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The Effects Of Acidic Conditions And Tannins On The Survival, Development, And Behavior Of Cope's Gray Treefrog (Hyla Chrysoscelis)

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THE EFFECTS OF ACIDIC CONDITIONS AND TANNINS ON THE SURVIVAL, DEVELOPMENT, AND BEHAVIOR OF COPE'S GRAY TREEFROG (*HYLA CHRYSOSCELIS*)

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ABSTRACT

As amphibians decline around the world, freshwater acidification resulting from pollutants and acid rain may be a contributor. The ability of organisms to cope with environmental changes is greatly mediated by behavior, and recent studies indicate that anthropogenic acidification impairs behavioral responses by impacting olfactory abilities of aquatic organisms. Responding appropriately to novel stimuli is important for individual performance and survival, and pollutants may cause organisms to behave maladaptively. In this study I sought to: a) determine whether the oviposition site choices of adult female frogs correspond with the pH and tannin conditions that maximize tadpole survival and performance in the laboratory, and b) investigate the impacts of mildly acidic conditions, with and without the added stress of tannins, on the survival, development, and anti-predator behavior of *Hyla chrysoscelis* tadpoles. I conducted a field oviposition study to determine adult female site choice, and reared tadpoles in acidic and tannic conditions to investigate survival and antipredator behaviors. I found that female oviposition site choice did not correspond with conditions that maximize offspring survival. Tadpole mortality was highest in tannic treatments, yet tannic treatments received a high proportion of eggs in the oviposition experiment. Trends in tadpole antipredator behaviors suggested that mildly acidic conditions impaired predator recognition, though this was not statistically significant. My results suggest that tannic conditions reduce tadpole fitness, yet adult females appear to respond maladaptively to elevated tannins by failing to avoid tannic treatments when ovipositing.

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First and foremost, I would like to thank my thesis advisor, Dr. Richard Buchholz, for the patience, guidance, and advice he has provided throughout my time as a student. I have been extremely fortunate to have a supervisor who cared so much about my work and willingly sacrificed his time to ensure my success as a graduate student.

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I. GENERAL INTRODUCTION

As we enter in what many believe to be the sixth mass extinction, amphibians are experiencing dramatic declines worldwide (Wake et al., 2008). Major threats impacting amphibians include climate change, disease, and habitat destruction; however, many amphibian populations also face additional subtle anthropogenic stressors, such as freshwater acidification. Many contaminants can influence aquatic pH including agricultural and industrial wastes, and acid rain (Bhargava and Bhargava, 2013). Acid precipitation is the primary source of freshwater acidification, affects large geographical regions, and remains a serious threat to ecosystems and biodiversity (Driscoll et al., 2001). Globally, acid precipitation remains a threat to aquatic species because some countries lack rigorous emission regulations and enforcement (Driscoll et al., 2001; Greaver et al., 2012). For example, China is suffering intense air pollution caused by anthropogenic emissions of nitrogen and sulfur (Liu et al., 2013). It is anticipated that the continuous increase in emissions of both sulfur and nitrogen will intensify acid precipitation (Wang and Wang, 1995; Streets and Waldhoff, 2000) that could negatively impact amphibians worldwide (Stuart et al., 2004).

Since the US Clean Air Act was implemented, reduced emissions have resulted in decreased acidic precipitation throughout the United States (Likens et al., 1979; Driscoll et al., 2001), but problematic regional deposition trends persist particularly in the Eastern states (Leduc et al., 2008; National Atmospheric Deposition program, 2017). Some ponds in Vermont, for example, remain highly acidic (i.e. pH ~4) (Sherman and Munster., 2012). Though some freshwater ecosystems show signs of recovery after acidification, previous exposure to acid rain can deplete the buffering capacity of aquatic ecosystems making them more vulnerable to future bouts of acid precipitation (Clair et al., 2004; Leduc et al., 2008).

It is perhaps self-evident that heavily acidic conditions (pH <5.0) will negatively affect aquatic organisms (Pierce, 1985; Freda and Dunson, 1986); however, recent studies indicate that weakly acidic conditions (pH >5.9), typically not considered lethal or threatening, can impair the survival of certain fishes by interfering with their ability to detect and respond appropriately to conspecific chemical alarm cues (Brown et al., 2002; Leduc et al., 2008). In aquatic environments, prey animals, including many amphibians, frequently rely on semiochemical signals to reduce the risk of predation (Chivers and Smith, 1998; Ferarri et al., 2010). These chemicals are comprised of multiple components including kairomones and alarm cues which emanate from predators and injured prey, respectively (Ferarri et al., 2010). Potential prey may exhibit anti-predator behaviors, such as hiding, when they detect these chemicals, or may reduce the frequency of behaviors, such as foraging or courtship, that can attract predators (Ferarri et al., 2010). If acidic conditions impact the chemoreception capability of amphibians as they do in fishes, even low levels of freshwater acidification may be harming amphibian recruitment and survival.

Although anthropogenic pollution is the primary cause of freshwater acidification, acidic conditions can also occur naturally due to the introduction of organic acids (Nelson et al., 2011; Dangles et al., 2004; Barth and Wilson, 2010). Unlike anthropogenic acids, including those found in acid deposition events (i.e. nitric and sulfuric acids), organic acids (i.e. humic and tannic acids) occur naturally in soils and plants and are often leached into aquatic ecosystems following both the weathering of adjacent soils and bedrock, and the decomposition of biological materials (Barth and Wilson, 2010). Organic acids are large complex compounds, and their impacts on aquatic organisms are not well understood (Barth and Wilson, 2010). Because certain plants possess high levels of secondary compounds including hydrolysable phenolics (e.g. tannins) that leach into waterways during decomposition it is unclear whether low pH alone or other chemical properties of organic acids increase amphibian mortality (Maerz et al., 2005; Leonard, 2008; Martin and Blossey, 2013). For example, water from acidic bogs increases amphibian mortality above that seen in plain water with a similar pH in laboratory experiments (Freda and Dunson, 1986; Picker et al., 1993). Because plant communities and canopy composition vary greatly across landscapes, nutrient input and secondary chemicals also differ across ecosystems (Niinemets and Tamm, 2005). Several invasive plant species in the United States, including the Chinese tallow tree (*Triadica sebifera*) and purple loosestrife (*Lythrum salicaria*), possess particularly high levels of tannins, and have been linked to local declines in animal populations (Maerz et al., 2005; Leonard, 2008). Conversely, other studies indicate that organic acids may mitigate the negative impacts of acidic conditions and increase survivorship (Barth and Wilson, 2010; Holland et al., 2013; Holland et al., 2014), suggesting that further research is needed to understand how natural organic acids and anthropogenic acidification contribute to amphibian declines.

The goal of my study is to investigate the impact of an ecologically relevant level of acidification (pH=5.5) on oviposition, pre-metamorphic mortality, and larval anti-predator responses of a treefrog, *Hyla chrysoscelis*, with and without a suspected secondary stressor, plant tannins.

II. ACIDIC CONDITIONS AND TANNINS ALTER TADPOLE SURVIVAL, ANTIPREDATOR BEHAVIOR, AND ADULT OVIPOSITION SITE CHOICE OF COPE'S GRAY TREEFROG (*HYLA CHRYSOSCELIS*)

One-third of amphibian species are threatened with extinction, and pollutants are believed to be a major cause of amphibian population declines (Wyman, 1990; Hoffman et al., 2010). Acid rain, a common pollutant, can greatly increase the acidity of aquatic ecosystems, and amphibians are particularly sensitive to acidic conditions (Wake et al., 2008). It is evident that heavily acidic conditions affect amphibian survival, but little research has been conducted regarding ecologically relevant, subtler impacts of mildly and weakly acidic conditions (Åtland, 1998). Furthermore, the impacts of sublethal acidic conditions may be compounded by the presence of additional, naturally-occurring stressors (Sih et al., 2004). Maladaptive developmental and behavioral responses to acidification, with or without other stressors, are likely to precede population declines and thus should be a priority for investigations into the causes of amphibian decline.

