HP and AH executed the experiments with help from CG and ZF. CG and AA analyzed the data. All authors contributed to writing the manuscript. All authors have read and approved the final version of the manuscript.

FUNDING

This research was funded in part by U.S. Department of Education, HSI STEM Program (84.031c), Award #P031C160036 and the Francis Rose Undergraduate Award for Excellence in Biological Research to HP and Texas State University Honors program funding to AH.

ACKNOWLEDGMENTS

We thank Diana Kim, Cory Goff, Chloe Trevino for help with the experiment.

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Conflict of Interest Statement: 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.

frontiers
in Ecology and Evolution

REVIEW
published: 17 May 2019
doi: 10.3389/fevo.2019.00155

Behavior in a Changing Landscape: Using Movement Ecology to Inform the Conservation of Pond-Breeding Amphibians

*Pierre Joly**

UMR LEHNA, CNRS, ENTPE, Université de Lyon, Lyon1, Villeurbanne, France

Movement, both within an individual's home range and at the scale of dispersal, is a fundamental aspect of an animal's life. The field of movement ecology has established a conceptual framework to analyze the lifetime movement of an organism, offering a sound basis for conservation actions since the movement range of many species has been altered by habitat fragmentation and degradation. An organism's lifetime movement is organized around three main functions—exploitation, exploration, and relocation—which are associated with specific behavioral mechanisms and spatio-temporal scales. The movement ecology framework is a valuable tool as applied to amphibians, as managing these spatially structured populations requires in-depth knowledge of the behavioral mechanisms that determine movement. In terms of exploitation, these animals have a complex lifecycle, which involves migrating between different types of habitat, thus requiring them to cross a landscape matrix that may be more or less inhospitable. In terms of exploration and relocation, between-pond movements within the pond archipelagoes of a given population are frequent and strongly contribute to population resilience. Relocation also occurs at a larger scale, through long-distance dispersal to colonize new patches, exposing the individuals to unknown environments. Each function, at each scale, involves specific interactions between individual motivation (phenotype dependence) and environmental quality (context dependence) that determine decision-making and fitness outputs. Long-term exposure to local selective pressures can lead to differentiation in coping types that could be considered as Evolutionarily Significant Units (ESUs) for conservation. At the scale of a patch, the optimal direction of migration can be inherited, thus allowing the optimization of migration routes for juveniles. At the regional scale, a dispersal syndrome resulting in a greater propensity for boldness and exploration could be a response to unpredictable breeding sites or the high benefits of colonizing a rich habitat. Greater knowledge about such behavioral adaptations to specific situations would allow more targeted development of conservation measures or help to stop the spread of invasive species. The evolutionary context of movement behavior is thus of primary interest in designing effective conservation actions in a changing world.

Keywords: movement ecology, landscape, habitat fragmentation, spatially structured populations, migration, dispersal

Edited by:
Caitlin R. Gabor,
Texas State University System,
United States

Reviewed by:
David Charles Droney,
Hobart and William Smith Colleges,
United States
Monique De Jager,
Netherlands Institute of Ecology
(NIOO-KNAW), Netherlands

***Correspondence:**
Pierre Joly
pierre.joly@univ-lyon1.fr

Specialty section:
This article was submitted to
Behavioral and Evolutionary Ecology,
a section of the journal
Frontiers in Ecology and Evolution

Received: *19 December 2018*
Accepted: *23 April 2019*
Published: *17 May 2019*

Citation:
Joly P (2019) Behavior in a Changing Landscape: Using Movement Ecology to Inform the Conservation of Pond-Breeding Amphibians. Front. Ecol. Evol. 7:155. doi: 10.3389/fevo.2019.00155

INTRODUCTION

Since the advent of agriculture 9,000 years ago, with all of its by-products (e.g., industry, urbanization, transportation networks, etc.), many landscapes around the world have been dramatically modified, converting wild ecosystems into artificial environments (agrosystems, urban areas, etc.). The use of fossil fuels as an energy source since the nineteenth century has significantly hastened this "artificialization," leading to agricultural intensification (Mazzerole and Villard, 1999), expansion of urban areas (Hamer and McDonnell, 2008), and the construction of extensive transportation infrastructure (Fahrig et al., 1995). Unable to inhabit these artificial environments, most species are constrained to live in the remaining more or less wild fragments, suffering the effects of reduced population numbers, loss of genetic diversity, and increased extinction risks (Pereira et al., 2010).

Since high human densities often make it impossible to "rewild" areas by reversing the artificialization process, frequently the only way to mitigate the deleterious effects of habitat fragmentation is to maintain or enhance demographic flows between habitat patches. One of the major themes of conservation biology has been to explore ways these objectives could be reached, resulting in hundreds of studies in recent decades that address questions that require multidisciplinary integrative approaches (Fahrig and Merriam, 1985; Moilanen and Hanski, 1998; Gilbert-Norton et al., 2010; Baguette et al., 2013). Of these questions, the movements of animals across the landscape are central, and movement ecology has become a crucial field of investigation for conservation actions (Nathan et al., 2008; Fraser et al., 2018). One of the tools proposed by movement ecology is a multi-scale conceptual framework that connects movement processes with life histories, population dynamics, and functional outputs (Morales et al., 2010).

Amphibians are recognized as one of the animal groups most threatened by human-driven landscape modification, and their conservation is an urgent task (Houlahan et al., 2000; Beebee and Griffiths, 2005). There are many arguments for conserving amphibians based on the main values motivating biodiversity conservation (utility, amenity, functional, and intrinsic values) (Blaustein and Kiesecker, 2002). Pond-breeding amphibians are especially concerned by landscape threats, as their breeding habitats have historically been, and continue to be, destroyed because of lack of immediate economic profitability (Lehtinen and Galatowitsch, 1999; Becker et al., 2007). Their complex lifecycles also involve seasonal migrations between aquatic breeding habitats (i.e., ponds and wetlands) and terrestrial maintenance habitats (e.g., forests and moorlands) (Cushman, 2006). The success of these migrations depends on interactions between movement capacity (e.g., locomotion performance, orientation mechanisms, and stress management) and the harshness (e.g., aridity, toxicity, danger) of the landscape to be crossed. These interactions determine landscape connectivity (Joly et al., 2001), which in turn determines metapopulation functioning through dispersal flows between populations or the colonization of empty patches (Marsh and Trenham, 2008). As movement and connectivity are the core issues in pond-breeding amphibian conservation, the aim of this review is to focus on amphibian conservation in light of movement ecology. Since the work in this area by Sinsch (2014) and Pittman et al. (2014), numerous studies have been published that bring new insights, especially regarding amphibian responses to landscape fragmentation and dispersal syndromes.

Because of physiological and ecological specific features, amphibian fitness depends on physical and ecological characteristics of their life milieu, making them valuable indicators of anthropogenic modifications of the environment. Moreover, because they also are small predators, they can be used as umbrella species in the assessment of conservation actions for many taxonomic groups inhabiting wetlands and land/wetland ecotones (Joly and Morand, 1996). Humidity, heat, and predation are the primary factors that constrain amphibian movement. Amphibians rely on moisture because they are subject to permanent evaporative water loss and have to continuously replenish their water reserves by actively taking up water through the ventral skin (Shoemaker et al., 1992; Brunelli et al., 2007). This process is greatly impeded when the substrate water potential reaches high values. Heat influences amphibians because they are ectothermic, so external heat is necessary to enhance metabolism and movement. Finally, most amphibians are exposed to high predation pressure by reptiles, birds, and mammals due to their small body size. The response to predation, whatever its nature (the secretion of toxins or hiding strategies), has a cost that potentially impacts habitat use and movement potential (Winandy et al., 2017). Added to these constitutive factors, there are now a range of human-driven constraining factors, including the presence of pesticides, the expansion of new pathogens (e.g., *Ranavirus*, *Batrachochytrium*) and new predators (e.g., fish, crayfish, *Xenopus*), as well as increasing landscape fragmentation (Cushman, 2006; Swanson et al., 2018; Tornabene et al., 2018). Empirical studies on the movement ecology of amphibians are multiplying, taking advantage of technical progress in the miniaturization of tags and transmitters, the simplification of genetic tools, and the emergence of long-term monitoring data (Leskovar and Sinsch, 2005; Heard et al., 2012; Connette and Semlitsch, 2013; Ousterhout and Semlitsch, 2014).

In this review, after a short introduction to the theoretical framework of movement ecology, I will consider three spatial-temporal scales from that of exploitation activities corresponding to the familiar home range to that of exploration corresponding to information sampling around the home range to lastly that of long-distance relocation corresponding to dispersal. I will then consider the evolution of movement in response to selective pressures coming from anthropogenic modifications of the environment, from variation of personality frequencies to the emergency of dispersal syndromes at the population level. In a last section I will explore the use of this conceptual and empirical framework in amphibian conservation, from landscape management to reintroduction strategies.

THEORETICAL FRAMEWORK OF MOVEMENT ECOLOGY

The lifetime movement of an individual is structured around three main activity types: exploitation, exploration, and relocation. These are defined by timing, distance amplitude, pathway characteristics (e.g., linear and angular speeds, sinuosity), behavioral mechanisms (e.g., orientation/navigation, informed or exploratory, reactive or proactive), and functional consequences (e.g., food acquisition, breeding success, avoidance of inbreeding, level of competition), which act as rewards validating or invalidating previous decisions (Nathan et al., 2008). Each movement type occurs at different spatial/temporal scales around which the lifetime functions of an individual are organized. **Figure 1** proposes a template structured according to movement characteristics, from slow and sinuous to rapid and straight (Y-axis), and related spatial scale (X-axis). The spatial scales are from familiar home range to long-distance dispersal, including near relocation, exploration and migration. The movement linked to each animal's activity can be assigned a place on this template. Most paths can be distributed along an axis from slow and sinuous to fast and straight. Slow and sinuous paths are related to restricted area, usually corresponding to familiar home range, while fast and straight paths are related to long trips between different habitat patches, or dispersal. The functional significance of these characteristics will be detailed in the following sections. **Figure 2** provides the conceptual framework of movement ecology, showing the interactions between behavioral factors (phenotype dependence) and environmental factors (context dependence) that determine activity types and the subsequent characteristics of associated movements, the plasticity of the system and its evolutionary potential.

EXPLOITATION BEHAVIOR

At the local scale of a familiar home range, movements obey a reactive mode, exhibiting high sinuosity and low linear speed, and correspond to exploitation activities, such as foraging, breeding, or ensuring safety or physiological comfort. The involved mechanisms are an interaction of motivations related to homeostasis, allostasis, or reproduction, and require the processing of information obtained by thorough environment scanning and saving processes. However, when resources are spread over large distances, patch exploitation can require individuals to cross more or less hostile environments, leading to direct movements from one resource patch to another (i.e., resource supplementation). In addition, annual cycles that alternate between cold and warm or dry and wet seasons can involve long migrations between regions with contrasting climatic conditions, resulting in multi-nuclear home ranges (this is the case for many butterflies, birds, bats, large herbivores, and whales, for example) (Holt and Fryxell, 2011). In species with complex lifecycles, larval habitats differ significantly from those occupied at the juvenile and adult stage, requiring seasonal migrations between these habitats (i.e., habitat complementation, which is found in anadromous fish and pond-breeding amphibians, for example; Pope et al., 2000). When migrating, the animals follow straight and fast movements, governed by more or less complex orientation mechanisms (navigation, cognitive maps, dead reckoning) (Wiltschko et al., 1981; Wallraff, 1983; Berthold, 1991; Able, 1993).

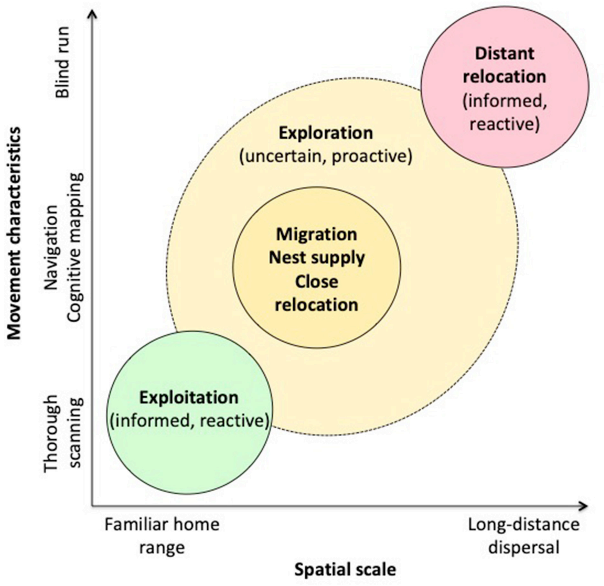

FIGURE 1 | Relationships between movement characteristics and spatial scale for different types of activity in animals, based on the movement ecology concepts used in this paper. Movement characteristics are distributed along a gradient from slow and sinuous strongly organized by stimuli associated with resources (food, sexual partners, social partners, commodities) to more rapid and less sinuous movement organized by complex orientation mechanisms (navigation, cognitive map) associated with migration between habitat patches or exploration to lastly rapid and straight movement across unknown areas associated with dispersal and definitive long-distance relocation (emigration). X-axis gives the related spatial scales from familiar home range to long-distance dispersal. In species with habitat supplementation or complementation (patchy resources, complex lifecycles, exploitation of contrasted habitats), movements between habitat patches are governed by complex orientation mechanisms, such as navigation or cognitive mapping that reduce sinuosity and increase speed (migrations, nest supply). Exploratory movements have also intermediate characteristics since they are related to environment sampling usually for resources linked to homeostasis and they can lead to near relocation. They can be promoted by specific motivation (proactive behavior with latent learning).

In pond-breeding amphibians, the pond is an essential site around which the population is organized. Ponds are often clustered in archipelagoes in which movements from one pond to another are frequent, leading to panmictic, multinuclear populations (Trenham et al., 2001; Petranka and Holbrook, 2006; Decout et al., 2012; Heard et al., 2012; Sinsch, 2014). A patch in this case consists of a set of ponds, terrestrial habitats for juveniles and adults, and wintering habitats, corresponding to the concept of habitat complementation (Pope et al., 2000). Both the aquatic and terrestrial habitats exhibit highly variable physical conditions, mainly due to climate and rainfall stochasticity. The ponds are usually in a process of ecological succession, and the degradation of the quality of these habitats selects for dispersal propensity. The terrestrial habitats are often forested, but some species prefer open environments, such as dunes, grasslands, steppes, or croplands (e.g., *Acris blanchardi*, *Epidalea calamita*, *Anaxyrus americanus*; Youngquist and Boone, 2014; Frei et al., 2016; Koumaris and Fahrig, 2016), while others show great

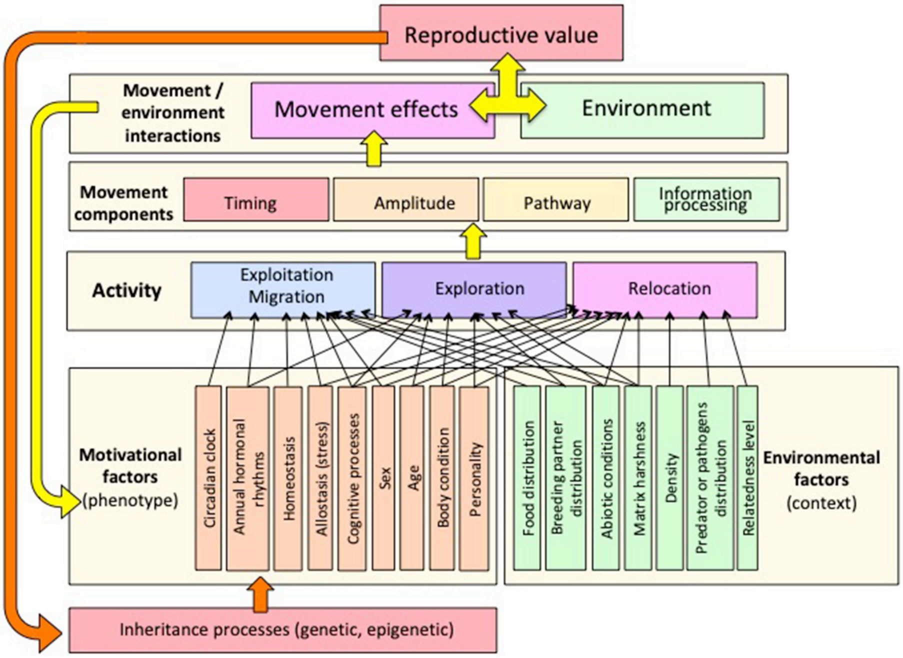

FIGURE 2 | Conceptual framework of movement ecology, organized around a multi-scale typology (exploitation, exploration, and long-distance relocation—see **Figure 1**). A complex interplay between phenotype-dependent variables and environmental variables (context-dependence) determines activity type and movement characteristics. Movement is characterized through four basic components [timing, distance amplitude (spatial scale), pathway (speed and sinuosity), and information processing (orientation mechanisms)]. The yellow arrow expresses plasticity feedback at the scale of the individual, including learning processes. The orange arrow expresses the evolutionary feedback at an intergenerational scale based on selection or on epigenetic processes.

flexibility in habitat use (e.g., *R. sphenocephala*, *R. temporaria*, *B. terrestris*; Vos et al., 2007; Graeter et al., 2008). Landscape structure and fragmentation have a direct influence on the costs of migration, and subsequently on individual fitness and the population growth rate. Migratory movements often follow a straight line (*Ambystoma maculatum*: Pittman and Semlitsch, 2013; *Lithobates sylvaticus*: Groff et al., 2017), but biased random walks or lateral movements have also been described in forests (Pittman and Semlitsch, 2013; Coster et al., 2014). The straightness of migratory movements is likely due to navigation mechanisms that rely on a combination of geomagnetic location and target-emanating olfactory cues, without any evidence of the use of a cognitive map (Rodda and Phillips, 1992; Joly and Miaud, 1993; Phillips, 1996; Sinsch, 2006; Diego-Rasilla et al., 2008; Landler and Gollmann, 2011). Tadpoles' learned knowledge of the different chemical characteristics of their natal pond could be used in later migratory movements, either positively in philopatric individuals, or negatively in dispersers (Ogurtsov and Bastakov, 2001). As a consequence of straight movements, the availability of terrestrial habitats could depend on the width of biological corridors; this hypothesis has been supported in European newts (Joly et al., 2001). A specific puzzle is that of juvenile migration, since juveniles have no experience of the route to take at the end of the metamorphosis process. Stevens et al. (2006) and Pittman and Semlitsch (2013) hypothesize that juveniles are primarily guided by aversion to water through a move-away process, until the individual reaches a favorable environment, where a random walk is adopted. However, recent studies have shown that juveniles also express an inherited migratory direction, thus improving migration success (Miaud et al., 2005; Janin, 2010).

The success of migration depends on movement costs when crossing a more or less hostile landscape matrix. This has been investigated through both simulation modeling and experimental approaches. In percolation models, the erosion of the migratory potential of an individual is related to the resistance costs of each type of land cover encountered along the migratory path, which is used to simulate friction maps and migration areas (Ray et al., 2002; Joly et al., 2003). In Janin et al. (2009), these resistance costs were estimated through a calibration-validation procedure, which identified urbanized areas and intensive farmland as the most resistant types of land cover for the common toad, *Bufo bufo*. In Decout et al. (2012), this approach was combined with graph theory to design functional patches at the regional scale. Experimental approaches can also provide empirical data that can be used in models. For example, measuring stress hormone levels demonstrated the harshness of a farmland matrix for common toads (Janin et al., 2011, 2012a), while other studies have revealed the negative influence of long travel distances (Marsh et al., 2004), desiccation risks (Mazzerole and Desrochers, 2005; Nowakowski et al., 2015), or unsuitable land cover, including cultivated crops and roads (Rittenhouse and Semlitsch, 2006;

Consentino et al., 2011; Trochet et al., 2019). A key conclusion to draw from all these studies is that movements linked to habitat complementation at the scale of a patch are essential components of population persistence and are strongly impacted by multiple human-driven landscape modifications. When it is not possible to preserve a buffer zone of favorable terrestrial habitats around a pond (Semlitsch, 1997; McDonough and Patton, 2007), the best way to restore connectivity at this scale is to maintain a permeable matrix or straight-line corridors between essential habitat components (i.e., ponds, terrestrial habitats, and wintering habitats) (Groff et al., 2017). Stable corridors should serve to enhance the inheritance of migration direction, increasing connectivity effectiveness in the long term.

EXPLORATION BEHAVIOR

The second movement type is exploration, in which individuals regularly move outside their usual exploitation range before finally returning to this familiar area, or exploit a new zone in the vicinity of the home range (near relocation). These movement pathways differ from those of exploitation activities, exhibiting reduced sinuosity and increased speed. They can be elicited in laboratory conditions; for instance, through complex maze experiments in which a well-fed rat creates a cognitive map of the maze without receiving any kind of reward when exploring it (the concept of latent learning, Tolman, 1948). This behavior is linked to a specific motivation in which the exploratory action itself, and the expected information gained along the journey, constitutes the reward. It is a proactive behavior (as opposed to a response to a stimulus) to cope with environmental uncertainty (Bartumeus et al., 2016).

