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EVALUATING THE IMPACT OF TEMPERATURE AND ARTIFICIAL LIGHT AT
NIGHT ON THE GROWTH, SWIMMING PERFORMANCE, AND
CORTICOSTERONE LEVELS OF SOUTHERN LEOPARD FROG (*LITHOBATES*
SPHENOCEPHALUS) TADPOLES

by
Skylar Grace Alexander

A thesis submitted to the faculty of The University of Mississippi in partial fulfillment of
the requirements of the Sally McDonnell Barksdale Honors College.

Oxford
May 2023

Approved by

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Dedicated to my late grandmother, Bebe - my biggest fan and encourager. Thank you for my educational opportunities. I love and miss you very much.

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Abstract

Anthropogenic activities caused by urbanization have considerably altered our world's ecosystems which has led to a global amphibian population decline. Encroaching and unyielding urbanization by humans has contributed to a rise in artificial light at night due to light pollution as well as increased temperature due to climate change. Both rising temperatures and artificial light at night (ALAN) have been shown to individually have negative effects on amphibian physiology such as increased stress and decreased behavior. However, due to the drastic nature of human imposition, amphibians typically encounter these anthropogenic stressors in combination. Therefore, it is important to understand how both individual and multiple stressors impact amphibian physiological responses and fitness so that we can predict how future populations will be affected and improve conservation efforts. To determine the relationship between anthropogenic stress and physiological fitness, we designed an experiment that exposed 60 southern leopard frog (*Lithobates sphenoccephalus*) tadpoles for three weeks to elevated temperatures and artificial light at night both individually, combined, and with a control of normal temperatures and light/dark conditions. We then determined their predator evasion abilities by recording their swimming performance via turn-around time and burst swimming speed with a high-speed go-pro camera and a simulated predator. We then determined their total stress hormone levels by performing a whole-body corticosterone (CORT) assay using an ELISA to acquire the corticosterone concentrations within each tadpole. We found that neither ALAN nor increased temperature had any significant

impact on either turn-around time or burst swimming speed ($P>0.05$). However, we found that ALAN caused an increase in whole-body corticosterone concentrations, but increased temperature had no effect. Finally, we found that increased temperature significantly decreased body mass, and increased temperature combined with ALAN marginally decreased body mass. Overall, we found that an increase in temperature had a stronger negative impact on growth, while ALAN increased whole-body corticosterone. However, swimming performance was largely unimpacted after three weeks of exposure to both stressors. An increase in whole-body corticosterone levels with only three-weeks of exposure to ALAN may indicate that longer-term exposures could be more detrimental to larval amphibians.

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Introduction

Our ecosystems have been drastically altered by anthropogenic activities, causing a substantial decline in biodiversity worldwide (Jackson *et al.*, 2016; McKee *et al.*, 2003). In part due to their physiology, amphibians are unfortunately at the forefront of biodiversity loss. In 2013, amphibian population loss was estimated to be around 41% coupled with around one-third of amphibian species endangered and facing extinction, and this number is currently steadily rising (Stuart *et al.*, 2004; Hoffmann *et al.*, 2010, Whittaker *et al.*, 2013). These drastic declines are concerning as amphibians are widely considered indicator species for overall ecological health, and thus may highlight more serious environmental issues with possible systematic effects (Welsh and Ollivier, 1998; Estes-Zumpf *et al.*, 2022; Hocking and Babbitt, 2014). Amphibians also play a crucial role in our healthcare system due to their skin secretions that are widely used in pharmaceuticals and antiviral drugs. With the decline of amphibians, we may also lose a broad range of ecosystem services such as insect regulation and nutrient cycling (Hocking and Babbitt, 2014). Amphibians are unfortunately enduring many stressors from anthropogenic activities due to the introduction of invasive species (Nunes *et al.*, 2019), habitat destruction (Fisher and Shaffer, 1996), disease spread (Daszak *et al.*, 1999; Sabin *et al.*, 2020), and fertilizer and pesticide runoff (Baker *et al.*, 2013). Along with these stressors, an increase in urbanization is giving rise to light pollution such as artificial light at night (Guetté *et al.*, 2018) and an increase in temperatures due to climate change (Blaustein *et al.*, 2010). Both artificial light at night and increased temperature

individually have been correlated with an increase in stress and altered behavior in amphibians and reptiles (Touzot *et al.*, 2019; Thawley and Kolbe, 2020; Blaustein *et al.*, 2010) as well as a decrease in growth and development (Forsburg, 2010; Goldstein, 2017; Weerathunga and Rajapaksa, 2020). Given the drastic changes that humans have imposed on ecosystems, organisms are extremely likely to face a multitude of stressors rather than individual stressors. Currently, there is a lack of understanding of how multiple stressors impact amphibians' physiological responses and behavior (Stuart *et al.*, 2004; Butchart *et al.*, 2010; Horner *et al.*, 2017).

