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The Effects of Sulfuric Acid Deposition on the Growth And Development of Pond Breeding Salamanders in the Genus Ambystoma

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THE EFFECTS OF SULFURIC ACID DEPOSITION ON THE GROWTH AND DEVELOPMENT OF POND BREEDING SALAMANDERS IN THE GENUS AMBYSTOMA

A Thesis
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Master of Science

By
Kenneth J. Anderson

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THE EFFECTS OF SULFURIC ACID DEPOSITION ON THE GROWTH AND DEVELOPMENT OF POND BREEDING SALAMANDERS IN THE GENUS AMBYSTOMA

Date Recommended 11/18/2016

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I dedicate this thesis to the salamanders.
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THE EFFECTS OF SULFURIC ACID DEPOSITION ON THE GROWTH AND DEVELOPMENT OF POND BREEDING SALAMANDERS IN THE GENUS AMBystoma

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In terrestrial habitats with a history of mining activity and previous or ongoing reclamation efforts, understanding the effects of acidification on the ecology of amphibians is an important part of the restoration process and the conservation of local amphibian populations. Pond-breeding amphibians spend much of their postmetamorphic life history in direct contact with the soil in upland habitat adjacent to aquatic breeding sites. I reared recently metamorphosed marbled salamanders (Ambystoma opacum) to evaluate the role of soil acidity on determinants of fitness such as growth and survival. My results indicate that a substrate of pH 4 was lethal to recent A. opacum metamorphs. Among animals surviving the higher pH treatments, we found that individuals reared on a pH 5 substrate suffered a reduction in total length and snout vent length by the end of the experiment.

The mechanisms of acidity are complex; both hydrogen ions and anions contribute to negative effects on amphibians. Sulfuric acid has larger negative effects than other acids and sulfates can cause reductions in growth without a change in pH. I reared larval spotted (Ambystoma maculatum) and Jefferson salamanders (Ambystoma jeffersonianum) to evaluate the effects of pH and sulfates on two species with differential acid resistances. My results indicate that a pH of 4 is lethal to larval salamanders of both species. In high sulfate treatments there was an early reduction in growth in the spotted
salamander, but not in the Jefferson salamander showing that acid resistance applies to the effects of sulfates as well as hydrogen ions. Together, our results suggest that acid and sulfate deposition can affect the fitness of *Ambystoma* salamanders through direct mortality and a decrease the growth rate of salamanders both as larvae and subsequent to metamorphosis.
CHAPTER ONE

THE EFFECTS OF SUBSTRATE pH ON GROWTH AND SURVIVAL OF RECENTLY METAMORPHOSED MARBLED SALAMANDERS (*AMBYSTOMA OPACUM*)

The importance of salamanders in forested ecosystems has been historically underestimated (Davic & Welsh, 2004; Burton & Likens, 1975). However, their importance to ecosystem function is becoming more apparent, with salamanders making up large proportions of forest biomass (Welsh & Droge, 2001). Salamanders drive important forest ecosystem processes such as leaf litter retention and carbon sequestration as predators on small invertebrates (Semlitsch et al., 2014; Best & Welsh Jr, 2014). Leaf litter acts as a store of nutrients and protects established plants from competitors by forming a physical barrier against seedling growth (Facelli & Pickett, 1991).

Salamanders, along with other amphibians, have been commonly viewed as 'indicator species' of environmental damage (Wilson & McCranie, 2003; Welsh & Droge, 2001), and the importance of studies aimed at understanding the ecology of salamanders is amplified by evidence of widespread decline in amphibian species (Adams et al., 2013; Rovito et al., 2009; Boone et al., 2007; Stuart et al., 2004).

Declines in amphibian species have been linked to a variety of factors, but foremost is habitat loss and fragmentation (Cushman, 2006). Habitat loss is especially harmful to migrating amphibians, which have breeding sites separated from terrestrial foraging areas or overwintering sites (Gibbs & Shriver, 2005). The removal of tree cover is a major contributor to habitat loss, and has been shown to significantly reduce amphibian populations, with recovery to original population levels taking up to 50 years (Semlitsch et al., 2009; Ash, 1997; Petranka et al., 1993). However, the degree to which
different amphibian species are affected by activities such as logging varies (Felix et al., 2010). In addition to habitat loss caused by land use activities such as logging, a variety of other human activities affect amphibian diversity by degrading existing habitats. For example, chemical runoff from fertilizers and pesticides has been shown to decrease larval growth and survival rates in amphibians (Boone et al., 2004, 2007).

One activity that combines the effects of habitat loss due to deforestation and habitat degradation due to leaching of chemical contaminants is strip mining. The process of mining consists of the removal of vegetation, followed by the removal and homogenization of soil (Indorante et al., 1981). After mining is complete, reclamation efforts begin. Sites are commonly planted with early successional species, leading to faster initial plant growth, but a slower overall development of the reclaimed forest, leading to a reduction in total plant diversity (Holl, 2002). In addition to the reduction in plant diversity, soil from reclaimed mine sites is commonly acidic (Thurman & Sencindiver, 1986; Plass & Vogel, 1973), with low levels of organic material and nutrients (Palmer et al., 2010) and a notable amount of heavy metal pollution (Yao et al., 2010). The problems faced with mining are especially apparent in western Kentucky, which is conspicuous for large shallow deposits of coal (Guernsey, 1960).

Current reclamation efforts in the Appalachian region are increasingly coordinated by the Appalachian Regional Reforestation Initiative (ARRI), a coalition of groups formed of the industry, private citizens, and the government. The ARRI promotes the Forestry Reclamation Approach (FRA) as the basis of their reclamation efforts, which emphasizes five primary steps in a successful reclamation process (Angel et al., 2009). The first step involves the selection of a suitable rooting media at least 4 feet deep. The
suggested media is one with low to moderate levels of salts, low sulfur, appropriate
texture for drainage, and with a pH between 5 and 7, considered to be the best pH for
native hardwood diversity. The second step is to loosely grade topsoil to avoid
compaction of the soil. This is important for tree root penetration and it has been shown
that compaction of the soil impairs tree growth and survival (Angel et al., 2006; Torbert
& Burger, 1990; Torbert et al., 1988). The third step is to use compatible ground cover
species. Appropriate species will control erosion but not compete with desired tree
species. Step four includes selecting the right mix of tree species, while step five
highlights proper tree planting techniques (Burger et al., 2005). The FRA as a whole
highlights the importance of creating proper soil conditions for the growth of tree species
in order to restore a functioning forest ecosystem.

While current reclamation processes focus on the soil conditions necessary for the
growth of tree species, explicit attention is not paid to other forms of life essential to the
forest ecosystem, such as amphibians. Ambystomatid salamanders have been found to
select among alternative microhabitats, for varying soil properties including pH
(Mushinsky, 1975), moisture (Sugalski & Claussen, 1997), and between soils from
different habitat types (Rittenhouse et al., 2004). Microhabitat discrimination suggests
that selected sites confer advantages and implies some kind of fitness benefit in terms of
reproduction or growth.

While water acidity has been shown to affect oviposition site selection in various
pond breeding salamanders (Fairman et al., 2013), as well as the growth and survival of
larval anurans (Beattie & Tyler-Jones, 1992; Rowe et al., 1992; Tyler-Jones et al., 1989)
and caudates (Brodman, 1993; Ireland, 1991; Ling et al., 1986; Cook, 1983; Pough &
Wilson, 1976), research on the effects of substrate acidity post-metamorphosis is limited. However, the fully terrestrial, direct-developing salamander, *Plethodon cinereus*, showed reduced levels of growth and oxygen consumption in chronic acidic conditions (Wyman & Hawksley-Lescault, 1987). However, sensitivity to acidic substrates was shown to vary across species (Wyman, 1988; Wyman & Hawksley-Lescault, 1987). These studies concluded that levels of acidity that are able to reduce the growth and distribution of forest salamanders have the potential to change the makeup of the forest floor ecosystem, of which salamanders play an essential role as a top level predator (Best & Welsh Jr, 2014). It has also been shown that marbled (*Ambystoma opacum*) and spotted (*Ambystoma maculatum*) salamanders will select for a higher pH when given an option between pH 5.5 and 7.7 (Mushinsky, 1975). Additionally, acidity has been shown to affect the distribution of adults; in New York, spotted salamanders were only found at sites with soil pH of 4 or higher (Wyman, 1988).