Exploratory behavior has been deeply neglected in studies on amphibians. In the many recent studies on monitoring movement in the wild using telemetry, any movement is qualified as exploratory behavior, while the term itself is never used (Miaud et al., 2000; Muths, 2003; Indermaur et al., 2009; Constible et al., 2010; Heemeyer and Lannoo, 2012; Humphries and Sisson, 2012; Sinsch et al., 2012; Liang, 2013; Browne and Paszkowski, 2014; Timm et al., 2014; Frei et al., 2016; Groff et al., 2017; Pitt et al., 2017). As the main objectives of these studies were to establish the length of landward migrations, the size of the home range, and the components of terrestrial habitats, the movement characteristics themselves (e.g., the pathway or function) were most often not analyzed. Most studies have found adult individuals to be highly sedentary. However, it is known that individuals can move toward a new place in response to abiotic (e.g., humidity or flooding) or biotic (e.g., availability of prey) factors, without any previous exploration. In this case, near relocation results in the use of elementary orientation mechanisms, such as kineses or taxes, immediately followed by settlement, without any previous sampling of the environment (Fraenkel and Gunn, 1961). Movements from one pond to another within a pond archipelago could result from these elementary mechanisms.

Exploration is a complex behavior that is expected to contribute to fitness if the individual is able to memorize the information gained. Such storing of spatial information requires constructing a cognitive representation of the environment based on specific information processing. The question of the ability of lower vertebrates (such as fish and amphibians) to elaborate such cognitive maps is a subject of investigation. Some positive results have been obtained in fish using basic mazes (with 2 or 4 branches) (Rodriguez et al., 1994; Gomez-Laplaza and Gerlai, 2010), nevertheless there is no evidence that these results could be extrapolated to larger scales requiring more complex configurations to be memorized on the long term. In amphibians, exploratory behavior relies on the navigation mechanism involved and depends on the balance between the pay-offs of exploration, the costs of the neural mechanisms it requires, and the complexity (grain) of the landscape (Benhamou, 1997; Fagan et al., 2013). At present, no findings support the hypothesis of location-based navigation involving cognitive mapping and exocentric memory in pond-breeding amphibians, thus explaining the paucity of observations of exploratory behavior that returns to the starting point. However, location-based navigation seems to have evolved in species in which males carry the tadpoles from the ground to small waterbodies located in the forest canopy (Pasukonis et al., 2016), thus calling for more attention to be paid to navigation and memory processing in amphibian movement.

DISPERSAL

The third movement type occurs at a larger scale and corresponds to long-distance relocation or dispersal. This is exhibited when an individual breeds in a place that is different than that of its birth or of its previous breeding events (Ronce, 2007). In a lifetime, the frequency of this type of relocation behavior is usually low, except in nomadic individuals. It can be described as informed and reactive, since the individual decides to definitively leave the home range of its parents, which contains suitable resources, as attested by its own birth. This type of movement is typically rapid, extensive and ballistic (Bartumeus et al., 2016).

The term “philopatry” describes the absence of dispersal, i.e., when an individual breeds in the site where it was born. In all animal species, dispersal is expected to occur more frequently during the juvenile stage (natal dispersal) than during the adult stage (breeding dispersal) due to the absence of breeding costs and because juveniles have an immediate appraisal of the quality of their birthplace through their own body condition, which reflects current food availability and the level of competition. In iteroparous species, natal dispersal also allows the avoidance of kin competition, especially with parents (Harts et al., 2015). However, the costs of natal dispersal can outweigh these advantages if the small body size of juvenile individuals restricts their movement capacity, affords low physiological resilience, or exposes them to high predation risks. Moreover, as juveniles do not yet have knowledge of the environment to be crossed, dispersal behavior may involve simply moving away from the birthplace rather than following a pathway with a particular target. In contrast, adult (breeding) dispersal could benefit from the higher movement capacity of a larger body and a better knowledge of the location. Furthermore, in iteroparous species, it is hypothesized that adults would use dispersal as a strategy at an age when their own reproductive value becomes inferior to the sum of the reproductive value of their offspring (Morris, 1982). All these factors point to the coexistence of both natal and breeding dispersal in long-lived iteroparous species.

One of the results of dispersal is gene flows between populations (Baguette et al., 2013). By spreading advantageous alleles, gene flows contribute to a population's genetic diversity and adaptation. They also reduce the risks of inbreeding and genetic drift (Lowe and Allendorf, 2010). Additionally, dispersal has demographic consequences: for example, through rescue effects that could preserve a population from local extinction (Brown and Kodric-Brown, 1977). Unfortunately, obtaining empirical evidence of emigration and immigration rates to model the demographic functioning of spatially structured populations remains a difficult task (Lowe and Allendorf, 2010; Benton and Bowler, 2012). However, the increasing miniaturization of tags and radio transmitters, together with advances in modeling techniques, are improving the reliability of quantitative estimates of demographic flows (Cayuela et al., 2018a).

The dispersal process consists of three phases: departure, transfer, and settlement. Each involves different behavioral mechanisms (Ims and Yoccoz, 1997). Today we have substantial insights into departure decisions, which result from a number of interacting causes, from phenotype state (e.g., age, sex, physical condition, hormonal state, personality) to current environment quality (e.g., genetic relatedness with other residents, density, presence of predators or parasites, food shortage, degradation of the physical milieu) (see Matthysen, 2012 for a review) (**Figure 2**).

The transfer phase is the least understood, as it has not been described with sufficient accuracy to allow experimental investigation. Tracking dispersing individuals is a difficult task, firstly because it is impossible to forecast which individual will disperse and when the process will start. Secondly, because technical devices are lacking that allow small juvenile individuals to be tracked, which restricts monitoring to the identification at the arrival site by means of the recapture of marked individuals. While this gives insights into traveled distance and overall direction through the calculation of dispersal kernels (Nathan and Muller-Landau, 2000), it provides only indirect information about the route that has been followed and the behavioral mechanisms involved. If dispersal is considered a reactive process, the objective of which is to get away from the departure site, we would expect dispersal movement to follow a straight path that avoids any doubling back through random wandering (Selonen and Hanski, 2006). However, a "biased random walk" dispersal pathway is also a working hypothesis (Barton et al., 2009). Here an individual's indirect knowledge of the direction of potential target habitats is an important question. Airborne olfactory cues could inform an individual about a distant habitat patch and contribute to orienting dispersal movement. The direction of a distant patch could also be indicated by the arrival point of immigrating individuals, who may also provide information about the potential quality of the patch they departed from through their apparent body condition (Cote and Clobert, 2007). The transfer phase in dispersal would be expected to be highly costly, as an individual must cross unfamiliar and hostile environments where it may be exposed to predation risks and harsh physical conditions. However, empirical estimates of mortality during the transfer phase remain scanty.

The settlement phase, i.e., settling in the arrival site, assumes an evaluation of habitat quality by the disperser. Numerous studies have shown that the presence of conspecifics and/or their reproductive success provide reliable cues about habitat quality (Boulinier and Danchin, 1997; Stamps, 1998; Doligez et al., 2002). However, other characteristics of a niche (physical condition, food availability, absence of predators) can also attract disperser individuals, thus allowing the colonization of empty habitat patches (Le Galliard et al., 2005). Two behavioral phenotypes (pioneer and joiner) can coexist as a stable evolutionary strategy in the same population (Clobert et al., 2009). The strength of habitat selection for settlement is negatively influenced by the duration of the transfer phase, suggesting that increasing habitat fragmentation could lead to a decrease in the fitness of immigrants (Stamps et al., 2005).

Considering the three phases simultaneously is crucial, as the selective forces acting on each may diverge. Furthermore, the decision to emigrate is also likely to depend on tradeoffs between the necessary investment in mobility (e.g., energy reserves, limb length, muscle strength) and forthcoming fecundity. The form of this tradeoff is expected to interact with mortality risks during the transfer phase to determine a more or less safe movement pathway. When mobility costs are low and the distance to be covered is short, the emigration rate is expected to be high and movements tortuous (e.g., exploratory prospections), leading to density variation at the metapopulation level (Delgado et al., 2011; Travis et al., 2012). In contrast, when mortality risks during the transfer phase increase, movement is expected to follow a straighter, more direct path.

BEHAVIORAL ECOLOGY OF DISPERSAL IN AMPHIBIANS

Philopatry and site fidelity have long been commonly accepted in amphibian biology (Twitty, 1959; Heusser, 1968). Site fidelity appears to be a statistical property of adult migration since many individuals return to the breeding site used the previous year, and this choice is deliberate (Joly and Miaud, 1989; Sinsch and Kirst, 2016). However, a non-negligible proportion of adult individuals do not exhibit site fidelity in homing experiments. Moreover, a detectable level of transience in mark–recapture (MR) studies confirms the reality of nomadism in natural populations, showing that breeding dispersal can be frequent (Perret et al., 2003; Muths et al., 2018). Dispersal rates vary between amphibian species and populations, from near zero to 50% (Reading et al., 1991; Hamer et al., 2008; Garwood, 2009; see Cayuela et al., 2018 submitted for review). The respective proportions of natal and breeding dispersal also vary greatly according to species and populations: natal dispersal dominates in Ranid frogs (Berven and Grudzien, 1990; Garwood, 2009), while the data is not consistent in other taxa. The distribution of dispersal distance is strongly skewed, with many species moving very long distances—up to 16 km in some anurans (Smith and Green, 2005) (**Table 1**). Indeed, long-distance dispersal is probably more frequent than currently observed, since we lack the methodological means to detect it. For instance, while the longest movement that has been observed in the natterjack toad is 4.4 km, the estimated dispersal distance based on a leptokurtic dispersal function is 12.2 km: a distance that is in line with estimated gene flows in this species (Oromi et al., 2012; Sinsch et al., 2012).

In contrast to most dispersal studies for other animal groups, the departure phase of amphibian movement has not received detailed attention. Findings from the studies that have been done show that the emigration of adult individuals is negatively related to density; individuals leave less-populated sites in favor of the most-populated ones (Gamble et al., 2007; Cayuela et al., 2019). The influence of sex on dispersal is not consistent, varying between species and populations (Cayuela et al. submitted). Little is known about the influence of kin competition and inbreeding on emigration propensity, aside from the recognized ability of kin recognition (Blaustein and Waldman, 1992; Masters and Forester, 1995). Dispersing individuals are often larger than residents (Denoël et al., 2018) and can exhibit a morphology adapted to movement, with longer forelimbs (in a salamander: Lowe and McPeek, 2012) or longer hindlimbs (in a toad: Phillips et al., 2006; Hudson et al., 2016). Their survival is higher than that of resident individuals (Lowe, 2009, 2010; Denoël et al., 2018). It is likely that dispersers also differ from residents in personality traits. In Australia's cane toad (*Rhinella marina*) invasion, dispersers at the front lines are more aggressive and less social than residents, and exhibit more exploratory and risk-prone behavior (Fogarty et al., 2011; Gruber et al., 2017). Despite a relative paucity of data, these results converge with the body of knowledge that has been established for other groups (Clobert et al., 2012).

The transfer phase has received more attention, probably because moving across the landscape matrix is a crucial topic in conservation biology. However, dispersal behavior, especially natal dispersal, has never been observed and monitored, so we have very little data about the followed pathways and the orientation processes involved. Most knowledge in this area relies on the analysis of gene flows or recapture data that indirectly provides information about movement pathways (Rowe et al., 2000). For example, Mark-Recapture data suggests that in the boreal toad, breeding dispersers follow straight pathways (Muths et al., 2018). As predicted by the theory of population genetics, the isolation of amphibian populations decreases genetic diversity as a result of gene flow impediment, genetic drift, and inbreeding when the effective population size is small (Andersen et al., 2004; Broquet et al., 2010; Rhoads et al., 2017). Several studies have established a negative relationship between gene flow and the resistance of the landscape matrix, which is related to agricultural intensification and the expansion of urbanization (Stevens et al., 2006; Van Buskirk, 2012; Crawford et al., 2016; Sawatzky et al., 2019). The barrier role of highways varies depending on the studied species, the age of the infrastructure, and, probably, the density of tunnels under the roadway. While highways or high-speed railways were not found to affect the genetic structure of Alpine newt populations (Prunier et al., 2014; Luqman et al., 2018), they did impact gene flows in the cricket frog and the Japanese brown frog (Youngquist et al., 2017; Kobayashi et al., 2018). Large rivers or concrete-banked waterways can act as barriers to gene flows in some species (*Ambystoma maculatum, Rana japonica*), but not in others (*Hyla arborea, Ichtyosaura alpestris, Lithobates sylvaticus*) (Angelone et al., 2011; Richardson, 2012; Kobayashi et al., 2018; Luqman et al., 2018). While valuable, this indirect evidence of large-scale movements provides imperfect information about the behavioral mechanisms involved in the transfer phase, which is of crucial importance in restoring connectivity. This is especially vital for the design of underpasses that allow the safe crossing of transportation infrastructures (Jarvis et al., 2019).

During the settlement phase, studies have shown that the presence of conspecifics can exert an attractive influence (Aragon et al., 2000). In the few species for which this question has been addressed, immigrants were attracted to sites occupied by a large population (*Ambystoma opacum*: Gamble et al., 2007; *Litorea aurea*: Bower et al., 2013; *Triturus cristatus*: Cayuela et al., 2019). Attraction by conspecifics could be related to Allee effects, such as sharing public information about food location (Martin and Caillère, 1982; Aragon, 2009) or finding sexual partners. Yet if kin are present, recognized by olfactory cues, this may act as a repellent (Ogurtsov, 2004).

The influence of heterospecifics on settlement is more debatable (Buxton and Sperry, 2017). Great crested newts select ponds where the density of both Alpine and palmate newts is high (Cayuela et al., 2018b). Newts can also be attracted by auditory cues from toads and frogs (Diego-Rasilla and Luengo, 2004; Pupin et al., 2007; Madden and Jehle, 2017). Anurans often use chorus calls as an attractor (Gerhardt and Klump, 1988; Swanson et al., 2007). Dispersers are also attracted by non-social cues, since newly created ponds devoid of any amphibians are rapidly colonized (Joly and Grolet, 1996; Baker and Halliday, 1999; Rannap et al., 2009; Weinbach et al., 2018). To add further complexity, these non-social cues can be specific to the local population (Sanuy and Joly, 2009). However, these results concern adult individuals that are typically involved in migration or breeding dispersal. No studies have focused on natal dispersal, probably because marking small individuals remains difficult, and low recapture probability entails a lot of fieldwork effort for uncertain results.

EVOLUTION OF DISPERSAL AND DISPERSAL SYNDROMES

Each of the three phases of the dispersal process is under strong selective pressure due to the variability of local environmental and global environmental changes. As dispersal is determined by a combination of phenotype-dependent and context-dependent causes, the selection targets are multiple, and one might expect that changes in one element of the phenotype involve changes

TABLE 1 | Some examples of the distance differences between migration (intrapopulation round-trip movements to and away from breeding sites) and dispersal (inter-population one-way movements from a birthplace to a breeding site) in selected amphibians.

Species	Terrestrial habitat	Migration distance or width of buffer zone	Dispersal distance	References
Ambystoma maculatum	Forest	Males: 100 m Females: 200 m	2,000–4,000 m	Zamudio and Wieczorek, 2007; Purrenhage et al., 2009
Ambystoma opacum	Forest	200–400 m	1,300 m	Gamble et al., 2007
Salamandra salamandra	Forest	50–300 m	1,000–4,000 m	Schulte et al., 2007; Bani et al., 2015
Bufo bufo	Forest	550 m	1,000–3,600 m	Moore, 1954; Daversa et al., 2012
Anaxyrus hemiophrys	Forest	50–100 m	10,000 m	Constible et al., 2010; Muths et al., 2018
Epidalaea calamita	Open environment	700 m	3,000–12,000 m	Miaud et al., 2000; Sinsch et al., 2012; Frei et al., 2016
Rana luteiventris	Mixed	Males: 200 m Females: 1,030 m	5,750 m	Pilliod et al., 2002; Funk et al., 2005

in the others. However, the overall evolutionary challenge is to predict the propensity of leaving the natal population. A first causation lies in phenotypic plasticity, which would confer to each individual a similar capacity to respond to habitat cues (Stearns and Koella, 1986; Via, 1993; Massot and Clobert, 2000; Tufto, 2000). In fact, several studies have shown that individuals differ in their motivation to leave a site, and that dispersal behavior could be predicted by particular phenotype traits (Bowler and Benton, 2005; Börger et al., 2008; Sih and Bell, 2008; Cote et al., 2010a). The concept of dispersal syndrome has been developed to establish a framework linking several internal-state components, such as physiology, behavior, morphology, and life history—in order to identify different phenotypes with respect to dispersal propensity (Careau et al., 2008; Clobert et al., 2009; Careau, 2012). A behavioral syndrome is a suite of correlated behaviors across situations (Boissy, 1995). It becomes an evolutionary solution to environmental variability when plasticity reaches its functional limits (Sih et al., 2004). Individual personality can be characterized according to five behavioral gradients: boldness, exploration, activity, sociability and aggression (Réale et al., 2010). From these, two fundamental coping styles have been detected in fish and birds: a proactive behavioral style characterized by aggressiveness, boldness, exploratory propensity, dispersal propensity, and mating success, and a reactive style characterized by shyness, neophobia, and an ability to adjust to the current environment. These coping styles are heritable and could influence population differentiation (Drent et al., 2003; Dochtermann et al., 2014). Behavioral styles have profound ecological implications on a range of outcomes—from population performance to biotic interactions—that could determine colonization ability and adaptation to human-modified environments (Sih et al., 2012). Different personality types can coexist in a population, with individuals expressing consistent differences in behavior, or suites of behaviors (functional types), and plasticity (Stamps, 2004, 2016; Dingemanse and Wolf, 2010). From an evolutionary perspective, different personalities can coexist when the fitness payoffs of each type depend on the frequency of the competing strategies—a result of frequency-dependent selection (Dall et al., 2004). This explanation is fairly well supported by empirical observation, suggesting a tradeoff along a boldness/shyness gradient (Smith and Blumstein, 2008; Stamps and Groothuis, 2010). The coexistence of several personalities within a population could thus contribute to the species' ability to persist. Further empirical investigation on the evolution of the personality spectrum under the selective pressures of habitat fragmentation would be valuable. Lastly, within the field of movement ecology, recent advances in the modeling of dispersal evolution need to be incorporated at the population level.

The dispersal syndrome concept assumes genetic correlations between traits through linkage disequilibrium or the pleiotropic effects of genes linked with dispersal (Saastamoinen et al., 2018). At the scale of personality, the traits related to dispersal propensity are aggressiveness, low sociability, low neophobia, readiness to explore, risk proneness, and boldness (Ronce and Clobert, 2012). The coexistence of two contrasting dispersal personalities within a population could be enhanced by habitat fragmentation, which concurrently increases both dispersal costs and inbreeding risks (Cote et al., 2010b, 2017; Sih et al., 2012). Looking for other correlations with life-history traits, large-scale meta-analyses have found a positive association of dispersal with early maturation and longevity, as well as a trend with fecundity (Stevens et al., 2014; Comte and Olden, 2018). Whereas, dispersal distance is positively related to body size, dispersal willingness is higher in generalist than in specialist species. High investment in reproduction (e.g., parental care, large eggs) is negatively related to dispersal propensity, identifying a conflict in resource allocation between dispersal and reproduction.

The coexistence of contrasting personalities could also act as a safeguard against habitat fragmentation, affording a population greater capacity to mitigate an increase in dispersal costs. Bold individuals can act as keystones, ensuring long-distance dispersal, colonizing new habitat patches, or gathering information about food distribution (Sih et al., 2012). A directional selection toward one coping style could induce a behavioral syndrome at the population level; a step toward local adaptation and the emergence of an ecotype, which may become an Evolutionarily Significant Unit for conservation (ESU). In contrast, directional selection could weaken the resilience of a population by leading to the impoverishment of personality diversity, restricting the population's ability to cope with temporal environmental variation and/or local habitat conditions (Sih et al., 2012; Cornelius et al., 2017).

As in other animal groups, amphibians exhibit variation in individual personality types (see Kelleher et al., 2018 for a review of this topic). Tadpoles vary in boldness and activity, characteristics that can be correlated (Koprivnikar et al., 2012; Wilson and Krause, 2012; Brodin et al., 2013; Urszan et al., 2015). Juveniles and adults also show differences in personality traits. Juvenile *Epidalea calamita* toads exhibit correlations between activity, movement speed, and exploratory behavior related to their ability to use corridors (Maes et al., 2012). In the invasive cane toad in Australia, individuals occur along a shyness/boldness gradient, with the more social being on the shy side (Gonzalez-Bernal et al., 2014). *Xenopus tropicalis* captured in the wild show strong differences in exploration intensity, though without any clear correlation to activity rate or morphology (Videlier et al., 2015).

