THE ROLE OF CONSERVATION PHYSIOLOGY AND ENVIRONMENTAL CHANGE IN AMPHIBIAN DECLINES

by

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TABLE OF CONTENTS

Page

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

ABSTRACT

Environmental change associated with anthropogenic alterations to habitat and the synergistic effects with climate change and disease can render habitats unsuitable and affect the physiological health of species. This can ultimately affect survival and fitness. Amphibians are one group of vertebrates especially vulnerable to changes in environmental quality. Using multiple measures of physiological health can aid in identifying populations at increased risk of declines. This dissertation focuses on research that examines the effects of environmental variables on the health of larval amphibians through field sampling, laboratory experiments, and outdoor mesocosms. The first chapter is an introduction to the main topics addressed in my research. The second chapter includes the results of field sampling to measure environmental variables at multiple spatial scales and their effects on three physiological health metrics of ornate chorus frog (*Pseudacris ornata*) tadpoles to identify potential correlates of population declines. The third chapter examines the effects of increased water temperatures on multiple physiological health metrics in a larval anuran, the Rio Grande leopard frog (*Rana berlandieri*) by manipulating water temperatures via tank heaters in the lab and from a lack of shade cover in outdoor mesocosms. Together, my research shows that lower environmental quality, such as increased water temperatures from climate change or habitat alteration, may result in chronic stress in larval amphibians, reducing physiological health and leading to declines. However, depending on the species and aquatic environment, some individuals may benefit from warmer water temperatures. My

research suggests multiple health metrics are needed to understand the complex effects of environmental change on larval amphibian health and provides suggestions for the management of these species.

I. INTRODUCTION

Species Declines

The Earth is undergoing what has been called the sixth mass extinction event, as species and populations are rapidly declining and being extirpated even with goals set to reduce biodiversity loss (Butchart et al. 2010; Ceballos et al. 2017). Many factors affect global species decline including invasive species, disease, overexploitation, habitat loss and climate change (Brook et al. 2008; Ceballos et al. 2017; Daszak et al. 2000; Hof et al. 2011; Mainka & Howard 2010; Vitousek et al. 1997), but the greatest threats to biodiversity are the synergistic effects of multiple factors, especially habitat loss and climate change (Brook et al. 2008; Mantyka-Pringle et al. 2012; Segan et al. 2016; Travis 2003). Habitat loss generally includes habitat fragmentation, or the breaking up of continuous habitat into smaller patches (Fahrig 2003). Habitat loss and fragmentation by habitat loss both have large, negative impacts on biodiversity because of reduced habitat quality, smaller patch sizes, and increased isolation, which affect species distributions, abundance, and genetics (Fahrig 2003). A big contributor to habitat loss and habitat degradation is land-use conversion, or the increase in human-modified landscapes and the fragmentation and loss of habitat by roads and other developments (Grimm 2008; McKinney 2002). Land-use conversion causes drastic changes to the local habitat and landscape where a gradient is created from areas of lower habitat loss, fragmentation and reduced physical changes in rural areas to high habitat loss, fragmentation, impermeability, and physical changes in the urban center (Grimm 2008; McKinney 2002). Because converted areas alter environmental conditions and reduce habitat

suitability at both the landscape-level and local water quality (Meybeck 2004; Vitousek et al. 1997), biodiversity is generally lost and shifts towards more non-native species as the amount of converted area increases (Grimm 2008; McKinney 2002).

Climate change has also become a significant driver in ecology. The Intergovernmental Panel on Climate Change (IPCC) has predicted higher global mean surface temperatures and increased precipitation extremes with decreased precipitation in mid-latitude and equatorial dry regions, increased precipitation in mid-latitude wet regions and high latitudes, and increased severity and frequency of extreme precipitation events and storms (IPCC 2014). As a result of these changes, sea levels are expected to rise, impacting many coastal ecosystems from habitat loss and saltwater inundation (IPCC 2014). Species are expected to shift their ranges more northward or higher in elevation in response to increased temperature and to change timing of spring events, as has been seen in plants, insects, birds, mammals, fish, and amphibians (Beebee 1995; Chen et al. 2011; Parmesan and Yohe 2003). Ultimately, environmental alterations due to climate change affect species' interactions, phenology, range shifts, and abundance, impacting evolution and causing extinctions (reviewed in Parmesan 2006). The geographic distributions of species are related to many ecological and evolutionary factors including physical barriers, climate, species interactions, and the degree of adaptive ability (Sexton et al. 2009). Species can only inhabit areas that meet certain environmental conditions within their tolerance limits, therefore climate is often a major abiotic force affecting distributions (Sexton et al. 2009).

As climate change and anthropogenic disturbances affect environmental habitat factors, and tolerance limits are reached, populations will be unable to persist in the

altered conditions and become more isolated, leading to increased population declines (Mantyka-Pringle et al. 2012; Parmesan 2006; Segan et al. 2016). Ultimately, climate change and habitat loss, along with the other factors associated with declines (e.g. contaminants, invasive species, disease), are all stressors that can have physiological impacts on the individuals in a population, leading to increased susceptibility to declines (Figure 1; Collins 2010; Jeffrey et al. 2015). Measuring the physiological response of an organism to changing conditions which might lead to declines is the basis for the increasingly popular discipline known as 'conservation physiology' (Dantzer et al. 2014; Jeffrey et al. 2015; Sheriff et al. 2011; Wikelski & Cooke 2006) which has shown significant promise in informing management (Madliger et al. 2016). These stressors can mediate natural changes in physiology and behavior, but over prolonged periods can increase physiological stress and disrupt homeostasis (Sapolsky et al. 2000; Wingfield 2013; Wingfield & Romero 2001). Specifically, increased stress from prolonged direct stressors have been linked to inhibited reproductive behavior, a suppressed immune system, suppressed growth and development, reduced body condition, and reduced competitive ability (Romero et al. 2009; Wingfield & Romero 2001; Wingfield & Sapolsky 2003).

The Stress Response

In response to acute or short-term stressors, vertebrates increase the circulation of glucocorticoid hormones in the blood (McEwen & Wingfield 2003; Sapolsky et al. 2000). These hormones are released by the hypothalamo-pituitary-adrenal (HPA; hypothalamo-pituitary-interrenal, HPI, in amphibians) axis to promote the quick conversion of stored

proteins and fats to useable energy to assist in enduring or moving away from a stressor ("fight-or-flight" response) and to return the body to allostasis, a physiological balance of the systems (McEwen & Wingfield 2003). At different points and at different concentrations, glucocorticoids show permissive actions, prepping the body for activity, stimulatory actions, causing an increase in activity, preparative actions, sustaining activity to prepare for another stressor, and suppressive actions, reducing activity of unnecessary functions for survival (Sapolsky et al. 2000). Under more prolonged or chronic stressors, however, the HPA axis may become dysregulated and individuals can no longer mount a stress response to external stressors (McEwen & Wingfield 2003). This can result in several different responses to chronic stress: release of high levels of glucocorticoids resulting in the inability to increase further in response to a stressor (homeostatic overload), or very low glucocorticoid levels at or below baseline. These low levels of glucocorticoids may be a result of homeostatic failure and HPA exhaustion when individuals are close to death (Romero et al. 2009). However, low levels of glucocorticoids near baseline may also indicate downregulation/desensitization of the stress response, or habituation to the chronic stressors, where the event or conditions are no longer perceived as a stressor and glucocorticoid levels are not elevated (Cyr & Romero 2009). One way to differentiate between low glucocorticoid levels due to chronic stress or habituation is to introduce a test involving an acute stressor and examine whether the individuals are able to show a normal response to stressors, indicating habituation, or an inability to mount a response, indicating chronic stress (Cyr & Romero 2009; Rich & Romero 2005). Chronic stress can have deleterious effects on vertebrate health including suppressed growth and development, reduced reproduction, and reduced

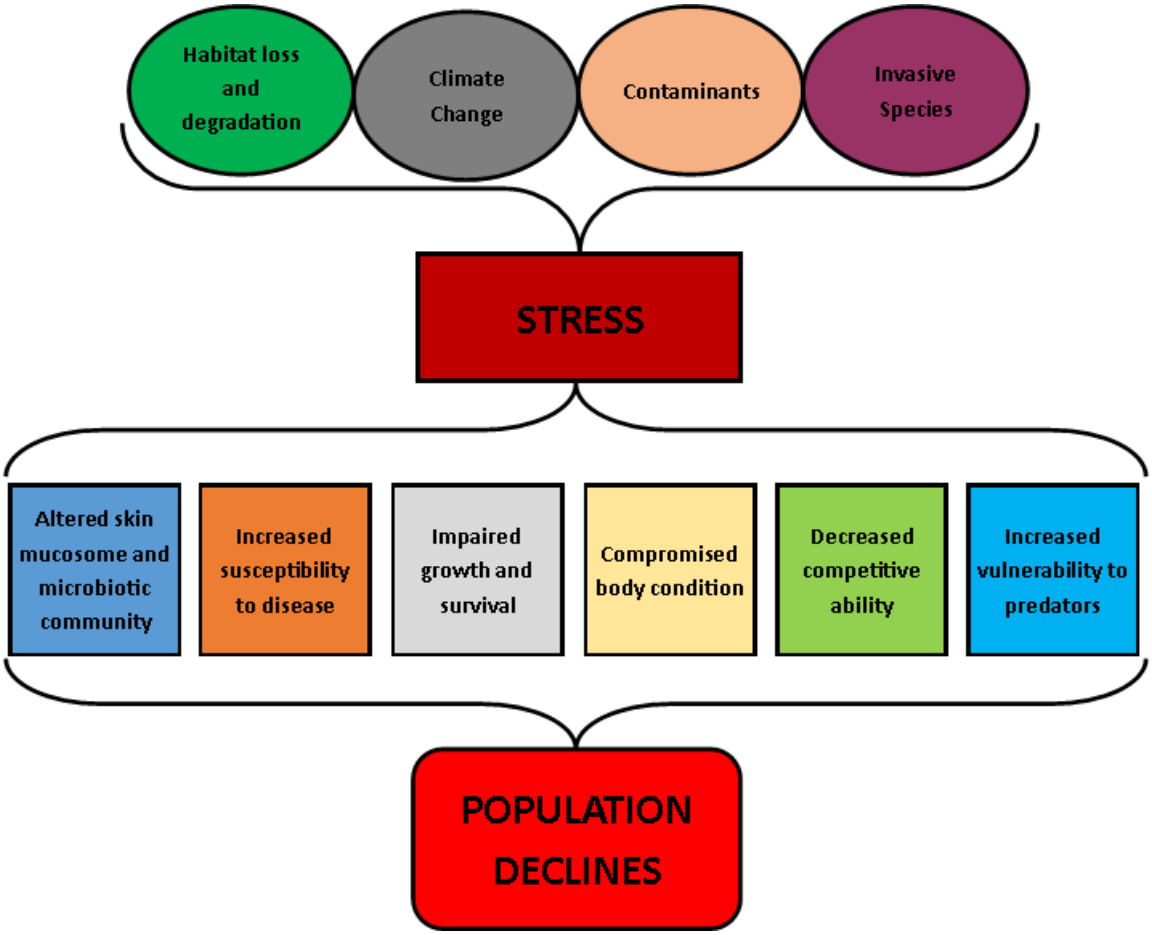

Figure 1. Conceptual model indicating links between environmental stressors, physiological stress, and potential responses that ultimately lead to population declines. Modified from diagram by S. Walls and reproduced with permission.

immune function (McEwen & Wingfield 2003; Rollins-Smith 2017; Romero 2004; Sapolsky et al. 2000). Comparing "baseline" GC levels with those in response to acute stressors has been used to identify populations under chronic stress, which may be at an increased risk of declines (Baugh et al. 2018; Dantzer et al. 2014; Gabor et al. 2018; Homan et al. 2003; Janin et al. 2011; Jeffrey et al. 2015).

Amphibian Stress Response

Corticosterone (CORT) is the main glucocorticoid released by amphibians and is a key hormone involved in the stress response, energy mobilization, development, and metamorphosis under normal levels (Denver 2009; Glennemeier & Denver 2002). Corticosterone increases in some amphibians during breeding season to assist in energy mobilization during courting and reproduction (Moore & Jessop 2003). Environmental conditions associated with decreased habitat quality may be perceived as stressors, which can increase GC hormones chronically, inhibit the stress response to additional stressors, or dysregulate the HPI axis function, affecting physiological health (Burraco & Gomez-Mestre 2016; Chambers et al. 2013; Gabor et al. 2018; Homan et al. 2003; Janin et al. 2011, 2012). Amphibians release CORT in response to many environmental stressors such as pond drying, high salinity, extreme pH and temperature, and other water quality variables, as well as changes in landscape characteristics such as forest fragmentation (Burraco & Gomez-Mestre 2016; Chambers et al. 2013; Denver 1998; Gomez-Mestre et al. 2013; Janin et al. 2011; Narayan & Hero 2014a, 2014b). Following acute stressors, amphibians activate the HPI axis which releases CORT to assist with energy mobilization, to stimulate immune function, and to synergize with thyroid hormone, speeding up metamorphosis (Denver 2009; Moore & Jessop 2003; Rollins-Smith 2017). However, depending on timing of events, some stressors suppress the HPI axis and subsequent CORT release (Crespi & Denver 2005; Middlemis-Maher et al. 2012). Under chronic stressors, amphibians may have abnormally low or elevated CORT concentrations which can suppress the HPI axis, affect growth and development, antipredator behavior and suppress reproductive behaviors (reviewed in Denver 2009;

Glennemeier & Denver 2002; Homan et al. 2003). Increased CORT in amphibians inhibits growth and development in larval amphibians and can also accelerate or decelerate metamorphosis in certain contexts (Denver 2009). Elevated CORT during metamorphosis and under chronic stress may be immunosuppressive and may make individuals more vulnerable to disease (reviewed in Rollins-Smith 2017, discussed below).

Stress and Immunity

Populations under chronic stress due to environmental factors, such as higher temperatures, are at increased risk of disease (Rollins-Smith 2017), which may lead to faster declines. Elevated CORT during metamorphosis and under chronic stress is immunosuppressive and may make individuals more vulnerable to disease (Rollins-Smith 2017). Elevated CORT inhibits the production of amphibian skin peptides (Rollins-Smith et al. 2011) and affects gut microbial communities in other taxa (Clarke et al. 2014). Amphibian skin is the primary line of defense against disease (Harris et al. 2009; Woodhams et al. 2007). Skin secretions including mucosal antibodies and antimicrobial peptides, combined with the native skin microbial community, create a mucosal layer on the skin that aids in immunity (Harris et al. 2009; Woodhams et al. 2014). Microbiota on the skin and in the gut aide in the amphibian immune response (Harris et al. 2009; Woodhams et al. 2016). The microbiota may vary with abiotic factors (Bletz et al. 2017a; Jani and Briggs 2018; Kohl et al. 2016; Varela et al. 2018) across populations (Hernandez-Gomez et al. 2017; Jani and Briggs 2018; Kueneman et al. 2013) and species (Belden et al. 2015; Kueneman et al. 2013; Varela et al. 2018). The various skin peptides

and microbiota that make up this micro-ecosystem of the mucus, or "mucosome" (Woodhams et al. 2014) can vary between species and affect innate immunity (McKenzie et al. 2012). Because of the variation in resistance between these mucosome components, examining the ability of the mucosome to fight infection (mucosome function) can aid in predicting populations at higher risk of disease (Blaustein et al. 2012; Woodhams et al. 2014). Exposure to disease not only affects microbiota but also induces a stress response in amphibians (Gabor et al. 2013b, 2015; Kindermann et al. 2017; Peterson et al. 2013; Warne et al. 2011). Therefore, increased stress from reduced habitat quality and/or disease may be associated with altered immune function and could lead to increased declines.

Changing environmental conditions, disease, stress from both, and their synergistic effects may play a role in amphibian declines (reviewed in Rollins-Smith 2017). Environmental changes, like increased temperature, increase stress hormone levels (Chambers et al. 2013; Millikin et al. 2019; Narayan & Hero 2014a, 2014b; Novarro et al. 2018) and increased stress can reduce immune function (Clarke et al. 2014; Rollins-Smith et al. 1997). Furthermore, disease itself can increase stress in amphibians (Warne et al. 2011; Gabor et al. 2013b). However, no studies to date have examined all of these factors in conjunction. If chronic stress induced by environmental changes can alter the microbiotic makeup and mucosome function in amphibian populations, these populations may be more vulnerable to disease, which can lead to further amphibian declines. In the same way, disease may increase stress, potentially altering the microbiotic makeup and mucosome function of amphibians, making them more vulnerable to other stressors. Therefore, multiple variables most likely drive amphibian declines and therefore these

factors and their synergistic effects need to be considered together.

Research Experiments

Measuring stress hormone levels (CORT profile) and the ability to mount a response to an acute stressor is one mechanism to assess physiological health and how populations respond to changing environmental conditions, providing a potential early-warning indicator of declines (Birnie-Gauvin et al. 2017; Dantzer et al. 2014; Homan et al. 2003; Sheriff et al. 2011). Furthermore, integrative measures of stress hormones, rather than traditional measures that use plasma or whole-body concentrations, provide quick, non-invasive methods which further aid in early identification of populations at higher risk of declines (Dantzer et al. 2014; Sheriff et al. 2011). Because increased stress affects the immune function, body condition, and survival of amphibians, examining these other physiological health metrics provides a more complete picture of the effects of environmental conditions. The research presented in this dissertation aimed to examine the effects of habitat quality and environmental factors on multiple measures of amphibian physiological health including the CORT profile, condition, immune function, and survival by incorporating both field and laboratory experiments.

II. CHANGES IN PHYSIOLOGY AND MICROBIAL DIVERSITY IN LARVAL ORNATE CHORUS FROGS ARE ASSOCIATED WITH HABITAT QUALITY

Abstract

Environmental change associated with anthropogenic disturbance can render habitats unsuitable, especially for sensitive species such as many amphibians. Variation in environmental quality may affect an organism's physiological health and, ultimately, survival and fitness. Using multiple health measures can aid in identifying populations at increased risk of declines. Our objective was to measure environmental variables at multiple spatial scales and their effect on three indicators of health in ornate chorus frog (*Pseudacris ornata*) tadpoles to identify potential correlates of population declines. To accomplish this, we measured a glucocorticoid hormone (corticosterone) profile associated with the stress response, as well as the skin mucosal immune function (combined function of skin secretions and skin bacterial community) and bacterial communities of tadpoles from multiple ponds. We found that water quality characteristics associated with environmental variation, including increased water temperature, conductivity, and pH, as well as percent developed land nearby were associated with increased corticosterone release rates. However, mucosal immune function, although highly variable, was not significantly associated with water quality or environmental factors. Finally, we examined skin bacterial diversity as it aids in immunity and is affected by environmental variation. We found that skin bacterial diversity differed between ponds and was affected by land cover type, canopy cover, and pond proximity. Our results indicate that both local water quality and land cover characteristics associated

with low habitat quality are important determinants of population health for ornate chorus frogs. Moreover, these proactive measures of health over time may aid in early identification of at-risk populations which could be used to prevent further declines and aid in management decisions.

Keywords: bacterial communities, environmental stress, water-borne hormones

Introduction

As the human population continues to increase, land-use conversion has altered habitat suitability for many species (McKinney 2002; Vitousek et al. 1997). Habitat loss, fragmentation and degradation, coupled with climate change and other anthropogenic factors, are among the most significant drivers of population declines and species' extinctions (Brook et al. 2008; Ceballos et al. 2017; Mantyka-Pringle et al. 2012; Segan et al. 2016). Landscape-level disturbances such as habitat loss and degradation affect populations by altering habitat connectivity, composition, and quality (Fahrig, 2003). For aquatic organisms, local impacts on water quality such as changes in temperature, pH, contaminant, nutrient and sediment levels (Meybeck, 2004) can also pose significant consequences to population health and resilience. Together, the additive or synergistic effects are especially troubling for species, especially those with complex life cycles that require multiple habitats to complete development (Blaustein et al. 2011; Hayes et al. 2010). Examining physiological responses of individuals to such factors can provide insight into the mechanisms by which environmental stressors can lead to population declines. In turn, understanding the consequences of these factors on populations could aid in better management practices for declining species.

Glucocorticoid (GC) hormones can be useful tools to assess the physiological response of organisms to environmental and anthropogenic stressors which, when combined with other metrics (e.g. immune function), can be indicators of individual and population health (Dantzer et al. 2014; Jeffrey et al. 2015; Sheriff et al. 2011). In response to stressors, GC hormones are released by the hypothalamo-pituitary-interrenal (HPI) axis above baseline levels as an adaptive response that assists in energy

mobilization, mediates natural changes in physiology and behavior, and helps return the organism to homeostasis (Cyr and Romero 2009; Romero et al. 2009; Sapolsky et al. 2000). However, under prolonged (chronic) stress, vertebrates may have elevated or lowered baseline GCs. In such cases, the HPI axis can become dysregulated (where individuals have a muted response to additional stressors), resulting in reduced physiological health, pathology, and ultimately death (Cyr and Romero 2009; McEwen and Wingfield 2003; Romero et al. 2009; Wingfield and Romero 2001). Vertebrates under chronic stress have suppressed growth and development, reduced reproduction, and reduced immune function (McEwen and Wingfield 2003; Rollins-Smith 2017; Romero 2004; Sapolsky et al. 2000). Pre-stressor baseline GC levels and the response to acute stressors (HPI axis responsiveness) can be measured to aid in identifying whether populations are under chronic stress, which may be associated with higher risk of declines (Baugh et al. 2018; Dantzer et al. 2014; Gabor et al. 2018; Homan et al. 2003). Measuring glucocorticoid hormones may identify populations to target for management but may not provide a full picture of environmental effects on population health. Using additional metrics such as immune function and microbial diversity are needed for a more holistic view of how environmental stressors impact population health (Breuner et al. 2013).