Oviposition site selection has tremendous effects on the fitness of many anurans, because parental investment is often limited to the decision of where to lay eggs (Wilbur and Alford, 1985; Resetarits and Wilbur, 1989). Habitat quality frequently governs offspring performance and survival, thus the location where a female chooses to place her eggs can influence reproductive success (Resetarits and Wilbur, 1989). Throughout natural landscapes different habitats possess varying levels of both resources and risks providing a complex mosaic of breeding site options. The importance of oviposition site choice is evident in the response of several species of anurans to certain biotic factors. For example, treefrogs (*Hyla* spp.) increase offspring survival by preferentially laying eggs in habitats with fewer predators and competitors (Resetarits and Wilbur, 1989). No comparable studies have elucidated the role of pH in oviposition site selection by frogs.

The largest impact that acid rain has on amphibians is reproductive failure (Freda, 1986; Carey and Bryant, 1995). The embryonic and larval (i.e. tadpole) life stages of amphibians are almost always aquatic and thus are especially susceptible to acidic conditions (Lefcort et al., 1998). Extreme acidity can cause direct mortality in anurans by preventing hatching and altering ion-regulatory processes (Freda, 1986). Additionally, acidic conditions cause delayed development, and physical deformities in tadpoles (Schlichter, 1981; Carey and Bryant, 1995; Devi et al., 2016) that interfere with metamorphosis and consequently prolong exposure to aquatic predators (Smith, 1987; Brunelli et al., 2009).

Acidic conditions may also reduce the survival of tadpoles that otherwise show normal development by interfering with predator-prey encounters. For example, many amphibians depend on chemical cues, such as kairomones, to detect predators and respond with antipredator behavior that increases their probability of survival (Chivers and Smith, 1998; Ferrari et al., 2010). For anurans, particularly tadpoles, a trade-off exists between activity levels and predator avoidance (Lefcort and Blaustein, 1995; Kiesecker et al., 1996). Chemical alarm cues cause tadpoles to minimize activity when the risk is high, thereby reducing detection by the predator

(Lefcort, 1996). Water acidification can impact chemosensory pathways, thus affecting alarm cue recognition by prey species (Leduc et al., 2007; Leduc et al., 2008; Ferrari et al., 2010; Leduc et al., 2013). Antipredator responses in tadpoles are known to be affected by both pollutants (Lefcort et al., 1998; Polo-Cavia et al., 2016) and water quality parameters including the type and concentration of dissolved solids (Troyer and Turner, 2015), but it remains unknown if the ability of tadpoles to detect predator alarm cues is affected by acidic conditions.

Aquatic ecosystems are complex and often contain numerous stressors including predators, competitors, and diseases. In addition to the impacts caused exclusively by acidic conditions, other stressors may have additive effects and influence the toxicity of acidity on amphibians (Sih et al., 2004). Human activities including habitat modification and the introduction of invasive plant species are altering the type and quantity of plant secondary compounds entering aquatic environments, potentially exposing amphibians to novel chemicals and concentrations that threaten local populations (Leonard, 2008; Martin and Blossey, 2013). For example, exotic plants like the Chinese Tallow tree (*Triadica sebifera*) possess high concentrations of tannins which have been shown to negatively impact amphibian populations by decreasing larval performance and reducing recruitment (Maerz et al., 2005). Tannins, including tannic acids, are large phenolic compounds produced and stored in the leaves and bark of many plant species as a defense against herbivory, and are often leached into adjacent aquatic ecosystems during the decomposition of leaf litter (Maerz et al., 2005). Tannic compounds readily dissolve in water; thus, aquatic organisms may literally bathe in tannins in areas where they are present (Maerz et al., 2005; Leonard, 2008). Somewhat surprisingly, other studies have shown that similar organic acids ameliorate the negative effects of acidic conditions (Barth and Wilson, 2010; Holland et al., 2013; Holland et al., 2014). Because most aquatic ecosystems are heavily influenced by surrounding plant communities, it is crucial to investigate if plant compounds, like tannins, compound or ameliorate the impacts of acidity.

The two objectives of my study were to: a) determine whether the oviposition site choices of adult female *Hyla chrysoscelis* correspond with pH and tannin conditions that maximize tadpole survival and performance in the laboratory and b) investigate the impacts of mildly acidic conditions, with and without tannins, on the survival, development, and anti-predator behavior of tadpoles. I hypothesized that acidification has negative consequences for frogs, especially in the presence of tannins, and thus is contributing to the population decline of amphibians. First, I predicted that acidic and tannic conditions reduce tadpole survival. Second, I predicted that acidic and tannic conditions influence oviposition site selection in adult females; and, if females respond maladaptively by ovipositing in conditions that do not optimize tadpole performance, offspring survival could be reduced.

Cope's gray treefrog (*Hyla chrysoscelis*) is a common subject of ecological study but its susceptibility to contaminants is rarely investigated (Kerby et al., 2010). This species breeds from April through August in the southeastern United States. Females prefer to oviposit in small ponds and ephemeral pools where predators are absent or uncommon (Resetarits and Wilbur, 1989). Because larvae develop quickly (Ritke, 1990) and are sensitive to the presence of predators (Petranka et al., 1987), it is well suited to serve as a model to investigate how acidic conditions impact an amphibian's ability to recognize predator cues.

MATERIALS AND METHODS

Oviposition Site Preference

Five separate blocks were established with each block consisting of 5 plastic wading pools (diameter = 1.5 m, depth = 0.29 m) at the University of Mississippi Field Station (UMFS; 34°250 4″ N, 89°230 32″ W) located near Oxford, Mississippi, USA. Blocks were separated by ≥ 5 m with a total of 25 pools used. In each block, the pools were placed in a straight line, 2 m parallel to the forest's edge with each pool spaced 1 m apart (Fig. 2.1). Contrary to other frog oviposition experiments (Resetarits and Wilbur, 1989; Pintar and Resetarits, 2017), leaf litter was not added to the pools to prevent the addition of naturally occurring tannins found in the leaves. Within each block, four treatments were randomly assigned to the pools: 1) control (C), 2) acidic (A), 3) tannic (T), 4) acidic and tannic (A&T). Control pools contained only well-water and possessed a neutral pH (~7). For the acidic treatments, nitric acid (A200-212, Fisher Chemical) was diluted with well-water to achieve an acidic pH of 5.5 and mimic the impacts of acid rain (McHale et al., 2017). Tannin treatments received 11 mg/L of tannic acid (202425000, Acros Organics), a commercially purified form of tannins, to mimic naturally elevated tannins (Maerz et al., 2005). Lastly, acidic and tannic pools were first treated with 11 mg/L of tannic acid and then treated with nitric acid to achieve a pH of 5.5. The pH of tannic acid treatments did not differ from that of controls.

