ABIOTIC FACTORS UNDERLYING STRESS HORMONE LEVEL VARIATION AMONG LARVAL AMPHIBIANS

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ABSTRACT

Anthropogenic disturbances can alter the abiotic composition of freshwater systems. These compositional changes can act as physiological stressors towards system inhabitants. However, little is known about how these altered abiotic factors influence stress hormones (corticosterone) in larval amphibians. Throughout the following chapters, I examined the effects of several abiotic factors on baseline and stress-induced corticosterone levels in the larvae of four amphibian species: Jefferson salamander (*Ambystoma jeffersonianum*), spotted salamander (*A. maculatum*), wood frog (*Rana sylvatica*), and grey treefrog (*Hyla versicolor*). Chapter II examined corticosterone level differences throughout development in *A. jeffersonianum* and *R. sylvatica* larvae under field, mesocosm, and laboratory venues. Baseline corticosterone levels in *R. sylvatica* increased near metamorphic climax in all venues, but not in *A. jeffersonianum*. Rather, baseline corticosterone levels differed with respect to venue throughout development in *A. jeffersonianum*. Chapter III examined corticosterone level differences among free-living *A. jeffersonianum* populations and possible abiotic factors underlying these hormone differences. Corticosterone levels significantly differed across populations. Increased baseline corticosterone levels significantly correlated to low pH. There was also a trend for increased baseline corticosterone levels to be positively correlated with chloride levels and negatively correlated with conductivity. Chapter IV examined the effects of laboratory manipulated pH on

corticosterone levels in *A. jeffersonianum*, *A. maculatum*, *R. sylvatica*, and *H. versicolor*. There was a significant correlation between increased baseline corticosterone levels to low pH in all four species. Prey consumption (in both *Ambystoma* species) and survival (in *A. jeffersonianum*, *A. maculatum*, and *R. sylvatica*) were also negatively correlated to low pH. Chapter V examined the effects of increased conductivity on corticosterone levels in *A. jeffersonianum*, *R. sylvatica*, and *H. versicolor*. Increased conductivity exposure significantly correlated to increased baseline corticosterone levels in *A. jeffersonianum* and *R. sylvatica*. Prey consumption in *A. jeffersonianum* was also negatively correlated to increased conductivity. My dissertation shows that abiotic factors, such as pH and conductivity, can influence corticosterone levels in larval amphibians. These results suggest that corticosterone levels in larval amphibians may be a suitable biomarker reflective of altered freshwater habitat quality. However, my results also suggest that one should use a high degree of caution when using corticosterone levels in larval amphibians as a means to infer the health status of a population.

DEDICATION

To my mother and my father. Your unwavering love, guidance, and support have kept me afloat. Without you, I would have surely sunk and none of this would have been possible.

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And to the one lost along the way. I came out of ice hockey retirement this past spring. After scoring my first goal and skating back to the bench, my eyes wandered into the stands. It is there that I swear I saw you cheering with the rest of the crowd. I knew it was not possible, but for that split second, the oceans in between us vanished and things felt like what they once were. You are missed, my friend. You are missed.

ATTRIBUTIONS

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Dr. Lisa K. Belden served as my advisor and chair of my dissertation committee. Chapters II, III, and IV are presented as separate manuscripts, on which she is a coauthor.

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CHAPTER I: INTRODUCTION AND DISSERTATION OVERVIEW

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Introduction

The primary objective of my dissertation was to examine environmental sources of corticosterone level variation, particularly baseline levels, in larval amphibians. Both field observations, mesocosm-based, and laboratory-based experiments were used in this research. I used larval amphibians to address the complex relationship between an individual's stress physiology and surrounding environment. The larval amphibian species I used were Jefferson salamanders (*Ambystoma jeffersonianum*), spotted salamanders (*A. maculatum*), wood frogs (*Rana sylvatica*), and gray treefrogs (*Hyla versicolor*). Specifically, my dissertation goals were:

- to determine corticosterone level variation based upon life-history stage, specifically developmental stage, in the larval amphibians species of interest
- to identify potential abiotic factors underlying corticosterone level differences among free-living amphibian populations
- to experimentally manipulate abiotic factors correlated to increased corticosterone levels in free-living amphibian populations to determine any scale effects of stressor exposure
- to link exposure to several stressors (not at the same time) to a decrease in individual fitness, specifically a decrease prey capture efficiency and survival

Anthropogenic threats to freshwater systems

Anthropogenic disturbances are capable of exerting lethal and sub-lethal effects upon freshwater fauna by altering both biotic and abiotic system components (Bronmark and Hanson 2002; Riccardi and Rasmussen 1999). In this section, I discuss anthropogenic factors that alter freshwater quality and that may affect amphibian species. There are several ways in which

freshwater system quality can be altered by anthropogenic disturbances. Freshwater systems can become altered due to chemical contamination. For instance, approximately 9.98 million tons of nitrogen-based compounds are used annually in the United States, mainly in the form of fertilizer (Schuytema and Nebeker 1999) and approximately 83 million tons are used on an annual basis globally (Guillette and Edwards 2005). Fertilizer and waste water from industries and municipalities are common sources of nitrogen pollution to water bodies (Schuytema and Nebeker 1999). Nitrate exposure has been associated with amphibian growth and development inhibition, swimming speed reduction, feeding behavior alteration, and mortality (Baker and Waights 1993 and 1994; Griffis-Kyle 2007; Hecnar 1995; Marco et al. 1999; Oldham et al. 1997; Ortiz et al. 2004; Watt and Oldham 1995; Xu and Oldham 1997).

Acid deposition, and subsequent pH reduction, is also a common anthropogenic disturbance (Driscoll et al. 2001). Several anthropogenic events can lead to pH reduction (McCoy and Harris 2003). One common depository pathway is acid precipitation, which results from fossil fuel burning. Acidification can be most pronounced in the spring as rainfall and snow melt increases, causing a water table rise (Driscoll et al. 2001). Several effects following low pH exposure have been identified in amphibians, including delayed hatching (Horne and Dunson 1994), larval deformation (Beattie and Tyler-Jones 1992), immunological alterations (Brodkin et al. 2003), altered oviposition/reproductive behavior (Ortiz-Santaliestra et al. 2007; Rowe and Dunson 1993), and decreased survival (Blem and Blem 1989; D'Amen et al. 2007; Dunson and Connell 1982; Rowe et al. 1992; Saber and Dunson 1978).

Another major anthropogenic threat to freshwater systems is conductivity alteration. Conductivity is a measure of electrical conductance (Wetzel and Likens 2000). In freshwater systems, conductivity is influenced by the presence of ions in the water column (Wetzel and

Likens 2000). Chemical applications to environments, such as the use of de-icing agents, are a major mechanism by which conductivity is altered in freshwater systems (Collins and Russell 2009; Sanzo and Hecnar 2006). In North America alone, an estimated 14 million tons of salt compounds are applied to roads each year (Environment Canada 2001). Exposure to altered conductivity can have profound impacts on several taxa (Cheong and Yun 2007; Grosell et al. 2007). Concerning amphibians, studies have shown exposure to altered conductivity to negatively impact growth and development (Gomez-Mestre and Tejedo 2002; Snodgrass et al. 2008), behavior (Collins and Russell 2009; Haramura 2007; Karraker et al. 2008), and survival (Collins and Russell 2009; Sanzo and Hecnar 2006).

Physiological stress response

One method in which vertebrates respond to stress is by activating the highly conserved physiological stress response (Romero 2004; Selye 1936). Glucocorticoid hormones are released from the adrenal cortex upon activation of the hypothalamic-pituitary-adrenal axis following stress (McEwen and Wingfield 2003). Upon release, glucocorticoids trigger energy movement and metabolism of lipids, proteins, and carbohydrates to promote survival (Dallman et al. 1993; Romero 2002; Weber 1992). Long term physiological effects of glucocorticoid exposure include the suppression of growth, reproduction, and immune system components (Moore and Jessop 2003). Corticosterone, the main glucocorticoid in amphibians, also has integral roles in reproduction, metamorphosis, and immunity (Belden and Kiesecker 2005; Moore and Jessop 2003; Shi 2000). Corticosterone is an integral component of various events in amphibian life cycles, such as anuran tail fin reabsorption and metamorphosis (Hayes and Wu 1995; Kaltenbach 1958). I discuss specific corticosterone level changes throughout development in two amphibian

species, *R. sylvatica* and *A. jeffersonianum*, in Chapter II. Several studies have shown corticosterone to be influenced by diel, seasonal, and social changes (Burmeister and Wilczynski 2000; Romero et al. 2000 and 2008; Wright et al. 2003).

There are two main models that attempt to elucidate patterns of the physiological stress response and integrate the term "stress" with homeostasis. They are the Allostasis Model and the Reactive Scope Model. The Allostasis Model, proposed by McEwen and Wingfield (2003), is an extension of a biomedical term "allostasis" (Sterling and Eyer 1988). Allostasis, or the accumulation of daily and seasonal physiological changes within an individual to maintain optimal set-points for internal processes, is comprised of two main phases – allostatic load and allostatic overload (McEwen and Wingfield 2003). Allostatic load is the cumulative costs of allostasis that an individual incurs (McEwen and Wingfield 2003). Generally, allostatic load costs do not lead to pathological effects because an individual has sufficient energy income and reserves to overcome a stressor. However, an individual may incur an allostatic overload when there is a lack of sufficient energy reserves needed to cope with a stressor. During allostatic overload, an individual may enter an "emergency life history" phase, associated with an increase in glucocorticoid levels.

The Reactive Scope Model, proposed by Romero et al. (2009), is broader than the Allostasis Model. For example, the Reactive Scope Model can be applied to behavioral changes and several physiological endpoints (such as heart rate, blood pressure, antibody titers, neurotransmitters, glucocorticoid levels) while the Allostasis Model is rather specific to glucocorticoid levels. The Reactive Scope Model is comprised of four phases – predictive homeostasis, reactive homeostasis, homeostatic overload, and homeostatic failure (Romero et al. 2009). While the Reactive Scope Model can be applied to several physiological endpoints

(previously discussed), I will use glucocorticoid levels as the physiological endpoint of interest when defining each of the four phases. Predictive homeostasis comprises all circadian rhythms of glucocorticoid levels of an individual (Romero et al. 2009). Glucocorticoid level variation can be due to seasonality effects or the specific life-history phase of an individual (Romero 2002; Romero et al. 2008; Wada 2008). Reactive homeostasis is the phase that entails glucocorticoid level increases after experiencing an unpredictable stressor, such as droughts, harsh storms, or anthropogenic disturbances (Wingfield 2008). Homeostatic overload can occur when glucocorticoid levels are elevated over a long period of time, potentially leading to pathological effects (previously discussed). Homeostatic failure can occur when glucocorticoid levels fall below their optimal range for homeostasis to occur, and death can result (Romero et al. 2009).

Environmental Impacts on Corticosterone in Vertebrates

As previously discussed, anthropogenic disturbances are altering freshwater systems (Bronmark and Hanson 2002). These alterations can act as stressors upon system inhabitants (Riccardi and Rasmussen 1999; Wingfield and Romero 2001). Wikelski and Cookie (2006) emphasize the importance of understanding how anthropogenic disturbances can cause stress within system inhabitants. Thus, it seems plausible that stress hormones could be used as a physiological biomarker to assess the impact of anthropogenic disturbances (see Homan et al. 2003). Unfortunately, only a few studies have used corticosterone in this context despite the call for such investigations (Carey 2005; Walker et al. 2005). One study showed that fecal corticosterone levels in male northern spotted owls (*Strix occidentalis caurina*) were higher in areas with logging-road traffic and timber harvest activities (Wasser et al. 1997). Snowmobile use has been shown to elevate glucocorticoid levels in elk (*Cervus elaphus*) and wolves (*Canis*

lupus) (Creel et al. 2002). Exposure to cadmium and zinc in brown trout (*Salmo trutta*) significantly influenced confinement-induced corticosterone levels (Norris 2000; Norris et al. 1999).

Several studies have been able to use corticosterone levels as direct predictors of some ecologically relevant effect in free-living vertebrates. For example, Romero and Wikelski (2001) used increased stress-induced corticosterone levels to predict survival in marine iguanas (*Amblyrhynchus cristatus*) during El Niño events in the Galápagos Islands. Bonier et al. (2007) used increased maternal corticosteroids to predict female-biased offspring sex ratios in white-crowned sparrows (*Zonotrichia leucophrys*).

Concerning amphibians, studies have shown environmental stressors to significantly increase corticosterone levels. For example, exposure to coal combustion waste has been shown to elevate corticosterone levels in Southern toads (*Bufo terrestris*) and Southern leopard frogs (*R. sphenocephala*) (Hopkins et al. 1997; Peterson et al. 2009). Altered terrestrial habitat significantly elevated baseline corticosterone levels in migrating male spotted salamanders (*A. maculatum*) (Homan et al. 2003). Experimental pond drying was significantly correlated with increased corticosterone levels in Western spadefoot toad tadpoles (*Scaphiopus hammondii*) (Denver 1998). However, some known environmental stressors such as exposure to ultraviolet-B radiation, coal fly ash, and atrazine did not significantly increase corticosterone levels in several amphibian species (Belden et al. 2003; Larson et al. 1998; Ward and Mendonca 2006). It is partially because of these mixed results that corticosterone level interpretations are subject to great debate. Romero (2004) cautioned investigators that higher corticosterone levels do not necessarily suggest that an individual is in poor health. Rather, variations in corticosterone levels

can naturally occur, and can be mediated by diel, seasonal, or developmental changes (Romero 2002; Wright et al. 2003).