Artificial Light at Night (ALAN) as an Anthropogenic Stressor

Due to an ever-increasing human population, our world is becoming even brighter at night. An acceleration in urbanization and economic growth has triggered a rise in the prevalence and density of light pollution in our natural environments due to changes in natural light levels from artificial light at night (Gaston *et al.*, 2013; Longcore and Rich, 2004). Artificial light at night, or ALAN, can be seen in a myriad of ways: car lights, public streetlights, public buildings, commercial lighting, and even light reflections (Kyba *et al.*, 2011). In the last century, it has been estimated that artificial light at night has increased approximately 6% every year globally (Hölker *et al.*, 2010). Despite the idea that the development and widespread use of artificial light at night has significantly improved our quality of life, the benefits of this technological advancement have now been widely acknowledged to have negative impacts on our biosphere (Hölker *et al.*, 2010; Wang *et al.*, 2023). Artificial light at night is essentially the exposure of our natural and semi-natural ecosystems to direct or indirect artificial illumination at night by either

surrounding light sources or artificial skyglow (Bennie *et al.*, 2016). Artificial skyglow in our biosphere is caused by the combined effects of ALAN elevating the luminosity of our nightscapes (Davies and Smyth, 2018; Falchi *et al.*, 2016). Based on data from 2016, it was revealed that 23% of the world and ~50% of the United States' surface had unnatural nighttime light values generated by artificial skyglow (Falchi *et al.*, 2016). As a result, organisms in exposed ecosystems experience an altered natural night period and this can contribute to widespread impacts on the behavior, physiology, and development of different organisms (Gaston *et al.*, 2014; Longcore and Rich, 2004; Swaddle *et al.*, 2015) producing significant ecological ramifications and necessary conservation efforts.

While the literature on ALAN is increasing, few studies have explored how it specifically affects amphibians. Artificial light at night affects the behavior, growth, and physiology of amphibians (Baker and Richardson, 2006; Doren *et al.*, 2017). Artificial light at night altered the breeding periods and decreased fertilization rate in male common (*Bufo bufo*) toads (Touzot *et al.*, 2020) and reduced the number of male northern green frogs' (*Rana clamitans melanota*) mating calls thus impacting recruitment rates (Baker and Richardson, 2006). In a separate study, decreased activity, altered energy allocation, and increased standard energy expenditure have also been observed in adult male common (*Bufo bufo*) toads when exposed to ALAN, but had no effects on body mass (Touzot *et al.*, 2019). Contrarily, wood frog (*Lithobates sylvaticus*) size (Shidemantle *et al.*, 2022) and juvenile American toad (*Bufo americanus*) growth and metamorphosis rate decreased with ALAN (Dananay and Bernard, 2018). Rio Grande leopard frog (*Rana berlandieri*) tadpoles were exposed to ALAN and subsequently experienced elevated corticosterone release rates (Forsburg *et al.*, 2021). ALAN was also

observed to impact the expression of genes linked to the innate immune system in male common toad (*Bufo bufo*) tadpoles (Touzot *et al.*, 2021). By examining larval amphibian's behavioral and physiological responses to ALAN, we can begin to understand their susceptibility to environmental stressors and overall well-being (Wikelski and Cooke, 2006). Similarly, ALAN increases stress-mediated glucocorticoid levels (Forsburg *et al.*, 2021), and chronically elevated CORT levels have been linked to decreased size at metamorphosis in larval amphibians (Kirschman *et al.*, 2017; Glennemeier and Denver, 2002). An increase in glucocorticoid levels can have many negative ramifications such as the decreased ability to respond effectively to other environmental stressors including increased temperature and predation (McCormick and Romero, 2017) and a decreased mass at metamorphosis can lead to lower survival rates (Székely *et al.*, 2020). Overall, the impact of ALAN on the behavior, growth, and physiology of amphibians is complex and varies among different species and life stages.

Increased Temperature as an Anthropogenic Stressor

Among the many environmental factors that could potentially have a large impact on the physiology and behavior of amphibians, temperature has garnered considerable attention, especially in the context of global climate change. Amphibians are ectothermic organisms which means they rely on surrounding environmental conditions to internally regulate their body temperature and thus changing temperature can pose significant ramifications (Zuo *et al.*, 2012). Climate change is affecting biodiversity and the functionality of ecosystems globally due to increasing temperatures and overall climate variability (Weiskopf *et al.*, 2020). Climate change can induce more extreme temperatures such as

warmer winters, hotter summers, and decreased transitions between seasons resulting in drastic implications for organisms (McMenamin *et al.*, 2008). An increase in temperature due to climate change can have various effects on organisms due to species specific variability and the scope of temperature change. However, an increase in temperature has been widely researched among various organisms and reveals direct and indirect implications on metabolic rate (Hawkins, 1995; Mehdi, 2019; Zhu *et al.*, 2023), migration (Cattaneo and Peri, 2016), physiological stress (Easterling *et al.*, 2000), reproductive success (Somero, 2010), and interactions among species (García *et al.*, 2018).

Many larval amphibians are susceptible to climate change via the effects of warming and drying on their aquatic environments (for a review, see Edge *et al.*, 2016; Shoo *et al.*, 2011). Stressors experienced during this critical life stage could have large scale implications for population viability. An increase in temperature impacts larval amphibian's immune function which influences disease dynamics and increased susceptibility (Bradley *et al.*, 2019; Brand *et al.*, 2016). Furthermore, higher temperatures can negatively affect larval growth, particularly by significantly delaying their development and decreasing morphometrics of total body length, snout-vent length, tail length, and body width (Weerathunga *et al.*, 2020). The growth and survival of larval amphibians to metamorphosis is crucial for reproductive success and overall population fitness (Denver, 1997). Longer larval periods increase the risk of predation and death by desiccation due to pond drying (Crump, 1989). Chronically elevated corticosterone levels associated with decreased size have also been positively linked to frequent high temperature exposure in larval amphibians, which can have overarching effects on

survival when combined with additional stressors (Blaustein *et al.*, 2012). In Sri Lankan whipping frog (*Polypedates cruciger*) tadpoles, elevated temperatures significantly delayed development and growth, reduced swimming speed, decreased white blood cell counts, and resulted in several developmental deformities (Weerathunga and Rajapaksa, 2020). Thus, the impacts of increased temperature due to climate change could greatly affect the survival and fitness of larval amphibians.

