EFFECTS OF ARTIFICIAL LIGHT AT NIGHT (ALAN) ON THE PHYSIOLOGY, GROWTH, AND BEHAVIOR OF TWO COMMON TEXAS ANURANS

by

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ABSTRACT

Artificial light at night (ALAN) alters the natural light dark patterns in ecosystems and is a growing problem, as 40% of the world's population lives in areas continually illuminated and area exposed to ALAN is increasing at an estimated 6% per year. ALAN can have a suite of effects on the behavioral, developmental, and physiological traits of organisms. Amphibians are the most imperiled vertebrate class, as over 40% of known species are threatened with extinction, yet our understanding of how ALAN affects amphibians is lacking compared to our knowledge of how ALAN affects other organisms. My dissertation research explored how ALAN affects the physiology, growth, and behavior of the larvae of two common Texas anurans, the Rio Grande leopard frog (*Rana berlandieri*) and the Gulf Coast toad (*Bufo valliceps*). Chapter 1 is an introduction to ALAN, how ALAN affects other groups of organisms, and an overview of the main topics of my research. I validate a non-invasive water-borne hormone collection method for *Rana berlandieri*, explore the physiology of *R. berlandieri* tadpoles, and compare rearing methods in Chapter 2. Chapter 3 discusses the results of laboratory reaction norm studies showing that short term exposure to ALAN affects the physiology, but not the behavior or growth of *R. berlandieri* and *B. valliceps* tadpoles. The last chapter explores if ALAN acts synergistically with a natural environmental stressor for tadpoles, the presence of predators, using a semi-natural outdoor mesocosm experiment. Additionally, I examined how long-term exposure to ALAN affected the growth and anti-predator behavior of *R. berlandieri* Rio Grande leopard frog tadpoles. My results show that both

short-term and long-term exposure to environmentally relevant levels of ALAN affect the physiology of anuran larvae. Short-term ALAN exposure did not affect behavior, but long-term exposure to ALAN diminished anti-predator behavior. Long-term exposure decreases the growth of tadpoles and increases the rate of metamorphosis. My research also suggests that ALAN interacts synergistically with other environmental stressors to negatively affect the physiology of tadpoles. Together, my research suggests that urban planners should include ways to remove ALAN, or mitigate the effects of ALAN, when building around wildlife areas to decrease the consequences of ALAN on amphibian populations.

I. INTRODUCTION

Background

Humans have significantly altered much of the Earth's surface, waters, and atmosphere over the past 300 years, such that we are now in a new geological epoch, the Anthropocene (Ellis, 2011). The human population is currently estimated at 7.2 billion and is projected to reach 10.9 billion by 2100 (Gerland et al., 2014). Continued exponential human population growth will lead to increased urbanization and anthropogenic changes to the environment, resulting in a decrease of natural habitat for other organisms. In the United States alone, over 295,000 square kilometers of land was classified as urbanized in 2012 by the United States Department of Agriculture (USDA, 2015). Globally, there were over 652,000 square kilometers of land classified as urbanized in 2000 with a projected increase to 1.2 million km^2 of urbanized land by the year 2030 (Seto et al., 2012). Anthropogenic changes to the environment include habitat alteration/loss, global climate change, spread of disease and exotic species, overharvesting and exploitation of natural resources, and many forms of pollution (Pelletier & Coltman, 2018; Sih et al., 2011; Swaddle et al., 2015; Tillman et al., 2017), and can act as selective pressure on organisms, inducing rapid evolutionary responses (Pelletier & Coltman, 2018; Sih et al., 2011). Continued human population growth, urbanization, and other anthropogenic changes pose a significant threat to global biodiversity (McKee et al., 2003). It is difficult to estimate a current extinction rate, though it has been predicted that between 20% to 50% of known species will become extinct within the next 30 years (Stork, 2010), and it is now widely accepted that we are at the beginning of the 6^{th} mass extinction (Barnosky et al., 2011; Ceballos et al., 2015).

On a global scale, amphibians are the most imperiled vertebrate class and amphibian populations are declining at alarming rates (Clulow et al., 2014; Collins & Halliday, 2005; Grant et al., 2016). Of the known amphibian species, it is estimated that 43% are experiencing declining populations and 32% are globally threatened (Wake & Vrendenburg, 2008), compared to just 12% and 25% of avian and mammal species, respectively (Clulow et al., 2014). It is also estimated that 3.79% of local amphibian populations are being lost yearly (Grant et al., 2016). Early research efforts focused on finding a single cause of amphibian declines, but it is now recognized that there are multiple factors contributing to declining populations (Grant et al., 2016). The six main causes of amphibian declines are habitat loss/alteration, invasive species, over exploitation, emerging infectious diseases, pesticides/pollution, and global climate change (Blaustein et al., 2010; Collins & Storfer, 2003; Wake & Vrendenburg, 2008). There is no universally applicable approach to halting amphibian declines, so research to understand how individual stressors affect species regionally, as well as how synergism between stressors may affect amphibians, will aid conservation efforts (Grant et al., 2016).

Hormone responses may be repeatable and or flexible. Hormone levels fluctuate from day to night and may change in response to variation in abiotic and biotic factors in a changing environment (Hau et al., 2016). Modulating hormonal responses help mediate behavior, physiology, and morphology in changing environments (Nelson, 2011), particularly glucocorticoid (GCs) response, as they help to maintain energy needs in both predictable and unpredictable events (Sapolsky et al., 2000). Variation in circulating levels of GCs and hormonal and behavioral responses to changing environments have

been observed within and among individuals (reviewed in Hau et al., 2016) and this variation is mediated by phenotypic flexibility (Piersma & Drent, 2003). It is important to evaluate within- and between- individual variation in behavior and physiology and how the variation affects how individuals respond to stressors or changing or unpredictable environments (Lendvai et al., 2014).

A non-invasive method is required for repeatedly measuring hormones over time, as methods to measure GCs and other hormones typically are invasive or require euthanizing individuals (reviewed by Baugh et al., 2018; Gabor et al., 2016; Sheriff et al., 2011). Recently, several studies have validated the use of a non-invasive water-borne hormone collection protocol for both larvae and adults of several amphibian species that facilitates repeated measure experimental designs and works in field and laboratory (Baugh et al., 2018; Forsburg et al., 2019; Gabor et al., 2016; Narayan et al., 2019). This water-borne hormone collection method was developed for use in fish, and measures steroid hormones passively diffused into the water via gills, urine, and feces (Scott et al., 2008). By repeatedly measuring hormones from the same individuals over time, one can parse out the within and among individual variation of hormone levels and calculate the repeatability of those hormone levels. The repeatability of hormone levels provides insight into how individuals respond to changing environments or stressors (Hau et al., 2016; Lendvai et al., 2014) and is considered an upper bound estimate of heritability of a trait (Lessells & Boag, 1987; but see Dohm 2002).

Organisms respond to environmental stressors and cope with changing environments in many ways, including altering their physiology to maintain homeostasis (McEwen & Wingfield, 2003; Wikelski & Cooke, 2006). The amphibian neuroendocrine

response to a stressor involves the hypothalamic-pituitary-interrenal, HPI, axis (Cyr & Romero, 2009). When something is perceived as a stressor by the brain, the hypothalamus secretes corticotropin releasing hormone (CRH) which induces the pituitary gland to release adrenocorticotropic hormone (ACTH). ACTH is transported by blood to the adrenal cortex, which then releases glucocorticoids (GCs) (Denver, 2009). Physiological changes in response to stressors, or stressful environments, include increased circulating levels of GCs above normal "baseline" levels (Romero et al., 2009). A short-term elevation of circulating GCs can be advantageous as it mediates gluconeogenesis and mobilizes energy (Hau et al., 2016; Romero et al., 2009; Sapolsky et al., 2000), however unpredictable and long-term perturbations can lead to persistently elevated or down regulated (suppressed) GC levels which can be deleterious (Romero et al., 2009). Corticosterone (cort) is the main GC in amphibians (Forsburg et al., 2019; Idler, 1972) and elevated levels of cort can reduce growth in amphibian larvae, alter tadpole morphology, and hasten metamorphosis (Crespi & Warne, 2013; Denver et al., 1998; Glennemeier & Denver, 2002a; Hu et al., 2008), and reduced mass at metamorphosis can affect growth and survivorship later in life (Cabrera-Guzman et al. 2013; Chelgren et al., 2006; Crespi & Warne, 2013; Earl & Whiteman, 2015; Rohr et al., 2013).

Artificial light at night as an anthropogenic stressor

Of the many anthropogenic factors contributing to amphibian population declines, only recently has artificial light at night (ALAN; Haim & Zubidat, 2015) started gaining attention as a potential stressor to organisms and a threat to biodiversity (Garret et al., 2019; Gaston et al., 2019; Secondi et al., 2020; Seymoure et al., 2019). ALAN is artificial

light that alters the natural light dark patterns in ecosystems (Swaddle et al., 2015), and sources include street lamps, lights on buildings, vehicles, and sky glow from cities (Navara & Nelson, 2007; Longcore & Rich, 2004). “Light trespass” occurs when light from a streetlamp, flood light, or any other source of artificial lighting enters an area not intended to be illuminated (Schreuder, 1987). It is estimated that over 80% of the world population live in areas that are considered polluted by ALAN (Falchi et al., 2016) and about 40% of the world population lives in areas that are continually illuminated at night (Swaddle et al., 2015). Seymoure et al. (2019) estimate that over 50% and 75% of key biodiversity areas and global protected area units, respectfully, are affected by ALAN. With continued human population growth and urbanization, ALAN is estimated to be increasing at a rate of 6% yearly (Hoelker et al., 2010), and thus the impact of ALAN on organisms may be of great importance and potential negative effects may have significant ecological consequences and conservation implications (Garret et al., 2019; Gaston et al., 2015; Secondi et al., 2020; Seymoure et al., 2019).

ALAN affects the behavior, physiology, and development of many taxa (see reviews: Gaston et. Al., 2014; Longcore & Rich, 2004; Navara & Nelson, 2007; Secondi et al., 2020; Swaddle et al., 2015). ALAN reduces active foraging (Bird et al., 2004; Wise & Buchanan, 2006) and may also increase the risk of predation (Rydell, 2006) and the incidence of road mortalities (Mazerolle et al., 2005). Shifts in the calling behavior (Miller, 2006) and clutch lay date (De Jong et al., 2015) of birds have been observed in areas with ALAN. Exposure to ALAN can suppress immune function (Bedrosian et al., 2011; Bedrosian et al., 2013), and can elevate levels of corticosterone (Ouyang et al., 2015). Exposure to ALAN can accelerate sexual maturation (Dominoni et al., 2013) and

pupation (Van Geffen et al., 2014), but can also reduce levels of gonadotropin (Bruning et al., 2016). Thus, ALAN has far reaching effects on organisms.

Effects of ALAN on amphibians

Artificial light at night (ALAN) affects both the behavior and physiology of amphibians. Reduced foraging and activity have been observed in red-back salamanders, *Plethodon cinereus,* exposed to ALAN (Wise & Buchanan, 2006). Low levels of artificial light reduce the ability of grey treefrogs *Hyla chrysoscelis,* to detect and capture prey (Buchanan, 1993) and when exposed to artificial light, common green frogs, *Rana clamitans,* reduce calling (Baker & Richardson, 2006). A field study by Dias et al. (2019) showed that anurans in continuously lit wetlands started calling earlier in the year, and the calling season was shorter, compared to anurans in un-lit wetlands. Leopard frog tadpoles, *Rana pipiens,* reared under constant light metamorphosed faster and weighed less at metamorphosis than tadpoles on a control light cycle (Eichler & Gray, 1976). Similarly, leopard frog, *R. pipiens,* (Wright et al., 1988) and American toad, *Bufo americanus,* (Dananay & Benard, 2018) tadpoles metamorphosed faster when exposed to 18 hours of light a day or constant light, respectfully. In contrast, May et al. (2019) showed wood frog, *Rana sylvatica,* tadpoles exposed to ALAN weighed more at metamorphosis, though ALAN did not affect time to metamorphosis or survival. When reared under constant light, tiger salamanders, *Ambystoma tigrinum,* did not show the typical oscillating melatonin rhythm associated with a normal light/dark cycle (Gern et al., 1983). Captive *Melanophryniscus rubriventris* toads reared under constant light had higher neutrophils proportions and a higher ration of neutrophils to lymphocytes than toads reared under a normal light cycle (Gaston et al., 2019). Relative to other taxa, the

effects of ALAN on amphibians are still understudied (Dutta, 2018; Gaston et al., 2019: Ouyang, et al., 2017; Perry et al., 2008), particularly, there is a lack of studies investigating how ALAN may affect behavior and stress physiology in larval amphibians, yet is important, as amphibians are the most imperiled taxa (Grant et al., 2016), and knowledge gained from these studies can be applied to conservation and management efforts to protect global amphibian populations.

Study species

Rana berlandieri

Rio Grande leopard frogs (Anura: Ranidae: *Rana berlandieri* Baird, 1859) are common anurans in the *Rana pipiens* complex that are found throughout Northeastern Mexico and Southern Texas (Fig. 1; Hillis, 1981; Hughes & Meshaka, 2018; Lanoo, 2005; Zaldívar-Riverón et al., 2004). *R. berlandieri* inhabit a range of habitats, associated with rivers, springs, ponds, and artificial bodies of water, including cattle tanks (Hughes & Meshaka, 2018). Males are smaller than females, with an average snout-vent length (SVL) of ~70mm, while females have an average SVL of ~77mm (Hughes & Meshaka, 2018). Mating occurs primarily in the Spring, from February to May, though mating has been observed in the Fall, from October through December (Hughes & Meshaka, 2018). Females lay clumps of eggs, ranging in size from several hundred to several thousand eggs, in calm streams, ponds, or temporary bodies of water (Hughes & Meshaka, 2018). Tadpoles feed on several types of algae and diatoms (Hillis, 1982) and the larval stage is typically 4 to 9 months (Hughes & Meshaka, 2018), though tadpoles do overwinter,

particularly if a result of a Fall mating (Hillis, 1982).

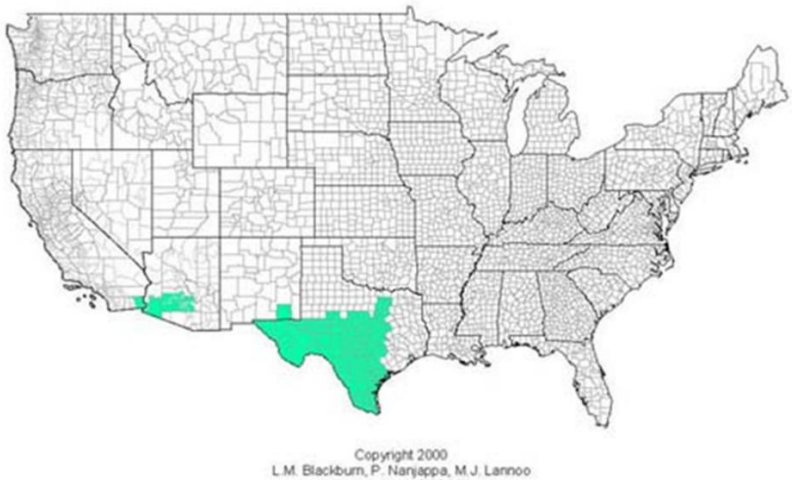

Figure 1. Distribution (in green) of the Rio Grande leopard frog, *Rana berlandieri*, in the United States. Map borrowed from Lannoo, M. (2005) and published online (AmphibiaWeb. 2020).

Bufo valliceps

Gulf Coast toads (Anura: Bufonidae; *Bufo valliceps,* Wiegmann, 1833) are common anurans found throughout the Gulf Coast plain from Mexico through Texas and East to Mississippi (Fig 2; Lanoo, 2005; Porter, 1970). *Bufo valliceps* inhabit a range of habitats throughout its range (Dixon, 2000; Dundee & Rossman, 1989). Males are smaller than females, with a snout-vent length (SVL) up to 98mm, while females have an SVL up to 130mm (Blair, 1953; Porter, 1970; Wright & Wright, 1949). Mating occurs from March to August (Thornton, 1960). Females lay strings of up to 20,000 eggs in still, shallow, water (Blair, 1960). Tadpoles feed on algae and the larval stage ranges from 1 to 2 months (Thornton, 1955; Wright & Wright, 1949).

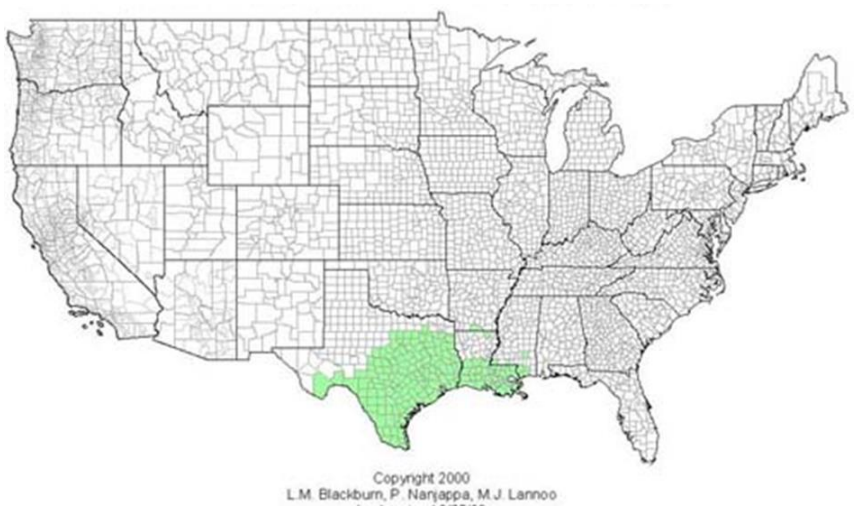

Figure 2. Distribution (in green) of the Gulf Coast toad, *Bufo vallicpes*, in the United States. Map borrowed from Lannoo, M. (2005) and published online (AmphibiaWeb. 2020).

Research experiments

Artificial light at night (ALAN) is a stressor to many taxa and a threat to biodiversity (Garret et al., 2019; Gaston et al., 2019; Secondi et al., 2020; Seymoure et al., 2019), yet, relative to other taxa, the effects of ALAN on amphibians, the most imperiled vertebrate taxa (Grant et al., 2016), are still understudied (Dutta, 2018; Gaston et al., 2019: Ouyang et al., 2017; Perry et al., 2008). Particularly, there is a lack of studies investigating how ALAN may affect behavior and stress physiology in larval amphibians. By measuring an aspect of anuran physiology, such as glucocorticoid levels, over time, and how individuals respond to acute stressors, one can assess how individuals are responding to environmental perturbations, such as ALAN, and changing environments (Dantzer et al., 2014; Sheriff et al., 2011). ALAN can elevate levels of corticosterone in birds (Ouyang et al., 2015) though it is unclear how ALAN affects corticosterone levels in amphibians. I first explored the physiology of glucocorticoids in larval *Rana berlandieri* and different laboratory housing methods. I also validated the non-invasive

water-borne hormone collection protocol (Gabor et al., 2016) for *R. berlandieri*. Through a series of experiments with differing sequence of exposure to ALAN on larval *R. berlandieri* and *Bufo valliceps*, I tested the hypothesis that exposure to 190 lx to 250 lx ALAN would affect corticosterone release rates in tadpoles, and predicted tadpoles would show either elevated or decreased cort release rates. I explored potential synergistic effects between ALAN and predator presence on the physiology and growth of *R. berlandieri* using a semi-natural mesocosm experiment. I hypothesized that the presence of dragonfly larvae and exposure to ALAN would be more stressful than either exposure to ALAN or dragonfly larvae alone, with tadpoles showing elevated levels of corticosterone. Additionally, I examined how long-term exposure to ALAN would affect growth and anti-predator behavior in leopard frog tadpoles in a laboratory setting, predicting that tadpoles in ALAN treatments would metamorphose faster and would be smaller at metamorphosis than tadpoles reared on a natural light cycle.

II. VALIDATION OF WATER-BORNE CORTISOL AND CORTICOSTERONE IN TADPOLES: RECOVERY FROM AN ACTURE STRESSOR, REPEATABILITY, AN EVALUATING REARING METHODS[†]

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Abstract

Amphibian populations are declining globally, so understanding how individuals respond to anthropogenic and environmental stressors may aid conservation efforts. Using a non-invasive water-borne hormone assay, we measured the release rates of two glucocorticoid hormones, corticosterone and cortisol, in Rio Grande Leopard frog, *Rana berlandieri*, tadpoles. We validated this method pharmacologically and biologically using an adrenocorticotropic hormone (ACTH) challenge, exposure to exogenous corticosterone, and an agitation test. We calculated the repeatability of hormone release rates, the recovery time from an acute stressor, and explored rearing methods for tadpoles. Tadpole corticosterone release rates increased following an ACTH challenge, exposure to exogenous corticosterone, and agitation, validating the use of water-borne hormone methods in this species. After exposure to an acute stressor via agitation, corticosterone release rates began to decline after 2h and were lowest after 6h, suggesting a relatively rapid recovery from an acute stressor. Tadpoles reared in groups had higher corticosterone release rates than tadpoles reared individually, and lost mass by Day 7, while tadpoles reared individually did not show a stress response, therefore either rearing method is viable, but have differing physiological costs for tadpoles. Repeatability of corticosterone release rates was moderate to high in *R. berlandieri* tadpoles, indicating that this species can show a response to selection and potentially respond to rapid environmental change. Our results show that the water-borne hormone assay is a viable

way to measure glucocorticoids in this species and is useful in the field of conservation physiology for rare and endangered species.