This paper reports a laboratory study of the effect of pH on the growth and survival of recently metamorphosed marbled salamanders (*A. opacum*). I hypothesized that as substrate acidity increased I would see a decline in growth in recently metamorphosed salamanders assumedly indicating a future reduction in reproductive fitness. The results of this work indicate the importance of soil pH as an abiotic characteristic when assessing the recovery of mined sites, and could affect decisions about the selection of soils for forest reclamation, especially considering the importance of salamanders to North American forests, and specifically to the Appalachian forest ecosystem.
MATERIALS AND METHODS

Study Species — The marbled salamander (*Ambystoma opacum*) is found throughout most of Kentucky, excluding only the far northern and eastern portions of the state (Petranka, 1998). Breeding occurs in the fall and winter when males will typically court females prior to or during migration to breeding sites (Krenz and Scott, 1994). Eggs are laid in dried pond beds and are then inundated with water when the ponds fill during late winter and early spring (Petranka, 1998). Larvae feed primarily on macro-zooplankton, and larger larvae will eat other amphibian larvae and eggs (Petranka and Petranka, 1980). In Kentucky, metamorphosis typically occurs in mid to late May (Keen, 1975; Petranka, 1998). After metamorphosis, juveniles disperse beneath leaf litter and debris adjacent to ponds (Stenhouse, 1985).

Experimental Design — Free-swimming larval marbled salamanders (20–25mm, 0.5–0.8g) were collected from an autumnal pool in Hart County Kentucky, near the Western Kentucky University Green River Preserve during the spring of 2015. Larvae were maintained individually in 16-oz. deli cups and fed brine shrimp (*Artemia sp.*) and California blackworms (*Lumbriculus variegatus*) until metamorphosis. Upon metamorphosis, individuals were sequentially assigned to one of three reduced pH treatments or a neutral pH control group, so that as each individual metamorphosed there were equal numbers across treatments. This was done to account for differences in fitness between early and late metamorphosis. Each individual was housed in a deli cup with a foam substrate saturated with ~80ml modified Holtfreter’s solution (40%), and the three
reduced pH treatments were adjusted to pH 4, 5, or 6 with 1M sulfuric acid. All treatments were changed every three days to account for changes in pH. A total of 100 individuals were raised in their enclosures at 20˚C on a 12L:12D cycle. I assigned 25 salamanders to each treatment. All metamorphosed animals were fed flightless fruit flies (*Drosophila hydei*) for the first 60D and were fed half-inch crickets (*Acheta domesticus*) for the remaining 90D of the study. In total, individuals were exposed to their treatment substrates from metamorphosis in June 2015 until I terminated the experiment in November 2015. The decision to terminate the experiment was determined by the date at which the ambient temperature near their natal pond dropped below 0˚ C, at which time metamorphs in the wild should have moved to underground burrows for overwintering (Semlitsch, 1983). Snout-vent length (SVL) and total length were determined for each individual by measuring the length with a ruler through a clear container, and individuals were then weighed on a digital scale. Measurements were taken every two weeks.

**Statistical Analyses** — Growth was analyzed in terms of change in weight and length over time with Growth Curve Analysis (Mirman et al., 2008). For each measurement, a series of models were constructed to simulate the growth curve, and models were compared to observe differences in growth patterns between treatments. Before models were constructed, time data were transformed into first and second order orthogonal polynomials in order to avoid problems of collinearity between data points (Mirman et al., 2008). The base model was constructed with the measurement (SVL, total length or weight) and compared to time with the random effect of individuals included. Three subsequent models were constructed: one adding treatment, one adding the interaction
between treatment and the first order orthogonal polynomials, and one adding the interaction between treatment and second order orthogonal polynomials. Models were created using the lme4 (Bates et al., 2015) package in R with growth as the dependent variable and time as the independent comparison, fixed effects of pH treatments, and the interaction between treatment and time were then added along with the random effects of individual variation. A model was created for each treatment and model comparisons were performed with analysis of variance (ANOVA). Model comparisons were tested for three measures of growth: SVL, total length, and weight. Growth curve models were fitted and graphed using the ggplots2 package in R (Wickham, 2009). The analysis was performed using R version 3.2.4 (R Core Team, 2015).

RESULTS

By day 38 of the experiment, all individuals in the pH 4 treatment had died, but survival was similar for pH 5, pH 6, and control (pH 7; Figure 1A; Table 1). Individuals exposed to pH 5 showed a significantly slower growth rate (Table 2), and expressed a shallower growth curve in terms of SVL (ANOVA on GCA models, df = 14, $X^2 = 13.63$, $P = 0.001$; Figure 1B) and total length (ANOVA on GCA models, df = 14, $X^2 = 8.63$, $P = 0.01$; Figure 1C) when compared to the control treatment. For groups at both pH 5 and pH 6, growth was not significantly different in terms of mass (ANOVA on GCA models, df = 14, $X^2 = 4.21$, $P = 0.12$; Figure 1D). The growth, in terms of mass, at pH 5 does however seem to indicate a decreased trend compared to the control and pH 6 treatments.

Regarding growth, individuals in the pH 5 treatment were shorter in terms of both total
length and SVL, but maintained similar mass to the pH 6 and 7 treatments (ANOVA, df = 2, F = 7.14, P < 0.004; Figure 2; Tables 3 & 4). In the pH 5 treatment individuals tended to be shorter and stouter than individuals in the control and pH 6 treatments.

**DISCUSSION**

This study has demonstrated that a substrate pH of 4 is lethal to newly metamorphosed marbled salamanders. This means that recovering previously mined landscapes with a soil pH of 4 or less are likely prohibitive to the establishment of salamander populations supporting the best practices recommendations laid out in the Forestry Reclamation Approach (Burger et al., 2005). Even if individuals disperse to the area, post–metamorphosis survival is essential to the continued survival of the population. For example, in the marbled salamander, catastrophic failure of larval cohorts, where all larvae die prior to metamorphosis, has been observed as a somewhat common occurrence in natural populations. In the protected Rainbow Bay population of South Carolina, events of catastrophic failure with no larval survival occurred in 6 of the 22 years on record (Taylor et al., 2006). The population, however, remained stable due to the persistence of terrestrial long-lived adult populations. Taylor et al. (2006) hypothesized that the survival in terrestrial stages is more important for the maintenance of populations than larval survival. The survival of juveniles post-metamorphosis is directly linked the ability of the population to sustain larval failure through the presence of a persistent terrestrial population (Taylor et al., 2006). The time directly following metamorphosis has been shown to be a critical period in determining the chance of survival to the first
reproduction and vulnerability during this life stage is essential for the recruitment of individuals to the breeding population (Rothermel & Semlitsch, 2006).

In addition to the increased mortality at pH 4, I have also demonstrated that substrate acidity at pH 5 is detrimental to the growth of juvenile marbled salamanders in the terrestrial environment. Reduced growth rates during the post-metamorphic juvenile period is likely to lead to a reduction of reproductive fitness by increasing the time to reach maturity and/or limiting the fecundity of individuals (Semlitsch et al., 1988; Smith, 1987). Reduced fitness of individuals residing on acidic substrates has distinct implications for the reclamation of mined lands, especially in light of the importance of salamanders to the forest ecosystem.

A reduction in growth rates during early ontogenetic stages has previously been associated with reductions in fitness. This has commonly been studied by observing the effects of size at metamorphosis on overall fitness. In the marbled (Ambystoma opacum) and spotted salamanders it has been shown that juveniles that weighed more during the initial months post-metamorphosis had a significantly higher chance of survival than smaller individuals (Rothermel & Semlitsch, 2006). In chorus frogs (Hylidae), there are carry-over effects from the larval environment; larvae that metamorphose early to escape hostile conditions will face higher mortality rates alongside a longer time to maturity (Tarvin et al., 2015; Allen et al., 2010). In seasonally breeding amphibians, reduced time to maturity can be especially detrimental as individuals are restricted to breeding at a certain time of year. Chorus frogs will commonly reach maturity a year after metamorphosis, however if their time to sexually maturity is delayed even by a few months they will miss an entire breeding season (Smith, 1987). A similar phenomenon
may occur in the marbled salamander, which can also reach sexual maturity within a year of metamorphosis (Taylor et al., 2006). There may also be a sexually dimorphic effect on the reduction in fitness. In the salamander *Ambystoma talpoideum*, age at first reproduction is only linked to size at metamorphosis for females. Females that are larger at metamorphosis reproduce at a younger age than smaller individuals (Semlitsch et al., 1988).