Amphibian Species Used

My dissertation used four amphibian species to examine environmental sources of corticosterone level variation**.** These species were *Ambystoma jeffersonianum* (Jefferson salamander), *A. maculatum* (spotted salamander), *Rana sylvatica* (wood frog), and *Hyla versicolor* (gray treefrog). The reason I selected these species was because they represent a broad range of breeding times for amphibians, thus acting as a representative group encompassing most of spring and summer breeding for amphibians. Having such a representative group is important in understanding interactions between an individual's environment and the stress response since the type and intensity of an environmental stressor may vary throughout spring and summer. Specific breeding times for each species are discussed below.

Ambystoma jeffersonianum

Ambystoma jeffersonianum (Jefferson salamander) is locally uncommon, with its historic southern-most range being in Giles County, Virginia (Hutchison 1956). However, recent herpetological surveys have extended the southern-most range of *A. jeffersonianum* to Scott and Wise counties, Virginia (Mitchell and Reay 1999; Roble and Hobson 1995). Its entire geographic range is from Vermont to Virginia's New River Valley and west to Indiana.

In most instances, *A. jeffersonianum* is the first salamander to break hibernation, emerging as early as mid to late-February despite possible snow cover. Egg masses are deposited

in small clusters containing an average of 16 eggs, ranging from 7 to 60 (Bishop 1941). However, there are reports of *A. jeffersonianum* eggs being laid singly (Bleakney 1957; Stille 1954). Larvae hatch approximately 30 to 45 days after egg mass deposition, measuring 10 to 14 mm in total length (Hulse et al. 2001). Typical of ambystomatid larvae, *A. jeffersonianum* have feather-like external gills and balancers early in development. Metamorphosis usually occurs in July or early August.

Ambystoma maculatum

Ambystoma maculatum (spotted salamander) is also locally uncommon. Mitchell and Reay (1999) report them having a state-wide occurrence in Virginia, except in a few areas including southwestern Virginia. Its entire geographic range is quite widespread. It covers a majority of the eastern United States, going as far south as Georgia and as far north as Quebec, Canada (Hulse et al. 2001).

Egg masses are commonly attached to aquatic vegetation typically during early to mid-spring months. Individual egg masses typically contain 60 to 200 eggs (Bishop 1941). Depending on water temperature, larvae typically hatch 30 to 50 days after egg mass deposition. Emerging *A. maculatum* larvae are typically 11 to 13 mm in total length, similar to *A. jeffersonianum* (Hulse et al. 2001). As with *A. jeffersonianum* and other ambystomatid larvae, *A. maculatum* larvae have feather-like external gills and balancers early in development. Metamorphosis occurs in August or September, depending on hatching time (Hulse et al. 2001).

Rana sylvatica

Rana sylvatica (wood frog) is locally common in the New River Valley. Its state-wide occurrence in Virginia is generally restricted to most western and some northern counties (Mitchell and Reay 1999). Its entire geographic range covers most of Canada into Alaska, the northeastern United States, and as far south as Georgia (Hulse et al. 2001). *Rana sylvatica* typically emerge from hibernation in February or March (Hulse et al. 2001), depending on location and temperature. Unlike *A. jeffersonianum* and *A. maculatum*, *R. sylvatica* tend to stay active on the surface long after breeding with hibernation beginning in October and November (Hulse et al. 2001).

Like *A. jeffersonianum* and *A. maculatum*, *R. sylvatica* generally prefer temporary ponds over permanent ponds for breeding. *Rana sylvatica* display explosive breeding, with egg masses being laid communally (Waldman 1982). Clutch sizes average between 572 to 745 individual eggs (Berven 1988). Tadpoles hatch approximately 20 days after egg mass deposition and complete development 80 to 113 days later (Hulse et al. 2001).

Hyla versicolor

Hyla versicolor (gray treefrog) is locally common in the New River Valley. It has a spotty state-wide distribution in Virginia (Mitchell and Reay 1999). Its entire geographic range can be difficult to establish since it looks similar to *H. chrysoscelis* (Cope's gray treefrog). They can be found in the northeastern United States, as far west as Texas, and in parts of Ontario and Manitoba, Canada. Adults emerge from hibernation in late spring/early summer, depending on temperature and precipitation (Hulse et al. 2001). Their breeding season is longer than the other amphibian species I examined, extending into August.

Egg masses are laid in temporary and permanent water bodies. Individual egg masses contain approximately 50 eggs. Incubation and development time is short, with complete transformation taking only 45-65 days (Hulse et al. 2001). Metamorphosed individuals range in snout-urostyle length from 17 to 20 mm (Hulse et al. 2001).

Dissertation Composition

This dissertation is comprised of six chapters. Chapter II examines how stress hormone levels change throughout larval amphibian development under field, mesocosm, and laboratory settings. Chapter III examines stress hormone differences among free-living salamander populations and possible abiotic parameters underlying these hormone differences. Chapters IV and V experimentally manipulate two water quality parameters (pH and conductivity, respectively) found to be correlated with baseline stress hormone levels in the field (Chapter III) and examines the effect of these two factors on stress hormones, prey consumption, and survival. Chapter VI presents concluding remarks and proposes directions for future research.

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Corticosterone level changes throughout development in *Rana sylvatica* and *Ambystoma jeffersonianum*

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Abstract

Endocrine pathways are arguably the most studied processes in amphibian metamorphosis. Yet some aspects still remain understudied, namely glucocorticoid changes throughout development. What we do know about corticosterone (the main glucocorticoid in amphibians) levels throughout amphibian metamorphosis is based only on a few species held under laboratory conditions. Our objective was to examine corticosterone level changes throughout development in free-living, mesocosm-held, and laboratory-held amphibians using anuran (*Rana sylvatica*) and caudate (*Ambystoma jeffersonianum*) models. *Ambystoma jeffersonianum* larvae showed different baseline corticosterone level patterns throughout development in regards to sample venue. Meanwhile, baseline corticosterone levels in *R. sylvatica* experienced an increase close to metamorphic climax in all sample venues. Confinement-induced corticosterone levels in *A. jeffersonianum* and *R. sylvatica* significantly increased throughout development in all sample venues, suggesting that the stress axis is still developing throughout metamorphosis. In addition, we evaluated the effectiveness of two commonly used stress-inducing techniques (confinement and adrenocorticotropic hormone (ACTH) injection) in A. *jeffersonianum* larvae. Both confinement and ACTH significantly increased corticosterone levels in *A. jeffersonianum*. Specifically, treatment exposure (both confinement and ACTH injections) lasting 30 min elicited the maximum hormonal response level. Our results support studies on other vertebrate taxa suggesting that corticosterone levels can significantly vary between life-history phases, specifically developmental stages.

Introduction

Metamorphosis has long been an intriguing biological phenomena for investigators (Shi 2000), and is common in many taxa including insects, crustaceans, echinoderms, cnidarians, and fish (Dufour and Rousseau 2007; Heyland et al. 2004; Leitz 1997; McWilliam and Phillips 2007; Truman and Riddiford 2002). However, amphibian metamorphosis is one of the most dramatic among all organisms (Shi 2000). Most mechanistic studies examining amphibian metamorphosis have focused on only a few species as models, mainly anurans (frogs and toads; Shi 2000).

Anuran metamorphosis has three general phases: pre-metamorphosis, pro-metamorphosis, and metamorphic climax (Etkin 1968; Dodd and Dodd 1976). Pre-metamorphosis embodies embryogenesis and early growth stages. Pro-metamorphosis is comprised of morphogenesis, specifically limb development. Metamorphic climax consists of rapid growth, development, and differentiation in preparation for adulthood. Caudate (salamander) metamorphosis is not as dramatic as anuran metamorphosis in terms of morphological changes (Just et al. 1981), but is comprised of similar general developmental phases.

Several hormones have integral roles in amphibian metamorphosis, including prolactin, thyroid hormones, gonadal steroids, and glucocorticoid hormones (Kikuyama et al. 1993; Shi 2000). Corticosterone is the main glucocorticoid in amphibians (Idler 1972). Tail fin absorption, hepatic enzyme activity, and cell differentiation are all mediated by corticosterone (Shi 2000). Elevations of corticosterone can also accelerate metamorphosis in some instances (Hayes 1997). Fewer studies have addressed corticosterone levels throughout development in caudates as compared to anurans. Carr

and Norris (1988) examined corticosterone changes throughout development in the tiger salamander (*Ambystoma tigrinum*). They observed corticosterone levels at the lowest level during pre-metamorphosis, significantly increasing towards mid-metamorphosis, followed by a significant decrease upon completing metamorphosis. Similar patterns have been seen in laboratory studies of anurans (Wada 2008), such as *Rana catesbeiana* (Jaffe 1981; Krug et al. 1983; Wright et al. 2003), *R. pipiens* (Glennemeier and Denver 2002a), and *Xenopus laevis* (Jolivet-Jaudet and Leloup-Hatey 1984; Kloas et al. 1997). These reported corticosterone levels and observed trends have never been confirmed in free-living individuals. One of our objectives was to examine these patterns in free-living Jefferson salamander (*A. jeffersonianum*) and wood frog (*R. sylvatica*) larvae. We also examined corticosterone throughout development under laboratory and mesocosm conditions in the same amphibian species, as both settings are commonly used in amphibian-based research.

In addition, we examined the responsiveness of the stress axis throughout development. Glucocorticoid hormones are released from the adrenal cortex with activation of the hypothalamic-pituitary-adrenal (HPA) axis following perceived stress (McEwen and Wingfield 2003). In amphibians, both confinement and adrenocorticotropic hormone (ACTH) injections can activate the HPA axis (Belden et al. 2005). Again, most of these studies have examined anurans. For instance, Glennemeier and Denver (2002a) compared several confinement times and varying ACTH doses in triggering the HPA axis at different developmental timepoints in anurans, *X. laevis* and *R. pipiens*. Few studies have compared the effectiveness of these two methods in eliciting the stress response in caudates. One example however, Gendron et al. (1997), assessed

the functionality of the HPA axis using confinement and ACTH injection in male and female mudpuppies (*Necturus maculosus*) from polluted and nonpolluted sites. However, they only used a single confinement time (60 min) and a single ACTH injection dose (4 IU/100 g). Another objective of our study was to determine the optimal confinement time and ACTH dose that can maximize resulting corticosterone levels in the caudate, *A. jeffersonianum*.

Materials and Methods

Experimental species

Both *A. jeffersonianum* and *R. sylvatica* breed in ponds, often ephemeral, in early spring months (Hulse et al. 2001). They are typically the first amphibians to break hibernation within their ranges. Depending on temperature, *A. jeffersonianum* has incubation and development periods of 30 to 45 days and two months, respectively (Petranka 1998). *Rana sylvatica* tadpoles typically hatch after 20 days and complete development 80 to 113 days later (Hulse et al. 2001). Amphibian development can be viewed as a series of morphological changes, and several staging schemes are used depending on the species (McDiarmid and Altig 1999). We used the Watson and Russell (2000) staging scheme for *A. jeffersonianum*. During stages 1-9, forelimb development occurs, followed by hindlimb growth in stages 10-18, and gill reduction leading to metamorphosis in stages 19-22. We used the Gosner (1960) staging scheme for *R. sylvatica*. It consists of 46 stages, with embryogenesis during stages 1-19, hatchling development (including tail fin differentiation, skin pigmentation, and gill atrophy)

during stages 19-25, tadpole growth and limb formation during stages 25-41, and metamorphosis during stages 42-46.

Corticosterone throughout development

Free-living stress series

We collected *A. jeffersonianum* and *R. sylvatica* from a single pond in Montgomery County, Virginia. Sampling occurred on a bi-weekly basis throughout development and stopped just prior to complete metamorphosis. Baseline and confinement-induced corticosterone levels were sampled for each species. To minimize diel effects on corticosterone (Wright et al. 2003), all sampling occurred during the same time of day (between 1100 and 1500 hrs). For baseline corticosterone samples, $N = 5$ individuals/species were collected using dip-nets and immediately frozen in a dry ice/ethanol slurry. Freezing within three min of collection likely reflects baseline corticosterone levels measurement (Romero and Reed 2005). The confinement stress series protocol consisted of placing individual larvae ($N = 5$/species) in 120 mL cups filled with approximately 60 mL of pond water for 30 min, with gentle agitation every three min (Belden et al. 2007). After 30 min, larvae were frozen in a dry ice/ethanol slurry and stored at -80°C until radioimmunoassay (RIA) could be performed.

Mesocosm stress series

Recently deposited *A. jeffersonianum* ($N = 26$) and *R. sylvatica* ($N =$ parts of 8 masses) egg masses were collected from a single pond in Montgomery County, Virginia. After collection, egg masses of each species were housed in separate outdoor 1000 L

mesocosm stock tanks containing approximately 250 g deciduous leaf litter and 1 L wild-caught zooplankton. After egg masses were placed in their respective stock tank, we covered each tank with a shade-cloth lid to prevent colonization by other species, mainly gray treefrogs (*Hyla versicolor*) and dragonflies (Odonata). Egg masses and hatchling amphibian larvae were monitored at least three times/week for nearly four weeks prior to experimentation.