Keywords: ACTH; amphibian; conservation physiology; stress; water-borne hormones; non-invasive endocrinology

Introduction

Continued human population growth, urbanization, and other anthropogenic changes pose a significant threat to global biodiversity (McKee et al., 2003), contributing to the 6th mass extinction (Barnosky et al., 2011; Ceballos et al., 2015). Amphibians are the most imperiled vertebrate class, with an estimated 43% of species declining in numbers (Clulow et al., 2014; Collins and Halliday, 2005; Grant et al., 2016; Wake and Vredenburg, 2008). Stressors that contribute to amphibian population decline include global climate change, invasive species, over exploitation, emerging infectious diseases, pesticides/pollution, and habitat loss/alteration (Blaustein et al., 2010; Collins and Storfer, 2003; Hof et al., 2011; Wake and Vredenburg, 2008).

Measuring glucocorticoid (GC) hormones associated with the stress response in vertebrates, provides a way to quantify physiological responses to stressors. The stress response is one of the mechanisms organisms use to maintain physiological stability (homeostasis) during perturbations or to cope with changing environments (McEwen and Wingfield, 2003). The higher vertebrate neuroendocrine stress response involves the hypothalamic-pituitary-adrenal axis (hypothalamic-pituitary-interrenal, HPI, axis in amphibians; Cyr and Romero, 2009). When a perturbation is perceived as a stressor by the brain, the hypothalamus secretes corticotropin releasing hormone (CRH) which induces the pituitary gland to release adrenocorticotropic hormone (ACTH). ACTH is

transported by blood to the adrenal cortex, which then releases glucocorticoids (GCs) (Denver, 2009). Physiological changes during a stress response include increased circulating levels of GCs above normal "baseline" levels to promote gluconeogenesis and to mobilize energy (Hau et al., 2016; Romero et al., 2009). Corticosterone is the main glucocorticoid associated with stress in amphibians (Idler, 1972) and while many studies focus on corticosterone in amphibians (Belden and Kiesecker, 2005; Belden et al., 2010; Glennemeier & Denver, 2002a; Glennemeier and Denver, 2002b; Narayan et al., 2010), cortisol has been measured in multiple species of amphibians but it has not consistently been studied (Krug et al., 1983; Baugh et al., 2018; Santymire et al., 2018). A short-term elevation of GCs in response to an acute stressor can be advantageous as it mediates the mobilization of energy stores (Sapolsky et al., 2000). However, unpredictable, and long-term, perturbations can lead to chronic stress, which is associated with persistently elevated or down regulated GC levels which can be deleterious (Romero et al., 2009). Elevated levels of corticosterone can negatively affect growth in tadpoles, alter morphology, and shorten time to metamorphosis (Crespi &Warne, 2013; Denver, 2009; Glennemeier and Denver, 2002a; Hu et al., 2008), and reduced mass at metamorphosis can affect growth and survivorship later in life (Cabrera-Guzman et al. 2013; Chelgren et al., 2006; Crespi and Warne, 2013; Earl and Whiteman, 2015; Rohr et al., 2013).

Understanding how stressors affect organisms, how individuals respond to stressors, and how flexible individuals are in response to environmental changes is at the core of conservation physiology (Wikelski and Cooke, 2006). Until recently, methods to measure GCs and other hormones were invasive or required sacrificing individuals (reviewed by Baugh et al., 2018; Gabor et al., 2016; Sheriff et al., 2011). Conservation

studies often focus on threatened and endangered species, therefore being able to non-invasively measure hormone levels, to minimize stress from handling/blood-sampling or the need for sacrifice, is imperative. Narayan et al. (2010, 2013) validated a novel, non-invasive, method for measuring steroids in amphibians using urinary corticosteroid metabolites in adult frogs. Recently, several studies have validated the use of a water-borne hormone collection protocol for both larvae and adults of several amphibian species of varying sizes and this method works in the field and laboratory (Baugh et al., 2018; Gabor et al., 2013, 2016). This water-borne hormone collection method was developed for use in fish, and measures steroid hormones passively diffused into the water via gills, urine, and feces (Scott et al., 2008). More recently, Santymire et al. (2018) examined GC concentrations collected from swabs of amphibian skin secretions but did not fully validate the method. Following this, Hammond et al. (2018) validated the use of salivary secretions to measure GCs in three species of adult frogs, but this method is limited to adults of a relatively large size and requires more handling. These non-invasive methods facilitate repeated sampling from the same individual and by utilizing a repeated measures design, one can measure within and among individual variation of hormone levels and stress responses and calculate the repeatability of hormone levels.

The repeatability of hormone levels provides insight into how individuals respond to changing environments or stressors (Hau et al., 2016; Lendvai et al., 2014) and is considered an upper bound estimate of heritability of a trait (Lessels and Boag, 1987; but see Dohm 2002). Therefore, it is important to include repeatability analysis of hormonal traits into experimental designs. In a review that had a limited sample size, amphibians show higher repeatability for baseline and stress-induce GC levels compared to other taxa

(Shoenemann and Bonier, 2018). An experimental design that allows for repeated measures from the same individual often requires tagging or marking organisms (Bainbridge et al., 2015), however, the additional handling and invasive procedures of marking can contribute to the stress of individuals. Housing subjects individually alleviates the need for marking, but it is unclear whether this also contributes to stress because some species of amphibians, especially at the larval stage, form aggregations. Gabor et al. (2013; 2016) developed a non-invasive water-borne method for measuring physiological responses to stressors in amphibians and validated the use of water- borne hormones and the positive relationship between water-borne CORT and plasma CORT in multiple amphibian species. Here we provide additional validations to test the efficacy of this method for an additional species, as circulating GC levels show great variability within and between individuals (Miles et al., 2018; Schoenermann and Bonier, 2018). We validated the water-borne hormone collection technique in the Rio Grande leopard frog, *Rana berlandieri*, pharmacologically using both an adrenocorticotropic hormone (ACTH) challenge and exposure to exogenous corticosterone, and biologically using an agitation test. Individuals should show elevated corticosterone release rates after each challenge, as ACTH stimulates the production of glucocorticoids, resulting in elevated corticosterone levels in several frog species (Baugh et al., 2018; Narayan et al., 2010a,b; 2011a,b), exogenous corticosterone is absorbed through the amphibian skin and results in increased endogenous GCs in tadpoles of several frog species (Belden et al., 2005; Glennemier and Denver, 2002a,b; Middlemis Maher et al. 2013), and agitation elevates corticosterone levels in several species of amphibians (Belden et al., 2003; Belden, et al., 2010; Chambers et al., 2011; Gabor et al. 2016). Further, we examined the repeatability of

water-borne corticosterone release rates and the rate of recovery from a stressor using repeated sampling of the same individuals. Lastly, we compared two rearing techniques that facilitate repeated measures without relying on invasive marking techniques.

Methods

Study species

Rio Grande leopard frogs, *Rana berlandieri*, are common anurans in the *Rana pipiens* complex that are found throughout Northeastern Mexico and Southern Texas (Zaldívar-Riverón et al., 2004). We collected egg masses of *R. berlandieri* from an ephemeral pond in San Marcos, Texas on 23 February 2017 (3 masses) and 5 March 2018 (4 masses), (29°52'28.86"N, 97°57'45.86"W) and transported half of each mass back to our laboratory on the Texas State University Campus. Egg masses collected in 2017 were used for the exogenous corticosterone experiment conducted in 2017 and egg masses collected in 2018 were used for the ACTH challenge experiment that was conducted in 2018 (see below). In both years, we reared the eggs in de-chlorinated, aged, tap water until tadpoles were free swimming (approximately 1 week from collection of eggs). Once tadpoles were free swimming, we mixed tadpoles from each egg mass, and housed tadpoles in groups of 12 in 6 L plastic tanks filled with aged de-chlorinated water at 19°C. We also collected free swimming *Rana berlandieri* tadpoles from an artificial pond at the USFWS San Marcos Aquatic Resources Center (SMARC) in San Marcos, Texas (29°50'26.39"N, 97°58'36.17"W) on 8 March 2018 for use in the agitation/recovery and housing experiments. In both years, we fed tadpoles a mixture of spirulina powder and Tetramin fish flakes in an agar base *ad libitum,* housed them under a natural light 14L:10D cycle, and changed water at least once per week and as needed. All protocols

and housing were approved by the Institutional Animal Care and Use Committee of Texas State University (IACUC #201563714 and #5636).

Validation- ACTH challenge on corticosterone and cortisol & exogenous corticosterone

To pharmacologically validate water-borne hormone collection in *Rana berlandieri*, we conducted an ACTH challenge on free-swimming tadpoles that were reared in the laboratory from egg masses (eggs collected 5 March 2018, n = 17; Gosner stages 30-35) on 13 July 2018. We weighed each tadpole one day prior to the ACTH challenge and used the mass to calculate individual ACTH doses for the experiment (Mean ± SE: 1.218 ± 0.088 grams). We collected "baseline" water-borne hormones from all tadpoles using a non-invasive water-borne hormone method (Gabor et al., 2016). Briefly, we placed each tadpole in a clean plastic insert (a perforated plastic lab bottle with the top cut off to facilitate removal of tadpoles from beakers) in a 250 ml glass beaker filled with 100ml of spring water for 60 minutes. We wore non-powdered nitrile gloves throughout the hormone collection process and cleaned beakers and inserts with 95% ethanol and rinsed them with de-ionized (DI) water before each use. Immediately following the collection of baseline hormones, we intraperitoneally injected each tadpole with a mass specific dose of 0.5 µg ACTH (Sigma Chemical Co., A-0298) per gram bodyweight, dissolved in Ringer's solution, using a 31-gauge needle on a 0.3cc syringe. Similar doses have been used in other species (Baugh et al., 2018; Narayan et al., 2010a, b; 2011a, b). Immediately following injection, we collected water-borne hormones following the methods we used to collect baseline levels. Each sample was used to measure corticosterone and cortisol release rates (one cortisol value had >34 CV so was removed from analysis) because Baugh et al. (2018) found substantial levels of cortisol in

water-borne hormones and plasma water of frogs. For a pooled sample, water-borne cortisol started at 998.45 pg/ml pre-ACTH injection and decreased to 193.6 pg/ml post-ACTH injection, when measured using an HPLC-MS (Baugh et al., 2018).

We also exposed free-swimming tadpoles (Gosner 26-29; Mean ± SE: 0.316 ± 0.012 grams), that were reared in the laboratory from egg masses (collected 23 February 2017) to exogenous corticosterone (Sigma Chemical Co. 27840) on 17 – 23 May 2017. We filled twenty, 5.7 L polypropylene shoe boxes each with 3 L aged, de-chlorinated, water. We had 10 control containers (dosed with 75 µl ethanol vehicle) and dosed 10 containers with 75 µl of 5 mM stock corticosterone solution dissolved in ethanol to create a final concentration of 43,308 ng/L (125 nM exogenous corticosterone using the same dose as Glennemeir & Denver 2002b). The volume of ethanol added to each tank was 0.0025% of the total water volume. We haphazardly assigned 4 tadpoles to each container in each treatment (n = 40 per treatment) and then added the appropriate treatment. We reared tadpoles in treatments for 7 days at 19 °C with water changes every third day and reapplication of hormone/control treatments. Water-borne hormones were then collected on day 7 from 2 random tadpoles from each tub for each treatment (n = 20 per treatment) following collection methods of Gabor et al. (2016) and outlined above. After hormone collection, we weighed each tadpole.

Validation-agitation stress test, recovery, and repeatability

To biologically validate water-borne hormone collection in *Rana berlandieri*, we conducted a standard agitation test and then quantified recovery rate using a repeated measures design on 14 March 2018. We used free swimming *R. berlandieri* tadpoles (Gosner 26-29; Mass ± SE: 0.893 ± 0.078 grams) from SMARC (collected 8 March

2018, see above). For the agitation test, we placed tadpoles (n = 20) in individual clean plastic perforated inserts within 250ml beakers filled with 100ml of spring water then manually agitated the *R. berlandieri* tadpoles following Gabor et al. (2016). Briefly, tadpoles in the beakers were placed in a cardboard box with dividers and then the box was manually agitated for 1 minute, every 3 minutes, for 60 minutes total. We then removed the insert with the tadpole, saved the water samples, and then moved tadpoles to a new beaker with fresh 100 ml of spring water to collect the first "recovery" hour of water-borne hormones. We collected agitation and then recovery water-borne hormones for each tadpole for 6 subsequent hours, collecting 7 hourly hormone samples (one from each beaker) from each tadpole. After the last hormone collection, we weighed each tadpole. We processed hormone samples from n = 14-17 individuals per time step (some samples were lost due to spillage, and we were limited on plate space due to minimal funding).

Non-invasive rearing conditions: individually vs. in groups

To examine whether housing conditions affect stress levels, we explored the effects of two different rearing methods using *R. berlandieri* tadpoles from SMARC (collected 8 March 2018). On 3 April 2018, we randomly assigned tadpoles (Gosner 26-29; Mean ± SE: 1.344 ± 0.092 grams) to one of two housing treatments: (1) individually housed in clear plastic polyethylene cups (Fig. 1C) filled with 0.5 L conditioned water (n = 24) or (2) housed in groups of 6 individuals in 5.7 L polypropylene shoeboxes filled with 3.0 L aged, de-chlorinated, water (4 replicates, n = 24 tadpoles in total), with the tadpoles isolated from each other with fiberglass screening to allow contact by visual and chemical cues (Fig. 3A, 3B). We reared all of the tadpoles in a growth chamber set at 21

ºC with a 14L:10D cycle. We allowed tadpoles 2 days to recover from being moved from their group housing to individual spaces to assay "baseline" hormone levels. After 2 days in treatments, we collected baseline corticosterone release rates from all the tadpoles following Gabor et al. (2016) and outlined previously. We collected baseline corticosterone release rates from each tadpole again after 7 days in the housing treatments and then immediately conducted an agitation stress test (see methods above) on each tadpole. We then weighed each tadpole. We analyzed data from 21 tadpoles reared individually and 19 tadpoles reared in groups (several samples were lost due to test tube breakage).

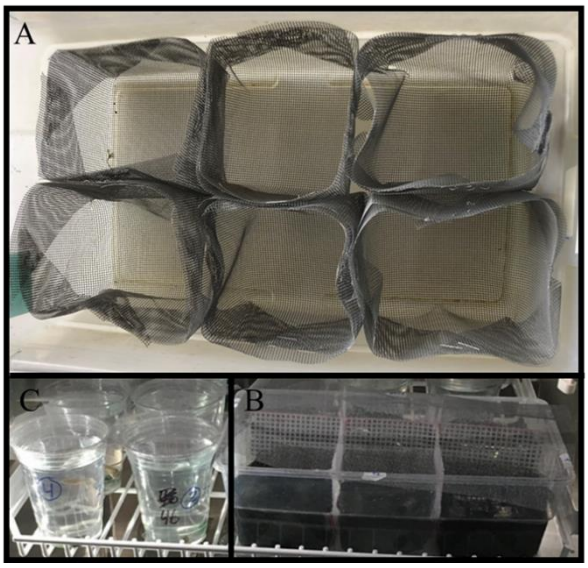

Figure 3. Experimental housing set up for individual vs. group reared tadpoles of *Rana berlandieri*. (A) and (B) are top and side views, respectively, for group housing, and (C) are individual housing containers.

Hormone extraction, reconstitution, and enzyme immunoassays (EIA)

We stored water-borne hormone samples at -20 °C until we thawed them for extraction following methods of Gabor et al. (2016). We extracted corticosterone (and cortisol) from water samples following Gabor et al. (2016) by pulling water samples under vacuum through Tygon tubing into C18 solid phase extraction (SPE) columns

(SepPak Vac3 cc/500 mg; Waters, Inc., Milford, MA, USA) primed with 100% HPLC grade methanol (4 ml) and distilled water (4ml). Following extraction, we eluted columns with 4ml 100% HPLC grade methanol into borosilicate vials, which we then evaporated under a gentle stream of nitrogen gas (approx. 2 hours) while samples were placed in a hot-water bath (37 °C) to facilitate evaporation of the methanol. Following drying, we re-suspended the residue in 5% ethanol (95% lab grade) and 95% EIA buffer to a total volume of 300 or 600 µl depending on the experiment. The resuspension volumes were based on previous experiments in our lab to ensure that sample values were within the assay range of the EIA kits. For the recovery experiment samples were resuspended at 300µl and the first three hours were diluted at 1:8 and all others were not diluted. For the group vs isolated experiment, the samples were resuspended at 300µl and diluted 1:4. For the exogenous CORT test, samples were resuspended at 600 µl and did not dilute before plating. For the ACTH challenge, samples were resuspended at 300 µl and diluted 1:4 for baseline samples and 1:8 for ACTH samples. All corticosterone and cortisol values were standardized for re-suspension volume before statistical analysis. EIA buffer was made following a published Cayman Chemical, Inc. protocol, by mixing 10ml ELISA buffer concentrate (№ 400060, 1 M phosphate, containing 1% BSA, 4 M sodium chloride, 10 mM EDTA, and 0.1% sodium azide) with 90ml Millipore water.

We measured corticosterone release rates in duplicate for all samples using EIA kits (№ 501320, Cayman Chemical Company, Inc., assay has a range of 8.2-5,000 pg/ml and a sensitivity (80% B/B0) of approximately 30 pg/ml) and cortisol release rates in duplicate for the ACTH challenge using an EIA kit (№ 500360, Cayman Chemical Company, Inc., assay has a range from 6.6-4,000 pg/ml and a sensitivity (80% B/B0) of

approximately 35 pg/ml). We used a pooled control sample from previously collected hormones from a large sample size of *Eurycea tonkawae* salamanders for corticosterone plates and a pooled control sample from previously collected hormones from a large sample of *Poecilia latipinna* fish for the cortisol plate. Sample absorbance was read on a spectrophotometer plate reader at 405nm (BioTek 800XS). Inter-plate variation for agitation stress test, recovery, and repeatability experiments was 9.98% (4 plates) and for the rearing condition experiment was 5.42% (4 plates). Intra-plate variation for both experiments ranged 0.41-5.74%. Intra-plate variation for cortisol and corticosterone release rates for the ACTH challenge were 1.97% and 2.69%, respectively (Only one corticosterone and one cortisol plate were used for the ACTH experiment). Inter-plate variation for the exogenous corticosterone experiment was 15.18% (5 plates) and intra-plate variation ranged from 0.87-4.13%. Inter-plate variation for the agitation/recovery and repeatability experiment was 9.98% (4 plates) and for the rearing condition experiment was 5.42% (4 plates). Intra-plate variation for both experiments ranged from 0.41-5.74%.

Statistics

We multiplied corticosterone release rates (pg/ml) by the final resuspension volume (0.3 -0.6 ml) and then standardized the value by dividing by mass of the respective individual. All hormone release rates were natural log transformed before data analysis (though untransformed data are presented in figures). We analyzed corticosterone and cortisol release rates in response to an ACTH challenge using repeated measures ANOVA. We analyzed response to exogenous corticosterone using a generalized linear mixed model (GLMM) with tank as the random effect. To examine the

time it takes to recover from a stressor (agitation) on the fixed effect corticosterone, we used a repeated measures GLMM with individual as a random factor to account for repeated measures. We assessed the effect of housing (treatment) and time on corticosterone release rates and mass using a repeated measures GLMM with individual and tank as random effects to account for repeated measures. When there was a significant difference, we ran a *post hoc* Tukey's (HSD) comparison between treatments. We used a matched pairs t-test to examine if corticosterone release rates increased after tadpoles were agitated on day 7. All tests were performed using JMP 14 software (SAS Institute, Inc). Using the R package rtpR in R version 3.2.3 (R Core Development Team), we calculated an adjusted repeatability (r) with a linear mixed model (LMM) based approach using the Restricted Maximum Likelihood (REML) method (Dingemanse and Dochtermann, 2013; Nakagawa and Schielzeth, 2010). We calculated repeatability of corticosterone release rates across time for tadpoles in the recovery and housing experiments included in this paper (for the housing experiment, we calculated repeatability separately for each housing treatment). Corticosterone release rate was our response variable, with sampling hours or days (for the recovery and housing experiments respectively) as the fixed variables, and individual identity as the random slope and intercepts effect.

Results

Validation- ACTH Challenge on corticosterone and cortisol & exogenous corticosterone

Intraperitoneal injection of ACTH at a dose of 0.5 µg/g significantly increased corticosterone release rates above baseline release rates in *Rana berlandieri* tadpoles (repeated measures ANOVA: $F_{1,16} = 13.65$, $p = 0.002$; Fig. 4a) and significantly

decreased cortisol release rates below baseline release rates ($F_{1,15}$ =6.34, p = 0.0236; Fig. 4b). Exposing tadpoles of *Rana berlandieri* to 125nM exogenous corticosterone induced a significant increase in corticosterone release rates ($F_{1,17}$ = 45.52, p < 0.0001; Fig. 5).