Impact of Anthropogenic Stressors on Corticosterone Levels

In response to external stressors in their changing environment, organisms can alter their physiological responses in order to cope with stress and preserve homeostasis (McEwen and Wingfield, 2003; Touzot *et al.*, 2022; Hau *et al.*, 2016). Organisms can moderate physiology, behavior, and morphology in response to variation within their environment by modulating their hormonal responses (Nelson, 2011). The hypothalamo-pituitary-interrenal (HPI) axis regulates the production and release of corticosterone (CORT) in amphibians. This HPI axis also plays an important role in the regulation of amphibian metamorphosis, and CORT can profoundly impact development (Denver, 2021). CORT is the main glucocorticoid stress hormone in amphibians that is responsible for the physiological and behavioral responses to external stressors (Denver, 2009). Circulating CORT levels often become temporarily elevated above “normal” baseline levels when acute stressors are present and can alter behavior and physiology by decreased foraging and mating and by modifying energy allocation in order to maintain energy needs (Sapolsky *et al.*, 2000; Romero *et al.*, 2009; Hau *et al.*, 2016). When chronic stressors are present, a sustained CORT elevation can cause organisms to ineffectively counter further

challenges imposed by the environment (Romero *et al.*, 2009). An elevation in baseline CORT levels is correlated with anthropogenic stressors such as environmental pollution, invasive species, habitat degradation, and alterations of temperature in the atmosphere (Gabor *et al.*, 2015; Blaustein *et al.*, 2012). Chronic CORT elevation results in reduced growth, modified morphology, and accelerated metamorphosis in larval amphibians (Forsburg *et al.*, 2020; Crespi and Warne, 2013; Denver *et al.*, 1998). Reduced growth and modified morphology at early metamorphosis can lead to increased predation risk and even survivorship for larval amphibians (Crespi and Warne, 2013).

Impact of ALAN and Increased Temperature on Swimming Performance and Predator Evasion

Anthropogenic stressors, such as ALAN and increased temperature, can influence the swimming performance of larval amphibians and ultimately reduce fitness (Crespi and Warne, 2013; Watkins, 2000). An organisms' ability to swim is a crucial factor for its survival in an aquatic environment as it affects factors such as predator evasion, reproduction, and food capture (Gui *et al.*, 2014; Ohlberger *et al.*, 2006). The physiological aspects of swimming performance in many species have received a majority of attention primarily in the realm of increased temperature (Heuer *et al.*, 2021), corticosterone levels (Lipowska *et al.*, 2019), morphological effects (Rouleau, *et al.*, 2010), and behavior (Dabiri *et al.*, 2010). It is important to consider the impact of environmental stressors on the early life stages of organisms as any changes in survival can amplify repercussions at the population level (O'Connor *et al.*, 2014).

Swimming performance in larval amphibians can be measured in turn-around time and burst swimming speed. Burst swimming speed is a measure of the maximum speed a tadpole can reach over a short distance. Burst swimming speeds are generally exhibited during startle behaviors during times of predator evasion (Fleming and Bateman, 2015; Dayton *et al.*, 2005). Both turn-around time and burst swimming speed are important measures of tadpole performance and survival. By measuring swimming performance under different conditions in the laboratory, we can experimentally assess the impacts of various stressors on this fitness-related trait (Watkins, 1996; Dayton *et al.*, 2005).

Studies have shown that ALAN increases predator abundance and swimming performance of organisms and therefore predation risk (Becker *et al.*, 2013). Although we have data on how ALAN affects the swimming performance of other organisms, there is limited research on how ALAN specifically impacts the swimming performance of tadpoles. A laboratory study performed by (May *et al.*, 2019) exposed wood frog (*Lithobates sylvaticus*) tadpoles to three different light treatments: a natural light-dark cycle, intensified daytime illuminance, and ALAN. They found that the tadpoles in the ALAN treatment had a significant reduction in overall swimming behavior and activity levels when compared to the other two treatments. This research suggested that a reduction in swimming activity in tadpoles may be related to the disruption of tadpoles' circadian rhythms and their ability to respond properly to predator cues. In order to regulate their circadian rhythms, amphibians primarily rely on light cues and natural light/dark cycles from their environment. Disruptions of these light/dark cycles by ALAN affects the natural circadian rhythms of amphibians and alters their behaviors,

physiological processes, and growth which are all critical for survival and evasion of predators (Fonken and Nelson, 2014; Falchi *et al.*, 2016).

Considering that swimming performance often serves as a whole-body indicator of survival because it reflects smaller scale physiological changes (Binning *et al.*, 2017), it is unsurprising that increasing temperature strongly impacts larval amphibians' swimming performance as well (Kent and Ojanguren, 2015; Wilson *et al.*, 2000; Watkins, 2000). One such study found reduced common hourglass tree frog (*Polypedates cruiger*) tadpole activity, in terms of their swimming speed, when exposed to elevated temperatures (Weerathunga and Rajapaksa, 2020). Tadpoles are ectotherms which means their body temperature is regulated by the temperature of their environment. Generally, swimming performance will increase with increasing acute temperature exposure. Once the temperature of the water becomes too high, however, it will negatively affect swimming performance, as seen in multiple studies. In a laboratory experiment, wood frog (*Rana sylvatica*) tadpoles had a decreased burst swimming speed at increased temperatures (Watkins and Vraspir, 2006). An additional laboratory experiment found that Pacific chorus frog (*Ambystoma macrodactylum*) tadpoles had altered activity and locomotor performance in warmer temperatures and these behavioral modifications resulted in increased predation (Jara *et al.*, 2019). These findings suggest that if temperatures increase above an optimal range, tadpoles can become stressed and elicit modified activities causing a decrease in tadpole swimming performance. Overall, the impact of increased temperature on different aspects of tadpole swimming performance is complex and highly dependent on species-specific thermal biology.