To examine the response to stress in mesocosm-reared individuals during development, we stocked two 1000 L outdoor cattletank mesocosms with $N = 50$ *A. jeffersonianum* larvae in one tank and $N = 50$ *R. sylvatica* larvae in the other. Prior to larval additions, we added approximately 250 g deciduous leaf litter and 1 L wild-caught zooplankton to each mesocosm. From these tanks, we sampled both baseline ($N = 4$) and confinement-induced ($N = 4$) corticosterone levels for each species on a bi-weekly basis until just prior to metamorphosis. All methods used for baseline and confinement-induced sample collection and storage were identical to those previously described for free-living individuals.

Laboratory stress series

For this experiment, we collected *A. jeffersonianum* and *R. sylvatica* larvae from our 1000 L stock tanks (previously described) and brought them back to the laboratory. To examine the response to stress in laboratory-reared individuals during development, *A. jeffersonianum* and *R. sylvatica* larvae were housed in 54.9 L tubs (63.2 cm x 43.7 cm x 30.5 cm) in a 17°C temperature controlled room. Each tub contained $N = 25$ larvae, with $N = 2$ tubs/species. Sampling for both baseline ($N = 4$) and confinement-induced (N

= 4) corticosterone levels for each species from each tub occurred on a bi-weekly basis until just prior to metamorphosis. *Ambystoma jeffersonianum* larvae were fed live brine shrimp *ad libitum* until large enough to consume live black worms. *Rana sylvatica* tadpoles were fed ground-up rabbit chow (Rabbit Ration Pellets, Big Spring Mill, Inc., Elliston, Virginia) *ad libitum*. Water changes occurred at least once/week. All methods used for baseline and confinement-induced sample collection and storage were identical to those previously described for free-living individuals.

ACTH and confinement to induce the stress response

Ambystoma jeffersonianum egg masses (N = 5) were collected in early spring from a Montgomery County, Virginia pond. Once in the laboratory, egg masses were housed individually at room temperature (~ 22°C) in 14 L plastic tubs (39.5 cm x 27 cm x 15 cm) until larvae hatched. Newly hatched larvae were fed live brine shrimp *ad libitum* until large enough to consume live black worms. After 11 days, all larvae (N = 100) were transferred to individual 0.5 L plastic cups and housed at 17°C in a 12:12 h (light: dark) photoperiod room. Larvae were then fed live black worms *ad libitum* until reaching developmental stage 15 for testing (Watson and Russell 2000).

Ambystoma jeffersonianum larvae (N = 80 total, with N = 5 larvae/ACTH dose/time treatment) were randomly assigned to one of four different ACTH dose treatments: 0, 2.5, 25, or 250 mIU/g ACTH (Sigma-Aldrich, St. Louis, MO, USA). Intraperitoneal injections via tail muscle were used to deliver the ACTH dose in phosphate buffered saline (PBS) in a total volume of 20 µL. After injection, larvae were returned to individual 0.5 L plastic cups for 0, 15, 30, or 60 min (randomly determined).

Following the appropriate time period, larvae were immediately frozen in a dry ice/ethanol slurry and stored at -80°C until RIA could be performed.

In addition to ACTH injections, a separate test examined if confinement/agitation for different confinement times (0, 15, 30 or 60 min) could stimulate the stress response. There were $N = 5$ larvae/time treatment ($N = 20$ total larvae). Confinement occurred in 120 mL cups, filled with 60 mL dechlorinated water. Throughout confinement, cups were gently agitated every three min. This is the typical protocol that we use for field studies (e.g., Belden et al. 2007). After time expiration, larvae were frozen in a dry ice/ethanol slurry and stored at -80°C until RIA could be performed.

Corticosterone extraction and radioimmunoassay

Corticosterone was measured using whole-body RIA procedures, as in Belden et al. (2003) with the following modifications. Larval homogenization occurred in mass adjusted distilled water (mass of individual x 10 mL + 0.5 mL rinse, with a minimum of 1.5 mL and maximum of 5 mL). After extraction, samples were centrifuged at 3000 rpm for 10 mins to break the emulsion. Three standard curves were prepared for each of six assays. The free-living *A. jeffersonianum* samples ($N = 40$; limit of assay detection = approximately 1.19 ng/g), free-living *R. sylvatica* samples ($N = 29$; limit of assay detection = approximately 0.89 ng/g), all laboratory-held samples ($N = 52$; limit of assay detection = approximately 0.89 ng/g), and all mesocosm-held samples ($N = 52$; limit of assay detection = approximately 0.89 ng/g) required one assay each. Our ACTH and confinement samples required two assays ($N = 50$ samples/assay; limit of detection for both assays = approximately 0.89 ng/g). Inter-assay variation was 23 %. Intra-assay

variation averaged 25 %. Based upon individual recoveries, extraction efficiency averaged 48 %.

Statistical analyses

Based on the structure of the data, either linear or 2nd order polynomial regressional analyses were used to assess the relationship between confinement time (baseline or 30 min) and developmental stage for each species. We used an analysis of variance (ANOVA) to determine if confinement for 30 min significantly increased corticosterone levels as compared to baseline levels for each species for each sampling origin. Statistical procedures were conducted using SAS JMP (version 7.0).

For our ACTH and confinement experiment, corticosterone levels were analyzed using an ANOVA, followed by Fisher's Least Squares Difference (LSD) pairwise comparisons to compare corticosterone levels from confinement or ACTH dose injection to their respective control group. These statistical analyses were conducted using XLSTAT (version 2008.7.03).

Results

Corticosterone throughout development in *Ambystoma jeffersonianum*

In free-living *A. jeffersonianum* larvae, there was a significant negative linear relationship between baseline corticosterone levels and developmental stage ($R^2 = 0.52$, $P < 0.001$; Fig. 1A). There was a significant, positive linear relationship between confinement-induced corticosterone levels and developmental stage ($R^2 = 0.43$, $P =$

0.002; Fig. 1A). As expected, baseline corticosterone levels significantly increased as a result of confinement ($F_{1, 39} = 35.04$, $P < 0.001$; Fig. 1A).

In mesocosm-held *A. jeffersonianum* larvae, there was a significant, slightly positive linear relationship between baseline corticosterone levels and developmental stage ($R^2 = 0.35$, $P = 0.01$; Fig. 1B). There was a significant, positive linear relationship between confinement-induced corticosterone levels and developmental stage ($R^2 = 0.60$, $P = 0.003$; Fig. 1B). As expected, baseline corticosterone levels significantly increased as a result of confinement ($F_{1, 27} = 42.69$, $P < 0.0001$; Fig. 1B).

In laboratory-held *A. jeffersonianum* larvae, there was no relationship between baseline corticosterone levels and developmental stage ($R^2 = 0.15$, $P = 0.14$; Fig. 1C). There was a significant, positive linear relationship between confinement-induced corticosterone levels and developmental stage ($R^2 = 0.67$, $P = 0.001$; Fig. 1C). As expected, baseline corticosterone levels significantly increased as a result of confinement ($F_{1, 27} = 18.7$, $P = 0.0002$; Fig. 1C).

Corticosterone throughout development in *Rana sylvatica*

In free-living *R. sylvatica* tadpoles, there were significant, positive relationships between baseline corticosterone levels (2^{nd} order polynomial relationship; $R^2 = 0.55$, $P = 0.008$; Fig. 2A) and confinement-induced corticosterone levels (linear relationship; $R^2 = 0.40$, $P = 0.02$; Fig. 2A) with developmental stage. Overall, there was a trend of increased corticosterone levels as a result of confinement ($F_{1, 28} = 3.37$, $P = 0.07$; Fig. 2A).

In mesocosm-held *R. sylvatica* tadpoles, there were significant, positive relationships between baseline corticosterone levels (2^{nd} order polynomial relationship;

$R^2 = 0.93$, $P < 0.001$; Fig. 2B) and confinement-induced corticosterone levels (linear relationship; $R^2 = 0.70$, $P < 0.001$; Fig. 2B) with developmental stage. Overall, there was a trend of increased corticosterone levels as a result of confinement ($F_{1, 23} = 3.59$, $P = 0.07$; Fig. 2B).

In laboratory-held *R. sylvatica* tadpoles, there were significant, positive 2nd order polynomial relationships between baseline corticosterone levels ($R^2 = 0.94$, $P < 0.001$; Fig. 2C) and confinement-induced corticosterone levels ($R^2 = 0.78$, $P = 0.001$; Fig. 2C) with developmental stage. Overall, baseline corticosterone levels were not significantly influenced by confinement ($F_{1, 23} = 2.48$, $P = 0.13$; Fig. 2C).

ACTH and confinement to induce the stress response

Overall, both confinement ($F_{1, 18} = 7.64$, $P = 0.01$) and ACTH injection ($F_{1, 64} = 9.99$, $P = 0.002$) significantly influenced corticosterone levels (Fig. 3). There was a significant increase in corticosterone levels compared to respective controls following 30 min confinement (Fisher's LSD pairwise comparison, $P = 0.0002$), and 60 min confinement (Fisher's LSD pairwise comparison, $P = 0.001$), but not following 15 min confinement (Fisher's LSD pairwise comparison, $P = 0.45$) (Fig. 3). Concerning ACTH injections, no significant differences were detected among all 0 min groups (2.5 mIU Fisher's LSD pairwise comparison, $P = 0.94$; 25 mIU Fisher's LSD pairwise comparison, $P = 0.47$; 250 mIU Fisher's LSD pairwise comparison, $P = 0.56$) compared to saline controls (Fig. 3). However, corticosterone levels did significantly increase compared to saline control during the following exposure times and ACTH treatments (Fig. 3): 15 min following 2.5 mIU ACTH (Fisher's LSD pairwise comparison, $P = 0.002$), 25 and 250

mIU ACTH (all Fisher's LSD pairwise comparisons, $P \leq 0.0001$); 30 min following 2.5, 25, and 250 mIU ACTH (all Fisher's LSD pairwise comparisons, $P \leq 0.0001$); 60 min following 2.5 mIU ACTH (Fisher's LSD pairwise comparison, $P = 0.004$), 25 and 250 mIU ACTH (all Fisher's LSD pairwise comparisons, $P \leq 0.0001$).

Discussion

We found an inconsistent pattern of baseline corticosterone levels throughout development in *A. jeffersonianum* based on sample venue. Baseline corticosterone levels decreased across development in free-living *A. jeffersonianum* larvae. Yet, baseline corticosterone levels slightly increased across development in mesocosm-held larvae and remained unchanged across development in laboratory-held larvae. Absolute corticosterone level values also differed between sample venues. For instance, laboratory-held *A. jeffersonianum* larvae had up to four-fold lower confinement-induced corticosterone levels as compared to the other two sample origins across similar developmental stages. Using the laboratory to conduct amphibian-based research allows a certain degree of control over several variables that are not easily controlled in field studies. Some of these variables, such as food availability (Crespi and Denver 2005), water quality (Gendron et al. 1997; Hopkins et al. 1999), larval density (Hayes 1997), and intraspecific competition (Glennemeier and Denver 2002b) have all been shown to influence corticosterone levels in amphibians. Since these corticosterone-influencing variables were controlled for in our laboratory experiment, it may explain why our absolute corticosterone levels were lower in the laboratory than in free-living and mesocosm-reared *A. jeffersonianum*.

Our results convincingly show that there was no significant increase in baseline corticosterone levels in *A. jeffersonianum* during metamorphic climax, regardless of sample venue. These results are interesting because they conflict with the common finding among past studies showing that corticosterone levels peak at metamorphic climax in larval amphibians. However, most of these past studies focused on anurans (but see Carr and Norris 1988). More studies examining steroid levels during salamander development will need to be completed before a general pattern can be identified.

One factor making cross-species comparisons difficult is that corticosterone levels can have diel cycles (Dupont et al. 1979; Thurmond et al. 1986). For example, Wright et al. (2003) showed corticosterone levels ranging from 22 ng/mL at 0900 hr to 1.2 ng/mL at 1300 hr in *R. catesbeiana* tadpoles. Thus, sampling for corticosterone levels at approximately the same time is crucial for comparisons. However, sampling times in studies examining corticosterone levels in amphibians have greatly varied (if sampling time is even reported) from early morning (Krug et al. 1983) to mostly late morning (this study) to afternoon (Kikuyama et al. 1986; Jolivet-Jaudet and Leloup-Hatey 1984).

In contrast to our findings for *A. jeffersonianum*, baseline corticosterone levels in *R. sylvatica* significantly increased towards metamorphic climax under free-living, laboratory, and mesocosm conditions. This pattern also occurs in other anuran species, including *R. catesbeiana* (Jaffe 1981; Krug et al. 1983; Wright et al. 2003), *R. pipiens* (Glennemeier and Denver 2002a), and *X. laevis* (Jolivet-Jaudet and Leloup-Hatey 1984; Kloas et al. 1997). Increasing circulating corticosterone levels at metamorphic climax serves several purposes. Anuran metamorphic climax consists of dramatic changes in morphology and physiology (Shi 2000). Corticosterone mediates several events during

these metamorphic changes in anurans. For instance, several studies have shown corticosterone to have integral roles in tail reduction (Kaltenbach 1985; Kikuyama et al. 1983). Corticosterone can also stimulate larval tissue degeneration in the epidermis, spleen, thymus, and intestine (Hayes et al. 1995). Caudate metamorphic climax does not entail these dramatic morphological and physiological changes. Rather, caudates retain their tail into adulthood and do not have drastic internal organ turnover since little dietary changes occur from larvae to adult. Thus, a rise in corticosterone levels at this stage may not be as necessary in caudates as in anurans.