The mechanism for this increased time to sexual maturity is likely driven by tradeoffs in development seen in the growth of amphibians. In *Xenopus*, there are tradeoffs between somatic and gonadal growth that occur when resources are restricted, however while the growth of both gonads and somatic tissue is reduced by worse conditions, the development of tissues remains constant (McCoy et al., 2007). Even if sexual maturity is reached, size has a clear effect on the number and quality of offspring produced (Salthe, 1969). Specifically, in the marbled salamander, size is directly related to the number and size of eggs produced by females (Scott & Fore, 1995). These tradeoffs and effects of size at metamorphosis are analogous to the reduction in growth during the juvenile stage. Both have the same effect in increasing the time required to reach sizes necessary for maturity. It is clear that a reduction in growth during the juvenile stage, as observed during this study, can lead to a decrease in fitness across an individual’s lifetime.

Currently, the most successful reclamation approach for Appalachian forests, the forestry reclamation approach (FRA), recommends soil at a pH between 5 and 7 (Burger et al., 2005). Successful implementation varies between tree types; pines prefer more acidic soils than oaks, oaks grow poorly on soils with a pH level of 4.7 (Burger et al.,
Therefore, contemporary mining companies employing the forestry reclamation approach may be creating forest ecosystems that are less conducive to the establishment of salamander populations. Salamanders have been repeatedly shown to be important in the function of North American forested ecosystems by providing ecosystem services as mid-level predators, distributing energy and nutrients between aquatic and terrestrial environments during migration, contributing to soil dynamics in the form of burrowing, and as an abundant source of high quality nutrients for higher level consumers (Davic & Welsh, 2004). Additionally, forest ecosystems developed on previously mined landscapes are less efficient at carbon sequestration and specifically showed a reduction in soil carbon (Amichev et al., 2008). The plethodontid salamander *Ensatina eschscholtzii* was found to increase litter retention, likely through its role as a consumer of leaf litter invertebrates. The retention of leaf litter keeps carbon in the soil rather than being released to the atmosphere (Best & Welsh Jr, 2014). While salamanders may not be the only cause of reduced carbon sequestration it is clear that their presence in forest ecosystems would help maintain carbon in the reforestation of Appalachian coal spoils.

This study shows that at a pH of 5 or less there is a significant reduction in the growth and survival of the marbled salamander, which can lead to slower development and a lower fitness over the course of an individual’s life. This loss in fitness has the potential to reduce salamander populations in recovering forests and decrease the potential for salamanders to increase forest carbon sequestration. As such, in forestry reclamation projects with an interest in aiding the recolonizations of salamanders, I recommend targeting a soil pH of 6 or higher in order to provide better conditions for post–metamorphic growth.
CHAPTER TWO

THE EFFECTS OF PH AND SULFATE ANIONS ON THE GROWTH AND SURVIVAL OF AMBYSTOMA MACULATUM AND AMBYSTOMA JEFFERSONIANUM

The degree to which acidified substrates affect amphibians appears to vary by species. For example, Ambystoma opacum larvae are only found in ponds with a pH of 4 or higher, while A. mabeei was found to be unaffected by pond acidity (Fairman et al., 2013). It is unclear whether the association between acidity and salamander presence is due to differences in embryonic/larval survival or if acidic sites are being selected against as oviposition sites. Other salamanders in the family Ambystomatidae have been shown to select oviposition sites for various factors but it is unclear whether they select sites based on acidity (Kern et al., 2013). Both of these studies illustrate both the effects of acidity on a landscape scale and species-specific vulnerability to acidification of breeding pools (Fairman et al., 2013).

Amphibian eggs deposited in acidic conditions have been shown to be vulnerable to the effects of acidity both in isolation and in conjunction with other factors. In the European common frog (Rana temporaria), embryonic mortality increases in acidic water only in conjunction with an increase in aluminum concentration in the water. Similarly gill damage to brook trout (Salvelinus fontinalis) in acidified lakes was exacerbated by high levels of aluminum (Beattie & Tyler-Jones, 1992; Chevalier et al., 1985). There is much variety in resistance to acidity in amphibian eggs, with some individuals demonstrating tolerances as low as pH 4.5. For example, embryonic mortality in the African clawed frog (Xenopus laevis) is largely unaffected until acidity reaches pH 4, which is prohibitive to hatching (Dunson & Connell, 1982). In Ambystomatid
salamanders, embryonic mortality is affected by pond acidity but at different levels. Spotted salamander eggs survive until hatching at pH levels ranging from 6–10 but are most successful from pH 7–9 while Jefferson salamander eggs survive at pH levels from 4–8 but are most successful between pH 5–6 (Pough & Wilson, 1976). These closely related species have very different acid–tolerance ranges and, despite the difference in pH preference the species commonly co-occupy ponds (Petranka, 1998).

At the post-embryonic larval stage, amphibians continue to be affected by acidity. For example, in the lab, pH levels of 3 and below are lethal to larval spotted salamanders (Ling et al., 1986) and individuals reared at pH 4.5 suffer a noted reduction in growth and diminished prey–capture ability (Preest, 1993). An increase in acidity can also change interspecific dynamics. When Jefferson and spotted salamanders are raised together in an acidic environment (pH 5.3), spotted salamanders show a significant decrease in survival compared to neutral acid levels due to predation by Jefferson salamanders (Brodman, 1993). Spotted salamander larvae were observed to be more sluggish and this is believed to have accounted for the increase in predation (Brodman, 1993).

It is clear that acidity shows some effect on many species of amphibians at multiple developmental stages, but the levels of acidity necessary to induce a negative effect vary across species and within the range of a single species. To understand this variability in response to acidity, investigation regarding the mechanisms that cause the detrimental effects of acidity is necessary. There are a number of hypotheses related to observed reductions in growth, including the reduction of zooplankton populations, a reduction in feeding success, and a disruption of metabolic functions (Bardwell et al., 2007; Preest, 1993; Ireland, 1991).
Larval salamanders feed largely on zooplankton, and if pond acidification affects zooplankton assemblages, it could reduce food availability and limit growth (Bardwell et al., 2007; Freda, 1983). Information on the effects of acidity on the zooplankton of temporary amphibian breeding pools is limited, but the effects of acidity on zooplankton populations have been studied in various other freshwater ecosystems. In lakes subject to acidification, invertebrate community diversity decreases with decreasing pH, with many species limited by acidity but with some, including some copepods and cladocerans, showing a high level of tolerance to acidification (Roff & Kwiatkowski, 1977; Almer et al., 1974). Additionally, aquatic crustaceans from Canadian tundra ponds are unable to tolerate acidic water and will quickly die off in acid environments. Other species, such as midge larvae, are extremely resistant to acidity and can be found in ponds with pH levels as low as 3 (Havas & Hutchinson, 1982). While a reduction in prey populations may lead to a decrease in growth due to food availability, there is also evidence for a physiological impact on feeding ability. Such impacts may be due to a reduction in prey–capture ability, as the number of lunges at food attempted by larval salamanders at pH 4 was nearly half of those in a neutral pH (Preest, 1993). This reduction in prey–capture ability may be linked to a disruption in metabolic function, and likely works in conjunction with it to cause decreases in growth.

Numerous studies show an acid-mediated reduction in metabolic function in fishes, but studies of the effects on amphibians have been limited. In freshwater fish, low environmental pH has been linked to epithelial damage to the gills along with a significant increase in chloride cells, which are used to pump excess chloride out of the body and are typically associated with habituation to increased salinity (Chevalier et al.,
1985; Leino & McCormick, 1984). This change indicates a reduction in the osmoregulatory ability of fish affected by acidic water, and is accompanied by a reduction in Na\(^+\) and Cl\(^-\) concentration in the blood, the magnitude of which is correlated with Ca\(^{2+}\) concentration (Evans, 1987). In soft (low ion concentration) water, rainbow trout (Salmo gairdneri) shows a greater reduction in Na\(^+\) and Cl\(^-\) blood levels than in harder waters (McDonald, 1983). The increase in Na\(^+\) and Cl\(^-\) efflux is likely due to an increase in permeability across the brachial epithelium. Exposure to acid conditions leads to the loss of membrane bound Ca\(^{2+}\) at two binding sites, one of which is involved in membrane permeability, leading to the leakage of Na\(^+\) and Cl\(^-\) in acid conditions (McWilliams, 1983).