Specific Scope

Due to the continual detrimental effects of urbanization and anthropogenic activity on our world's ecosystems, amphibians are most likely to endure multiple human-induced stressors at once. It is important to understand how individual and multiple stressors impact the physiological responses and overall fitness of amphibians currently and for future populations. In this study, we set out to understand how multiple stressors, both individually and combined, might impact whole-body corticosterone levels and burst swimming performance in southern leopard frog (*Lithobates sphenoccephalus*) tadpoles. Specifically, we experimentally investigated the impacts of increased temperatures and artificial light at night (ALAN) on tadpoles in controlled environmental chambers over a three-week exposure period using a 2x2 factorial design. Then, we tested burst swimming performance, which related to amphibian predator evasion behavior (Watkins, 1996; Dayton *et al.*, 2005), and measured whole-body corticosterone (CORT) values as an indicator of stress in these animals. We hypothesized that the tadpoles exposed to the combined environmental stressors of increased temperature and ALAN will have an increase in corticosterone levels and a decrease in burst swimming performance, therefore decreasing their ability to evade predators. Amphibians are currently in a state of global decline. By investigating how individual and multiple stressors impact larval amphibian physiology and performance, we can further understand and provide insight into the effects of anthropogenic pollution on critical life stages.

Methods

Collection and Setup

We collected 60 southern leopard frog (*Lithobates sphenoccephalus*) tadpoles from the University of Mississippi Field Station and Center for Water and Wetland Resources located in the southeastern region of the United States (34.4324° N, 89.3904° W). In order to reduce stress and prepare for experimental trials, the tadpoles were housed together for a week at the University of Mississippi Field Station and received 30% water changes each day until a full water change of dechlorinated tap water took place.

Tadpoles were transported to the University of Mississippi and housed individually in 2.1L plastic containers filled with 1.5L of dechlorinated water in order to reduce competition. We used four Conviron GEN 1000 controlled environmental chambers to allow for the programming of specific temperature settings and light conditions. Fifteen tadpoles were placed into each chamber, and then allowed one week to acclimate before experimental treatments began. The tadpoles were fed every other day with rabbit chow and pleco wafers and their containers were routinely cleaned and received chemically-aged water changes. All experiments were conducted with the approval of the University of Mississippi IACUC committee (22-001) and the Mississippi Dept. of Wildlife, Fisheries, and Parks (0103222).

Experimental Design

We designed a 2x2 factorial experiment to test the singular and combined effects of increased temperature and ALAN on tadpole performance and physiology. Each

treatment experienced either average fall maximum temperatures for Oxford, MS (25°C) or elevated maximum temperature (27°C) based on the 2050 climate projection for the southeastern region of the United States (obtained from the 2022 IPCC report). In addition, each treatment experienced either a 12 h light:dark cycle or ALAN. Daytime light levels were 2000 lux in all treatments, while overnight light levels were 100 lux in the ALAN treatments only. The temperatures in the chambers fluctuated from 25°C-10°C or 27°C-12°C to represent daily temperature fluctuations in Oxford, MS (obtained from NOAA). ALAN levels were selected to represent natural conditions in local urban areas, in which 100 lux was the average amount emitted by lighted parking lots, homes, and urban glow (Gaston *et al.*, 2013).

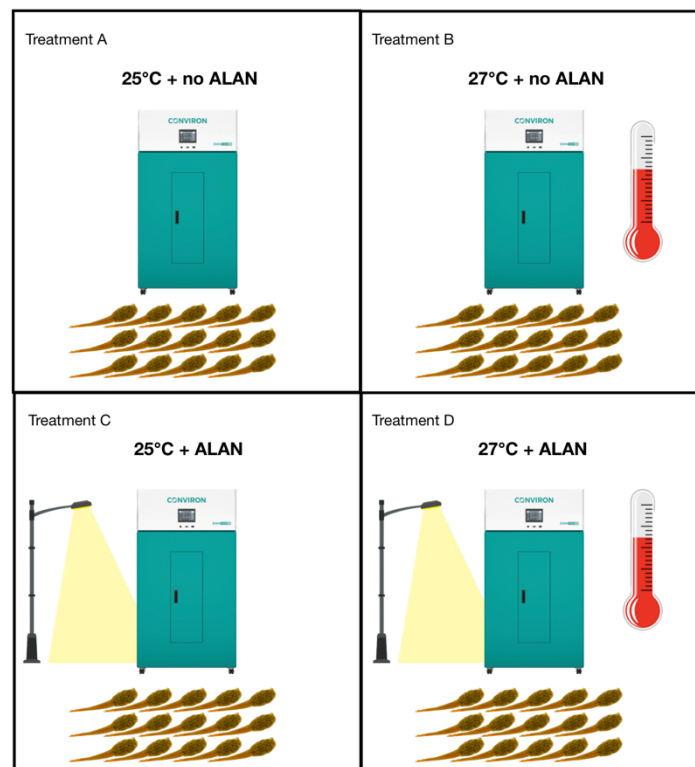


Figure 1. 2x2 factorial design of experimental treatments with artificial light at night and increased temperature.