Collecting baseline and confinement-induced corticosterone samples in our laboratory and mesocosm experiments decreased the density of the remaining *A. jeffersonianum* larvae and *R. sylvatica* tadpoles. High population densities might act as a stressor; hence a physiological response to a high density stressor should occur (Belden et al. 2007). Indeed, Glennemeier and Denver (2002b) showed a high density of 40 *R. pipiens* tadpoles/4 L to significantly increase corticosterone levels. Mixed results after exposure to high densities have been reported in *R. sylvatica*. Rot-Nikcevic et al. (2005) showed corticosterone levels to significantly increase after high density exposure in *R. sylvatica* in the laboratory (40 tadpoles/ 10 L), while Belden et al. (2007) did not show any significant relationship in mesocosms (low density = 80 tadpoles/ 1000 L; medium density = 160 tadpoles/ 1000 L; high density = 320 tadpoles/ 1000 L) and the field (<20 egg masses = low density; >100 egg masses = high density). Concerning our laboratory (25 larvae/ 54.9 L) and mesocosm (50 larvae/ 1000 L) experiments, if the initial density (acting as the highest density larvae were exposed to) was acting as a stressor, we would have expected to see high corticosterone levels at early developmental stages/early

sampling events. However, our results do not show this for *A. jeffersonianum* and *R. sylvatica* in our laboratory and mesocosm experiments (Figs. 1 and 2), thus we do not believe density was confounding our corticosterone throughout development results.

One possible explanation for a lack of a stress response to likely stressors is that the HPA axis may not be fully operational at early developmental stages. Our results for both *A. jeffersonianum* and *R. sylvatica* show that confinement-induced corticosterone levels significantly increased throughout development regardless of sample venue (Figs. 1 and 2). This may suggest that the development of the HPA axis and subsequent stress response continues throughout metamorphosis. Thus, individuals exposed to a stressor early in development, such as high density, may not be capable of mounting a full stress response at that time compared to being exposed to a stressor at later developmental stages.

Interestingly, laboratory *A. jeffersonianum* larvae appeared to have a diminished stress response to 30 min confinement/agitation compared to conspecifics in our free-living and mesocosm experiments (Fig. 1). There are several explanations that may explain this result. First, laboratory *A. jeffersonianum* may not have perceived our 30 min confinement/agitation as a stressful event. While this is highly unlikely since 30 min confinement/agitation resulted in a stress response in conspecifics in free-living and mesocosm settings, it is still a possible explanation. Cyr and Romero (2009) proposed four explanations that could cause a diminished stress response in an individual. These explanations include perceiving a stressor differently at different life-history stages or during different seasons, habituation to a stressor, desensitization of the stress response, and physiological exhaustion (Cyr and Romero 2009). Three of the four explanations

proposed by Cyr and Romero (2009) are unlikely in regards to our laboratory *A. jeffersonianum* results. Perceiving a stressor differently at different life-history stages is not likely because *A. jeffersonianum* larvae at identical developmental stages in our free-living and mesocosm settings responded to 30 min confinement/agitation. Furthermore, since *A. jeffersonianum* larvae experiencing a diminished stress response were held in the laboratory, seasonality effects should be negligible. Habituation to a stressor is unlikely because individuals were only subjected to 30 min confinement/agitation once before being frozen in a dry ice/ethanol slurry for hormone analysis. Desensitization to a stressor is also unlikely because that term also implies repeated exposure. Physiological exhaustion leading to a diminished stress response may be occurring in our laboratory *A. jeffersonianum*. Physiological exhaustion can occur if an individual is too fatigued to mount a stress response when confronted with a stressor (Cyr and Romero 2009). *Ambystoma jeffersonianum* larvae may have been fatigued from being housed in the laboratory over a long period of time.

Ambystoma jeffersonianum is believed to be one of the most sensitive salamander species to environmental alterations in North America (Petranka 1998). Our results suggest that the environmental sensitivity of *A. jeffersonianum* can carry over into the laboratory. If this is the case, future laboratory studies involving *A. jeffersonianum* for prolonged periods of time need to closely monitor the larvae because pathological effects (e.g., immune system failure, reproduction suppression, muscle deterioration, and decreased survival) can occur when individuals are physiologically exhausted (Romero et al. 2009).

Our results with *A. jeffersonianum* show that confinement and ACTH injection are both capable of stimulating the HPA axis and significantly increasing corticosterone levels. Corticosterone levels after ACTH injection and confinement in this study are similar to the corticosterone levels after confinement in free-living and mesocosm-reared *A. jeffersonianum* of similar developmental stage (approximately stage 15, Watson and Russell (2000)) (Figs. 1A, 1B, and 3). Examining stress-induced corticosterone levels is important for understanding the functional activity of an individual's hormonal stress response (Belden et al. 2007; Gendron et al. 1997). Several studies examining stress in amphibians have used various confinement times to trigger the HPA axis. For instance, Homan et al. (2003a and 2003b) used 30 min confinement in adult *A. maculatum*. Belden et al. (2005 and 2007) used both 30 and 60 min confinement in *Hyla regilla* and *R. sylvatica*. Highly varying doses of ACTH have also been successful in activating the HPA axis (see Belden et al. 2005; Rich and Romero 2005). Our results suggest that a 30 min time exposure typically elicits the maximal and most consistent hormonal response in *A. jeffersonianum*, regardless of whether confinement or ACTH injection is used.

Our results also suggest that developmental stages should be as homogenous as possible when conducting stress-related studies using amphibian as models. Indeed, others have shown similar results of varying baseline corticosterone levels throughout development in other amphibian species (Carr and Norris 1988; Glennemeier and Denver 2002a; Wright et al. 2003). In further support of our suggestion, Belden et al. (2007) showed that corticosterone levels significantly varied based on mass in *R. sylvatica* with larger tadpoles having lower corticosterone levels. Developmental stage homogeneity

should become more imperative as there is an increasing trend of using amphibians as models to study environmental change (Hopkins 2007).

It has been suggested that corticosterone levels could be an indicator of habitat quality (Homan et al. 2003a), with high corticosterone levels being indicative of an environment exerting "stress" upon its inhabitants (Homan et al. 2003b). However, our study suggests that a certain degree of caution should be used when examining baseline corticosterone levels. High baseline corticosterone levels may not necessarily be indicative of an individual being "stressed". Rather, our results show that it could be reflective of the individual's developmental stage. Romero (2004) issued a similar caution in that higher corticosterone levels do not necessarily suggest an individual is in poor health. However, these cautions should not deter investigators from examining corticosterone levels. Baseline corticosterone data are invaluable, despite their rarity in the literature (Wikelski and Cooke 2006), because they provide an understanding of the natural stress hormone profile of an individual (Belden et al. 2005).

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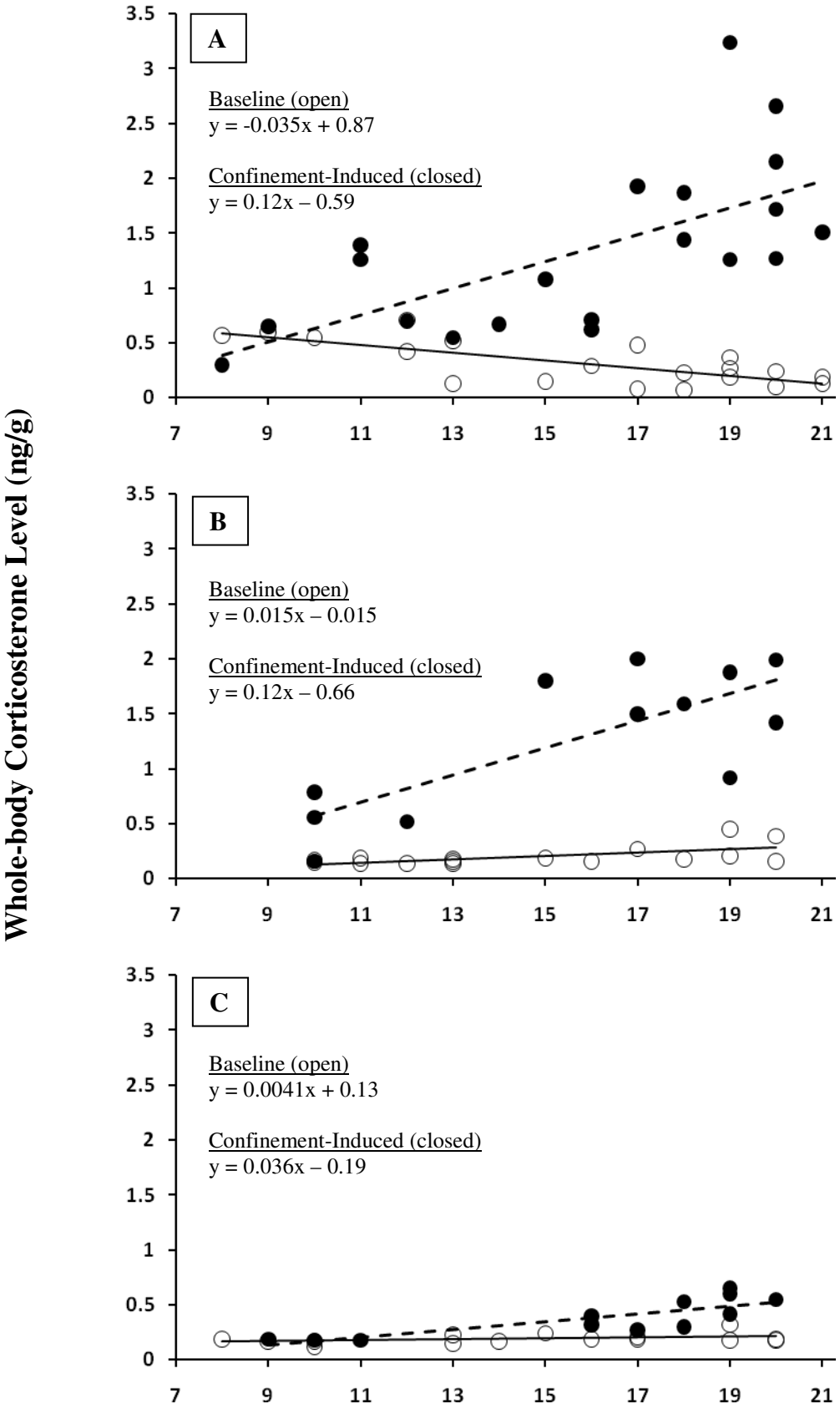
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Baseline (open)
y = -0.035x + 0.87
Confinement-Induced (closed)
y = 0.12x – 0.59
B
Baseline (open)
y = 0.015x – 0.015
Confinement-Induced (closed)
y = 0.12x – 0.66
C
Baseline (open)
y = 0.0041x + 0.13
Confinement-Induced (closed)
y = 0.036x – 0.19
Whole-body Corticosterone Level (ng/g)
Developmental Stage

Figure 1. Whole-body corticosterone levels (ng/g) throughout development in *Ambystoma jeffersonianum* under free-living (A), mesocosm (B), and laboratory (C) conditions. Staging scheme of Watson and Russell (2000). Open points represent baseline corticosterone levels. Closed points represent confinement-induced corticosterone levels. Regression equations and lines are shown for baseline (solid line) and confinement-induced (dashed line) corticosterone levels.