Similar effects are seen in amphibians, suggesting that acid acts through the same mechanism. In frogs of the family Ranidae, a reduction of sodium influx and an increased sodium efflux is seen at low experimental pH treatments (Freda & Dunson, 1984). This change in osmoregulatory ability is likely responsible for the reduction in growth seen with chronic exposure to low pH of Rana sylvatica in ponds with pH at 4–5, showing lower body sodium, chloride and water content than those in ponds with a pH of 6–7 (Freda & Dunson, 1985). The increase in sodium efflux has also been seen in terrestrial salamanders on acidic substrates (Frisbie & Wyman, 1991). Sodium and potassium are essential in various cell functions as part of sodium/potassium ATP mediated pumps and channels, and the impairment of their function could lead to increased energy expenditure for the maintenance of homeostasis leading to less energy expenditure on growth.

In spotted salamanders from Virginia, it has been shown that in acid concentrations at a pH of 5.5 and below and sulfate adjusted to -log anion concentrations,
in order to provide an analogous system to pH, of 5.5 and below, larvae show a decrease in growth in terms of wet mass (Ireland, 1991). This study showed sulfates to be an important factor in the reduction of growth by showing a reduction in both solutions with sulfuric acid as well as solutions with sulfate salts, but not in salt solutions without sulfates. Additionally acid solutions made without sulfates (acetic acid) showed no reduction in growth until pH 4.5 (Ireland, 1991). Similarly, sulfuric acid shows an increased ability to displace membrane–bound calcium compared to nitric and hydrochloric acids, suggesting a link between growth loss, membrane bound calcium, and sulfates (McWilliams, 1983).

This study reports a laboratory experiment of the effect of pH compared to that of sulfates on the growth and survival of larval spotted (Ambystoma maculatum) and Jefferson salamanders (A. jeffersonianum). I hypothesized that there would be a difference between the effects of acidity and sulfates on the growth of two species of Ambystomatid salamanders due to the more acidic preferences in the Jefferson salamander compared to the spotted. The results of this work will further clarify the mechanism behind the decreases in growth and survival seen among larval salamanders when exposed to acidic water and could provide further data on regional tolerances to differing levels of acidity.

**Materials and Methods**

*Study Species* — The spotted salamander is found almost throughout the state of Kentucky (Petranka, 1998). Courtship and oviposition in Kentucky occur between mid-February and early March (Keen, 1975). Egg masses of up to 250 eggs are typically laid
in ephemeral ponds, including vernal pools, roadside ditches and larger puddles (Kern et al., 2013). In the southern portion of its range, including Kentucky, larvae metamorphose between June and August (Petranka, 1998). Juveniles disperse post-metamorphosis under leaf litter around their natal pond and eventually overwinter underground (Stenhouse, 1985).

The Jefferson salamander is found in the northern interior plateau of Kentucky (Petranka, 1998). In Kentucky, breeding occurs between January and February (Douglas, 1979). Egg masses consist of up to 60 eggs with an average of 16 per egg mass (Petranka, 1998). Similarly to spotted salamanders, post-metamorphic juveniles disperse through and reside in forest habitats under woody debris (Rothermel & Semlitsch, 2006).

Experimental Design — Ten Jefferson salamander egg masses were collected from an autumnal pool in Hart County, Kentucky, in the Western Kentucky University Green River Preserve, during the spring of 2016. Three spotted salamander egg masses were collected from an autumnal pool in Warren County Kentucky. Eggs were hatched in 4-foot-diameter plastic pools on March 13th and were maintained there until April 7th to account for initial unexplained intrinsic mortality. During this period, hatchlings were fed zooplankton collected from vernal pools in Warren County, KY. On April 7th larvae were haphazardly assigned to one of three reduced pH treatments, three increased sulfate anion treatments or a neutral pH control treatment. Each treatment began at a pH of approximately 7 and was gradually adjusted to the targeted pH over the course of three weeks. Each individual was housed in a 90ml plastic bathroom cup filled with approximately 80ml of modified Holtfreter’s solution for the first 80 days of the
experiment, at which time they were transferred to deli cups filled with approximately 400ml of modified Holtfreter’s solution (40%).

The three reduced pH treatments were adjusted to pH 4, 5, or 6 with 1M sulfuric acid. The sulfate anion treatments were adjusted to a -log sulfate concentration of 3, 4, 5, or 6 with 1M sodium sulfate, all of which were at neutral acidity near pH 7. The control was unadjusted for pH and was approximately pH 7 and there were no sulfates added. I assigned 10 animals to each treatment for a total of 70 animals per species. All individuals were raised at 20°C on a 12L:12D cycle. All animals were fed brine shrimp (Artemia sp.) for the first 70D and were fed California blackworms (Lumbricus variegatus) for the remaining 90D of the study or until metamorphosis. Individuals were exposed to their respective treatments from June 2016 until they metamorphosed between August and September 2016. Snout-vent length (SVL) was measured by taking each individual out of their respective container using a piece of fiberglass mesh and measuring the length with a ruler. Individuals were then placed into a cup of water on a digital balance to measure their mass. Measurements were taken every two weeks.

Statistical Analyses — Growth was analyzed by comparing the change in both mass and SVL over the course of the experiment with Growth Curve Analysis (Mirman et al., 2008). A series of models were constructed for both change in mass and SVL in order to simulate the growth curves of each treatment group. The simulated growth curves were compared to elucidate differences caused by individual treatment groups. The measurements of mass and SVL were compared with time and random effects were included in order to create the base model. In each model, growth was used as the
dependent variable and time was included as the independent variable. Treatment was included as a fixed effect term in the models and individual ID was included as a random effect term. To avoid problems with collinearity, time data were transformed into orthogonal polynomials prior to model construction. Models were constructed adding treatment, interaction between treatment and the first order orthogonal polynomials constructed for time, and interaction between treatment and second order polynomials constructed for time. The lme4 package was used to create the mixed-effect models in R (Bates et al., 2015). Models were constructed for each treatment and comparisons were performed by analysis of variance (ANOVA). Model comparisons were tested in terms of both SVL and mass, and were visualized using the R package ggplots2 (Wickham, 2009). The analysis was performed using R version 3.2.4 (R Core Team, 2015).

RESULTS

Within 14 days at pH 4, all individuals of both species had died. For spotted salamanders, the pH 5 and pH 6 treatments showed an increase in mortality with only 50% survival in each treatment compared to 90% in the control treatment (pH 7; Figure 3A; Table 5). Survival in spotted salamanders was not affected by any sulfate treatment (Figure 3B; Table 7). For Jefferson salamanders, survival was not affected by the higher pH treatments (pH 5 and 6; Figure 3C; Table 6). Jefferson salamanders in the –log 6 treatment experienced a 50% reduction in survival (Figure 3D; Table 7).

In Jefferson salamanders there was no significant reduction in growth rate in acid treatments for either SVL (ANOVA on GCA models, $X^2 = 0.85$, df = 6, P = 0.65; Figure
4A; Table 8A) or mass (ANOVA on GCA models, $X^2 = 3.3$, df = 6, $P = 0.19$; Figure 4B; Table 8B) or in sulfate treatments for either SVL (ANOVA on GCA models, $X^2 = 5.14$, df = 6, $P = 0.27$; Figure 4C; Table 9A) or mass (ANOVA on GCA models, df = 6, $X^2 = 5.54$, $P = 0.23$; Figure 4D; Table 9B).