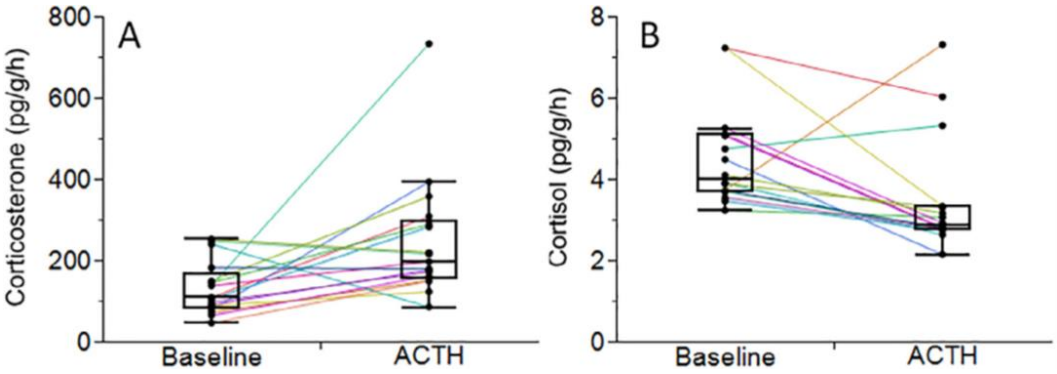

Figure 4. Corticosterone (A) and cortisol (B) release rates (pg/g/h) obtained before (baseline) and after ACTH injection (ACTH) from *Rana berlandieri* tadpoles (n=17). Each line color represents a different individual across time. Box plots indicate median, range and first and third quartiles.

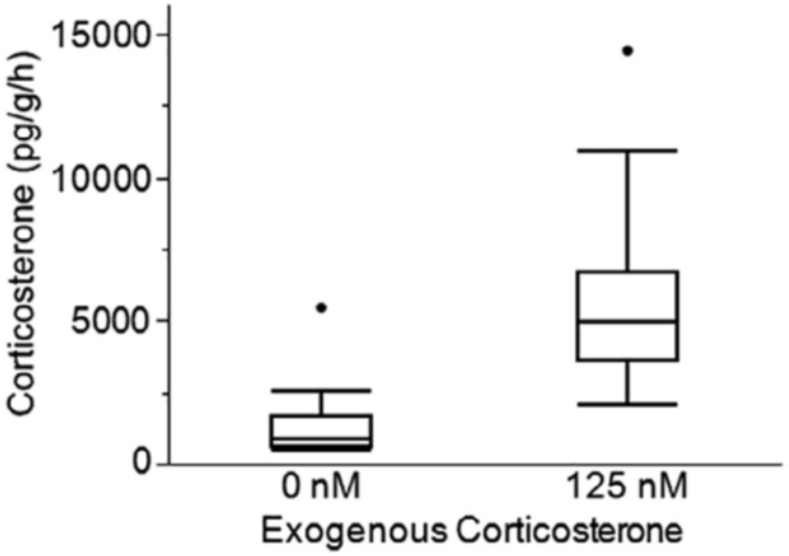

Figure 5. Corticosterone release rates (pg/g/h) obtained from *R. berlandieri* tadpoles exposed to no exogenous corticosterone (n=18) and 125 nM corticosterone(n=20) for 7 days. Each line color represents a different individual across time. Box plots indicate median, range and first and third quartiles. Dots indicate outliers.

Validation-agitation stress test, recovery, and repeatability

Corticosterone release rates in tadpoles of *Rana berlandieri* differed over time ($F_{6,85} = 14.50$, $p < 0.001$; Fig. 6): they were significantly lower than agitation by two hours post agitation and had the lowest values 6 hours post agitation. Corticosterone release rates were repeatable across measurements (r = 0.365 ± 0.111, 95% CI: 0.128, 0.561; $p < 0.001$).

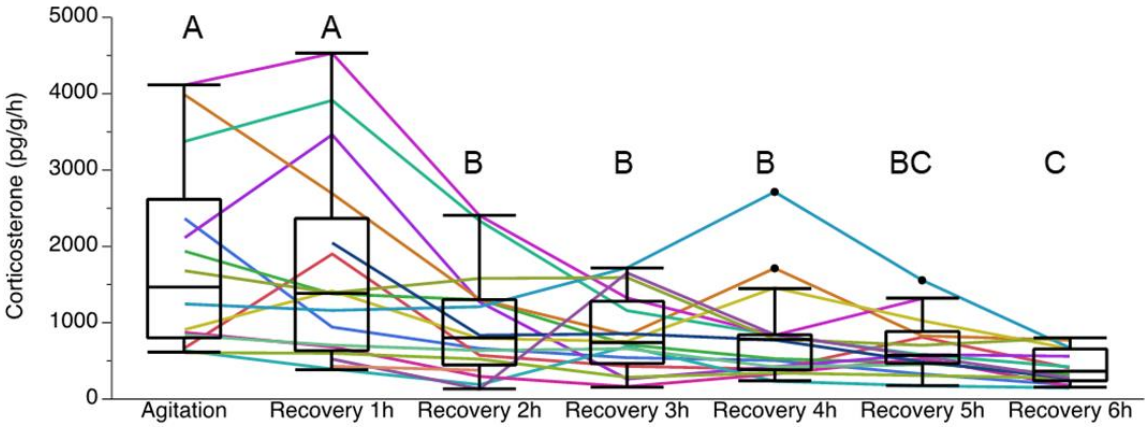

Figure 6. Corticosterone release rates (pg/g/h) obtained from *R. berlandieri* tadpoles after 60 min of agitation and recovery 1 to 6 h post agitation (n=14–17/time step). Each line color represents a different individual across time. Box plots indicate median, range and first and third quartiles. Dots indicate outliers. Different letters indicate significant differences.

Non-invasive rearing conditions: individually vs. in groups

Tadpoles reared in groups had higher corticosterone release rates on day 7 than on day 2 but not those reared individually (time × treatment: $F_{2,76} = 2.94$, $p = 0.0589$; Fig. 7a, b; isolated: df = 20, t = -0.5, $p = 0.311$; group: df = 18, t = -3.2, $p = 0.005$). Tadpoles reared in groups mounted a stress response after 7 days in response to an agitation test (df =18, t = 2.26, $p = 0.036$; Fig. 5a) but not those that were reared individually (df =20, t = 1.71, $p = 0.103$; Fig. 7b). Tadpoles reared in groups lost mass over time but not those

reared individually (treatment × time: $F_{2,38} = 30.64$, $p < 0.0001$; Fig. 8). Corticosterone release rates were repeatable for tadpoles reared in groups (r = 0.300 ± 0.144, 95% CI: 0, 0.562; $p = 0.022$) and for tadpoles reared individually (r = 0.588 ± 0.116, 95% CI: 0.315, 0.776; $p < 0.001$).

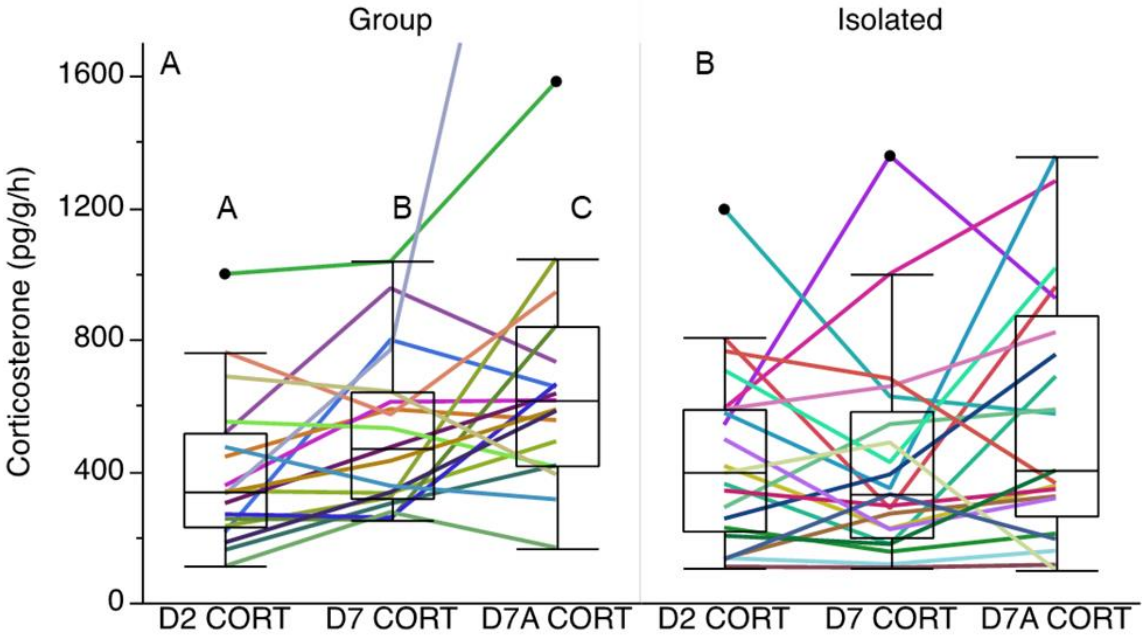

Figure 7. Corticosterone release rates (pg/g/h) obtained from *R. berlandieri* tadpoles after 2 days (D2) and 7 days (D7) in treatments and after an agitation test (D7A) for those reared in (A) groups (n=19) or (B) individually (n=21). One point in the agitation group data at 3000 pg/g/h was left out for ease of view. Each line color represents a different individual across time. Box plots indicate median, range and first and third quartiles. Dots indicate outliers. Different letters indicate significant differences.

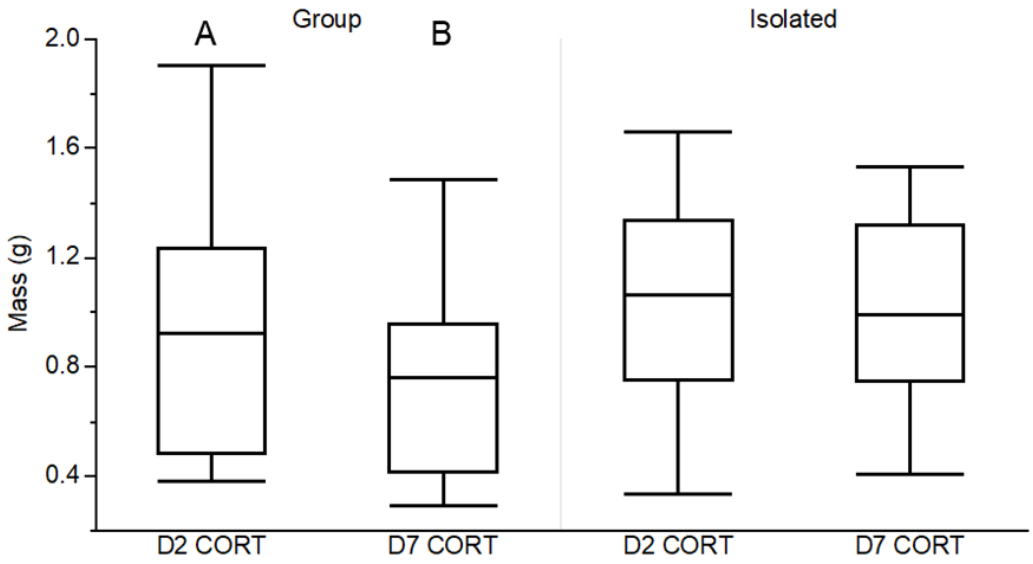

Figure 8. Mass (g) after 2 days (D2) and 7 days (D7) in treatments. Box plots indicate median, range and first and third quartiles. Different letters indicate significant differences.

Discussion

As the loss of amphibian populations continues, it is important to develop methods for early warning indicators of population declines and to find non-invasive methods for measuring physiological health. These methods also need to be validated. Further, to understand the ability of a population to respond to rapid environmental change, it is necessary for these methods to allow for repeated hormone measures, to allow for the calculation of repeatability (a proxy for the upper level of heritability). Our results demonstrate that the waterborne-hormone collection method is a valid method for sampling glucocorticoids and assessing physiological changes in *Rana berlandieri*. As predicted, pharmacological challenges with ACTH and exogenous corticosterone increased corticosterone release rates in tadpoles. Additionally, the use of a biological challenge, in the form of an agitation test, also increased corticosterone release rates significantly in tadpoles. Our results are congruent with findings in other amphibian

species (Baugh et al., 2018; Gabor et al., 2013, 2016; Glennemeier and Denver 2002a; Narayan et al., 2010). Importantly, we found that our integrated measures of GCs are moderately repeatable, indicating a heritable component to the GC response in this population of *R. berlandieri*.

We measured release rates of two excreted glucocorticoids (corticosterone and cortisol) from tadpoles after the ACTH challenge. Although we observed elevated corticosterone release rates after ACTH injection in *R. berlandieri*, those values may not represent the peak of the reactive range (Romero, 2002) as we collected hormones immediately following injection, and Baugh et al. (2018) recently showed in *Physalaemus pustulosus* that maximum levels may be released 2 hours post injection. In contrast to corticosterone, cortisol release rates decreased with ACTH challenge. In fish, for example, ACTH challenge results in an increase in cortisol release rates, but that is the major glucocorticoid in fish (Kim et al., 2018). To the best of our knowledge, this is the first study to validate a water-borne hormone collection method for cortisol in an anuran species. Baugh et al. (2018) measured cortisol in amphibians using HPLC-MS and found that one paired pooled sample showed a decrease in cortisol levels after an ACTH challenge. Our results suggest that corticosterone better represents the stress response in amphibians rather than cortisol, as corticosterone increased while cortisol decreased after ACTH injection, though the biological implications of why cortisol decreased after an ACTH challenge are not clear, therefore further study is merited.

Exposure to exogenous CORT has been used previously to illicit a hormonal response and increase endogenous CORT in amphibians (Belden et al., 2005; Glennemeier and Denver, 2002a, b; Middlemis Maher et al., 2013). Our results suggest

this method is also viable to raise water-borne CORT release rates in *R. berlandieri*. Tadpoles were not rinsed in water to remove surface CORT prior to placing in beakers, which may have contributed to the higher CORT release rates observed. However, based on the CORT concentration calculated from a 100 ml sample of tub water (43308 pg/ml) resuspended at 0.60 ml prior to plating, only about 13 pg of CORT was introduced to the sample with each tadpole. So, the possible addition of up to 13 pg of CORT per tadpole would still not account for the difference in CORT observed between control and exogenous CORT exposed tadpoles.

The non-invasive water-borne hormone collection method provides an accurate way to repeatedly measure hormones from individuals while minimally stressing the organism and eliminating the need for blood sampling or sacrifice. This method is a valuable tool for conservation studies to assess stress physiology in threatened and endangered species. Further, lower sample sizes are required for experiments because individuals can be resampled across time. Typically, rearing facilities for endangered or threatened species of amphibians have enough individuals to achieve sample sizes necessary for this protocol, and while larger sample sizes may not be easily obtainable in the field, Gabor et al. (2018) were able to collect sufficient sample sizes in the field to sample hormones in the Federally threatened Jollyville Plateau salamander, *Eurycea tonkawae*. Additionally, this method alleviates the need to transport individuals to the lab as water-borne hormone samples can be collected in the field and transported back to the lab on ice. A potential shortcoming of this method is the need for individuals to be confined to a beaker for one hour, as confinement has been used as an acute stressor in previous studies (Middlemis Maher et al., 2013). However, our results show that baseline

levels of corticosterone release rates after one hour in a beaker are statistically lower than ACTH challenged or agitated corticosterone release rates. Additionally, tadpole corticosterone release rates for *R. berlandieri* were significantly lower 6 hours after agitation, which is a more rapid recovery than observed in other species using a different method (Narayan et al., 2010).

Repeatability of glucocorticoids have been observed in many free-living individuals (reviewed by Hau et al., 2016) and often significantly higher in amphibians compared to other taxa (Schoenmann and Bonier, 2018). Repeated measure designs are often difficult in free-living organisms (Hau et al., 2016) and repeated measure laboratory or mesocosm studies require marking individuals, which may add additional stress or variation in physiological responses. Our results demonstrate that baseline levels of corticosterone release rates, measured using our non-invasive water-borne collection method, are repeatable over time and our values ranging from $r = 0.300$ to 0.588 for stress and recovery and the rearing experiment are moderate to high (Hau et al., 2016). Narayan et al. (2013) measured repeatability in *Platymantis vitiana* using an older statistical method and found very high repeatability for baseline values ($r = 0.973$) and for the stress response values (range $r = 0.82$-0.92; Narayan and Hero, 2013). In cane toads, *Rhinella marina*, Narayan et al. (2012) found high repeatability for baseline and corticosterone metabolite responses ranging from $r = 0.630$ to $r = 0.793$. In our studies, the highest repeatability values, not surprisingly, came from when the tadpoles were maintained in close to the same conditions across time in the individual cups. This is the first time that repeatability of corticosterone release rates using water-borne hormones has been quantified in tadpoles. We also note that we had large among individual

variance which indicates that there are multiple phenotypes in the population of which some may respond better in a given environment. Because repeatability can be viewed as the upper bound of heritability at the population level, our results indicate that corticosterone release show enough variation that, in theory, there is potential to evolve in response to selection (Hau et al., 2016).

Repeated measure experimental designs require marking individuals or rearing them individually. However, it may be difficult (or require special permitting) to mark individuals and rearing individually may be stressful for social species. Our results indicate that rearing tadpoles individually may contribute to stress of tadpoles, though quantifying the impacts of housing type are difficult. Interestingly, tadpoles reared in groups, isolated by mesh screen allowing for visual and chemical cues to be perceived by individuals, showed higher corticosterone release rates than the individually reared tadpoles. Yet, individually reared tadpoles did not show a stress response whereas those reared in groups did show a stress response to agitation, suggesting tadpoles reared individually may have a dysregulated HPI axis. Given these findings, it is difficult for us to make any strong conclusion about whether one method or the other is better for rearing tadpoles of *R. berlandieri.* Further investigation into housing design is warranted, such as experiments with larger containers with more water volume per tadpole to reduce potential effects of crowding, as rearing methods should be an important consideration of experimental design. We also did not see a difference in the variance of stress hormone levels on each of the three days we measured CORT across treatments (Day 2 baseline Levene's test: $p = 0.13$; Day 7: $p = 0.13$; Day 7 Agitation: $p = 0.09$). Prior studies on rearing found that zebrafish, *Danio rerio*, a shoaling species, show higher and more

variable cortisol levels when housed individually compared to in groups (Pagnussat et al., 2013). Additionally, Narayan et al. (2013) found that urinary corticosterone concentrations were higher in adult cane toads housed in groups, but corticosterone declined after toads were moved to individual enclosures. Similar observations were made in endangered adult harlequin Frogs, *Atelopus spp*., (Cikanek et al., 2016). When designing an experiment, it is important to consider whether the species you are working with is social or not. In another species of leopard frog, *Rana pipiens,* it was found that tadpoles of this species do not aggregate (Golden et al., 2001) but many species may benefit from being in larger groups in the presence of predators (Kelly, 1994) and leopard frogs were found to be more active in larger groups (Golden et al., 2001). We did find that individuals reared with other tadpoles lost mass over time and showed elevated corticosterone release rates, which is similar to findings by Glennemeier and Denver (2002b) in *Rana pipiens* tadpoles. Lower body mass is associated with elevated corticosterone levels in toads reared in captivity over time (Titon et al., 2018). Together, our results on rearing tadpoles of *R. berlandieri* indicate that the possible benefits to being reared in groups are offset by the reduced growth, whereas the benefit of individual rearing may be offset by additional stress of being solitary, resulting in a lack of adaptive stress response. These findings indicate that no one method of rearing is best, but housing decisions will depend on the question being asked. It is important to note that physiological responses to housing is likely species specific and will depend on whether the species is generally social, but housing methods are important to consider, particularly if repeated measures are needed in studies of threatened or endangered species.

Conclusions

We validated that the water-borne hormone method reliably measures the GC response of *Rana berlandieri* tadpoles to stressors both pharmacologically and biologically. Further, using this water-borne hormone method, we found that it takes up to two hours for corticosterone release rates to start to decline post stressor and by six hours corticosterone were even lower. We found that this species could be reared alone or individually in groups if repeated measure designs are being used, however the optimal rearing method will depend on the question being asked and the species being tested. Finally, we also found that the water-borne hormone collection method provides repeatable measures of GCs in *Rana berlandieri* indicating that this species can show a response to selection on stress hormones, and thus this species could evolve in response to environmental stressors. Together, our results indicate that using the non-invasive water-borne hormone method allows for studying threatened or endangered species (both in terms of minimal sample sizes and minimal invasiveness) and determining whether they can show a response to selection in stressful environments, an important conservation tool given the rapid decline in amphibian populations to date.