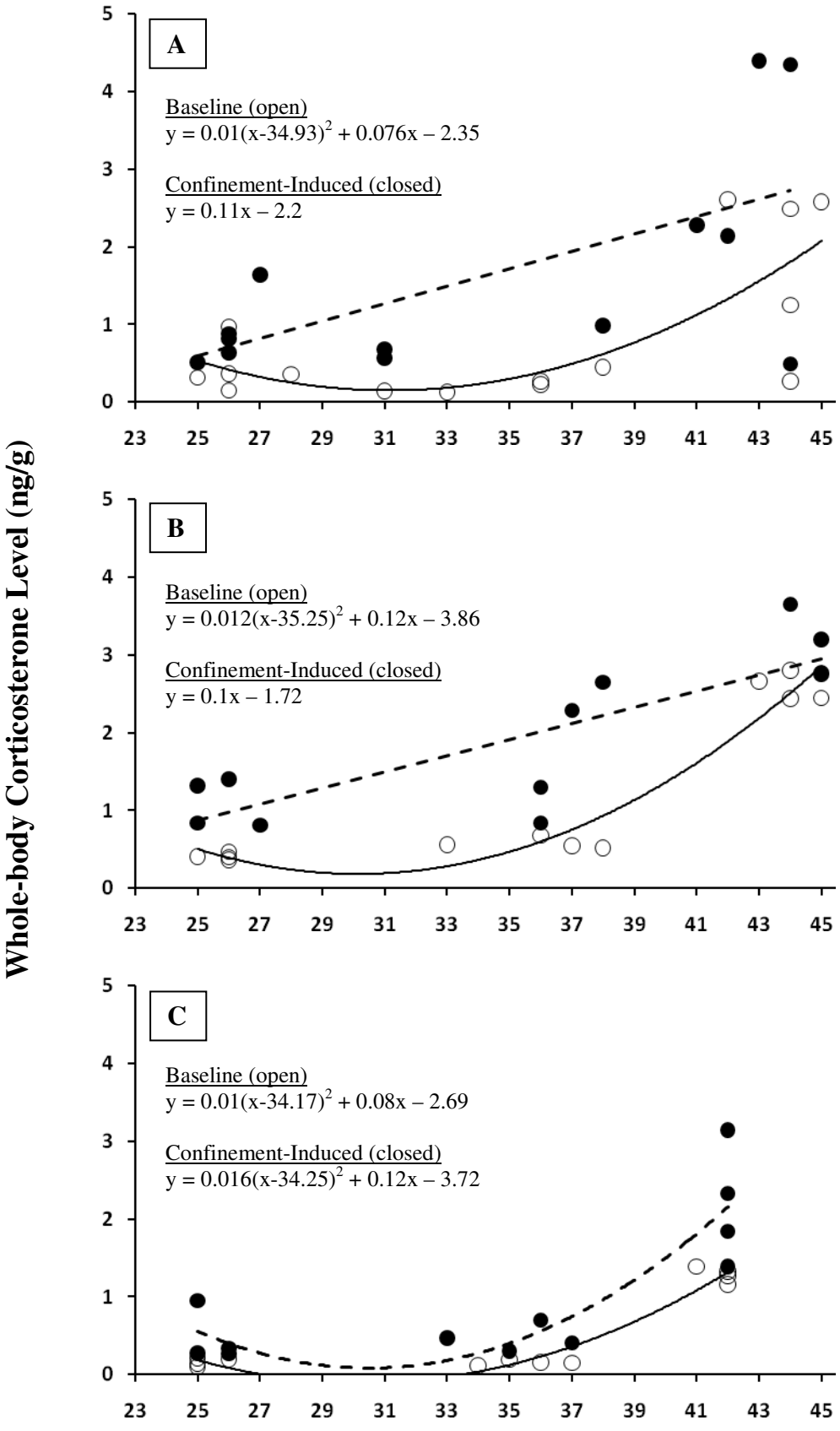
A
Baseline (open)
y = 0.01(x-34.93)2 + 0.076x – 2.35
Confinement-Induced (closed)
y = 0.11x – 2.2
B
Baseline (open)
y = 0.012(x-35.25)2 + 0.12x – 3.86
Confinement-Induced (closed)
y = 0.1x – 1.72
C
Baseline (open)
y = 0.01(x-34.17)2 + 0.08x – 2.69
Confinement-Induced (closed)
y = 0.016(x-34.25)2 + 0.12x – 3.72
Whole-body Corticosterone Level (ng/g)
Developmental Stage

Figure 2. Whole-body corticosterone levels (ng/g) throughout development in *Rana sylvatica* under free-living (A), mesocosm (B), and laboratory (C) conditions. Staging scheme of Gosner (1960). Open points represent baseline corticosterone levels. Closed points represent confinement-induced corticosterone levels. Regression equations and lines are shown for baseline (solid line) and confinement-induced (dashed line) corticosterone levels.

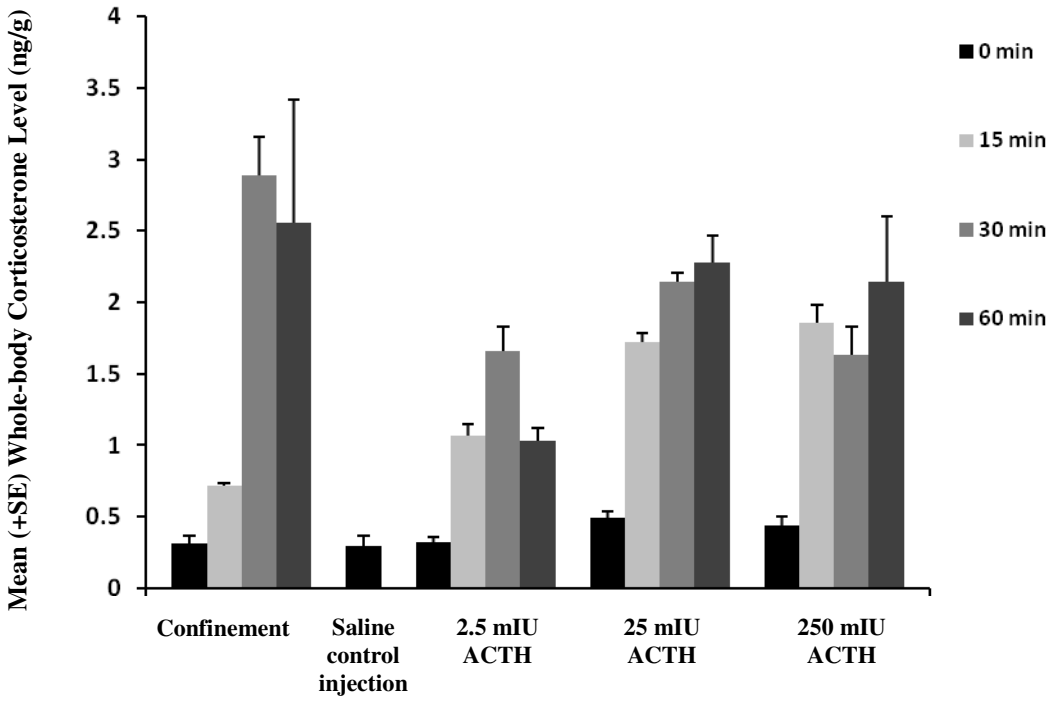

Figure 3. Mean (+SE) whole-body corticosterone levels (ng/g) in *Ambystoma jeffersonianum* after confinement or ACTH injection to stimulate the stress response. Specific exposure times for confinement and ACTH injection are noted.

Proximate factors underlying corticosterone level differences among salamander populations

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Abstract

Anthropogenic disturbances are altering nearly every habitat type on a global scale. In freshwater systems, these changes have resulted in one of the highest extinction rates in the world. In addition, habitat alterations can generate significant changes in biotic and abiotic components of these systems that can act as stressors upon system inhabitants. Vertebrate organisms possess several highly conserved physiological mechanisms for coping with stress, including the hormonal stress response. We examined levels of the stress hormone, corticosterone, in salamander larvae (Jefferson salamander – *Ambystoma jeffersonianum*) among eight different populations. We used the field survey and a subsequent mesocosm experiment to examine correlations between water quality parameters and corticosterone levels in *A. jeffersonianum* populations. We found that corticosterone levels were significantly different across ponds. Specifically, we found a significant negative correlation between pH and baseline corticosterone levels. There were also trends for baseline corticosterone levels to be positively correlated with chloride levels and negatively correlated with conductivity. Mesocosm experiments manipulating pH added further support to our field results of acidic conditions significantly increasing baseline corticosterone levels. Our findings suggest that abiotic factors, such as pH, could be a driving force behind corticosterone level differences among amphibian populations.

Introduction

Understanding how organisms interact with their environment is a central theme in ecology. Unfortunately, increasing anthropogenic disturbances to environments (Sala et al. 2000) can act as stressors upon system inhabitants by altering biotic and abiotic components of the environment (Bronmark and Hanson 2002; Riccardi and Rasmussen 1999; Wingfield and Romero 2001). The impact of environmental stressors, defined as any perturbation that alters an individual's homeostatic state, on an individual can range from sub-lethal behavioral (Walker et al. 2006) and physiological changes (Wikelski and Cooke 2006) to death (Alford and Richards 1999).

Vertebrate organisms possess a highly conserved physiological mechanism for coping with stress – the hormonal stress response (Romero 2004). Glucocorticoid hormones are released from the adrenal cortex with activation of the hypothalamic-pituitary-adrenal (HPA) axis following perceived stress (McEwen and Wingfield 2003). Physiological effects of glucocorticoids include energy mobilization, along with potential suppression of growth and reproduction over longer time frames (Moore and Jessop 2003). Glucocorticoids are essential in general energy economics, metabolism, behavior, reproduction, and survival (Dallman et al. 1993; Emerson and Hess 2001; Moore and Jessop 2003; Norris 1997). Despite their importance to all vertebrates, less is known about glucocorticoids in free-living amphibians than in other vertebrates.

Studies examining corticosterone, the main glucocorticoid in amphibians, typically analyze baseline and stress-induced corticosterone levels (Romero and Reed 2008). In general, baseline corticosterone levels are examined to provide an understanding of an individual's current physiological state. Stress-induced

corticosterone levels provide an understanding of the functioning of an individual's stress response (Gendron et al. 1997). Variation in baseline corticosterone levels may occur naturally within individuals, and can be mediated by diel, seasonal, or developmental changes (Romero 2002; Wright et al. 2003). Baseline corticosterone levels may also vary at the population level. Population differences in baseline corticosterone levels have been reported in birds (Lindstrom et al. 2005; Silverin 1998; Williams et al. 2008), reptiles (Wilson and Wingfield 1994), and amphibians (Belden et al. 2007; Homan et al. 2003a; Hopkins et al. 1997). However, some studies have reported no population differences in baseline corticosterone levels (Breuner et al. 2003; Pravosudov et al. 2004; Romero and Wikelski 2002). Though it is still unclear and debated among investigators, differing baseline corticosterone levels among populations may be due to the populations experiencing varied stressors. For instance, Hopkins et al. (1997) showed baseline corticosterone levels being significantly higher in a southern toad (*Bufo terrestris*) population exposed to coal combustion waste than a population from a non-polluted site.

One of our goals was to examine possible variation in corticosterone levels among larvae from multiple free-living populations of Jefferson salamander (*Ambystoma jeffersonianum*). We also examined whether corticosterone levels (baseline and confinement-induced) correlated with water quality parameters that could act as stressors. We selected pH, conductivity, water temperature, chloride (Cl), phosphate (PO_4), sulfate (SO_4), and nitrate (NO_3) as our water chemistry variables of interest. We selected these variables for several reasons. For instance, approximately 9.98 million metric tons of nitrogen-based compounds are used annually in the United States (Schuytema and Nebeker 1999). These compounds have been linked to both lethal and non-lethal effects

(reduced growth and development) in amphibians (Griffis-Kyle 2007; Ortiz et al. 2004). Other agrochemicals and chemical pollution (such as acid deposition) have also been linked to amphibian community disturbance (Beebee and Griffiths 2005). Considering their potential effects on amphibians, it seems likely that our selected water quality parameters could influence corticosterone levels. We followed up the field survey with a mesocosm experiment that further examined the significant relationship between corticosterone and low pH in *A. jeffersonianum* that we found in the field. We selected *A. jeffersonianum* because Petranka (1998) suggests that *A. jeffersonianum* is one of the most sensitive North American salamanders to environmental alterations. While its current distributions do not radically differ from its historic distributions, some *A. jeffersonianum* populations are currently declining or disappearing from areas entirely (Brodman 2005).

Materials and Methods

Field sample collection

We sampled a total of eight ponds in Maryland (MD) ($N = 2$), Pennsylvania (PA) ($N = 5$), and West Virginia (WV) ($N = 1$). To minimize diel, developmental, and seasonal effects on corticosterone (Romero 2002; Wright et al. 2003), all sampling occurred within three weeks during the same time of day (between 1100 and 1500 hrs). *Ambystoma jeffersonianum* larvae were collected using dip-nets. Both baseline ($N = 5$ larvae/pond) and confinement-induced ($N = 5$ larvae/pond) corticosterone levels were examined. Baseline corticosterone levels were sampled by freezing larvae individually in a dry ice/ethanol slurry within three mins of handling to reflect unstressed levels (Belden et al.

2003). To induce the stress response, we held individual larvae in 120 mL cups filled with 80 mL pond water for 30 mins, with cups being gently agitated every three mins (e.g., Belden et al. 2007). After 30 mins, larvae were frozen using a dry ice/ethanol slurry. Larvae were then returned to the laboratory and transferred to a -80°C freezer until radioimmunoassays could be performed. An additional N =10 larvae from each pond were collected to determine body mass and developmental stage (Watson and Russell 2000) homogeneity, and were subsequently released. Differences between mean larval mass and mean developmental stage (Watson and Russell 2000) across all ponds were assessed using an analysis of variance (ANOVA). Mean larval mass (g) (±SD) and developmental stage (Watson and Russell 2000) (±SD) for each sampled site were, respectively: 0.249 ± 0.04 and 15.6 ± 1.26 (WV1), 0.249 ± 0.05 and 14.8 ± 1.32 (MD1), 0.281 ± 0.05 and 16.3 ± 0.67 (MD2), 0.264 ± 0.08 and 15.2 ± 1.03 (PA1), 0.280 ± 0.05 and 14.7 ± 1.34 (PA2), 0.273 ± 0.02 and 15.7 ± 1.57 (PA3), 0.276 ± 0.04 and 16.8 ± 1.03 (PA4), and 0.279 ± 0.03 and 16.4 ± 1.07 (PA5). No significant differences were detected among all ponds for mean larval mass ($F_{1, 7} = 0.94$, $P = 0.37$) or developmental stage ($F_{1, 7} = 0.56$, $P = 0.48$).

Water chemistry analysis

A 90 mL water sample was collected from each sampled pond, frozen, and taken back to the laboratory for chemical analysis. Both pH and conductivity (mg/L) were measured in the field at each pond using a YSI 60 and YSI 550A probe (YSI Incorporated, Yellow Springs, Ohio), respectively. Water temperature (°C) was measured in the field using the YSI 60 probe.