In spotted salamanders there was no significant reduction in growth rate in acid treatments for either SVL (ANOVA on GCA models, $X^2 = 2.47$, df = 6, $P = 0.29$; Figure 5A; Table 10A) or mass (ANOVA on GCA models, $X^2 = 3.0$, df = 6, $P = 0.22$; Figure 5B; Table 10B). In Jefferson salamanders the -log 3 and -log 4 sulfate treatments however showed a significant reduction in growth for SVL (ANOVA on GCA models, $X^2 = 15.3$, df = 6, $P = 0.004$; Figure 5C; Table 11A). However, there was no reduction in terms of mass (ANOVA on GCA models, df = 6, $X^2 = 5.91$, $P = 0.20$; Figure 5D; Table 11B). The growth based on SVL, in both species, was the highest in the control treatments (Figures 6A & C and 7A & C), while mass growth is the lowest in the control treatment (Figures 6B & D and 7B & D). In the Jefferson salamander there was a difference between the mass of treatments (ANOVA, df = 2, $F = 4.8$, $P = 0.02$; Figure 7; Table 12). Mass was significantly increased in the pH 5 treatment (Tukey HSD, df = 2, $P = 0.02$; Table 12). There was no significant effect of pH or sulfates on total growth in spotted salamander larvae (Tables 13 & 14). There was no significant effect of sulfates on the total growth of Jefferson salamander larvae (Table 15). There was no significant effect of treatment on time to metamorphosis in either spotted (ANOVA, df = 5, $F = 1.28$, $P = 0.23$; Figure 8; Table 16B & D) or in Jefferson salamanders (ANOVA, df = 5, $F = 1.05$, $P = 0.51$; Figure 9; Table 16A & C).
DISCUSSION

In both species I tested, water at pH 4 is prohibitory to the survival of larvae. Therefore, natural breeding pools that have had acid deposition lowering pool acidity in the pH 4-5 range will likely be detrimental to the growth and survival of salamander larvae and reduce recruitment from ponds to the terrestrial environment. Populations inhabiting acidified pools will face reproductive failure events, but those events can be buffered by the persistence of long-lived individuals at the terrestrial stage (Warner et al., 1991) if conditions are only transiently acidic. In long-lived ambystomatid salamanders, terrestrial individuals can safeguard against local extinction for a longer period of time, however if acidic conditions are persistent, then repeated reproductive failures could lead to local extinction events. In a long-term study of *Eurycea quadridigitata*, a pond-breeding plethodontid salamander, multiple years of reproductive failure led to the local extinction of the species at Rainbow Bay (Semlitsch et al., 1996). With the increasing fragmentation of habitats, however, the size of local populations are being diminished, leaving each individual population more prone to a local extinction event (Taylor et al., 2006; Cushman, 2006).

In the acid experiment on spotted salamanders I see a reduction in survival to 50% at pH 5 and pH 6. Previous studies have shown a comparable survival rate in embryos and additionally the same differential survivorship between eggs of spotted and Jefferson salamanders (Pough & Wilson, 1976). In the sulfate trials I see a reduction in survival to 50% at the -log 6 anion concentration. This reduction is likely due to sampling error in the -log 6 anion concentration. I see unaffected survival levels at both higher and lower sulfate levels, so I cannot confidently provide a plausible context for this result.
My study supports previous work showing a slight reduction in growth in spotted salamanders, however I see a lesser effect from acidity than has previously been reported (Preest, 1993; Ireland, 1991). This difference may be due to variation in feeding regimens, as my animals were fed *ad libitum* compared to more restricted diets in other studies, which may have increased the ability to resist acid stress. In the pH 5 treatment, there was no initial reduction in growth, but instead growth was reduced near the end of larval stage, resulting in the pH 5 treatment generating the smallest animals at metamorphosis. It has been repeatedly shown that a smaller size at metamorphosis causes a series of carry-over effects from the larval period to terrestrial life stages; individuals who were smaller at larval stages show reduced lifetime fecundity and an increased time to sexual maturity (Tarvin et al., 2015; Allen et al., 2010; Scott, 1994). This study also reinforces studies showing acid tolerance in Jefferson salamanders as I saw no change in growth or development across any of my treatments, but lethal acid levels remain similar to those in spotted salamanders (Brodman, 1993; Pough & Wilson, 1976). My data also seems to show a decrease in growth caused by the dual effects of hydrogen ions and sulfate anions as the mechanism of acid damage. I see a reduction in growth in in sulfate treatments, as has been seen previously (Ireland, 1991), but additionally shows how reductions in growth vary over the course of the larval period.

My findings in -log3 and -log4 treatments in the sulfate experiment support sulfates as a driver of reductions in growth caused by acidification of breeding ponds. For example, in spotted salamanders I saw a change in length when individuals were exposed to sulfate levels of -log 4 anion concentration; growth is slowed initially, but individuals experienced an uptick in growth close to metamorphosis. This slower initial growth
would have the effect of limiting prey availability in natural ponds, as larvae are gape limited and will incorporate new prey into their diet as they grow (Petranka, 1998). This in turn could lead to a further decrease in growth caused by a decrease in food availability resulting in smaller larvae more vulnerable to predation. Similar phenomena have been shown in single-factor studies on insecticides which showed minimal effects, but led to a large scale change when predators were present in full ecosystems (Boone & Semlitsch, 2001).

The effect of sulfates is very similar to the effect seen with acidic environments; in fish, it has been shown that the acid acts by depleting body sodium and chloride ions (Evans, 1987). This depletion in acid environments is due to the removal of calcium ions bound to the cell wall, leading to leaky gill membranes (McWilliams, 1983). Sulfates seem to be important as more calcium is removed in acidic conditions with sulfuric acid than in acids without sulfates (McDonald, 1983). Sulfates may in fact strip membrane calcium to form calcium sulfate without the addition of acidity; however further studies are needed to determine whether sulfates will reduce membrane–bound calcium independent of acidity and additionally if sulfate action will reduce blood concentrations of sodium and chloride.

In contrast to previous studies, I saw a change in the length rather than in the growth in terms of mass (Ireland, 1991). In my treatments, I see a differential allotment of resources, as individuals in an acid or sulfate–heavy environment apportion resources to growth in terms of weight rather than in growth in terms of length. I saw this same phenomenon post-metamorphosis in the marbled salamander, where individuals exposed to acid conditions expend more energy in growing longer, but maintain mass similar to
shorter individuals, showing some change in resource allocation (Chapter 1). There are many examples of differential resource allocation in response to environmental stressors (Congdon et al., 2001; Relyea, 2001; Emerson, 1986). For example, marbled salamanders will invest more heavily in egg lipid levels in high food conditions, showing adaptive levels of lipid allocation based on environmental stressors (Scott & Fore, 1995).

The von Bertalanffy model (von Bertalanffy, 1938) of somatic growth shows an inverse relationship between optimal age to maturity and the adult mortality rate where an increase in length directly competes with an investment in reproduction (Lester et al., 2004). My data suggest a similar scenario, where lipid deposition for energy storage might compete with somatic growth (i.e., changes in length). In the presence of the environmental stressor presented by increased acidity, nutrients may be allocated to the storage of energy rather than somatic growth. This may allow for individuals to store energy for more efficient growth in a less stressful environment post–metamorphosis, or to prepare for an increase in the stressor if contaminant concentrations increase during pool drying. This relationship is however complicated as time to metamorphosis is not changed. The change in length may be unrelated to time to metamorphosis and could indicate progress towards the development of reproductive structures.

My data show a very limited effect of acidity in the rage of pH 5-7 on the growth and development of salamander larvae, however, even a small effect can work in conjunction with other environmental stressors to have greater negative consequences (Beattie & Tyler-Jones, 1992). In my study system, animals were fed ad libitum in order to provide each individual as much food as possible to see the pure effects of acidity on individual organisms. One potential factor that will work in conjunction with acidity is
food availability, and the interaction may be complex. First, food may be limited due to changes in prey populations after pond acidification (Roff & Kwiatkowski, 1977; Almer et al., 1974). Food availability may work together with the exclusion of larval cohorts from available food sources due to an initial depression in growth as smaller individuals would be unable to consume certain food sources due to gape limitation (Harris, 1995). Additionally, available food levels determine levels of lipid storage during larval growth, and in the absence of excess nutrients, individuals may not have energy stores necessary to handle acid stress (Scott & Fore, 1995). My data may show a compensatory growth response in which individuals are able to grow at faster rates after growth depression if favorable conditions are restored in order to return to the ideal growth trajectory (Ali et al., 2003). In my sulfate treatments, individuals seem to show an initial reduction in growth followed by a compensatory growth period. Without ample food resources this early depression in growth could remain throughout the rest of the larval period. Further studies are required to fully understand the relationships between acids, sulfates and food levels in pond communities and ecosystems.

This study provides a starting point for looking further into the effects of acid deposition on pond ecosystems. It highlights the importance of looking at effects of acid deposition on multiple levels. The effects of sulfates present an important concern for conservation efforts to restore pools, as even in pools where acid has been neutralized sulfate deposition could be a limiting factor to the growth of pond breeding amphibian populations. If populations are further stressed, limitation of growth by sulfate deposition could lead to catastrophic reproductive failure events, which can eventually lead to local extinction events (Taylor et al., 2006; Semlitsch et al., 1996). It is also possible that pools
with high sulfate concentrations may prohibit establishment of amphibian populations, however further research is required to look into lethal sulfate concentrations.