Amphibians have the highest threat status of all vertebrate classes (Foden et al. 2013; Stuart et al. 2004) and are particularly susceptible to changes in habitat quality. Species that are especially vulnerable use ponds or other water bodies to breed but occupy terrestrial habitats outside the breeding season (Becker et al. 2007). Environmental changes and conditions associated with lower quality habitat may be

perceived as stressors, which elevate or dysregulate GC hormones and affect physiological health (Chambers et al. 2013; Gabor et al. 2018; Homan et al. 2003; Janin et al. 2011, 2012). Amphibians release the hormone corticosterone (CORT; the primary amphibian GC) when exposed to various stressors, including increased pond drying, high salinity, extreme pH and temperature, and other water quality variables (Burraco and Gomez-Mestre 2016; Chambers et al. 2013; Denver 1998; Gabor et al. 2018; Gomez-Mestre et al. 2013; Narayan and Hero 2014a, 2014b). In addition, landscape characteristics such as the extent of canopy cover and forest fragmentation around breeding ponds, as well as substrate type, are significant predictors of CORT in adult common toads (*Bufo bufo*, Janin et al. 2011, 2012) and spotted salamanders (*Ambystoma maculatum*, Homan et al. 2003), and increased CORT in Jollyville Plateau salamanders (*Eurycea tonkawae*) has been associated with more urbanized streams (Gabor et al. 2018). These findings suggest a link between habitat quality at multiple spatial scales and physiological health in amphibians.

Microbiota play a role in amphibian immune response to disease (Harris et al. 2009; Woodhams et al. 2016) and may vary with abiotic factors (Bletz et al. 2017a; Jani and Briggs 2018; Varela et al. 2018), across populations (Hernandez-Gomez et al. 2017; Jani and Briggs 2018; Kueneman et al. 2013), and among species (Belden et al. 2015; Kueneman et al. 2013; Varela et al. 2018). Microbiota living on the skin combined with skin secretions make up the mucosome, or micro-ecosystem of the skin, providing the first line of defense in amphibian disease resistance (Harris et al. 2009; Woodhams et al. 2007, 2014). Identifying the bacterial community of amphibians in a population and the ability of their mucosome to fight infection (mucosome function; Woodhams et al. 2014)

can be used to predict disease risk across populations (Woodhams et al. 2014). Disease in amphibians is associated with increased CORT (Gabor, Fisher, et al. 2013, 2015, 2017; Kindermann et al. 2017; Peterson et al. 2013; Warne et al. 2011) and elevated CORT is immunosuppressive (reviewed in Rollins-Smith 2017), inhibits production of amphibian skin peptides (Rollins-Smith et al. 2011), and affects gut microbial communities in other taxa (Clarke et al. 2014). Further, the microbiome of amphibians shifts with temperature (Kohl et al. 2016), soil pH, precipitation (Varela et al. 2018), and infection intensity (Jani and Briggs 2018). Therefore, reduced habitat quality and increased stress from environmental stressors may be associated with altered skin bacterial communities and immunity in amphibians, which could lead to increased declines.

To explore potential underlying causes of population declines in the ornate chorus frog (*Pseudacris ornata*), we measured environmental variables at multiple spatial scales, including pond water quality and surrounding landscape characteristics, and examined their effect on three health metrics in tadpoles. Because of the known interactions between habitat quality and population health, we measured and examined the relationships of these variables with CORT release rates, mucosome function, and bacterial community diversity from populations of *P. ornata* across their range. We tested several hypotheses to assess whether environmental quality affects tadpole health: first, we quantified baseline and acute stress-induced CORT release rate profiles for tadpoles collected across the species' range. We hypothesized that reduced habitat quality at multiple spatial scales affects CORT release rates and the CORT profile in *P. ornata* larvae, as environmental conditions are associated with increased stress in amphibians (Chambers et al. 2013; Gabor et al. 2018; Homan et al. 2003; Janin et al. 2011, 2012).

We also tested the hypotheses that environmental conditions and CORT release rates are associated with altered immune function and an altered skin bacterial community, as both environment and stress are associated with altered immune defenses (Bletz et al. 2017a; Jani and Briggs 2018; Rollins-Smith 2017; Varela et al. 2018). Our examination of multiple metrics of population health allows us to potentially identify links between environmental stress from reduced habitat quality and population declines.

Methods

Study species

The ornate chorus frog (*Pseudacris ornata*) is endemic to the southeastern Coastal Plain and longleaf pine (*Pinus palustris*) ecosystem of the southeastern United States (U.S.; Means, 2006). This frog has a disjunct range that extends from North Carolina south to Florida and west to Louisiana (Enge et al. 2014; Powell et al. 2016). The ornate chorus frog is a longleaf pine specialist common throughout the panhandle and northern regions of Florida in mesic and xeric habitats, but this species has been declining along its southern range edge in the Florida peninsula and western edge in Louisiana (Bartlett and Bartlett, 2011; Enge et al. 2014). Declines are potentially caused by increased temperatures, droughts, and reduced habitat quality associated with fire suppression (Enge et al. 2014; Means and Simberloff, 1987). Fire suppression and insufficient management can reduce environmental quality for species endemic to pyrogenic systems, such as *P. ornata* and the longleaf pine-wiregrass (*Pinus palustris-Aristida* spp.) ecosystem of the southeastern U.S. (Noss 2013, 2018). Hydroperiod is also considered an important factor associated with *P. ornata* population persistence, as this species requires

fishless, seasonally-inundated water bodies with a 3 to 4-month hydroperiod for complete tadpole development (Enge et al. 2014; Semlitsch et al. 1996). Though little is known about the adult frog, it is known to be fossorial, using loose, sandy soils to burrow, and has been found over 400 m from breeding ponds (Brown and Means 1984). Adults are winter breeders, with males calling as early as November, with actual breeding occurring December or January through March, when females deposit eggs on submerged vertical vegetation (Dorcas and Gibbons 2008; Enge et al. 2014). Aquatic breeding sites range from roadside ditches, flooded fields and marshes adjacent to forested areas, as well as pine and mixed-woodland ponds (Bartlett and Bartlett 2011; Dorcas and Gibbons 2008; Enge et al. 2014; Powell et al. 2016). However, most breeding ponds are found within sandhills, flatwoods, wetlands, and pine forest/plantations, and are associated with open-canopy sites with herbaceous understory, characteristic of a short-interval fire regime (Enge et al. 2014; Gorman et al. 2013; Noss 2013, 2018).

Field sampling

We collected tadpoles (stages 30-40; Gosner 1960) across seven properties and up to four ponds (sites) per property for a total of 16 ponds throughout the species' range in Florida, Georgia, and South Carolina (Table 1, Fig. 2). Ponds were sampled 2 March – 23 March 2016 and 23 February – 14 March 2017 (Table 1). At each pond, we recorded water temperature, conductivity, and total dissolved solids (TDS); pH was also collected from ponds in 2017 (Table 2). Additionally, we conducted a Geographic Information Systems (GIS) analysis of land cover at each pond using land cover class and percent canopy cover data from the National Land Cover Database (NLCD 2011). Using ArcMap

Table 1. Pond property, state, sites within each property, and dates sampled.

Region	State	Site	Dates Sampled
Eglin Airforce Base	FL	EG1	2 March 2016 and 23 February 2017
		EG2	24 February 2017
Apalachicola National Forest	FL	AP1	3 March 2016 and 26 February 2017
		AP2	4 March 2016 and 25 February 2017
		AP3	4 March 2016
St. Marks National Wildlife Refuge	FL	SM1	8 March 2016
		SM2	8 March 2016
		SM3	28 February 2017
		SM4	1 March 2017
Joseph W. Jones Ecological Research Center at Ichauway	GA	JC1	22 March 2016 and 4 March 2017
		JC2	22 March 2016 and 2 March 2017
		JC3	23 March 2016 and 3 March 2017
Lafayette Forest Wildlife Environmental Area	FL	LF1	15-16 March 2016
Orianne Society Preserve	GA	OS1	9 March 2017
		OS2	10 March 2017
James W. Webb Wildlife Center	SC	WC1	14 March 2017

Table 2. Water quality variable ranges and average values across all sites sampled for each year.

Variable	Range		Average	
	2016	2017	2016	2017
Water Temperature (°C)	13.6 - 23.1	11.3 - 24.3	17.2	19.2
Conductivity (µs/cm)	12.1 - 49.4	13.0 - 48.7	22.8	25.5
pH	N/A	4.12 - 5.95	N/A	5.17

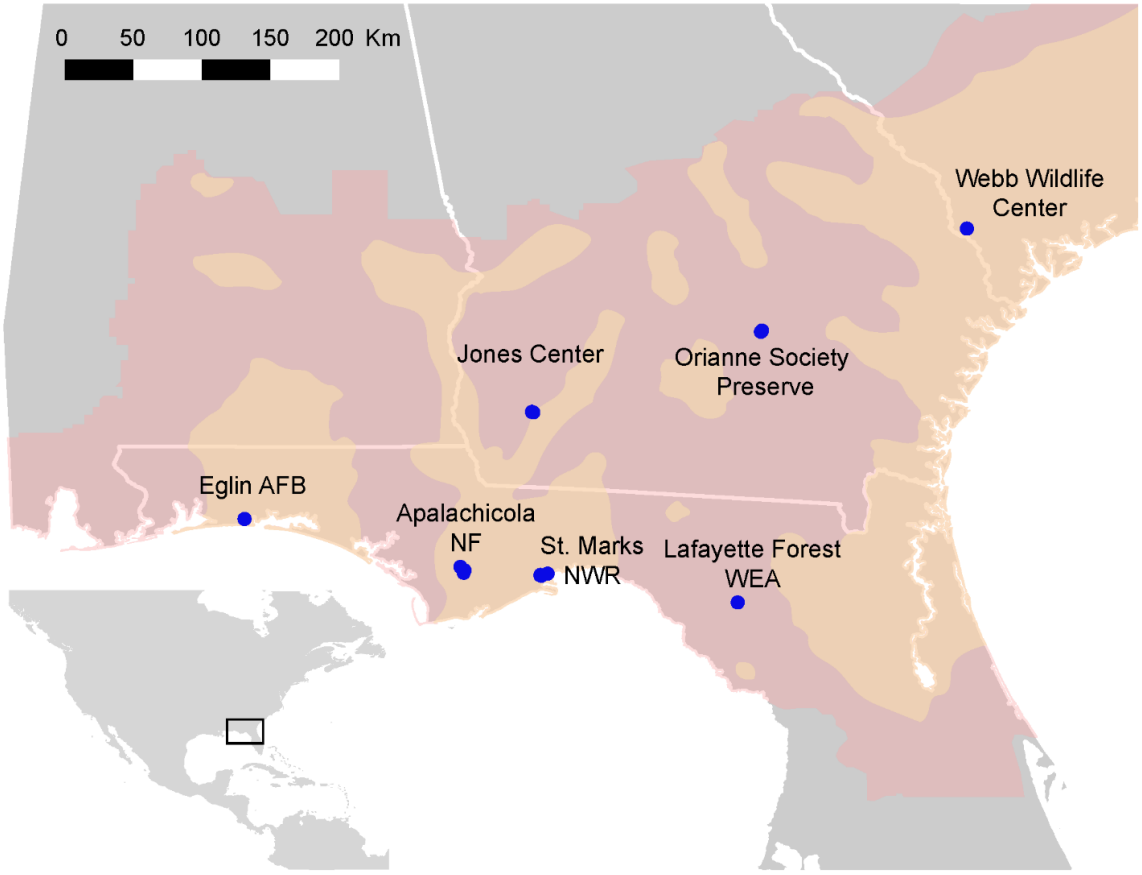

Figure 2. Approximate pond locations (blue dots) throughout the range of *Pseudacris ornata*. Pink shading represents historical range (International Union for the Conservation of Nature [IUCN] 2015) and orange shading represents newest range map (Powell et al. 2016). Ponds are located on the following properties: Apalachicola National Forest (NF), Eglin Air Force Base (AFB), Joseph W. Jones Ecological Research Center at Ichauway, Lafayette Forest Wildlife Environmental Area (WEA), St. Marks National Wildlife Refuge (NWR), Orianne Society Preserve, and the James W. Webb Wildlife Center.

(ESRI, Redlands, CA), we created a 100 m, 500 m, and 1000 m buffer around each pond. Land cover classes were determined for each 30 m pixel within the three spatial scales. The 16 land cover types recognized by the NLCD were combined into six classes for analysis: Agriculture, Developed, Forest, Shrub, Water, and Wetlands. The Agriculture class included grasslands, pasture, and cultivated crops; Developed included all intensities of developed and barren land; Forest included deciduous, evergreen, and mixed forest cover; Shrub consisted of short, woody plants; Water was characterized by open ponds; and Wetlands included woody and emergent herbaceous wetlands. We then quantified the percent of each land cover type and the percent canopy cover within the three spatial scales using the zonal statistics as a table tool in ArcMap. We used the primary land cover class, percent developed land, and percent canopy cover for each of the three spatial scales in analyses.

Water-borne CORT release rates

In 2016 and 2017, we used a non-invasive water-borne hormone method (Gabor, Bosch, et al. 2013, 2016) to measure CORT release rates from up to 40 individual tadpoles from each pond. This method measures CORT secreted through the skin, urine, and feces, which provides an integrated measure of the cumulative effects of chronic stress (Dantzer et al. 2014; Sheriff et al. 2011). We captured tadpoles using dipnets and then placed individuals in 250 ml beakers, one individual per beaker, containing 100 ml of bottled spring water and a perforated Nalgene liner. This liner allowed us to remove the tadpole but leave the water sample in the beaker. Half of the individuals were unmanipulated for 1 hour to obtain baseline CORT release rates and the other half were agitated by shaking the beakers for 1 minute every 3 minutes for 1 hour to measure the

response to the acute agitation stressor (Forsburg et al. 2019; Gabor et al. 2016). These baseline and agitation CORT release rates represent the treatment categories. After 1 hour, we removed the liner with the individual from each beaker and poured the water containing leached hormones into HDPE plastic cups. Samples were maintained on ice and transported to the laboratory for immediate extraction or frozen at -20 °C to be processed at a later time (Ellis et al. 2004). We photographed each tadpole from the side with a ruler for scale before releasing it back into the pond. Snout-vent length (SVL, in mm) was measured from photographs using the program ImageJ (Rosband, 1997). In 2017, all tadpoles from which water-borne hormones were collected were also swabbed for skin bacterial community analysis. After the water samples containing leached hormones were collected, each individual was swabbed ventrally from the mouth to the vent using a single, sterile swab. The cotton tips of each swab were placed in Eppendorf tubes, frozen, and transported back to the lab for analysis.

Laboratory analysis

Once defrosted, we filtered each water sample through standard coffee filters (equivalent to grade 4 filter paper) to remove large debris and fecal material. All samples were then drawn through C18 solid phase extraction (SPE) columns (SepPak Vac 3 cc/500 mg; Waters, Inc.) primed with 4 ml methanol and 4 ml distilled water (Gabor et al. 2016). After hormone extraction, we eluted columns with 4 ml methanol into borosilicate test tubes. Eluted samples were dried using nitrogen gas flowing through an Evap-O-Rac (Cole-Palmer) while sitting in a hot water bath (37 °C). Each dried sample was then resuspended in a mixture of 5% ethanol and 95% enzyme-immunoassay (EIA) buffer (Cayman Chemical Company, Inc.) to a final volume of 260 µl. We also ran water

samples from each pond and blank controls of bottled spring water resuspended to a final volume of 130 µl. Each sample was run in duplicate on corticosterone EIA plates (n=26; Cayman Chemical Company, Inc.) and absorbance was read using a spectrophotometer plate reader (BioTek ELX 800) set to 405 nm. Final CORT values (pg/ml) were multiplied by amount resuspended (0.260 ml) and divided by SVL for final unit of pg/SVL/h. Water samples were multiplied by reconstitution volume (0.130 ml) and the relevant spring water values (5.4 to 15.6 pg/sample) were subtracted from the CORT release rates of each tadpole.

The water-borne CORT collection method was validated for *P. ornata* using a pooled sample of hormones from 7 non-experimental tadpoles serially diluted (Gabor et al. 2016). We examined parallelism of the serial dilution curve (1:1–1:32) of the pooled sample to the known standard curve (comparison of slopes, $t_{11} = 1.304$, $p = 0.22$). We also assessed quantitative recovery by spiking the pooled sample with each of eight standards. The minimum observed recovery was 62%, and we found a linear relationship between observed and expected slopes ($\beta = 0.77$, $F_{1,6} = 1817.75$, $R^2 = 0.997$, $p < 0.0001$). Using a different pooled control sample run in quadruplicate on each plate, we examined the intra- and inter-plate variation of the 26 plates. Intra-plate variation ranged from 0.29-16.33% and mean inter-plate variation was 13.11%. The sensitivity of the CORT EIA plates ranged from 41.47 to 1390.60 pg/ml on average.

In 2017, 1 ml water was removed from each baseline CORT sample and stored at -20 °C to assess mucosome function. Using the Cell Titer Glo 2 kit (Promega), a 25 µl water sample – containing host skin peptides, mucosal antibodies, and bacterial communities – was combined with a 25 µl solution of *Batrachochytrium dendrobatidis*

(*Bd*) zoospores with known concentration (~25,000 total spores per 25 µl) on 96-well plates (Barnhardt 2018). *Bd* zoospores were collected from plate cultures of the Global Panzootic Lineage (BdGPL), JEL423, by flooding 3-5 day old culture plates with autoclaved water to stimulate zoospore release from sporangia. This *Bd* strain was used because it was readily available and easy to grow in the laboratory. Each mucosome sample was then plated in triplicate to assess *Bd* viability in the presence of the mucosome. In addition, six heat-killed *Bd* standards (0%, 20%, 40%, 60%, 80%, 100%) were plated in triplicate. After incubation for 1 hr at room temperature, 50 µl of Cell Titer Glo Reagent was added to each well and placed on an orbital shaker at 200 rpm for 2 min and incubated at room temperature for an additional 15 min. A luminescent plate reader was then used (Biotek Synergy H1) to assess the percent cell viability from a ratio of live:dead *Bd* to determine the mucosome function against the pathogen. The gain of the plate reader was set at 150.

We extracted DNA from skin swabs of 10 tadpoles per pond using 50 µl of PrepMan (Applied Biosystems, Inc.) following the manufacturer's protocol. Swabs and extracts were spun down briefly then the swab was inverted with sterile forceps and spun down again at 2,348 x g for 1 min. The swab was removed from the tube with sterile forceps and the remaining extract was centrifuged at 21,130 x g for 5 mins to pellet any precipitates. Without disturbing the pellet, 40 µl of extract was transferred to a new 1.5 ml centrifuge tube (adapted from Becker et al. 2015). The purified extracts were used to generate PCR amplicons of bacterial 16S V4 properties using 515F and GOLAY barcoded 806R primers (Caporaso et al. 2011) following the Earth Microbiome Protocol (Gilbert et al. 2014). Amplicons were quantified using a Qubit dsDNA Broad sensitivity

Assay Kit (Invitrogen) and, after normalizing and pooling, amplicons were size selected on a BluePippen (SageScience, Inc.) using a 2% agarose 100-600 bp cartridge, the resulting fraction was cleaned using AMPure XP magnetic beads (Beckman Coulter, Inc). The pooled library size (~350bp) and concentration were verified on a TapeStation 2200 (Agilent Technologies, Inc.) using a D1000 screen tape and reagents. Sequencing was performed on a MiSeq instrument (Illumina, Inc.) using the 600-cycle MiSeq Reagent Kit v3. The resulting reads from the MiSeq were trimmed of adapters and parsed in BaseSpace (Illumina, Inc.) according to their respective barcode. The dada2 version 1.5.0 pipeline (Callahan et al. 2016) in R (version 3.5.2; R Core Team 2018) was used to first inspect read quality profiles and then filter and trim reads. Reverse reads were not used in downstream analyses owing to low quality and, thus, paired read merging was skipped after dereplication. Forward sequence reads were trimmed to 220 bp based on their quality profile. Taxonomy assignment was performed using dada2 formatted fasta file from the Silva taxonomic training data (version 132). The resulting dada2 tables were merged with the sample metadata and analyzed using phyloseq (McMurdie and Holmes 2013). Using phyloseq, samples were rarified to 25000 sequences (average number of sequences = 229663) and alpha diversity indices (Richness, Shannon, and Simpson) were calculated. The final data set consisted of 8337 OTUs across 98 samples.

Statistical analysis

All analyses were run using R (version 3.5.2; R Core Team 2018). First, to determine if there was spatial autocorrelation in CORT profiles across ponds, distance-based Moran's Eigenvector Mapping (dbMEM) was used to map longitude and latitude for each pond and build spatial predictors using the following packages: adespatial (Dray

et al. 2018), geosphere (Hijmans 2017), and vegan (Oksanen et al. 2019). Permutation ANOVA on the distance-based Moran axes identified 2 of 3 spatial axes as significant predictors of CORT variation. These two spatial axes, MEM2 and MEM3, corresponded to scaled distance measures of longitude and latitude, respectively. These axes were extracted and incorporated into model building. Eigenvalues for these two positive spatial axes were -0.78 and 0.24, respectively, indicating they represent autocorrelation between ponds at a fine spatial scale.