Tadpole burst and swimming speed

Tadpole swimming performance trials occurred after 14 and 21 days of exposure to the temperature and ALAN treatments. To begin, tadpoles were placed individually in a 13" x 16.5" x 6.5" plastic container filled with water from their and their respective treatment to a 3 cm depth. Each tadpole was then allowed 30 seconds to acclimate to the new environment before we began the trial. A 1 cm x 1 cm grid was placed under the plastic arena and a high-speed Go-Pro camera was used record turn-around time and the burst speed 3cm after the turn-around occurred (Watkins, 1996; Watkins and Vraspir, 2006; Van Buskirk and McCollum, 2000; Bridges, 1997). We used a blunt probe to simulate a predator by lightly tapping the nose of the tadpole to initiate a response. Each tadpole was tapped up to 3 times in succession to ensure there was successful video footage of the turn-around response. We then weighed and measured the tadpoles after each trial. We used ImageJ to measure the turn-around time and time traveled for 3cm post turn-around for each tadpole. We converted the frame rate to 0.0083 seconds global and set the scale according to the grid under the arena. The turn-around time was measured as the time it took the tadpole to turn 180° away from the probe and straighten its body. The time it took to travel the first 3 cm after the turn-around was measured from the tip of the nose when it began swimming to the tip of the nose at the stopping point of 3 cm. After our swimming performance trials, the tadpoles were humanely euthanized in MS-222 and stored at -80°C for future whole-body corticosterone measurements.

Whole-body corticosterone measurements

To measure corticosterone, we used a whole-body CORT assay protocol developed for use in tadpoles (McMahon *et al.*, 2017, with modifications by Dr. Rebecca Cramp, University of Queensland, pers. comm.). To extract CORT from the tadpole tissues, we placed tadpole carcasses along with two steel beads in 2mL tubes on dry ice for ~30 min. Frozen tadpoles were reweighed in order to use this measurement as a covariate in our analyses. We added 1.3mL of ice cold 0.01M PBS to the 2mL tubes and homogenized the samples for 14 minutes at max oscillation speed (50 1/s) using a QIAGEN bead mill homogenizer. The samples were then centrifuged at 3000 rpm for 5 minutes. A 1mL subsample of the homogenate was extracted and placed into an 8 mL glass vial.

We then added a 3mL cocktail of 7:3 ethyl ether-petroleum ether and vortexed for 3 minutes. The samples were allowed to settle and were placed at -80°C for 15 minutes in order to freeze the aqueous layer. After 15 minutes, the solvent layer was poured off into a clean glass test tube and the process was repeated again by adding an additional 2mL of 7:3 ethyl ether-petroleum ether to each sample, freezing the aqueous layer, and pouring off the solvent layer into the same test tube. The samples were then evaporated at 40°C under a gentle stream of nitrogen gas under a fume hood. Afterwards, the dry samples were sealed with parafilm and placed at -20°C until the ELISA CORT assay could be conducted (within 24-48 h).

In order to test for extraction efficiency, we created four extra 1 ml samples from leftover pooled tadpole homogenate. We then added 1 ml of activated charcoal to each efficiency sample and vortexed every 15 minutes for an hour to remove all hormones from the sample. After 1 hour, we centrifuged the samples at 2200 rpm for 10 minutes.

The homogenate was then transferred to 8 ml glass vials and either spiked with a known amount of CORT standard, or not spiked to determine any non-specific binding.

In preparation for the ELISA assay, we re-suspended all samples in 250 μ l extraction buffer (Assay Buffer 15, ENZO Corticosterone Kit) and ran all samples, efficiency samples, and standards in duplicate following the manufacturer's guidelines (ENZO Corticosterone Kit). The optical density was read on a plate reader (BioTek) at 405 nm, with a blank correction at 590 nm, and we determined CORT concentration in samples using a standard curve.

Statistical Analyses

In order to maximize the duration of exposure to the temperature and ALAN treatments, I only analyzed the second round of swimming performance trials. All statistical analyses were completed using R version 4.1.2 in R-Studio (2023). I analyzed the change in mass (g) and total length (TL, mm) using separate linear models, with treatment as the independent variable. I compared whole-body corticosterone (pg/mg) values using a linear mixed model with body mass (frozen) in mg as a covariate and plate ID as a random effect. Burst and swimming speed (s) were first log transformed due to non-normality, then compared across treatments using separate linear models, with log-transformed mass (g) included as a covariate. Finally, I analyzed the relationship between burst speed or swimming speed and whole-body corticosterone using a linear model. All models met assumptions for linear models, including normality of residuals and homogeneity of variance

Results

Survival and growth

During the three-week exposure to the temperature and ALAN treatments, there were very few mortalities (n=3), which were evenly distributed across treatments. However, there was a significant difference in change in mass, with the tadpoles from the 27°C + no ALAN treatment demonstrating stunted growth and even mass loss during the experiment compared to the control treatment (Figure 1, $\beta = -0.093$, SE = 0.031, $p = 0.0041$). The 27°C + ALAN treatment had marginally lower growth than the control treatment as well (Figure 1, $\beta = -0.060$, SE = 0.032, $p = 0.066$). There was also lower growth in total length (TL) in the 27°C + no ALAN treatment compared to the control treatment ($\beta = -0.29$, SE = 0.13, $p = 0.024$). Due to the measurement error inherent in measuring small tadpoles, the change in length was negative for some individuals. Given that the measurement error was likely equal across the treatments, we did not remove these negative measurements from our analyses.