All other water quality parameters were obtained using ion chromatography methods similar to those proposed by the United States Environmental Protection Agency (USEPA 1993; USEPA 1997). Prior to analysis, all water samples were filtered using a Gelman 0.45 µm Acrodisc filter. Approximately 4 mL of each sample was screened using a Dionex DX500 ion chromatograph with an EG50 Eluent Generator, AD20 absorbance detector, ED40 conductivity detector, Ionpac AS19 anion analytical column (4 mm), AG19 anion guard column, and a ASRS anion self-regenerating suppressor, all with a 500 µL sample loop. We measured Cl, PO_4, SO_4, and NO_3 levels (all ppb). Peak Net software (revision 8, version 5.1) was used to analyze water quality data output.

Mesocosm pH experiment

To examine the correlation between pH and corticosterone, we followed up the field survey by experimentally manipulating pH in mesocosms and examining changes in baseline and confinement-induced corticosterone levels in *A. jeffersonianum*. We collected recently deposited *A. jeffersonianum* egg masses ($N = 26$) from a single permanent pond in Montgomery County, Virginia. Once collected, we transported the egg masses to a 1000 L mesocosm stock tank at our outdoor research facility. All egg masses were placed in a single 1000 L stock tank filled with well water, approximately 250 g deciduous leaf litter and wild-caught zooplankton (screened from 2 L water). After egg masses were placed in the stock tank, we covered the tank with a cloth lid to prevent colonization by other species.

For the experiment, we setup a 3x3, 1000 L mesocosm array arranged in a randomized block design. pH treatments were pH = 5.0-5.8, 6.0-6.8, and 7.9-8.2

(control), with three replicates per level. Desired pH ranges were reached by adding dilute sulfuric acid (Rowe et al. 1992). Each mesocosm contained well water, approximately 250 g deciduous leaf litter and wild-caught zooplankton (screened from 2 L water), and were covered with a cloth lid to prevent colonization by other species (gray tree frog – *Hyla versicolor*, and dragonflies – Odonata). It took five weeks to achieve stable pH levels before $N = 20$ larvae were added to each mesocosm. An additional $N = 10$ *A. jeffersonianum* larvae were brought back to the laboratory to determine starting mass and developmental stage (Watson and Russell 2000), and were subsequently released. Starting mean (±SD) mass (g) and mean (±SD) developmental stage (Watson and Russell 2000) for *A. jeffersonianum* larvae were, respectively: 0.265 ± 0.04 g and 13.5 ± 1.27. Both baseline and confinement-induced corticosterone levels were sampled after 4 weeks of pH exposure by taking $N = 3$ larvae/mesocosm for each confinement time (baseline and 30 min). We used the same methodology described above for confinement techniques, sample storage, and pre-radioimmunoassay procedures.

Whole-body corticosterone radioimmunoassay

Prior to radioimmunoassays, all field-collected samples thawed overnight and were subsequently refrozen following failure of the -80°C freezer. As all of the samples are compared only amongst themselves, any variation from this freeze-thaw-freeze cycle should be consistent. In addition, observed levels are within the range we have observed previously for this species (Belden, unpublished data). Corticosterone was measured using whole-body radioimmunoassay procedures of Belden et al. (2003) with the following modification. Larvae were homogenized after weighing and developmental

staging (Watson and Russell 2000). Homogenization occurred in mass adjusted distilled water (mass of individual x 10 mL + 0.5 ml rinse, with a minimum of 1.5 mL and maximum of 5 mL). Samples were centrifuged at 3000 rpm for 10 mins to break the emulsion. Three standard curves were prepared for each assay. In total, we conducted three assays. Our field survey samples comprised two assays. Specifically, one assay consisted of all Maryland, all West Virginia, and two Pennsylvania pond samples ($N = 49$ total samples; limit of assay detection = approximately 0.89 ng/g). The remaining three Pennsylvania pond samples ($N = 30$ total samples; limit of assay detection = approximately 0.89 ng/g) comprised another assay. Our mesocosm samples ($N = 54$) were analyzed in a single assay (limit of assay detection = approximately 0.89 ng/g). Inter-assay variation was 30.2%, and intra-assay variation averaged 12.1%. Based upon individual recoveries, whole-body extraction efficiency averaged 49%.

Statistical analyses

We used a two-way ANOVA to assess differences between mean corticosterone levels among confinement time (baseline and 30 min) and ponds ($N = 8$). A correlation matrix was used to determine if corticosterone levels (baseline or confinement-induced) were correlated with Cl (ppb), PO_4 (ppb), SO_4 (ppb), NO_3 (ppb), water temperature (°C), pH, and conductivity (mg/L). For our outdoor mesocosms, we used corticosterone level means from each mesocosm across all pH treatments in our analyses. An ANOVA was used to determine if mean corticosterone levels (baseline and confinement-induced) varied among pH treatments. Identical to our field survey, we used an ANOVA to determine if confinement for 30 min significantly increased corticosterone levels as

compared to baseline levels. All statistical analyses were conducted using SAS JMP (version 7.0).

Results

Corticosterone levels significantly varied across all ponds ($F_{8, 15} = 6.89$, $P = 0.001$; Fig. 1). Corticosterone levels significantly increased in response to confinement in the field ($F_{1, 7} = 17.54$, $P = 0.006$; Fig. 1). Baseline corticosterone levels were negatively correlated with pH ($r = 0.73$, $P = 0.04$; Fig. 2A). There was a trend for baseline corticosterone levels to be positively correlated with chloride levels ($r = 0.65$, $P = 0.08$; Fig. 2C) and negatively correlated with conductivity ($r = 0.62$, $P = 0.09$; Fig. 2B). Phosphate levels ($r = -0.01$, $P = 0.97$), sulfate levels ($r = -0.06$, $P = 0.88$), nitrate levels ($r = 0.22$, $P = 0.61$), and water temperature ($r = -0.23$, $P = 0.58$) did not correlate to baseline corticosterone levels in *A. jeffersonianum*. There was a trend for confinement-induced corticosterone levels in *A. jeffersonianum* to be negatively correlated with pH ($r = -0.71$, $P = 0.05$), but not to conductivity ($r = -0.59$, $P = 0.12$), chloride levels ($r = 0.27$, $P = 0.52$), phosphate levels ($r = 0.25$, $P = 0.55$), sulfate levels ($r = -0.25$, $P = 0.54$), nitrate levels ($r = -0.06$, $P = 0.89$), and water temperature ($r = 0.16$, $P = 0.71$). In our outdoor mesocosms, pH significantly influenced baseline corticosterone levels ($F_{2, 8} = 335.81$, $P < 0.0001$; Fig. 3) and confinement-induced corticosterone levels ($F_{2, 8} = 12.48$, $P = 0.007$; Fig. 3). In addition, confinement significantly increased corticosterone levels compared to baseline levels ($F_{1, 8} = 31.31$, $P = 0.0008$; Fig. 3).

Discussion

Baseline corticosterone levels were correlated to acidic conditions in both the field and mesocosms. Acid deposition, and subsequent pH reduction, is a known environmental stressor in aquatic systems (Driscoll et al. 2001). Monette and McCormick (2008) showed a 4.3 fold increase in plasma cortisol levels after exposure to pH 5.0-5.4 in Atlantic salmon smolt (*Salmo salar*). However, no study has directly measured the physiological stress response associated with low pH exposure in amphibians until ours. Several studies have shown potential negative impacts of acidic conditions on amphibians. For instance, exposure to acidic conditions has been linked to decreased developmental rates (Horne and Dunson 1994), and increased mortality in *A. jeffersonianum* (Rowe et al. 1992). Thus, a physiological response to this potential stressor is a reasonable expectation. Another possible explanation for increasing corticosterone levels in larval salamanders after exposure to acidic pH is an indirect effect of acidic pH on the food web, specifically on zooplankton. Zooplankton biomass can significantly decrease after exposure to acidic conditions (Hogsden et al. 2009; Markovic et al. 2009). Thus, if *A. jeffersonianum* larvae have less food available, we would expect to observe an increase in corticosterone levels as they mobilize energy reserves to negotiate through periods of low prey availability.

An individual may also respond to acidic conditions by increasing corticosterone, as we have shown, because of an increased demand for energy. Vertebrates, including amphibians, have optimum pH ranges for internal processes (Burton 2002). If pH differs from their optimum range in these systems, functionality could be altered. Thus, more energy could be required to repair and maintain these systems during exposure to acidic

conditions. This could explain our observed increase in baseline corticosterone levels, as corticosterone is released to initiate energy mobilization (Moore and Jessop 2003). In addition, amphibian skin assists in maintaining internal pH homeostasis (Candia and Yorio 1997). pH imbalances are resolved by excreting H^+, NH_4^+, or HCO_3^- from epithelial cells, depending on specific pH conditions, to restore pH balance (Vanatta and Frazier 1981). Again, such activity could require an increase in energy that is mobilized by increasing circulating baseline corticosterone levels.

In most amphibians, confinement and adrenocorticotropic hormone (ACTH) injections can activate the stress response (Belden et al. 2005; Glennemeier and Denver 2002). As expected, confinement increased circulating corticosterone above baseline levels in most instances in our study. In instances where this did not occur, it is likely that elevated baseline levels negated any possible significant difference. Hopkins et al. (1999) saw a similar pattern of insignificance because of elevated baseline corticosterone levels in *B. terrestris* exposed coal combustion waste.

Another possibility that could explain why 30 min confinement/agitation did not significantly increase corticosterone levels is altered HPA axis functionality. Habituation, desensitization, or physiological exhaustion after prolonged exposure to a stressor could possibly explain our diminished stress response results (Cyr and Romero 2009). Future studies could examine HPA axis functionality after prolonged exposure to an environmental stressor in *A. jeffersonianum* to determine whether habituation, desensitization, or physiological exhaustion is occurring. Such studies would be invaluable not only because some *A. jeffersonianum* populations are disappearing in some areas (Brodman 2005), but because habituation, desensitization, and physiological

exhaustion can lead to pathological effects including decreased survival (Romero et al. 2009).

We found evidence of a trend for baseline corticosterone levels to be positively correlated with chloride levels and negatively correlated with conductivity**.** In general, the effects of conductivity on amphibians are greatly understudied and subsequently poorly understood. While some studies have shown conductivity to be a limiting factor of amphibian diversity (Smith et al. 2007), others have shown either mixed results (Welch and MacMahon 2005) or no effects of conductivity whatsoever on species abundance (Dodd and Dorazio 2004) and survival (Loman and Lardner 2006). Our results suggest that amphibian larvae may physiologically respond to altered ion content by increasing baseline corticosterone levels. Whether this response significantly deviates from normal baseline corticosterone levels or if it has any significant fitness effects remains unknown.

Studies examining baseline corticosterone levels are relatively rare in the literature despite the contributions of corticosterone to several physiological processes (Sapolsky et al. 2000; Wikelski and Cooke 2006). Baseline corticosterone data, especially from natural populations, are also critical for understanding the natural stress hormone profile and how individuals respond to environmental stressors (Belden et al. 2005). Our field survey results show corticosterone levels significantly differing among free-living *A. jeffersonianum* populations. One of the authors (LKB) has also shown variations in baseline corticosterone levels among free-living wood frog (*Rana sylvatica*) populations (Belden et al. 2007). Differing baseline corticosterone levels could suggest that these populations are experiencing varied environmental stressors in their aquatic systems. In support, Homan et al. (2003b) proposed that high corticosterone levels could be

indicative of an environment exerting stress upon its inhabitants. However, it is critically important to verify that any variations in baseline corticosterone levels are caused by actual stressors and are not a by-product of natural variations due to season or diel variation (Rich and Romero 2008). This study addresses the concerns of Rich and Romero (2008) by correlating increased baseline corticosterone levels among *A. jeffersonianum* populations to low pH under experimental conditions. Future studies could examine the direct and indirect effects of multiple stressors, at the same, on corticosterone levels in amphibians. Our study and others suggest that exposure to a suite of environmental stressors is more likely to occur than exposure to a single stressor (Alford and Richards 1999). However, this study was only able to correlate abiotic parameters and propose an indirect effect of food webs on corticosterone levels in larval amphibians. Determining direct and indirect cause/effect relationships between multiple environmental stressors and corticosterone levels could provide more ecologically relevant insight into how amphibians cope with environmental change.

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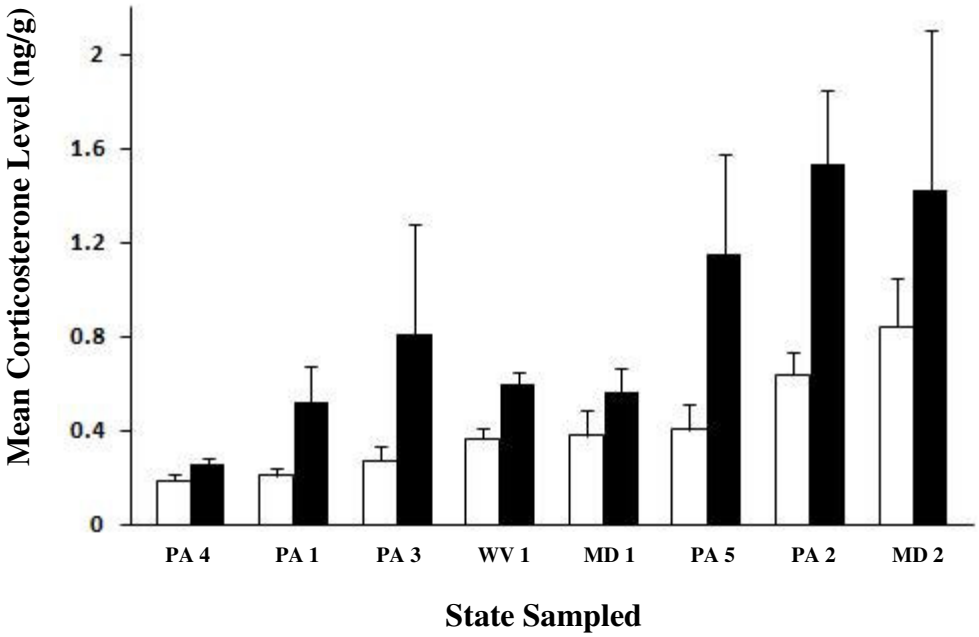

Figure 1: Mean (± SE) whole-body baseline and confinement-induced corticosterone levels of *Ambystoma jeffersonianum* across multiple states. Baseline corticosterone levels are indicated by white bars. Confinement-induced corticosterone levels are indicated by black bars. PA=Pennsylvania, MD=Maryland, WV=West Virginia.