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III. ARTIFICIAL LIGHT AT NIGHT (ALAN) AFFECTS THE PHYSIOLOGY BUT NOT THE BEHAVIOR OR GROWTH OF *RANA BERLANDIERI* AND *BUFO VALLICEPS*

Abstract

Artificial light at night (ALAN) alters the natural light dark patterns in ecosystems. ALAN can have a suite of effects on community structure and is a driver of evolutionary processes that influences a range of behavioral and physiological traits. Our understanding of possible effects of ALAN across species amphibians is lacking and research is warranted as ALAN could contribute to stress and declines of amphibian populations, particularly in urban areas. We tested the hypothesis that exposure to constant light or pulsed ALAN would physiologically stress Rio Grande leopard frog (*Rana berlandieri*) and Gulf Coast toad (*Bufo valliceps*) tadpoles. We reared tadpoles under constant or pulsed (on and off again) ALAN for 14 days and measured corticosterone release rates over time using a non-invasive water-borne hormone protocol. ALAN treatments did not affect behavior or growth. Tadpoles of both species had higher corticosterone (cort) release rates after 14 days of constant light exposure. Leopard frog tadpoles had lower cort release rates after exposure to pulsed ALAN while toad tadpoles had higher cort release rates. These results suggest that short-term exposure to constant or pulsed light at night may contribute to stress in tadpoles but that each species differentially modulated their cort response to ALAN exposure and a subsequent stressor. This flexibility in the upregulation and downregulation of hypothalamic-pituitary-interrenal axis response may indicate an alternative mechanism for diminishing the deleterious effects of chronic stress. Nonetheless, ALAN should be considered in management and conservation plans for amphibians.

Keywords: ALAN, light pollution, conservation physiology, stress physiology, corticosterone, amphibians

Introduction

Artificial light at night (ALAN, Haim & Zubidat, 2015) is a significant threat to biodiversity (Gaston et al., 2019; Secondi et al., 2020; Seymoure et al., 2019) particularly in urban areas. Sources of ALAN include light trespass from streetlamps, lights on buildings, vehicles, and sky glow from cities (Aube et al., 2015; Longcore & Rich, 2004; Navara & Nelson, 2007). Natural environmental daytime light levels range from 800 lx at sunset up to 100,000 lx on a clear day, while nighttime light levels range from 0.3 lx on a full moon night down to 0.001 lx on a clear, moonless, night (Hänel et al., 2018). Sources of ALAN can increase light levels many kilometers from urban centers (Kyba et al., 2011; Secondi et al., 2020) so ALAN also impacts rural areas adjacent to urban centers. Over 80% of the world population live in areas polluted by ALAN (Falchi et al., 2016), 40% of which experience constant ALAN (Swaddle et al., 2015), and Seymoure et al. (2019) estimate that over 50% and 75% of key biodiversity areas and Global protected rea units, respectfully, are affected by ALAN. With continued human population growth and urbanization, ALAN is estimated to be increasing at a rate of 6% yearly (Hoelker et al., 2010), and thus the impact of ALAN on organisms may be of great importance and potential negative effects may have significant ecological consequences and conservation implications (Gaston et al., 2019; Secondi et al., 2020; Seymoure et al., 2019).

Amphibian populations are experiencing global population declines (Clulow et al., 2014; Collins & Halliday, 2005; Grant et al., 2016) and ALAN may be contributing to their population declines, ALAN affects the behavior, physiology, and development of

many taxa (see reviews: Gaston et. al., 2014; Longcore & Rich, 2004; Navara & Nelson, 2007; Secondi et al., 2020; Swaddle et al., 2015), but our understanding of the ecological impacts of ALAN is limited relative to that of other anthropogenic perturbations (Desouhant et al., 2019). ALAN affects amphibian behavior, physiology, and growth (Dananay & Benard, 2018; Gern et al., 1983; May et al. 2019; Wise & Buchanan, 2006). When exposed to 3.8-12.0 lx ALAN, adult grey treefrogs, *Hyla chrysoscelis*, reduce foraging (Buchanan, 1993) and adult red-back salamanders, *Plethodon cinereus,* exposed to ALAN from string lights (lux not reported but likely low light) reduce activity and foraging (Wise & Buchanan, 2006). Similarly, adult common green frogs, *Rana clamitans*, reduce advertisement calling when exposed to 52 lx – 120 lx ALAN (Baker & Richardson, 2006). Constant light (lux not reported) affects the physiology of tiger salamanders, *Ambystoma tigrinum*, by disrupting normal oscillating melatonin rhythms associated with normal light/dark cycles (Gern et al., 1983). Larval leopard frogs, *Rana pipiens* (lux not reported; Eichler & Gray, 1976), and larval American toads, *Bufo americanus* (22 lx – 1.3 lx; Dananay & Benard, 2018), metamorphosed faster and weighed less at metamorphosis when reared under constant light. Contrastingly, May et al. (2019) found larval wood frogs, *Rana sylvatica*, metamorphosed faster but weighed more at metamorphosis when exposed to 300 lx ALAN. Additionally, May et al. (2019) did not see an effect of ALAN on corticosterone release rates in *R. sylvatica* larvae, however ALAN affects glucocorticoid concentrations in other taxa (Ouyang et al., 2015). Understanding how ALAN affects amphibians is important, as ALAN may be potentially contributing to stress and declines of amphibian populations, particularly in urban areas.

Anuran larvae are likely stressed by ALAN as many species are nocturnal and have dark adapted eyes (Baker & Richardson, 2006), circadian rhythms are tied to natural light/dark cycles (Azzi et al., 2014; Botha et al., 2017; Ciarleglio et al., 2011) and changes to circadian rhythms can affect physiological processes (Fonken & Nelson, 2014). ALAN disrupts natural light/dark cycles (Falchi et al., 2016), and disrupts circadian rhythms (Bedrosian et al., 2013; Botha et al., 2017; Dominoni et al., 2013), which can alter glucocorticoid (GC) levels (Ouyang et al., 2015). The amphibian neuroendocrine response to a stressor involves the hypothalamic-pituitary-interrenal, HPI, axis (Cyr & Romero, 2009) and often results in increased circulating levels of corticosterone (cort), the main GC in amphibians (Forsburg et al., 2019; Idler, 1972), above normal "baseline" levels (Romero et al., 2009). A short-term elevation of circulating GCs can be advantageous as it mediates gluconeogenesis and mobilizes energy (Hau et al., 2016; Romero et al., 2009; Sapolsky et al., 2000), however unpredictable and long-term perturbations can lead to persistently elevated or down regulated (suppressed) GC levels which can be deleterious (Romero et al., 2009). Elevated levels of cort can reduce growth in amphibian larvae, alter tadpole morphology, and hasten metamorphosis (Crespi & Warne, 2013; Denver et al., 1998; Glennemeier & Denver, 2002a; Hu et al., 2008), and reduced mass at metamorphosis can affect growth and survivorship later in life (Cabrera-Guzman et al. 2013; Chelgren et al., 2006; Crespi and Warne, 2013; Earl & Whiteman, 2015; Rohr et al., 2013).

We can gain a better understanding of how individuals physiologically cope with changing environments by measuring cort release rates over time and by measuring how individuals respond to an acute stressor, such as agitation (Wikelski & Cooke, 2006). The

physiological responses of individuals can give us insight into individual and population health (Dantzer et al., 2014; Gabor et al., 2018; Sheriff et al., 2011; Wikelski & Cooke, 2006) and may change over time or show flexibility as the environment changes. Hormone responses may be repeatable and or flexible. Hormone levels fluctuate from day to night and may change in response to variation in abiotic and biotic factors in a changing environment (Hau et al., 2016). Modulating hormonal responses help mediate behavior, physiology, and morphology in changing environments (Nelson, 2011), particularly glucocorticoid (GCs) responses, as they help to maintain energy needs in both predictable and unpredictable events (Sapolsky et al., 2000). Variation in circulating levels of GCs and hormonal and behavioral responses to changing environments have been observed both within and among individuals (reviewed in Hau et al., 2016) and this variation is mediated by phenotypic flexibility (Piersma & Drent, 2003). By repeatedly measuring hormones from the same individual over time using a non-invasive, water-borne, hormone collection protocol (Gabor et al., 2016), one can measure within and among individual variation of hormone levels and calculate the repeatability of hormone levels.

We explored the consequences of exposure to 190 lx – 250 lx ALAN on the physiology and growth of two common anurans, the Gulf Coast toad (*Bufo valliceps*) and the Rio Grande leopard frog (*Rana berlandieri*), using laboratory experiments across three years. We also examined how ALAN affects the behavior of *R. berlandieri*. First, in 2017, we exposed pre-metamorphic *Rana berlandieri* tadpoles to different light conditions for 14 days to explore how ALAN affects corticosterone release rates, tadpole behavior, and growth. We then reared the tadpoles on a natural light cycle for an

additional 7 days, post treatments, to explore if tadpoles recover from any effects of ALAN treatments. We then reared pre-metamorphic larval *R. berlandieri* and *Bufo valliceps*, in 2018 and 2019 respectfully, under a control light cycle for 7 days and then exposed them to a either pulsed ALAN or constant ALAN for 14 days to explore if tadpoles respond to a changing environment by altering cort release rates and to test if ALAN exposure affects growth. We hypothesized that both constant and pulsed ALAN would be a stressor for tadpoles and tadpoles cort release rates be affected by the light treatments.

Methods

Species Collection & Husbandry

We collected portions of three Rio Grande leopard frog, *Rana berlandieri*, egg masses from a local pond in San Marcos, Hays Co., Texas (29.874695, -97.962733) on 23 February 2017, four *R. berlandieri* egg masses from the same pond on 5 March 2018, and collected several strands of eggs from four different groupings of Gulf Coast toad, *Bufo valliceps*, eggs from two different local ponds in San Marcos, Hays Co., Texas (29.874695, -97.962733 and 29.903373, -97.966839) on 10 April 2019. In each year, we transported the eggs to our laboratory on the Texas State University Campus, where we reared eggs in aged tap water until tadpoles were free swimming (approximately one week from collection of eggs). In each year, and for each species, once tadpoles were free swimming (Gosner stage 25; Gosner, 1960), we mixed tadpoles from each clutch together and housed them in 6 L polypropylene plastic tanks filled with aged tap water at room temperature (12 per tank for *Rana berlandieri* and 24 per tank for *Bufo valliceps*). We checked for mortality daily, fed tadpoles *ad libitum* a mixture of spirulina powder and

ground fish flakes in an agar powder base (food cubes), and changed water as needed, but at least once per week, using aged tap water.

Water-borne Hormone Collection

To examine how cort release rates changed over time and across lighting environments, we used a non-invasive water-borne hormone collection method (Gabor et al., 2016) that has been validated for *Rana berlandieri* (Forsburg et al., 2019). Briefly, we placed each tadpole in a clean low-density polyethylene (LDPE) plastic insert (a perforated plastic lab bottle with the top cut off to facilitate removal of tadpoles from beakers) in a 250 ml glass beaker filled with 100ml of spring water for 60 minutes. We also obtained the mass of each tadpole by placing individual tadpoles on tared pieces of moistened sponge and photographed them (to measure SVL and TH using ImageJ) after we collected hormones. We wore non-powdered gloves throughout the hormone collection process and cleaned beakers and inserts with 95% ethanol and rinsed them with DI water before each use. We also performed an agitation stress test on each tadpole after we collected the unmanipulated, baseline, hormone samples. We moved tadpoles in plastic inserts to clean 250 ml glass beakers with 100 ml of spring water and agitated them by hand for 60 seconds every three minutes for an hour. At the end of the experiment, we euthanized tadpoles using an overdose of benzocaine (20%).

We stored the water-borne hormone samples at -20° C until we thawed them for extraction following methods of Gabor et al. (2016). Briefly, we extracted hormones (n = 20 per treatment in 2018, and n = 18 per treatment in 2019) by running water samples through Tygon tubing into methanol (4 ml) and distilled water (4 ml) primed C18 solid phase extraction (SPE) columns under vacuum. Following extraction, we eluted columns

with 4 ml methanol into borosilicate vials, which we then evaporated under nitrogen gas. Following drying, we re-suspended the residue in 25 µl of 95 % ethanol, followed by 5 min of shaking, and 475 µl of enzyme-immunoassay (EIA) buffer (Cayman Chemical Company, Inc.) for a total re-suspension volume of 500 µl. We measured cort release rates in duplicate for all samples using EIA kits (№ 501320, Cayman Chemical Company, Inc.; assay range of 8.2 pg/ml – 5,000 pg/ml; sensitivity (80 % B/Bo) of 30 pg/ml) on a spectrophotometer plate reader at 405 nm (BioTek 800XS). For the 2017 experiment, inter-plate (11 total plates) variation was 13.75 % and intra-plate variations ranged from 1.01 – 8.58 %. For the 2018 experiment, inter-plate (5 plates) variation was 9.19 % and intra-plate variations ranged from 0.14 % – 3.65 %. For the 2019 experiment, inter-plate (3 plates) variation was 12.4 % and intra-plate variations ranged from 0.35 % – 2.28 %.

2017 Rana berlandieri: Reared under ALAN treatments then moved to control

In 2017, we placed tadpoles into individual 0.5 L clear containers filled with 0.4 L of aged tap water. We randomly assigned tadpoles (n=30/treatment) to one of three light cycle treatments: (1) 12L:12D (control), (2) 24L:0D (constant light), and (3) 12L: with light pulsed on and off every hour for 12 hours (pulsed light) through the night. We maintained all tadpoles in a growth chamber set at 20^{o} C and exposed them to each light treatment for 14 days. We placed LED strip lights (3000K, 80 CRI) set on timers 30cm from the top of the tadpole containers and used opaque black plastic sheeting to compartmentalize each treatment in the growth chamber. Light level, measured with a Dr. Meter LX1330B digital light meter, at the water surface was 250 lx, which is in the environmentally relevant range we observed in San Marcos, Texas. We checked tadpoles

daily for mortality, fed tadpoles food cubes *ad libitum*, and changed water on days 2, 7, and 14 of the experiment. After 14 days of ALAN treatments, we examined their recovery by maintaining all tadpoles on the control light cycle (12L:12D) for an additional 7 days (Day 21 of the experiment). We collected baseline water-borne hormones on days 2 and 14 during ALAN treatments and on day 21 of the experiment (after 7 days on a control cycle). We also conducted an agitation stress test (outlined above) on day 14 after we collected baseline levels.

To examine how ALAN affected behavior in tadpoles, we completed 3 sets of focal observations during the experiment (on days when hormones were not collected). Using a mirror on a pole, we observed every tadpole 6 times during 1 hour on days 4, 6, and 13 of the experiment, recording whether each tadpole was actively foraging, moving, or not moving. We counted foraging and moving as "active" and calculated the proportion of time a tadpole was "active" for each set of focal observations.

2018 Rana berlandieri: Reared under control then moved to ALAN treatments

In 2018, we set up twelve 5.7 L polypropylene plastic containers with plastic mesh inserts forming three compartments (Fig. 9), which permitted repeated measurements from the same tadpoles, while also allowing tadpoles to communicate with visual and chemical cues. We filled the containers with aged tap water and placed them in a growth chamber set at 20^{o} C. We placed 3000K LED strip lights, to provide ALAN, and 5000K full spectrum LED strip lights, to provide daytime lighting, 30 cm above the tanks on timers. ALAN light level at the water surface was 190 lx (Dr. Meter LX1330B digital light meter), which is in the environmentally relevant range we observed in San Marcos, Texas., and the daytime light level was 2800 lx (within the normal range). We

randomly assigned 36 Rio Grande leopard frog, *Rana berlandieri*, tadpoles to each treatment (n = 3 / tank; 12 replicates per treatment) and allowed a two-day acclimation in the tanks within the growth chamber before the start of the experiment. We then reared the tadpoles on a control light cycle, 12L:12D, for 7 days, after which we changed their environment by rearing the tadpoles for 14 days under constant ALAN. We then repeated this experiment with 36 new tadpoles by placing them in the control environment for 7 days followed by changing their environment to 14 days of a pulsed ALAN (12L: light on for an hour, then off for an hour, for 12 hours). We checked tadpoles daily for mortality, fed them *ad libitum* food cubes, and changed water weekly with aged tap-water. We collected baseline water-borne hormones on day 7 (after one week under control light cycle) and on day 21 (after 14 days of ALAN treatments). We also conducted an agitation stress test (outlined above) on day 21 after we collected baseline levels.

2019 Bufo valliceps: Reared under control then moved to ALAN treatments

In 2019, we repeated the 2018 above experiment with Gulf Coast toad, *Bufo valliceps* tadpoles, but we exposed the groups of tadpoles to the constant and pulsed ALAN treatments concurrently, rather than consecutively. We randomly assigned Gulf Coast toad tadpoles to either the pulsed ALAN or constant ALAN treatments (n=18 per treatment) and placed them in 5.7 L polypropylene plastic tanks with plastic mesh inserts filled with aged tap water. We partitioned the shelves with opaque black plastic sheeting to compartmentalize each treatment in the incubator. After a 2-day acclimation period, we reared the tadpoles under the control light cycle (12L:12D) for 7 days, and then under either the constant or the pulsed ALAN (12L: light on and off each hour for 12 hours) for

14 days. We checked tadpoles daily for mortality, fed them *ad libitum* (food cubes). We also conducted an agitation stress test (outlined above) on day 21 after we collected baseline levels.

Figure 9. Polypropylene tank with plastic mesh divided insert used for the 2018 and 2019 experiments with *Rana berlandieri* and *Bufo valliceps* tadpoles.

Statistical Analysis

We calculated body condition by regressing the residuals of mass and SVL measurements for each time step (MacCracken, 2005; Rohr et al., 2004). We multiplied corticosterone concentrations (pg/ml; from the EIA plate reads) obtained from the tadpoles in an hour (h) by the final resuspension volume (0.5 ml) and then standardized the value by dividing by SVL (cm) of the respective individual, giving us the corticosterone release rate (pg.ml/h). All hormone release rates were natural log transformed before data analysis. In 2017, we compared baseline cort release rates (pg/cm/h), mass, SVL, body condition, and mean tadpole behavior (time spent moving)

across time and between treatments using separate mixed models for each response variable, with treatment and time as fixed effects and individual as the random effect. All significant treatment effects were further explored with post-hoc tests t-tests. In 2018 and 2019, we compared baseline cort release rates (pg/cm/h) across time and between treatments using a mixed model with treatment and time as fixed effects and individual nested within tank as the random effect. All significant treatment effects were further explored with post-hoc t-tests. In all years, we compared baseline cort release rates (pg/cm/h) with agitation cort release rates (pg/cm/h) using separate mixed models for each treatment in each year, with time (baseline or agitation) as the fixed effect, and individual as the random effect. In 2019, we compared mass, snout-vent length, body condition, tail height, and robustness between treatments using pooled t-tests. Note that we did not compare mass, SVL, nor tail height of *Rana berlandieri* tadpoles between treatments in 2018 as the experiments were conducted consecutively and not concurrently so any differences in growth would be attributed to the tadpoles in the pulsed ALAN group being older. All tests were performed using JMP 14 software (SAS Institute, Inc). Using the R package rtpR in R version 3.2.3 (R Core Development Team), we calculated an adjusted repeatability of baseline cort release rates (pg/cm/h) with a linear mixed model (LMM) based approach using the Restricted Maximum Likelihood (REML) method (Dingemanse & Dochtermann, 2013; Nakagawa & Schielzeth, 2010), for tadpoles separately for each treatment. Corticosterone release rate was our response variable and sampling day was the fixed variable.

Results

2017 Rana berlandieri: Reared under ALAN treatments then moved to control

All tadpoles survived to the end of the experiment and there was no significant treatment effect on mass, snout-vent-length (SVL), tail height (TH), or body condition (Tables 1 & 2). Tadpole activity did not differ across treatments (Table 2). There was no significant interaction of treatment and time on cort release rates, though there were significant effects of treatment and time (Table 3). Cort release rates were significantly lower on days 2 and 14 for tadpoles in the pulsed ALAN treatment compared to the control and constant ALAN treatment tadpoles (Fig. 10). All tadpoles recovered to the same cort release rate by day 21. Tadpoles in the control and pulsed ALAN treatments did not show a significant change in cort release rates after agitation, while tadpoles in the constant ALAN treatment showed significantly lower cort release rates after agitation (Table 4, Fig. 11). Cort release rates were repeatable in the pulsed ALAN treatment, but not for control or constant ALAN (Table 5).

2018 Rana berlandieri: Reared under control then moved to ALAN treatments

All tadpoles survived to the end of the experiment in the constant ALAN treatment, while four tadpoles in pulsed ALAN died before the end of the experiment. There was no significant interaction between time and treatment (Table 3). Tadpoles in the pulsed ALAN treatment had significantly lower cort release rates on day 21 compared to day 7 and tadpoles in the constant ALAN treatment had significantly higher cort

release rates on day 21 compared to day 7 (Fig. 12). Tadpoles in both the constant and pulsed ALAN treatments showed significantly higher cort release rates after agitation (Table 4, Fig. 12). Cort release rates were repeatable for tadpoles in both ALAN treatments (Table 5).

Table 1. Mean ± SE mass, snout-vent length (SVL), and tail height (TH) for *Rana berlandieri* tadpoles (2017) exposed to light treatments (Control: 12L:12D, Constant ALAN: 24L:0D, or Pulsed ALAN: (3) 12L: with light pulsed on and off every hour for 12 hours through the night) for 14 days, and then reared on a controlled light cycle for 7 days (until Day 21 of the experiment).