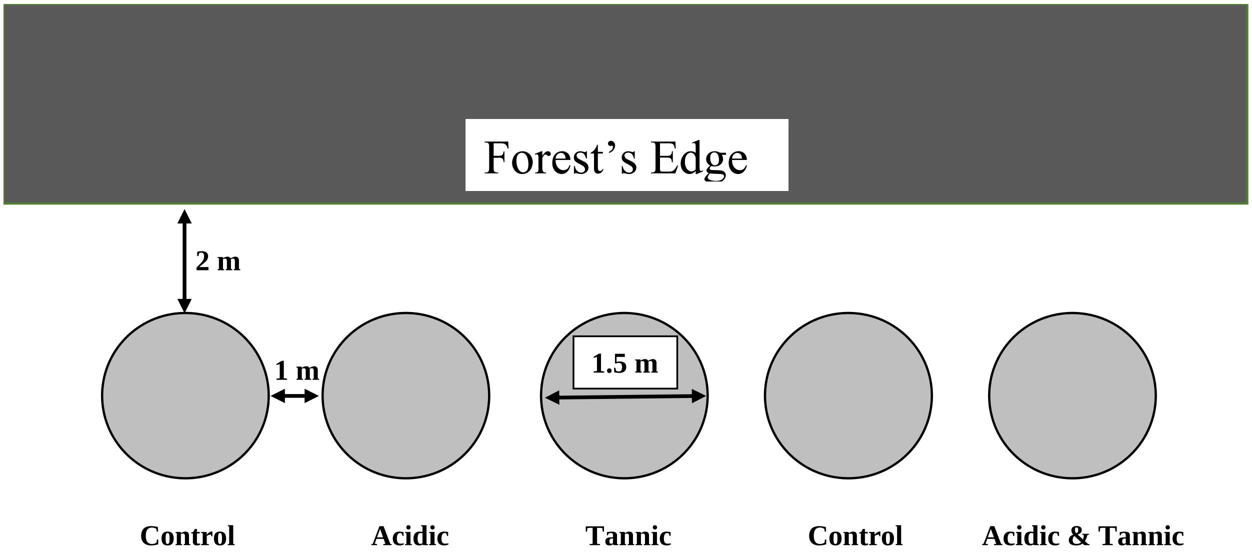


Figure 2.1. Experimental Design for Oviposition Site Choice. This figure portrays a single block in an experiment testing for treefrog oviposition in response to acidic and tannic conditions. Light gray circles indicate wading pools.

All pools were maintained at an equal volume (300 L) with well-water. The pH was monitored daily and adjusted before dark when necessary. Pools were checked for eggs each morning, and all *H. chrysoscelis* eggs were removed and counted by hand at that time. *Hyla chrysoscelis* is one of the only species of anuran at the UMFS to readily oviposit in wading pools. Other species (i.e. *Hyla cinerea*, *Rana sphenocephala*) rarely oviposit in pools, and their eggs are easily discernable because they sink as opposed to the floating egg masses of *H. chrysoscelis*. A swimming pool net was used to remove leaf litter and insects daily. Treatment pools were diluted with well-water before the treatment water was disposed of to minimize impacts on UMFS amphibians.

Tadpole Survival, Development and Anti-Predator Behavior

Egg Collection

Cope's gray treefrog egg masses were collected by hand from wading pools on July 1, 2017 at the UMFS with a permit from the Mississippi Department of Wildlife, Fisheries and Parks (permit 0520161). To ensure genetic diversity, about 6,000 eggs from 3 separate locations were collected. All eggs were similarly aged (Gosner, 1960), and represented at least 3 distinct egg masses from different females. All eggs were combined into one 5-L plastic container and homogenized. Twenty-five eggs were chosen haphazardly and randomly assigned to a treatment using a number generator. Eggs were moved using a turkey baster to minimize damage to the gelatinous coating of the eggs.

Treatment Groups

Twelve, 37.85-L aquaria were filled with 8-L of unchlorinated well-water from the UMFS. The aquaria were not aerated, were randomly assigned a treatment, and then were randomly distributed among 3 blocks with each comprised of 4 treatments: control (C), acidic (A), tannic (T), and acidic and tannic combined (A&T). Treatments were made using the same procedures mentioned in the previous (oviposition) methods. Controls possessed only well-water with a pH of ~7.0. For acidic treatments (A and A&T), eggs were added to neutral well-water before the pH was slowly adjusted downward with a nitric acid solution over the course of an hour until it reached the desired acidity (5.5). The pH was monitored daily and adjusted as necessary. The tannin content of the two tannic treatments (T and A&T) was not monitored after establishment of the treatment aquaria. To maintain both water volume and quality, treatment water was replenished weekly with 50% water changes, and all aquaria were cleaned, and treatment water completely replaced biweekly.

Once free swimming, tadpoles were fed boiled lettuce (0.25g/tadpole) twice weekly. The room temperature was maintained at 22 degrees Celsius under a 12:12 light and dark schedule. Mortality was monitored, and dead individuals were removed daily. At 44 days of age, after the behavioral tests (see below), the study subjects were humanely euthanized, weighed (after blotting dry with a paper towel) and photographed aside a ruler to obtain snout-vent length (SVL) and total length (TL) using ImageJ (Rasband, 1997).

Experimental Design

Behavioral responses to predator cues were tested using techniques derived from Hayden et al. (2015). This experiment was a 4 x 2 factorial design examining the impacts of acidity (treatments: C, A, T, A&T), and predator cues (present or absent) on *H. chrysoscelis* tadpole behavior that tested tadpole responses to 8 conditions: 1) control with predator cues; 2) control without predator cues; 3) acidic with predator cues; 4) acidic without predator cues; 5) tannic with predator cues; 6) tannic without predator cues; 7) acidic + tannic with predator cues; and 8) acidic + tannic without predator cues.

Predator Cues Preparation

Predator cues were obtained from dragonfly nymphs (*Anax junius*) and small fish (golden shiners (*Notemigonus crysoleucas*) for separate tests of tadpole response to each of these stimuli. Dragonfly nymphs (N=12) were collected from natural ponds and housed in pairs in polyethylene containers filled with 0.5-L of well-water in a room separate from the tadpoles.

Each nymph was fed one tadpole each day starting 2 days prior to the initiation of the experiment to allow the alarm cues to permeate the water (Peacor, 2006; Troyer and Turner, 2015; Polo-Cavia et al., 2016). All the water from the nymphs' containers was collected and combined (3 L) to use as cues on days of testing. Golden shiners (N=12) were purchased from a supplier, and housed in groups of 4 in polyethylene containers containing 3 L of well-water. Eight tadpoles were placed in each container for the fish to consume daily, beginning 2 days prior to the beginning of cue collection. On the day of the behavioral tests 1 L of water containing alarm cues was removed from each container and combined (3 L).

Behavioral Tests

To test if pH affects how tadpoles respond to predator cues, the following methods derived from Hayden et al. (2015) were used. Trials took place in 20 L aquariums (41 cm x 20 cm x 25 cm) filled half-way (10 L) with unchlorinated well-water. Water was replaced, and tanks thoroughly rinsed between test subjects. Since additional tadpoles were available from the control rearing conditions, a subset of these individuals was tested in the 4 treatment conditions (C, N=12; A, N=12; T, N=12; A&T, N=12) using dragonfly nymph cues. Tadpoles that were reared in treatment conditions (C, N=20; A, N=20; A&T, N=14) were tested for their response to fish predator cues in water with their own rearing treatment conditions; however, too few tadpoles were raised to also test the T only condition with fish cues. Different predators were used in these studies because dragonfly naiads became increasingly difficult to collect, thus requiring the use of a more obtainable predator species, *Notemigonus crysoleucas*.

The aquariums were covered by white paper on three sides to reduce any influence from outside movement on tadpole behavior. A line on both the front and back of the aquariums demarcated equal top and bottom halves. To begin each trial, 80-mL of water, containing either predator cues or no predator cues, was gently poured across the surface of the water. A single tadpole (Gosner stage 28-35) was then placed in the aquarium and allowed to acclimate for 5 minutes. Once acclimated, the tadpole was observed continuously for 20 minutes. The duration of tadpole observation was determined both by previous studies (Polo-Cavia et al., 2016), and preliminary behavioral trials.

During each trial, the duration of 2 common antipredator behaviors were measured: tadpole position (above or below midline) and tadpole activity (moving or still). Tadpole position indicates the diving response that tadpoles use to hide from predators (Fraker, 2008).

Ethical Statement

This research was conducted with the approval of the University of Mississippi Institutional Animal Care and Use Committee (protocol 16-029).

STATISTICAL ANALYSIS

Statistical analyses were performed with R version 3.4.1 (R Core Team, 2017). Data were checked for normality prior to analysis and transformed if necessary as described in each section below.

