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

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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

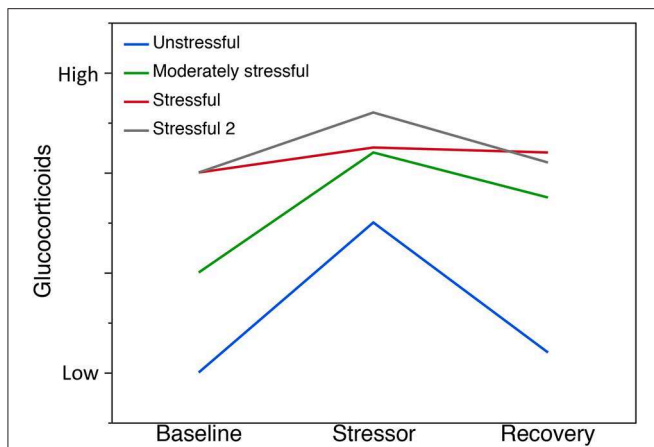


FIGURE 1 | Capacity to cope with environmental stressors. A schematic illustration of putative stress responses. Here, we present scenarios based on the expectation of chronic exposure in benign (unstressful), moderate stress, or severe stress environments. (Note: based on the origin of the study populations, 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 adrenocorticotrophic 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 enclosure-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; Pukazhenthil 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- β 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- β 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 enclosures 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.

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

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