We developed linear mixed effect models (lme) in the package nlme (Pinheiro and Bates 2018) to examine predictors of (1) CORT release rates, (2) mucosome function, and (3) skin bacterial diversity indices (Richness, Shannon, Simpson) among ponds as separate response variables. All models for all response variables included Site as a random effect. CORT models examined natural log-transformed CORT release rates standardized by SVL (pg/SVL/h) as the response variable Prior to building models, we first ran a preliminary analysis of models containing each land cover characteristic calculated from NLCD variables (Land cover type, percent urban development, and percent canopy cover) at each spatial scale as sole predictors for each of the response variables. To determine the most important scale for the three predictors, the models containing the landcover characteristic at the different scales were ranked according to Akaike's Information Criterion adjusted for small sample size (AICc) and the scale included in the highest ranked model was retained for subsequent models. Water quality variables (water temperature, conductivity, TDS, pH) from all ponds were combined using a principle components analysis (PCA) to reduce the number of variables in analyses. We ran analyses to assess predictors of CORT release rates for each year (2016

and 2017) separately, as not all ponds were sampled in both years and pH was an additional pond characteristic added in 2017. For 2016, PCA revealed that PC1 accounted for 83.3% of the variation in the data and was driven by water temperature, conductivity, and TDS, whereas PC2 accounted for 16.7% of the variation and was mainly driven by water temperature (Table 3). In 2017, PC1 accounted for 50% of the variation and was driven by conductivity, TDS, and pH, whereas PC2 accounted for 30.1% of the variation and was driven by water temperature and DO (Table 3). To test the relationship of

Table 3. Eigenvectors and principle components for PCA of water quality variables collected at ponds in 2016 and 2017. Variables that loaded heavily on components are in bold.

Variable	2016		2017	
	PC1	PC2	PC1	PC2
W_Temp	0.503	**-0.858**	-0.206	**0.876**
Conductivity	**0.620**	0.274	**-0.630**	0.116
TDS	**0.602**	0.435	**-0.614**	-0.091
pH			0.429	0.460

predictors to each of the three response variables, models incorporated main effects including the dominant land cover type within 100 m of ponds (Landcover100), the percent developed land within 1000 m of ponds (Urban1000), the percent canopy cover within 500 m of ponds (Canopy500), PC1 and PC2 for each year, spatial axes (MEM2 and MEM3), as well as additive models. We created a total of 27 models for analyses (Table S1). Additionally, we analyzed each treatment (Baseline or Agitation) separately within each year. The same list of models was used to examine predictors of mucosome function (Table S2) and skin microbial diversity (Table S3), substituting the correct scale of the landscape predictors as determined by separate preliminary analyses, and adding

baseline CORT release rates (BCORT) as a sole predictor in an additional model. This resulted in 28 models for mucosome and skin bacterial diversity analyses. We used each bacterial diversity index (Richness, Shannon, and Simpson) as a separate response variable in separate analyses involving the entire model set. All models were ranked according to Akaike information criterion corrected for small sample size (AIC_c) using the package MuMIn (Barton 2018). We calculated parameters using the maximum-likelihood estimation during the model-selection process. Model-averaged parameter estimates, unconditional standard error (SE) and unconditional 95% confidence intervals were calculated for candidate models ($\Delta AICc < 2$) using the package AICcmodavg (Mazerolle 2019). In addition, we ran independent t-tests to compare baseline and agitation-induced CORT release rates for each pond and year separately to specifically address which populations might be chronically stressed, indicated by an inability to mount a CORT response above baseline levels. We also ran an ANOVA and Levene's test using the package lawstat (Gastwirth et al. 2017) to examine differences in mean mucosome function and mean variance across Sites.

Means of each of the three alpha diversity indices were compared across Site and Property. We analyzed mean Shannon diversity across Sites and Properties using an analysis of variance (ANOVA). Because data were non-normal, we used a Kruskal-Wallis test to compare the mean Richness and Simpson diversity (evenness) across Sites and Properties. The relative contribution of Site and Property to microbiome diversity (beta diversity) were analyzed using PERMANOVA (adonis; 999 permutations) using the package vegan (Oksanen et al. 2019). All pairwise comparisons were assessed for significant factors using the package pairwiseAdonis (Martinez Arbizu et al. 2019). Beta

diversity of skin bacterial communities (OTUs) were plotted using the Bray-Curtis method of non-metric multidimensional scaling (NMDS) and the following packages: phyloseq (McMurdie and Holmes 2013) and ggplot2 (Wickham et al. 2016).

Results

Water-borne CORT release rates

AICc model selection indicated six candidate models (ΔAICc < 2) to predict baseline CORT release rates and five candidate models to predict agitation CORT release rates for sites sampled in 2016 (Table S4). Model averaged parameter estimates indicated both PCs and both spatial axes were top predictors of baseline CORT release rates, though no predictor was significant (CI did not overlap 0; Table 4). Model averaged parameter estimates indicated both PCs and MEM3 were significant predictors of agitation CORT release rates in 2016 (Table 4). Agitation CORT release rates were higher in ponds with higher conductivity and TDS (positive loading on PC1; Fig. 3a) and lower in ponds with lower water temperature (negative loading on PC2; Fig. 3b). Additionally, agitation CORT release rates were higher at lower MEM3 values (Fig. 4). For 2017, two models predicting baseline CORT release rates and two models predicting agitation CORT release rates had a ΔAICc < 2 (Table S5). Model averaged parameter estimates indicated the percent developed land within 1000 m of ponds was a significant predictor of both baseline and agitation CORT release rates for ponds sampled in 2017 (Table 5). Baseline and agitation CORT release rates were both higher in ponds with more developed land within 1000 m (Fig. 5). In 2016, the marginal R^2 for the top model explaining baseline and agitation CORT release rates was 0.24 and 0.26, respectively.

The conditional R^2 for these same models were 0.34 and 0.32, respectively. The marginal R^2 is calculated using only the fixed effects, whereas the conditional R^2 includes both fixed and random effects. Therefore, the inclusion of random effects and fixed effects explained more variation in CORT release rates than fixed effects alone. The difference between marginal and conditional R^2 values was greater for 2017, where the marginal R^2 for the top model explaining baseline CORT release rates was 0.23, and for agitation was 0.21, with the conditional R^2 values of 0.38 and 0.60, respectively. Average CORT release rates across both years were highest at the site in Lafayette Forest Wildlife Environmental Area (LF1; Fig. 6). Examining CORT release rates within each treatment for each pond indicated 40% (n = 4) of ponds sampled in 2016 and 50% (n = 6) of ponds sampled in 2017 did not show significant differences between baseline and agitation CORT release rates, though only one population (JC3) did not show a difference across both years (Table S6; Fig. 6). However, not all ponds were resampled both years owing to a lack of tadpoles or complete loss of the pond in one case.

Mucosome function

There was no significant difference in the mean mucosome function across all ponds (ANOVA: $F_{11,109} = 0.476$, $p = 0.914$; Fig. S1). Levene's test further determined no difference in the mean variance across all ponds ($W = 1.19$; $p = 0.302$). Mean *Bd* viability in the presence of the mucosome was 88.1%. Five candidate models (ΔAICc < 2) were selected from AICc model selection to explain the variation in mucosome function (Table S5). These models included PC1, PC2, percent developed land within 100 m (Urban100) and the percent canopy within 500 m (Canopy500) as top predictors, though model averaged parameter estimates did not indicate any significant predictor (Table 5).

Table 4. Model averaged parameter estimates, unconditional standard error (SE), and unconditional 95% confidence intervals (CI) of environmental variables on baseline and agitation corticosterone (CORT) release rates of *Pseudacris ornata* tadpoles from 10 sites sampled in 2016. Only parameters from the top ranking models (ΔAICc < 2) are included. Bolded terms indicate parameters used for inference whose 95% confidence intervals do not overlap 0.

Response	Predictor	Estimate	SE	Lower 95% CI	Upper 95% CI
Baseline CORT	PC1	0.1798	0.094	-0.0045	0.3641
	PC2	-0.2249	0.1167	-0.4537	0.0039
	MEM2	0.1981	0.1097	-0.0169	0.4131
	MEM3	0.0068	0.2336	-0.4511	0.4647
Agitation CORT	**PC1**	**0.1902**	**0.0447**	**0.1026**	**0.2778**
	PC2	**-0.2857**	**0.098**	**-0.4777**	**-0.0936**
	MEM2	0.0808	0.0814	-0.0787	0.2403
	MEM3	**-0.2326**	**0.0517**	**-0.334**	**-0.1313**
	Canopy500	-0.008	0.0059	-0.0194	0.0035

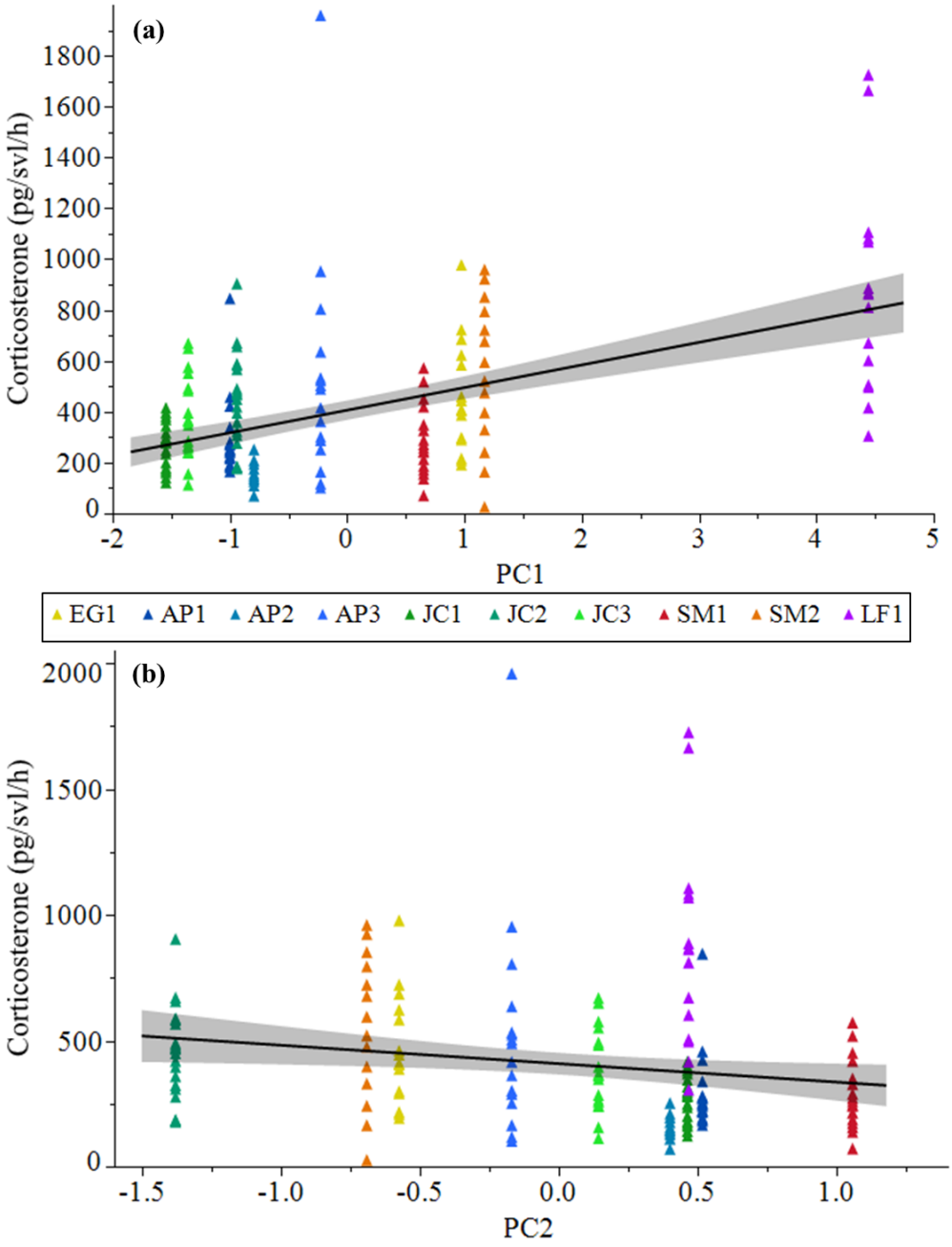

Figure 3. Relationship between corticosterone release rates (pg/SVL/h) and (a) PC1 or (b) PC2 values. Data were collected from *Pseudacris* ornata tadpoles and ponds sampled in 2016. Untransformed data are shown. Ponds: AP = Apalachicola National Forest, EG = Eglin Air Force Base, JC = Joseph W. Jones Ecological Research Center at Ichauway, LF = Lafayette Forest Wildlife Environmental Area, SM = St. Marks National Wildlife Refuge.

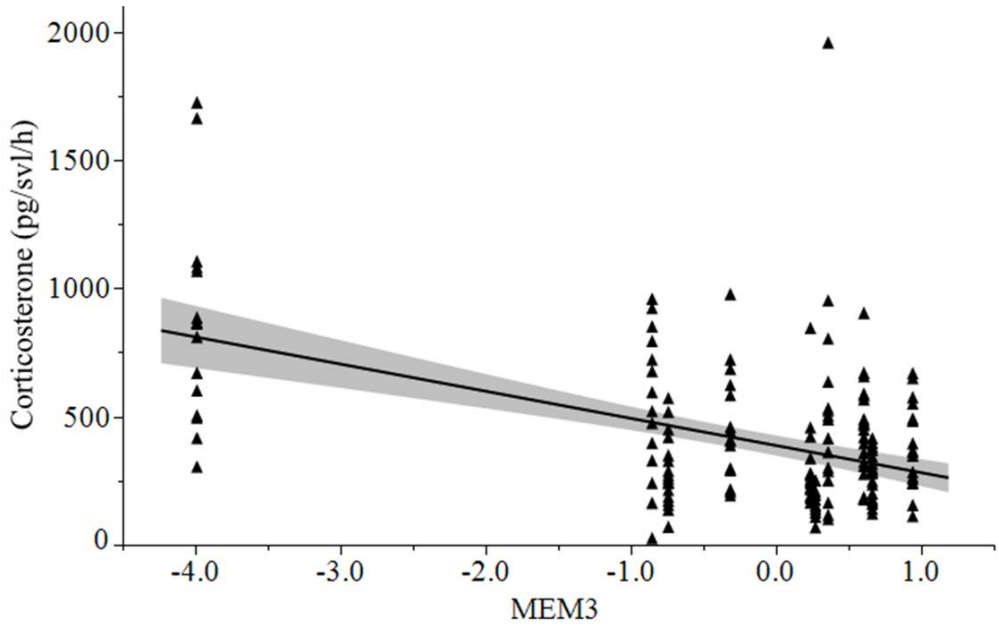

Figure 4. Relationship between corticosterone release rates (pg/SVL/h) and MEM3 values. Data were collected from *Pseudacris* ornata tadpoles and ponds sampled in 2016. Untransformed data are shown.

Table 5. Model averaged parameter estimates, unconditional standard error (SE), and unconditional 95% confidence intervals (CI) of environmental variables on baseline and agitation corticosterone (CORT) release rates, mucosome function, and bacterial diversity (Richness, Shannon and Simpson) of *Pseudacris ornata* tadpoles from 12 sites sampled in 2017. Only parameters from the top ranking models (ΔAICc < 2) are included. Bolded terms indicate parameters used for inference whose 95% confidence intervals do not overlap 0.

Response	Predictor	Estimate	SE	Lower 95% CI	Upper 95% CI
Baseline CORT	**Urban1000**	**0.117**	**0.037**	**0.046**	**0.189**
	PC2	0.082	0.103	-0.120	0.284
Agitation CORT	**Urban1000**	**0.126**	**0.053**	**0.022**	**0.231**
	PC1	-0.039	0.086	-0.206	0.129
Mucosome	PC1	-0.396	0.770	-1.905	1.113
	PC2	-2.279	1.320	-4.866	0.309
	Urban100	-0.095	0.076	-0.244	0.054
	Canopy500	-0.718	0.096	-0.259	0.116
Bacterial Richness	**Landcover1000: Forest**	**386.824**	**32.137**	**323.836**	**449.811**
	Landcover1000: Wetlands	**-265.525**	**89.063**	**-440.085**	**-90.965**
	Landcover1000: Shrub	**-147.176**	**69.089**	**-282.588**	**-11.763**
	PC1	12.786	16.991	-20.516	46.087
Shannon diversity	**Urban500**	**0.060**	**0.027**	**0.006**	**0.114**
	Canopy100	**-0.012**	**-0.006**	**-0.023**	**-0.002**
	PC1	0.093	0.076	-0.056	0.241
	PC2	-0.167	0.096	-0.356	0.021
	MEM2	**0.251**	**0.110**	**0.036**	**0.466**
Simpson diversity	**MEM2**	**0.046**	**0.014**	**0.019**	**0.073**
	MEM3	0.024	0.031	-0.036	0.084

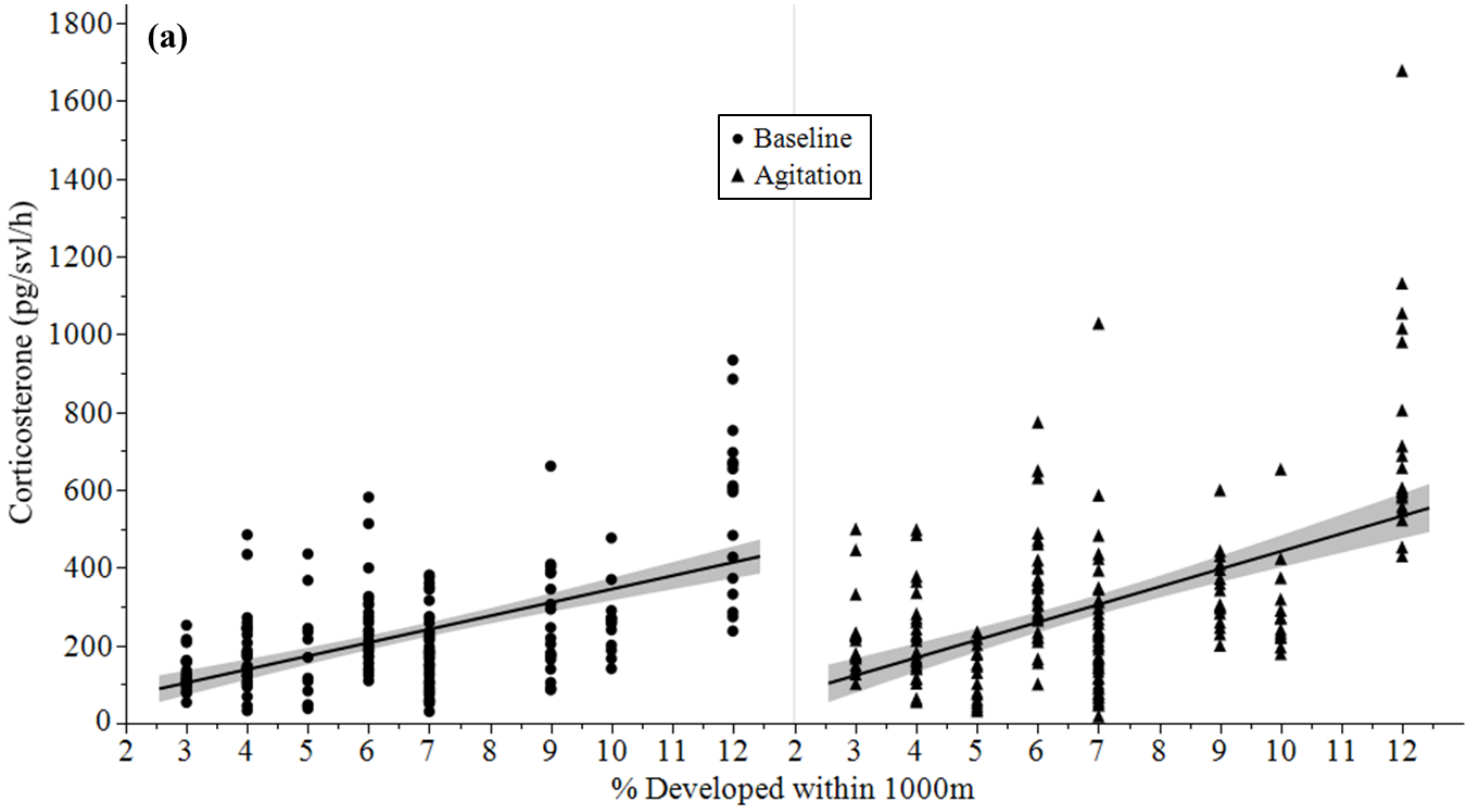

Figure 5. Relationship between (a) baseline and (b) agitation corticosterone release rates (pg/SVL/h) and the percent developed land within 1000 m. Data were collected from *Pseudacris* ornata tadpoles and ponds sampled in 2017. Untransformed data are shown.