In conclusion, this study presents novel evidence showing the dual effects of sulfates anions and hydrogen ions on the growth and development of two species of salamander larvae. The spotted salamander shows sensitivity to acid and sulfate deposition at pH 5 and below as well as at -log 3 and 4 anion concentrations. In Jefferson salamanders I demonstrated greater tolerance, as I saw no reductions in growth at any pH or sulfate levels. Regardless of acid tolerance, water at pH 4 is restrictive to the survival of amphibian larvae. Further research is required to ascertain if sulfates work similarly to acid deposition with the reduction of body sodium and chloride ions, due to the stripping of calcium from cell walls. Additionally, more studies investigating the effects of acidity alongside other stressors are important to identify compound effects with acid deposition.
LITERATURE CITED


R Core Team. 2015. R: A language and environment for statistical computing.


APPENDIX A

Tables

Table 1. A) ANOVA table for analysis of time to survival across pH treatments for recently metamorphosed marbled salamanders (*Ambystoma opacum*); B) Tukey HSD table for analysis of time to survival across pH treatments. Significance codes: ‘***’ 0.001, ‘**’ 0.01, ‘*’ 0.05.

A. | df | SS   | MS   | F     | P          |
---|----|------|------|-------|------------|
Treatment | 3  | 141356 | 47119 | 31.49 | <0.001*** |
Residuals  | 97 | 145122 | 1496  |       |            |

B. | Diff | lwr | upr | p adj |
---|------|-----|-----|-------|
$pH4$-Control | -92.20 | -120.52 | -63.87 | <0.001*** |
$pH5$-Control | -14.51 | -42.84 | 13.81  | 0.54   |
$pH6$-Control | -4.83  | -33.16 | 23.49  | 0.97   |
$pH5$-$pH4$  | 77.68  | 49.08 | 106.28 | <0.001*** |
$pH6$-$pH4$  | 87.36  | 58.76 | 115.96 | <0.001*** |
$pH6$-$pH5$  | 9.68   | -18.92| 38.28  | 0.81   |
Table 2. Parameter estimates for analyses of variance on growth curve models. Models were constructed with SVL, total length or mass compared with the fixed effects of both linear and quadratic time points and the random effects of individual ID. A) Effect of acidity on change in length (SVL) over 113 days of recently metamorphosed marbled salamanders (*Ambystoma opacum*); B) effect of acidity on growth in terms of change in total length; C) effect of acidity on growth in terms of change in mass.

<table>
<thead>
<tr>
<th>Estimate</th>
<th>Std. Error</th>
<th>t value</th>
</tr>
</thead>
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<td>Linear</td>
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<td>Quadratic</td>
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<td>pH6 treatment</td>
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<td>pH6 treatment: Linear</td>
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<td></td>
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<tr>
<td>Intercept</td>
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Table 3. ANOVA table for analysis of recently metamorphosed marbled salamander (*Ambystoma opacum*) in terms of A) total change in SVL across pH treatments; B) total change in total length across pH treatments; C) total change in mass across pH treatments. Significance codes: ‘****’ 0.001, ‘***’ 0.01, ‘*’ 0.05.

<table>
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Table 4. Tukey HSD table for analysis of A) total change in SVL across pH treatments in recently metamorphosed marbled salamanders (*Ambystoma opacum*); B) total change in total length across pH treatments. Significance codes: ‘***’ 0.001, ‘**’ 0.01, ‘*’ 0.05.

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<th>upr</th>
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Table 5. A) ANOVA table for analysis of time to survival across pH treatments in spotted salamanders (*Ambystoma maculatum*); B) Tukey HSD table for analysis of time to survival across pH treatments. Significance codes: ‘***’ 0.001, ‘**’ 0.01, ‘*’ 0.05.

A. | df | SS  | MS  | F   | P   |
---|----|-----|-----|-----|-----|
Treatment | 3  | 70490 | 23497 | 8.46 | <0.001*** |
Residuals  | 38 | 105487 | 2776  |      |      |

B. | Diff | lwr | upr | p adj |
---|------|-----|-----|-------|
pH4-Control | -113.66 | -147.27 | -53.06 | <0.001*** |
pH5-Control | -52.97  | -113.57 |  7.64 |  0.11  |
pH6-Control | -51.27  | -111.87 |  9.34 |  0.12  |
pH5-pH4    |  60.70  |  -2.60 | 124.00 |  0.06  |
pH6-pH4    |  62.40  |  -0.90 | 125.70 |  0.05* |
pH6-pH5    |  1.70   |  61.60 |  65.00 |  0.99  |
Table 6. A) ANOVA table for analysis of time to survival across pH treatments in Jefferson salamanders (*Ambystoma jeffersonianum*); B) Tukey HSD table for analysis of time to survival across pH treatments. Significance codes: ‘***’ 0.001, ‘**’ 0.01, ‘*’ 0.05.

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Table 7. ANOVA table for analysis of time to survival across sulfate treatments in A) Jefferson salamanders (*Ambystoma jeffersonianum*) and B) spotted salamanders (*Ambystoma maculatum*).

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Table 8. Parameter estimates for analyses of variance on growth curve models. Models were constructed with SVL or mass compared with the fixed effects of both linear and quadratic time points and the random effects of individual ID. **A)** Effect of pH on change in length (SVL) over 113 days of larval Jefferson salamanders (*Ambystoma jeffersonianum*); **B)** effect of acidity on growth in terms of change in mass.

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<td>Linear</td>
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<td>pH 6 Treatment</td>
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<td>pH 6 Treatment</td>
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Table 9. Parameter estimates for analyses of variance on growth curve models. Models were constructed with SVL or mass compared with the fixed effects of both linear and quadratic time points and the random effects of individual ID. A) Effect of sulfates on change in length (SVL) over 113 days of larval Jefferson salamanders (*Ambystoma jeffersonianum*); B) effect of acidity on growth in terms of change in mass.

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<td>-log anion 4 Treatment</td>
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<td></td>
<td>-log anion 5 Treatment</td>
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<td></td>
<td>-log anion 6 Treatment</td>
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Table 10. Parameter estimates for analyses of variance on growth curve models. Models were constructed with SVL or mass compared with the fixed effects of both linear and quadratic time points and the random effects of individual ID. A) Effect of sulfates on change in length (SVL) over 113 days of larval spotted salamanders (*Ambystoma maculatum*); B) effect of acidity on growth in terms of change in mass.

<table>
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<td><strong>B.</strong> Intercept</td>
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Table 11. Parameter estimates for analyses of variance. A) Effect of pH on change in length (SVL) over 113 days of larval spotted salamanders (*Ambystoma maculatum*); B) effect of acidity on growth in terms of change in mass.

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<td>pH 6 Treatment</td>
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<td>B. Intercept</td>
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Table 12. ANOVA table for analysis of growth of larval Jefferson salamanders (*Ambystoma jeffersonianum*) in terms of **A)** total change in SVL across pH treatments; **B)** total change in mass across pH treatments; **C)** Tukey HSD table for analysis of total change in SVL across pH treatments. Significance codes: ‘***’ 0.001, ‘**’ 0.01, ‘*’ 0.05.

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<td><strong>B.</strong></td>
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<td><strong>C.</strong></td>
<td>Diff</td>
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<td>upr</td>
<td>p adj</td>
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<td>pH5-Control</td>
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Table 13. ANOVA table for analysis of growth of larval spotted salamanders (`Ambystoma maculatum`) in terms of **A**) total change in SVL across pH treatments; **B**) total change in mass across pH treatments

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Table 14. ANOVA table for analysis of growth of larval spotted salamanders (*Ambystoma maculatum*) in terms of A) total change in SVL across sulfate treatments; B) total change in mass across sulfate treatments

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Table 15. ANOVA table for analysis of growth of larval Jefferson salamanders (*Ambystoma jeffersonianum*) in terms of **A**) total change in SVL across sulfate treatments; **B**) total change in mass across sulfate treatments

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Table 16. ANOVA table for analysis of time to metamorphosis across pH treatments in A) Jefferson salamanders (*Ambystoma jeffersonianum*) and B) spotted salamanders (*Ambystoma maculatum*) and across sulfate treatments in C) Jefferson salamanders (*Ambystoma jeffersonianum*) and D) spotted salamanders (*Ambystoma maculatum*).