	Day 2		Day 14			Day 21	
Treatment	**Mass (g ± SE)**	**SVL (cm ± SE)**	**Mass (g ± SE)**	**SVL (cm ± SE)**	**TH (cm ± SE)**	**Mass (g ± SE)**	**SVL (cm ± SE)**
Control	0.185 ± 0.016	0.97 ± 0.21	0.361 ± 0.026	1.23 ± 0.29	0.64 ± 0.020	0.373 ± 0.026	1.27 ± 0.30
Constant	0.185 ± 0.018	0.96 ± 0.21	0.385 ± 0.031	1.21 ± 0.27	0.65 ± 0.020	0.412 ± 0.032	1.29 ± 0.33
Pulsed	0.183 ± 0.009	0.98 ± 0.22	0.350 ± 0.024	1.24 ± 0.28	0.64 ± 0.019	0.367 ± 0.024	1.28 ± 0.40

Table 2. Results of mixed effects models comparing each response variable across treatments of *Rana berlandieri* tadpoles exposed to either a control, constant ALAN, or pulsed ALAN for 14 days in 2017.

Variable	**d.f.**	***F***	***p*-value**
Tail Height	2,42	0.161	0.852
Body Condition	2,42	2.563	0.089
Activity	2,42	1.459	0.244

2019 Bufo valliceps: Reared under control then moved to ALAN treatments

All *Bufo valliceps* tadpoles survived to the end of the experiment. There was no significant treatment effect on body condition, tail height, SVL, or body mass (Tables 6 & 7). Time significantly affected cort release rates (Table 3) with tadpoles in both the constant and pulsed ALAN treatments showing significantly higher cort release rates on day 21 compared to day 7 (Fig. 13). Tadpoles in both the constant & pulsed ALAN treatments showed significantly lower cort release rates after agitation (Table 4, Fig. 14). Cort release rates were repeatable for tadpoles in both ALAN treatments (Table 5).

Table 3. Fixed effects table for baseline cort release rates (pg/cm/h) with treatment and time across years using mixed models with the random effect as individual (2017) or individual nested within tank (2018 & 2019).

Year	Species		d.f.	*F*	*p*-value
2017	*Rana berlandieri*				
		Treatment	2,42	6.103	0.005
		Time	2,84	6.07	0.003
		Treatment*Time	4,84	1.507	0.208
2018	*Rana berlandieri*				
		Constant	1,19	33.826	< 0.001
		Pulsed	1,19	59.774	< 0.001
2019	*Bufo valliceps*				
		Treatment	1,34	1.168	0.288
		Time	1,34	74.16	< 0.001
		Treatment*Time	1,34	0.015	0.904

Table 4. Results of separate mixed effect models, for each treatment within each year, comparing baseline cort and agitation cort release rates (pg/cm/h) with time (baseline or agitation) as the fixed effect and the random effect as individual (2017) or individual nested within tank (2018 & 2019).

Year	Species	Treatment	d.f.	*F*	*p*-value
2017	*Rana berlandieri*				
		Treatment	1,14	0.429	0.523
		Constant	1,14	9.57	0.008
		Pulsed	1,14	2.01	0.178
2018	*Rana berlandieri*				
		Constant	1,19	15.541	< 0.001
		Pulsed	1,19	16.051	< 0.001
2019	*Bufo valliceps*				
		Constant	1,17	369.216	< 0.001
		Pulsed	1,17	576.865	< 0.001

Table 5. Adjusted repeatability values (*R*) of baseline cort release rates (pg/cm/h) over 3 time periods calculated using the Restricted Maximum Likelihood (REML) method (Dingemanse & Dochtermann, 2013; Nakagawa & Schielzeth, 2010). Corticosterone release rate was our response variable and sampling day as the fixed variable.

Year	Species	Treatment	*R* (SE)	95% CI	*p*-value
2017	*Rana berlandieri*				
		Control	0.104 (0.104)	0.0, 0.361	0.122
		Constant	0.095 (0.095)	0.0, 0.339	0.144
		Pulsed	0.294 (0.129)	0, 0.644	0.009
2018					
		Constant	0.541 (0.288)	0.0, 0.865	< 0.001
		Pulsed	0.598 (0.301)	0.0, 0.904	< 0.001
2019	*Bufo valliceps*				
		Constant	0.616 (0.304)	0.0, 0.889	< 0.001
		Pulsed	0.593 (0.298)	0.0, 0.809	< 0.001

Table 6. Mean ± SE mass, snout-vent length (SVL), and tail height (TH) for *Bufo valliceps* tadpoles (2019) exposed to a control (12L:12D) light cycle for 7 days, and then exposed to either Constant ALAN (24L:0D) or Pulsed ALAN (12L: with light pulsed on and off every hour for 12 hours through the night) for 14 days (Day 21 of the experiment).

	Day 7		Day 21		
Treatment	**Mass (g ± SE)**	**SVL (cm ± SE)**	**Mass (g ± SE)**	**SVL (cm ± SE)**	**TH (cm ± SE)**
Constant	0.117 ± 0.005	0.85 ± 0.016	0.140 ± 0.007	0.94 ± 0.015	0.42 ± 0.012
Pulsed	0.117 ± 0.005	0.87 ± 0.015	0.142 ± 0.009	0.96 ± 0.013	0.41 ± 0.011

Table 7. Results of pooled t-tests comparing each response variable across treatments of *Bufo valliceps* tadpoles exposed to either constant ALAN or pulsed ALAN for 14 days in 2019.

Variable	**d.f.**	**t**	***p*-value**
Tail Height	34	-0.497	0.622
Body Condition	34	-0.476	0.637

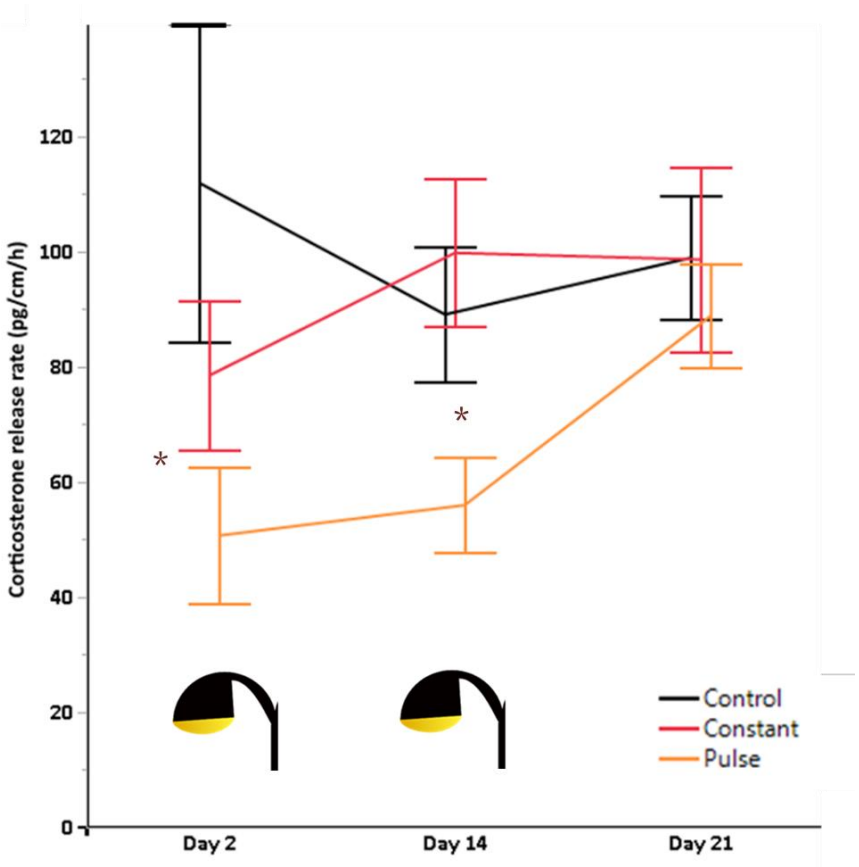

Figure 10. Corticosterone release rates (pg/cm/h) ± SE of *Rana berlandieri* tadpoles (2017) exposed to one of three light treatments, Control: 12L:12D, Constant ALAN: 24L:0D, or Pulsed ALAN: 12L: with light pulsed on and off every hour for 12 hours through the night, for 14 days, and then reared on a controlled light cycle (recovery) until Day 21 of the experiment. Significant differences between treatments based on post-hoc t-tests indicated by asterisks.

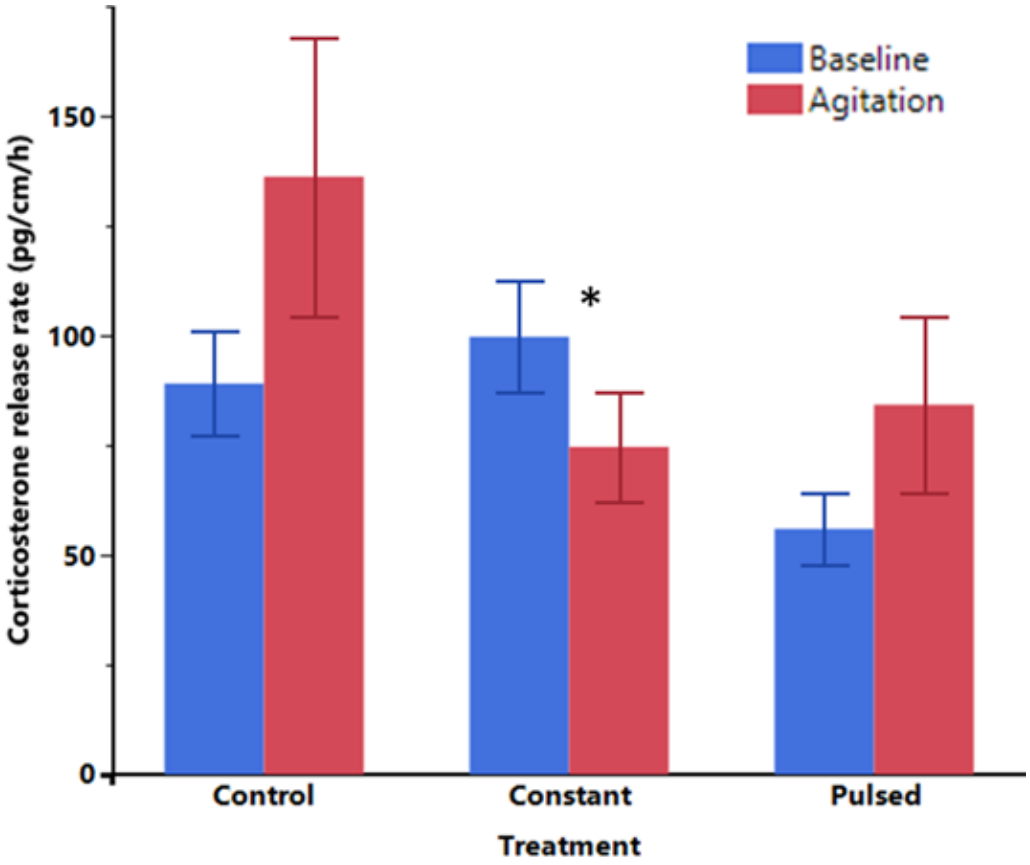

Figure 11. Baseline (blue) and agitation (red) corticosterone release rates (pg/cm/h) ± SE of *Rana berlandieri* tadpoles exposed to one of three treatments, Control: 12L:12D, Constant ALAN: 24L:0D, or Pulsed ALAN: 12L: with light pulsed on and off every hour for 12 hours through the night, for 14 days (Day 14) in 2017. Significant differences indicated by asterisks.

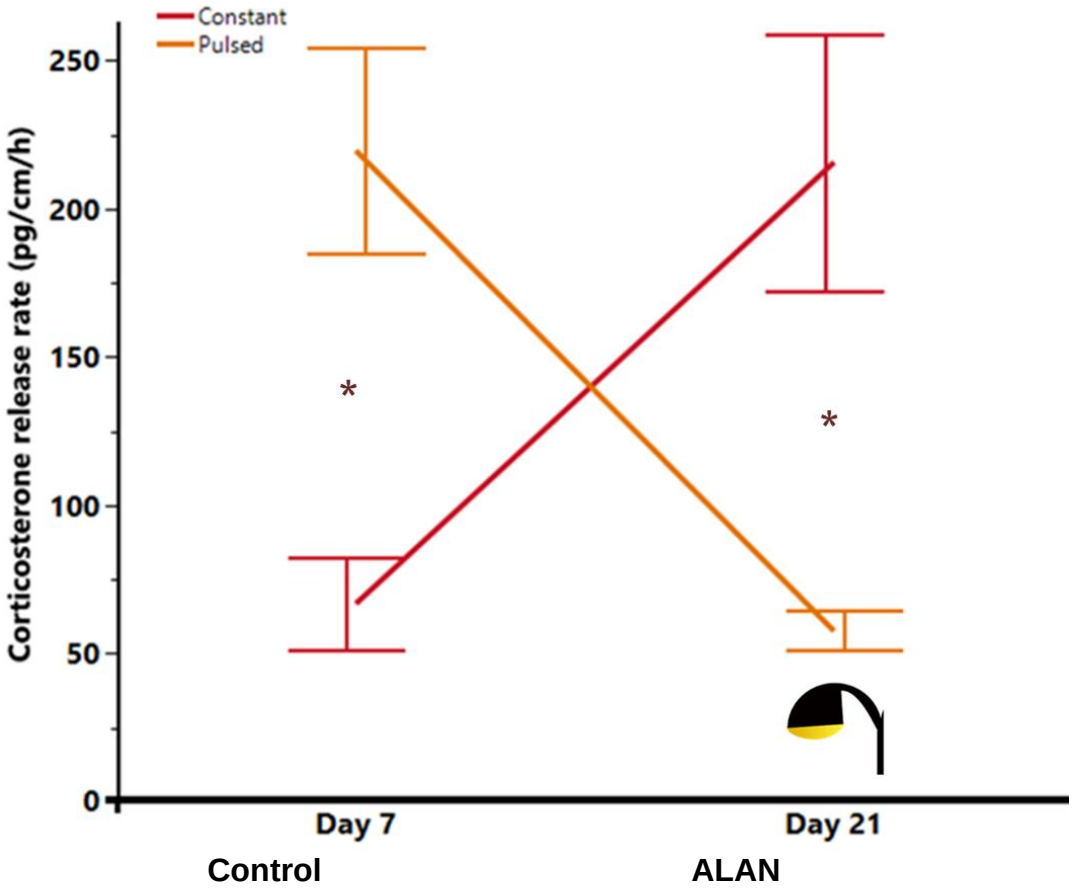

Figure 12. Corticosterone release rates (pg/cm/h) ± SE of *Rana berlandieri* (2018) tadpoles reared under a control light cycle (12L:12D) until Day 7, then reared under either Constant ALAN (24L:0D) or Pulsed ALAN (12L: with light pulsed on and off every hour for 12 hours through the night), for 14 days (until Day 21). Significant differences between treatments based on post-hoc t-tests indicated by asterisks.

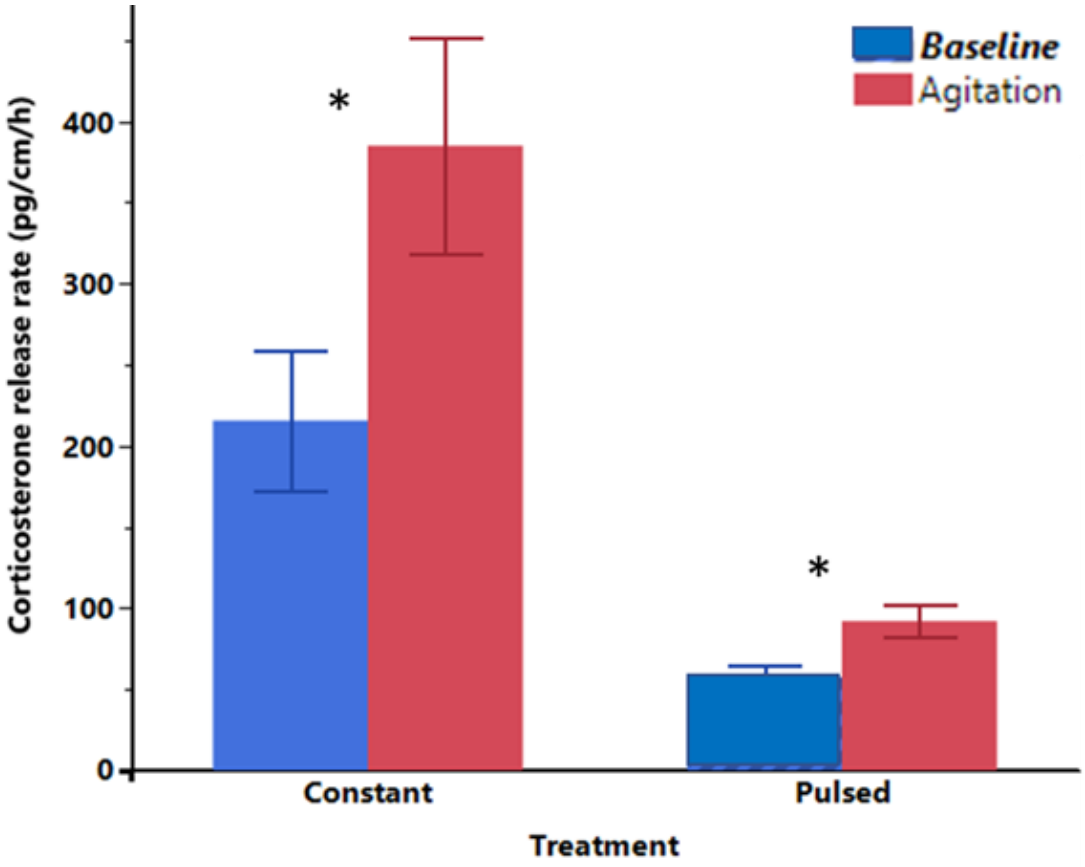

Figure 13. Baseline (blue) and agitation (red) corticosterone release rates (pg/cm/h) ± SE of *Rana berlandieri* tadpoles reared under either Constant ALAN (24L:0D) or Pulsed ALAN (12L: with light pulsed on and off every hour for 12 hours through the night), for 14 days (Day 21) in 2018. Significant differences indicated by asterisks.

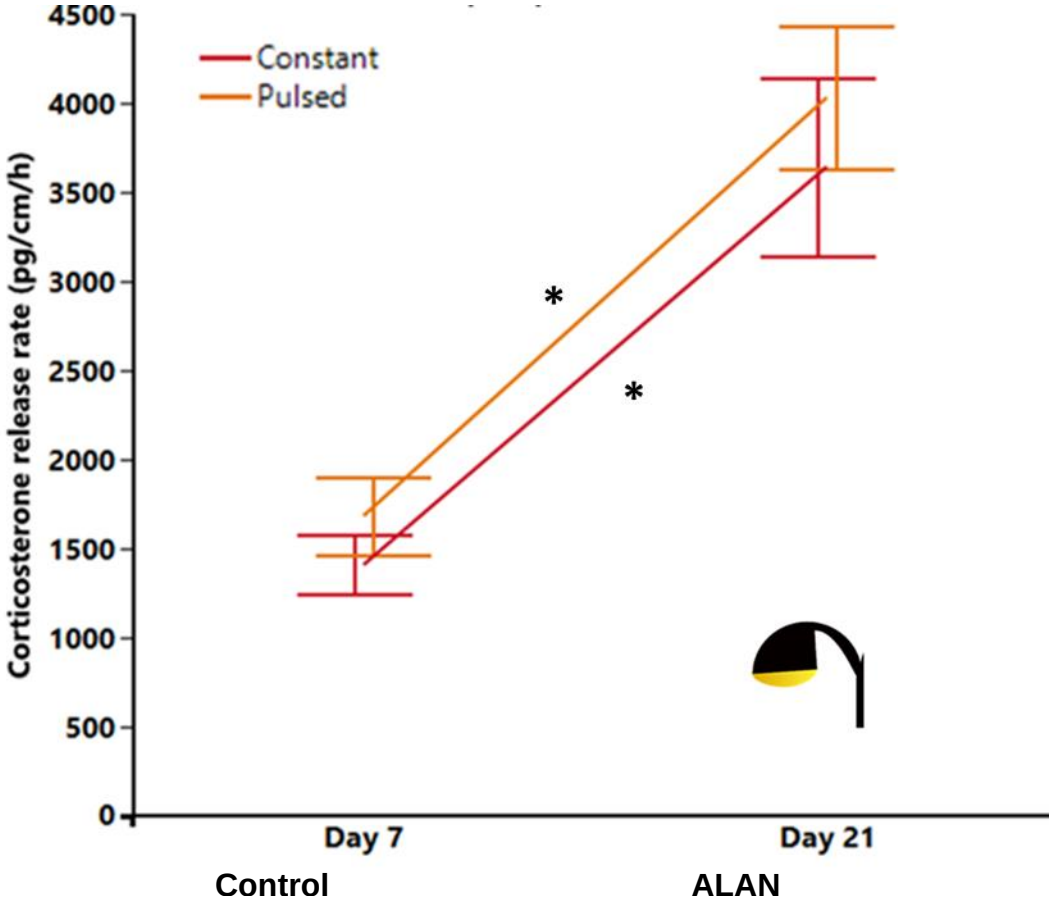

Figure 14. Corticosterone release rates (pg/cm/h) ± SE of *Bufo valliceps* (2019) tadpoles reared under a control light cycle (12L:12D) until Day 7, then reared under either Constant ALAN (24L:0D) or Pulsed ALAN (12L: with light pulsed on and off every hour for 12 hours through the night), for 14 days (until Day 21). Significant differences between treatments based on post-hoc t-tests indicated by asterisks.