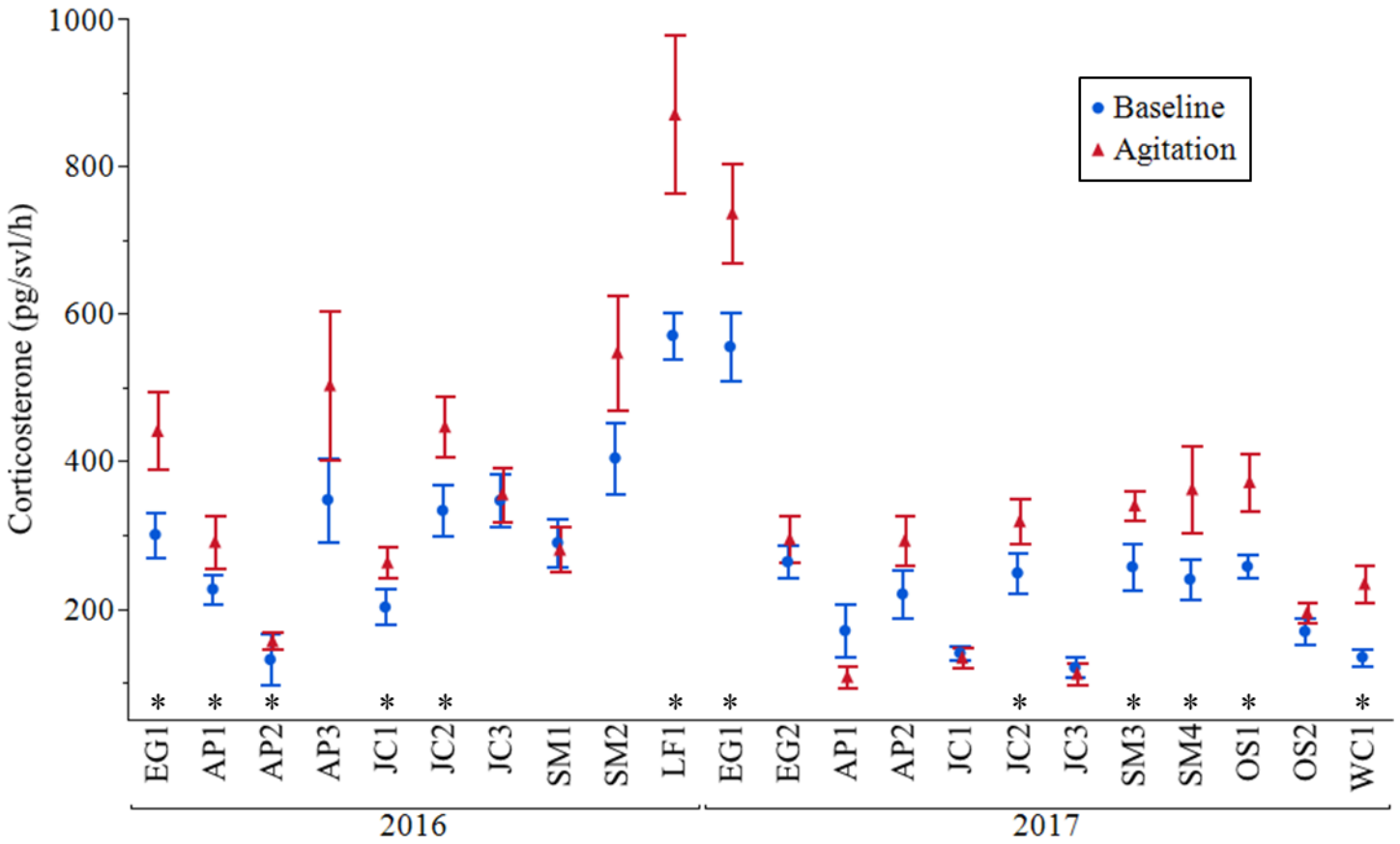

Figure 6. Mean (± SE) corticosterone release rates (pg/SVL/h; untransformed data shown) of *Pseudacris ornata* tadpoles for both treatments (Baseline or Agitation) at each pond across both years. Blue circles = Baseline values, Red triangles = Agitation values. Asterisks indicate significant differences. Ponds: AP = Apalachicola National Forest, EG = Eglin Air Force Base, JC = Joseph W. Jones Ecological Research Center at Ichauway, LF = Lafayette Forest Wildlife Environmental Area, SM = St. Marks National Wildlife Refuge, OS = Orianne Society Preserve, WC = James W. Webb Wildlife Center. Ponds are ordered geographically from west to east.

Skin bacterial diversity

Alpha diversity of skin bacterial communities differed across Sites and Properties in both richness and Shannon diversity (Richness, Site: χ^2= 42.80, df = 9, $p < 0.001$; Property: χ^2= 35.60, df = 5, $p < 0.001$; Shannon, Site: $F_{9,88} = 2.32$, $p = 0.022$; Property: $F_{5,92} = 3.18$, $p = 0.011$), but not in evenness (Simpson, Site: $\chi^2 = 13.88$, df = 9, $p = 0.127$; Property: $\chi^2 = 7.61$, df = 5, $p = 0.179$). Measures of diversity tended to be highest at sites within the Jones Center, and lowest at sites within Eglin AFB, with the exception of Richness which was lowest within Apalachicola National Forest (Table S7). Two candidate models (ΔAICc < 2) were selected to explain the variation in Richness across sites (Table S5). Model averaged parameter estimates indicated dominant land cover within 1000 m of each pond was a significant predictor of bacterial richness (Table 5; Fig. 7), with richness being highest in forest and lower in large wetlands and in ponds surrounded by shrubby vegetation. The top model explaining Richness had a marginal R^2 of 0.24 and a conditional R^2 of 0.36, indicating some of the variation was attributed to the random effect of Site. Six candidate models were selected to explain Shannon diversity (Table S5). Model averaged parameter estimates indicated that the percent developed land within 500 m (Urban500) and the percent canopy cover within 100 m (Canopy100) were significant predictors of Shannon diversity (Table 5; Fig. 8a), with diversity being higher in ponds surrounded by more developed land, and lower in ponds with more canopy cover. Additionally, Shannon diversity was higher as MEM2 increased (Table 5; Fig. 8b). The marginal and conditional R^2 for the top model explaining Shannon diversity was 0.07 and 0.10, respectively. Two candidate models were selected to explain Simpson diversity (evenness) across ponds (Table S5), with MEM2 being the only significant

predictor (Table 5; Fig. 9). As MEM2 increased, evenness also increased. The marginal and conditional R^2 for the top model explaining Simpson diversity was 0.14. Prominent bacterial families included Burkholderiaceae, Sphingomonadaceae, Xanthomonadaceae, Pseudomonadaceae, Enterobacteriaceae, and Desulfovibrionaceae (Fig. 10). Both Site and Property accounted for significant variation in microbiome beta diversity (Site: ADONIS R^2 = 0.47, p = 0.001; Property: ADONIS R^2 = 0.32, p = 0.001; Fig. 11). All pairwise comparisons between Sites were significant ($p < 0.05$) except for the comparison of site AP1 to AP2 (F = 1.46, p = 0.144) and AP1 to SM4 (F = 1.58, p = 0.117; Table S8). All pairwise comparisons between Properties were significant ($p < 0.001$) except for the comparison of the Apalachicola Property to St. Marks (F = 1.80, p = 0.066; Table S9).

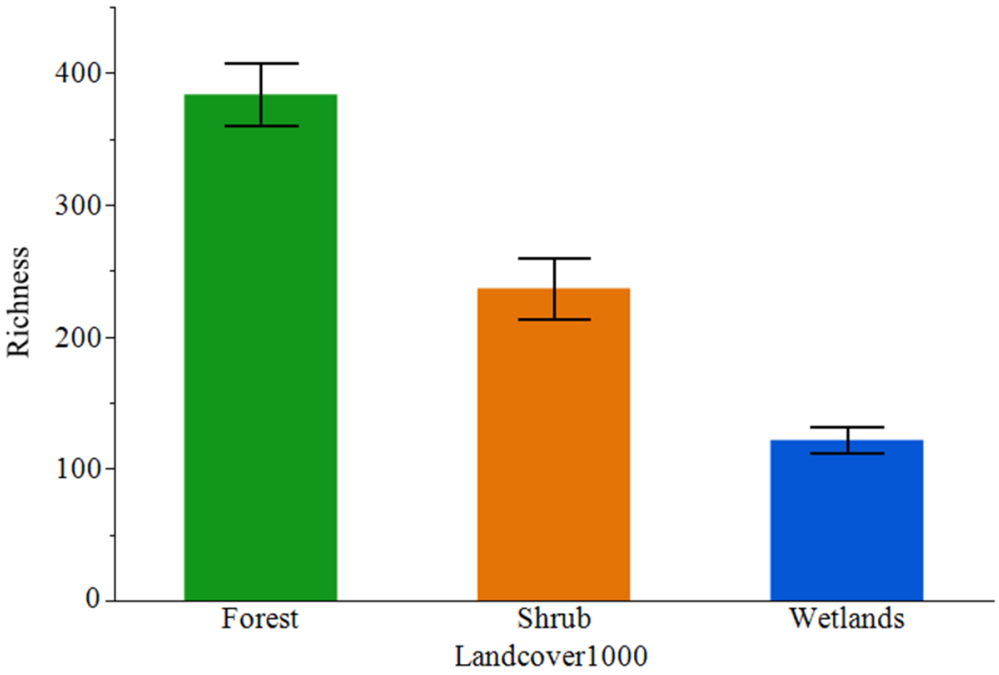

Figure 7. Skin bacterial richness of *Pseudacris ornata* tadpoles and the relationship with the dominant land cover type within 1000m (Landcover1000) of each pond.

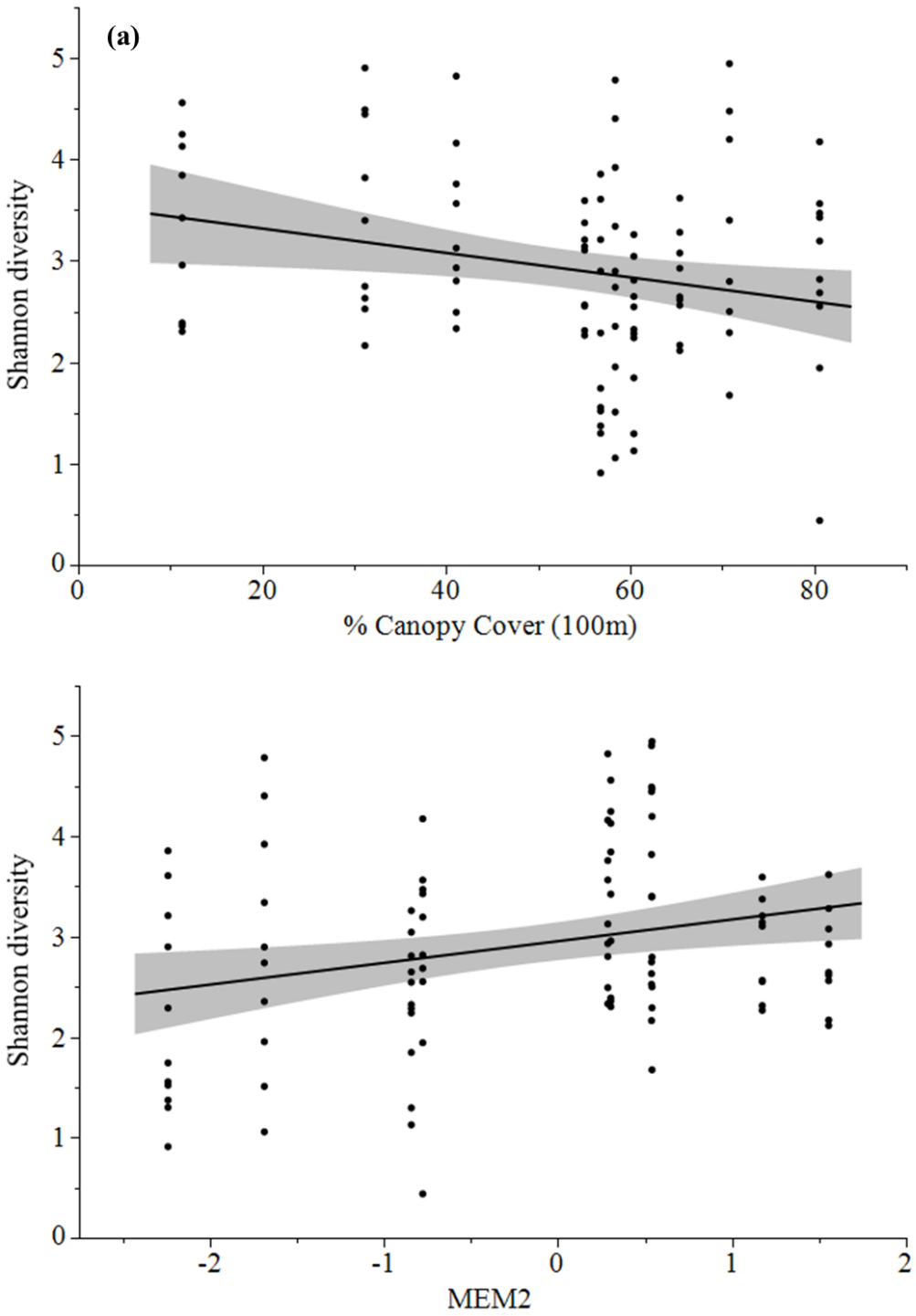

Figure 8. Shannon diversity of skin bacterial communities of *Pseudacris ornata* tadpoles and the relationship with (a) % canopy cover within 100m of each pond and (b) Moran's Eigenvector Map axis 2 (MEM2).

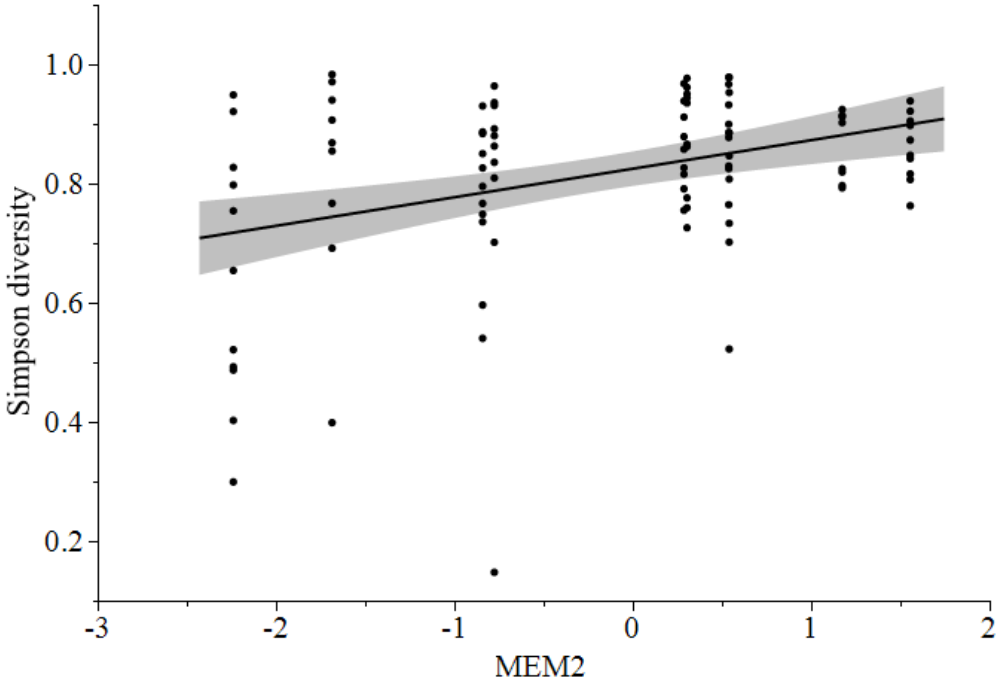

Figure 9. Simpson diversity of skin bacterial communities of *Pseudacris ornata* tadpoles and the relationship with Moran's Eigenvector Map axis 2 (MEM2).

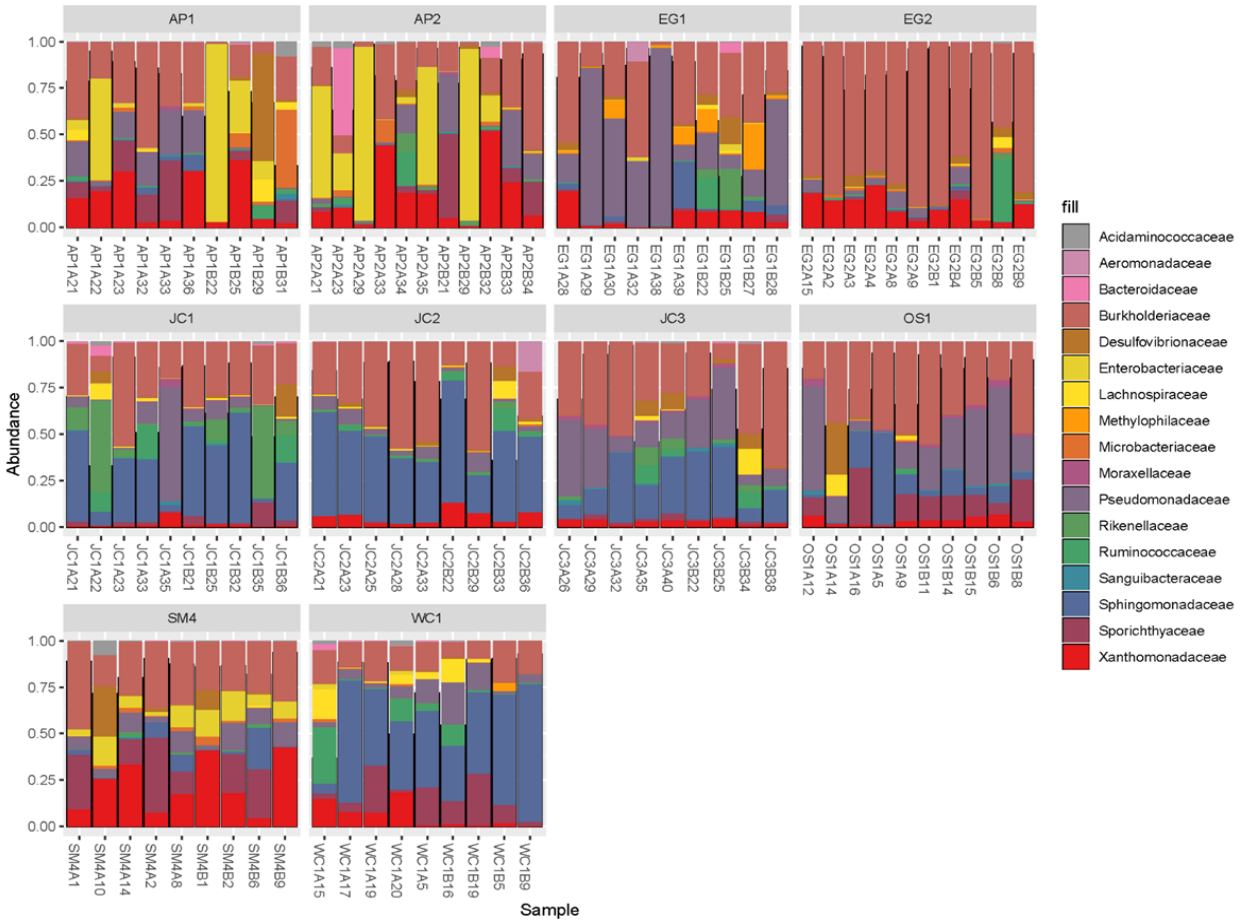

Figure 10. Mean relative abundance of bacterial families from the 50 most abundant taxa found on the skin of *Pseudacris ornata* tadpoles collected from 10 ponds in 2017. Ponds: AP = Apalachicola National Forest, EG = Eglin Air Force Base, JC = Joseph W. Jones Ecological Research Center at Ichauway, LF = Lafayette Forest Wildlife Environmental Area, SM = St. Marks National Wildlife Refuge, OS = Orianne Society Preserve, WC = James W. Webb Wildlife Center. Sample names on x axis are individual tadpole identifiers.