Oviposition Site Preference

A one-way analysis of variance (ANOVA) was conducted to test the effects of treatment (i.e. acidic and tannic conditions) on oviposition site preference. The dependent variable (mean number of eggs/night) was square root transformed to achieve a normal distribution. A Tukey's *post hoc* test was conducted to determine if the treatment means differed.

Tadpole Survival and Development

A one-way ANOVA was used to investigate overall differences in mortality across treatments. The dependent variable (number of deceased tadpoles) was square root transformed to achieve a normal distribution. Pairwise comparisons of the mortality means were then analyzed using a *post hoc* Tukey HSD test (α=0.05). To determine if tadpole survival over time differed between treatments, a survival analysis was performed using the non-parametric Kaplan-Meier estimate. The survival curves were then interpreted using a Log-Rank pairwise comparison.

All three body measurements (mass, snout-vent length, and total length) were analyzed using a multivariate analysis of variance (MANOVA).

Tadpole Response to Predators

Two-way MANOVA tests were conducted to determine the effects of treatment type and predator cues on tadpole activity and tadpole position. Due to differences in rearing history of the tadpoles used in the behavioral tests of the two cue types, separate statistical tests were conducted. I then used separate ANOVAs for each dependent variable as a follow-up analysis.

RESULTS

Oviposition

A total of 26,885 eggs were collected over 30 days. Given that the observed average clutch size for *H. chrysoscelis* is ~885 (Chalcraft and Resetarits, unpublished data), about 30 separate clutches were collected over the duration of this experiment. The mean number of eggs oviposited per night was affected by treatment (one-way ANOVA, $F_{3,21} = 5.28$, p= 0.007), with significantly more eggs laid in the A&T treatment than in the control (C) and acidic (A) treatments ($p<0.05$) but not the T treatments ($p>0.1$; Fig 2.2).

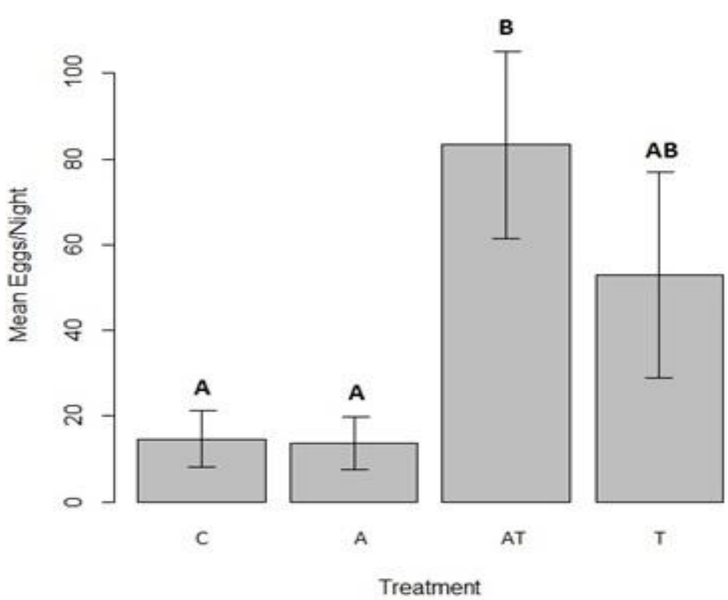


Figure 2.2. Oviposition Site Choice in Response to Acidic and Tannic Conditions. Significantly more eggs were oviposited per night in the acidic and tannic (AT) treatment than in the control (C) and acidic (A) treatments. For ease of interpretation the untransformed dependent variable values are shown. Columns are shown with SE bars, and columns with dissimilar letters above them are significantly different.

Tadpole Survival and Development

Total tadpole mortality over the 44-day study period was influenced by rearing treatment (one-way ANOVA, $F_{3,6} = 10.77$, p=0.008; Fig 2.3). Mortality was higher in the T treatment than in the C and A&T treatments (p<0.05) but not the A treatments (p=0.06). Additionally, the probability of tadpole survival over time was affected by treatment ($X^2 = 39.6$, df = 3, p <0.001). The probability of tadpole survival was less in the T treatment than all others (p<0.001; Fig 2.4) with survival declining rapidly around day 17.

Surviving tadpole mass, snout-vent length, and total length were not influenced by treatment (MANOVA, $F_{3,95} = 1.22$, p = 0.28; Table 2.1).

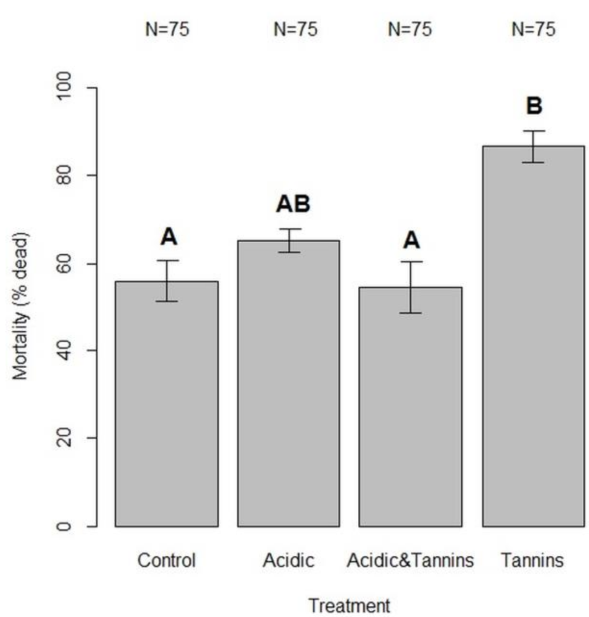


Figure 2.3. Tadpole Mortality in Response to Treatments. Tadpole mortality (percent of dead tadpoles) was higher in the tannic treatment (T) than in both the control (C) and the acidic and tannic treatments (AT) (p=0.008). Columns with dissimilar letters above them are significantly different. For ease of interpretation the untransformed dependent variable values are shown.

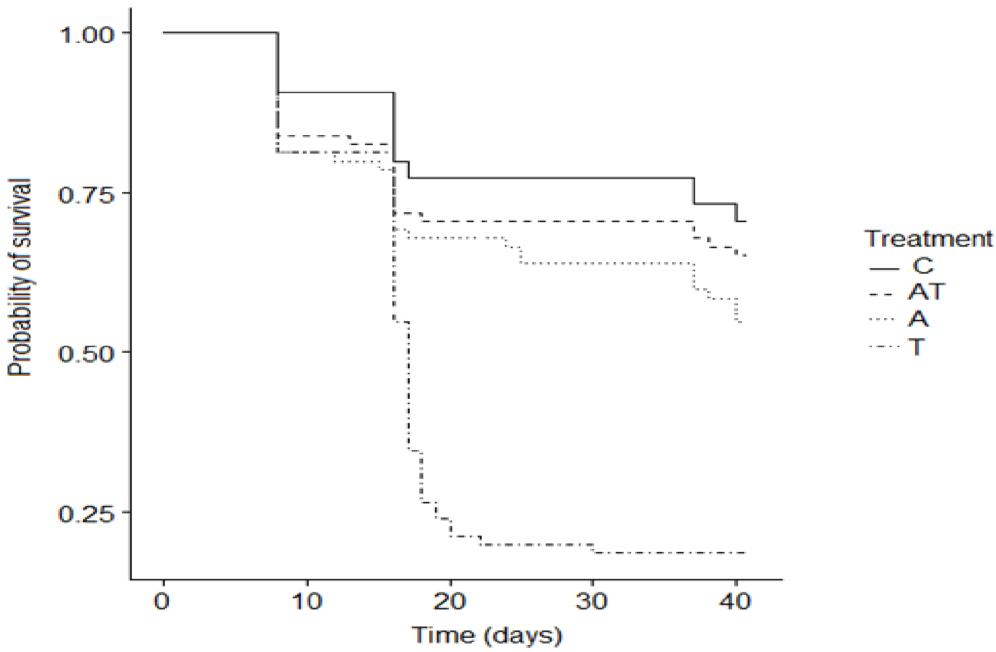


Figure 2.4. Tadpole Survival Curves Across Treatments. Daily survival probability drops suddenly for tadpoles in the tannic treatment starting on day 17 ($p < 0.001$).