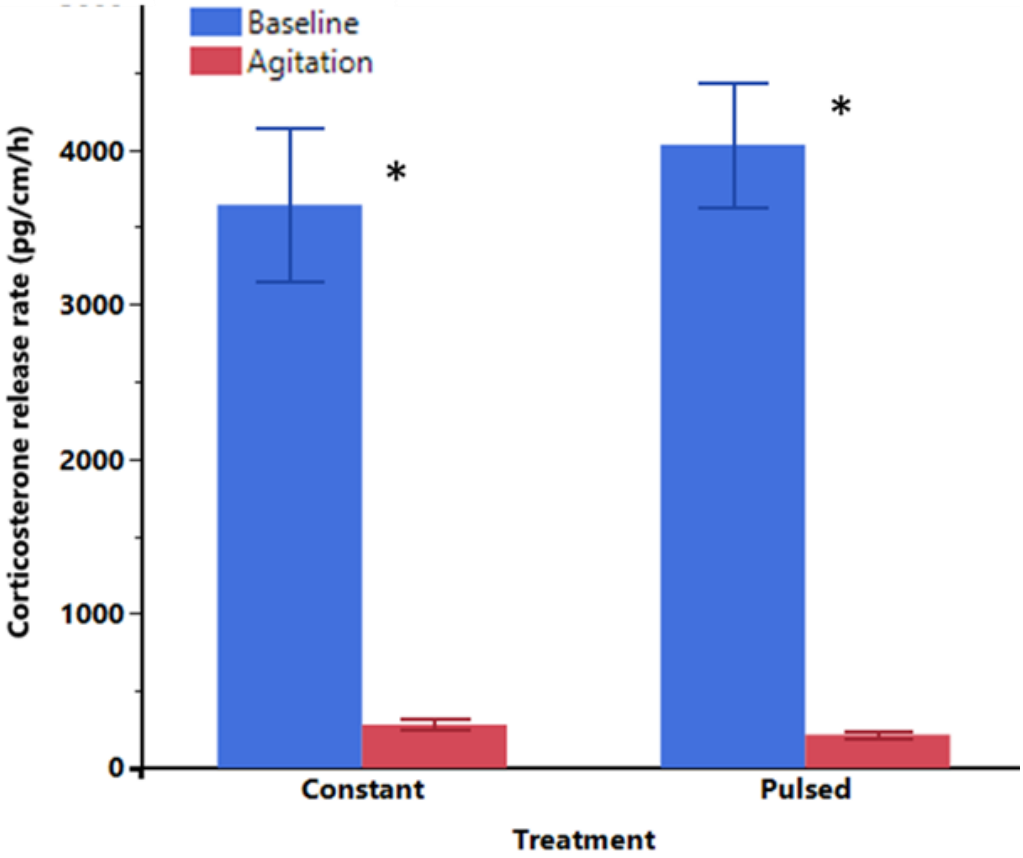

Figure 15. Baseline (blue) and agitation (red) corticosterone release rates (pg/cm/h) ± SE of *Bufo valliceps* tadpoles reared under either Constant ALAN (24L:0D) or Pulsed ALAN (12L: with light pulsed on and off every hour for 12 hours through the night), for 14 days (Day 21) in 2019. Significant differences indicated by asterisks.

Discussion

We explored how ALAN affects the physiology, growth, and behavior of Rio Grande leopard frog and Gulf Coast toad tadpoles. We found that exposure to environmentally relevant levels of constant or pulsed ALAN (190 lx - 250 lx) did not affect growth of two species of tadpoles, nor the behavior of *Rana berlandieri* tadpoles, however, we did see elevated and suppressed baseline cort release rates. This indicates that tadpoles are affected by ALAN but their physiological response varies by species and ALAN exposure. Further, all tadpoles showed repeatability of their cort response to ALAN indicating the potential to respond to environmental change when under selection by ALAN.

Our results indicate that both pulsed and constant ALAN are stressors for two species tadpoles, but that tadpoles of different species differentially modulate their stress response. We found that cort release rates were significantly lower in *R. berlandieri* tadpoles exposed to pulsed ALAN in both years, while cort release rates were significantly higher in tadpoles exposed to constant ALAN in 2018. In 2017, *R. berlandieri* tadpoles from the constant ALAN treatment showed a significant increase in cort release during an agitation stressor, whereas tadpoles from the pulsed ALAN treatment showed a non-significant decrease in cort. In 2018, tadpoles in both constant and pulsed ALAN treatments showed a significant increase in cort release after agitation. Pulsed ALAN appears to be a stressor for *R. berlandieri* tadpoles as they showed reduced cort levels after exposure, which is congruent with the response observed in cane toads, *Rhinella marina*, in response to thermal stressors (Narayan & Hero, 2014). Tadpoles in the pulsed ALAN treatment could be responding to the ALAN stressor by modulating

their glucocorticoid response to ALAN (lowering cort release rates), becoming more resistant to the repeated on and off again ALAN, to mediate deleterious effects of elevated cort levels (Wingfield, 2013; Bókony et al., 2021). Similarly, the pulsed ALAN could be acting as a repeated acute stressor with periods of relief between, and tadpoles could be showing physiological desensitization (Cyr & Romero, 2009). However, in 2017, tadpoles from the pulsed ALAN treatment recovered 7 days later to similar CORT levels as tadpoles in the control and constant ALAN treatments indicating that they were not chronically stressed. Our data also suggest that constant ALAN is a stressor for *R. berlandieri* tadpoles, evidenced by elevated levels of cort observed in the 2018 experiment, as elevated cort levels is associated with natural and environmental stressors in larval amphibians (Gabor et al., 2019; Middlemis-Maher, 2013). Increasing cort levels is a coping mechanism and can mediate deleterious effects of a stressful environment and aids in energy mobilization (Nelson, 2011; Romero et al., 2009), however persistent elevated levels and a suppressed HPI axis can be deleterious and lead to homeostatic failure (Romero et al., 2009). Similarly, *B. valliceps* tadpoles in both the constant and pulsed ALAN showed significantly elevated cort release rates after 14 days of exposure (and higher overall than *R. berlandieri)* and downregulated cort upon an agitation stressor. A possible reason why we did not observe a significant difference between baseline and agitation cort release rates in 2017 for *R. berlandieri*, while we did in 2018, is due to different maternal effects transferred to eggs across years (Donelan et al. 2020) or the different experimental exposures differentially affected the tadpoles. Our results highlight differential response of species to light but at the same time indicate that ALAN affects the stress physiology differently.

Little has been studied about differential regulation of HPI axis within and across species. here we found that *R. berlandieri* tadpoles down-regulate cort production in response to pulsed ALAN and up-regulate cort production in response to constant ALAN, indicating different forms of ALAN elicit different coping mechanisms in larval frogs that aid in avoiding homeostatic failure. One hypothesis may be that amphibian larvae respond differently than many other well studied species such as birds and reptiles. Because elevated cort can hasten metamorphosis (Denver et al., 1998), tadpoles may differentially downregulate cort in response to stressors instead of up-regulate as is found in most species (Romero 2009). This may allow the tadpole more time to grow before metamorphosing because the cost of a smaller size at metamorphosis can be high (Crespi & Warne, 2013; Denver et al., 1998; Glennemeier & Denver, 2002a; Hu et al., 2008). More work is needed to understand the basis to these varied coping mechanisms in tadpoles.

Repeatability is the proportion of the variation of a phenotype within a population that is explained by among individual variation (Hau et al., 2016). Corticosterone release rates were significantly repeatability for leopard frogs in the pulsed ALAN treatments in both years and in the 2018 constant ALAN treatment. Toad tadpoles also had significantly repeatable cort release rates in both ALAN treatments. Congruent with the findings of these experiments, other studies have found that cort levels were repeatable in amphibians (Forsburg et al., 2019; Narayan & Hero, 2013; Schoenemann & Bonier, 2018), In theory, for populations to evolve in response to selection, there must be significant levels of phenotypic variation within the population (Hau et al., 2016). Since repeatability has been used as a proxy for the upper limit of heritability when it cannot be

measured directly (Hau et al., 2016; Lessells & Boag, 1987; but see Dohm, 2002), cort release rates, in response to ALAN, show enough variation that there is potential to evolve in response to selection. However, we emphasize the need for heritability studies involving amphibians responding to ALAN to explore the potential for an evolutionary response to selection from ALAN exposure.

Constant and pulsed ALAN treatments did not affect growth or body condition in *Bufo valliceps* or *Rana berlandieri* tadpoles and ALAN did not affect behavior in *R. berlandieri* tadpoles. Our results contrast an early study that found larval northern leopard frogs, *Rana pipiens*, exposed to constant light for up to 100 days metamorphosed faster and weighed less at metamorphosis (Eichler & Gray, 1976; lux level not reported). May et al. (2019) also found wood frog tadpoles, *Rana sylvatica*, had higher mass and longer SVL at Gosner stage 25 when exposed to 300 lx ALAN, though they started treatments during the egg stage. We also did not observe a significant difference in tail height, yet previous research has shown that deeper tails is associated with higher cort levels and stress in larval amphibians (Gabor et al., 2019; Glennemeier & Denver, 2002b; Middlemis-Maher, 2013). We may not have observed differences in growth or morphology due to the shorter 14-day duration of treatments in our study combined with a relatively long larval period of 4 to 9 months for *Rana berlandieri* (Hughes & Meshaka, 2018) compared to the shorter 2 to 4 month larval period of *Rana sylvatica* (Wright & Wright, 1949). Also, we did not start treatments at egg stage, when they may be more sensitive to ALAN treatments. Our results for *Bufo valliceps* are similar to Dananany & Benard (2018) who found ALAN did not affect growth in American toad, *B. americanus,* tadpoles. *R. berlandieri* tadpole activity was also not affected by the ALAN treatments in

our experiment, yet May et al. (2019) showed differences in activity of tadpoles exposed to 300 lx ALAN. Additionally, newly metamorphic *B. americanus,* that were exposed to 3 lx ALAN during the larval period showed marginally increased activity (Dananay & Benard, 2018), however they observed metamorphs, and not larval activity. We only observed tadpoles during daylight hours and may have missed shifts in activity during twilight hours when control tadpoles were in the dark and ALAN treatment tadpoles were exposed to light, similar to what May et al. (2019) found.

Conclusions

The results of this experiment support the hypothesis that both pulsed and constant ALAN act as a stressor for both *R. berlandieri* and *B. valliceps* tadpoles and contribute to our understanding of how ALAN affects amphibians. Interestingly, both species differentially modulated their cort response to ALAN exposure and a subsequent stressor. Such flexibility in the HPI axis response has not been found in other non-amphibian species and may indicate an alternative mechanism for diminishing the deleterious effects of chronic stress. This may explain why we saw no differences in growth within the time period of our experiment. Because elevated cort can drive metamorphosis, tadpoles may differentially downregulate cort in response to stressors instead of upregulating as is found in most species. This may allow the tadpole more time to grown before metamorphosing because the cost of metamorphosis at a small size can be high (Crespi & Warne, 2013; Denver et al., 1998; Glennemeier & Denver, 2002a; Hu et al., 2008). One limitation of this study is that it only focused on short-term exposure of larval amphibians to ALAN. It is important to understand how long-term ALAN exposure affects tadpoles, as sources of ALAN are typically permanent, and individuals

will be exposed from egg through the duration of the larval period, and other studies have shown negative effects on growth in other species that were exposed for longer than 14 days (Eichler & Gray, 1976; May et al., 2019).

These results, combined with previous studies (Cope et al., 2020; Dananay & Benard, 2018; Eichler & Gray, 1976; May et al., 2019), suggest that there are species specific differences in how anurans respond to ALAN. Other studies have shown species specific differences in response to environmental stressors (Bernabo et al., 2013; Gabor et al., 2017) and species-specific differences in host susceptibility (Searle et al., 2011). More vulnerable species or species with narrow niches may be at higher risk than less vulnerable or habitat generalist species. But the ability to show flexible HPI response may help modulate the response to ALAN. Additionally, there are other anthropogenic stressors, such as chemical pollutants, and natural stressors (e.g., predators) in the environment that are also affecting amphibians. Exploring potential synergisms between ALAN and other stressors is warranted. Finally, the results of this experiment suggest that ALAN is contributing to stress in larval amphibians and therefore land and wildlife managers and urban planners should incorporate ways to mitigate the effects of ALAN on organisms into plans.

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IV. EFFECTS OF ALAN AND PREDATOR PRESENCE ON THE PHYSIOLOGY, GROWTH, AND ANTI-PREDATOR BEHAVIOR OF *RANA BERLANDIERI*

Abstract

Organisms are exposed to many natural and anthropogenic stressors in the environment. Artificial light at night (ALAN) affects the behavior, growth, and physiology of many taxa, including amphibians, and is a threat to biodiversity. A common natural stressor for organisms is predator presence, which affects the physiology of many organisms. Stress from predators and aquatic pollutants act synergistically, resulting in greater effects. I tested whether ALAN and the presence of a common aquatic predator, dragonfly larvae (*Anax sp*.), act synergistically, affecting the growth and physiology of Rio Grande leopard frogs, *Rana berlandieri*, tadpoles more than either stressor alone. I reared tadpoles in outdoor mesocosms for 14 days in one of four treatments: (1) Control, (2) predator, (3) constant ALAN, or (4) ALAN + predator. In a second experiment, I exposed tadpoles to constant ALAN in the lab for the duration of the larval period to test if long-term ALAN exposure affects growth and anti-predator behavior. Tadpoles in the ALAN + predator mesocosms had higher corticosterone release rates than tadpoles in the other treatments, though growth was not affected. In the second experiment, tadpoles metamorphosed faster and were smaller at metamorphosis under constant ALAN and showed reduced anti-predator behavior. These results suggest ALAN acts with other stressors synergistically and long-term ALAN exposure alone negatively affects tadpole growth. I found that ALAN can reduce fitness and the effect of ALAN on physiology is exacerbated by interaction with other stressors and management plans should include ways to mitigate the effects of ALAN on vulnerable taxa.

Keywords: Artificial light at night, ALAN, Light pollution, Conservation physiology, Stress physiology, Amphibians

Introduction

The Anthropocene (Ellis, 2011) is associated with what is considered the sixth mass extinction, as we observe a loss of global biodiversity due to anthropogenic changes to the environment (Barnosky et al., 2011; Butchart et al., 2010; Ceballos et al., 2017; McKee et al., 2003; Pelletier & Coltman, 2018). Anthropogenic changes to the environment include habitat alteration/loss, global climate change, spread of disease and exotic species, overharvesting and exploitation of natural resources, and many forms of pollution (Pelletier & Coltman, 2018; Sih et al., 2011; Swaddle et al., 2015; Tillman et al., 2017). Many of these perturbations act as individual stressors, contributing to population declines (Jeffrey et al., 2015), but it is the synergism between multiple stressors that may be the greatest threat to global biodiversity (Brook et al., 2008; Mantyka-Pringle et al., 2012; Segan et al., 2016).

Organisms respond to environmental stressors and cope with changing environments in many ways, including altering their physiology to maintain homeostasis (McEwen & Wingfield, 2003; Wikelski & Cooke, 2006). Physiological changes in response to stressors, or stressful environments, include elevated circulating levels of glucocorticoids (GCs), such as corticosterone or cortisol, above normal "baseline" levels (Romero et al., 2009). A short-term elevation of circulating GCs can be advantageous as it mediates gluconeogenesis and mobilizes energy (Hau et al., 2016; Romero et al., 2009; Sapolsky et al., 2000), however unpredictable and long-term perturbations can lead to persistently elevated or down regulated (suppressed) GC levels which can be deleterious

(Romero et al., 2009). By measuring GC levels over time and by measuring how individuals respond to an acute stressor, such as agitation, one can assess how individuals physiologically cope with changing environments (Wikelski & Cooke, 2006). Understanding how individuals physiologically respond to stressors or changing environments can give us insight into individual and population health (Dantzer et al., 2014; Gabor et al., 2018, Sheriff et al., 2011, Wikelski & Cooke, 2006) and can aid management and conservation efforts to mitigate biodiversity loss (Madliger et al., 2016).

Artificial light at night (ALAN; Haim & Zubidat, 2015) is an anthropogenic stressor that is a threat to biodiversity (Garret et al., 2019; Gaston et al., 2019; Secondi et al., 2020; Seymoure et al., 2019) and affects the behavior, physiology, and development of many taxa (see reviews: Gaston et. al., 2014; Longcore & Rich, 2004; Navara & Nelson, 2007; Swaddle et al., 2015). It is estimated that over 80% of the world population live in areas that are considered polluted by ALAN (Falchi et al., 2016) and over 50% and 75% of key biodiversity areas and global protected area units, respectfully, are affected by ALAN (Seymoure et al., 2019). ALAN reduces foraging activity (Bird et al., 2004; Wise & Buchanan, 2006), alters calling behavior (Miller, 2006) and affects reproductive behavior (De Jong et al., 2015). Additionally, exposure to ALAN can suppress immune function (Bedrosian et al., 2011; Bedrosian et al., 2013) and can alter levels of GCs such as corticosterone (Forsburg et al., in prep; Cope et al., 2020; Ouyang et al., 2015). ALAN is estimated to be increasing at a rate of 6% yearly (Hoelker et al., 2010), and thus the impact of ALAN on organisms may be of great importance (Garret et al., 2019; Gaston et al., 2015; Secondi et al., 2020; Seymoure et al., 2019) and may be

acting synergistically with other environmental stressors exacerbating global biodiversity loss.

The presence of predators is a natural biotic stressor that affects the behavior and physiology of a range of taxa (Bourdeau & Johansson, 2012; Burraco & Gomez-Mestre, 2013; Clinchy et al., 2013; Davis & Gabor 2015; Fraker et al., 2009; Middlemis Maher et al., 2013; Relyea & Mills, 2000; Vitousek et al., 2014). In the presence of a predator, an organism has the choice to actively defend itself from the predator (Arca et al., 2014) or alter behavior to avoid being detected or depredated (Breed et al., 2017; Fraker et al., 2009). For example, when an intruder is detected in a honeybee, *Apis mellifera*, colony, bees may directly attack the predator (Arca et al., 2014), or use a fanning behavior to prevent depredation (Yang et al., 2010). San Marcos salamanders, *Eurycea nana*, reduce activity (anti-predator behavior) when they detect the presence of predatory fish chemical cues (Davis & Gabor, 2015). Predator presence can elevate corticosterone levels, as observed in male song sparrows, *Melospiza melodia*, (Clinchy et al., 2011) and San Marcos salamanders, *Eurycea nana* (Davis & Gabor, 2015). Predators directly reduce populations via depredation, negatively affect populations through deleterious impacts on physiology (Middlemis Maher et al., 2013; Relyea & Mills, 2000) and predator presence can act synergistically with anthropogenic stressors (Burraco & Gomez-Mestre, 2013; Gabor et al., 2019; Relyea et al., 2004).

The physiology and behavior of amphibians is affected by both predator presence (Burraco & Gomez-Mestre, 2013; Davis & Gabor, 2015; Fraker et al., 2009; Gabor et al., 2019; Middlemis Maher et al., 2013; Relyea & Mills, 2000) and ALAN (Dananay & Benard, 2018; Forsburg et al., in prep; Gaston et al., 2019; May et al., 2019). Amphibian

larvae detect predators using chemical and visual cues, and typically reduce movement when a predator is detected (Miko et al., 2017; Middlemis Maher et al., 2013; Moore et al., 2015). Dragonfly, *Anax* sp. and *Aeshna* sp., larvae are common predators of aquatic tadpoles and Takahara et al. (2012) observed a reduction in tadpole activity in response to chemical cues from dragonfly larvae, *Anax junius*. When reared with caged *Anax sp*. dragonfly larvae, leopard frog, *Rana sylvatica*, tadpoles showed elevated corticosterone levels (Middlemis Maher et al., 2013) and similarly, spadefoot toad tadpoles, *Pelobates cultripes*, exposed to chemical cues of a predacious aquatic beetle, *Dytiscus circumflexus*, showed elevated corticosterone (cort) levels (Burraco et al., 2013). Additionally, Fraker et al. (2009) observed a suppression of the hypothalamus-pituitary-interrenal (HPI) axis in *Rana sylvatica* and *R. clamitans* tadpoles after exposure to predator cues, associated with lower cort and reduced activity. Exposure to 190 lx ALAN also induces elevated cort levels in Rio Grande leopard frog, *Rana berlandieri*, and Gulf Coast toad, *Bufo valliceps*, tadpoles (Forsburg et al., *in prep*). Low levels of ALAN also hasten metamorphosis in American toad, *Bufo americanus*, (3 lx; Dananay & Benard, 2018) and leopard frog, *Rana pipiens*, tadpoles (Eichler & Gray, 1976; lux not reported). May et al. (2019) observed shifts in behavior of wood frog, *Rana sylvatica*, metamorphs reared in a 300 lx ALAN. Because tadpoles vary in whether they up-regulate or down-regulate baseline cort and agitation cort in response to stressors, it is necessary to consider this while exploring the effects of both ALAN and predator presence effects on HPI axis in tadpoles.