In amphibians, dispersal propensity and dispersal distance are thought to quickly evolve under the selective pressures of habitat availability and/or habitat fragmentation (Maes et al., 2012; Wilson and Krause, 2012; Kelleher et al., 2017). In Australia, the cane toad invasion has provided researchers with a mine of knowledge regarding the rapid evolution of the phenotype of a disperser at an invasion front. Compared to individuals at the back, toads in the front lines have longer hindlimbs, which allows them to move farther and faster. Moreover, they move for twice as long and follow more direct pathways, proving to be more exploratory and risk-prone, and less social (Phillips et al., 2006; Fogarty et al., 2011; Lindström et al., 2013; Hudson et al., 2016; Gruber et al., 2017). This well-documented invasion process shows how efficiently dispersal traits can evolve: they are inheritable and can accelerate the rate of dispersal by a factor of five (Phillips et al., 2010).

Habitat fragmentation and disturbance also exert selective pressures that can promote or inhibit dispersal depending on the cost–benefit tradeoff. Mean activity rate, exploratory propensity, and boldness can vary between populations. In the common frog (*Rana temporaria*), individuals from island populations (newly formed islands) exhibit more boldness and higher activity than individuals in mainland populations, probably as a result of founder effects when the islands have been colonized (Brodin et al., 2013). In contrast, salamanders that inhabit stands of old forest are more active than those that inhabit young stands (Consentino and Droney, 2016). When reared in the same garden, post-metamorphic toadlets from highly fragmented landscapes exhibit more risk-prone and exploratory behavior than toadlets from less-fragmented landscapes (Janin et al., 2012b). A comparison of populations of *Bombina variegata* breeding in forests with those breeding along riverbanks detected a dispersal syndrome in the forest-dwellers characterized by a high dispersal rate, high fecundity, and a short lifespan, probably linked to the unpredictability caused by logging activities (Cayuela et al., 2016). Similarly, fire salamanders have been found to respond to pond instability by exhibiting higher activity rates and dispersing farther distances than those breeding in more stable small streams (Hendrix et al., 2017).

Studies focusing on personality have been clearly overlooked in amphibians as compared to other vertebrate groups (Kelleher et al., 2018) and very little attention has been paid to the sociality and aggression gradient. The personality spectrum could have implications in the spread of pathogenic agents if behavior influences the number of contacts between individuals. However, results regarding this are contradictory since while social individuals are more susceptible to transmitting a disease to other individuals, the boldest individuals are more susceptible to spreading it over long distances (Koprivnikar et al., 2012; Araujo et al., 2016). Given contrasting observations, the role of personality gradients in the spatial use of patchy habitats is an important avenue to explore: the consequences of this polymorphism on the functioning of amphibian populations are far from understood. Further research into these topics would be valuable in understanding the problems linked to habitat fragmentation.

MOVEMENT ECOLOGY AND AMPHIBIAN CONSERVATION

For a given population, all the resources needed for breeding success are found in the habitat, which has a geographical structure. Land cover types that do not provide sufficient resources for each niche component are non-habitat areas, which make up the landscape matrix. The influence of this landscape matrix on functional connectivity has received much attention in ecology (Fahrig and Merriam, 1985; Moilanen and Hanski, 1998). A given matrix is composed of land cover with contrasting impacts on animal movement, from mildly resistant to impassable. Together with the risks taken when dispersing, land cover modulates the costs of movement at each step of the lifetime movement path. At the scale of a habitat patch, both resource supplementation and complementation processes drive movements across the landscape matrix (Pope et al., 2000). At a larger scale, movements are prospective and exploratory. If the environment is homogenous or fine-grained, these movements can lead to short-distance dispersal, resulting in a patchy population with high inertia (Harrison, 1991; Delgado et al., 2011). In contrast, if the environment is hostile or coarse-grained, movements from one patch to another result in long-distance dispersal with high mortality risks, which selects for straight pathways and high movement capacity (Travis et al., 2012; Cheptou et al., 2017). Fragmentation leads to reduced local population sizes, thus altering fitness by impeding Allee effects and increasing both genetic drift and inbreeding risks. When fragmentation dynamics are slow, these processes may be compensated by an increase in dispersal capacity (Thomas, 2000; Hanski et al., 2004). However, human-driven fragmentation is often a rapid process, characterized by the introduction of harsh landscape matrices (e.g., intensive agriculture or urbanization) that dramatically impede dispersal movements. As a consequence, the most dispersive individuals are counter-selected, driving the population toward the dominance of sedentary individuals (Massol et al., 2011). Genetic drift and inbreeding can then transitorily induce local differentiation that may promote local adaptation. However, the resulting decrease in evolutionary potential weakens the population's capacity to respond to environmental changes, thus increasing extinction risks. It is thus crucial to maintain a degree of connectivity to promote polymorphism through balanced fitness gains between disperser and resident phenotypes (Mathias et al., 2001). Empirical evaluations of this conceptual framework are nevertheless inconsistent and scanty, calling for further investigation. In the leaf beetle *Phaedon cochleariae,* for example, inbreeding is related to a bold personality, suggesting that risk-proneness may be promoted by a decline in reproductive value (Müller and Juskauskas, 2018).

Evaluating the impact of the matrix on movements is challenging due to technical limitations in direct movement tracking. Most knowledge comes from analyses of occurrence data and gene flows (Zeller et al., 2012) with a strong bias toward dispersal processes (Cosgrove et al., 2018). Different modeling options respond to different questions. Least-cost path analysis, graph theory and circuit analysis (e.g., Circuitscape) allow connectivity to be modeled and are usually carried out with gene flow data (Stevens et al., 2006; McRae and Beier, 2007; Foltête et al., 2012). However, these techniques are not based on behaviorally realistic rules, whereas individual-based simulation models, especially stochastic movement simulators, allow the integration of decision-making rules based on the perceptual range of the species. This enables them to draw powerful predictions (Knowlton and Graham, 2010; Palmer et al., 2011; Coulon et al., 2015). However, for a great majority of species, there is insufficient knowledge about movement mechanisms to effectively inform these models with realistic rules. It would also be valuable to integrate the increasing knowledge concerning the personality spectrum within a population in modeling procedures to improve prediction reliability (Fogarty et al., 2011; Chapple et al., 2012; Hirsch et al., 2017).

Understanding amphibian movement ecology is critical in developing effective conservation measures. These movements can be organized according to a spatial-temporal framework (**Figure 3**). At the scale of a patch, knowledge about habitat complementation processes used by individuals to seek resources would help to ensure functional connectivity, from the design of corridors to the mitigation of the barrier role of roads and railways with underpass technology (Bain et al., 2017). Safeguarding connectivity requires a better understanding of the orientation mechanisms involved in migration, and the potential inheritance of migration direction in juveniles. The putative negative influence of pesticides on orientation mechanisms must also be investigated (Janin, 2010).

At the scale of a population, it is important to find the optimal configuration of pond archipelagoes that ensures the greatest viability and resilience through rescue effects and spreading the risk of extinction, especially in the context of climate change (Petranka and Holbrook, 2006; Weinbach et al., 2018). The great disparity of demographic responses to climatic variation calls for detailed investigations into the relationships between pace of life, movement ecology, and local adaptation to improve the predictive power of population models (Cayuela et al., 2017; Muths et al., 2017). Allee effects can explain the positive dependence on density

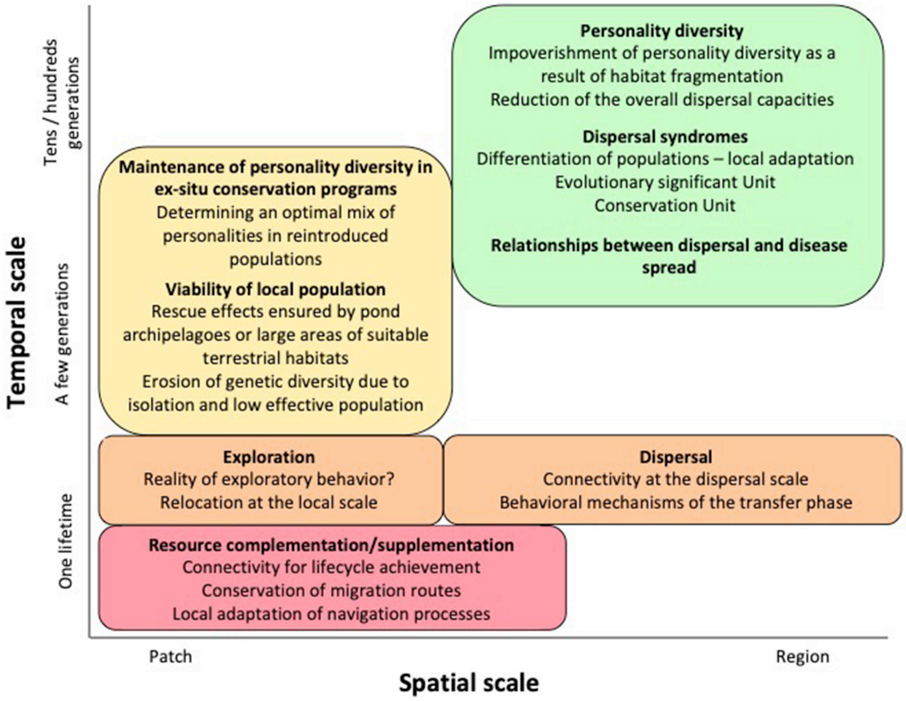

FIGURE 3 | Aspects of amphibian movement ecology at different spatial and temporal scales that need to be better understood for conservation planning.

of adult amphibians for fitness components, such as sexual selection or information sharing (Cayuela et al., 2019). In metapopulations, these effects can drive a population toward extinction ("evolutionary suicide") unless these dynamics are counterbalanced by juvenile dispersal (Parvinen, 2007; Fowler, 2009). The knowledge juveniles have of a pond's current breeding conditions could promote such a compensatory process, but this cost–benefit tradeoff in breeding aggregations remains to be tested.

At the scale of a landscape, we do not know if the rules governing longer-distance dispersal movement could be extrapolated from knowledge regarding habitat complementation since the causes and functions of these two processes differ significantly. The fact that individuals, populations and species respond inconsistently to landscape components highlights the need for coupling an analysis of movement determinants with life histories, habitat preferences and the personality spectrum at different scales (Koumaris and Fahrig, 2016).

Another important issue for conservation is the assessment of the role of dispersal syndromes in local adaptation, including in response to human-modified landscapes, to test the hypothesis of Evolutionarily Significant Units and how to conserve these (Neal et al., 2018; Saastamoinen et al., 2018). While the conceptual framework of personality has opened the way to experimental approaches, approaches are also needed at the scale of dispersal to examine the determinants of emigration. This task is difficult since dispersal is likely to cover a bimodal distribution of traveled distances (exploration-based vs. long-distance dispersal), themselves expressing different behavioral processes.

In captive-breeding and reintroduction programs, a functional assessment of the personality spectrum should be a priority. By constraining captive individuals to live within restricted spaces, breeding programs may select for the most reactive individuals, leading to a deficit of dispersers in the reintroduced population (McDougall et al., 2006; Kelleher et al., 2018). This possibility makes it crucial to develop rearing methods that maintain an optimal mix of personalities in captive-breeding programs.

Greater knowledge of how amphibians move across landscapes is of critical interest for conservation biology. This information would allow demographic flows at the landscape scale to be modeled using graph theory (Foltête et al., 2014; Clauzel et al., 2015; Peterman et al., 2018) or individual-based modeling (Coulon et al., 2015). Movement ecology, by considering animal movement at different spatial and temporal scales, is an important step in improving this knowledge. The theoretical framework it has developed for assessing the distribution of biodiversity in changing landscapes is a sound basis for planning future studies and conservation actions.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

ACKNOWLEDGMENTS

This study received funding from the Institut Universitaire de France. I thank two referees for their helpful comments that have allowed to significantly improving the manuscript.

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

frontiers
in Ecology and Evolution

MINI REVIEW
published: 25 June 2019
doi: 10.3389/fevo.2019.00234

Integrating Behavior and Physiology Into Strategies for Amphibian Conservation

Susan C. Walls[1]* and *Caitlin R. Gabor*[2]

[1] U.S. Geological Survey, Wetland and Aquatic Research Center, Gainesville, FL, United States, [2] Department of Biology, Texas State University, San Marcos, TX, United States

OPEN ACCESS

Edited by:
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***Correspondence:**
Susan C. Walls
swalls@usgs.gov

Specialty section:
This article was submitted to Behavioral and Evolutionary Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: *24 December 2018*
Accepted: *05 June 2019*
Published: *25 June 2019*

Citation:
Walls SC and Gabor CR (2019) Integrating Behavior and Physiology Into Strategies for Amphibian Conservation. Front. Ecol. Evol. 7:234. doi: 10.3389/fevo.2019.00234

The amphibian decline crisis has been challenging to address because of the complexity of factors—and their multitude of interactive effects—that drive this global issue. Dissecting such complexity could benefit from strategies that integrate multiple disciplines and address the mechanistic underpinnings of population declines and extirpations. We examine how the disciplines of behavior and physiology could be used to develop conservation strategies for amphibians and identify eight research gaps that provide future directions for the emerging fields of conservation behavior and conservation physiology. We present two case studies on imperiled salamanders that show how studies of behavior and physiology may support amphibian conservation efforts. We found several applications of stress physiology to amphibian conservation, but long-term studies are needed to understand how stress ultimately affects individual fitness and population resilience. Additionally, multiple measures of physiological health are needed to provide a more holistic assessment of an individual's overall condition. Previous behavioral and physiological studies have been instrumental for understanding how amphibians respond to habitat modification, pathogens and parasites, contaminants, and invasive species. Some behavior-based approaches to mitigating invasive species issues have been successful in short-term studies with individual species. However, widespread application of these tactics has not yet been integrated into conservation and management strategies for ecologically similar species. A diversity of modeling approaches has enhanced understanding of how climate variability may impact amphibian populations, but model predictions need empirical tests to provide conservation managers with workable approaches to multiple perturbations associated with global environmental change. We illustrate that behavior and physiology can have broad utility for amphibian conservation, but evidence is scant that such studies have actually been used to inform strategies for amphibian conservation and management.

Keywords: amphibians, climate change, contaminants, disease, *ex situ* conservation, habitat change, invasive species, urbanization

INTRODUCTION

A substantial challenge in addressing the biodiversity crisis is the inherent complexity of environmental problems that drive population declines (Blaustein et al., 2011). Because of this complexity, there is a need to incorporate interdisciplinary approaches in conservation planning. This need has given rise to such fields as conservation genetics (Hedrick, 2001; Shaffer et al., 2015), conservation physiology (Wikelski and Cooke, 2006; Cooke et al., 2013, 2014), conservation behavior (Sutherland, 1998; Blumstein and Fernández-Juricic, 2004; Buchholz, 2007), and integrative behavioral ecotoxicology (Clotfelter et al., 2004; Peterson et al., 2017). Interdisciplinary approaches can directly address the mechanistic underpinnings of population processes (e.g., extinction risk and metapopulation connectivity), thus providing insight into the causes of biodiversity loss. The merger of physiology, behavior, and genetics with conservation, for example, has the potential to generate "cause-and-effect" relationships that can reveal how various stressors may contribute to population declines (Cooke et al., 2013; Birnie-Gauvin et al., 2017). Despite the promise that these emerging disciplines hold, a disconnect continues between basic research and applied wildlife conservation and management, particularly with behavior (Merrick and Koprowski, 2017; but see Fortin et al., 2005 for an illustrative counterexample). Moreover, the knowledge and tools that interdisciplinary approaches produce need to not only be used to document problems, but to also develop and test management strategies to solve complex conservation problems.

Integrative approaches could be particularly relevant in the case of amphibians which, among the vertebrate classes, have experienced the highest rates of diversity loss with an estimated 43% of all known species declining globally (Wake and Vredenberg, 2008). Amphibian population declines have been well-documented and are attributed to various stressors—and their synergistic interactions—such as habitat modification, disease, contaminants, competition and predation from both non-indigenous and native species, climate change, and overexploitation (Egea-Serrano et al., 2012; Grant et al., 2016; Blackburn et al., 2019). Because of the compounding effects of interacting stressors, the causes for amphibian declines are complex (Hayes et al., 2010; Blaustein et al., 2011). Studies of amphibian population declines traditionally have not addressed this complexity but, rather, have often focused on single factors (e.g., disease) affecting particular amphibian species (Blaustein et al., 2011). Because of their interactive nature, multidisciplinary approaches are well-suited to disentangle this complexity, uncover the behavioral, physiological, and other mechanisms that drive population declines and extirpations, and reveal pathways forward to developing conservation solutions.

Stressors are biotic or abiotic factors that can challenge individual homeostasis, resulting in an acute glucocorticoid (GC) stress response. During such reactions, energy stores are mobilized and rapid behavioral and physiological changes typically occur (Greenberg and Wingfield, 1987). However, severe or prolonged exposure to stressors can negatively influence reproduction, immune function, and growth, and can impair subsequent responses to additional stressors because the hypothalamic–pituitary–adrenal/interrenal axis (HPA/HPI) becomes non-responsive (Sapolsky et al., 2000; McEwen and Wingfield, 2003). As the magnitude of a perceived stressor increases, GC levels may be upregulated or downregulated (Gendron et al., 1997; Gabor et al., 2018a), indicating that GCs may correlate with physiological health, population declines, and loss of genetic variation (Dantzer et al., 2014).

In addition to physiological indicators of environmental stress, behavioral "proxies" of population viability can also provide early indications of population decline, allowing proactive management and conservation *before* extinction risk escalates (Gerber, 2006; Janin et al., 2011; Gabor et al., 2018a; Madliger et al., 2018). Thus, given the unprecedented population declines and extirpations that amphibians are experiencing, we believe that the integration of behavior and physiology with conservation is both timely and likely urgent for this taxonomic group. We address the question: how can these disciplines be integrated and used to develop conservation strategies for amphibians? We review key studies in behavior and physiology to examine their potential to inform amphibian conservation. We then present two case studies with imperiled salamanders—one of a long-term program of behavioral research and the other from Gabor's research on physiology and behavior—that show how such studies may support conservation efforts. Finally, we identify research gaps and potential directions that could enable future behavioral and physiological studies to help develop conservation approaches for declining amphibian populations.

EXAMPLES OF BEHAVIORAL AND PHYSIOLOGICAL STUDIES THAT SUPPORT CONSERVATION EFFORTS

We compiled studies of conservation behavior/physiology with amphibians, using Google Scholar and our knowledge of the literature (**Table 1**). We performed a search for publications on "conservation behavior and amphibians" and "conservation physiology and amphibians" in Google Scholar, primarily from 2000 to present (unless otherwise indicated). We additionally searched the Thomson Reuters Web of Science^{TM} database across all years for literature on contaminants, behavior, and physiology, using the search terms "amphibian behavior and contaminants" and "amphibian physiology and contaminants." We compiled selected studies that are relevant to five known stressors that impact amphibian populations [climate change; pathogens and parasites; invasive species; habitat modification (including urbanization); and contaminants: (Hayes et al., 2010)] and one tactic that is widely used as part of recovery strategies for threatened species (*ex situ* conservation/captive breeding). Although we include captive breeding here, we caution that captive breeding is a measure of last resort for critically endangered species that should not usurp protection of animals in the wild. This compilation, though not exhaustive, provides insight into ways behavioral and physiological studies can reveal amphibian responses to various factors, thus helping

TABLE 1 | Selected examples of studies in behavior and physiology that could help inform amphibian conservation.