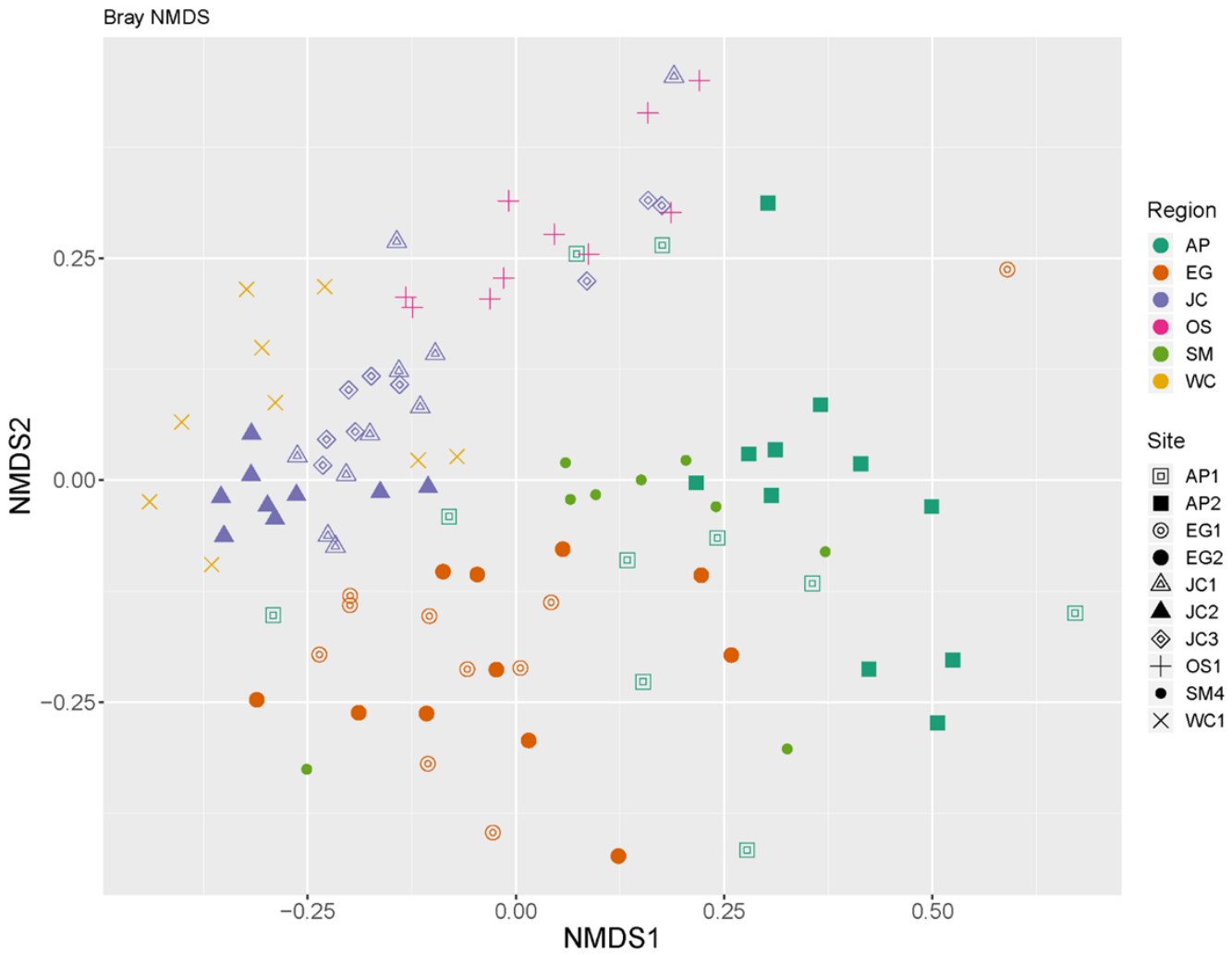

Figure 11. Ordination plot showing beta diversity (OTUs) of microbiota sampled from the skin of *Pseudacris ornata* tadpoles collected from 10 ponds in 2017 based on the Bray-Curtis method of non-metric multidimensional scaling (NMDS). Colors represent Properties, and shapes represent Sites. Each point represents a single individual.

Discussion

We found that both water quality and land cover characteristics were associated with increased CORT release rates and altered skin bacterial communities among populations of *Pseudacris ornata*. We found both baseline and agitation CORT profiles were linked to pond water quality, as well as the amount of developed land within 1000 m of ponds. These results indicate that environmental variation in two different settings—the aquatic and surrounding terrestrial habitats— operates to influence the physiology (GCs) of larval *P. ornata*. Similarly, pond water quality, land cover, and spatial location of ponds were significant predictors of mucosome function and skin bacterial diversity, indicating environmental variation may affect larval *P. ornata* immune defenses through changes in bacterial communities, though we did not see significant effects of these predictors directly on mucosome function. Therefore, perturbations and changes to environmental conditions at multiple spatial scales may significantly impact population health in this species. Given that some of these populations differed from year to year in their CORT profile, the impact of environmental variation—along with the ability to recover from stressor effects—likely varied over time, showing a need for continued monitoring (Blaustein et al. 2011). Taken together, the effects of environmental variation on glucocorticoid function and bacterial communities across years suggests that persistent deviations from optimum conditions may affect population health, thus setting the stage for population declines.

Water-borne CORT release rates

Water quality variables and nearby urban development were important predictors of larval ornate chorus frog CORT profiles. In 2016, both baseline and agitation CORT

release rates were higher in ponds with higher water temperature, conductivity, and total dissolved solids (TDS), as shown by the relationship with PC1 and PC2, whereas the percent urban development within 1000 m was positively associated with CORT release rates in 2017. Additionally, agitation CORT release rates in 2016 were higher at lower MEM3 values, indicating ponds closest to each other were most similar and latitude had an effect on CORT, probably from the warmer water temperatures at the southernmost site (LF1). Several studies involving salamanders also found elevated water-borne CORT release rates associated with higher temperatures (Millikin et al. 2019; Novarro et al. 2018). Both acute and chronic thermal stressors are associated with increased urinary CORT in *Rhinella marina*, the invasive cane toad (Narayan and Hero 2014a, 2014b; Narayan et al. 2012). Elevated CORT can assist with energy mobilization and metabolic increases associated with higher temperatures, but may also indicate stress (Chambers et al. 2013; Millikin et al. 2019; Narayan and Hero 2014a, 2014b; Novarro et al. 2018). Several studies show increased developmental rate and body condition of tadpoles in warmer water (Schiesari 2006; Reading 2010), though stress is generally associated with lower body condition and reduced growth in amphibians (Crespi and Warne 2013; Denver 2009; Glennemeier and Denver 2002a; Hu et al 2008; Janin et al. 2011). Therefore, water temperatures may be an environmental stressor for this winter-breeding amphibian, as warmer water temperatures may result in a shorter hydroperiod, which is a known environmental stressor itself (Denver 1998; Gomez-Mestre et al. 2013). Our results suggest environmental variation in water quality characteristics are important determinants of the CORT profiles of larval *P. ornata*, potentially affecting their physiological health.

Pond breeding frogs may be affected by habitat alterations at multiple scales due to their use of both ponds, as larvae and to breed, and terrestrial habitats as adults. Janin et al. (2011) found that body condition was lower and urinary CORT metabolites were higher in adult *Bufo bufo* from ponds characterized by low forest availability and high fragmentation within 500 m, suggesting effects of surrounding habitat on amphibian health. We found that ponds with higher percentages of developed land within 1000 m were associated with higher water-borne CORT release rates in 2017. Gabor et al. (2018) found Jollyville Plateau salamanders (*Eurycea tonkawae*) had higher water-borne CORT release rates in more urbanized streams as indicated by greater percent impervious cover within the watershed. We also found that CORT release rates were highest in tadpoles from a pond within Lafayette Forest Wildlife Environmental Area (LF1) which had some of the warmest water temperatures, and was dominated by shrubby vegetation, a result of infrequent prescribed fires. Of the properties sampled, this land was the most recently acquired and managed by the state of Florida (purchased in 2008, management began in 2010) with the first prescribed fires conducted in 2013 (N. Lambert, Florida Fish and Wildlife Conservation Commission, pers. comm.). Prescribed fire is vital to management of longleaf pine uplands and their embedded wetlands because it controls the growth of invasive, shrubby, mid-story vegetation such as saw palmetto (*Serenoa repens*) and gallberry (*Ilex coriacea*;; Enge et al. 2014; Means 2006; Noss 2013, 2018). These results suggest habitat quality at a larger scale may affect the CORT profile and subsequent physiological health of *P. ornata* tadpoles.

Increased CORT in response to an acute stressor is indicative of a healthy endocrine response. Tadpoles that elevate CORT release in response to an acute agitation

stressor indicates the HPI axis is still active and not dysregulated due to chronic stress. Therefore, the ability to mount a response to an acute stressor provides one component that can indicate a healthy population. Comparing the average baseline and agitation CORT release rates from each pond over both years showed four ponds in 2016 and six ponds in 2017 without a significant difference in their response (Fig. 6). The lack of a difference in response in these populations may be indicative of chronic stress, as indicated by their HPI axis not responding to an acute stressor (Cyr and Romero 2009; Romero 2004). These populations may be able to recover if environmental suitability increases and/or if individuals show compensatory growth once they leave the pond and possibly find suitable habitat (Metcalfe and Monaghan 2001). When individuals return as adults to the pond to lay eggs, their resulting larvae may not show health effects if the water and environmental quality have improved. However, stressful conditions during larval development may have negative post-metamorphic carry-over effects on juvenile and adult frogs (Crespi and Warne 2013). Therefore, these populations that do not show a healthy CORT response may fail to recover when exposed to numerous consecutive years of low environmental quality, or if the stressful conditions experienced during the larval stage have carry-over effects in the adults. Populations of *P. ornata* in the peninsular region of Florida are declining and undergoing local extirpations (Enge et al. 2014). We were unable to find *P. ornata* tadpoles (and therefore measure CORT) in this region, and it is possible that these declines may be the consequence of an inability to recover from past harsh environmental conditions (or repeated harsh conditions) such as the drought (and associated warmer temperatures) experienced by this region from 2006 to 2008 and from 2010 to 2012 (Enge et al. 2014).

Though our results indicate environmental variation at multiple spatial and environmental scales (i.e. water quality vs. terrestrial habitat) impact the CORT profiles of larval *P. ornata*, some of the variation in CORT release rates was accounted for by the random effects in the models (as indicated by the conditional R^2). This suggests additional variability between ponds within each property associated with unmeasured factors. The difference between the marginal and conditional R^2 was greater in 2017, indicating more variation between sites not accounted for by the predictors. This is likely due to natural differences in pond characteristics (i.e. size, depth, hydroperiod) and other habitat factors not examined. This variation contributes to the uncertainty that enshrouds management decisions for *P. ornata* and ecologically-similar species. However, the consequences of development and water quality on the health of *P. ornata*, indicated in this study, lends additional support for the conservation and appropriate management of longleaf pine forests, including frequent prescribed fire during the growing season, to maintain high-quality pine savannahs for this and other longleaf pine endemic species (Enge et al. 2014; Means 2006; Noss 2013, 2018)

Mucosome function

The amphibian mucosome, including both skin secretions and microbial communities, are important factors in amphibian health and immunity. The ability of the mucosome to fight infection has been used as a holistic measure of amphibian skin defenses (Woodhams et al. 2014). We found no differences in the mean mucosome function across ponds, though the *Bd* cell viability within a pond was quite variable. Additionally, there were no significant predictors of mucosome function in our models, though water quality and landscape variables were included in the top models. These

results indicate no difference in the bacterial and mucosal defenses across ponds. In this study, mucosome function only reduced *Bd* cell viability to 88.1%, indicating this species may be susceptible to *Bd* infection. Indeed, Gervasi et al. (2017) found that *P. ornata* had the highest average *Bd* infection load of 20 species studied and a high rate of mortality following infection. Similarly, Horner et al. (2017) found *P. ornata* had a high infection intensity and a high *Bd* prevalence across ponds. However, due to logistics, we were unable to use the strain of *Bd* to which these frogs are naturally exposed, which may be why we did not see any difference in mucosome function across ponds. Woodhams et al. (2014) found a significant correlation between mucosome function and both field and laboratory *Bd* infection prevalence for Swiss and European frogs (for a Swiss *Bd* strain); thus, future studies could repeat exposure experiments using local isolates.

Skin bacterial diversity

Alpha and beta skin bacterial diversity varied across both Sites and Properties. Primary predictors of bacterial diversity were the dominant land cover type within 1000 m, the percent developed land within 500 m, the percent canopy cover within 100 m, and the spatial location of ponds. All alpha diversity indices were highest at sites located within the Jones Center property, and these wetlands were the largest sampled and surrounded by mature longleaf pine forest with short-interval prescribed fires to maintain high quality pine savannah. Though a site in Apalachicola National Forest (AP2) had the lowest observed richness, both Shannon and Simpson diversity indices were lowest within the Eglin Air Force Base (EG) property. The ponds sampled at Eglin were along a powerline right-of-way, and thus were composed of more disturbed habitat. Variation in skin bacterial communities are generally associated with pond characteristics including

conductivity, pH, temperature, dissolved oxygen and precipitation (Krynak et al. 2015, 2016; Kueneman et al. 2013; Varela et al. 2018). The significant association of bacterial diversity with land cover suggests landscape-scale features also affect bacterial communities. Bacterial richness, the number of species present, was positively associated with ponds surrounded by forest. Interestingly, shannon diversity, a measure of the abundance and variety of bacteria present, was higher in ponds with more developed land within 500 m and with less canopy cover within 100 m. Enge et al. (2014) found that most wetlands inhabited by *P. ornata* lacked a canopy or had an open canopy that allowed growth of grasses during dry down periods, a result of short-interval fire management. Our results suggest these open canopy ponds with greater environmental variation may have higher bacterial diversity.

In the southeastern USA, land-use conversion, fire suppression, and droughts are important factors in the decline of amphibian species, especially those reliant on the savannah-like longleaf pine-wiregrass (*Pinus palustris-Aristida* spp.) ecosystem. In addition to its control of invasive shrubs and hardwoods, summer growing season fire, when ponds are typically dry, is required to maintain grasses and sedges in ephemeral wetlands used by those pond-breeding amphibians that are endemic to this ecosystem (Noss 2013, 2018). Within the pine savannahs of the southeastern Coastal Plain, a short-interval fire regime maintains herbaceous vegetation, reduces canopy cover in breeding ponds, and increases amphibian abundance and diversity (Noss 2013, 2018). Our findings that landcover type, the percent developed land, and increased canopy cover were associated with altered skin bacterial diversity indicate environmental variation may also contribute to changes in skin bacterial diversity of larval *P. ornata*. These effects may

ultimately impact the immune defenses of species like *P. ornata* that are specialists of the longleaf pine ecosystem. The sole significant predictor of Simpson diversity, and an additional significant predictor of Shannon diversity, was the spatial Moran's eigenvector map (MEM) axis MEM2. The eigenvalue for this axis was -0.78, indicating autocorrelation among sites at a fine spatial scale. This autocorrelation indicates both Shannon and Simpson diversity are most similar in nearby ponds, which is expected, and that longitude has an effect on the diversity observed. This relationship is also suggested in the results of our distance-based ordination plot of bacterial diversity (Fig. 11). However, the pairwise comparison of beta diversity between sites showed that all comparisons between sites except two showed significant differences (Table S8) and all comparisons between properties were significantly different except for those that were geographically the closest (Apalachicola National Forest and St. Marks National Wildlife Refuge; Table S9). As we have seen, environmental characteristics can influence bacterial diversity. Additionally, maintaining suitable ponds and wetlands across a species range can aid in establishing a higher bacterial diversity. Together, these results suggest site location and pond characteristics play an important role in the skin bacterial community composition, which can affect immune defenses, ultimately influencing the health of these larval amphibians.

Though bacterial communities are known to vary across sites and even among species within the same site (see Belden et al. 2015), some bacterial families have high proportions of *Bd* inhibitory isolates. Bletz et al. (2017) examined skin bacteria from Madagascar amphibians and found three families with 75% or more of the isolates having inhibitory effects on *Bd* infection, suggesting these may be good candidates for broad

probiotic treatment. These three families (Enterobacteriaceae, Pseudomonadaceae, and Xanthamonadaceae) were also well represented in our site diversity, though *P. ornata* have high *Bd* prevalence (Gervasi et al. 2017) and the mucosome function in our study only reduced *Bd* cell viability to about 88% (see section 4.2 above), suggesting other factors may be contributing to the infection of this species. Continued research should consider the skin bacterial community to aid in developing probiotics to fight known pathogens such as *B. dendrobatidis* and *B. salamandrivorans* (Woodhams et al. 2016).

Conclusions

Amphibian populations have been declining rapidly worldwide, and this decline is likely tied to their need for multiple habitats and their susceptibility to environmental changes (Becker et al. 2007; Stuart et al. 2004). There is a growing need to monitor population health and factors that influence their health to provide early warning signs for proactive management decisions. Our results suggest the conservation of *P. ornata* could be enhanced by management actions that address water quality and forest composition to maintain high-quality aquatic and terrestrial habitat. Natural resource managers could consider habitat quality at multiple spatial scales to best manage larval amphibian populations and the bacterial communities present. By examining baseline and stress (agitation) induced CORT profiles and other health metrics across locations and for several years in succession, researchers and managers may be able to use this method proactively to identify populations at increased risk of declines to focus management efforts. Additionally, little is known about the adult life history of this cryptic and fossorial species (Bartlett and Bartlett 2011; Brown and Means 1984), warranting

additional research and implementing management practices that maintain suitable habitat for both aquatic and terrestrial life stages. By examining environmental variables, the CORT profiles, and skin bacterial communities of populations of *P. ornata* across the range of the species, we identified factors at multiple spatial scales that affect population health. Though some populations showed an ability to mount a CORT response in different years, without habitat remediation, other populations may no longer be able to recover from changes in environmental conditions.

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III. POSITIVE AND NEGATIVE EFFECTS OF HIGHER TEMPERATURES ON THE PHYSIOLOGICAL HEALTH OF RIO GRANDE LEOPARD FROG (*RANA BERLANDIERI)* TADPOLES

Abstract

Anthropogenic alterations to habitat and the synergistic effects with climate change and disease affect the physiological health of amphibian populations and contribute to their declines globally. I examined the effects of increased water temperatures on multiple physiological health metrics in a larval anuran, the Rio Grande Leopard Frog (*Rana berlandieri*). I performed a laboratory experiment to test the effects of increased water temperatures on corticosterone release rates and body condition and a field mesocosm experiment to test the effects of increased water temperatures, specifically from a lack of canopy cover, on corticosterone profiles, body condition and immune function. CORT release rates were lower and body condition was greater in warmer water in the lab. Warmer water temperatures in the mesocosms without cover were also associated with lower CORT release rates, lower survival, but reduced body condition. Immune defense, measured as mucosome function, was higher in warmer water temperatures. Repeatability of CORT release rates was low, indicating that CORT cannot show a response to selection in warmer temperatures in this population. Together, increased water temperatures from climate change or habitat alteration, such as canopy loss, may result in chronic stress in tadpoles, reducing physiological health. However, warmer water may also improve body condition and immune health. My results suggest multiple health metrics are needed to understand the complex effects of environmental

change on larval amphibian health. Additionally, management practices should consider canopy cover as an important factor in *Rana berlandieri* population health, especially under a changing climate.

Key Words: amphibians, conservation physiology, corticosterone, water-borne hormones, stress

Introduction

Amphibians are declining globally due to many factors including habitat loss/fragmentation, climate change, disease, contaminants, invasive species, and their synergistic effects (Blaustein et al. 2011; Brook et al. 2008; Collins 2010; Cushman 2006; Hof et al. 2011). Both landscape-level disturbances such as habitat loss and degradation, as well as local water quality changes such as temperature, pH, contaminant, nutrient, and sediment levels associated with these disturbances, are especially important for aquatic or semi-aquatic organisms, such as amphibians. Amphibians have the highest threat status of any vertebrate class (Foden et al. 2013; Stuart et al. 2004; Wake and Vredenburg 2008) and may be especially vulnerable to environmental changes because they generally require both aquatic and terrestrial habitats to complete development across larval and adult life stages (Becker et al. 2007; Blaustein et al. 2011; Hayes et al. 2010).

Examining the physiological responses of organisms to changes in environmental conditions can be used as indicators of individual and population health (Dantzer et al. 2014; Gabor et al. 2018; Jeffrey et al. 2015; Sheriff et al. 2011; Wikelski and Cook 2006). Amphibians activate the hypothalamo-pituitary-interrenal (HPI) axis in response to stressors, increasing the circulation of corticosterone (CORT; the main amphibian glucocorticoid) above baseline levels, which assists in energy metabolism and aids in physiological and behavioral changes that help individuals return to homeostasis (Cyr and Romero 2009; Sapolsky et al. 2000). Under prolonged (chronic) stress, vertebrates may have elevated or lowered baseline glucocorticoids, which can lead to reduced physiological health, pathology, and ultimately death (Cyr and Romero 2009; McEwen

and Wingfield 2003; Romero et al. 2009; Wingfield and Romero 2001). Lower CORT can indicate downregulation/desensitization of the stress response, exhaustion of the HPI axis, or habituation to a stressor (Cyr and Romero 2009). To fully understand changes in CORT, measuring the response to an acute stressor and/or examining some other physiological health metric is needed to identify possible causes (Dantzer et al. 2014; Janin et al. 2011; Wikelski and Cooke 2006). Though glucocorticoids can be adaptive in the short term and aid in development and metamorphosis in larval amphibians (Denver 1998, 2009), under chronic stress, amphibians show reduced growth and development, suppressed reproduction, and altered immune function (Belden et al. 2005; Moore and Jessop 2003; Rollins-Smith 2017). Environmental changes such as increased pond drying, high salinity, extreme pH and temperature, and forest fragmentation can be perceived as stressors, which can elevate or dysregulate glucocorticoid hormones in amphibians and affect physiological health (Burraco and Gomez-Mestre 2016; Chambers et al. 2013; Gabor et al. 2018; Gomez-Mestre et al. 2013; Janin et al. 2011; Narayan and Hero 2014b). Additionally, environmental conditions themselves are associated with variation in body condition, altered immune function, and changes in microbial communities in amphibians (Janin et al. 2011; Kohl and Yahn 2016; Krynak et al. 2016; Reading 2010; Rollins-Smith et al. 2017; Varela et al. 2018). In this way, examining CORT profiles and body condition under different environmental conditions and across developmental stages may aid in identifying long-term effects of stressors.