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</table>
APPENDIX B

FIGURES

Figure 1. Survival and growth of recently metamorphosed marbled salamanders. A) Failure-time curve indicating percent of individuals surviving over the course of 113 days. Recently metamorphosed marbled salamanders (*Ambystoma opacum*) were raised individually on substrates at pH4, pH 5, pH 6 and an unadjusted control substrate at pH 7. Survival was recorded daily. (B-D) Observed growth data and growth curve model fits for change in B) snout vent length, C) mass and D) total length over 113 days of recently metamorphosed marbled salamanders (*Ambystoma opacum*). Each individual was raised independently on substrates at pH 5, pH 6 and an unadjusted control substrate at pH 7. Symbols indicate observed data points; dashed lines indicate growth curve model fits. Vertical lines indicate +/- standard error (SE).
Figure 2. Growth of recently metamorphosed salamanders over the course of 113 days in terms of A) SVL, B) total length and C) mass. Each individual was raised independently on substrates at pH 5, pH 6 and an unadjusted control substrate at pH 7. Boxed areas represent interquartile range, while dashed lines represent a minimum or maximum of 1.5x the interquartile range. The dark central line indicates the median and the dark closed circles represent the mean. Open circles represent outlier values.
Figure 3. Failure-time curve indicating percent larval survival. **A)** Spotted salamander larvae exposed to pH treatments, **B)** spotted salamander larvae exposed to sulfate anion treatments, **C)** Jefferson salamander larvae exposed to pH treatments, and **D)** Jefferson salamander larvae exposed to sulfate anion treatments. Larvae were raised individually in solutions adjusted with sulfuric acid or sodium sulfate. Acid treatments were adjusted pH 4, pH 5, pH 6, while sulfate treatments were adjusted to -log anion concentrations at -log 3, -log 4, -log 5 and -log 6. One treatment group was left unadjusted at a pH of 7 without the addition of sulfates. Survival was recorded daily.
Figure 4. Observed growth data and growth curve models of (A-B) Jefferson salamanders exposed to pH treatments recording change in A) snout vent length and B) mass. (C-D) Jefferson salamanders exposed to sulfate treatments recording change in C) snout vent length and D) mass. Each individual was raised independently in solutions adjusted with sulfuric acid or sodium sulfate. Acid treatments were adjusted to pH 5 or pH 6, while sulfate treatments were adjusted to -log anion concentrations at -log 3, -log 4, -log 5 and -log 6. One treatment group was left unadjusted at a pH of 7 without the addition of sulfates. Symbols indicate observed data points; dashed lines indicate growth curve model fits. Vertical lines indicate +/- standard error (SE).
Figure 5. Observed growth data and growth curve models of (A-B) spotted salamanders exposed to pH treatments recording change in A) snout vent length and B) mass. (C-D) spotted salamanders exposed to sulfate treatments recording change in C) snout vent length and D) mass. Each individual was raised independently in solutions adjusted with sulfuric acid or sodium sulfate. Acid treatments were adjusted to pH 5 or pH 6, while sulfate treatments were adjusted to -log anion concentrations at -log 3, -log 4, -log 5 and -log 6. One treatment group was left unadjusted at a pH of 7 without the addition of sulfates. Symbols indicate observed data points; dashed lines indicate growth curve model fits. Vertical lines indicate +/- standard error (SE).
Figure 6. Growth of larval spotted salamanders for acid treatments recording change in A) snout vent length and B) mass and sulfate treatments recording change in C) snout vent length and D) mass. Each individual was raised independently in solutions adjusted with sulfuric acid or sodium sulfate. Acid treatments were adjusted to pH 5 or pH 6, while sulfate treatments were adjusted to -log anion concentrations at -log 3, -log 4, -log 5 and -log 6. One treatment group was left unadjusted at a pH of 7 without the addition of sulfates. Boxed areas represent interquartile range, while dashed lines represent a minimum or maximum of 1.5x the interquartile range. The dark central line indicates the median and the dark closed circles represent the mean. Open circles represent outlier values.
Figure 7. Growth of larval Jefferson salamanders for acid treatments recording change in A) snout vent length and B) mass and sulfate treatments recording change in C) snout vent length and D) mass over 112 days. Each individual was raised independently in solutions adjusted with sulfuric acid or sodium sulfate. Acid treatments were adjusted to pH 5 or pH 6, while sulfate treatments were adjusted to -log anion concentrations at -log 3, -log 4, -log 5 and -log 6. One treatment group was left unadjusted at a pH of 7 without the addition of sulfates. Boxed areas represent interquartile range, while dashed lines represent a minimum or maximum of 1.5x the interquartile range. The dark central line indicates the median and the dark closed circles represent the mean. Open circles represent outlier values.
Figure 8. Failure-time curve indicating percent of spotted salamander individuals remaining in the larval stage for A) acid treatments and B) sulfate treatments. Boxplots depicting average time to metamorphosis for C) acid treatments and D) sulfate treatments. Boxed areas represent interquartile range, while dashed lines represent a minimum or maximum of 1.5x the interquartile range. The dark central line indicates the median and the dark closed circles represent the mean. Open circles represent outlier values.
Figure 9. Failure-time curve indicating percent of Jefferson salamander individuals remaining in the larval stage for A) acid treatments and B) sulfate treatments. Boxplots depicting average time to metamorphosis for C) acid treatments and D) sulfate treatments. Boxed areas represent interquartile range, while dashed lines represent a minimum or maximum of 1.5x the interquartile range. The dark central line indicates the median and the dark closed circles represent the mean. Open circles represent outlier values.
APPENDIX C

CATTLE TANK MESOCOSM METHODS

My laboratory data presented evidence of the potential for acid deposition to contribute to variation in salamander community dynamics, so I initiated a study of the effects of pH in experimental pond mesocosms. There is evidence that acid deposition can cause changes in community interactions. For example, in acidic vernal pools Jefferson salamanders are able to produce a greater number of juveniles than spotted salamanders (Pough & Wilson, 1976), which is supported by my experiment showing Jefferson salamanders to have a higher resistance to acid conditions (Chapter 2). Variation in tolerance or resistance may lead to changes in community dynamics in environments of varying acidity, allowing Jefferson salamanders to act as a predator on spotted salamanders in acidic ponds (Brodman, 1993).

Pond mesocosms represent an important step towards understanding the effects of stressors on community dynamics, allowing for a better simulation of effects than laboratory studies can provide (Rowe & Dunson, 1994). Previous work has shown that observing the joint effects of multi-species interactions and varied conditions in mesocosms present a better understanding of community dynamics than similar studies conducted in laboratory settings (Boone et al., 2004, 2007; Figiel Jr & Semlitsch, 1990; Semlitsch, 1987), and the effects of pH have been studied in pond mesocosms previously. Warner et al. (1991) used 7 various pH treatments to look at the effects of acidity on the growth and development two species of hylid frog tadpoles. Sadinski and Dunson (1992) looked into the effects of acidity on community composition including Jefferson and spotted salamanders, and showed that all Jefferson salamanders died at pH 4.2 regardless
of community composition, and most spotted salamanders died at the same pH. Jefferson salamanders have typically been shown to exhibit greater acid tolerance than spotted salamanders so this result may represent a significant difference indicated by simulated mesocosms compared to lab studies (Pierce, 1985).

The objective of my experiment was to evaluate the effects of acidity on two species of salamander that commonly share breeding ponds, the spotted salamander (A. maculatum) and Jefferson salamanders (A. jeffersonianum). I have determined that animals raised in the lab are equally affected by pH (Chapter 2), which is not in agreement with the existing literature. Therefore, I was interested in evaluating the effects under the more natural, yet still controlled, conditions that cattle tank mesocosms provide. However, my investigation failed due to methodological issues involving the set up and maintenance of appropriate acidity treatments. This appendix provides an account of these initial experimental methods and a modified strategy that proved more successful. The intent is to provide a framework for further mesocosm studies investigating the effects of acidity on community dynamics.