Factor	Approach used/Finding	Behavior, Physiology, or Both?	Taxon	Source
CLIMATE CHANGE				
	Used knowledge of microhabitat preferences to enhance climate refugia	Both	Cane Toad (*Rhinella marina*)	Seebacher and Alford, 2002
	Used portable irrigation sprayers to artificially wet and manipulate water potentials at breeding sites because: (1) high substrate water potentials induce male calling and female oviposition and (2) could ameliorate dehydration, thus elevating resting metabolic rates	Behavior	Bibron's Toadlet (*Pseudophryne bibronii*)	Mitchell, 2001
	Studied foraging, food conversion efficiency, and stress release rates in response to increasing temperatures	Both	Eastern Red-backed Salamander *(Plethodon cinereus)*	Novarro et al., 2018
	Studied change in terrestrial moisture on stress, foraging, water uptake, and growth	Both	Spotted Salamander (*Ambystoma maculatum*)	Charbonnier et al., 2018
	Examined the effects of temperature and food availability on the timing of hibernation behavior	Behavior	Black-spotted Frog (*Pelophylax nigromaculatus*)	Gao et al., 2015
	Studied change in response to water reduction on resource allocation and stress	Physiology	Wood Frog (*Lithobates sylvaticus*)	Crespi and Warne, 2013
	Used thermal physiology, habitat associations, and biophysical models to estimate maximum body temperatures and, thus, composition of species assemblages, using simulated landcover change and climate warming	Physiology	Numerous species	Nowakowski et al., 2016
	Integrated physiological acclimatization and behavioral avoidance into simulation-based species distribution models to predict extinction risk due to climate change	Both	Southern Gray-cheeked Salamander (*Plethodon metcalfi*)	Riddell et al., 2018
PATHOGENS AND PARASITES				
	Documented how infections from pathogens and parasites may be mediated by altered thermoregulatory behavior ("behavioral fever") in amphibian hosts	Behavior	American Bullfrog (*Lithobates catesbeianus*); Red-legged Frog (*Rana aurora*); Boreal Toad (*Bufo boreas*); Panamanian Golden Frog (*Atelopus zeteki*)	Lefcort and Eiger, 1993; Lefcort and Blaustein, 1995; Richards-Zawacki, 2010; Murphy et al., 2011
	Emphasized that relationship between host behavior and infectious disease can (1) inform reserve design; (2) help predict how infectious diseases will be influenced by global changes; and (3) help develop network models to understand how parasites spread through a population	Behavior	Not specific	Hoverman and Searle, 2016
	Documented that host behavior can affect disease response	Behavior	Not specific	Woodhams et al., 2011
	Measured disease prevalence, body condition and two steroid hormones as bioindicator of health	Physiology	Wood Frog (*Lithobates sylvaticus*)	Crespi et al., 2015
	Documented the association between stress hormones, disease levels and behavior	Both	Midwife Toad *(Alytes obstetricans)*	Gabor et al., 2015
	Explored physiological mechanisms through which ultraviolet B radiation (UVBR) may affect host immune function and, thus, amphibian susceptibility to disease	Physiology	Not specific	Cramp and Franklin, 2018
	Demonstrated that individual host behavior, in particular, may influence infection success and parasite aggregation	Behavior	Pacific Chorus Frog (*Pseudacris regilla*)	Johnson and Hoverman, 2014
	Demonstrated that predators elicited behavioral (reduced activity) and morphological changes in tadpole hosts which, in turn, increased parasite transmission	Behavior	Pacific Chorus Frog (*Pseudacris regilla*)	Orlofske et al., 2014

(Continued)

TABLE 1 | Continued

Factor	Approach used/Finding	Behavior, Physiology, or Both?	Taxon	Source
	Explored the role of a contaminant as a stressor in mediating susceptibility to infection	Physiology	Cuban Treefrog (*Osteopilus septentrionalis*)	Gabor et al., 2018b
INVASIVE SPECIES				
	Demonstrated that a native mammal (Northern Quoll, *Dasyurus hallucatus*) has adapted to presence of poisonous invasive toads in some areas of Australia	Behavior	Cane Toad (*Rhinella marina*)	Kelly and Phillips, 2017
	Documented that anuran species with calls similar to those of invasive species modify their calling behavior when masked by chorus of invasive competitor	Behavior	Green Treefrog (*Hyla cinerea*); Cuban Treefrog (*Osteopilus septentrionalis*)	Tennessen et al., 2016
	Implemented predator recognition training	Behavior	Ozark Hellbender (*Cryptobranchus alleganiensis bishopi*)	Crane and Mathis, 2011
	Studied embryonic learning to novel fish predator chemical cues	Behavior	American Bullfrog (*Lithobates catesbeianus*)	Garcia et al., 2017
	Measured stress responses to evaluate effects of an invasive species on body condition, stress hormones, sex steroids, and reproductive success	Physiology	Cane Toad (*Rhinella marina*)	Narayan et al., 2015
	Used male calling behavior to attract, capture and remove an invasive toad	Behavior	Cane Toad (*Rhinella marina*)	Muller and Schwarzkopf, 2017
	Measured stress hormones and behavior to evaluate response to native and introduced fish predators	Both	San Marcos Salamander (*Eurycea nana*)	Epp and Gabor, 2008; Davis and Gabor, 2015
	Measured antioxidant enzyme activity to evaluate response to an invasive predatory snake	Physiology	Majorcan Midwife Toad (*Alytes muletensis*); Balearic Green Toad (*Bufotes balearicus*)	Pinya et al., 2016
	Measured antipredator behavior in response to aquatic invertebrates to gain insights on habitat restoration	Behavior	Barton Springs Salamander (*Eurycea sosorum*)	Davis et al., 2017
	Measured behavioral responses of salamanders to odors of native and introduced invertebrate predators/competitors	Behavior	Eastern Red-backed Salamander (*Plethodon cinereus*)	Anthony et al., 2007
HABITAT MODIFICATION (INCLUDING URBANIZATION)				
	Used conspecific call playbacks to alter amphibian distributions in ponds	Behavior	Green and Golden Bell Frog (*Litoria aurea*)	James et al., 2015
	Used chorus sounds to locate breeding habitat for anuran amphibians	Behavior	Multiple species	Buxton et al., 2015, 2018
	Used stress hormones and habitat selection to assess connectivity	Both	European Toad (*Bufo bufo*)	Janin et al., 2011, 2012
	Determined individual spatial requirements, home range size, and habitat needs	Behavior	European Toad (*Bufo bufo*); Green Toad (*Bufo viridis*)	Indermaur et al., 2009
	Measured heat tolerance and evaporative water loss to evaluate sensitivity to habitat modification	Physiology	Multiple species	Nowakowski et al., 2018
	Discovered that cognition and site fidelity in an arboreal salamander can help inform forest management strategies (clearcutting vs. retention of individual shelter trees)	Behavior	Spectacled Salamander (*Salamandrina perspicillata*)	Piraccini et al., 2017
	Studied movement behavior in fragmented landscapes vs. old forest stands and measured repeatability of behavior to understand behavioral divergence among populations in response to forest fragmentation and desiccation risk	Behavior	Eastern Red-backed Salamander *(Plethodon cinereus)*	Cosentino and Droney, 2016
	Sampled blood from individuals to test whether differences in corticosterone levels were associated with different qualities of breeding and migration habitat	Physiology	Spotted Salamander (*Ambystoma maculatum*)	Homan et al., 2003

(Continued)

TABLE 1 | Continued

Factor	Approach used/Finding	Behavior, Physiology, or Both?	Taxon	Source
	Used behavior to design irrigation and drainage systems	Behavior	Multiple species	Hou et al., 2010
	Examined movement and spatial distribution patterns to inform habitat selection and refuge construction for use in future reintroductions of captive animals	Behavior	Mexican Axolotl (*Ambystoma mexicanum*)	Ayala et al., 2019
	Used behavioral choice experiments to design road crossing structures	Behavior	Green Frog (*Lithobates clamitans*); Northern Leopard Frog (*Lithobates pipiens*)	Woltz et al., 2008
	Used learning to mitigate road mortality	Behavior	General	Proppe et al., 2016
	Measured stress hormones and activity across urban and rural streams to evaluate effect of urbanization	Both	Jollyville Plateau Salamander (*Eurycea tonkawae*)	Gabor et al., 2018a; this paper
	Measured behavioral responses to lights of various colors to understand effects of color on nocturnal migration across roads	Behavior	European Toad (*Bufo bufo*)	van Grunsven et al., 2017
	Reviewed physiological and behavioral effects of artificial night lighting in urban environments	Both	Multiple species	Perry et al., 2008
	Measured stress hormones to assess effect of intensive traffic	Physiology	Yellow-bellied Toad (*Bombina variegata*)	Cayuela et al., 2017
	Measured stress hormones to evaluate effect of traffic noise on stress and vocal sac coloration, an important cue in mate choice	Physiology	European Treefrog (*Hyla arborea*)	Troïanowski et al., 2017
CONTAMINANTS				
	Reviewed physiological and behavior responses to contaminants	Both	Multiple Species	Sparling, 2003; Shuman-Goodier and Propper, 2016
	Used activity as a tool to study ecotoxicological effects of various contaminants	Behavior	3 Iberian anuran species; European Frog (*Rana temporaria*)	Denoël et al., 2010, 2012, 2013; García-Muñoz et al., 2011
	Documented that a herbicide adversely affected neurotransmission and swimming performance in tadpoles	Both	Montevideo Tree Frog (*Hypsiboas pulchellus*)	Peltzer et al., 2013
	Investigated contaminant effects on tadpole activity and survival when exposed to different predator treatments	Behavior	Gray Treefrog (*Hyla versicolor*); American Bullfrog (*Lithobates* [*Rana*] *catesbeianus*); Green Frog (*Lithobates clamitans*); Spotted Marsh Frog (*Limnodynastes tasmaniensis*); Striped Marsh Frog (*Limnodynasates peronii*)	Bridges, 1999; Hanlon and Relyea, 2013; Sievers et al., 2018b,c
	Measured glycogen in tadpoles to assess stress response to aquatic pollution (nutrients and pesticides)	Physiology	Common Frog (*Rana temporaria*)	Strong et al., 2017
	Used movement and foraging behavior to inform effects of mercury contamination	Behavior	Northern Two-lined Salamander (*Eurycea bislineata*)	Burke et al., 2010
	Used water-conservation behaviors and foraging efficiency to inform effects of a contaminant (atrazine), temperature, and moisture	Behavior	Streamside Salamander (*Ambystoma barbouri*)	Rohr and Palmer, 2013
	Reviewed behavioral responses of amphibians to contaminants that are endocrine disruptors	Behavior	Various species	Clotfelter et al., 2004
	Used male calling behavior to inform effects of exposure to EE2 (an endocrine disruptor that is a component of contraceptives) on reproductive success	Behavior	African Clawed Frog (*Xenopus laevis*)	Hoffmann and Kloas, 2012
	Demonstrated that exposure to endocrine disrupting compounds alters male reproductive behavior	Behavior	Western Clawed Frog (*Xenopus tropicalis*)	Schwendiman and Propper, 2012

(Continued)

TABLE 1 | Continued

Factor	Approach used/Finding	Behavior, Physiology, or Both?	Taxon	Source
	Demonstrated that increased exposure to copper and elevated water temperatures increased the number of dragonfly attacks on tadpoles 3-fold	Behavior	Wood Frog (*Lithobates sylvaticus*)	Hayden et al., 2015
CAPTIVE BREEDING/*EX SITU* CONSERVATION				
	Evaluated group housing strategies	Both	Darien Stubfoot Toad (*Atelopus certus*); Pirri Range Stubfoot Toad (*Atelopus glyphus*)	Cikanek et al., 2014
	Used captive populations to develop non-invasive tools to monitor glucocorticoid in wild populations and to evaluate physiological acclimation of wildlife brought into captive environments	Physiology	Not specific	Narayan, 2017
	Encouraged the assessment of animal behavior and personality syndromes to improve: (1) mating and reproductive success of captive animals by ensuring that breeding pairs are behaviorally compatible and (2) post-reintroduction survival and reproductive potential by informing the selection of optimal behavioral types for release	Behavior	Not specific	Kelleher et al., 2018
	Evaluated stress of captivity using multiple measures of physiology, two hormones and body mass change	Physiology	Brazilian Toad (*Rhinella schneideri*)	Titon et al., 2017
	Quantified behavior in captivity during successful breeding events to pin-point critical time periods and conditions for successful reproduction	Behavior	Ozark Hellbender (*Cryptobranchus alleganiensis bishopi*)	Settle et al., 2018

to design and improve approaches to their conservation and management.

Understanding how amphibians may cope with climatic variability has involved examining microhabitat preferences, as well as species' behavioral and physiological responses to increasing temperatures and decreasing water availability (Griffis-Kyle, 2016; **Table 1**). Such studies have helped develop a climate adaptation plan for at least one declining species (Mitchell, 2001). Understanding host behavior has been instrumental in revealing how amphibians can sometimes mediate (through altered thermoregulatory behavior) infections from parasites and pathogens, and physiological studies have helped elucidate how abiotic factors may affect host immune function and susceptibility to disease (**Table 1**). Vocalization behavior has been used to attract individuals to newly created habitats to ameliorate the effects of habitat loss and fragmentation and several physiological metrics—e.g., stress responses, heat tolerance, and evaporative water loss—have helped evaluate amphibian responses to habitat modification (including urbanization). Both behavioral and physiological studies have revealed how species respond to invasive predators and findings have generated novel ways to reduce predation risk (**Table 1**).

In addition to direct lethal impacts, contaminants and disease can have numerous sublethal effects—including behavioral and physiological responses—that, while not overtly causing mortality, may weaken an animal and make it more vulnerable to other stressors (Sparling, 2003; Shuman-Goodier and Propper, 2016; Rollins-Smith, 2017). For example, behavior and physiology can be sensitive indicators of endocrine disruption (Schwendiman and Propper, 2012). Behavioral studies have provided insight into strategies for reducing the impact of emerging infectious diseases (Hoverman and Searle, 2016). Experimental studies have illustrated the role that contaminants could play in compromising reproductive success, therefore potentially contributing to the global problem of amphibian decline (**Table 1**). In the context of *ex situ* conservation, physiological assays have helped evaluate acclimation of animals brought into captive environments (Narayan, 2017). Likewise, an understanding of animal behavior and personality syndromes can inform the selection of optimal behavioral types for release and ensure that captive breeding pairs are behaviorally compatible, thus improving mating success, post-reintroduction survival and reproductive potential (Kelleher et al., 2018). Evidence is growing that cognitive behavior, decision making, and personality syndromes occur in amphibians (Jaeger et al., 2016; Kelleher et al., 2018) and could have many conservation and management applications (Blumstein and Berger-Tal, 2015; Owen et al., 2016; **Table 1**).

Despite the large body of evidence that these various stressors unequivocally affect amphibian populations, the application of this information to conservation issues—i.e., to the development of specific conservation strategies—remains uncommon. Similarly, Mahoney et al. (2018) found that an increase in conservation physiology research has not led to an increased application of physiological tools in recovery planning for threatened and endangered species in the United States. For example, for recovery plans developed over an 11-year period, only 17% incorporated current physiological methodologies as recovery or monitoring activities. Thus, the need to integrate such knowledge into concrete conservation actions remains a critical need in recovery planning for imperiled species.

CASE STUDY 1: LONG-TERM BEHAVIORAL STUDIES INFORM CONSERVATION OF AN EXTINCTION-PRONE SPECIES

The Eastern Red-backed Salamander (*Plethodon cinereus*: hereafter, Red-backed Salamander) is widespread on forest floors of northeastern North America. By comparison, the Shenandoah Salamander (*P. shenandoah*) is restricted to only three populations, isolated on three mountain tops in Shenandoah National Park, Virginia, United States (Jaeger, 1980). These three populations inhabit Pleistocene-age talus slopes that contain sparse shade and are drier than the surrounding, deeper soil in well-shaded forests (Jaeger et al., 2016). Red-backed Salamanders are abundant in these forests and completely surround each Shenandoah Salamander population. Also, the talus slopes are slowly eroding, with Red-backed Salamanders found in these intruding soils. Jaeger (1970, 1971a,b); Jaeger (1972) hypothesized that the Shenandoah Salamander is an extinction-prone species based on these observations. He then plotted the distributions of both species and found that Red-backed Salamanders rarely entered the talus whereas Shenandoah Salamanders moved into the surrounding deeper, moister soil, but seldom are able to progress more than 3 m from the talus' edge into the forest on Hawksbill Mountain. Laboratory experiments indicated that both species prefer moist soil vs. drier rocky areas and that the Shenandoah Salamander is more tolerant of drier habitats than is the Red-backed Salamander.

Experiments in outdoor enclosures indicated that the Red-backed Salamander competitively excludes Shenandoah Salamanders from the deep-soil forest and, thus, essentially confines it to talus-refugia. Additional research indicated that the two species compete for prey on the forest floor (Jaeger et al., 2016). On Hawksbill Mountain, several small areas of talus occur below the large talus on top of the mountain. Jaeger (1980) conducted a 14-year census in these isolated talus patches and found that in one of them, the Shenandoah Salamander became extinct during a long drought. In general, amphibians have numerous adaptations that minimize water loss but, regardless, dehydration ultimately occurs when water loss exceeds an individual's physiological tolerance and ability to store water internally (Griffis-Kyle, 2016). To prevent mortality from dehydration during droughts, one management tactic could be to water talus patches during extended dry periods, as has been suggested to extend pond hydroperiods for aquatic-breeding amphibians (Griffis-Kyle, 2016).

Behavioral experiments in the laboratory showed that both species avoided pheromones of each other and that individuals preferred to reside on their own substrate pheromones (Jaeger and Gergits, 1979). This suggested that both species behaved as if territorial. Wrobel et al. (1980) also conducted behavioral experiments in the laboratory; the two species aggressively displayed and often bit each other, again suggesting interspecific competition as a mechanism driving the spatial distributions of the two species. Later research (Griffis and Jaeger, 1998) on Hawksbill Mountain found that Red-backed Salamanders successfully defend territories (under rocks and logs) against the Shenandoah Salamander. This inhibits the latter species' movements from the source talus on top of the mountain to the sink populations below: a rare example of how interspecific competition—mediated by aggressive interference and territoriality—can lead to the endangerment of one of the competing species. Ultimately, this research contributed to listing the Shenandoah Salamander as a federally endangered species in 1989 [U.S. Fish and Wildlife Service USFWS (1989)]. Therefore, the use of observations and experiments in both laboratory and natural habitats provided the necessary information to protect and manage this vulnerable species but, to our knowledge, no specific management actions (related to interspecific competition as a factor in declines) have yet been implemented (USFWS, 2013).

CASE STUDY 2: PHYSIOLOGICAL STUDIES REVEAL THE EFFECTS OF URBANIZATION ON A THREATENED STREAM DWELLING SALAMANDER

Land-use modification (e.g., urbanization and agriculture) is often linked to higher environmental temperatures, pollution, and eutrophication in water bodies (Smith et al., 2006). Changes in water quality can affect the behavior and endocrine systems of aquatic organisms, and nitrogen and phosphorous are among the top contributors to waterway degradation (Cook et al., 2018). Nitrogen release—from fertilizers in agriculture and suburban settings, animal waste, and wastewater effluents—results in elevated levels of active forms of nitrogen (e.g., nitrate, nitrite, ammonia, and ammonium). Nitrate is a major contaminant in freshwater aquatic environments and is especially toxic to amphibians (Rouse et al., 1999; Kellock et al., 2018). Thus, understanding risks, susceptibility, and behavioral and physiological responses of aquatic species to changes in land use is necessary to mitigate potential stressors, especially as habitat conversion continues (Pauchard et al., 2006).

Gabor et al. (2018a) examined the relation between corticosterone (the main amphibian GC) and urbanization in a stream dwelling amphibian, the Jollyville Plateau Salamander (*Eurycea tonkawae*). This plethodontid is a neotenic (completely aquatic) species found in karst-associated groundwater in the Edwards-Trinity aquifer system of central Texas, United States. This spring water is oligotrophic, has constant temperatures, and supports a unique assemblage of aquatic species adapted to these conditions. A recent phylogenomic analysis revealed extensive cryptic species diversity in the salamander assemblage endemic to this aquifer system (Devitt et al., 2019). However, because of overexploitation of this groundwater resource, regional climate and hydrologic models project that these salamanders are at high risk of extinction within the next century (Devitt et al., 2019). Indeed, the Jollyville Plateau Salamander has already been listed as threatened under the Endangered Species Act of 1973 (USFWS, 2019) due to threats from urbanization. Counts of this species have declined in areas that had the largest increases in urbanization (measured by residential development) and salamander densities were negatively correlated with residential development throughout the species' range (Bendik et al., 2014).

Gabor et al. (2018a) examined baseline corticosterone and stress response (as an indication of chronic stress) for 3 years in urban sites (≥25% impervious cover) vs. rural sites. They obtained corticosterone release rates using a non-invasive water-borne hormone assay and found that corticosterone was higher in urban sites than in rural ones in 2 of the 3 years of the study. Salamanders showed general stress responsiveness (to agitation) across all years and populations, indicating that even if salamanders were physiologically stressed they were not necessarily chronically stressed. They also found that "background corticosterone," measured directly from the stream in which the salamanders were sampled, was higher in urban than rural streams and was positively correlated with baseline corticosterone across populations and years. Background corticosterone provides an efficient, indirect method of evaluating stress levels and physiological health in aquatic vertebrates, allowing for more expeditious management decisions and evaluation of their effectiveness for imperiled species.