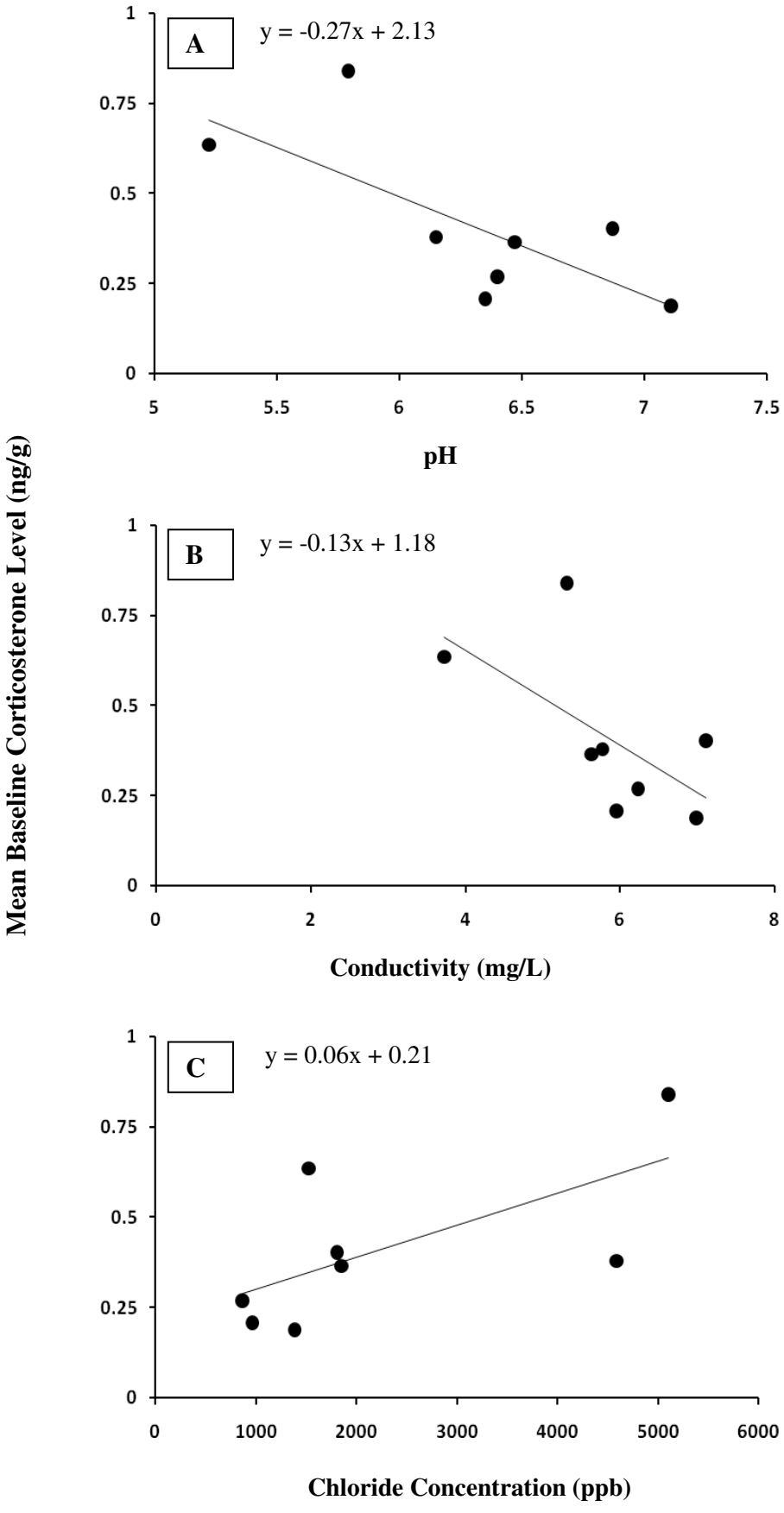
A
y = -0.27x + 2.13
1
0.75
0.5
0.25
0
5
5.5
6
6.5
7
7.5
pH
Mean Baseline Corticosterone Level (ng/g)
B
y = -0.13x + 1.18
1
0.75
0.5
0.25
0
0
2
4
6
8
Conductivity (mg/L)
C
y = 0.06x + 0.21
1
0.75
0.5
0.25
0
0
1000
2000
3000
4000
5000
6000
Chloride Concentration (ppb)

Figure 2: Mean whole-body baseline corticosterone levels (ng/g) of *Ambystoma jeffersonianum* plotted against water quality parameters: (A) pH, (B) conductivity (mg/L), and (C) chloride concentration (ppb). Regression equations are shown for each water quality parameter.

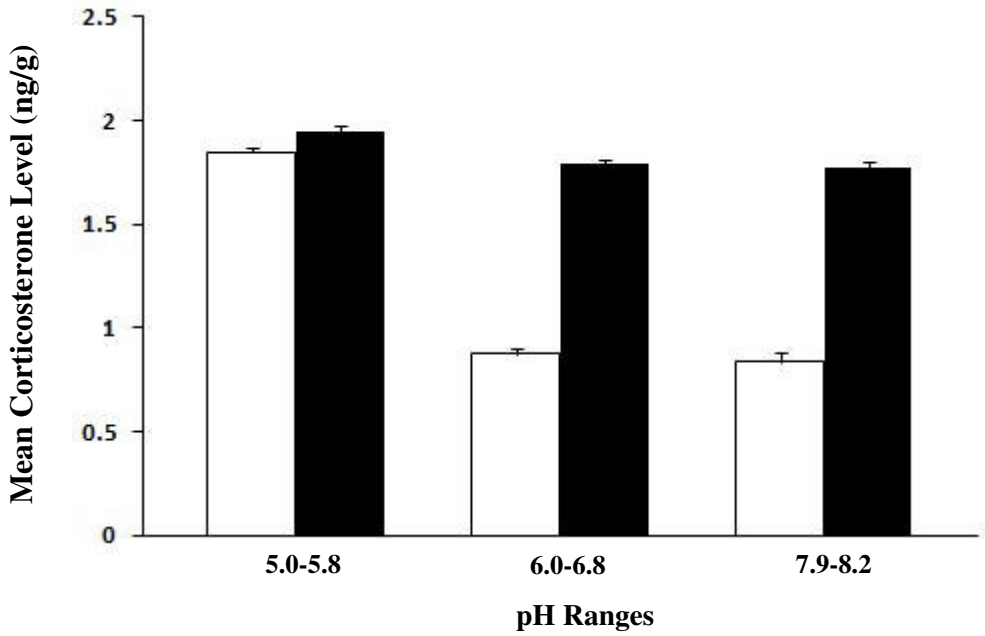

Figure 3: Mean (± SE) whole-body corticosterone levels (ng/g) of *Ambystoma jeffersonianum* after exposure to low pH in mesocosms. Baseline corticosterone levels are indicated by white bars. Confinement-induced corticosterone levels are indicated by black bars.

Effects of pH on corticosterone, prey consumption, and survival in larval amphibians under laboratory conditions

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Abstract

Anthropogenic disturbances in freshwater systems are responsible for one of the highest extinction rates in the world. These disturbances can also exert sub-lethal effects upon freshwater system inhabitants by acting as stressors. Vertebrate organisms possess a highly conserved physiological mechanism for coping with stress – the hormonal stress response. We examined the hormone stress response to acidification, a prevalent freshwater disturbance, by manipulating pH levels in the laboratory and examining the response in the larvae of four amphibian species: Jefferson salamander (*Ambystoma jeffersonianum*), spotted salamander (*A. maculatum*), wood frog (*Rana sylvatica*), and grey treefrog (*Hyla versicolor*). Both baseline and confinement-induced corticosterone levels were examined following one week exposure to pH levels ranging from 5.53 to 8.16 (control), depending on the species. We also examined survivorship (all amphibian species) and prey consumption (*Ambystoma* only) following low pH exposure. Exposure to acidic conditions significantly increased baseline corticosterone levels in all four amphibian species, and significantly increased confinement-induced corticosterone levels in *A. jeffersonianum*, *A. maculatum*, and *R. sylvatica*. In addition, low pH exposure significantly decreased survivorship in *A. jeffersonianum*, *A. maculatum*, and *R. sylvatica*, and decreased prey consumption in *A. jeffersonianum* and *A. maculatum*. Our results convincingly show that acidic conditions can be an important stressor for larval amphibians.

Introduction

Anthropogenic disturbances are altering biotic and abiotic components of nearly all habitats, particularly freshwater systems (Bronmark and Hansson 2002). As a result, organisms living in freshwater systems have one of the highest extinction rates in the world (Riccardi and Rasmussen 1999). Anthropogenic disturbances are also capable of exerting a variety of sub-lethal effects upon system inhabitants (Bronmark and Hansson 2002). One such anthropogenic disturbance, acidification, is prevalent in many freshwater systems (Driscoll et al. 2001).

There are several mechanisms that can lead to freshwater acidification. Atmospheric deposition of sulfur and nitrogen-based compounds, and subsequent acid precipitation, from fuel emissions is one common depository mechanism (Driscoll et al. 2001). Freshwater systems can also be subject to acidification through surface runoff (Rowe et al. 1992; Vuori 1995). Closed freshwater systems, such as vernal pools, can also become acidic from plant material decomposition (Colburn 2004). Since vernal pools typically lack continuous water flow (Colburn 2004) and have poor buffering capacities (Freda 1986), organic acids released from decaying plant material can accumulate and become concentrated. Acidification of freshwater systems is most pronounced during spring months when rainfall and snow melt rates increase (Driscoll et al. 2001). This time period coincides with most amphibian breeding activity in freshwater systems. As a result, amphibians can be exposed to acidic conditions during peak amphibian activity periods.

Many negative effects have been observed in amphibians following acidic pH exposure, including larval deformation (Beattie and Tyler-Jones 1992), altered timing of egg hatching (Bradford et al. 1992; Horne and Dunson 1994), decreased growth and development (Beattie and Tyler-Jones 1992; Sadinski and Dunson 1992), altered species diversity and density (Wyman and

Jancola 1992), and decreased survival (Blem and Blem 1989; Rowe et al. 1992; Sadinski and Dunson 1992). Comparatively fewer studies have identified behavioral modifications as a result of exposure to acidic conditions in amphibians, although pH as low as 2.75 has been linked to altered reproductive behavior (Ortiz-Santaliestra et al. 2007; Rowe and Dunson 1993), altered habitat preference (Freda and Taylor 1992; Wyman and Hawksley-Lescault 1987), altered swimming behavior (Green and Peloquin 2008; Kutka 1994), and altered foraging behaviors (Griffiths et al. 1993; Preest 1993; Rasanen et al. 2002).

Amphibians, and other vertebrates, physiologically respond to environmental stressors by releasing glucocorticoids from the adrenal cortex after hypothalamic-pituitary-adrenal axis activation (McEwen and Wingfield 2003). Increasing glucocorticoid circulation mobilizes energy reserves to cope with stressful events (Romero 2002). Throughout this energy mobilization, glucocorticoids may temporarily halt non-essential processes such as reproduction and growth (Moore and Jessop 2003). Most studies investigating corticosterone, the main glucocorticoid in amphibians, typically examine baseline and stress-induced corticosterone levels (Romero and Reed 2008). Baseline corticosterone levels reflect an individual's current physiological state, while stress-induced corticosterone levels reflect the functionality of an individual's stress axis (Gendron et al. 1997). Prolonged exposure to increased corticosterone may have associated fitness costs. Concerning amphibians, several studies have experimentally linked elevated corticosterone levels to altered behavior (Moore and Miller 1984), immune system components (Belden and Kiesecker 2005; Ducoroy et al. 1999), growth and development (Belden et al. 2005; Glennemeier and Denver 2002), and survival (Hayes et al. 1993).

In this study, we examined the effects of exposure to acidic pH on corticosterone levels (baseline and stress-induced) under laboratory conditions using the larvae of four amphibian

species: Jefferson salamander (*A. jeffersonianum*), spotted salamander (*A. maculatum*), wood frog (*Rana sylvatica*), and grey treefrog (*Hyla versicolor*). We have previously shown baseline corticosterone levels in larval *A. jeffersonianum* to be negatively associated with acidic conditions in the field and in mesocosms (Chapter III). Thus, we hypothesized that pH would negatively influence baseline corticosterone levels in our four species under laboratory conditions. We also examined the effects of acidic conditions on prey consumption (for *Ambystoma* only) and survival (all amphibian species). We hypothesized that exposure to acidic conditions would negatively impact prey consumption rates (*Ambystoma* only) and survival in our four species.