The amphibian mucosome, or micro-ecosystem of the skin, is made up of skin secretions and microbiota, providing the first line of defense in amphibian immunity (Harris et al. 2009; Woodhams et al. 2014). Examining the mucosome function (ability to

fight infection) of individuals can be used to predict population disease risk (Smith et al. 2018; Woodhams et al. 2014). Further, the production of amphibian skin peptides are inhibited by elevated CORT (Rollins-Smith et al. 2011). This suggests a link between stressors, microbiota and ultimately amphibian immunity. Consequently, increased temperatures from climate change or habitat alteration, such as reduced canopy cover and land-use changes, may have varying effects on larval amphibian health. Tadpoles from warmer ponds and open canopy show faster metamorphosis and higher body condition than those from cooler ponds (Reading et al. 2010; Schiesari 2006). However, warmer temperatures may result in shorter pond hydroperiods, which can be perceived as a stressor to aquatic amphibians (Denver 1998; Gomez-Mestre et al. 2013) and can affect development, survival, and immune function of tadpoles and juveniles (reviewed in Kohli et al. 2019). Exposure to higher temperatures have been associated with elevated CORT in salamanders (Chambers et al. 2013; Millikin et al. 2019; Novarro et al. 2018), as well as toads (Narayan et al. 2012; Narayan and Hero 2014a, 2014b). Though increased temperatures may be associated with increased stress, immune function may be enhanced, as increased temperatures alter gut microbiota (Kohl and Yahn 2016) and are associated with a greater microbial diversity (Bletz et al. 2017). Elevated CORT stimulates immune function in the short term, but chronically elevated CORT is immunosuppressive (reviewed in Rollins-Smith et al. 2017), affects gut microbial communities in other taxa (Clarke et al. 2014), and is associated with lower body condition in frogs (Leary and Harris 2013; Narayan et al. 2013). Therefore, identifying relationships between stress, environmental conditions, and immune function may aid in detecting populations at increased risk of declines.

I performed both laboratory and mesocosm experiments to identify the effects of increased water temperatures on multiple measures of physiological health in Rio Grande leopard frog, *Rana berlandieri*, tadpoles. Using tank heaters in the laboratory, and by manipulating water temperatures using shade cloth over mesocosms, I examined the effects of increased water temperatures on tadpole CORT profiles, body condition, mucosome function, and survival. My research aimed to answer two questions: first, what is the effect of water temperature on CORT and body condition in *R. berlandieri* tadpoles under controlled lab conditions; and second, what is the effect of water temperature as a result of altered pond conditions (reduced canopy cover) on CORT, body condition, immune function, and survival in *R. berlandieri* tadpoles? I predicted that prolonged elevated water temperatures, would affect CORT release rate profiles and body condition, alter immune function, and lower survival in tadpoles.

Materials and Methods

Study species

The Rio Grande Leopard Frog (*Rana berlandieri*) is a common species found throughout central and southwestern Texas and northeastern Mexico (Powell et al. 2016; Zaldivar-Riveron et al. 2004). This species takes advantage of Spring rains and breeds in streams, ponds, or temporary pools, though breeding may occur at almost any time of year (Powell et al. 2016). The Rio Grande Leopard Frog has adapted to live in arid climates (Powell et al. 2016) and tadpoles develop lungs early, gulping air to facilitate living in low-oxygen environments such as ponds and vernal pools (Feder 1983). I collected egg masses of *R. berlandieri* from an ephemeral pond (29°52'28.86"N,

97°57'45.86"W) in San Marcos, TX on 23 February 2017 (3 masses) and 5 March 2018 (4 masses). I used eggs collected in 2017 for the laboratory experiment, and eggs collected in 2018 for the mesocosm experiment (see below). I transported one half of each egg mass back to the laboratory on the Texas State University campus and reared them in plastic tanks with aged, de-chlorinated tap water until tadpoles were free swimming (stages 26–29, following Gosner 1960). Once tadpoles were free swimming, I mixed individuals from each clutch and housed them in groups of 12 in plastic tanks with 6 L of aged, de-chlorinated tap water. I housed tadpoles under a natural 14:10 light:dark cycle and fed a mixture of Spirulina powder and ground Tetramin fish flakes in an agar base *ad libitum* with water changes at least once weekly or as needed.

Laboratory experiment

On 9 May 2017, I set up 5.4 L plastic tubs (n = 20), each with 3 L of aged, de-chlorinated tap water and four *R. berlandieri* tadpoles, on racks in the lab with a 12:12 light:dark cycle. Half the tubs (n = 10) received individual 15 w tank heaters set at 27° C as the temperature treatment, and the other tubs (n = 10) were left at a room temperature of 19° C for the control treatment. I allowed all tadpoles were 24 h to acclimate to the new tubs at 19° C before I turned on the tank heaters to start the treatments. Tadpoles remained in their respective treatments for seven days, with full water changes twice during this time, and I fed tadpoles a spirulina and fish flake mixture in an agar base *ad libitum*. I performed water changes by moving tadpoles into a clean tub containing water maintained at each treatment's respective temperature. After seven days in their respective treatments, I removed two tadpoles from each tub (n = 20/treatment) and collected baseline water-borne hormones from each individual following Gabor et al.

(2016). This non-invasive method measures glucocorticoids secreted through skin, urine and feces, providing an integrative measure of stress (Dantzer et al. 2014; Sheriff et al. 2011). After one hour, I collected water containing the hormones in individual sample cups and stored them at -20° C for later hormone extraction. I then then weighed and photographed tadpoles from the side with a ruler for scale before euthanizing them. I measured snout-vent length (SVL, mm) of each tadpole from the photographs using ImageJ (Rosband 1997).

Statistical analysis

To examine the effect of increased laboratory water temperatures on both CORT release rates and body condition (mass/SVL) of *R. berlandieri* tadpoles, I used separate generalized linear mixed effect models (GLMM). I examined natural log-transformed CORT release rates standardized by mass (pg/g/h) with Treatment (19° C or 27° C) as the main effect. I examined body condition with Treatment and CORT release rates as main effects. All models included individual nested in tank as the random effects. I ran all statistical tests in JMP Pro 14.0.0 (SAS Institute, Inc.).

Laboratory experiment results

R. berlandieri tadpoles after one week in 27° C water had significantly lower CORT release rates than tadpoles at 19° C ($F_{1,38} = 12.40$, $p = 0.001$; Fig. 12a). Additionally, tadpoles from the higher water temperature treatment had higher body condition compared to those from the lower temperature treatment ($F_{1,37} = 7.46$, $p = 0.010$; Fig. 12b). Body condition was not associated with CORT release rates ($F_{1,37} = 1.79$, $p = 0.189$).

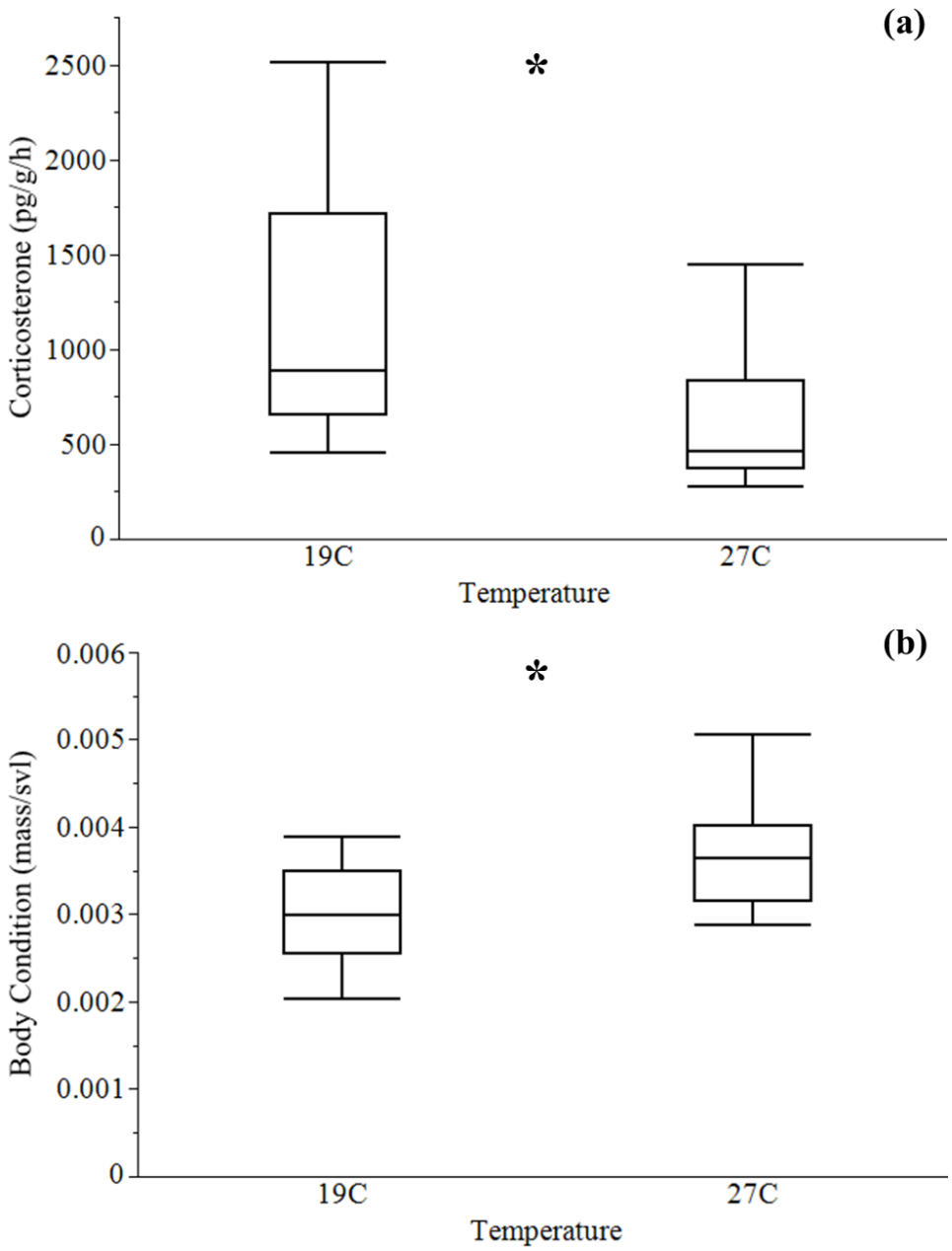

Figure 12. Mean (± SE) (a) corticosterone release rates (pg/g/h) and (b) body condition of Rio Grande Leopard Frog (*Rana berlandieri*) tadpoles after one week in a lab exposed to 19° C or 27° C water temperature. Untransformed data are shown.

Mesocosm experiment

To further examine the relationship between water temperatures on the health of larval *R. berlandieri*, I performed an outdoor mesocosm experiment. On 26 April 2018, I

set up twenty outdoor mesocosms consisting of 65 L clear, plastic tubs, each containing 49 L of harvested rainwater, 1 L of pond water (to inoculate), 50 g of dried mixed deciduous leaves, 15 g of dried grass, and 15g of Purina rabbit chow. I collected the pond water, leaves, and grass from around the same pond from which the egg masses were collected, with leaves consisting primarily of cedar elm (*Ulmus crassifolia*) and live oak (*Quercus fusiformis*), and grasses consisting primarily of switchgrass (*Panicum virgatum*). I divided the twenty tubs into two treatments: I placed ten in the open without cover (Open treatment), and I placed another ten under a 60% shade cloth to mimic canopy cover (Canopy treatment). In this way, I indirectly manipulated water temperature by placing tubs in the shade or open to the sun. I cut six, 4cm. holes around the top edge of each tub and covered each hole in fiberglass window screen. I also placed a modified lid covered in fiberglass window screen, to allow sunlight in and excess water to drain, while preventing tadpole escape and unwanted colonization. Additionally, I adhered glass slides to the inside of each tub, below the water line, to accommodate algal growth for later periphyton analysis. I allowed 2 weeks for tubs to form self-sustaining ecosystems, after which I added 12 *R. berlandieri* tadpoles (stage 25–29; Gosner 1960) to each tub: 6 unmarked and 6 marked subcutaneously with a unique visual implant elastomer (VIE; Northwest Marine) for identification. I placed a temperature data logger (Onset) into two tubs within each treatment to collect water temperatures. I caught tadpoles using nets and I collected baseline water-borne hormones (following Gabor et al. 2016) from all VIE marked individuals in both treatments during at least 4 time points: after 2 days in their respective treatments, after 7 days (both during premetamorphosis; Gosner stages 25–29), and at least once more during both prometamorphosis (Gosner stages 30–40) and

metamorphic climax (Gosner stages 41–45) when possible. I collected the water samples containing the hormones in individual sample cups and stored them at -20° C for later hormone extraction. I then weighed (g) tadpoles before releasing them back into their respective tubs. Tadpoles remained in the treatments until they metamorphosed, died, or until the end of the experiment on 30 August 2018. I added aged tap water at treatment temperatures to each tub as needed to maintain water levels. On each hormone sampling day, I weighed and photographed each tadpole. I later used the photographs to measure SVL (mm) through the program ImageJ (Rosband 1997). I also recorded the number of surviving tadpoles within each treatment on each sampling day. By repeatedly sampling the same individuals, I was also able to examine the repeatability of CORT release rates over time. Repeatability is used as an upper bound estimate of heritability (Lessels and Boag 1987) and measuring the repeatability of hormones can indicate how well individuals are able to respond to changing environments or stressors (Forsburg et al. 2019; Hau et al. 2016; Lendvai et al. 2014).

Hormone extraction and analysis

To extract hormones from each water sample, I first thawed frozen water samples overnight and then filtered all samples through standard coffee filters (equivalent to grade 4 filter paper) before extraction. Corticosterone extraction from water samples followed Gabor et al. (2016) and consisted of pulling water through C18 solid phase extraction (SPE) columns (SepPak Vac 3 cc/500 mg; Waters, Inc.) Primed with 4ml of 100% HPLC grade methanol and 4ml of distilled water. I then eluted columns with 4ml of 100% HPLC grade methanol into borosilicate test tubes and stored them at -20° C until drying. To dry eluted samples, I placed them in a hot water bath (37° C) under a gentle stream of

nitrogen gas flowing through an Evap-O-Rac (Cole-Palmer). I then resuspended the dried precipitate in a mixture of 5% ethanol (95% lab grade) and 95% enzyme-immuno-assay (EIA) buffer (from CORT EIA kits; No. 501320, Cayman Chemical Company, Inc.) To a final volume of 600 µl (laboratory experiment) or 1200 µl (mesocosm experiment). I ran each sample in duplicate on CORT enzyme immuno-assay (EIA) plates from the kit (Cayman Chemical Company, Inc.). I read the absorbance of each sample using a spectrophotometer plate reader (Biotek ELX 800) set to 405 nm. I then multiplied the final CORT concentrations (pg/ml) by the reconstitution volume (0.600ml, or 1.20 ml) and then divided by the tadpole mass (g) for a final unit of pg/g/h. I also multiplied the water samples by the reconstitution volume (0.13 ml) and subtracted the relevant spring water values (pg/sample) from the CORT release rates of each tadpole. Intra-plate variation for ranged from 0.87–7.69% for the laboratory experiment, and 0.00–11.86% for the mesocosm experiment. Average inter-plate variation was 14.59% for the lab (5 plates) and 9.26% for the mesocosm (10 plates) experiments. Sensitivity of the CORT EIA plates ranged from 26.96–895.67 pg/ml and 25.77–885.99 pg/ml on average for the laboratory experiment and mesocosm experiment, respectively.

Mucosome analyses

For all samples collected during the mesocosm experiment, I removed 1 ml of each sample prior to filtering and stored the subsamples in microcentrifuge tubes at -20° C. To assess mucosome function, I plated a 25 µl aliquot of each sample on opaque, white 96-well flat-bottom plates (No. 655075, Greiner Bio-One) with either a 25 µl solution of *Batrachochytrium dendrobatidis* (*Bd*, TM016 strain) zoospores at a concentration of ~30,000 zoospores per 25 µl, or 25 µl of autoclaved spring water as a

control for each sample to determine background fluorescence. To quantify the ratio of live:dead cells, I added a 50 µl sample of Cell Titer-Glo 2.0 reagent (Promega) to each well and incubated the plate for 1 hr at room temperature. After 1 hr, I placed the plate on an orbital shaker for 2 minutes at 200 rpm and then read the luminescence on a plate reader (Biotek Synergy H1) with the gain set at 150. I then subtracted the luminescent values of each individual control from the luminescent values of each sample + *Bd* to obtain background controlled luminescent values. Higher luminescent values indicate higher cell viability (more ATP) and therefore represent lower mucosome function (higher *Bd* viability in the presence of the mucosome).

Periphyton measures

To compare tadpole food availability across treatments, I analyzed periphyton by measuring the chlorophyll *α* concentration of samples scraped from glass slides. I collected one slide from each tub once a month and slides were pooled for each treatment. Using a razor blade, I scraped periphyton growth from all glass slides for a given treatment into a graduated Falcon tube and rinsed each slide with Millipore water and diluted to a final volume of 10 ml. I homogenized these samples using a biohomogenizer and further mixed them in a beaker on a stir plate. I pipetted a subsample (200 µl) of this solution into a microvial, and added 1 ml of methanol to extract the chlorophyll. I vortexed samples before plating 200 µl of each solution in triplicate onto clear, flat-bottomed 96-well plates (No. 269620, Thermo Fisher Scientific). I then read the absorbance of each well at 652 nm and 665 nm using a plate reader (Biotek Synergy H1). I corrected the absorbance values for 1-cm pathlength by subtracting the absorbance of a methanol blank and then dividing by 0.51 (Warren 2008). I calculated the

chlorophyll α concentration from a 1-cm corrected pathlength using the formula of Ritchie (2006). I corrected chlorophyll calculations for dilution volume before running the analysis.

Statistical analysis

All tadpoles in the Open treatment died by 21 June 2018 (after 43 days in treatment) due to extremely high weather temperatures. Therefore, I limited analyses examining the effects of treatment on response variables to the first four sampling days (11 May, 18 May, 12 June, 19 June). I compared the average water temperatures over this time period between treatments using a t-test. All CORT analyses used natural log-transformed CORT release rates standardized by mass (pg/g/h). I examined the effects of sampling day on CORT release rates and body condition (mass/SVL) using separate repeated measures GLMMs. I also examined the effects of treatment and metamorphic stage (prematamorphosis = stages 25–30, prometamorphosis 1 = stages 30–35, prometamorphosis 2 = stages 36–40, and metamorphic climax = stages 41–45; Gosner 1960) on CORT release rates and body condition using separate repeated measures GLMMs. Including data from all sampling days (16 sampling times across 113 days), I ran repeated measures GLMMs to examine body condition and CORT release rates across general metamorphic stages for the Canopy treatment alone. I used individual nested within tub number as the random effect for all mixed effect models to account for repeated measures. I used the Tukey's HSD *post hoc* test to compare groups when there was a significant difference across sampling days or metamorphic stages. I also calculated an adjusted repeatability (r) of CORT release rates for the Canopy treatment using a GLMM approach and a Restricted Maximum Likelihood (REML) approximation

(Dingemanse and Dochtermann 2013; Nakagawa and Schielzeth 2010). This analysis was run using the rtpr package in the program R version 3.5.2 (R Core Development Team). Sample size in the Open treatment was too small for analysis. I calculated repeatability of CORT release rates across time for tadpoles in the outdoor mesocosms and analyzed each treatment separately. CORT release rate was the response variable, with sampling day as the fixed effect, and tadpole ID as the random effect. I also compared tadpole survival between the two treatments using a logrank Mantel-Cox survival analysis, censoring for individuals that metamorphosed (no longer in the treatments). I also assessed the mucosome function of tadpoles using a repeated measures GLMM with CORT release rates, body condition, as well as date sampled, treatment, and their interaction as fixed effects, and mucosome plate number as a random effect to account for any variation due to plating. Finally, to compare food availability within each treatment, I analyzed the chlorophyll α concentrations between the two treatments using a Mann-Whitney test, because the data was not normally distributed. All statistical tests except for the repeatability analysis were run in JMP Pro 14.0.0 (SAS Institute, Inc.).

Results

Mesocosm experiment

Water temperatures over the first 43 days of the experiment (first four sampling days) were significantly different between the two treatments ($t = 8.67$, $df = 52$, $P < 0.0001$) and ranged from 26.1–32.3° C in the Open treatment and from 25.3–29.1° C in the Canopy treatment, with water temperatures averaging 29.9° C and 26.8° C for the Open and Canopy treatments, respectively. CORT profiles differed across the first four

sampling days (11 May – 19 June 2018), where CORT release rates were significantly higher on 11 May and significantly lower on 19 June than on either 18 May or 12 June ($F_{3,128}$ = 43.04, P < 0.0001; Fig. 13a). I also found a significant effect of sampling day on body condition of tadpoles ($F_{3,127}$ = 144.70, P < 0.0001), where body condition on 18 May was significantly higher than 11 May, but lower than 12 June and 19 June (Fig. 13b). Treatment had a significant effect on the CORT profiles and body condition of tadpoles over the first four sampling days. CORT release rates were higher in the Canopy treatment than the Open treatment ($F_{1,67}$ = 9.61, P = 0.003; Fig. 14a) and body condition was also higher in the Canopy treatment than the Open treatment ($F_{1,69}$ = 7.25, P = 0.009; Fig. 14b). There was no significant interaction between sampling day and treatment for either CORT release rates ($F_{1,1369}$ = 1.14, P = 0.337) or body condition ($F_{1,129}$ = 1.80, P = 0.150). CORT release rates over this time period were not repeatable for the canopy treatment (r = 0.074 ± 0.068, 95% CI: 0, 0.229, P = 0.187). Average luminoscity (indicating *Bd* zoospore viability in the presence of the skin mucosome) was significantly higher in the Canopy treatment than in the Open treatment ($F_{1,157}$ = 7.92, P = 0.006; Fig. 15). There was no significant effect of body condition ($F_{1,156}$ = 0.01, P = 0.953), sampling day ($F_{3,7}$ = 3.52, P = 0.076), or the interaction between sampling day and treatment ($F_{3,152}$ = 0.66, P = 0.581) on the mucosome function of tadpoles. Average chlorophyll α concentrations ranged from 0.20–3.32 µg/ml and were not significantly different between the two treatments (Mann-Whitney: U = -0.64, P = 0.523).