Mondelli (2016) used a behavioral assay (activity) in combination with a corticosterone assay to further examine the effects of urbanization on the Jollyville Plateau Salamander. Corticosterone and activity were positively related in two urban sites yet negatively related in two rural ones (**Figure 1**). The relation between activity and corticosterone release rates in urban and rural sites (albeit in opposite directions) demonstrates that measures of activity provide an additional indication of salamander responsiveness to their environment; for this species, salamanders in more urban habitats that release higher levels of corticosterone will be more active. Yet, higher activity can make individuals more vulnerable to visual predators (Epp and Gabor, 2008). This suggests a possible trade-off in consequences of behavioral activity, particularly in urban environments, for some aquatic amphibians.

These results have not yet been used directly in conservation and management strategies for amphibians. Moreover, we are unaware of any other studies in which behavioral and/or physiological findings with respect to urbanization, in general, have been used in this manner. However, because aquatic urban habitats often accumulate toxic contaminants that can affect amphibians at sublethal levels (Sievers et al., 2018a,b), behavioral and physiological assays can be additional tools in the conservation toolbox with which to assess effects of urban-associated contaminants and may serve as early warning signals of potential population declines. Although the specific contaminants that may have affected corticosterone in Mondelli (2016) are not known, exposure to nitrate and nitrite affects behavior, physiology, and survival in some aquatic species (Hecnar, 1995; Jannat et al., 2014; Pottinger, 2017). An important next step in this research will be to examine potential links among specific contaminants, physiology and behavior. A combination of laboratory and field studies that examine the relationship between corticosterone and behavior across a range of environmentally-relevant levels of active nitrogen could aid in understanding the consequences of urbanization on population health. If indeed nitrate affects behavior and/or physiology, then a management plan could include the use of vegetated buffer zones around water to reduce the amount of nitrate entering the water through runoff, as suggested by Rouse et al. (1999).

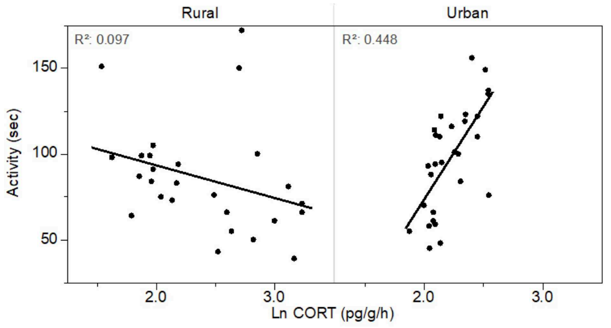

FIGURE 1 | Relationship between total movement activity (in seconds) of Jollyville Plateau Salamanders (*Eurycea tonkawae*) and natural log of corticosterone release rates for salamanders collected in more rural vs. more urban streams. Modified from Mondelli (2016).

RESEARCH GAPS AND FUTURE DIRECTIONS

Herein we have highlighted representative examples of how studies of behavior and physiology could help inform amphibian conservation efforts, but substantial deficiencies remain. Studies with amphibians are generally under-represented in the conservation behavior and physiology literature, indicating a clear need for greater focus on amphibians in future research (Madliger et al., 2018). Long-term studies could help elucidate how behavior and physiological stress ultimately affect individual fitness and population resilience. Considerable advancements have been made in conservation behavior: for example, the scientific community now has a greater understanding of how native amphibian species respond to invasive predators, and novel behavior-based approaches have been designed to minimize the impact of invasive species (**Table 1**). However, widespread application of actions has not yet been integrated into conservation and management strategies for a broad array of ecologically-similar species. Other than the species-specific climate adaptation plan implemented by Mitchell (2001), it is unclear what targeted conservation strategies have been developed as a consequence of many physiological and behavioral studies. Thus, fully integrating the information gained from research in behavior and physiology into concrete conservation actions is the most critical need at the interface of these three disciplines.

In addition, there are several areas of research in amphibian behavior and physiology that could be strengthened to better integrate these disciplines with conservation. For example:

1. The terms "stress" and "stressor" are often used without a clear physiological basis in the conservation literature. Species' threats are often identified as "stressors," even though the physiological effects of such factors on individuals and populations may not have been empirically established (Mahoney et al., 2018) or, if examined, may have been found to not occur (Gunderson et al., 2017). Contrary to the expectation of stressor effects on amphibians, there are several examples of how pathogens (Kiesecker and Blaustein, 1999), contaminants (Boone et al., 2004; Boone, 2018), and certain predators (Werner and Anholt, 1996; Davis and Gabor, 2015) can have positive (indirect) or otherwise unanticipated effects on populations. Thus, the effects of stressors can vary within and among populations (e.g., Hopkins et al., 2016) and species, and across scales (Grant et al., 2016; Muths et al., 2017). This variation underscores the need to test assumptions about the physiological basis of stress, especially when used in the context of species' threats.
2. Anthropogenic change can often occur over short time periods, and physiological indicators of how well a species may be able to respond to environmental disruptions could help reveal the potential for populations to persist. Steroid levels can fluctuate rapidly with environmental modifications and variation in individual responses can be high. Repeatability of these variable responses (multiple phenotypes) could indicate that endocrine traits exhibit heritable variation and, thus, the ability to evolve in response to environmental change (Hau et al., 2016). Miles et al. (2018) documented support for this hypothesis with respect to corticosterone but more work is needed. Thus, measuring repeatability in stress hormone activity is an important area of future research because it could provide an indication of how well a given species may be able to respond to environmental change.
3. Multiple measures of physiological health (e.g., glucose and lactate concentrations, metabolic rate or telomere length as a measure of metabolic cost) would provide a more holistic assessment of an individual's condition and overall resilience of a population than would individual measures.
4. Physiological and behavioral attributes of relatively healthy populations—both of endangered and common species—need to be documented. Doing so could provide a comparative basis for evaluating responses to environmentally-induced stress. Non-threatened species can also undergo localized population declines and losses, yet typically are not the beneficiaries of conservation interventions, as are imperiled species. Proactively implementing conservation of common species could lead to early detection of conservation problems *before* endangerment occurs.
5. Behavior-based approaches to climate change adaptation are still in their infancy (Caro, 2016). Phenological shifts are key responses of species to recent climate change, yet the use of experimental approaches to gain insights into phenology are still uncommon (Gao et al., 2015). Both behavioral and physiological responses to climate change have been simulated with biophysical and species distribution models (**Table 1**), but these predictions need empirical tests to provide conservation managers with workable solutions to climate change.
6. The potential for behavior to mitigate disease outbreaks also warrants further study, especially as it relates to informing the design of refugia from pathogens and parasites (Hoverman and Searle, 2016).
7. Nitrate has recently been considered an endocrine disruptor that has many negative effects on amphibians and other aquatic organisms (Poulsen et al., 2018). Indeed, cortisol release rates in fish were elevated below wastewater plants where nitrate levels were high compared to upstream sites (Pottinger, 2017). More studies are needed with amphibians to examine the relationship between corticosterone and active nitrogen. If there is a link, then just measuring active nitrogen in an aquatic system could provide a first approximation of population health.
8. Last, the fundamental questions in any mechanistic study are, first, how do behavioral and/or physiological phenomena "scale up" from the individual to that of the population and community levels (e.g., Jaeger et al., 2016; Saaristo et al., 2018)? Secondly, do responses observed under controlled, experimental conditions translate into measurable effects on population demography in nature (Saaristo et al., 2018)? Answers to these questions will ultimately determine the extent to which behavioral and physiological studies may influence conservation strategies for amphibians.

CONCLUSIONS

Our review and highlighted case studies illustrate the potential utility of studies in behavior and physiology for informing strategies for amphibian conservation and management. Caro (2016) stated that examples of useful behavioral applications for conservation practitioners are "principally restricted to *ex situ* conservation." However, we found numerous examples of behavioral and physiological studies that address key stressors known to impact amphibian populations—climate change, pathogens and parasites, invasive species, habitat change, and contaminants—in addition to *ex situ* conservation.

Our first case study highlighting Jaeger's research (Jaeger et al., 2016) illustrates the value of long-term investigations and, together with the studies by Gabor (Mondelli, 2016; Gabor et al., 2018a), shows the dynamic nature of relationships among environmental factors, physiology, and behavioral attributes as climate change and habitat alteration escalates. These highlighted research programs illustrate how examining a species' extinction risk through the lens of behavior and physiology can help elucidate why an extinction-prone species is currently declining, as well as provide early indicators of future population declines in at-risk species. In the future, longer-term studies, use of multiple measures of physiological health, and a focus on scaling-up from individual behavior and physiology to the population and community levels could help pinpoint how stressor impacts compound to affect population persistence and community organization. Understanding mechanisms by which various stressors may be contributing to population declines is a fundamental first step in fostering solutions to the amphibian decline crisis. However, in many cases, specific conservation strategies based on such knowledge have yet to be developed or implemented and are priorities for future conservation and management of this imperiled group of vertebrates.

AUTHOR CONTRIBUTIONS

Both authors contributed to the review through development of ideas, along with drafting, and revision of text. CG prepared the figure.

FUNDING

This work was funded, in part, by the U.S. Geological Survey's Amphibian Research and Monitoring Initiative (ARMI) to SW and a Texas State Wildlife Grant (#T-99-1) in cooperation with the U.S. Fish and Wildlife Service, Wildlife and Sport Fish Restoration Program to CG.

ACKNOWLEDGMENTS

We are grateful to C. D. Anthony, R. G. Jaeger, and J. C. Mitchell for comments on earlier drafts of this manuscript and J. T. Thibodeaux for administrative assistance. R. G. Jaeger generously summarized his work for inclusion in this paper. Any use of trade, firm, or product names is for descriptive purposes only and does not imply endorsement by the U.S. Government. This work is ARMI contribution number 687.

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Conflict of Interest Statement: 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.

frontiers
in Ecology and Evolution

PERSPECTIVE
published: 03 July 2019
doi: 10.3389/fevo.2019.00254

Mitigating Disease Impacts in Amphibian Populations: Capitalizing on the Thermal Optimum Mismatch Between a Pathogen and Its Host

Attila Hettyey[1,2]*, János Ujszegi[1], Dávid Herczeg[1], Dóra Holly[1,3], Judit Vörös[4], Benedikt R. Schmidt[5,6] and Jaime Bosch[7,8]

[1] Lendület Evolutionary Ecology Research Group, Centre for Agricultural Research, Plant Protection Institute, Hungarian Academy of Sciences, Budapest, Hungary, [2] Department of Ecology, Institute for Biology, University of Veterinary Medicine, Budapest, Hungary, [3] Department of Systematic Zoology and Ecology, Eötvös Loránd University, Budapest, Hungary, [4] Department of Zoology, Hungarian Natural History Museum, Budapest, Hungary, [5] Department of Evolutionary Biology and Environmental Studies, University of Zurich, Zurich, Switzerland, [6] Info Fauna Karch, UniMail, Neuchâtel, Switzerland, [7] Museo Nacional de Ciencias Naturales, Consejo Superior de Investigaciones Científicas, Madrid, Spain, [8] Research Unit of Biodiversity - Consejo Superior de Investigaciones Científicas/Oviedo University/PA, Universidad de Oviedo, Mieres, Spain

Understanding how animal behavior can influence the susceptibility of endangered hosts to emerging pathogens and using this knowledge to ameliorate negative effects of infectious wildlife diseases is a promising avenue in conservation biology. Chytridiomycosis, an emerging infectious disease caused by the fungal pathogen *Batrachochytrium dendrobatidis* (*Bd*) in amphibians has led to the most spectacular disease-borne loss of vertebrate biodiversity ever recorded in history. Unfortunately, the methods of mitigation that are available today are only practical in captive populations, and an effective method that could be applied in natural habitats without inflicting vast collateral damage is lacking. We suggest here that the thermal tolerance mismatch between *Bd* and its ectothermic hosts coupled with the thermoregulatory behavior of amphibians could be exploited in mitigation interventions combating *Bd* infection *in situ*. If microhabitats with elevated temperatures are made available in their natural environment, individuals taking advantage of the possibility to reach their preferred body temperature could critically lower their infection intensity or even clear the pathogen. We provide a basis for studying this approach by reviewing the evidence that supports the idea, describing how technical difficulties may be overcome, pointing out gaps in our knowledge that need to be filled by future studies, and listing presumable benefits and probable limitations of localized heating. The proposed approach has good potential to become an effective *in situ* mitigation method that can be easily employed in a wide taxonomic range of amphibians, especially in species that are warm-adapted, while causing less collateral damage than any other method that is currently available. If so, it may quickly become a widely applicable tool of biodiversity conservation and may contribute to saving many amphibian populations and species from extinction in the next few decades.

Keywords: ***Batrachochytrium dendrobatidis*, *Batrachochytrium salamandrivorans*, emerging infectious disease, heated shelter, mitigation, thermal tolerance**

Edited by:
Susan C. Walls,
United States Geological Survey,
United States

Reviewed by:
Jason Robert Rohr,
University of South Florida,
United States
Reid Harris,
James Madison University,
United States

***Correspondence:**
Attila Hettyey
hettyey.attila@agrar.mta.hu

Specialty section:
This article was submitted to Behavioral and Evolutionary Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: *22 February 2019*
Accepted: *18 June 2019*
Published: *03 July 2019*

Citation:
Hettyey A, Ujszegi J, Herczeg D, Holly D, Vörös J, Schmidt BR and Bosch J (2019) Mitigating Disease Impacts in Amphibian Populations: Capitalizing on the Thermal Optimum Mismatch Between a Pathogen and Its Host. Front. Ecol. Evol. 7:254. doi: 10.3389/fevo.2019.00254

INTRODUCTION

Emerging infectious diseases of wildlife pose a serious threat to biodiversity. They can have large economic costs via spill-over to livestock and, if zoonotic, they may also threaten human health (Daszak et al., 2000). Chytridiomycosis is an emerging infectious disease caused by the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*) in amphibians, where it has already led to the decline or extinction of hundreds of species (Skerratt et al., 2007; Wake and Vredenburg, 2008; Lips, 2016; Scheele et al., 2019). Chytridiomycosis continues to be one of the largest conservation issues because it is still spreading, it is highly virulent, and no widely applicable solution is in sight (Woodhams et al., 2011; Scheele et al., 2014, 2019; Garner et al., 2016). What makes this disease especially worrying is that it affects amphibians, which have been declining for decades and are already one of the most severely threatened vertebrate groups today (Houlahan et al., 2000; Stuart et al., 2004; Wake and Vredenburg, 2008), with 41% of species listed at least as threatened (IUCN, 2016).

Several lineages of *Bd* have been described (O'Hanlon et al., 2018), some of which have existed locally at least for decades without causing mass mortalities, but one hypervirulent lineage, *Bd*GPL, has spread throughout the world in recent years and caused extinctions across several continents (Farrer et al., 2011; James et al., 2015; Lips, 2016; O'Hanlon et al., 2018; Scheele et al., 2019). Spread is attributable to two main factors: human activities (especially transportation and animal husbandry; Weldon et al., 2004; Garner et al., 2006; Scheele et al., 2019) and natural processes (primarily via migrating animals, including non-amphibians carrying the fungus; Vredenburg et al., 2010; Garmyn et al., 2012; McMahon et al., 2013). When *Bd* arrives to a new area, it can spread in a wave-like front, often leading to local extinctions or resulting in sharp declines and leaving just dwindling remnants of amphibian populations (Rachowicz et al., 2005; Lips et al., 2008; Cheng et al., 2011). Moreover, due to climate change, or the appearance of new, more virulent lineages, *Bd* can also suddenly become devastating in locations where it has previously not led to disease outbreaks (Bosch et al., 2007; Rödder et al., 2010; Clare et al., 2016; Jenkinson et al., 2016; Cohen et al., 2018). Furthermore, these two mechanisms leading to mass mortalities in amphibians are not mutually exclusive and may act in concert (Fisher et al., 2009; Rohr and Raffel, 2010; Walker et al., 2010; Lips, 2016; Cohen et al., 2018, 2019).

Bd infects keratinous skin surfaces of amphibians (Berger et al., 1998). In larval amphibians, *Bd* infection usually causes only mild symptoms, including lethargy and poor swimming performance resulting in somewhat lowered body mass, but larval mortality due to chytridiomycosis is rare (Blaustein et al., 2005; Garner et al., 2009; Hanlon et al., 2015). In metamorphs and adults, clinical signs of the disease can include thickening of the outermost skin layer, reddening, ulceration and excessive shedding of the skin, lethargy and anorexia (Berger et al., 1998, 2005). Chytridiomycosis impairs breathing and osmoregulation, facilitates co-infection by other infectious agents, may induce immunopathology, and can ultimately lead to cardiac arrest (Voyles et al., 2009; Campbell et al., 2012; Whitfield et al., 2013).

Several mechanisms of defenses against infectious agents exist in amphibians. Individuals of many species excrete antimicrobial peptides (AMPs) onto their skin and these have been shown to depress *Bd* infection loads *in vitro* (Woodhams et al., 2007a; Rollins-Smith, 2009). However, some species possessing AMPs are highly susceptible to infection (e.g., Rollins-Smith et al., 2006, 2009), and many species lack AMPs completely (Conlon et al., 2009). The adaptive immune system of amphibians may also contribute to the suppression of chytridiomycosis, but results are mixed in this respect as well (Rollins-Smith et al., 2009; Ramsey et al., 2010; Stice and Briggs, 2010; Cashins et al., 2013; McMahon et al., 2014). Individuals may adopt behavioral patterns that prevent infection or lower pathogen burden (i.e., "behavioral fever"; Kluger, 1977; Sherman et al., 1991; Lefcort and Blaustein, 1995; Sherman, 2008; Rakus et al., 2017), but the number of studies reporting such behavioral alterations in response to *Bd* has remained very limited (Murphy et al., 2011; Karavlan and Venesky, 2016; but also see Han et al., 2008; Sauer et al., 2018). Finally, some symbiotic bacteria inhabiting amphibian skin produce antifungal metabolites that can hamper colonization by *Bd* and reduce its growth (Harris et al., 2006, 2009a,b; Lam et al., 2010, 2011). However, symbionts are not ubiquitous (Lam et al., 2010), their performance is environment-dependent (Daskin et al., 2014), and they do not protect against all *Bd* lineages (Antwis et al., 2015). Despite these variable and sometimes powerful defenses, many amphibian populations and species are threatened by chytridiomycosis and are likely to persist only if we implement effective measures against this deadly disease.

Several countermeasures to chytridiomycosis have been proposed as a result of intense research focusing on the biology of *Bd*, its interactions with amphibian hosts, and the factors influencing infection probability and disease progression (for reviews see Woodhams et al., 2011; Scheele et al., 2014; Garner et al., 2016). These include the application of salt and antifungals (Johnson et al., 2003; White, 2006; Pessier, 2008; Heard et al., 2014; Woodward et al., 2014; Bosch et al., 2015; Stockwell et al., 2015; Hudson et al., 2016; Geiger et al., 2017), the addition of probiotics (Woodhams et al., 2007b; Harris et al., 2009a; Muletz et al., 2012), immunization (Ramsey et al., 2010; McMahon et al., 2014), selection for *Bd*-tolerance in captive-bred populations (Garner et al., 2016), enhancement of the density of microscopic aquatic predators of *Bd* zoospores (Buck et al., 2011; Searle et al., 2013; Schmeller et al., 2014), general actions facilitating population persistence and recovery (Muths et al., 2011; Shoo et al., 2011; Heard et al., 2014), and elevating the temperature in the environment of infected individuals (Woodhams et al., 2003; Chatfield and Richards-Zawacki, 2011; Heard et al., 2014; Scheele et al., 2015). Indeed, some of these approaches proved to be highly effective when applied in captive populations. However, because of high costs of maintaining populations in captivity, only a few dozen species may be saved *ex situ* (Zippel et al., 2011), so that mitigation approaches suitable for treating infected amphibian populations in their natural habitats are needed (Scheele et al., 2014; Garner et al., 2016). Unfortunately, the methods listed above are in their current form not yet suitable for *in situ* application. They are either associated with vast collateral damage to the environment, may also harm amphibians, are impractical outside the laboratory, are associated with immense costs, are simply not effective under natural conditions, or their use cannot be permitted because of nature conservation legislation (Scheele et al., 2014; Garner et al., 2016). Consequently, a feasible mitigation strategy for lowering the threat posed by chytridiomycosis in natural populations is still lacking (Scheele et al., 2014, 2019; Garner et al., 2016).

Finding a suitable strategy for mitigation, including those mentioned above, will require further intense research into the basic ecology of the pathogen and of its interactions with amphibian hosts, but may contribute to saving hundreds of amphibian species from extinction. Our aim here is to draw attention to a presumably effective, safely applicable and rather simple method of *in situ* mitigation that conservation managers may readily employ in the fight against chytridiomycosis. We outline the basic idea, list evidence providing support for its potential, describe how it could be realized technically, point out knowledge gaps that need to be filled before its application, list presumable benefits and probable limitations, and thereby provide a basis for researching this promising approach.