Table 2.1**.** Measurements of Tadpole Development Across Treatments. Tadpole body measurements did not differ amongst treatments ($p = 0.28$). Body measurements consisted of mass, snout-vent length (SVL), and total length (TL).

Treatment	**Mass (g)**	**SVL (mm)**	**TL (mm)**
Control	0.23 ± 0.01	11.36 ± 0.20	29.71 ± 0.42
Acidic	0.19 ± 0.01	10.85 ± 0.26	28.81 ± 0.72
Acidic&Tannic	0.22 ± 0.01	11.08 ± 0.28	28.87 ± 0.56
Tannic	0.24 ± 0.04	11.20 ± 0.49	29.01 ± 1.01

Tadpole Response to Predators

In the dragonfly nymph cue exposure test neither tadpole activity nor position were affected by treatment or cue presence, or their interaction (MANOVA, $p > 0.05$; Table 2.2). When fish predator cues were present tadpole behavior was affected (MANOVA, $F_{1,48} = 3.88$, $p = 0.028$). Tadpole activity was reduced when fish cues were present, ($F_{1,48}=7.80$, $p=0.007$) but activity was not affected by treatment, nor by the interaction of treatment x cues (Table 2.3a). Additionally, tadpole position was not affected by treatment, cues, or their interaction (Table 2.3b).

Table 2.2. MANOVA Summary Table for Tadpole Anti-Predator Behavior. There was not a statistically significant effect of treatment, dragonfly predator cues (*Anax junius*), or treatment x cues interaction on tadpole activity, or time below midline.

Source	*df*	*Pillai*	*F*	*p*
Treatment	3	0.15	1.06	0.40
Cues	1	0.03	0.53	0.59
Treatment: Cues	3	0.09	0.65	0.69
Residuals	40			

Table 2.3. ANOVA Summary Tables for Tadpole Anti-Predator Behavior. Tadpole activity (a) was affected by fish predator cues (*Notemigonus crysoleucas*), but not by treatment or treatment x cues. Tadpole time below midline (b) was not affected by treatment, cues, or treatment x cues.

a)

Source	*df*	*SS*	*F*	*p*
Treatment	2	24518	0.65	0.52
Cues	1	146050	7.80	0.007*
Treatment: Cues	2	47671	1.27	0.29
Residuals	48	898980		

b)

Source	*df*	*SS*	*F*	*p*
Treatment	2	26.3	0.03	0.98
Cues	1	99.5	0.20	0.67
Treatment: Cues	2	30.8	0.03	0.97
Residuals	48	25319.4		

DISCUSSION

Female Cope's gray tree frogs did not prefer to lay eggs in water favorable to larval survival and development. On average, the A&T pools received ~80% more eggs per night than C or A pools (Fig. 2.2), even though the C, A and A&T treatments were equivalent developmental habitats for tadpoles under laboratory conditions (Fig. 2.3; Fig. 2.4). Females did lay some eggs in treatment pools with neutral and acidic conditions but the presence of tannins, with or without the mineral acids seem to be the major factor positively influencing site selection (Fig. 2.2). Although not statistically significant, T pools received the second most eggs even though T treatments had the highest tadpole mortality in the first experiment. Acid tolerance appears to be highly species-specific in amphibians with some species experiencing population declines in mildly acidic conditions (Freda, 1986), and other species, like the Wallum Sedgefrog (*Litoria olongburensis*) thriving in pH as low as 3.5 (Ingram and Corben, 1975). Because mildly acidic conditions did not affect hatching success or tadpole survival, and females did not avoid acidic pools (C vs. A), it is likely that *H. chrysoscelis* is an acid-tolerant species.

It remains unclear whether acidic conditions influence the ability of tadpoles to sense or respond to kairomones produced by predators. In the neutral pH treatment trials, overall tadpole activity was reduced 42% in response to cues from dragonfly larvae, and 34% when fish predator cues were present (Fig. 2.5a, 2.6a). In almost all other treatments, however, tadpole activity only declined by 15% or less in the presence of predator cues suggesting an impairment of their antipredator response, although this was not statistically significant. Given the power of these analyses, 0.51 and 0.67, respectively, a larger sample size of n=18 could possibly detect a statistically significant difference. Because high activity levels have been shown to elevate the risk of predation by increasing prey detectability (Sih, 1992; Skelly, 1994; Relyea and Edwards, 2010), tadpoles should reduce activity levels once they perceive evidence of a predator nearby.

Diving behavior has also been observed in tadpoles as a response to predators in previous studies (Peacor, 2006; Hayden et al., 2015); however, my study failed to replicate these findings (Fig. 2.5b; 2.6b). Hayden et al. (2015) discovered that diving behavior was most prominent immediately following a fatal attack by a predator while testing a group of tadpoles with predators present. Furthermore, another study only observed a difference in tadpole diving behavior hours after predator cues were introduced (Peacor, 2006). Thus, it appears tadpole diving behavior may rely upon very specific conditions that my experiment lacked (i.e. fatal attacks or extended periods of observation).

The tannic treatment was the most harmful to tadpoles in my study but surprisingly received the second highest number of eggs over the study period. Generally, amphibians are thought to be particularly sensitive to tannins (Temmink et al., 1989; Maerz et al., 2005; Earl et al., 2012). Anurans that are obligate gill breathers during larval stages, such as the American toad (*Bufo americanus)*, suffer gill damage from elevated concentrations of tannins (Maerz et el., 2005). Unlike *B. americanus*, however, *H. chrysoscelis* tadpoles have well-developed lungs early in development, and thus should be able to ameliorate the effects of gill damage by breathing air at the surface of the water (Ultsch et al., 1999). Therefore, it is unlikely that gill damage is the primary cause for high mortality in the tannic treatment in my study.

Alternatively, tannins may have impaired tadpole development by interfering with digestive enzymes and preventing the absorption of nutrients (Temmink et al., 1989; Maerz et al., 2005). Early in this experiment, general differences in fecal output were observed across treatments. Although no formal measurements were taken, fecal production in treatments containing tannins appeared to be much greater than in treatments not possessing tannins. Indeed, dietary tannic acid is known to increase fecal mass in rats (Bravo et al., 1994). Also, the fecal matter in tannic treatments was much darker, appearing almost black, and had a prominent rope-like appearance. The altered fecal properties suggest differences in digestion, absorption and/or the gastrointestinal microbiome that may have resulted in the tannin-related tadpole mortality that I observed.

Recent studies reveal how certain organic acids increase survivorship of aquatic organisms in low pH by binding to toxic metals and removing free ions, thus contributing to the success of species living in naturally acidic ecosystems (Barth and Wilson, 2010; Holland et al., 2013; Holland et al., 2014). However, in this study, it was the presence of acid that increased tadpole survival in tannic acid, not the contrary, suggesting that a complex dynamic exists between pH and tannic acid that may affect the toxicity of tannic acid to tadpoles.