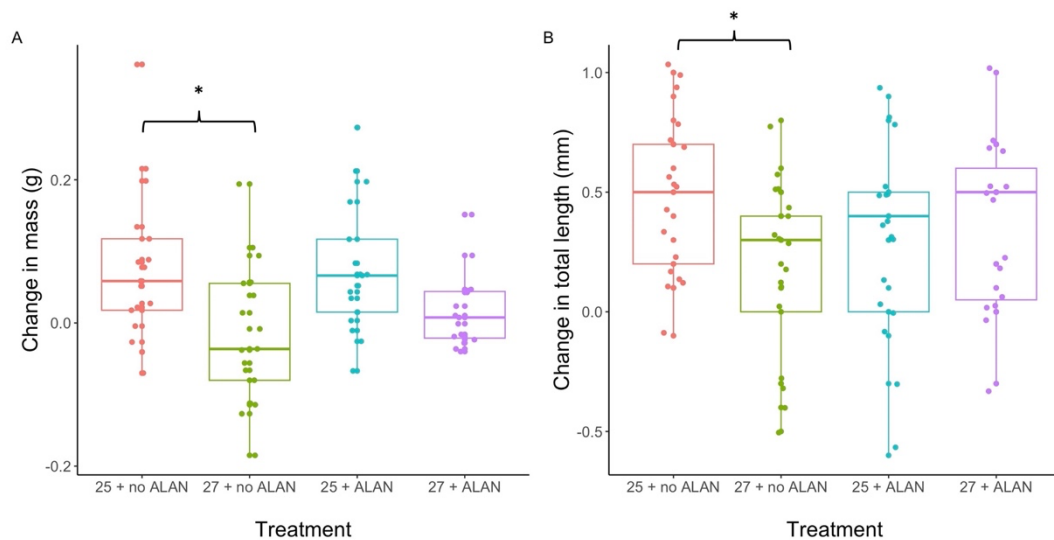


Figure 2. Change in mass (g) (A) and change in total length (mm) (B) of *Rana sphenoccephala* tadpoles after exposure to different temperature and artificial light at night (ALAN) treatments for three weeks. (A) The 27+ no ALAN treatment had a significantly smaller change in mass than the control treatment (25 + no ALAN; $p = 0.0041$). Circles represent individual data points. In the boxplots, the center line is the median, the top and bottom of each box represents third and first quartiles, respectively, and whiskers extend to extreme data points. Sample sizes in all treatments were $n = 17$, with the exception of the 25 + no ALAN treatment, where $n = 15$.

Whole-body corticosterone

There was relatively good concordance between the standard curve and the efficiency samples (Appendix 1, Supplementary Figure 1), with high recovery from CORT-removed

tadpole homogenate. The non-spiked sample indicated that there was about 78 pg of non-specific binding occurring in each sample, which is relatively low. Overall, the no ALAN treatments had significantly lower whole-body corticosterone than the ALAN treatments ($\beta = -266.00$, $SE = 121.56$, $p = 0.034$). The best supported model did not include interactive effects of ALAN and temperature.

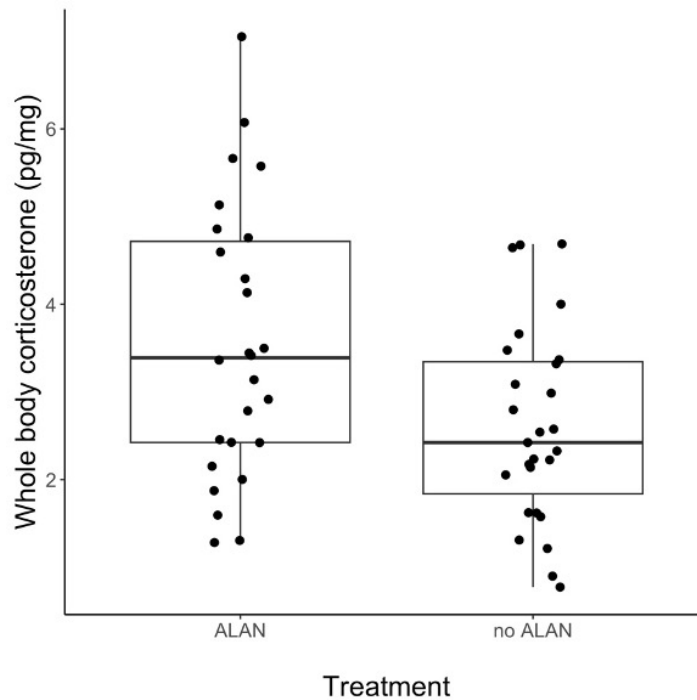


Figure 3. Whole body corticosterone concentrations (CORT; pg/mg) found in *Rana sphenoccephala* tadpoles after three weeks exposure to either 100 lux of artificial light at night (ALAN) or a normal 12 h light: dark cycle (no ALAN). ALAN treatment tadpoles had significantly higher whole-body CORT ($p = 0.034$). Circles represent individual data points. In the boxplots, the center line is the median, the top and bottom of each box represents third and first quartiles, respectively, and whiskers extend to extreme data points. $N = 27$ for each treatment.

Burst and swimming speed

There were no significant differences between the treatments in burst ($F_{3,52} = 0.54$, $p = 0.65$) or swimming speed ($F_{3,52} = 0.43$, $p = 0.73$), nor was there a relationship between whole-body corticosterone and burst ($F_{1,35} = 1.09$, $p = 0.30$) or swimming ($F_{1,35} = 0.03$, $p = 0.86$) speed. There was no relationship between burst ($\beta = -0.023$, $SE = 0.22$, $p = 0.92$) or swimming speed ($\beta = 0.15$, $SE = 0.18$, $p = 0.40$) and body mass.