Predatory stress acts synergistically with chemical contaminants and increases mortality in anuran larvae (Burraco & Gomez-Mestre, 2013; Relyea et al., 2004). These

contaminants include carbaryl, glyphosate, and malathion on mortality in wood frog, *Rana sylvatica*, tadpoles (Relyea, 2005), grey treefrog tadpoles, *Hyla versicolor*, (Relyea & Mills, 2001), and green frog, *Rana clamitans*, tadpoles (Relyea, 2003). Predator presence (Middlemis Maher et al., 2013) or ALAN (Forsburg et al., *in prep*) increase corticosterone, the main glucocorticoid in amphibians (Forsburg et al., 2019; Idler, 1972), levels in anuran larvae. Recently, Cope et al. (2020) tested for synergism between ALAN and predator presence by rearing American toad, *Anaxyrus americanus*, tadpoles with dragonfly larvae and exposing them to low level ALAN (3 lx at water surface) and found no significant treatment effects on cort levels or time to metamorphosis. It is possible they did not see a treatment effect due to the low level of ALAN used (3 lx), as we found exposure to an environmentally relevant level of ALAN (190 lx) significantly elevated cort levels in *Rana berlandieri* and *Bufo valliceps* tadpoles (Forsburg et al., *in prep*). Therefore, elevated cort levels from synergism between ALAN and predator stress would be detrimental to amphibian populations, as elevated levels of cort can reduce growth in amphibian larvae, alter tadpole morphology, and hasten metamorphosis (Crespi & Warne, 2013; Denver et al., 1998; Glennemeier & Denver, 2002a; Hu et al., 2008). Reduced mass at metamorphosis can affect growth and survivorship later in life (Cabrera-Guzman et al. 2013; Chelgren et al., 2006; Crespi & Warne, 2013; Earl & Whiteman, 2015; Rohr et al., 2013). Using amphibians as a model organism to further investigate synergism between an environmentally relevant level of ALAN and predator stress will further our understanding of how ALAN is affecting organisms and aid conservation and management decisions (Grant et al., 2016).

I explored potential synergistic effects between ALAN and predator presence on the physiology and growth of Rio Grande leopard frogs, *Rana berlandieri* using a semi-natural mesocosm experiment. I hypothesized that chemical and visual cues from dragonfly larvae combined with exposure to ALAN would be more stressful than either exposure to ALAN or dragonfly larvae alone, with tadpoles showing elevated levels of corticosterone and growth. Additionally, in a second experiment, I examined how long-term exposure to ALAN would affect growth and anti-predator behavior in leopard frog tadpoles in a laboratory setting, hypothesizing that tadpoles in ALAN treatments would metamorphose faster and would be smaller at metamorphosis than tadpoles reared on a natural light cycle.

Methods

Animal collection and husbandry

I collected portions of five *Rana berlandieri* egg masses from two different local bonds in San Marcos, Hays Co., Texas (29.874695, -97.962733 and 29.903373, -97.966839) on 10 April 2019. I transported eggs to a laboratory on Texas State University Campus, where I reared eggs in aged tap water until tadpoles were free swimming (approximately one week from collection of eggs). Once tadpoles were free swimming (Gosner stage 25; Gosner, 1960), I mixed tadpoles from each clutch and housed them in 6 L polypropylene plastic tanks filled with aged tap water at room temperature (12 per tank). I checked for mortality daily, fed tadpoles ad libitum a mixture of spirulina powder and ground fish flakes in an agar powder base (food cubes), and changed water as needed, but at least once per week, using aged tap water. The

experiments were conducted from June to August 2019 on Texas State University campus in San Marcos, Hays Co., Texas.

Experiment 1: Outdoor mesocosms with ALAN and predator presence

Four weeks prior to the mesocosm experiment, I set up 32 plastic 65 L tubs on flat ground under a 40% shade cloth canopy behind a Texas State University greenhouse that was not exposed to ALAN. I filled each tub with 49 L of rainwater and added 15 g of dry, native, oak leaves, 15 g of dry grass vegetation collected from around the ponds where I had collected the frog eggs, and 15 g of alfalfa rabbit chow to each tub. I also inoculated each tub with 1 L of fresh pond water with concentrated zoo plankton (I repeatedly swept through the ponds with a zooplankton net and pooled the concentrated zooplankton in a large tub of pond water- I agitated the water in the tub to evenly distribute the zooplankton and made 1 L aliquots from this tub). I covered the mesocosms with lids with mosquito screening to keep out any predators. Two weeks after I set up the mesocosms, I added a second 1 L aliquot of concentrated zooplankton to each mesocosm. I let the mesocosms sit for a total of four weeks before the start of the experiment.

One week before the experiment, I marked tadpoles in the lab with one of six different colors of visual implant elastomer (VIE) to facilitate repeated measures in the mesocosm. I also collected dragonfly larvae (*Anax sp*.) from the same two ponds I collected the frog eggs from. I maintained dragonfly larvae in the laboratory in individual plastic aquaria filled with aged tap water and a stick for a perch. I fed dragonfly larvae two, non-experimental, tadpoles every other day. I randomly assigned six VIE marked tadpoles (one each of six colors) to each mesocosm and randomly assigned six non-marked tadpoles to each mesocosm, with a final density of twelve tadpoles per

mesocosm. I started treatments after a two-day acclimation period for the tadpoles and ended after 14 days of exposure to the treatments. I had eight replicates of each of four treatments: (1) control, (2) constant artificial light at night (ALAN), (3) caged dragonfly larvae predator, and (4) ALAN and caged dragonfly larvae predator. For the ALAN treatments, I placed 35 W 5000 K white LED flood lights on timers 2 m above groups of four mesocosms. Light levels were 200 lx (in the range of environmentally relevant levels I measured in San Marcos, Hays Co., Texas) measured at the surface of the water of each mesocosm with a Dr. Meter LX1330B digital light meter. I placed an opaque black plastic barrier between ALAN and non-ALAN mesocosms to prevent any light trespass. The predator treatment consisted of dragonfly larvae (*Anax sp*.) housed individually in floating clear 0.5 L plastic bottles with holes (Fig. 16) that allowed for the tadpoles to visually and chemically sense the dragonfly larvae but prevented the dragonfly larvae from depredating tadpoles. I provided a perch and air space for each dragonfly larvae within the bottle and I fed them 2 non-experimental tadpoles three times per week. Non-predator treatment mesocosms had empty floating bottles and I opened each empty bottle every time I fed the dragonfly larvae to control for disturbance. I maintained water levels in the mesocosm by adding rainwater as needed.

To examine how cort release rates changed over time, I collected hormones from each VIE marked tadpole on days 7 and 14 of the experiment using a non-invasive water borne hormone method (Gabor et al., 2016) validated for this species (Forsburg et al., 2019). Briefly, I placed each tadpole in a clean LDPE (low density plastic..) insert (a perforated lab bottle with the top cut off to facilitate removal of tadpoles from beakers) in a 250ml glass beaker filled with 100ml of spring water for 60 minutes. I also measured

the mass of each tadpole by placing tadpoles on a tared piece of moistened sponge on a digital balance and photographed them (to measure SVL and TH) after I collected hormones. I wore non-powdered gloves throughout the hormone collection process and cleaned beakers and inserts with 95% ethanol and rinsed them with DI water before each use. On day 14, I also performed an agitation stress test on each tadpole after I collected the unmanipulated, baseline, hormone sample. I moved tadpoles in plastic inserts into clean 250 ml glass beakers with 100 ml of spring water and agitated them for 60 seconds every three minutes for an hour. At the end of the first water-borne collection period I returned tadpoles to their original mesocosms.

I stored the water-borne hormone samples at -20°C until we thawed them for extraction following methods of Gabor et al. (2016). Briefly, we extracted hormones (n = 24 per treatment) by running water samples through Tygon tubing into methanol (4ml) and distilled water primed C18 solid phase extraction (SPE) columns under vacuum. Following extraction, I eluted columns with 4ml methanol into borosilicate vials, which I then evaporated under nitrogen gas (approx. 2 hours). Following drying, I re-suspended the residue in 50 µl 95% ethanol and 950 µl enzyme-immunoassay (EIA) buffer (Cayman Chemical Company, Inc.) for a total re-suspension volume of 1 ml. I measured cort release rates in duplicate for all samples using EIA kits (№ 501320, Cayman Chemical Company, Inc.; assay range of 8.2 pg/ml – 5,000 pg/ml; sensitivity (80 % B/Bo) of approx. 30 pg/ml on a spectrophotometer plate reader at 405 nm (BioTek 800XS). Inter-plate (9 plates) variation was 8.97% and intra-plate variations ranged from 0.45 – 2.87%.

I calculated body condition by regressing the residuals of mass and SVL measurements for each time step (MacCracken, 2005; Rohr et al., 2004). I standardize

corticosterone release rates (pg/ml) by dividing by SVL (cm) of the respective individual. All hormone release rates were natural log transformed before data analysis. I compared cort release rates (pg/cm/h) across time and between treatments using a mixed model with treatment and time as fixed effects and individual nested within mesocosm as a random factor. I compared body condition and tail height of tadpoles on day 14 across treatments using pooled t-tests.

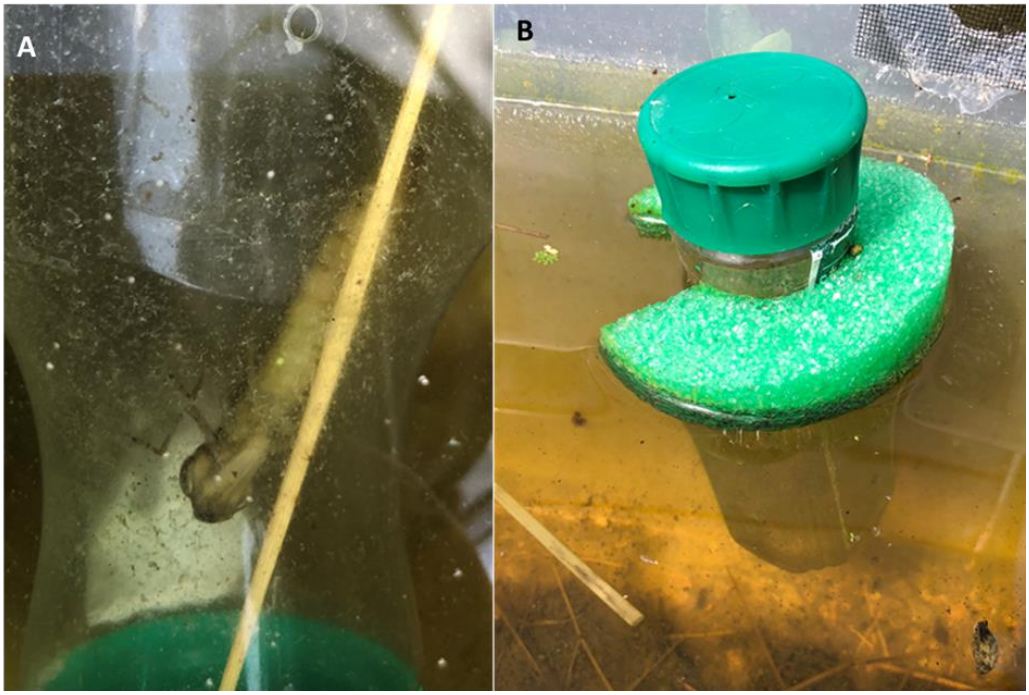

Figure 16. Dragonfly larva (*Anax sp.*) on perch (A) inside floating housing within mesocosm (B).

Experiment 2: Long-term ALAN exposure

To examine how long-term exposure to ALAN affects time to metamorphosis (TTM) and size at metamorphosis, I reared *Rana berlandieri* tadpoles in the laboratory under a control light cycle (12L:12D) or constant ALAN (24L:0D) for the duration of metamorphosis. I set up twelve 5.7 L polypropylene plastic containers with plastic mesh inserts forming three compartments, which permitted repeated measurements from the

same tadpoles, while also allowing tadpoles to sense each other visually and chemically. I filled the containers with aged tap water and placed them in a growth chamber set at 20^{o} C. I placed 3000K LED strip lights, to provide ALAN, and 5000K full spectrum LED strip lights, to provide daytime lighting, 30 cm above the tanks on timers. The intensity of the ALAN lights at the surface of the tank water was 190 lx (in the range of environmentally relevant levels we measured in San Marcos, Hays Co., Texas) and the intensity of the full spectrum lights during the day was 2800 lx at the surface level. I partitioned the shelves with opaque black plastic sheeting to compartmentalize each treatment in the growth chamber. I randomly assigned 36 leopard frog tadpoles to tanks (n = 3/tank; 6 replicates per treatment) and allowed a two-day acclimation in the tanks/growth chamber before the start of the experiment. I then reared half the tadpoles on a control light cycle and half in the ALAN treatment. I checked tadpoles for mortality daily, fed them ad libitum food cubes, and changed water weekly with aged tap-water. I photographed each tadpole and used ImageJ (Schneider et al., 2012) to calculate the snout vent length (SVL) and tail height (TH), and also obtained the mass of each tadpole by placing tadpoles on a tared piece of moistened sponge on a digital balance on day 21 and day 49 of the experiment. I checked tadpoles daily once they reached metamorphic climax (Gosner, 1960) and removed metamorphs when the tail was 5mm or less in length, recorded the date of metamorphosis, and weighed/photographed each metamorph. I euthanized each metamorph with benzocaine (20%).

To examine if exposure to constant ALAN affects anti-predator behavior of *Rana berlandieri* tadpoles, I first collected diet-based chemical cues from *Anax sp*. dragonfly larvae, common aquatic predators of anuran larvae. I collected dragonfly larvae from the

same ponds I collected frog eggs in July 2019. I kept dragonfly larvae individually in plastic aquaria filled with aged tap water and a perch. I started feeding the dragonfly larvae 2 conspecific tadpoles per day for 7 days. After 7 days of feeding, I placed 6 dragonfly larvae in separate, clean, beakers with 230 ml of spring water per gram of dragonfly larvae for 24 hours. I pooled and mixed the water samples before freezing aliquots of the water-based "diet cue" at -20^{o} C (Epp and Gabor, 2008). I defrosted the amount of diet cue needed for the antipredator trials by thawing the aliquots to room temperature before use. On day 28 of the long-term ALAN exposure experiment, I set up 6 individual 1.5 L tanks, each with 1 L of aged tap water and two 60 ml syringes connected to tubing with a two-way spot that were placed inside each tank. One syringe was filled with room temperature aged tap water and one was filled with room temperature predator diet cue. I mounted webcams connected to laptop computers above pairs of tanks (3 camera/laptop set ups) so that I could film the trials. I then placed individual tadpoles in the tanks and allowed them to acclimate for 8 minutes. During each round of trials, 3 tadpoles from the control treatment and 3 tadpoles from the ALAN treatment were filmed using ManyCam software. After the 8 min acclimation period, I injected 10 ml of aged tap water into each tank at a rate of 1 ml per second and filmed "pre" exposure activity (time spent moving) for 8 minutes. After 8 minutes, I then injected 10 ml of the diet cue at a rate of 1 ml per second and filmed "post" exposure activity for 8 minutes. I cleaned syringe tips, tubing, and tanks between trials with 3 % hydrogen peroxide and rinsed with deionized water. I repeated this until all 36 tadpoles were filmed. I analyzed the videos using EthoVision XT (Noldus) for total activity (time spent moving) for "pre" exposure and "post" exposure for each tadpole.

I calculated body condition by regressing the residuals of mass and SVL measurements for each time step (MacCracken, 2005; Rohr et al., 2004). I compared mass, tail length, and tail height across time and between treatments for tadpoles in the long-term ALAN experiment using a mixed model with treatment and time as fixed effects and individual nested within tank as a random factor. I also compared body condition on day 49, time to metamorphosis, and size at metamorphosis between treatments using pooled t-tests. To analyze differences in anti-predator behavior, I first tested for any significant variation in pre-predator cue exposure activity between treatments using a student's t-test. I then tested for any significant differences in the activity response (post-exposure activity subtracted from pre-exposure activity) between treatments using a student's t-test. All tests were performed using JMP 14 software (SAS Institute, Inc).

Results

Experiment 1: Mesocosm experiment

All but one of the VIE marked tadpoles survived in the mesocosms through day 14 of the experiment (0.7% mortality). There was no significant treatment effect on body condition nor tail height (Tables 8 & 9). Time and treatment each significantly affected cort release rates but there was no significant interaction between the two factors (Table 10). Tadpoles in the dragonfly + ALAN treatment had significantly higher cort release rates on day 7 compared to tadpoles in the other treatments and significantly higher cort release rates on day 14 compared to the control and ALAN only treatments (Fig. 17). Tadpoles in the control group showed significantly higher cort release rates after

agitation, while tadpoles in all other treatments showed significantly lower cort release rates after agitation (Fig. 18).

Table 8. Mean ± SE mass, snout-vent length (SVL), and tail height (TH) of *Rana berlandieri* tadpoles exposed to one of four treatments, (1) control, (2) predator, (3) ALAN, (4) predator + ALAN (DF & ALAN), for 14 days in outdoor mesocosms.

Treatment	**Mass (g ± SE)**	**SVL (cm ± SE)**	**TH (cm ± SE)**
Control	1.837 ± 0.088	2.279 ± 0.039	1.102 ± 0.035
Dragonfly	1.668 ± 0.064	2.280 ± 0.030	1.076 ± 0.023
ALAN	1.674 ± 0.062	2.199 ± 0.031	1.025 ± 0.018
DF & ALAN	1.822 ± 0.094	2.349 ± 0.051	1.112 ± 0.024

Table 9. Results of mixed effect models comparing each response variable across treatments of *Rana berlandieri* tadpoles exposed to one of four treatments, (1) control, (2) predator, (3) ALAN, (4) predator + ALAN, for 14 days in outdoor mesocosms.

Variable	**d.f.**	***F***	***p*-value**
Tail Height	3,92	2.308	0.082
Body Condition	3,92	1.319	0.273

Table 10. Fixed effects table for corticosterone release rate (pg/cm/h) in response to treatment and time from a mixed model with the random effect as individual nested within mesocosm.

	d.f	***F***	***p*-value**
Treatment	3,92	5.062	0.003
Time	1,92	5.509	0.021
Treatment*Time	3,92	0.594	0.62

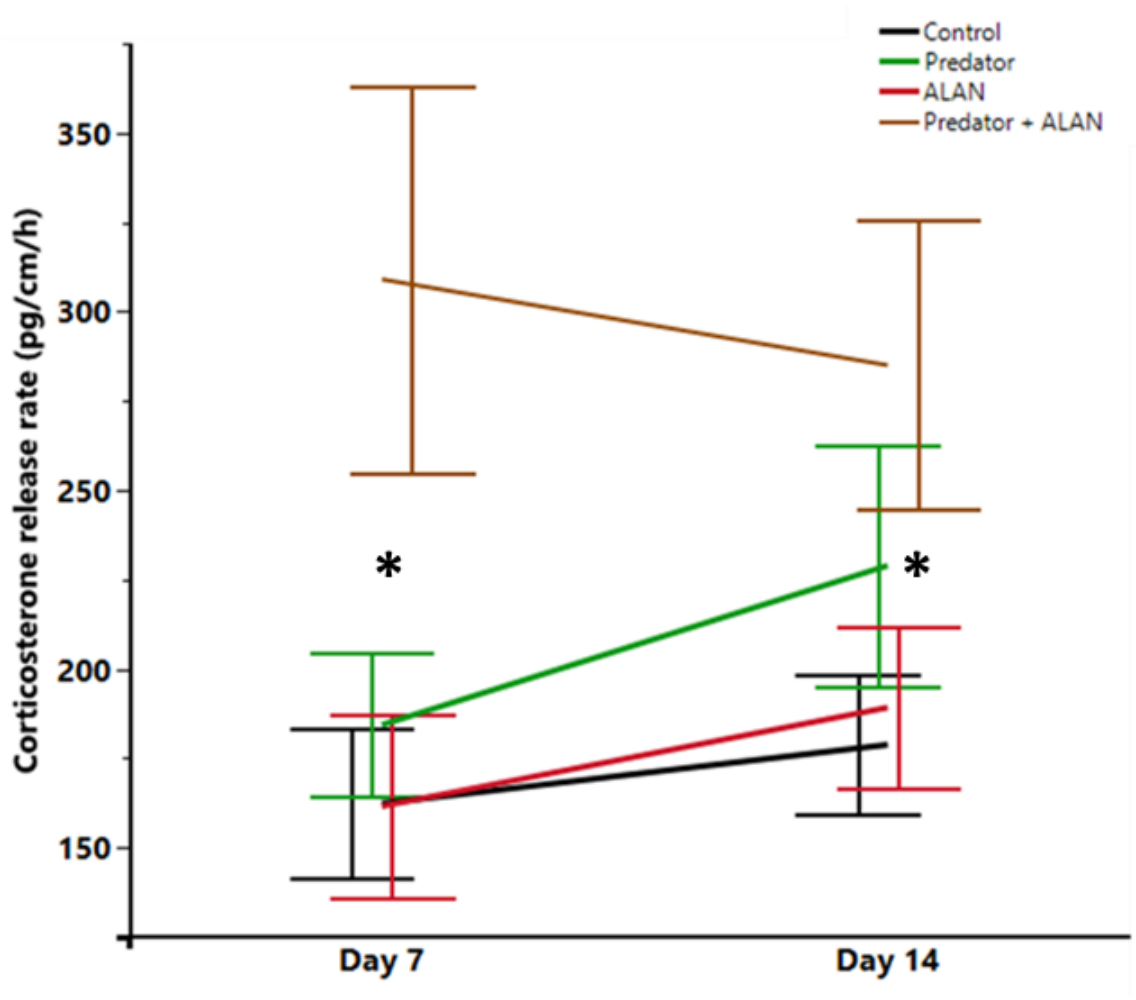

Figure 17. Corticosterone release rates (pg/cm/h) ± SEM of *Rana berlandieri* tadpoles exposed to one of four treatments, (1) control, (2) predator, (3) ALAN, (4) predator + ALAN, for 14 days in outdoor mesocosms. Significant differences between treatments based on post-hoc t-tests indicated by asterisks.