A SUGGESTION FOR FIGHTING *BD IN SITU*

We suggest exploiting the difference in the thermal optima and maxima between *Bd* and its amphibian hosts in their natural environment simply by providing opportunities for amphibians to reach their preferred body temperature. According to *in vitro* studies, the optimal temperature for *Bd* is around 20°C, it grows well below 24°C, and its critical thermal maximum (CT_{max}) is around 28°C (Johnson et al., 2003; Piotrowski et al., 2004; Stevenson et al., 2013; Cohen et al., 2017; Voyles et al., 2017; also see **Table 1**). However, a global dataset on physiological heat tolerance comprising relevant data on 91 amphibian species suggests that the CT_{max} is higher than 32°C in ca. 80% of amphibian species and lower than 30°C in just 7% (lower than 28°C in just one species; see supplementary dataset in Sunday et al. (2014a); also see Ultsch et al., 1999; Gutiérrez-Pesquera et al., 2016). Importantly, CT_{max} strongly depends on age (Sherman and Levitis, 2003; Turriago et al., 2015) and it is weakly related to altitude and latitude while the phylogenetic footprint is significant (Sunday et al., 2014a,b; Gutiérrez-Pesquera et al., 2016). Nonetheless, exposure to temperatures of 28–30°C for a few days only will not be hazardous to a large number of amphibian species, but caution needs to be applied in case of cold-adapted species (see below). If the CT_{max} of the targeted species, or at least of closely related species is known, it will be possible to set wide enough safety margins on the temperatures to be applied. Elevated temperatures can directly kill *Bd* zoospores and cells encysted in amphibian skin and sporangia. In addition, the immune function of amphibians and the antifungal activity of symbiotic bacteria living on amphibian skin may also increase toward higher temperatures (Raffel et al., 2006; Rollins-Smith et al., 2011; Daskin et al., 2014), especially in warm-adapted-species (Cohen et al., 2017, 2019; Sauer et al., 2018). These mechanisms may act synergistically, lowering the need for exposure of individuals to high temperatures for a prolonged time period (**Table 1**). In cold-adapted species the effectiveness of the immune system may decrease more quickly with increasing temperature than the performance of *Bd*, resulting in relatively low temperature optima for the hosts (Cohen et al., 2017, 2018, 2019; Sauer et al., 2018). Because these cold-adapted species may not endure temperatures that surpass the CT_{max} of *Bd*, applying elevated temperatures may be counterproductive in their case. Consequently, it is important to first assess temperature preferences and critical thermal maxima of the species to be treated and thereafter apply heating, where the applied temperature should ideally surpass the CT_{max} of *Bd*, which is around 28°C (**Table 1**). The use of ambient temperatures elevated to 28–30°C may thereafter be a safe and effective way of treating amphibians infected by *Bd* (Berger et al., 2010; Woodhams et al., 2011; Scheele et al., 2014).

Several lines of evidence provide ample support for the potential of the *in situ* chytridiomycosis-mitigatory use of elevated temperatures. A good number of studies report successful clearing of *Bd*-infection or at least significant lowering of infection prevalence and intensity in captive populations after application of elevated temperatures (**Table 1**). In addition, infection may be prevented in the first place by a warm environment (e.g., Blooi et al., 2015). Also, theoretical models and empirical studies on *Bd*-prevalence and infection load revealed that both increase toward cooler areas and cooler periods of the year in tropical as well as in temperate climate zones (Retallick et al., 2004; Woodhams and Alford, 2005; Kriger et al., 2007; Walker et al., 2010; Forrest and Schlaepfer, 2011; Puschendorf et al., 2011; Fernández-Beaskoetxea et al., 2015; Gabor et al., 2015). These patterns are in accord with reports that in the tropics and subtropics, chytridiomycosis can have devastating effects in cool areas, like highlands, but much less so in warmer lowlands (e.g., Retallick et al., 2004; La Marca et al., 2005; Lips et al., 2006, 2008; Pounds et al., 2006; Walker et al., 2010; Rodríguez-Brenes et al., 2016), and during cool winter months, but much less during warmer parts of the year (Bradley et al., 2002; Berger et al., 2004). It is worth noting, however, that while some species suffer extreme population declines due to chytridiomycosis, others are little affected. This variation in the susceptibility to chytridiomycosis may partly be due to interspecific differences in the effectiveness of the immune system. However, species-, population- and sex-specific thermal profiles may also play a role: individuals that more often experience temperatures higher than 25°C are less likely to carry *Bd* (Rowley and Alford, 2013; Stevenson et al., 2014). Hence, elevating environmental temperatures beyond 25°C or, preferably, to 28–30°C may help amphibians keep *Bd*-infection intensities low or even clear the infection, and, thus, may be an effective strategy of chytridiomycosis-mitigation (Berger et al., 2010; Woodhams et al., 2011; Scheele et al., 2014).

If elevated temperatures are so effective in lowering *Bd*-prevalence and -loads, why has this approach not been applied so far in natural populations? A plausible explanation may be the apparently vast energy demand. Heating up the aquatic environment of larval amphibians to a high enough temperature and also maintaining this temperature would

TABLE 1 | The effectiveness of elevated environmental temperature applied against *Bd* as reported by experiments performed on laboratory cultures of the fungus (*in vitro* studies) and on live and infected amphibians (*in vivo* experiments).

Type of experiment	Treatment	Effectiveness	References
In vitro	28 or 29°C for 14 days	lowered *Bd*-growth at 28°C, no growth at 29°C	Longcore et al., 1999
In vitro	32°C for 4 days OR 37°C for 4 h	*Bd* wipeout in 100% of cultures	Johnson et al., 2003
In vitro	30°C for 8 days	*Bd* wipeout in 50% of cultures	Piotrowski et al., 2004
In vitro	26,5°C for 8 days	no zoospore release	Woodhams et al., 2008
In vitro	33°C for 1 h daily	lowered *Bd* growth	Daskin et al., 2011
In vitro	23, 25, 26, 27, or 28°C for 14 days	lowered *Bd* growth beyond 26°C	Stevenson et al., 2013
In vitro	26, 27, or 28°C constantly	no *Bd*-growth beyond 28°C	Cohen et al., 2017
In vitro	26°C for 4 h daily	lowered *Bd* growth	Greenspan et al., 2017a
In vitro	26, 27, or 28°C constantly	lowered *Bd*-growth at 27°C, no growth at 28°C	Voyles et al., 2017
In vivo	37°C for 8 h on two consecutive days	clearance of *Bd* from 100% of juvenile frogs	Woodhams et al., 2003
In vivo	27°C for 98 days	clearance of *Bd* from 50% of juvenile frogs	Berger et al., 2004
In vivo	32°C for 5 days	clearance of *Bd* from 100% of adult frogs	Retallick and Miera, 2007
In vivo	17 or 22°C constantly	enhanced survival of juvenile frogs (from 5 to 50%) at 22°C	Andre et al., 2008
In vivo	26°C for 42 days	reduced growth of *Bd* on adult frogs	Ribas et al., 2009
In vivo	17 or 23°C constantly	enhanced survival of adult frogs (from 7 to 81%) at 23°C, at exposure to low zoospore density	Bustamante et al., 2010
In vivo	30°C for 10 days	clearance of *Bd* from 96% of adult frogs	Chatfield and Richards-Zawacki, 2011
In vivo	26°C for 5 days OR 30°C for 8 + 8 + 43 h	clearance of *Bd* from 63 to 88% of tadpoles	Geiger et al., 2011
In vivo	temperature increased from 15 to 18°C	doubling of time to death in juvenile toads	Murphy et al., 2011
In vivo	30°C for 12 h + 35°C for 24 h	ineffective in clearing *Bd* from adult frogs	Woodhams et al., 2012
In vivo	30°C for 11 days	clearance of *Bd* from 100% of adult frogs	McMahon et al., 2014
In vivo	22, 26, or 28°C constantly	growth of *Bd* ceases at around 28°C in adult frogs	Cohen et al., 2017
In vivo	29°C for 4 h/day	clearance of *Bd* after 68 days from adult frogs	Greenspan et al., 2017a
In vivo	20, 23, or 26°C for ca. 90 days	*Bd* load at 23 and 26°C one order of magnitude lower than at 20°C in adult frogs	Sonn et al., 2017
In vivo	26°C for 63 days	lowered *Bd* load and elevated survival in adult frogs	Robak and Richards-Zawacki, 2018

Please note that the effectiveness was usually assessed in in vivo studies using qPCR, which may underestimate the effect of the thermal treatment on Bd cells because DNA extracted from dead cells may still be intact enough to amplify, resulting in false positives.

require a power input that can realistically not be provided in the wild in water bodies exceeding just a few m^3 in size (ca. 12 kWh necessary for each m^3 of water just to increase the temperature by 10°C; calculated as $W(kWh) = \{V(l) \times c(kJ/kg°C) \times \Delta T\,(°C)/3600\}$, where V is the water volume, c is the specific heat of water, ΔT is the change in temperature). In the terrestrial environment of adults, on the other hand, animals live dispersed, so that heating up their entire habitat is obviously absolutely impossible. Decreasing canopy cover around ponds and streams may lead to elevated temperatures and thereby provide sufficiently warm thermal refuges in many environments (Raffel et al., 2010; Geiger et al., 2011; Heard et al., 2014), but the approach of felling or pruning trees and shrubs may often not be an acceptable measure due to the collateral damage inflicted, especially so in protected areas, and removal of shading vegetation may simply not elevate temperatures to a sufficient extent in many others.

The obstacle of a vast energy demand can, however, be circumvented in many amphibians, especially in pond-breeding species, by applying localized heating: amphibians may be provided with localities where they can reach their preferred body temperatures also during times and at places where this would otherwise be impossible. It is not necessary to heat up the entire water body, its immediate surroundings or large areas that cover the terrestrial habitat, because if individuals are provided with warm spots, they will use these thanks to their inherent warmth-searching drive (e.g., Heath, 1975; Wilbur, 1980; Dupré and Petranka, 1985). Larvae, juveniles and adults of anuran amphibians are known to select warm areas in their natural habitat (e.g., shallow areas of ponds and sun-lit spots on land) and generally select temperatures between 26 and 30°C (Wells, 2007), while urodelans prefer somewhat lower temperatures ranging from 18 to 26°C, depending on species and on developmental stage (e.g., Licht and Brown, 1967; Heath, 1975; Hutchison and Hill, 1976; Dupré and Petranka, 1985; Fontenot and Lutterschmidt, 2011). Because of their fossorial life history, thermal preferences in caecilians (Gymnophiona) are not well-understood, but Bd has also not been detected in this group yet. Consequently, individuals of many anuran species are likely to readily occupy areas with artificially elevated temperatures.

FIGURE 1 | Appliances that may be used for the realization of localized heating: **(a)** heat mat, **(b)** infrared heat lamp, as employed also for mammals in zoos (San Diego Zoo in this case), and **(c)** submersible heater [prototype: (1) skirting preventing heat dissipation via conductance; (2) insulation; (3) aquarium heaters fixed to bottom of appliance; (4) bottom with holes allowing amphibian larvae to enter warm spot; (5) swimmers under the upper edge stabilizing appliance and keeping it afloat; (6) electric cables; and (7) suspension facilitating in-pond placement of appliance].

If individuals can assess their *Bd*-infection status and use warmer locations when necessary (i.e., behavioral fever; Murphy et al., 2011; Karavlan and Venesky, 2016), the effectiveness of localized heating may further be enhanced. Breeding adults, aquatic larvae and newly metamorphosed individuals could be targeted relatively easily in most species, because they occur aggregated both in time and space. Also, amphibians contract *Bd* during these water-dependent events or life stages, and the disease most often leads to mass-mortality shortly after metamorphosis (Van Rooij et al., 2015). Hence, lowering disease transmission rates and infection loads in larvae, metamorphs and reproducing adults using localized heating is likely possible in many species, and this is a plausible objective for any *in situ* chytridiomycosis mitigation method.

The technical realization of localized heating is simple in theory. In the terrestrial environment artificial shelters built around thermostat-controlled heat mats (e.g., 20 × 20 cm, consuming 10 W) or infrared heat lamps (e.g., effectively lit area of 0.1 m^2 at a consumption of 100 W), as applied by terrarists and home gardeners, may be used (**Figure 1**). Heated terrestrial shelters may be dispersed around breeding ponds to target adults and freshly metamorphosed individuals leaving the water. In the aquatic environment, submersible aquarium heaters can be used to locally elevate water temperature. Heat dissipation through convection poses a problem here, but this can be overcome using an insulated skirting around heaters, which helps contain the warm water, but allows amphibians to enter from below (e.g., submersible heaters consuming just 500 W are sufficient to elevate the water temperature by 15°C in a 20 cm deep layer over a semi-isolated area of 0.5 m^2; **Figure 1**). As the power source, a landline is most convenient, but a power generator, a solar power system or their combination may also be suitable for supplying heaters (ca. 100 heated terrestrial shelters or 2 submersible heaters/kW). The requirements these devices have to meet are the ability to heat up their immediate environment and amphibians therein to temperatures between 28 and 30°C, to be portable and usable also in remote areas, to have relatively low power needs, to be cost-effective, be easy and safe to run also by non-academic personnel, and, importantly, to be attractive to amphibians. Solutions for several technical issues will have to be found, including the prevention of dehydration of amphibians using terrestrial warm spots, keeping individuals away from hot surfaces of heaters while still allowing efficient heat uptake, keeping predators away from amphibians aggregating on warm spots, or constructing funnel-trap-like one-way entrances for species or life-stages that would not use the provided warm spots for long enough voluntarily, but would endure these temperatures without damage. Finally, different climates and habitat types may require or allow for the use of different appliances. Consequently, the theoretical and technical expertise of electro technicians and energy engineers is likely to prove helpful during development and optimization, but none of these technical difficulties appear unsolvable.

KNOWLEDGE GAPS THAT NEED TO BE FILLED

Intensive research over the last two decades delivered detailed insights into several aspects of the biology of *Bd* and its interaction with amphibian hosts, but there have remained several important knowledge gaps that would first need to be filled before localized heating can be applied *in situ* routinely. One field where we lack sufficiently detailed information regards the combination of treatment duration and the temperature necessary to lower infection loads or clear *Bd* completely (for basic characteristics of the thermal ecology of *Bd* and of its interaction with amphibian hosts see **Table 1**). For the treatment of captive populations, where elevated temperatures can be provided for long periods without difficulty and immediate surveillance is possible, it may be sufficient to evaluate a combination of temperature and exposure time—which experimentation reveals will clear *Bd* from a treated population—then apply that preventively or in case infection is suspected. However, for the *in situ* application of localized heating, where the energy available for heating is likely limited and the direct observation of treated individuals will often not be possible, we need to know rather exactly the combinations of minimum treatment temperatures and exposure durations that effectively lower infection loads of *Bd*, or, preferably, lead to its complete clearance. In parallel, behavior of amphibians at various life-stages in relation to the use of warm spots will have to be studied to assess if individuals would voluntarily use warm areas for long enough (e.g., Sauer et al., 2018), or if repeated but short stays in warm spots may sufficiently lower *Bd* burdens

(Greenspan et al., 2017a). This is necessary for deciding if heated areas can be designed so that animals can come and go at their will, or if they have to have funnel-trap-like one-way entrances to keep animals inside for long enough. In the latter scenario, conservation officers or scientists would need to manage the process, but this would create the opportunity to mark treated animals and follow up on their future, thereby obtaining reliable estimates on the efficiency of the applied *in situ* treatment.

Temperatures that are effective against *Bd* are relatively low, so that treatment of amphibians with elevated temperatures is considered a safe approach, and has been used in captive populations. Nonetheless, knowing the basics of the thermal ecology of the species to be treated is important because exposure to temperatures around 30°C during the entire larval period can already lower survival and growth rate of tadpoles and metamorphs of some species (Harkey and Semlitsch, 1988; Bellakhal et al., 2014; Goldstein et al., 2017). This knowledge can, however, be obtained by applying simple tests of heat tolerance (e.g., assessment of CT_{max} by observing the temperature at which muscular coordination becomes disorganized; Hutchison, 1961; Huang et al., 2006; McCann et al., 2014; Greenspan et al., 2017b). Elevated temperatures may nonetheless also cause sublethal damage in treated individuals, which have remained largely ignored so far. High temperatures experienced during gametogenesis and gamete maturation have been proposed to impair gamete quality in adult amphibians (Woodhams et al., 2012), but we know of no report documenting such an effect at temperatures around 30°C. Even if remaining below CT_{max}, environmental temperatures maintained at around 28–32°C for several weeks or during the entire larval period can lead to lowered growth and development rates (Angilletta and Dunham, 2003; Bellakhal et al., 2014; Carreira et al., 2016; Goldstein et al., 2017; Phuge, 2017), potentially depressing fitness of treated individuals (Smith, 1987; Semlitsch et al., 1988; Berven, 1990; Altwegg and Reyer, 2003; Schmidt et al., 2012). Also, even brief exposure to 30°C can increase stress hormone levels in adult frogs (Juráni et al., 1973; Narayan and Hero, 2014). However, within pessimal temperature limits, growth rate and overall physiological performance is positively related to temperature in ectotherms (Angilletta and Dunham, 2003; Carreira et al., 2016), and exposure to mildly elevated temperatures applied for a few days only is unlikely to lead to intolerable decreases in fitness even in species with a relatively low CT_{max}. Whether temporally limited exposure to mildly elevated temperatures can have significant negative fitness-effects remains to be assessed.

Elevated temperatures experienced during the sensitive period of larval development can lead to sex reversal in amphibians and only a handful of studies have so far investigated this phenomenon in amphibians (Chardard et al., 2004; Eggert, 2004; Nakamura, 2009). These studies report sex reversal to rarely occur with an effectiveness of 100% and only if environmental temperatures exceeding 28–32°C are maintained for weeks (Hsü et al., 1971; Dournon et al., 1984, 1990; Wallace et al., 1999; Wallace and Wallace, 2000; Chardard et al., 2004; Sakata et al., 2005; Phuge, 2017). Also, sex-reversed individuals may be infertile in some cases but fertile in others (Dournon et al., 1984; Wallace et al., 1999) and the sex-biasing effect may depend strongly on the timing of exposure to elevated temperatures [Muto, 1961 reviewed in Chardard et al. (2004), Wallace et al. (1999) and Sakata et al. (2005)]. Further, temporal variation in temperature may disrupt the sex-reversing effect of heating (Neuwald and Valenzuela, 2011), while the *Bd*-clearing effect may be upheld (Woodhams et al., 2003; Stevenson et al., 2014; Greenspan et al., 2017a). As sex reversal in amphibians (generally masculinization) is likely caused by lowered estrogen synthesis paralleled by elevated androgen and testosterone synthesis (Nakamura, 2009; Kitano et al., 2012), sex reversal could also be avoided by treating individuals with estrogen during heating, as shown in medaka (Kitano et al., 2012). However, the scarcity of reports makes it difficult to draw general conclusions (Chardard et al., 2004). Hence, for a safe application of elevated temperatures, it has to be determined if the temperature necessary to lower *Bd*-burden in infected animals may affect life history traits negatively, lead to lowered fertilization success, to compromised offspring viability, or to high rates of sex reversal. It should also be assessed whether these malign effects may be prevented by allowing temporary fluctuations in body temperature of treated animals, by hormonal balancing or by careful selection of the timing of thermal treatment outside the sensitive developmental window.

The immune function of amphibians and the antibacterial and antifungal activity of their skin microbiome are generally assumed to be highest around 28–30°C (Raffel et al., 2006; Rollins-Smith et al., 2011; Paull et al., 2012; Daskin et al., 2014), while optimal temperatures may depend both on the species of amphibians and skin bacteria. However, elevated temperatures may also promote the replication of microparasites, such as Ranaviruses ((Echaubard et al., 2014; Price et al., 2019); but also see Rojas et al., 2005; Allender et al., 2013) or the growth, reproduction and infection intensity of macroparasites (Mouritsen, 2002; Thieltges and Rick, 2006; Studer et al., 2010; Tinsley et al., 2011; but also see Kluger, 1992; Lafferty, 2009). Relevant tests investigating whether exposure to elevated temperatures—as would be the case at warm spots—enhance the susceptibility of individuals to pathogens and parasites are scarce and contradictory (e.g., Rojas et al., 2005; Allender et al., 2013; Cohen et al., 2017; Price et al., 2019), and do not yet allow general conclusions. Studies on the effects of elevated temperature on disease progression in individuals co-infected with *Bd* and additional pathogens (especially thermophilic Ranaviruses) and parasites are lacking completely, and would be needed urgently. Also, the risk of infection can be positively related to density (Briggs et al., 2010), so that by using warm spots that are attractive to amphibians, individuals may suffer an elevated risk of contracting the disease at the resulting aggregations. However, it appears that disease transmission rates will likely be lowered in heated microhabitats (Blooi et al., 2015). Nonetheless, the net outcome of elevated temperature and high density as resulting from localized heating remains to be investigated.