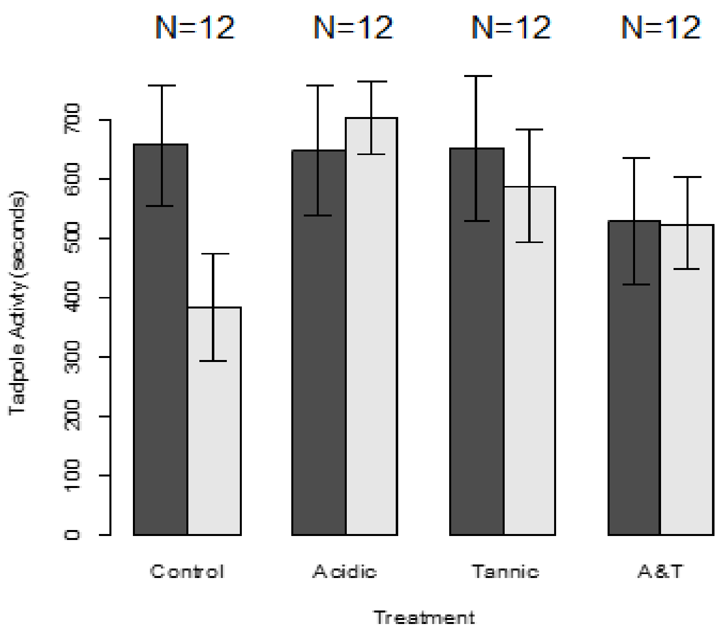
Ecosystems are comprised of interactions between organisms and their environments that form as organisms adapt to environmental conditions over time. Human induced rapid environmental changes (HIREC) are radically altering natural ecosystems and introducing evolutionarily novel conditions, like pollutants, that are disrupting biological communities (Sih et al., 2011). Behavioral responses to new threats mediate an organism's capacity to handle rapid changes; therefore, it is imperative to investigate organisms' behaviors in response to relevant novel stimuli to assess the impacts of potential environmental change (Sih et al., 2011; Shuker et al., 2016). If organisms fail to respond adaptively to an introduced threat, they may experience an increase in mortality (Cox and Lima, 2006). Such may be the case with elevated concentrations of tannins and oviposition site selection in *H. chrysoscelis*. At low level tannins may indicate the presence of leaf litter and detritus to serve as food and refuge for offspring; however, now as human-introduced plant species invade and elevate concentrations, tannins may begin to pose a novel threat to tadpole survival. As tannin-rich invasive plants make inroads into native ecosystems (Maerz et al., 2005; Leonard, 2008), ponds possessing high concentrations of tannins may act as ecological traps. An ecological trap is a low-quality habitat that fails to optimize survival or performance, yet is often preferred over available higher quality habitats (Gates and Gysel, 1978; Delibes et al., 2001). My results suggest that female frogs naïve to high levels of tannins will respond maladaptively to a cue previously positively associated with offspring survival, but now having the opposite effect on her reproductive success, and select habitats that fail to optimize offspring survival.

Conversely, other organic acids including humic substances can increase body size and overall health in fish (Meinelt et al., 2004), and since humic acids differ from tannins in molecular structure and origin, this suggests that effects may be specific to the type of organic acid, or the species being affected. Alternatively, the controlled lab conditions of my study may not accurately reflect the fitness consequences of these water conditions for tadpoles in the wild. Ovipositing females may assess multiple variables in addition to water quality, including predation risk and the density of larval competitors when determining which habitat will optimize offspring performance (Resetarits and Wilbur, 1989; Binckley and Resetarits, 2008; Pintar and Resetarits, 2017). Indeed, females may recognize tannins as a risk, but other benefits may outweigh the costs of tannin toxicity to larval development.

Conclusion

Few environments remain unscathed from anthropogenic alterations with human activities increasing the amounts of pollutants in the environment through practices including the generation of industrial and agricultural wastes, and combustion of fossil fuels (Cooper, 1993; Vitousek et al., 1997). Amphibian survivorship is often impacted by elevated levels of pollutants including reactive nitrogen (Ilha and Schiesari, 2014) and heavy metals (Zocche et al., 2014), yet few studies explore potential changes in behaviors that may influence how individuals respond to such changes. Recent findings regarding ocean acidification indicate that low pH is changing the feeding behaviors, homing abilities, and antipredator responses across many species impacting individual performance and fitness (Fabry et al., 2008; Munday et al., 2009). Similarly, a few studies indicate the olfactory impairment of ocean fishes by low pH may be mirrored in some fish species in freshwater ecosystems (Leduc et al., 2007; Leduc et al., 2008; Leduc et al., 2013). Although my study failed to demonstrate that acidic conditions hinder the chemosensory abilities of *H. chrysoscelis* tadpoles, relatively weak antipredator behavior responses of the non-control treatment suggest that further investigation is warranted. With amphibians continuing to decline, identifying the subtle behavioral effects of freshwater acidification will be important to identifying at risk populations, and implementing successful conservation strategies (Shuker et al., 2016). As little research has been conducted on the impacts of tannins and amphibians, studies like mine can be used to estimate potential impacts on unstudied amphibian populations. However, future studies should target populations likely to be affected by acidification or tannin rich invasive plants that have not been previously exposed to comparable conditions and use those data to identify at risk populations and aid in conservation prioritization.

a)



b)

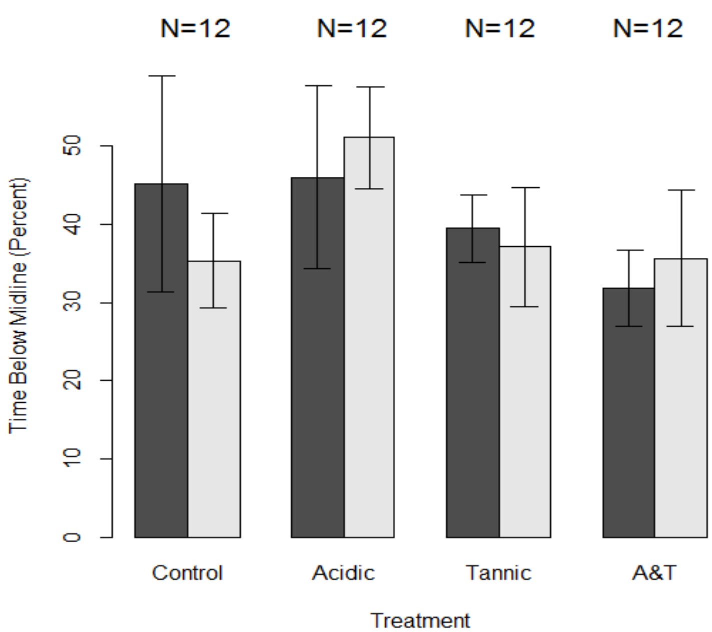
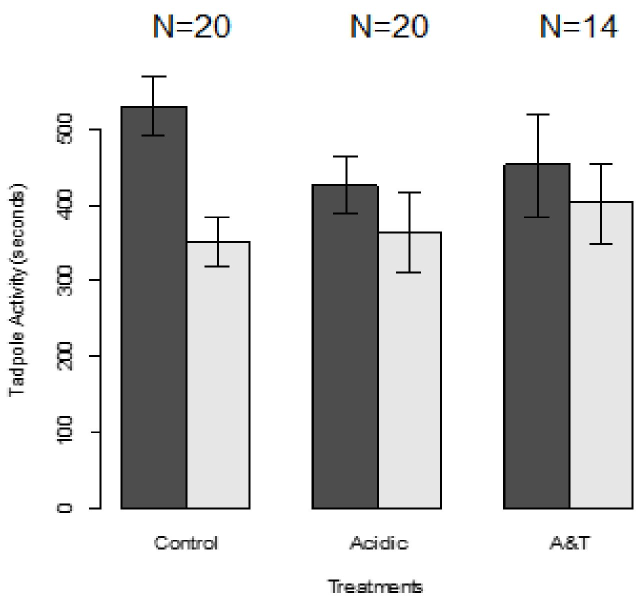


Figure 2.5. Tadpole Anti-Predator Behavior in Response to Dragonfly Predator Cues. Neither tadpole activity levels (a), nor time below midline (b) were affected by the absence (dark bars), or presence (light bars) of insect predator cues (*Anax junius*), although less reduction in activity did occur in all non-neutral treatments (a) as predicted.

a)



b)