Across all sampling days, CORT release rates in the Canopy treatment were highest during premetamorphosis and lowest during climax ($F_{3,156}$ = 66.91, P < 0.0001; Fig. 16a). Body condition in the canopy treatment was higher during prometamorphic

stages than either premetamorphosis or climax ($F_{2,189}$ = 136.56, P < 0.0001; Fig. 16b). Tadpole survival was significantly lower in the Open treatment than in the Canopy treatment (logrank Mantel-Cox: χ^2 = 101.15, P < 0.0001; Fig. 17).

Discussion

Increased temperatures as a result of climate change and synergistic interactions between temperature and other stressors may affect breeding phenology, immune responses, and pond hydroperiod, all important aspects to larval amphibians (Beebee 1995; Duarte et al. 2011; Hayes 2010; Parmesan 2006; Rollins-Smith 2017). However, the aquatic habitat preferences and developmental timeline of individual species may determine whether warmer water temperatures have negative effects or not. I found that higher water temperatures, whether directly from a heater or indirectly from no canopy cover, can affect the physiological stress, condition, immune function, and survival of *R. berlandieri* tadpoles. In the laboratory, I found that tadpoles housed for 1 week at 27° C had lower CORT release rates and higher body condition than those at 19° C. Similarly, tadpoles in outdoor mesocosms without shade (higher temperatures) had lower CORT release rates. These tadpoles also had reduced body condition and survival, though skin immune defenses were significantly higher. Together, these results indicate warmer water temperatures from both climate change and habitat alteration can impact *R. berlandieri* population health, though impacts may not necessarily be detrimental.

In response to stressors, vertebrates generally elevate glucocorticoid production as this helps metabolize energy and assist in coping with the threat (Romero et al. 2009;

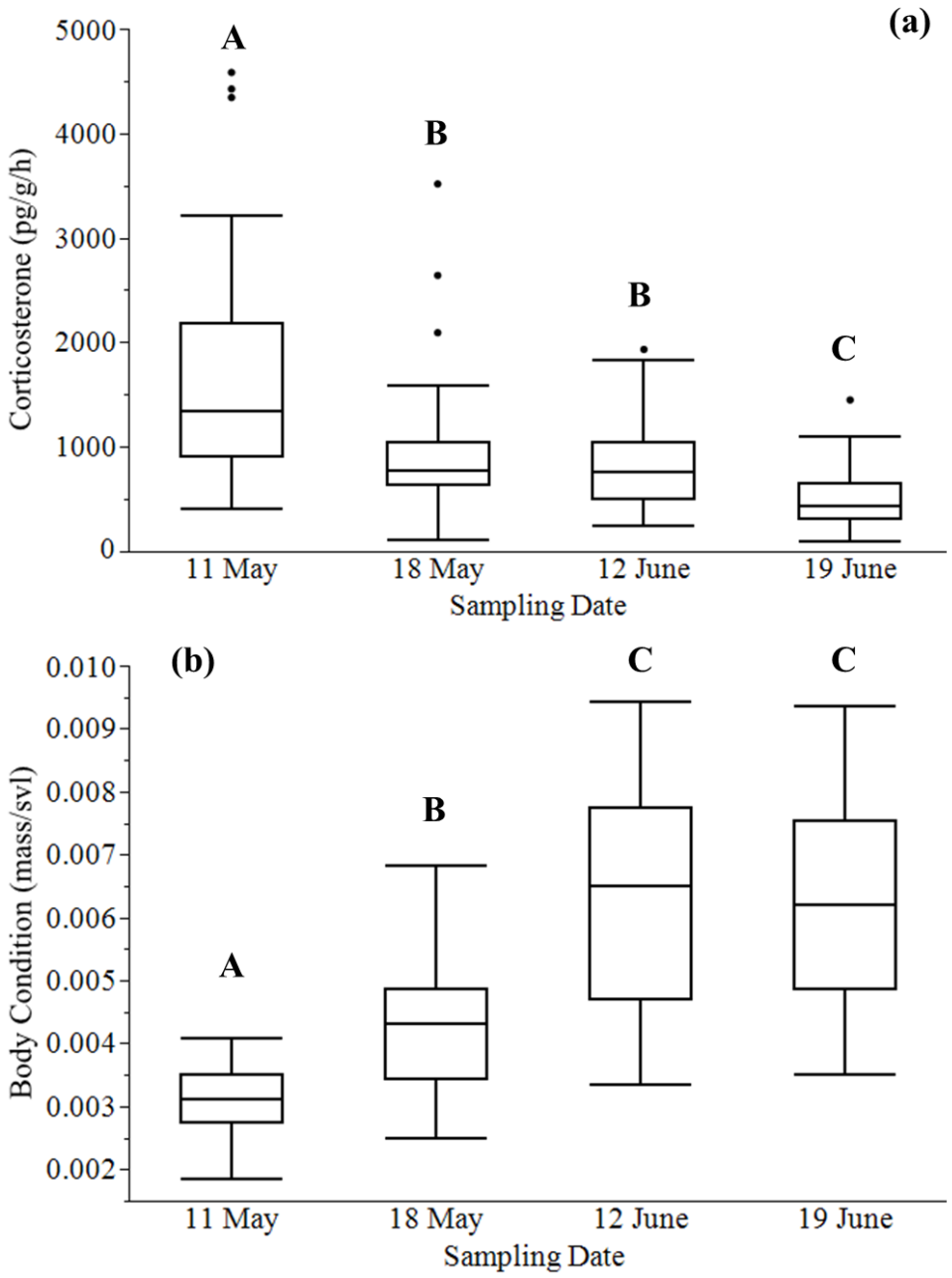

Figure 13. Mean (± SE) (a) corticosterone release rates (pg/g/h) and (b) body condition of Rio Grande Leopard Frog (*Rana berlandieri*) tadpoles from outdoor mesocosms across the first four sampling days. Different letters indicate significant differences. Untransformed data are shown.

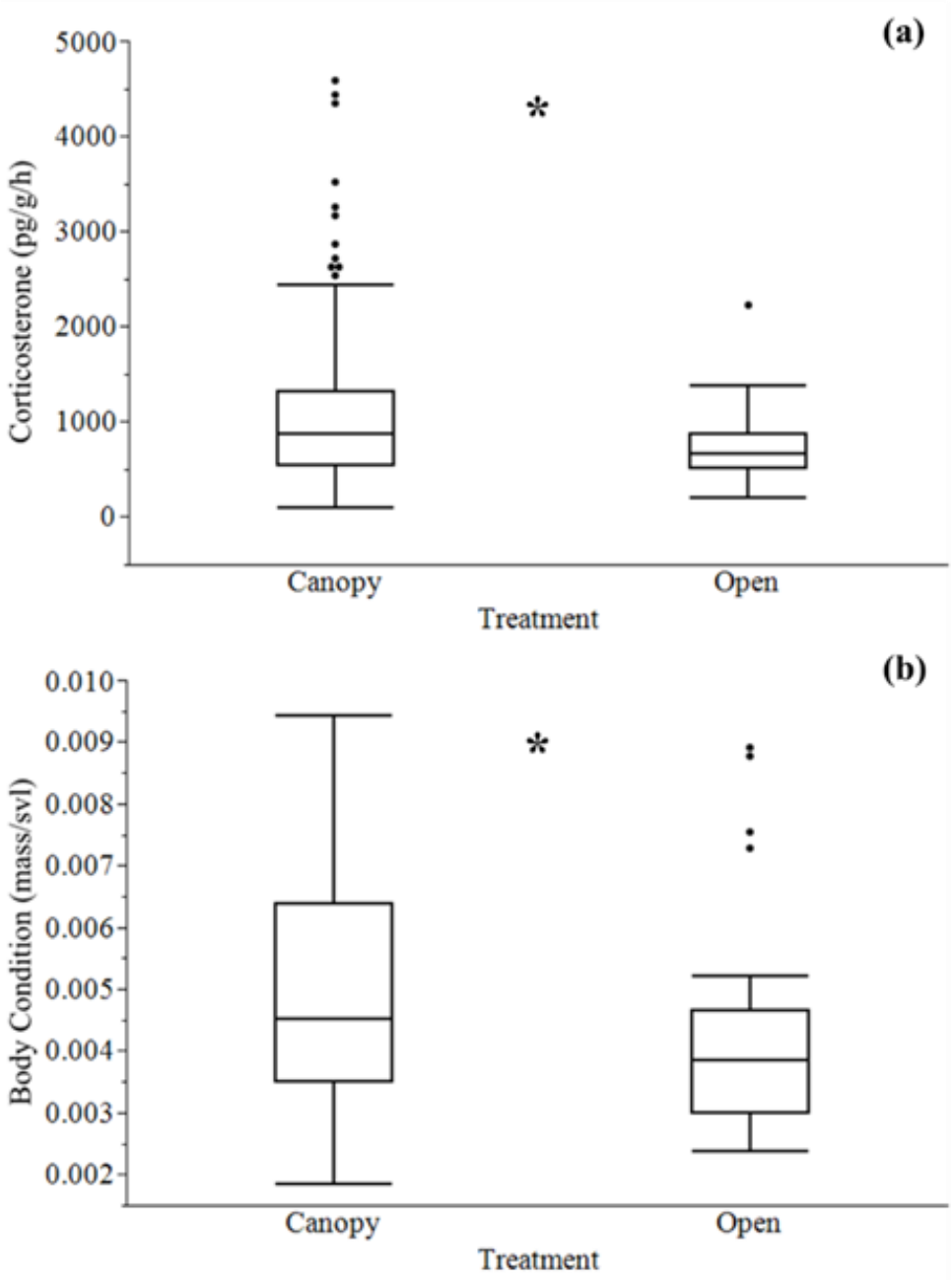

Figure 14. Mean (± SE) (a) corticosterone release rates (pg/g/h) and (b) body condition of Rio Grande Leopard Frog (*Rana berlandieri*) tadpoles from outdoor mesocosms in both Open (averaging 29.9° C) and Canopy (averaging 26.8° C) treatments across the first four sampling days. Untransformed data are shown.

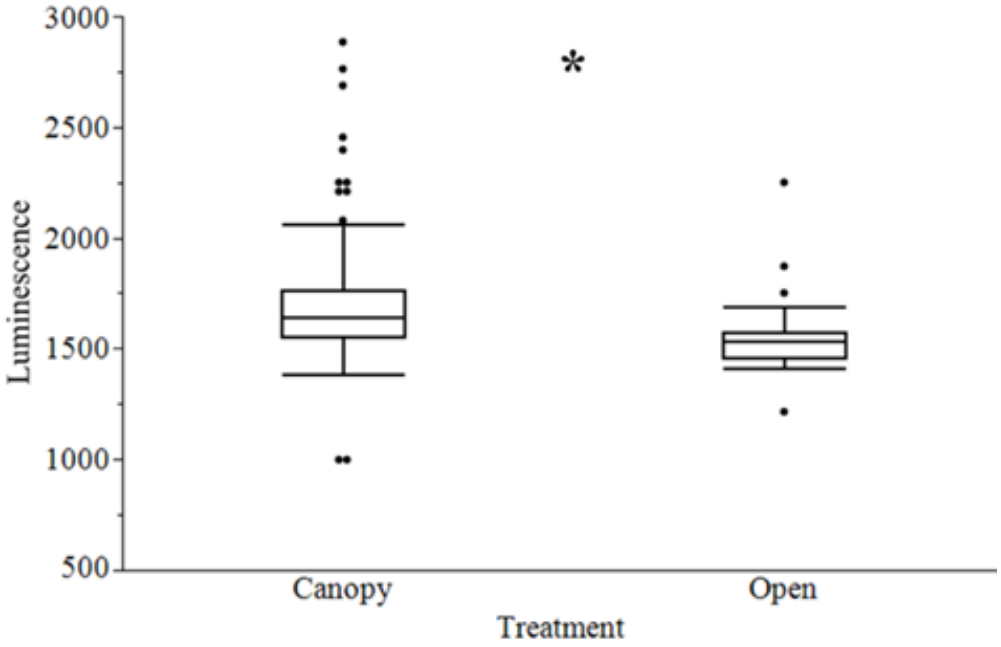

Figure 15. Mean (± SE) luminescence of Rio Grande Leopard Frog (*Rana berlandieri*) tadpole mucosome samples indicating *Batrachochytrium dendrobatidis* zoospore viability using the Promega CellTiter–Glo 2 assay. Samples are from both Open (averaging 29.9° C) and Canopy (averaging 26.8° C) treatments across the first four sampling days.

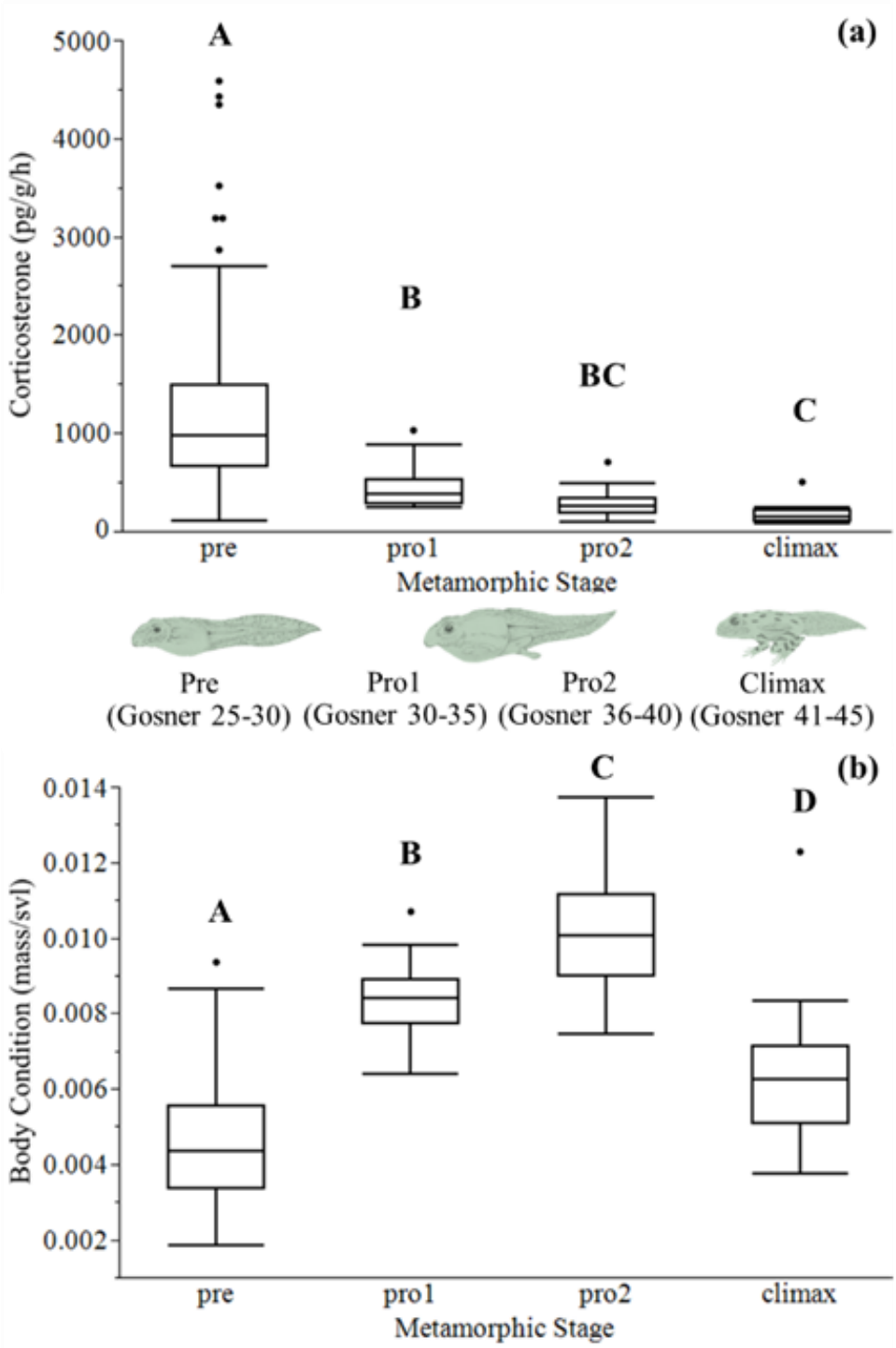

Figure 16. Mean (± SE) (a) corticosterone release rates (pg/g/h) and (b) body condition of Rio Grande Leopard Frog (*Rana berlandieri*) tadpoles across developmental stages within the Canopy treatment across the entire sampling period. Untransformed data are shown.

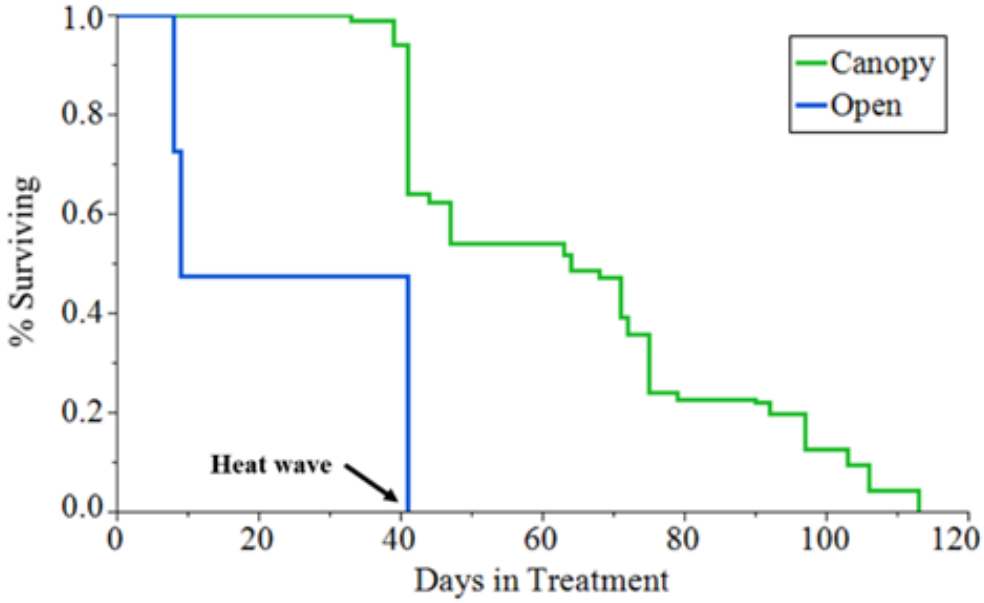

Figure 17. Logrank Mantel-Cox survival analysis of Rio Grande Leopard Frog (*Rana berlandieri*) tadpoles from both Open (averaging 29.9° C) and Canopy (averaging 26.8° C) treatments.

Sapolsky et al. 2000). Under prolonged (chronic) stress, vertebrates may show elevated or lower baseline GC levels (Cyr and Romero 2009) both of which could indicate habituation to a stressor, exhaustion of the HPI axis, or downregulation/desensitization of the response (Cyr and Romero 2009; McEwen and Wingfield 2003; Romero 2009; Wingfield and Romero 2001). Previous studies of amphibians generally show elevated CORT associated with higher temperature, presumably as a response to a stressor or to account for metabolic increases (Chambers et al. 2013; Millikin et al. 2019; Narayan and Hero 2014a, 2014b; Novarro et al. 2018). I found significantly lower CORT release rates at 27° C compared to 19° C in the lab, and in mesocosms without canopy cover (averaging 29.9° C) compared to mesocosms under shade cloth (averaging 26.8° C). Lower CORT can indicate the conditions are not perceived as stressful, however, this response may also indicate habitation or downregulation in response to chronic stress

(Cyr and Romero 2009; Rich and Romero 2005). Lower baseline and acute CORT levels following a chronic stressor have been documented in European starling (*Sturnus vulgaris*) nestlings (Cyr and Romero 2007; Rich and Romero 2005) and lower plasma CORT concentrations following food depravation have been observed in juvenile Western Spadefoot Toads (*Spea hammondi*; Crespi and Denver 2005). Further, downregulated CORT specifically in response to increased temperatures has been observed in other species of birds (Lobato et al. 2008) and a snake (Dupoue et al. 2018). These studies collected glucocorticoids from plasma, whereas the water-borne method I used to measure hormones is an integrative measure, measuring the cumulative effects of chronic stress (Dantzer et al. 2014; Sheriff et al. 2011). My results may suggest the high temperatures in these experiments are a chronic stressor, however I am unable to differentiate between habituation to a stressor, exhaustion of the HPI axis, or downregulation/desensitization of the GC response. I did not perform any test using an acute stressor (e.g. agitation, Gabor et al. 2016) to determine whether individuals were able to mount a response to additional stressors, though the reduction in body condition in the mesocosm experiment indicates that the lower CORT is downregulation or exhaustion due to chronic stress, not habituation.

Warmer water temperatures may have both positive and negative effects on amphibian health. Several studies show increased developmental rate and body condition of tadpoles in warmer water and open canopy (Reading 2010; Schiesari 2006), though stress is generally associated with lower body condition and reduced growth in amphibians (Crespi and Warne 2013; Denver 2009; Glennemeier and Denver 2002a; Hu et al 2008; Janin et al. 2011). Schiesari (2006) found that the Leopard Frog, *Rana pipiens*,

a species related to *R. berlandieri*, was an open-canopy specialist and had faster development and higher body condition in warmer water, though the temperature treatments only ranged from 14–24° C. Hillis (1981) found larval *R. berlandieri* in central Texas mostly along streams and rivers. This suggests *R. berlandieri* may prefer cooler water and canopy cover in this part of their range. Higher temperatures were associated with increased tadpole body condition in the laboratory experiment, but decreased body condition in the warmer mesocosm treatment without shade cover. Additionally, tadpoles from the mesocosms had reduced survival in the Open treatment when compared to the Canopy treatment, though body condition increased over time in both treatments. Periphyton growth was not different between the Open and Canopy treatments, indicating the reduced body condition observed in the mesocosms was not a factor of food availability. The reduced body condition further supports my hypothesis that the lower CORT release rates and altered CORT profiles observed in the warmer temperature treatments of both experiments are an outcome of downregulation or exhaustion of the HPI axis because of chronic stress, not habituation.