Discussion

Anthropogenic activities due to urbanization are contributing to widespread amphibian decline within our ecosystems. Larval amphibians are often exposed to a multitude of anthropogenic stressors in their natural environment which could have large-scale implications on their fitness. Therefore, the purpose of this study was to examine the effects of artificial light at night (ALAN) and increased temperature both individually and combined on southern leopard frog (*Lithobates sphenoccephalus*) tadpoles' growth, swimming performance, and whole-body corticosterone levels. I found that artificial light at night, but not increased temperature, resulted in increased whole-body corticosterone levels after only three weeks of exposure time. Furthermore, increased temperature alone significantly reduced growth in these tadpoles. When combined with ALAN, increased temperature also resulted in a borderline significant reduction in tadpole growth. However, I found that neither ALAN nor increased temperature had any effect on burst or swimming speed. Elevated corticosterone levels also showed no relationship between burst or swimming speed. Overall, these results suggest that even a short exposure to ALAN and increased temperature can impact different aspects of tadpole development and physiology, with elevated corticosterone levels potentially indicating that longer-term exposures to these stressors could lead to negative effects later in life.

Our results demonstrate that exposure to increased temperature, but not ALAN, significantly decreased growth of southern leopard frog tadpoles. Individually, elevated

temperatures decreased overall body mass and total length within our three-week exposure time. We also found that increased temperature in combination with ALAN marginally decreased total body mass in our treatments, but increased temperature had the greatest effect (Figure 2). Size can play a role in whole-body corticosterone measurements and swimming performance (Burraco *et al.*, 2015; Arendt, 2009), but the variation in tadpole size in this study was not a significant factor in determining whole-body corticosterone levels and swimming performance. These results provide valuable insight on how increased temperature due to future climate change may influence larval amphibians. Physiological processes are tightly regulated by environmental conditions and exhibit peak performance within a specific range (Somero, 2011). Because larval amphibians are ectothermic, their body temperature and biological processes are regulated by their environment and require specific environmental conditions for optimum growth and development (Angilletta *et al.*, 2004). Hence, changes in environmental conditions such as increased temperature can have a notable influence on larval amphibians' physiological processes (Weerathunga and Rajapaksa, 2020). Unfavorable environmental conditions can disrupt or slow physiological processes resulting in reduced growth, which can have overarching implications on survival (Foden *et al.*, 2013). Overall metabolism increases with temperature, increasing energetic demands, which can result in reduced growth (Clarke and Fraser, 2004; Neubauer and Anderson, 2019). Although the predicted increase in temperature due to climate change in our experiments may seem small in magnitude (2°C difference), it undoubtedly had appreciable impacts on southern leopard frog tadpole growth as seen in our results.

While we expect increased temperature to have a negative impact on larval growth based on previous studies (Weerathunga and Rajapaksa, 2020), we were surprised that ALAN had only a marginal effect on growth. Our observations of ALAN on tadpole growth contradict those of previous studies where exposure to artificial light at night resulted in decreased growth in amphibians (Shidemantle *et al.*, 2022; Dananay and Bernard, 2018) and larval amphibians (Forsburg, 2020). However, a few studies did find either no effects on body mass (Touzot *et al.*, 2019) and even increased body mass upon metamorphosis when exposed to ALAN (May *et al.*, 2019). Due to differing results of previous studies, it may be that effects are species-, duration-, or intensity-specific. Artificial light at night disrupts circadian rhythms and corticosterone levels in amphibians (May *et al.*, 2019), and within our specific experiment it may not have been a limiting factor in larval amphibian growth as previously expected. Additionally, species specificity could be a reason for a less significant impact of artificial light at night (Russart and Nelson, 2018).

While exposure to artificial light at night and increased temperature increased whole-body corticosterone levels and decreased growth respectively, we found no impact on either burst or swimming speed of southern leopard frog tadpoles. Within our experimental trials, we only tested the initial speed and not the swimming endurance of our larval amphibians. Thus, initial speed may not be as limited by chronic stressors, such as increased temperature (Kern *et al.*, 2015) or ALAN, as endurance. Moreover, our tadpoles were only exposed for three weeks, and longer exposure times might result in a decrease in swimming performance. This idea is congruent with results obtained from Forsburg (2020), where *Rana berlandieri* tadpoles experienced elevated corticosterone

levels after 14 days of ALAN exposure, but there were no effects on anti-predator behavior in relation to swimming performance. However, when they further exposed *R. berlandieri* tadpoles to artificial light at night for 28 days, they saw a significant decrease in anti-predator behavior due to decreased swimming performance. Perhaps some species are more sensitive than others, and this may be related to previous ALAN exposure in a given population.

Our results also indicate that exposure to ALAN is a stressor for southern leopard frog tadpoles. We found that whole-body corticosterone levels were significantly higher in tadpoles exposed to ALAN, while whole-body corticosterone levels were significantly lower in tadpoles not exposed to artificial light at night. These results are consistent with a previous study that exposed *R. berlandieri* tadpoles to ALAN at night for an acute 14-day period. They concluded that ALAN resulted in an increase in corticosterone release rate, indicating that short-term exposure to ALAN can increase the corticosterone levels of tadpoles. Elevated corticosterone levels within larval amphibians are known to be associated with abiotic and biotic stressors (Tornabene *et al.*, 2021; Denver, 2021), and this has been recognized to be a coping mechanism to mobilize their energy reserves to moderate harmful effects of a stressful environment. However, chronic elevated levels of corticosterone due to repeated stressors can lead to a decreased ability and capacity to cope and thus homeostatic overload (Romero *et al.*, 2009). With our exposure time being only three weeks, this indicates that ALAN is an added stressor to larval amphibians. Longer-term exposure to ALAN may lead to more chronic stress and thus extended elevated corticosterone levels in larval amphibians, which may decrease survival and fitness in the long term.