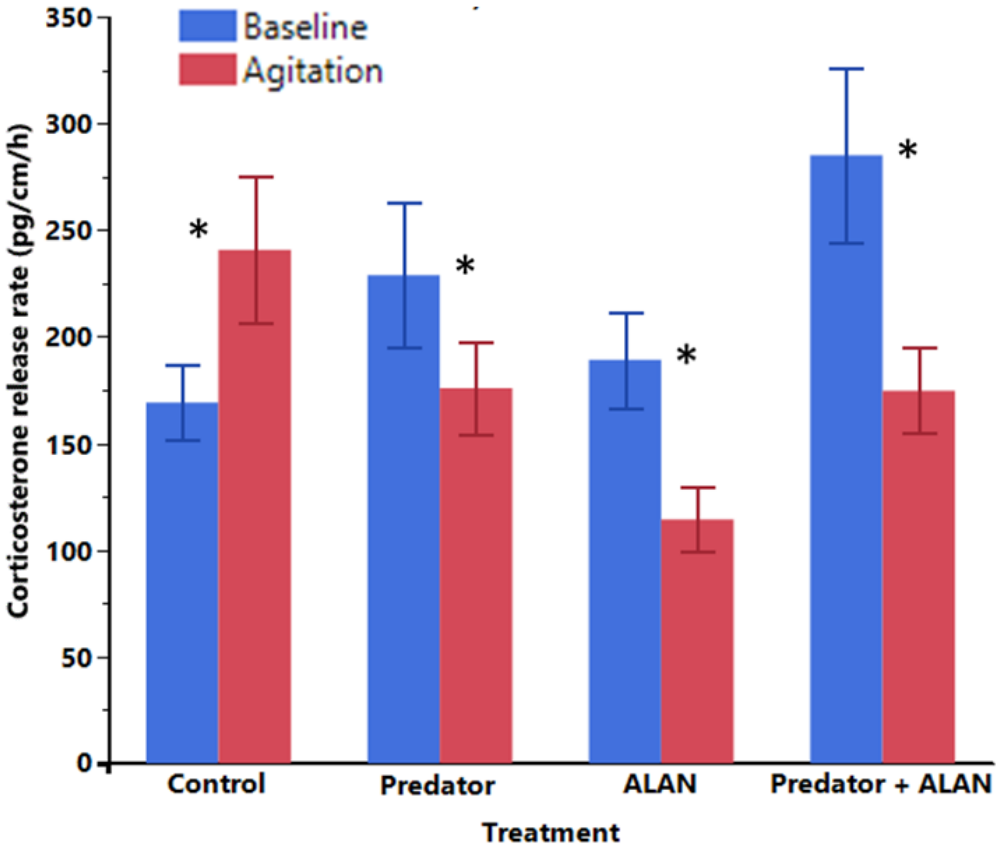

Figure 18. Baseline (blue) and agitation (red) corticosterone release rates (pg/cm/h) ± SE of *Rana berlandieri* tadpoles exposed to one of four treatments, (1) control, (2) predator, (3) ALAN, (4) predator + ALAN, for 14 days in outdoor mesocosms. Significant differences are indicated by asterisks.

Experiment 2: Long-term ALAN exposure

For the tadpoles in the long-term ALAN exposure laboratory experiment, body condition ($t = 0.669$, $DF = 34$, $p = 0.508$) did not significantly differ on day 49 of the experiment (Table 11). There was no significant treatment effect on snout-vent-length ($F_{1,55} = 0.092$, $p = 0.763$), tail height ($F_{1,55} = 3.228$, $p = 0.077$), tail length ($F_{1,55} = 3.118$, $p = 0.085$), or mass ($F_{1,55} = 0.659$, $p = 0.42$). Tadpoles in the constant ALAN treatment metamorphosed in an average of 69.56 days, significantly faster than the average time to metamorphosis of 84.33 days for tadpoles in the control group ($t = 9.253$, $DF = 34$, $p < 0.001$; Table 11). Metamorphs from the ALAN treatment weighed marginally less than metamorphs in the control group, (average 7% less, $t = 1.924$, $DF = 0.34$, $p = 0.063$; Table 12). Control group metamorphs had significantly longer SVL than ALAN treatment metamorphs (average of 1 mm, $t = 2.349$, $DF = 34$, $p = 0.0247$; Table 12), though there was no significant difference in body condition ($t = 1.280$, $DF = 34$, $p = 0.209$). After 28 days of exposure to ALAN or a control light cycle, but before exposure to any predator cues, there was no significant difference in the activity level of tadpoles ($t = 0.953$, $DF = 31$, $p = 0.343$). After exposure to predator cues, there was a significant difference in tadpole response activity between treatments ($t = 2.218$, $DF = 31$, $p = 0.034$; Fig. 19) with tadpoles exposed to ALAN not reducing activity after predator cue exposure.

Table 11. Mean ± SE mass, snout-vent length (SVL), tail height (TH), and time to metamorphosis (TTM) of *Rana berlandieri* exposed to either a control light cycle or constant ALAN in a laboratory growth chamber.

	Day 21			Day 49			____ Metamorphs		
Treatment	**Mass (g ± SE)**	**SVL (cm ± SE)**	**TH (cm ± SE)**	**Mass (g ± SE)**	**SVL (cm ± SE)**	**TH (cm ± SE)**	**Mass (g ± SE)**	**SVL (cm ± SE)**	**TTM (Days ± SE)**
Control	1.246 ± 0.048	1.964 ± 0.03	0.948 ± 0.023	2.878 ± 0.108	2.583 ± 0.03	1.287 ± 0.030	1.525 ± 0.034	2.569 ± 0.03	69.56 ± 0.074
ALAN	1.243 ± 0.042	1.991 ± 0.02	1.027 ± 0.018	2.941 ± 0.071	2.561 ± 0.06	1.329 ± 0.034	1.634 ± 0.045	2.691 ± 0.04	84.33 ± 1.41

Table 12. Results of pooled t-tests comparing each response variable of larval *Rana berlandieri* exposed to either constant ALAN or a control light cycle for 49 days in a laboratory growth chamber.

Variable	**d.f**	**t**	***p*-value**
Body Condition	34	1.28	0.209
Snout-Vent Length	34	2.349	0.025
Mass	34	1.924	0.063
Time to Metamorphosis	34	9.253	< 0.001

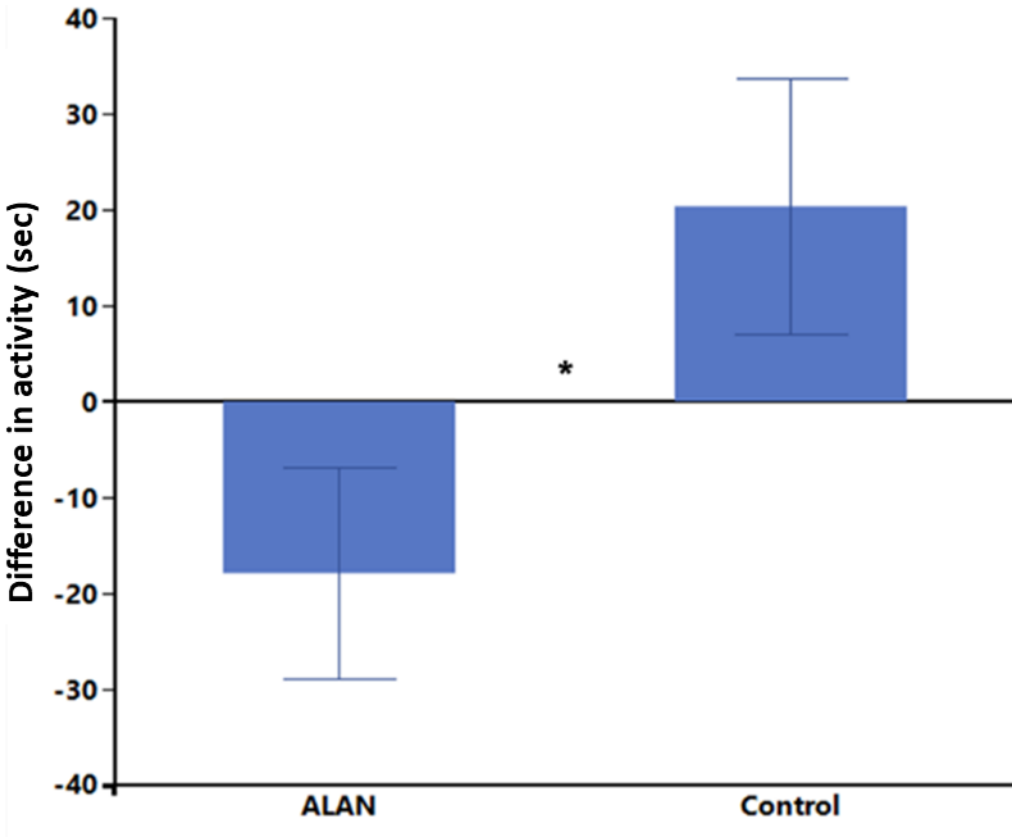

Figure 19. Mean difference in activity (post exposure time subtracted from pre-exposure time; sec ± SE) for *Rana berlandieri* tadpoles exposed to constant ALAN or a control light cycle for 28 days. Asterisk indicates significant difference.

Discussion

Organisms are often exposed to a suite of anthropogenic and natural stressors at one time, therefore I explored if there were any synergistic effects of an anthropogenic stressor, 200 lx ALAN, and a natural stressor, predator presence, on the physiology and growth of a model organism, the Rio Grande leopard frog, *Rana berlandieri*. Tadpole growth was not affected by the presence of dragonfly larvae or ALAN. Tadpoles exposed to ALAN and predator presence together (ALAN + predator) had significantly higher cort release rates on day 7 compared to all other treatments and had significantly higher cort release rates on day 14 compared to control tadpoles. Elevated cort levels are associated with natural and anthropogenic stressors in larval amphibians (Gabor et al., 2019;

Middlemis Maher et al., 2013). In response to agitation, tadpoles in all but the control treatments downregulated their cort release rates. Tadpoles also weighed less and metamorphosed quicker when exposed to ALAN. Together, these results indicate that exposure to ALAN, especially synergistically with a predator comes at a fitness cost to *R. berlandieri.*

Elevating circulating corticosterone is a way to mediate effects from the stressful conditions and suppression of the HPI-axis is a way to cope with additional stressors and to avoid deleterious effects of further elevated cort levels, as modulating hormonal responses help mediate behavior and physiology in changing environments (Nelson, 2011; Romero et al., 2009; Bókony et al., 2020). In response to agitation, unlike the control, tadpoles in all other treatments (predator, ALAN and ALAN + predator) showed significant downregulation of cort release rates. This could be indicative of a suppressed HPI axis, and can be deleterious, leading to homeostatic failure (Romero et al., 2009) or it could be a way for amphibians to modify their response to environmental stressors while avoiding the cost of early metamorphosis because elevated cort is associated with hastened metamorphosis (Denver et al., 1998). If metamorphosis is hastened by elevated cort released due to ALAN and or predator presence, then decreasing the stress response may minimize the rate at which tadpoles metamorphosize early. Indeed, tadpoles reared in the laboratory experiment under ALAN for the duration of the larval period did metamorphose faster and were significantly shorter in SVL and weighed marginally less. Though I did not measure cort in the laboratory experiment tadpoles, based on previous experiments (Forsburg et al., in prep) and the mesocosm study, we can assume that the laboratory tadpoles in the ALAN treatment had higher cort levels.

The elevated cort release rates after 14 days of exposure and the decrease of cort release after agitation experiment observed in tadpoles from the ALAN + predator treatment supports the hypothesis that ALAN and stress from predator presence acts synergistically. This is not surprising, as stress from predator presence acts synergistically with other forms of pollution (Burraco & Gomez-Mestre, 2013; Relyea & Mills, 2001; Relyea et al., 2004; Relyea, 2005), resulting in more severe effects than if either stressor was endured alone. While tadpoles were only exposed to treatments in mesocosms for 14 days, it would be useful to exposure tadpoles until metamorphosis. Cope et al. (2020) exposed tadpoles to 3 lx ALAN and found no significant effects on cort levels in tadpoles, however they did find a carry-over effect, as juveniles exposed to ALAN during the larval period had higher cort levels compared to the control group. If tadpoles were exposed to treatments for the duration of the larval period, I may have observed negative effects on growth, and time to metamorphosis, as I found in the laboratory experiment (and possibly mortality). These results strongly indicate that ALAN is acting synergistically with predator presence, therefore ALAN may also be acting synergistically with other biotic and anthropogenic stressors contributing to amphibian declines.

I also explored whether long-term exposure to 190 lx ALAN affected growth, development, and anti-predator behavior in tadpoles in a laboratory setting. There was no treatment effect on either tail height or body condition after 49 days of ALAN exposure, which is opposite of what was predicted. While I did not measure corticosterone in these tadpoles, *R. berlandieri* tadpoles exposed to the same intensity of ALAN showed elevated corticosterone levels in a previous study (Forsburg et al., in prep) and higher cort

levels are associated with deeper tails in larval amphibians (Gabor et al., 2019; Glennemeier & Denver, 2002; Middlemis Maher et al., 2013). Tadpoles exposed to ALAN were marginally smaller at metamorphosis, had significantly shorter snout-vent-lengths, and metamorphosed significantly faster than control tadpoles. These results are congruent with what has been observed in *Rana pipiens* (Eichler & Grey, 1976) and *Bufo americanus* (Dananay & Benard, 2018) reared with ALAN. Reduced mass at metamorphosis reduces juvenile survivorship (Chelgren et al., 2006; Denver et al., 1998; Semlitsch et al., 1988), hinders growth later in life, and lowers probability of survival of juvenile and adult amphibians (Crespi & Warne, 2013; Denver 2009; Rohr et al., 2013). ALAN exposure significantly reduced anti-predator behavior (reduction of movement) in experimental tadpoles, which is not advantageous. Exposure to another anthropogenic stressor, the herbicide RoundUp, also reduces anti-predator behavior in wood frog tadpoles, *Rana sylvatica*, (Moore et al., 2015). Additionally, exposure to endogenous corticosterone inhibits the anti-predator response of *Rana clamitans* tadpoles (Fraker et al., 2009), and as discussed above, the tadpoles exposed to ALAN in this study likely had higher cort levels based on previous findings (Forsburg et al., in prep). These results suggest that constant ALAN exposure during the duration of larval period have negative fitness consequences for amphibians via lower growth and reduced ability to avoid predators.

The results of the mesocosm and long-term laboratory study further support the hypothesis that ALAN is a threat to larval amphibians. Exposure to constant ALAN during the entire larval period hastened metamorphosis and reduced size at metamorphosis in *R. berlandieri* and tadpoles reared with predators and ALAN had

elevated cort levels and a suppressed HPI-axis. Though ALAN exposure did not lead to mortality, potential carry-over effects could negatively affect fitness or survival later in life (Braun et al., 2013; Charbonnier et al., 2018; Cope et al., 2020; Crespi & Warne, 2013; Crino et al., 2014) and synergism between ALAN and other anthropogenic or environmental stressors could lead to more severe effects (Burraco & Gomez-Mestre, 2013; Relyea & Mills, 2001; Relyea et al., 2004; Relyea, 2005).

Conclusions

There are many factors contributing to stress in free living organisms, and ultimately biodiversity loss, and the results of this study show that long-term exposure to ALAN and synergism between ALAN and a natural stressor may have negative consequences on fitness. Thus, ALAN is a significant threat to biodiversity, particularly in urban areas exposed to ALAN, where individuals and populations must cope with myriad other anthropogenic perturbations and natural stressors. With over 50% and 75% of key biodiversity areas and Global protected area units, respectfully, affected by ALAN (Seymoure et al., 2019), and an estimated yearly increase in ALAN of 6% (Hoelker et al., 2010), the negative impacts of ALAN on amphibians and other taxa (Gaston et al., 2019; Secondi et al., 2020; Seymoure et al., 2019) is far reaching. Since ALAN can exacerbate the effects of other stressors, urban planners should include ways to remove ALAN, or mitigate the effects of ALAN, when building around wildlife areas. Lastly, wildlife managers should include ways to mitigate the effects of ALAN in management plans, particularly for vulnerable species or for areas affected by other anthropogenic perturbations.

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V. CONCLUSION

Short term exposure to ALAN affects corticosterone release rates in two species of larval anurans, long term ALAN exposure affects the anti-predator behavior and growth of *Rana berlandieri* tadpoles, and ALAN acts synergistically with a natural biotic stressor to affect the corticosterone release rates of larval *R. berlandieri*. Gulf Coast toad, *Bufo valliceps,* and Rio Grande leopard frog, *Rana berlandieri*, tadpoles exposed to ALAN for 14 days showed either suppressed/down-regulated or elevated corticosterone release rates compared to cort release rates of tadpoles reared under a control light cycle. The differences in cort response to ALAN observed in these experiments could be species specific. Gulf Coast toads have a much shorter larval period than Rio Grande leopard frogs, and thus, Gulf Coast toad tadpoles could be down regulating cort production to additional stressors to avoid deleterious effects of persistently elevated cort levels. Further investigation of how ALAN affects other species is warranted, as ALAN may be more detrimental to habitat specialists or more vulnerable species, and less detrimental to habitat generalists or common/widespread species. I suggest additional studies exposing more species and genera starting at the egg stage and for the duration of the larval period, making sure that similar Gosner stages are compared between species. Exposure to ALAN for the duration of the larval period of *R. berlandieri* accelerates metamorphosis and reduces anti-predator behavior of tadpoles. Further, tadpoles reared in mesocosms with caged predators and ALAN showed significantly higher cort release rates compared to tadpoles reared with either perturbation alone or control tadpoles.

The results of these experiments show that ALAN is a stressor for both Rio Grande leopard frog, *Rana berlandieri*, and Gulf Coast toad, *Bufo valliceps,* tadpoles,

ALAN may have negative fitness consequences, and contribute to our understanding of how ALAN affects amphibians. Persistently elevated or down regulated cort levels can be deleterious (Romero et al., 2009), leading to death, and elevated levels of cort can reduce growth in amphibian larvae and hasten metamorphosis (Crespi & Warne, 2013; Denver et al., 1998; Glennemeier & Denver, 2002a; Hu et al., 2008). Reduced mass at metamorphosis can affect growth and survivorship later in life (Cabrera-Guzman et al., 2013; Chelgren et al., 2006; Crespi & Warne, 2013; Earl & Whiteman, 2015; Rohr et al., 2013) and stressors endured during the larval period in amphibians can have fitness consequences on later life history stages (Charbonnier et al., 2018; Dananay & Benard, 2018; May et al., 2019).

There are many factors contributing to stress in free living organisms, and ultimately biodiversity loss, thus, ALAN is a significant threat to biodiversity, particularly in urban areas exposed to ALAN, where individuals and populations must cope with other anthropogenic perturbations and natural stressors. With over 50% and 75% of key biodiversity areas and Global protected area units, respectfully, affected by ALAN (Seymoure et al., 2019), and an estimated yearly increase in ALAN of 6% (Hoelker et al., 2010), the negative impacts of ALAN on amphibians and other taxa (Gaston et al., 2019; Secondi et al., 2020; Seymoure et al., 2019) is far reaching. If ALAN severely reduces amphibian populations, there is likely to be further effects of ALAN on community structure and trophic dynamics, as amphibians control algae in aquatic ecosystems, and anurans at all stages are prey items for many other taxa. Additionally, if ALAN is negatively affected amphibians in an ecosystem, it is most likely negatively affected other taxa. Further, since ALAN can exacerbate the effects of

other stressors, urban planners should include ways to remove ALAN, or mitigate the effects of ALAN, when building around wildlife areas. Lastly, wildlife managers should be aware that ALAN may be negatively affecting amphibian populations, either directly or indirectly by impacting community assemblages, and should include ways to mitigate the effects of ALAN in management plans, particularly for vulnerable species and for areas affected by other anthropogenic perturbations.

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