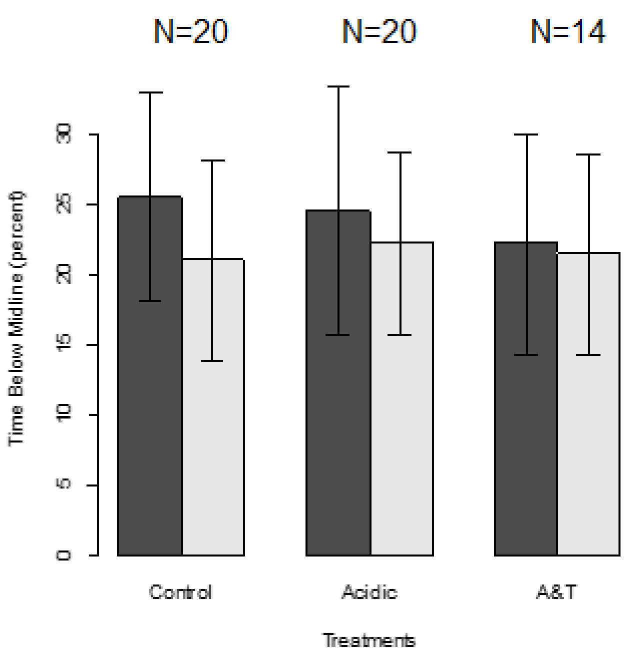


Figure 2.6. Tadpole Anti-Predator Behavior in Response to Fish Predator Cues. Neither tadpole activity levels (a), nor time below midline (b) showed treatment effects in the absence (dark bars), or presence (light bars) of fish predator cues (*Notemigonus crysoleucas*). Overall activity was significantly decreased by fish predator cues (p=0.007).

III. *HYLA CHRYSOSCELIS* AND EXTREME CONDITIONS: A PRELIMINARY STUDY

Though highly contaminated ecosystems are uncommon, they do occur, especially in areas heavily influenced by wastewater and runoff (Ali and Sreekrishnan, 2001; Igbinosa and Okah, 2009). The effects of heavily acidic conditions on amphibians are well understood (Freda and Dunson, 1986); however, few studies have examined the effects of severely high levels of tannins on amphibians. Investigating the impacts of extreme conditions on amphibians may serve as a reference and provide valuable information about environmental limits that will assist research focusing on more ecologically relevant conditions, like the one described in Chapter Two. In this chapter I describe a preliminary study, in which I gauged the impacts of severely elevated levels of acidity and tannins on *H. chrysoscelis* tadpoles.

The largest impact that acid rain has on amphibians is reproductive failure (Freda, 1986; Carey and Bryant, 1995). Embryos are more susceptible to acidic conditions than larvae and adults, because low pH often prevents hatching in developing embryos (Freda, 1986). Excess hydrogen ions are thought to protonate specific enzymes necessary for hatching, thus rendering them inactive (Freda, 1986). This causes the vitelline membrane to never lift off the embryo, frequently inhibiting hatching (Freda, 1986). By preventing hatching, acidic conditions can severely impact recruitment and contribute to population declines.

In larval and adult amphibians, acidic conditions can also cause direct mortality by affecting ion-regulatory processes. Low pH disrupts the balance of sodium and chlorine ions by depressing the influx of sodium and chlorine and accelerating sodium efflux causing mortality in adult frogs if the loss of sodium ions is great enough (Freda and Dunson, 1986).

Acidic conditions can cause many physiological problems for aquatic organisms including impaired development and physical deformities (Schlichter, 1981; Carey and Bryant, 1995; Devi et al., 2016). Environmental contaminants cause skeletal malformations, including scoliosis, in fishes and amphibians (Alvarez et al., 1995; Brunelli et al., 2009; Pimentel et al., 2014). Additionally, pesticides and agricultural wastes increase the incidences of tail abnormalities in larval frogs, with individuals possessing lateral flexure of their tail (Brunelli et al., 2009). Increased occurrences of deformities can affect activities such as foraging and predator avoidance, thus impacting larval survival and recruitment (Brunelli et al., 2009).

Furthermore, tannins are likely to impact amphibians during aquatic life stages (i.e tadpoles). Tannins impair bacteria and phytoplankton growth likely impacting organisms at higher trophic levels (Tuchman et al., 2002). Additionally, tannins cause gill damage in fish (Temmink et al., 1989), thus tannins may influence tadpole development.

Sub-lethal acidic conditions also impact locomotion in amphibians. Likely due to ionic disequilibrium, low pH reduces swimming speed and overall activity levels in salamanders (Kutka, 1994; Jung and Jagoe, 1995), potentially limiting the escape capabilities of amphibians and reducing survival.

The objective of this preliminary study was to explore how different rearing conditions (small and large groups), and acid and tannin concentrations affected tadpole survival. For a variety of reasons these rearing conditions were not successful, but I report my observations on tadpole performance and the effects of extreme acidic and tannic conditions on a) hatching success, b) tadpole development and physical malformations, c) and tadpole activity so that other researchers need not repeat this work.

MATERIALS AND METHODS

Egg Collection and Treatment Groups

Cope's gray treefrog egg masses were collected at the UMFS, as described in detail in Chapter Two, and assigned to small- or large-group rearing conditions. The small-group rearing condition consisted of 75, 0.95 L glass jars filled with 0.5 L of well-water from the UMFS. The jars were divided equally into 5 treatment groups (n=15) and placed randomly in a rectangular 3 X 25 grid. The treatments included: Control (C) =pH 7, Acidic (A) =pH 5.5, Heavily Acidic (HA) =pH 4.5, Tannic (T) =0.1 g L^{-1}, and Heavily Tannic (HT) =0.2 g L^{-1}. The tannic treatments (T, HT) lowered the pH of the water to 5.5 and 4.5, respectively. Three eggs were randomly assigned to each jar for a total of 225 eggs.

The second rearing condition consisted of twelve 37.85-L aquaria filled with 8-L of unchlorinated well-water from the UMFS. The aquaria were randomly distributed among 2 blocks with each comprised of the 5 original treatments (C, A, HA, T, HT), and an additional HA + HT treatment in which treatment waters from both HA and HT conditions were combined. Each tank received 50 eggs for a total of 600.

Treatment conditions for both rearing conditions were adjusted and monitored using methods described in Chapter Two. Additionally, tadpole husbandry remained identical to tadpole care in Chapter Two. This research was conducted with the approval of the University of Mississippi Institutional Animal Care and Use Committee.

Hatching Success and Tadpole development

All eggs were observed daily with notes taken on the general progress of egg and tadpole development. After 1 week, all unhatched eggs were identified as dead and removed. Unhatched eggs were an opaque yellow color, often lacking any discernable developing embryo. Tadpoles were observed daily for general activity, and physical deformities. Dead tadpoles were removed from tanks upon inspection.

Tadpole Activity

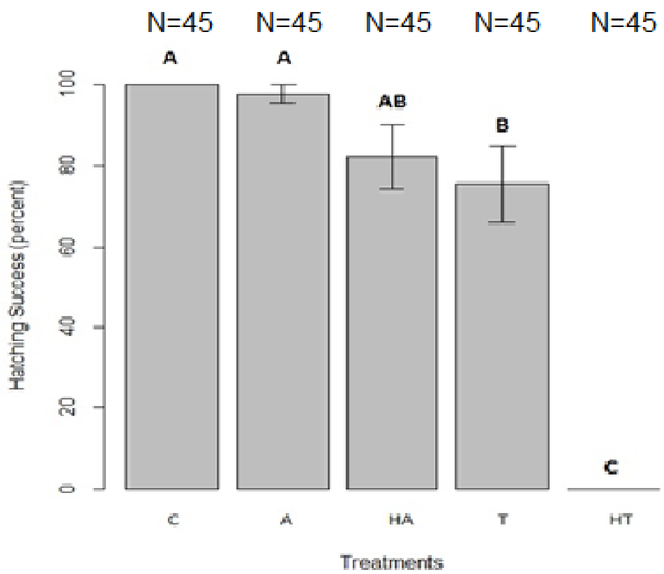
Experiments took place in plastic white rain gutters (101 × 11.4 × 6.4 cm) sealed with caps at both ends. Five lines were drawn on the bottom of the gutter visibly separating it into 5 equal parts. The gutter was filled with 3 L of the appropriate treatment water and a single tadpole (Gosner stage 25) was then placed in the center of the gutter and allowed to acclimate for 5 minutes. Activity was then recorded by documenting the number of quadrants a tadpole visited over 30 minutes. Due to high mortality in other treatments, only Control tadpoles were used during this experiment.