**Materials and Methods**

Jefferson salamander egg masses were collected on February 27\textsuperscript{th} from ‘Knob Pond’ in Hart County Kentucky, in the Western Kentucky University Green River Preserve, during the spring of 2016. Spotted salamander egg masses were collected on February 24\textsuperscript{th} from an ephemeral pool off of Hadley Cohron Rd. in Warren Co., KY. Eggs were hatched in 4-foot-diameter plastic pools on March 13\textsuperscript{th}, 2016.
An array of thirty-six Ace-Roto Mold 320 gallon cattle tanks were arranged in a 6x6 grid outside of the Hadley Research Station of Western Kentucky University near Hadley, KY. Mesocosms were filled using county water over the course of two weeks and were all filled by March 9th, 2016. Once all tanks were filled they were adjusted with sulfuric acid to nine replicates of one of four treatments: pH 4, pH 5, pH 6 or unadjusted at ~pH 7. Each set of nine treatments was broken up into three replicates with 12 spotted salamander (*Ambystoma maculatum*) larvae, three replicates with 12 Jefferson salamander (*A. jeffersonianum*) larvae and three mixed replicates with six spotted salamander larvae and six Jefferson salamander larvae. Acidity was adjusted by calculating the amount of acid required to reach the desired pH, and adding the determined volume of acid all at once. Pond acidity was monitored every three days with a THZY pH pen tester and adjusted to the desired pH with sulfuric acid. To monitor acidity, a deli cup of water was taken from each tank and tested. Acid was then diluted in the testing cup of water and added to the tank.

After each tank was acidified on March 9th, 2016, approximately 15 southern leopard frog (*Rana sphenocephala*) tadpoles were added to each tank to control algal levels. Planktonic invertebrates were collected from the Lock Bend Peninsula of the Barren River from ‘Lower Fairy Shrimp’ pond. The first batch was collected on March 10th, 2016. Invertebrates were collected by pouring 20 five-gallon buckets of pond water through a mesh strainer (100 microns). Five hundred milliliters of concentrated zooplankton were added to each cattle tank. Larval Jefferson salamanders were added upon hatching on March 13th, 2016. A second supplementary batch of concentrated
zooplankton was collected and added on March 17th, 2016. Larval spotted salamanders were added upon hatching on March 19th, 2016.

Tank pH levels crashed on March 23rd, 2016 after staying somewhat stable for a period of two weeks. The crash consisted of pH levels dropping suddenly below desired levels to around pH 3. All but one tank in both the pH 4 and pH 5 treatments crashed. After tank pH levels had crashed, surviving larvae were removed from the tanks and brought into the lab on April 7th, 2016. Around 75% of Jefferson salamanders were found for removal, but I was unable to find more than a few spotted salamanders. All tanks at pH 4 and 5 were drained and refilled by April 29th, 2016. All tanks were adjusted to a new pH configuration for a second attempt to implement the experiment.

It was too late in the year to obtain new egg masses, so I attempted to collect enough larvae from a natural pond to implement the second attempt. I could not obtain enough spotted and Jefferson salamander larvae, but I was able to locate enough larval marbled salamanders from an autumnal pool in Hart County Kentucky. For this second attempt using only a single species, tanks were adjusted to seven different treatment variants at pH 4.5, pH 5, pH 5.5, pH 6, pH, 6.5 and an unadjusted control around pH 7. Five replicates were created for each treatment to use a total of thirty-five tanks. Tanks were adjusted slowly adding 10ml of concentrated sulfuric acid per day until the pH was within 1 unit of the desired pH. Subsequently, 1-5ml were added per day until the pH reached the desired level. The newly adjusted tanks were seeded with zooplankton from neighboring control tanks. Acid pH was measured every three days with a THZY pH pen tester and adjusted with sulfuric acid if necessary. A supplementary batch of zooplankton was collected from Lower Fairy Shrimp pond and added on May 25th. Larvae were
weighed and assigned to treatment groups on June 1st, 2016, maintaining similar biomass in each treatment. To introduce larvae into the mesocosms, seven larvae were put into plastic containers with one part charcoal-filtered water and one part pond water. One box was floated in each tank and one part of water from the tank was added to the box. Larvae were floated overnight and then released into the tank. Tanks were checked daily for metamorphosis. Upon metamorphosis each individual was brought back to the lab and weighed.

RESULTS AND DISCUSSION

All tanks maintained pH levels for the next 52 days (with some addition of sulfuric acid) at which point the experiment was terminated because all larvae had either completed metamorphosis or died. All treatments exhibited at least 60% mortality, however there was no effect of pH treatment on survival. Time to metamorphosis was similarly unchanged, with all individuals metamorphosing by day 23 of the experiment. It is however notable that all individuals from the pH 7 control ponds had metamorphosed by day 8 of the experiment. There was additionally no difference in final mass between the various tank treatments.

In the first run of the experiment I experienced a crash, which I believe was due to rapid algal die off resulting from the initial acid shock during treatment preparation. Algae growth had been rapid and extensive during the time preceding the acidification of the pond water, and I hypothesize that the presence of large amounts of dead algae increased acid levels in the water and contributed to the crash in pH. For the second
experiment I showed that slowly adding acid to the tanks did not cause a crash. I believe that the slow addition of acid slowly killed off vulnerable algal populations, allowing the tank to adjust over time rather than immediately crash. Or possibly less algae had accumulated prior to acid addition during the second experimental iteration.

In my second trial I was successfully able to raise late stage larvae to metamorphosis and demonstrated a successful technique for the adjustment of cattle tank pH with sulfuric acid. However, because the larvae were already nearing metamorphosis before I put them in the cattle tanks, the data do not demonstrate any conclusive patterns. Nevertheless, following these modified protocols, future studies looking at the effects of acid deposition on the community structure of pond breeding amphibians should be more successful.

LITERATURE CITED

APPENDIX D

R CODE EXAMPLES

GROWTH CURVE ANALYSIS
Sal=read.csv(file.choose())
library(ggplot2)
library(lme4)
t <- poly(unique(Sal$day), 2)
Sal$day <- as.factor(Sal$day)
Sal[,paste("ot", 1:2, sep="")] <- t[Sal$day, 1:2]

ms.base <- lmer(SVL ~ (ot1+ot2) + (ot1+ot2 | ID), data=Sal, REML=FALSE)
ms.0 <- lmer(SVL ~ (ot1+ot2) + treatment + (ot1+ot2 | ID), data=Sal, REML=FALSE)
ms.1 <- lmer(SVL ~ (ot1+ot2) + treatment + ot1:treatment + (ot1+ot2 | ID), data=Sal, REML=FALSE)
ms.2 <- lmer(SVL ~ (ot1+ot2)*treatment + (ot1+ot2 | ID), data=Sal, REML=FALSE)
anova(ms.base,ms.0,ms.1,ms.2)

ggplot(Sal, aes(day, SVL, color=treatment)) +
  stat_summary(fun.data=mean_se, geom="pointrange")+
  stat_summary(aes(y=fitted(ms.2),linetype=treatment,group=treatment),
                fun.y=mean, geom="line")+
  labs(x="Day", y="SVL (cm)")+
  theme_classic(base_size = 20)+
  theme(legend.justification=c(0,1),legend.position=c(0,1),
        legend.background=element_rect(fill="transparent",color="transparent"),
        legend.title=element_blank())+
  theme(axis.title.x=element_text(face="bold", size=20),
        axis.title.y=element_text(face="bold", size=20))

BARPLOTS
Mar=read.csv(file.choose())
as.integer(factor(Mar$Treatment,c("Control","P6","P5")))
attach(Mar)
boxplot(SVL~treatment, horizontal = TRUE, col = c("darkgray"),las=1,
        xlab = "Change in SVL (cm)"
        points(y=c(1:3), x=c(9.09,6.66,9.05),pch=19)
SURVIVORSHIP CURVE

FT <- read.csv(file.choose())
summary(FT)
library(ggfortify)
library(survival)
library(rms)
FT$SurvObj <- with(FT, Surv(time, status == 2))
fit <- survfit(SurvObj ~ Treatment, data = FT, conf.type = "log-log")
plot(fit, xlab = "Day", ylab = "Percent Surviving", lty= c(1:4),col=c(1:4),lwd= c(2),
      font.lab=2, cex.lab=1.2)
legend(90,0.6, c("Control","pH4", "pH5", "pH6"),lty=c(1:4),col=c(1:4),lwd = c(2),
bty="n")

TREATMENT STATISTICS

attach(Mar)
a5 <- aov(weight ~ treatment)
summary(a5)
a6 <- aov(SVL ~ treatment)
summary(a6)
TukeyHSD(a6)
attach(MJeffph)
m1 <- aov(time ~ treatment)
summary(m1)
attach(SJeffph)
b1 <- aov(time ~ treatment)
summary(b1)
TukeyHSD(b1)