Exposure to increased temperature showed no effect on corticosterone levels although it did decrease the growth of our tadpoles. Ectotherms perform optimally within a range of temperatures, which varies by species and population. There is little information about the impact of elevated temperature on corticosterone levels in larval amphibians, but previous studies have found a positive correlation between increased temperature and corticosterone levels (Narayan *et al.*, 2012; Monroe *et al.*, 2023). While larval amphibians can acclimate to increased temperature, it constrains their ability to cope with further acute stress (Dallas and Warne, 2022). Our results are not congruent with previous studies, but since we used projected future temperature ranges for Oxford, MS during the fall, the elevated temperature may not have been extreme enough to induce stress. Our tadpoles may have also been able to acclimate to the elevated temperature regime after three weeks, in contrast to exposure to ALAN.

Conclusions

Our results demonstrate that ALAN is a stressor for southern leopard frog tadpoles and that increased temperature impacts their growth rate. While our experiment was limited to only a three-week exposure to ALAN and increased temperatures, we still found significant effects on crucial traits linked to fitness in amphibians. For example, amphibian size at metamorphosis, which is related to growth rate as a tadpole, is strongly associated with fecundity in adults (Semlitsch *et al.*, 1988). Also, chronically elevated corticosterone levels can decrease time to metamorphosis at the cost of growth, and even result in immunosuppression, leading to greater susceptibility to pathogens in amphibians (Warne *et al.*, 2011). Future work should investigate the impact of longer-term exposures to ALAN and increased temperature, because these stressors are typically long-term during development. Given global climate change and urbanization, these stressors are likely to only increase for larval amphibians. Interestingly, different stressors resulted in impacts on aspects of growth and physiology in this species. This work indicates that these stressors could potentially pose significant impacts on amphibian populations. Due to amphibians facing a myriad of biotic and abiotic stressors in their natural environments such as predation, chemical pollutants, and habitat destruction, understanding how different anthropogenic stressors, both individually and in combination, is key. This will ensure we can mitigate the negative effects of these stressors and work to prevent additional declines.

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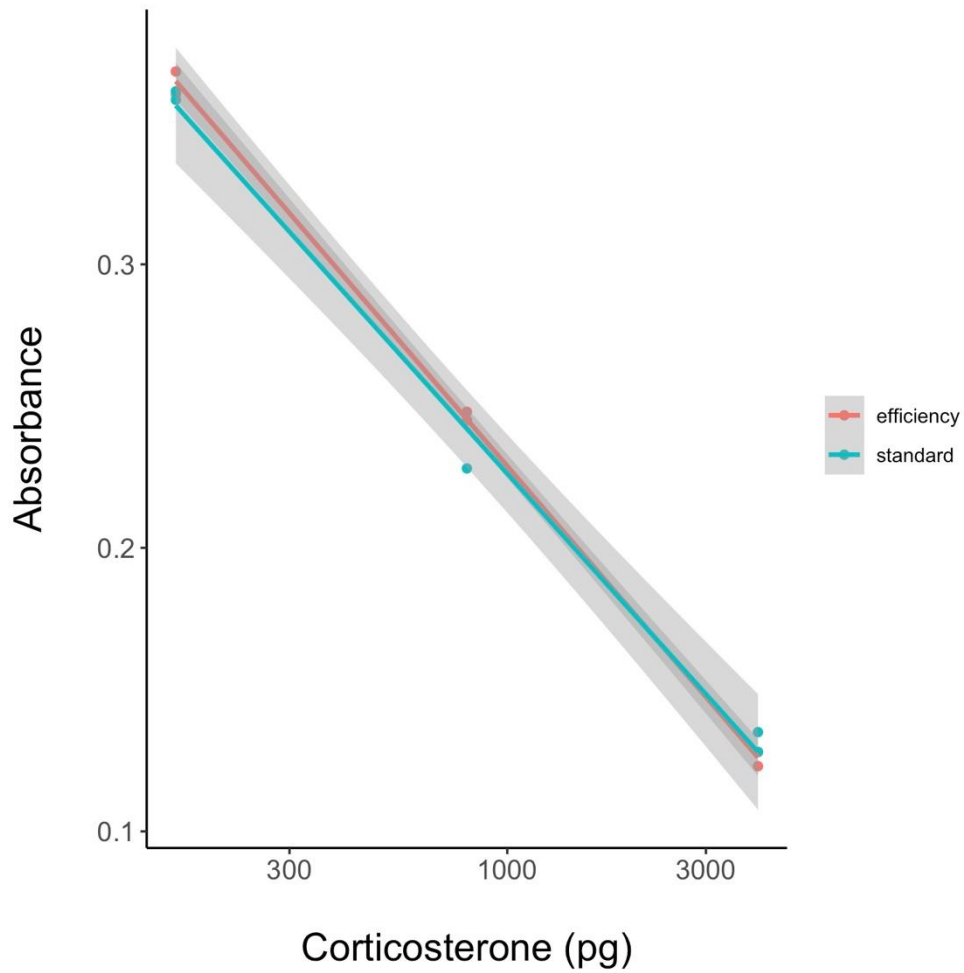
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Appendix 1.



Supplementary Figure 1. Concordance between efficiency samples (pooled tadpole samples spiked with known amounts of corticosterone) and corresponding standard curve samples from the sample plate. Multiple points are replicates (two per efficiency sample or standard).