BENEFITS AND LIMITATIONS

There are multiple benefits of using localized heating *in situ* against chytridiomycosis. It will presumably be effective also under natural conditions and is likely to pose no danger to amphibians because temperatures can be set with a wide enough safety margin (Sunday et al., 2014b). Also, localized heating will cause practically no collateral damage to the environment because it operates by only slightly elevating environmental temperatures in small spatial fractions of the habitat while leaving the microbiome, flora and fauna of the surrounding environment practically unaffected. Most importantly, however, as opposed to the *ex situ* approach, in case of localized heating applied *in situ*, individuals will come into contact with the pathogen and will be cured, often repeatedly, so that they may become immunized (Ramsey et al., 2010; McMahon et al., 2014; but also see Tobler and Schmidt, 2010; Hudson et al., 2016). Populations treated *in situ* may adapt to *Bd*-presence via altered life history traits (Palomar et al., 2016), by the spread of certain MHC class II alleles (Savage and Zamudio, 2011, 2016; Bataille et al., 2015), or by producing more potent skin secretions (Voyles et al., 2018), providing effective defenses against lethal chytridiomycosis. Consequently, via these mechanisms and by preserving enough genetic variation and maintaining infected populations for a sufficiently long time period for microevolutionary changes to occur, *in situ* mitigation using localized heating may often allow for effective adaptation to *Bd* presence, making long-term mitigation interventions obsolete. Based on preliminary studies we estimate that the re-usable equipment (including 25 heated refugia, 5 immersible heaters and a power generator) necessary to provide enough warm spots for treating amphibians in and around a small pond of 1 ha will cost ca. 4,000 *e*. Consumables (mainly petrol for running the power generator) for one season will amount to another 4,000 *e*, but this cost may be significantly lowered if electricity is available. Finally, transportation and personnel costs have to be added. The relatively low total costs of buying and running the equipment and a good transportability will allow for its application basically anywhere.

Nonetheless, there are also limitations to the application of localized heating. Species whose $\mathrm{CT}_{\max}$ is similar to that of *Bd* may avoid locations of high temperatures and will not profit from this mitigation approach (Nowakowski et al., 2016). Individuals of other species exhibiting a high enough $\mathrm{CT}_{\max}$, but a preferred body temperature that is lower than the upper bound of the optimal temperature range of *Bd*, would also not spend enough time voluntarily at areas with elevated temperatures. Funnel-trap-like, one-way entrances of heating appliances, coupled with attentive monitoring by personnel, may provide effective solutions. In very cold habitats, where the temperature stays below what is optimal for *Bd* (i.e., below ca. 12°C; Piotrowski et al., 2004; Stevenson et al., 2013), heating may be impractical because of a high energy demand and because heaters may be unable to create high enough temperatures homogeneously within appliances, thereby increasing the reproductive rate and pathogenicity of the fungus locally (Pounds et al., 2006; Bosch et al., 2007). If environmental temperatures are high enough (higher than ca. 12°C), both heated terrestrial shelters and submersible heaters can provide homogeneous warmth within appliances. However, the heat dissipating from them is not measurable 1 cm away from their walls, leading to essentially no temperature gradient around appliances. This has, however, the consequence that in cold habitats the heated areas may differ too much from the surroundings, so that they may be avoided by cold-habituated amphibians. Importantly, elevated temperatures may enhance replication rates and the spread of Ranaviruses, a group of globally emerging pathogens causing epidemics and mass-mortalities in fishes, amphibians and reptiles (Brunner et al., 2015; Duffus et al., 2015). Although results of the few existing studies are contradictory (Rojas et al., 2005; Allender et al., 2013; Echaubard et al., 2014; Price et al., 2019), a pre-screening for the presence of Ranaviruses is necessary before the application of localized heating, and in populations co-existing with a Ranavirus the application of localized heating can currently not be recommended. Finally, financing the necessary appliances, their transport to the target locations and the personnel running the equipment will be possible in many replicates, but an *en masse* employment of the proposed method will still remain limited by costs and logistics.

Eradicating *Bd* from entire geographic regions using localized heating, or, indeed, any mitigation method will often not be possible (Garner et al., 2016). It may, however, be suitable for the preservation of the most valuable populations. Also, it is an encouraging conclusion of previous studies that we do not have to eliminate *Bd* to prevent mass mortalities and extinctions due to chytridiomycosis (Briggs et al., 2010; Tobler et al., 2012; Schmeller et al., 2014; Hudson et al., 2016). For example, Vredenburg et al. (2010) observed in three North-American frog metapopulations that mass mortality only commenced once infection intensities passed a threshold. Experimental laboratory-based studies confirm that many amphibians do not show clinical signs and mortalities as long as infection intensities remain low (e.g., Carey et al., 2006; Cheng et al., 2011). Also, amphibian populations where the local climate allows individuals to elevate their body temperatures at least temporarily above the $\mathrm{CT}_{\max}$ of *Bd*, and can thereby lower infection intensities, have been shown to maintain their population sizes even at high *Bd* prevalence (Riley et al., 2013; Rowley and Alford, 2013). Consequently, suppressing infection intensities and thereby ensuring the survival of amphibian populations in the presence of *Bd* may represent an effective, and usually the only viable *in situ* mitigation strategy (Garner et al., 2016).

Besides fighting *Bd*, localized heating may also be applied against other emerging infectious diseases caused by agents whose $\mathrm{CT}_{\max}$ is lower than that of their hosts. For example, *Batrachochytrium salamandrivorans* (*Bsal*), a sister species of *Bd* that causes disease in urodelan amphibians (Martel et al., 2014) and has recently led to severe population declines in European newts and salamanders (Spitzen-van der Sluijs et al., 2013, 2016; Stegen et al., 2017), grows best at temperatures between 10 and 15°C and its $\mathrm{CT}_{\max}$ is around 25–26°C (Martel et al., 2013; Blooi et al., 2015; Laking et al., 2017; Beukema et al., 2018). Although these decisive temperatures are lower in *Bsal* than in *Bd*, the preferred temperatures and $\mathrm{CT}_{\max}$ of urodeles are in general also lower than those of most anurans, so that localized heating may not work for clearing *Bsal* from many cold-preferring urodeles just by the effect of elevated temperatures. Nonetheless, it may very well be effective as a supportive mitigation action in many salamander and newt species, because even mildly elevated temperatures (i.e., 20°C) may decisively limit the growth of *Bsal* (Martel et al., 2013; Blooi et al., 2015). Also, exogenous heat introduced into water bodies used by urodelans for reproduction accumulates via convection at the water-air interface, where *Bsal* cysts float out of reach for most aquatic predators (Stegen et al., 2017). If *Bsal* cysts exhibit similarly low CT_{max} as non-encysted cells, the application of localized heating may, as a beneficial side-effect, also critically reduce densities of these otherwise resistant and infective life-stages of *Bsal*.

SUMMARY

Widely deployable and effective measures of *Bd*-mitigation are urgently needed for averting the ongoing biodiversity crisis caused by chytridiomycosis. After an epizootic caused by *Bd* lead to sharp declines in previously stable amphibian metapopulations, a slow recovery may occur in some species (Newell et al., 2013; Knapp et al., 2016; Scheele et al., 2017; Voyles et al., 2018), but many others will be extinct. Also, new *Bd* strains, other infections or invasive alien predators may arrive, anthropogenic pollution, or simply chance events may cause the weakened amphibian populations to disappear (Murray et al., 2009; Puschendorf et al., 2011). We suggest a method that could be used far more easily and more widely while causing less collateral damage than any method which has been suggested so far for *in situ Bd*-mitigation. It is unlikely that one single method will solve the conservation problem posed by *Bd* (Garner et al., 2016), and research into other methods that have proven to be successful in captive populations and that are being tested and optimized for application in the field are urgently needed. Nonetheless, localized heating will likely prove to be a highly valuable approach, especially in the case of disease outbreaks and when the most threatened species or populations are to be saved. If mortality rates due to chytridiomycosis are suppressed by the application of localized heating, genetic variability may be retained in populations that selection can act upon: alleles promoting resistance or tolerance to *Bd* may not disappear during severe genetic bottlenecks and may spread so that prolonged mitigation may become obsolete. We do not propose that by researching and applying localized heating it will become possible to eradicate *Bd* from entire regions or save all extant amphibian species from extinction due to chytridiomycosis, but suggest that this method has a good potential for significantly contributing to the preservation of hundreds of populations and dozens of species that are likely to go extinct unless we find and employ an effective mitigation strategy against this deadly disease.

DATA AVAILABILITY

No datasets were generated or analyzed for this study.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

AH was supported by the Lendület programme of the Hungarian Academy of Sciences (MTA, LP2012-24/2012), by the National Research, Development and Innovation Office (NKFIH) of Hungary (grant no. K-124375), a János Bolyai Scholarships of the MTA, and a Bolyai+ Scholarship of the New National Excellence Programme of the Ministry of Human Capacities, Hungary (ÚNKP, EMMI), DH by the Young Investigators Programme (FiKut) of the MTA.

ACKNOWLEDGMENTS

We thank Susan C. Walls and two reviewers for providing insightful and detailed comments on earlier versions of the paper.

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Conflict of Interest Statement: 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 handling editor declared a past co-authorship with one of the authors, BS.

frontiers
in Ecology and Evolution

MINI REVIEW
published: 19 November 2019
doi: 10.3389/fevo.2019.00431

Non-invasive Methods for Measuring and Monitoring Stress Physiology in Imperiled Amphibians

Edward J. Narayan[1,2]*, **Zachery R. Forsburg**[3], **Drew R. Davis**[4] and **Caitlin R. Gabor**[3]

[1] School of Science and Health, University of Western Sydney, Penrith, NSW, Australia, [2] Faculty of Science, School of Agriculture and Food Sciences, The University of Queensland, St Lucia, QLD, Australia, [3] Department of Biology, Texas State University, San Marcos, TX, United States, [4] School of Earth, Environmental, and Marine Sciences, University of Texas Rio Grande Valley, South Padre Island, TX, United States

OPEN ACCESS

Edited by:
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***Correspondence:**
Edward J. Narayan
e.narayan@westernsydney.edu.au

Specialty section:
This article was submitted to Behavioral and Evolutionary Ecology, a section of the journal Frontiers in Ecology and Evolution

Received: *29 March 2019*
Accepted: *23 October 2019*
Published: *19 November 2019*

Citation:
Narayan EJ, Forsburg ZR, Davis DR and Gabor CR (2019) Non-invasive Methods for Measuring and Monitoring Stress Physiology in Imperiled Amphibians. Front. Ecol. Evol. 7:431. doi: 10.3389/fevo.2019.00431

Global climate change is negatively impacting global biodiversity and ectothermic vertebrates, with amphibians being the most imperiled vertebrate taxa. Increased mean global atmospheric temperatures, high rates of habitat degradation, and exposure to infectious diseases, such as chytridiomycosis, have contributed to population declines and extinctions of rare and endangered amphibian species. Field-based monitoring of physiological endocrine traits can help determine the sub-lethal effects of environmental stressors and provide early alerts when populations are chronically stressed. Recent advances in amphibian stress endocrinology include the development and use of non-invasive methods to quantify the glucocorticoid, or stress biomarker, corticosterone. Non-invasive methods, such as urinary, skin and buccal swabs, and water-borne hormone monitoring methods (suited for terrestrial and aquatic dwelling species), are available to quantify baseline and short-term physiological stress responses of amphibians under field settings. In this review, we illustrate, by using two case studies of aquatic and terrestrial amphibian species, the applications of non-invasive corticosterone monitoring methods to advance the ecological knowledge and conservation of imperiled amphibian species, discuss the limitations of these methods, and provide future directions for the use of non-invasive hormone monitoring methods. We highlight the use of non-invasive field endocrinology methods to monitor the impacts of environmental stressors on the physiology of amphibians, which can be applied to advance ecological research, conservation, and management of imperiled species.

Keywords: conservation, ecology, ecophysiology, glucocorticoids, invasion, stress physiology, threatened species

INTRODUCTION

Global biodiversity is threatened by many anthropogenic changes, including climate change, habitat alteration, and invasive species (Brook et al., 2008; Sigwart et al., 2018). At the forefront of the biodiversity crisis are worldwide amphibian population declines and extinctions (McCaffery et al., 2012), including species in relatively undisturbed environments, such as small islands. Despite existing on Earth for over 300 million years (Stuart et al., 2004), at least 168 amphibian species are believed to be extinct and 43% of extant populations are in decline (Stuart et al., 2004; Wake and Vredenburg, 2008; Lips, 2018). Given the current rapid rates of extreme environmental change, many amphibian populations globally are unable to cope with these disturbances, resulting in widespread declines (Stuart et al., 2004).

Endocrine titers, such as glucocorticoid hormones (GCs) associated with the physiological stress response, play an important function in animal growth and development, ecology, behavior, and in maintaining homeostasis (Mills et al., 2004; Romero et al., 2009; Lindsay et al., 2016). GCs are metabolic hormones associated with a stress response (and are considered a biomarker of stress), which act collectively with a large network of neurohormones and a suite of biochemical and physiological processes that aid organisms in maintaining homeostasis (MacDougall-Shackleton et al., 2019). A stress response of elevated GC levels is indicative of a functioning hypothalamus–pituitary–interrenal (HPI) axis and as individuals become chronically stressed, GC levels may become permanently elevated (homeostatic overload) or permanently suppressed (homeostatic failure; Romero et al., 2009). Exposure to chronic stressors, such as extreme drought and invasive species can lead to decreased physiological health, population declines, and loss of genetic variation (Dantzer et al., 2014). Baseline and stress-induced corticosterone is heritable based on studies with birds (reviewed by Hau et al., 2016). By repeatedly measuring baseline levels of circulating GCs over time, and quantifying how individuals respond to acute stressors, one can infer if an organism is stressed (acute or chronic) or indeed showing physiological acclimation and the capacity to cope with stressors over the long term (Łopucki et al., 2018; Vitousek et al., 2019a). Non-invasive methods to measure GCs can advance our knowledge of species' eco-physiological responses to environmental change, provide a metric of the physiological health of a population, which may provide an early indication of population declines, and thus support conservation and proactive management programs.

The primary aim of this mini-review is to explore the applications, benefits, and limitations of non-invasive methods for measuring corticosterone (the main GC in amphibians: Idler, 1972; Forsburg et al., 2019) for ecological and conservation research. Given the highly variable nature of GC levels across species and study systems (Hau et al., 2016), GCs alone cannot be used as stress indicators and the interpretation of whether a population is stressed certainly requires the input of multi-variate factors including repeated measures, evaluation of stress response, recovery, body condition, sex ratio, reproductive rates, time period, field site demographics, and climate (Sapolsky et al., 2000; Dickens and Romero, 2013). Together, these factors provide the basis for improving our understanding of the role of stress in ecological and evolutionary processes on amphibian population declines. Our mini-review explores the following key topics:

1. The role of glucocorticoids in stress physiology and the capacity to cope.
2. Using non-invasive methods vs. plasma or whole-body homogenate in amphibians.
3. Non-invasive sampling of corticosterone profile to the threat of predators in amphibians.
    a. *Fijian ground frogs and the threat of invasive cane toads.*
    b. *Spring-endemic salamanders and the threat of predation by introduced fish.*
4. Directions for future research.

THE ROLE OF GLUCOCORTICOIDS IN STRESS PHYSIOLOGY AND THE CAPACITY TO COPE

Hormone monitoring is part of the toolkit within the emerging field of conservation physiology (Cooke et al., 2013). Monitoring the physiological responses of wildlife to environmental changes by measuring GC stress biomarkers can guide management decisions, thus bolstering both *in-situ* and *ex-situ* ecological research and conservation programs (Madliger et al., 2018). In recent years, there have been vast developments in conservation physiology/endocrinology tools, however, further improvements are needed (Madliger et al., 2018). These improvements include a better understanding of the application of stress physiology, creating links between fitness, environmental, and physiological factors, validating techniques across multiple taxa, and establishing tool validation in conservation research programs (Madliger et al., 2018).

GCs help vertebrates respond and recover from challenges (Wingfield et al., 1998; Sapolsky et al., 2000). They may alter investments in growth, survival, and reproduction while mediating life-history trade-offs (Wingfield and Sapolsky, 2003). GC levels and responsiveness can also change through development (Glennemeier and Denver, 2002a) and be sex-specific (Narayan et al., 2012a).

Variation in individual GC levels can indicate the capacity to cope with challenging environments indicating that GCs may be under selection (reviewed by Vitousek et al., 2019b). Variation among individuals within populations also dictates how populations would respond to stress. The ability of individuals in a population to modulate their stress response provides an indication of that population's ability to cope with stressors. In addition to the activation of the GC stress response as part of coping with challenges, it is important that individuals in a population can activate the negative feedback after the perturbation has passed for rapid termination of the stress response (**Figure 1**). Rapid termination can prevent pathological outcomes of GCs that are elevated over long periods (Romero et al., 2009). In sum, examining the capacity to cope with environmental stressors should include evaluating both the GC stress response and recovery from the perturbation (Vitousek et al., 2019a). To examine these components of the capacity to cope, it is useful to be able to obtain repeated measures from individuals. There is also some evidence that coping capacity varies with components of fitness (reviewed by Vitousek et al., 2019a,b). Thus, the capacity to cope can be examined in relation to fundamental concepts, such as the CORT-Tradeoff, CORT-Fitness, or CORT-Adaptation hypotheses (Breuner et al., 2008; Bonier et al., 2009; Angelier et al., 2010; Escribano-Avila et al., 2013; Guindre-Parker, 2018; Vitousek et al., 2019a), to better understand the functional significance of GCs in

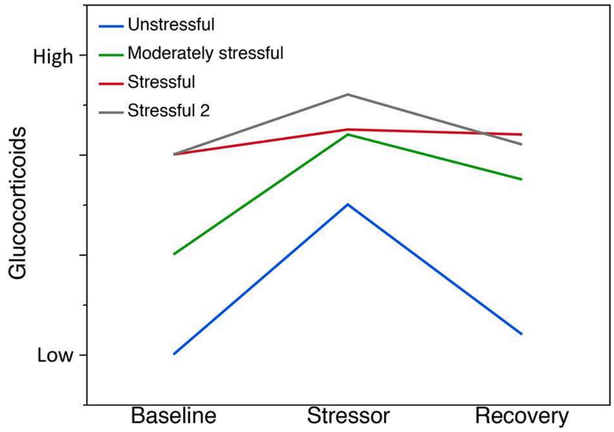

FIGURE 1 | Capacity to cope with environmental stressors. A schematic illustration of putative stress responses. Here, we present scenarios based on the expectation of chronic exposure in benign (unstressful), moderate stress, or severe stress environments. (Note: based on the origin of the study populations, the exact responses may take different forms). The ability to modulate the stress response provides an indication of the ability of a population to cope with stressors. Recovery indicates how quickly individuals in a population can activate negative feedback after the stressor has passed. For example, we propose that populations in unstressful environments will show, on average, low baseline GC, high stress response to an external stressor and high recovery (blue line). In the moderately stressful environment, populations may have higher average baseline GC, show a strong stress response to an external stressor but have a slower recovery (green line). In the stressful environment, populations may have higher average baseline GC, show none or very little stress response to an external stressor and no further change in GCs indicating some level of chronic stress or dysregulation (red line). Alternatively, a population may show the capacity to cope, even when average GCs are high, in the face of a stressful environment (Stressful 2; gray line). Further, GCs could be lower in more stressful environments.

amphibian populations and the responses to environmental change. By evaluating the costs associated with the capacity to cope via the relationship between fitness and CORT across high and low quality environments one may find that different hypotheses are supported in different environments and their associated populations.

USING NON-INVASIVE METHODS vs. PLASMA OR WHOLE-BODY HOMOGENATE IN AMPHIBIANS

There are a variety of ways to quantify physiological stress responses in animals (Madliger et al., 2018), including measuring GCs from tissues, blood, urine, feces, saliva, eggs, feather, fur, water, and respired air (Madliger et al., 2018). Blood collection from tadpoles and juvenile amphibians is generally challenging, if not infeasible, due to their small body size. Additionally, blood sampling requires sampling within 3 min of capture to obtain baseline GC levels, which may be difficult in wildlife studies (Romero and Reed, 2005). Further, plasma measures of GC only represent a single time point. Alternatively, GCs can be measured from whole-body homogenate, which may be a more integrated measure, but frequently require pooling multiple individuals to collect enough hormones (reviewed by Burraco et al., 2015). Sampling GCs via whole-body homogenate (and sometimes blood sampling) requires euthanizing individuals, which is often not permitted for threatened or endangered species and counterproductive to conservation efforts. Sequential blood sampling from small species of amphibians may also be difficult; however, minimally invasive techniques are available for amphibian hematology (Heatley and Johnson, 2009).