My results suggest that warmer water temperatures may be stressful for larval *R. berlandieri* in some environments, resulting in reduced physiological condition. Tadpoles that have lower body condition and size at metamorphosis may have reduced growth and survival after metamorphosis (Cabrera-Guzman et al. 2013; Earl and Whiteman 2015). For *R. berlandieri* tadpoles that develop in warmer, ephemeral ponds, the higher water temperatures may be beneficial in the short term, resulting in higher body condition and faster development, as seen in the related species, *R. pipiens* (Schiesari 2006). However, over longer periods, these warmer water temperatures may lower body condition, as seen

in the mesocosms. This trade-off between condition and development may be beneficial if the pond is rapidly drying. However, in more permanent and cooler systems, such as streams and rivers, *R. berlandieri* tadpoles may benefit from longer periods of development. *R. berlandieri* tadpoles are known to overwinter in central Texas (Hillis 1982) which may allow for more time to develop in the cooler, shaded environments, resulting in higher body condition at metamorphosis. In the Canopy treatment, I found that water-borne CORT release rates were not repeatable. Forsburg et al. (2019) previously found that water-borne CORT release rates showed a significant, moderate repeatability for *R. berlandieri* over time and in different housing types in the lab. Because repeatability can be used as a measure of heritability of a trait within a population, my results suggest CORT release may not be able to respond to higher temperatures. Therefore, cooler, more stable aquatic systems such as streams, or ponds with canopy cover may, be important for *R. berlandieri* tadpoles.

Measuring the ability of amphibian skin microbial communities to fight a known pathogen (mucosome function) has been used to predict disease risk across populations (Smith et al. 2018; Woodhams et al. 2014). Amphibian skin and gut microbial communities are influenced by environmental conditions, including temperature (Kohl and Yahn 2016; Krynak et al. 2016; Robak and Richards-Zawacki 2018). Both cooler temperatures and stress reduce amphibian immune defenses (reviewed in Rollin-Smith 2017), and cooler temperatures are associated with fewer gut microbial taxa (Kohl and Yahn 2016), skin microbiota (Bletz et al. 2017) and reduced blood-borne immune defenses in amphibians (Raffel et al. 2006). I found that the mucosome function of *R. berlandieri* tadpoles was higher in the warmer water of the Open treatment compared to

the cooler Canopy treatment. Therefore, warmer water temperatures may have a positive effect on the skin microbial community, regardless of individual host health. Increased water temperatures are also known to reduce infection by two main amphibian diseases: *Batrachochytrium dendrobatidis* (*Bd*) and *B. salamandrivorans* (*Bsal*) (Blooi et al. 2015; Robak and Richards-Zawacki 2018; Woodhams et al. 2008). Temperatures above 28° C kill the amphibian chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*; Woodhams et al. 2008) and those amphibians who are infected by *Bd* tend to survive better at warmer temperatures (Berger et al. 2004), yet warmer temperatures are associated with increased infection by Ranavirus (Brand et al. 2016). Thus, higher water temperatures, though detrimental to larval *R. berlandieri* health over long periods, may aid in resistance to some fungal pathogens by preventing pathogen growth and by harboring a more diverse skin and gut microbial community that assist in immune function.

The physiological responses of amphibians also differ across development. The developmental stage of *R. berlandieri* tadpoles were associated with differences in CORT profiles and body condition within the Canopy treatment. Chambers et al. (2011) showed an increase in whole-body CORT throughout development in laboratory-reared, mesocosm-reared, and free-living Wood Frog (*Rana sylvatica*) tadpoles. Glennemeier and Denver (2002b) found highest whole-body CORT levels at climax in Leopard Frog (*R. pipiens*) tadpoles, though premetamorphic stages of African Clawed Frog (*Xenopis laevis*) tadpoles had the highest whole-body CORT and was lowest during prometamorphosis. Metamorphosis is an energetically costly period in larval amphibians (Orlofske and Hopkins 2009) which may explain why CORT generally increases during climax. Unlike these studies, I found significantly lower water-borne CORT release rates

with increasing developmental stage. I used an integrated measure of CORT unlike the other studies which measured plasma, which may result in more CORT bound to support metamorphic climax and so less is released or metabolized in the urine, feces etc. that I measured.

Though body condition was highest during prometamorphic stages (Gosner 30–40), it was significantly lower at climax. The reduced CORT release rates observed in this species across development may also indicate downregulation due to higher water temperatures and/or chronic stress, as CORT should increase to mobilize energy stores required for development (Denver 2009). Though CORT aids in development in the short-term, increased CORT in response to stressors lowers body size and condition in amphibians (Crespi and Warne 2013; Denver 2009) and reduced size at metamorphosis can affect juvenile survival (Cabrera-Guzman et al. 2013; Earl and Whiteman 2015). The decrease in body condition as tadpoles reach metamorphic climax is not surprising, as the energetic demands of development would be highest at this time and fat stores would be used to complete metamorphosis, though increased stress during this time may exacerbate this response, affecting health. Together, these results indicate that more studies are needed to measure CORT across development using integrated measures to understand why our measures of CORT varied from prior studies.

Amphibian populations have been declining globally due to synergistic effects of multiple factors, though habitat loss/degradation, climate change, and disease are suggested to be significant drivers (Blaustein and Keisecker 2002; Blaustein et al. 2011, 2012; Collins et al. 2010; Stuart et al. 2004). My results suggest increased water temperatures over prolonged periods may dysregulate the CORT response, lower body

condition, and reduce survival in *R. berlandieri* tadpoles, though warmer water temperatures over shorter periods may aid in growth and warmer temperatures in general may increase skin immune defenses. Further, the CORT response was not repeatable, indicating a lack of ability of this population to respond to selection in higher temperatures\over time. By measuring multiple physiological health metrics, I was able to more clearly understand the effects of altered environmental characteristics on larval *R. berlandieri* health. These results show the need for researchers and managers to examine multiple metrics to fully understand population health and aid in management decisions. Additionally, these results indicate optimum habitat conditions may differ for larval *R. berlandieri* depending on their developmental timing and location. Cooler, shaded areas may facilitate better physiological health for those that take longer to develop, and warmer, open areas may facilitate growth (to get out of the pond sooner) and immune defenses.

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APPENDIX SECTION

APPENDIX A: Supplementary Material

Table S1. Baseline and agitation CORT release rates of *Pseudacris ornata* tadpoles from the 10 ponds sampled in 2016 and 12 ponds sampled in 2017 were modeled as a function of the following predictors.

Model number	Predictors
1	Landcover100
2	Urban1000
3	Canopy500
4	PC1
5	PC2
6	PC1+PC2
7	Landcover100+PC1
8	Landcover100+PC2
9	Landcover100+PC1+PC2
10	Canopy500+PC1
11	Canopy500+PC2
12	Canopy500+PC1+PC2
13	Urban1000+PC1
14	Urban1000+PC2
15	Urban1000+PC1+PC2
16	MEM2
17	MEM3
18	MEM2+MEM3
19	MEM2+PC1
20	MEM3+PC1
21	MEM2+MEM3+PC1
22	MEM2+PC2
23	MEM3+PC2
24	MEM2+MEM3+PC2
25	MEM2+PC1+PC2
26	MEM3+PC1+PC2
27	MEM2+MEM3+PC1+PC2

Table S2. Mucosome function of *Pseudacris ornata* tadpoles sampled in 2017 were modeled as a function of the following predictors.

Model number	Predictors
1	Landcover1000
2	Urban100
3	Canopy500
4	PC1
5	PC2
6	PC1+PC2
7	Landcover1000+PC1
8	Landcover1000+PC2
9	Landcover1000+PC1+PC2
10	Canopy500+PC1
11	Canopy500+PC2
12	Canopy500+PC1+PC2
13	Urban100+PC1
14	Urban100+PC2
15	Urban100+PC1+PC2
16	MEM2
17	MEM3
18	MEM2+MEM3
19	MEM2+PC1
20	MEM3+PC1
21	MEM2+MEM3+PC1
22	MEM2+PC2
23	MEM3+PC2
24	MEM2+MEM3+PC2
25	MEM2+PC1+PC2
26	MEM3+PC1+PC2
27	MEM2+MEM3+PC1+PC2
28	BCORT

Table S3. Skin bacterial diversity (Richness, Shannon, Simpson) of *Pseudacris ornata* tadpoles sampled in 2017 were modeled as a function of the following predictors.

Model number	Predictors
1	Landcover1000
2	Urban500
3	Canopy100
4	PC1
5	PC2
6	PC1+PC2
7	Landcover1000+PC1
8	Landcover1000+PC2
9	Landcover1000+PC1+PC2
10	Canopy100+PC1
11	Canopy100+PC2
12	Canopy100+PC1+PC2
13	Urban500+PC1
14	Urban500+PC2
15	Urban500+PC1+PC2
16	MEM2
17	MEM3
18	MEM2+MEM3
19	MEM2+PC1
20	MEM3+PC2
21	MEM2+PC1+PC2
22	MEM3+PC1+PC2
23	MEM3+PC2
24	MEM3+PC1+PC2
25	MEM2+MEM3+PC1
26	MEM2+MEM3+PC2
27	MEM2+MEM3+PC1+PC2
28	BCORT

Table S4. Top models explaining environmental influences on *Psuedacris ornata* natural log-transformed corticosterone release rates (CORT) from 10 sites sampled in 2016. Models are ranked according to Akaike's Information Criterion adjusted for small sample size (AICc). AICc score, change in AICc (ΔAICc), and AICc model weight (ω) for each model are shown for the top ranked models (ΔAICc < 2).

Response	Model	K	AICc	ΔAICc	ω
Baseline CORT	MEM2+PC1+PC2	6	315.76	0.00	0.150
	PC1+PC2	5	316.60	0.84	0.098
	MEM2+MEM3+PC1+PC2	7	317.20	1.44	0.073
	PC1	4	317.28	1.52	0.070
	MEM2+PC1	5	317.33	1.58	0.068
	MEM3+PC2	5	317.65	1.89	0.058
Agitation CORT	MEM3+PC2	5	303.25	0.00	0.166
	PC1+PC2	5	303.45	0.20	0.150
	Canopy500+PC1+PC2	6	303.90	0.65	0.120
	MEM2+PC1+PC2	6	304.18	0.93	0.104
	MEM2+MEM3+PC2	6	304.87	1.62	0.074

Table S5. Top models explaining environmental influences on *Psuedacris ornata* natural log-transformed corticosterone release rates (CORT), mucosome function, and bacterial diversity (Richness, Shannon, Simpson) from 12 sites sampled in 2017. Models are ranked according to Akaike's Information Criterion adjusted for small sample size (AICc). AICc score, change in AICc (ΔAICc), and AICc model weight (ω) for each model are shown for the top ranked models (ΔAICc < 2).

Response	Model	K	AICc	ΔAICc	ω
Baseline CORT	Urban1000	4	342.71	0.00	0.388
	Urban1000+PC2	5	344.18	1.48	0.185
Agitation CORT	Urban1000	4	316.85	0.00	0.226
	Urban1000+PC1	5	318.75	1.90	0.088
Mucosome function	PC2	4	950.14	0.00	0.145
	Urban100	4	951.13	0.99	0.088
	Urban100+PC2	5	951.34	1.20	0.080
	Canopy500+PC2	5	951.77	1.62	0.064
	PC1+PC2	5	952.06	1.92	0.056
Richness	Landcover1000	5	1289.06	0.00	0.314
	Landcover1000+PC1	6	1290.78	1.72	0.133
Shannon diversity	MEM2	4	270.95	0.00	0.124
	Canopy100+PC2	5	271.49	0.54	0.094
	Urban500+PC1	5	271.85	0.90	0.079
	Canopy100	4	271.90	0.95	0.077
	Urban500	4	272.58	1.63	0.055
	MEM2+PC1	5	272.65	1.70	0.053
Simpson diversity	MEM2	4	-96.70	0.00	0.312
	MEM2+MEM3	5	-95.10	1.60	0.140

Table S6. Results of Welch t-tests comparing agitation and baseline corticosterone release rates for each site across both sampling years. Values in bold indicate significant differences ($p < 0.05$). AP = Apalachicola National Forest, EG = Eglin Air Force Base, JC = Joseph W. Jones Ecological Research Center at Ichauway, LF = Lafayette Forest Wildlife Environmental Area, SM = St. Marks National Wildlife Refuge, OS = Orianne Society Preserve, WC = James W. Webb Wildlife Center.

Year	Site	df	t	*p*
2016	EG1	31.6	2.19	**0.018**
	AP1	32.4	1.75	**0.044**
	AP2	16.1	1.98	**0.032**
	AP3	32.9	1.22	0.115
	SM1	32.5	0.05	0.481
	SM2	20.3	0.52	0.304
	JC1	26.2	2.06	**0.024**
	JC2	35.4	2.01	**0.026**
	JC3	35.8	0.08	0.467
	LF1	19.8	2.43	**0.012**
2017	EG1	35.7	2.41	**0.011**
	EG2	27.9	0.08	0.207
	AP1	24.8	-1.16	0.871
	AP2	25.6	1.55	0.067
	SM3	27.0	2.87	**0.004**
	SM4	27.9	1.78	**0.043**
	JC1	25.6	-0.62	0.729
	JC2	36.9	1.81	**0.039**
	JC3	35.6	-0.58	0.718
	OS1	24.8	3.17	**0.002**
	OS2	33.3	1.48	0.074
	WC1	37.7	4.15	**<0.0001**

Table S7. Highest and lowest mean ± SE values for each of three alpha diversity indices (Richness, Shannon, and Simpson) for both Sites and Properties sampled in 2017. AP = Apalachicola National Forest, EG = Eglin Air Force Base, JC = Joseph W. Jones Ecological Research Center at Ichauway, WC = James W. Webb Wildlife Center.

Diversity index	Site	Mean ± SE	Property	Mean ± SE
Richness				
Highest	JC2	562.56 ± 48.23	WC	467.44 ± 64.67
Lowest	AP2	121.82 ± 10.08	AP	171.00 ± 23.10
Shannon				
Highest	JC3	3.46 ± 0.33	JC	3.41 ± 0.16
Lowest	EG2	2.21 ± 0.31	EG	2.53 ± 0.25
Simpson (evenness)				
Highest	JC3	0.89 ± 0.02	JC	0.87 ± 0.01
Lowest	EG2	0.65 ± 0.07	EG	0.73 ± 0.05

Table S8. Results of *post-hoc* pairwise comparisons among ponds to examine the similarity of OTU beta diversity from *Pseudacris ornata* skin microbial communities. AP = Apalachicola National Forest, EG = Eglin Air Force Base, JC = Joseph W. Jones Ecological Research Center at Ichauway, LF = Lafayette Forest Wildlife Environmental Area, SM = St. Marks National Wildlife Refuge, OS = Orianne Society Preserve, WC = James W. Webb Wildlife Center.

Comparison	F	*p*	Comparison	F	*p*
AP1 x AP2	1.46	0.144	EG1 X WC1	6.86	0.001
AP1 x EG1	3.68	0.001	EG2 X JC1	15.82	0.001
AP1 x EG2	7.73	0.001	EG2 X JC2	21.63	0.001
AP1 x JC1	6.48	0.001	EG2 X JC3	21.15	0.001
AP1 x JC2	9.11	0.001	EG2 X OS1	21.95	0.001
AP1 x JC3	7.78	0.001	EG2 X SM4	13.32	0.001
AP1 x OS1	6.66	0.001	EG2 X WC1	18.54	0.001
AP1 x SM4	1.58	0.117	JC1 X JC2	6.52	0.001
AP1 x WC1	6.46	0.001	JC1 X JC3	4.46	0.001
AP2 x EG1	4.06	0.001	JC1 X OS1	6.89	0.001
AP2 x EG2	9.75	0.001	JC1 X SM4	10.48	0.001
AP2 x JC1	7.08	0.001	JC1 X WC1	9.94	0.001
AP2 x JC2	9.87	0.001	JC2 X JC3	12.74	0.001
AP2 x JC3	8.34	0.001	JC2 X OS1	11.49	0.001
AP2 x OS1	8.38	0.001	JC2 X SM4	14.47	0.001
AP2 x SM4	2.31	0.033	JC2 X WC1	15.54	0.001
AP2 x WC1	7.75	0.001	JC3 X OS1	8.23	0.001
EG1 x EG2	9.04	0.001	JC3 X SM4	13.68	0.001
EG1 x JC1	4.91	0.001	JC3 X WC1	13.09	0.001
EG1 x JC2	6.34	0.001	OS1 X SM4	11.44	0.001
EG1 x JC3	6.63	0.001	OS1 X WC1	13.7	0.001
EG1 X OS1	6.89	0.001	SM4 X WC1	10.04	0.001
EG1 X SM4	4.96	0.001			

Table S9. Results of *post-hoc* pairwise comparisons among Properties to examine the similarity of OTU beta diversity from *Pseudacris ornata* skin microbial communities. AP = Apalachicola National Forest, EG = Eglin Air Force Base, JC = Joseph W. Jones Ecological Research Center at Ichauway, LF = Lafayette Forest Wildlife Environmental Area, SM = St. Marks National Wildlife Refuge, OS = Orianne Society Preserve, WC = James W. Webb Wildlife Center.

Comparison	F	*p*
AP X EG	6.09	0.001
AP X JC	11.74	0.001
AP X OS	8.35	0.001
AP X SM	1.80	0.064
AP X WC	7.82	0.001
EG X JC	10.58	0.001
EG X OS	9.93	0.001
EG X SM	5.65	0.001
EG X WC	8.60	0.001
JC X OS	6.46	0.001
JC X SM	10.85	0.001
JC X WC	10.60	0.001
OS X SM	11.44	0.001
OS X WC	13.70	0.001
SM X WC	10.04	0.001

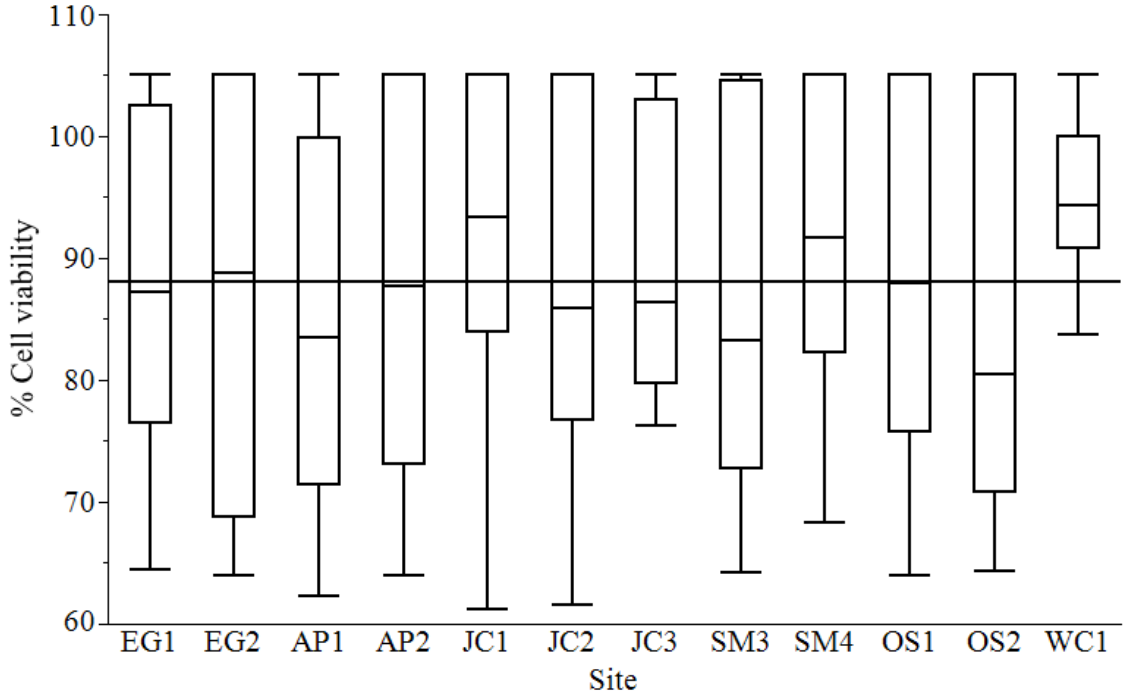

Figure S1. Box and whisker plots showing percent cell viability of *Batrachochytrium dendrobatidis* when plated along with a sample of the water containing the mucosome of 9 *Pseudacris ornata* tadpoles from each pond, representing the mucosome function. Box and whisker plots indicate median, interquartiles, and range for each pond. Horizontal line denotes average percent cell viability. Mucosome samples were only collected from ponds sampled in 2017. Ponds: AP = Apalachicola National Forest, EG = Eglin Air Force Base, JC = Joseph W. Jones Ecological Research Center at Ichauway, LF = Lafayette Forest Wildlife Environmental Area, SM = St. Marks National Wildlife Refuge, OS = Orianne Society Preserve, WC = James W. Webb Wildlife Center. Ponds are ordered geographically from west to east.

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