RESULTS

In the first rearing condition (small), hatching success was affected by treatment ($F_{4,70}$=53.84, $p<0.001$). Fewer eggs hatched in the tannic treatments (T, HT) than the other (C, A, HA) treatments ($p<0.05$; Fig 3.1a). Similarly, hatching success was dependent on treatment type in the second (large) rearing condition ($F_{5,6}$=6.51, $p=0.02$). More eggs hatched in the Control treatments than the heavily tannic (HT, HA+HT) treatments ($p<0.05$; Fig 3.1b).

Generally, tadpoles reared in tannic (T) treatments appeared to develop more slowly than other treatments, however all tadpoles, regardless of treatment, matured slowly and very little changes in body size were observed after week 3 across all treatments. About 16% of tadpoles exhibited physical malformations including lateral tail flexure in the tannic (T) treatments causing them to swim in small circles. No physical malformations were observed in any other treatments. Lastly, the activity of surviving tadpoles was not affected by treatment (Fig 3.2).

a)



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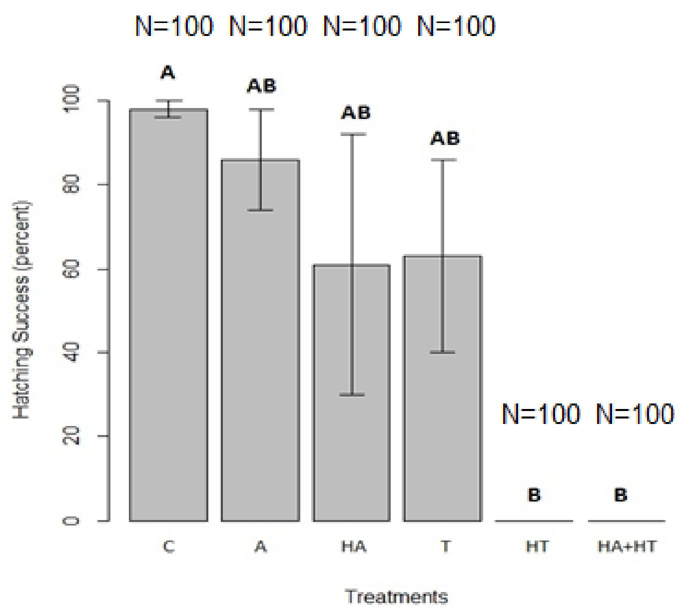

Figure 3.1. Egg Hatching Success in Acidic and Tannic Treatments. In both rearing conditions, a) small, and b) large, tadpole survival was affected by treatment. The letters above the bars represent statistically significant differences.

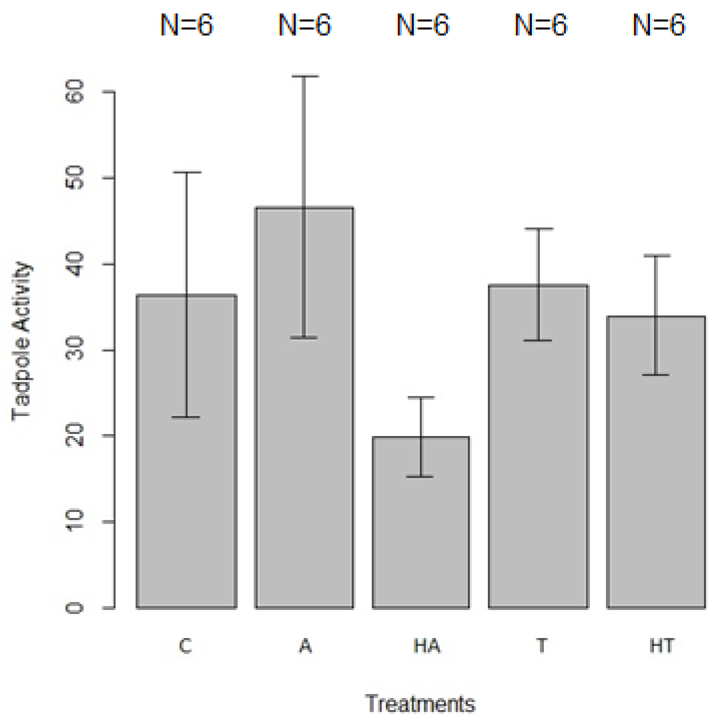

Figure 3.2. Tadpole Activity in Response to Acidic and Tannic Conditions. Tadpole activity (number of quadrants visited) was not affected by treatment type.

DISCUSSION

Recruitment is a crucial step in maintaining viable populations and is frequently the most vulnerable stage of amphibian development. Severe tannic conditions appear to pose a particular threat to hatching success with 100% of eggs failing to hatch in HT treatments (Fig. 3.1). Many amphibians lay their eggs in gelatinous egg masses that protect developing embryos by serving as a buffer from potentially harsh environmental conditions and altering the gas levels around embryos (Woods, 1999; Moran and Woods, 2007). Oxygen levels inside egg masses are often reduced as embryos develop due to respiration and metabolic processes, and oxygen must be replenished by diffusion from the external environment (Seymour and Roberts, 1991). At high enough concentrations, tannins can reduce dissolved oxygen concentrations in aquatic ecosystems (Maerz et al., 2005); and since the aquaria were not aerated, it is likely that low oxygen levels within egg masses likely contributed to the hatching failure in HT treatments.

Acidic conditions did not significantly affect embryo hatching success, though trends suggest heavily acidic conditions may impair hatching success (Fig 3.1; Fig 3.2). this was an exploratory study with small sample sizes and may have lacked the power necessary to detect a difference.

Anurans are particularly vulnerable to predation during the larval tadpole stage of development because tadpoles are small and often constrained to the environment they hatched in; therefore, they benefit from fast development to metamorphosis (Werner, 1986). Impaired growth and developmental abnormalities can prolong the time individuals are vulnerable to predation and limit the escape capabilities of individuals. Similar to other pollutant studies (Brunelli et al., 2009), this study indicates highly tannic environments may increase larval susceptibility to predation by slowing development and causing physical malformations.

Amphibian fitness is often associated with activity levels and locomotion as these variables indicate an individual’s ability to successfully evade predators (Skelly, 1994). Many pollutants can decrease overall activity and locomotion in amphibians, thus potentially increasing their susceptibility to predation (Kutka, 1994; Jung and Jagoe, 1995). Though tadpole activity levels appeared lower in HA conditions, no treatment had a significant effect on tadpole activity.

Over the course of this study, several extreme (±5º) temperature fluctuations occurred with temperatures dropping as low as 18º C. Temperature can influence tadpole development, especially in the presence of other stressors (Broomhall, 2004). When the laboratory ventilation was fixed and the temperature maintained at 22º C, no delayed development was observed; therefore, impaired tadpole development, could have been a result of low laboratory temperatures.

Many species around the world are declining, and knowing the environmental limits of an organism is imperative for understanding and predicting the impacts of anthropogenic changes (Shuker et al., 2016). My observations suggest that frog embryos are more vulnerable to tannins than they are to acidity. It would be wise to investigate how interspecific variation in egg mass properties (i.e. size, shape, and embryo density) and evolutionary history of exposure influence embryo tolerance to these and other pollutants before generalizing these results (Hamilton et al., 2017). This study confirms that treefrog eggs and tadpoles are especially susceptible to environmental pollutants, and indicates that tannins may pose a larger threat to amphibians than acidic conditions.

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