Traditional blood-sampling and whole-body homogenate methods are still useful in amphibian ecological monitoring. There are several experimental examples of endocrine changes associated with the end-effects of ecologically relevant stressors in amphibians. Studies have shown physiological changes (e.g., changes in plasma corticosterone, immune function, and behavior) associated with exposure to ecologically relevant stressors in amphibians, such as chytrid fungus (Fites et al., 2013), noise pollution (Tennessen et al., 2014), and change in habitat quality (Homan et al., 2003). The foundations for much of this work were built by Glennemeier and Denver (2002a,b,c), who used an adrenocorticotropic hormone (ACTH) challenge to validate the corticosterone response in tadpoles, exposed tadpoles to corticosterone and showed that they uptake corticosterone from the water, blocked corticosterone production with the corticoid synthesis inhibitor metyrapone, and demonstrated the role of corticosterone in mediating the negative effects of increased population density using whole-body homogenates.

Integrated measures of corticosterone profiles using non-invasive methods, such as fecal GC metabolites, water-borne, and urinary GC provide better, and earlier, indicators of chronic stress than do point measures, such as that from blood plasma (Dickens and Romero, 2013). Using GC hormone monitoring, we can obtain valuable information on the role of stress physiology in amphibian (and other organisms) breeding and survival across seasons and years (Boonstra, 2013; de Bruijn and Romero, 2018; Abdelrahman et al., 2019). Further, non-invasive methods of GC collection eliminate the need for euthanizing individuals and represent an integrated measure of episodic changes in cumulative stress over a time period through repeated sampling (Whitten et al., 1998; Touma et al., 2004). Individuals in laboratory, common garden, or exclosure-type studies can be marked and reared in ecologically relevant densities, permitting social interaction, and allowing repeated sampling from the same individual with little or no harm. Non-invasive sampling also allows for reduced sample sizes to estimate temporal patterns and control for individual variation in marked individuals or individually-recognizable individuals in the lab and wild. Hormone metabolites can be quantified through a variety of non-invasive biological samples, such as urine, water, skin swabs, and saliva, and have been used with success to monitor amphibian endocrine functions (Gabor et al., 2013; Narayan, 2013; Hammond et al., 2018; Santymire et al., 2018; **Table 1**). Measurement of adrenal GC metabolites in urine and feces is of considerable interest to conservation managers as it gives an indication of the link between stress and reproductive success in animals (Carroll et al., 1990; Creel et al., 1997; Wasser et al., 1997; Hopkins et al., 1999; Holt et al., 2003; Pukazhenthi and Wildt, 2004). For example, populations of

TABLE 1 | Comparison of non-invasive and traditional methods of corticosterone measurement in amphibians.

Method	Measurement timeline	Handling and time to obtain sample	Repeatability	Corrections	Extraction methods	Viability in field	Suitable species	Validation
Water-borne hormone	Integrated measure. Retrospective and not affected by initial sampling procedure	Requires little handling but 30–60 min in water	Allows repetitive sampling without anesthesia. No time between sampling needed	None	Requires single phase extraction column	Requires cooler and ice packs	Mostly smaller aquatic or semi-terrestrial species	Required for each species but has consistently been validated
Urinary glucocorticoid metabolites	Integrated measure. Retrospective and not affected by initial sampling procedure	Requires short handling to obtain sample	Allows repetitive sampling without anesthesia. May require time between sampling	Requires creatinine correction	None required	Requires cooler and ice packs	Mostly larger terrestrial species	Required for each species but has consistently been validated
Buccal swab	Somewhat integrated and not affected by initial sampling procedure	Requires short handling to obtain sample	Allows repetitive sampling without anesthesia. May require time between sampling	None	Requires TCA extraction	Requires −80°C (may be stable at −20°C)	Mostly larger mostly terrestrial species	Required for each new species
Blood sampling	Immediate snapshot. Requires <3 min sampling time-frame	Requires short handling to obtain sample	If animal is large allows for repeated sampling with time between samples. May require euthanizing	Requires measure of CBG to determine free or active GCs	Require centrifuging	Requires immediate centrifuge and −80°C	Larger aquatic or terrestrial individuals	Does not require validation
Whole-body	Somewhat integrated but may be affected by sampling timeframe (e.g., <3 min)	Requires little handling	No repeated sampling as euthanizing is required and may require pooling data from multiple individuals	None	Requires homogenization and centrifuging	Requires liquid nitrogen and −80°C	Smaller terrestrial or aquatic individuals	Requires validation for each species

For measurement timeline, we provide insight into how quickly a measurement needs to be taken to measure some sort of baseline concentration and whether the measurement includes GC metabolites or just plasma measures (immediate snapshot). For corrections, we indicate when the values obtained need correcting depending on the method. Extraction methods refers to how the hormone is extracted from the sample (if need be). Validation refers to whether an ACTH challenge or other method for showing that CORT increases when the HPI axis is stimulated is needed.

endangered Jollyville Plateau salamander (*Eurycea tonkawae*) had elevated water-borne corticosterone release rates in urban streams compared to rural streams in 2 out of 3 years (Gabor et al., 2018a). These results indicate that it is possible to explore environmental and ecological stressors on populations over time using non-invasive measures of GCs, however, it is important that these methods are biologically validated for any new study species.

Non-invasive GC monitoring has been biologically validated for several amphibian species (reviewed by Narayan, 2013; Baugh et al., 2018; Forsburg et al., 2019). Validation involves subjecting the animal to an ACTH challenge and/or an alternative physical stressor (e.g., capture-handling; Narayan et al., 2012a,b) and obtaining the appropriate non-invasive sample (e.g., feces, water, urine, saliva, skin swab) before and after the hormonal stimulation test with the prediction that GCs are elevated after exposure to an exogenous hormone. It is important to quantify the lag-time between the activation of the HPI axis, hormonal response, metabolism, and appearance of the hormonal metabolites of interest in the non-invasively obtained biological sample. Each non-invasive method of GC sampling has inherent benefits and limitations relative to each other and invasive methods (summarized in **Table 1**). In this table, we do not evaluate the non-invasive dermal swab method because the GCs detected in skin secretions could be independent of the HPI axis as the skin itself is recognized as an endocrine tissue. GC responses in dermal skin secretions are possibly a result of a much slower rate of GC secretion than GC metabolites detected via other non-invasive samples, such as water or urine (Santymire et al., 2018).

Validation of Urinary Corticosterone Profiling

Urinary GC metabolites (uGMs) are useful indicators of biologically active or plasma-free GCs (reviewed by Narayan, 2013). uGMs require capture and minimal handling (<5 min), causing slightly elevated metabolite concentrations, however, this allows baseline and short-term stress responses to be assessed over time (Narayan, 2013). Urine sampling devices for anurans are based on the size of each species (e.g., for large anurans, urine can be collected by gentle massage of ventral underbelly area over a sterile cup, blunt pipette tips, and micro-capillary tubes; this procedure is described in Narayan, 2013). Anurans are an ideal model for uGMs as they generally urinate frequently, and urine can be preserved at −20 or −80°C (Monfort, 2003). This method has been pharmacologically validated using an ACTH stimulation test in several anurans, including Fijian ground frogs (*Platymantis vitiana*; Narayan et al., 2010a), Australian Great Barred frogs (*Mixophyes fasciolatus*; Graham et al., 2013), Stony Creek frogs (*Litoria wilcoxii*; Kindermann et al., 2012), Bell frogs (*Litoria raniformis*; Germano et al., 2009), cane toads (*Rhinella marina*; Narayan and Hero, 2014), and Maud Island frogs (*Leiopelma pakeka*; Germano et al., 2012). The use of an ACTH stimulation test shows significant changes in uGMs 1–2 h post-injection. Thus, urine sampling provides a suitable method for assessing the physiological stress responses of anuran amphibians to short-term environmental challenges, such as transportation (Narayan et al., 2012c), physical marking (Narayan and Gramapurohit, 2019), or exposure to environmental stimulus (e.g., invasive competitor species; Narayan et al., 2013). Measuring GCs via urine is becoming more frequently used over fecal measurements and reduces labor costs (Millspaugh and Washburn, 2004), and uGMs are ideal for amphibian species where fecal samples may not be easy to obtain in the wild. By using uGMs, valuable information can also be obtained regarding the influence of the stress endocrine system on amphibian reproduction (Joshi et al., 2019a). For example, research in both captive and free-living amphibian species have successfully applied uGMs to study the interactions between corticosterone and reproductive hormones, body condition, and breeding behavior (e.g., vocalization in males and vitellogenesis in females; Narayan and Hero, 2013; Joshi et al., 2019b).

Validation of Water-Borne Corticosterone Profiling

A water-borne method of measuring GCs previously validated for fish (Scott and Ellis, 2007), was recently validated for use with amphibians (Gabor et al., 2013, 2016; Baugh et al., 2018; Forsburg et al., 2019). This method relies on extracting steroid hormones passively diffused into water through the gills, urine, skin, and feces (Scott et al., 2008). The relationship between water-borne corticosterone and plasma corticosterone has been validated in adult and larval common midwife toads (*Alytes obstetricans*; Gabor et al., 2013), captive-reared and field-collected adult San Marcos salamanders (*Eurycea nana*; Gabor et al., 2013), and larval Western Tiger Salamanders (*Ambystoma mavortium*; Davis et al., 2019). All studies found a significant positive correlation between blood plasma and water-borne corticosterone values. Hormones are collected by placing individuals in a standard volume of water (usually 100 mL) in an appropriately sized beaker for 30–60 min, and the GC levels are measured as a release rate per hour. Hormones are extracted from water samples using solid phase extraction columns and analyzed using enzyme immuno-assay (EIA) kits (following Gabor et al., 2013, 2016).

In addition to examining the relationship between plasma and water-borne corticosterone values it is also necessary to validate the stress response. Gabor et al. (2016) conducted an ACTH challenge on adult, captive-reared, *Eurycea nana*. Corticosterone release rates were significantly higher after an ACTH challenge compared to the non-injected control individuals, though corticosterone release rates were not significantly different between the ACTH and saline-injected individuals (likely due to not using high enough concentration of ACTH). Additionally, this method has been pharmacologically validated via ACTH challenges in eastern newts (*Notophthalmus viridescens*; Reedy et al., 2014) and Rio Grande leopard frogs (*Rana berlandieri*); Forsburg et al., 2019), and biologically validated using a stressor in *E. tonkawae* (Gabor et al., 2016), yellow-bellied toad (*Bombina variegata*), European tree frog (*Hyla arborea*; Gabor et al., 2017), spotted salamander (*Ambystoma maculatum*; Charbonnier et al., 2018), Gulf Coast toad (*Incilius nebulifer*; Gabor et al., 2019), and red-backed salamander (*Plethodon cinereus*; Novarro et al., 2018). Across all the species examined there is quite a bit of variation in average corticosterone release rates with salamanders showing the lowest values, specifically *P. cinereus* followed by *E. nana*. Nonetheless, water-borne measures of corticosterone show moderate to high repeatability for *R. berlandieri* when measured every hour for 6 h repeatedly (Forsburg et al., 2019). Baugh et al. (2018) pharmacologically validated water-borne hormones for Túngara frogs (*Physalaemus pustulosus*) and demonstrated that water-borne hormone values, measured using EIA, are correlated with those measured using high performance liquid chromatography-mass spectrometry (HPLC-MS). Like urine samples, water-borne extracts can also be used to measure other steroid hormones, such as 17-b estradiol, progesterone, and testosterone in addition to corticosterone from one sample (Narayan et al., 2010b; Mondelli, 2016; Baugh et al., 2018). Further Baugh et al. (2018) validated the use of water-borne extraction to measure 17-b estradiol and progesterone. For water-borne hormones, peak levels of corticosterone release rates require up to 2 h to be detected (*P. pustulosus*; Baugh et al., 2018) and begin to decline within 2 h of a stressor (*R. berlandieri*: Forsburg et al., 2019). Together these results demonstrate that the water-borne hormone method is a useful tool for monitoring populations to measure GCs as an indication of the physiological health of amphibian populations and for exploring the capacity of a population to cope with environmental variation. This non-invasive method is particularly useful for small species of amphibians that are rare or imperiled because it requires less handling. Water samples collected in the field can be stored in a cooler with ice packs until returning to the lab to be stored at −20°C, alleviating the need for immediate processing or freezing of blood samples. Further, this method does not require specialized equipment, such as needles and capillary tubes, and facilitates repeated sampling.

NON-INVASIVE SAMPLING OF CORTICOSTERONE PROFILE TO THE THREAT OF PREDATORS IN AMPHIBIANS

Upon recognition of predators, prey species respond in various ways that generally diminish the direct threat of predation (Lima and Dill, 1990). While these responses generally allow prey to survive encounters with predators, there are also nonlethal effects that predators have on prey species. These nonlethal effects are important considerations for the fitness of species, even though they may not be as obvious as direct predation upon individuals. One such nonlethal effect is an elevation in circulating GCs in prey, as found in a wide variety of organisms (Wingfield et al., 1998). Indirect predator interactions, such as competition are associated with nutritional stress and reduced body condition as a result of decreased foraging, triggering an elevation of GC levels (Boonstra et al., 1998; Brown et al., 1999; Creel et al., 2007; Travers et al., 2010; Clinchy et al., 2012; Anson et al., 2013). These circumstances can induce phenotypic dysregulation of an organism, leading to decreased fitness with reduced reproductive success and lower survival (Boonstra et al., 1998; Creel et al., 2007; Travers et al., 2010; Clinchy et al., 2012; Anson et al., 2013). While predators have an important influence on GCs in corticosterone-producing animals (Sapolsky et al., 2000; Romero, 2004), there has been little experimental evaluation of this in free-living amphibians. There are several published studies looking at the topic of amphibian endocrinology when facing predators, with most focused on tadpoles or metamorphs in the laboratory or mesocosms (Hossie et al., 2010; Middlemis Maher et al., 2013; Bennett et al., 2016; Gabor et al., 2019). However, to our knowledge there are no other published studies on predator–prey interactions in rare and endangered amphibian species. Herein we present two case studies that examined stress responses in terrestrial and aquatic amphibian species using non-invasive urine or water-borne GC sampling methods.

Fijian Ground Frogs and the Threat of Invasive Cane Toads

The island of Viwa within the Fiji Islands is home to the endemic Fijian ground frog (*Platymantis vitiana*), an IUCN Endangered species. Within this small (60 ha) island, ground frogs are negatively affected by invasive cane toads as they prey on young frogs, but also compete for habitat space and prey. Cane toads were introduced as a bio-control agent (Easteal, 1981), and are now regarded as one of the main threats for Fiji's endemic species (Narayan et al., 2008). Cane toads now persist on multiple Fiji Islands in very high densities and exhibit a strong spatial overlap with ground frogs, suggesting competition for prey and shelter (Thomas et al., 2011). The co-occurrence of ground frogs and cane toads is recent (within this century), making this study system particularly interesting to test the predator-stress hypothesis (Bonier et al., 2009) and to explore the topic of chronic stress in threatened frogs.

Using monthly sampling of urine from captive and free-living ground frogs, Narayan et al. (2010a,b) provided baseline data on the seasonal variation in uGMs and reproductive hormones in ground frogs. Narayan et al. (2013) demonstrated, using an open-field test set-up, that the presence of cane toads generated uGMs in the ground frogs and also increased tonic immobility (an index of fear response). To better understand the consequences of chronic stress, Narayan et al. (2015) conducted longitudinal trials using outdoor exclosures to study how the presence and absence of cane toads influenced the reproductive ecology of ground frogs. There was a distinct positive correlation between cane toad presence and decreased body condition in both male and female Fijian ground frogs, including sub-clinical changes in stress and reproductive hormones signifying chronic stress (Narayan et al., 2015). Thus, the application of non-invasive hormone monitoring methods in applied field research of an endangered amphibian species has allowed conservation biologists to better understand the consequences of an invasive species and helped guide management.

Spring-Endemic Salamander and the Threat of Predation by Introduced Fish

Central Texas, USA is home to a diverse group of fully-aquatic salamanders in the genus *Eurycea*, which inhabit spring and aquifer systems (Devitt et al., 2019). The San Marcos salamander (*E. nana*), is federally threatened and endemic to the headwaters of the San Marcos River (Nelson, 1993), an area with a diverse assemblage of native and introduced predatory fish. Previous studies have examined the antipredator response of *E. nana*, demonstrating innate, chemically-mediated predator recognition and that experience modifies these behaviors (Epp and Gabor, 2008; Davis et al., 2012), but little was known about how native and introduced predators influence corticosterone in these salamanders.

While the corticosterone response of prey species exposed to predators may be species-dependent, it may also differ depending on attributes of the predator. High-risk or native predators may elicit a different stress hormone response in prey than low-risk or introduced predators (Lima and Bednekoff, 1999; Chivers et al., 2001). By examining corticosterone levels in the presence of potential predators, a better understanding of how various types of predators (e.g., native, introduced, high-risk, low-risk) influence amphibians can be gained. To better understand the effects of introduced fish predators, Davis and Gabor (2015) exposed *E. nana* to chemical cues of the native largemouth bass and the introduced redbreast sunfish. Using water-borne hormone collection methods, combined with a repeated measures design, Davis and Gabor (2015) found that the corticosterone response (ratio of the post-stimulus to pre-stimulus corticosterone release rate) to the native predator was significantly greater than the response to both the introduced predator and blank water control, and that the corticosterone response to the introduced predator was not significantly different than the blank water control. Though previous studies have shown that *E. nana* can recognize redbreast sunfish (Davis et al., 2012), the lower corticosterone response observed by Davis and Gabor (2015) may reflect differences in the temporal variation in the risk between the two predators. The redbreast sunfish is more abundant and likely more frequently encountered compared to the largemouth bass (Perkin and Bonner, 2011) and may account for the muted corticosterone response. Elevated corticosterone release rates are associated with increased energetic expenditure and may aid in quicker escape behavior by salamanders. By incorporating non-invasive stress hormone measures and repeated measures, a more complete understanding of how native and introduced predators affect imperiled amphibians and the potential to respond to selection from introduced predators was gained.

DIRECTIONS FOR FUTURE RESEARCH

With amphibian population declines at the forefront of the global biodiversity extinction crisis, non-invasive methods for quantifying the physiological health of populations is a useful improvement to the field. Measuring hormones via water-borne, urinary GC metabolites, and buccal swab methods in amphibian populations are valuable conservation tools that are minimally invasive and can be completed in the field. Additionally, these methods allow for repeated measurements from the same individuals sequentially or over time. This makes it possible to evaluate the capacity of individuals to cope with environmental changes. By using repeated measures one can determine if individuals in populations are mounting responses to stressors to evaluate if populations are chronically stressed Further, it is essential to look at the rate of recovery from a stressor to explore variation in the capacity to terminate the stress response via negative feedback (Wingfield, 2013; Vitousek et al., 2019a; Zimmer et al., 2019). The faster individuals in a population are able to recover from acute stressors, the likelier the population is able to cope with stressors. When this information is evaluated in relation to fitness, it can provide insights into the costs associated with coping capacity in response to environmental change. Further, assessing whether the "GC phenotype" is a plastic or genetic response to environmental change by using common garden or cross-fostering experiments, whereby individuals can be followed, will aid in our understanding of how selection will act on this phenotype (Ouyang et al., 2019). Together, this information can be used to obtain insights into the overall potential for a population to respond to selection and to explore differences among populations of a given species. Another understudied aspect of stress physiology in amphibians is how corticosterone, and other hormone profiles, change as an organism develops, which could easily be measured using non-invasive methods (reviewed by Denver, 2009). Research on the relationship between corticosterone, disease, and the skin and gut microbiome across life-stages is also important as it may yield insights into the mechanisms associated with amphibian declines in some populations (e.g., Gabor et al., 2018b). By using non-invasive methods to measure GCs, further insight into the mechanisms associated with amphibian declines can be gained that were once out of reach.

CONCLUSION

In conclusion, non-invasive hormone monitoring tools can be applied to understand the ecology of amphibians, including rare and endangered species, especially in relation to environmental stressors, such as invasive species, pesticides, disease, and other anthropogenic perturbations. Non-invasive field endocrinology sampling can provide robust data to enable ecologists to make rapid and reliable assessments of the impacts of invasive species on native amphibians and assist with on-ground management and recovery of native species.

AUTHOR CONTRIBUTIONS

EN and CG synthesized this paper. ZF and DD expanded the discussion.

ACKNOWLEDGMENTS

We thank Stephanie Deering (University of Western Sydney) for conducting literature search and assisting with earlier drafts. We especially thank I. Gomez-Mestre and two reviewers for their insightful comments that have greatly improved this paper.

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