Materials and Methods

Amphibian egg mass collection/rearing

We collected recently deposited egg masses of *A. jeffersonianum* (N = 26) and *R. sylvatica* (N = parts of 8 masses) from Montgomery County, Virginia, and recently deposited *A. maculatum* egg masses (N = 5) from Augusta County, Virginia. Once collected, egg masses were transported to 1000 L mesocosm stock tanks at an outdoor research facility. For *H. versicolor*, we set out several 100 L plastic pools at the same outdoor research facility. Each pool was filled with well water and contained approximately 100 g deciduous leaf litter. *Hyla versicolor* usually breed in these 100 L pools under their own accord. Prior to egg mass deposition, each stock tank was filled with well water and we added approximately 250 g deciduous leaf litter and wild-caught zooplankton (screened from 2 L water). Egg masses of each species were housed in separate stock tanks. After egg masses were placed in their respective stock tank, we covered each tank with a shadecloth lid to prevent colonization by other species (particularly *H.*

versicolor and dragonflies). Egg masses and hatchling amphibian larvae were monitored at least three times/week until reaching desired mass (g) and developmental stage (Watson and Russell (2000) for *Ambystoma* species, Gosner (1960) for *R. sylvatica* and *H. versicolor*) for pH experiments. At the time we collected individuals for experiments, N = 10 additional larvae were collected from the stock tanks to determine mass and developmental stage. Starting mean (±SD) masses (g) and developmental stages (±SD), respectively, were: 1.43 ± 0.06 g and 17.8 ± 0.92 (*A. jeffersonianum*), 1.41 ± 0.06 g and 17.8 ± 0.79 (*A. maculatum*), 0.602 ± 0.02 g and 31.6 ± 1.26 (*R. sylvatica*), 0.432 ± 0.03 g and 33.0 ± 1.15 (*H. versicolor*).

pH manipulations

The pH experiments were completed using regression designs consisting of a control and four lowered pH levels. We examined the pH range of 5.5 to 8 because it reflected levels we have found in the field (Chapter III). Dilute sulfuric acid was added to our dechlorinated laboratory water (pH approximately 8 to 8.1) to lower the pH level in our four acidic treatments prior to adding the larval amphibians (Rowe et al. 1992). The actual pH levels at the start of experimentation were: 8.16, 7.22, 6.60, 6.38, and 5.59 (*A. jeffersonianum*); 8.03, 7.68, 7.15, 6.33, and 5.64 (*A. maculatum*); 8.14, 7.42, 6.54, 6.10, and 5.53 (*R. sylvatica*); 8.03, 7.72, 7.22, 6.53, and 5.74 (*H. versicolor*). Five 6 L plastic tubs were used for each species. Tubs were filled with 4.5 L dechlorinated water and placed in a temperature (~ 22°C) and photoperiod (12 h light: 12 h dark) controlled room. After reaching desired pH levels, N = 10 larvae were placed in each tub. Survivorship for each amphibian species in each container was recorded daily. After one week of exposure, N = 3 to 5 larvae (dependent upon survivorship) were removed and immediately (within 3 min) frozen in a dry ice/ethanol slurry for baseline corticosterone level measurement.

Confinement-induced corticosterone levels were sampled by taking N = 3 larvae from each tub and individually placing them in 120 mL cups filled with 80 mL dechlorinated water for 30 mins, with cups being gently agitated every three mins. After 30 mins, larvae were frozen. All samples were then stored in a -80°C freezer until radioimmunoassay (RIA) for corticosterone could be performed.

Prey consumption trials

We conducted prey consumption trials for *A. jeffersonianum* and *A. maculatum* after five days of exposure to acidic pH. We setup three pH levels for each *Ambystoma* species, with one control and two low pH treatments. pH levels used were: 8.14, 7.10, and 6.30 (*A. jeffersonianum*) and 8.03, 7.15, and 6.17 (*A. maculatum*). Each pH level had N = 5 larvae of each species. Dilute sulfuric acid was used to reach desired pH levels (Rowe et al. 1992). Larvae were individually housed in opaque white cups (approximately 946 mL) filled with approximately 800 mL dechlorinated water in the same temperature/light controlled room previously described. Larvae were not fed throughout the five day exposure. After 5 days, larvae were removed from respective their pH treatments and placed in another cup under pH neutral conditions so that pH would not affect prey activity. Each larva was given 1.5 g live black worms and allowed to feed for 24 hr. After time expiration, all remaining black worms from each cup were removed and re-weighed for wet mass.

Whole-body corticosterone radioimmunoassay

Corticosterone was measured using whole-body RIA procedures of Belden et al. (2003), with the following minor modifications. Larval homogenization occurred in mass adjusted

distilled water (mass of individual x 10 mL + 0.5 mL rinse, with a minimum of 1.5 mL and maximum of 5 mL). After sample extraction, centrifugation at 3000 rpm for 10 mins was conducted to break the emulsion. Three standard curves were prepared for each assay. In total, we conducted two assays. One assay was for the *A. jeffersonianum* and *R. sylvatica* samples (limit of assay detection = approximately 0.89 ng/g), the other for *A. maculatum* and *H. versicolor* samples (limit of assay detection = approximately 1.19 ng/g). Intra-assay variation was 16% and inter-assay variation was 2%. Based upon individual recoveries, average extraction efficiency was 63%.

Statistical analyses

We used 2nd order polynomial regression analyses to assess the relationship between confinement time (baseline or 30 min) and pH. The relationship between percent prey consumed and survivorship to pH for each species was also determined using regression (linear or 2nd order polynomial). We used an analysis of variance (ANOVA) to determine if confinement activated the stress response, subsequently increasing corticosterone levels compared to baseline levels. All statistical analyses were conducted using SAS JMP (version 7.0).

Results

Overall, there was a strong negative relationship between pH and corticosterone levels, with acidic conditions correlating to higher corticosterone levels. Specifically, there was a negative relationship between pH and corticosterone levels in *A. jeffersonianum* (baseline samples, $R^2 = 0.95$, $p < 0.0001$; confinement-induced samples, $R^2 = 0.65$, $p = 0.003$; Fig. 1A), *A. maculatum* (baseline samples, $R^2 = 0.89$, $p < 0.0001$; confinement-induced samples, $R^2 = 0.63$, p

= 0.002; Fig. 1B), *R. sylvatica* (baseline samples, $R^2 = 0.90$, $p < 0.0001$; confinement-induced samples, $R^2 = 0.48$, $p = 0.02$; Fig. 1C), and *H. versicolor* (baseline samples, $R^2 = 0.93$, $p < 0.0001$; Fig. 1D). There was a positive relationship between pH and confinement-induced corticosterone levels in *H. versicolor* ($R^2 = 0.89$, $p < 0.0001$; Fig. 1D). As expected, corticosterone levels were significantly elevated above baseline levels after confinement for all species combined ($F_{1, 19} = 4.38$, $p = 0.05$; Fig. 1).

Survival was significantly correlated to acidic pH in *A. jeffersonianum* ($R^2 = 0.86$, $p = 0.02$; Fig. 2A), *A. maculatum* ($R^2 = 0.84$, $p = 0.03$; Fig. 2B), and *R. sylvatica* ($R^2 = 0.86$, $p = 0.02$; Fig. 2C), with more acidic conditions resulting in decreased survival. No mortality resulted from acidic pH exposure in *H. versicolor* (Fig. 2D).

There was also a strong negative correlation between pH and prey consumption in both *A. jeffersonianum* ($R^2 = 0.94$, $p < 0.0001$; Fig. 3A) and *A. maculatum* ($R^2 = 0.97$, $p < 0.0001$; Fig. 3B).

Discussion

Based upon our previous work with *A. jeffersonianum* and pH in mesocosms and the field (Chapter III), we hypothesized that corticosterone levels would be significantly influenced by pH under laboratory conditions for the species tested in this study. Our results strongly support this, as elevated baseline corticosterone levels were significantly associated with decreased pH in *A. jeffersonianum*, *A. maculatum*, *R. sylvatica*, and *H. versicolor*. Several possible mechanisms may explain this relationship, including an increased energy demand to maintain and repair pH-altered internal systems and pH homeostasis. Accelerating metamorphosis to vacate sub-optimal conditions could be another possible explanation. Several studies have shown amphibians to

accelerate metamorphosis when experiencing food deprivation (Newman 1994 and 1998) and pond drying (Kiesecker and Skelly 2001; Newman 1989). Denver (1998) determined that accelerated metamorphosis in western spadefoot tadpoles (*Scaphiopus hammondii*) in response to pond drying was mediated by increases in thyroxine, triiodothyronine, and corticosterone. Exposure to sub-optimal pH levels may also activate the mechanisms needed to accelerate metamorphosis. Corticosterone is one of several hormones involved in metamorphosis (Shi 2000), with sharp increases typically associated with metamorphic climax (Wada 2008). Thus, when faced with sub-optimal conditions, an increase in circulating corticosterone levels to promote accelerated metamorphosis is a reasonable expectation.

As expected, confinement significantly increased corticosterone levels above baseline levels. The use of confinement has proven to be an effective method of eliciting a stress response in amphibians and other vertebrates (Belden et al. 2005; Glennemeier and Denver 2002; Romero et al. 2008). Our results also show that elevated confinement-induced corticosterone levels were significantly influenced by acidic pH exposure in *A. jeffersonianum*, *A. maculatum*, and *R. sylvatica*. This suggests that the integrity of the stress-axis had not been significantly compromised by exposure to acidic pH as these individuals are still capable of mounting a stress response. However, *H. versicolor* tadpoles appear to have a diminished stress response to confinement/agitation as pH becomes more acidic (Fig. 1D). Similar results of a diminished stress response after prolonged exposure to cadmium and zinc have been reported in brown trout (*Salmo trutta*) (Norris et al. 1999).

There are three possible mechanisms that could have led to a diminished stress response in our *H. versicolor* tadpoles – habituation to a stressor, desensitization of the stress response, and physiological exhaustion (Cyr and Romero 2009). Habituation can occur when an individual

becomes familiar with a stressor, thus not perceiving that stressor as noxious after repeated exposure (Cyr and Romero 2009). Desensitization without habituation can occur when an individual does not adapt to a repeated stressor and perceives that stressor as noxious (Cyr and Romero 2009). Physiological exhaustion occurs when an individual is too fatigued to maintain a physiological system after prolonged stressor exposure (Cyr and Romero 2009). Regardless of the underlying mechanism, a diminished stress response can lead to pathological effects including altered immune system functionality, inhibited reproduction, and decreased survival (Romero et al. 2009).

Survivorship was significantly influenced by acidic pH in *A. jeffersonianum*, *A. maculatum*, and *R. sylvatica*. Decreased survival after low pH exposure was expected, especially in the *Ambystoma* species. Several studies have shown *A. jeffersonianum* and *A. maculatum* to be particularly sensitive to acidic conditions (Blem and Blem 1989; Rowe and Dunson 1993; Rowe et al. 1992), which has led to claims of *A. jeffersonianum* being one of the most sensitive salamanders to acidification (Petranka 1998). For *A. maculatum*, acidification of breeding ponds is believed to be responsible for several population declines in Virginia (Blem and Blem 1989 and 1991). Interestingly, pH did not influence survival in *H. versicolor*, as no mortality resulted at any pH level. Cline (2005) suggested that *H. versicolor* is tolerant to pollutants and other anthropogenic disturbances. Our *H. versicolor* survival results support this claim.

Prey consumption was negatively influenced by low pH in *A. jeffersonianum* and *A. maculatum*. Decreased food consumption after exposure to acidic conditions have been reported in other amphibian species such as smooth newts (*Triturus vulgaris*), palmate newts (*T. helveticus*), tiger salamanders (*A. tigrinum*), and common frogs (*R. temporaria*) (Griffiths et al. 1993; Kiesecker 1996; Rasanen et al. 2002). One possible explanation lies within prey detection

ability. Olfaction is believed to play a role in prey detection among salamanders (Wake and Deban 2000). Salamanders possess a nasal olfactory system that is lined with specialized epithelial cells for sensing and detecting, among other things, chemical cues of prey (Dawley and Bass 1989; Dawley and Crowder 1995; Wake and Deban 2000). Exposure to acidic pH could alter the functionality of this chemical cue system. Thus, a decrease in prey capture efficiency is a reasonable expectation. Another possible explanation lies within muscle physiology. Preest (1993) proposed that altered muscle activity, such as decreased tension and increased latency, after acidic pH exposure could lead to a reduction in prey capture efficiency in *A. maculatum*. Exposure to acidic pH could also be rendering the *Ambystoma* larvae moribund, which could result in a reduction in prey capture efficiency.

Investigators are using corticosterone levels as a biomarker reflective of a population's health status (Romero 2004). For example, increased corticosterone levels were used to predict survival in marine iguanas (*Amblyrhynchus cristatus*) during El Niño events in the Galápagos (Romero and Wikelski 2001). Our results support this type of biomarker usage, as we have shown an environmental stressor to be significantly correlated to survival and corticosterone levels in several larval amphibians. However, unlike Romero and Wikelski (2001), our study does not directly link an environmental stressor to increased corticosterone levels and an ecologically relevant cost. Future studies should attempt to find direct cause/effect relationships between environmental stressors, increased corticosterone levels, and ecologically relevant costs associated with increased corticosterone levels. Such studies are of the utmost importance because environments are being altered at increasing rates by humans, and these alterations are capable of acting as stressors towards system inhabitants (Walker et al. 2005; Wikelski and Cooke 2006).

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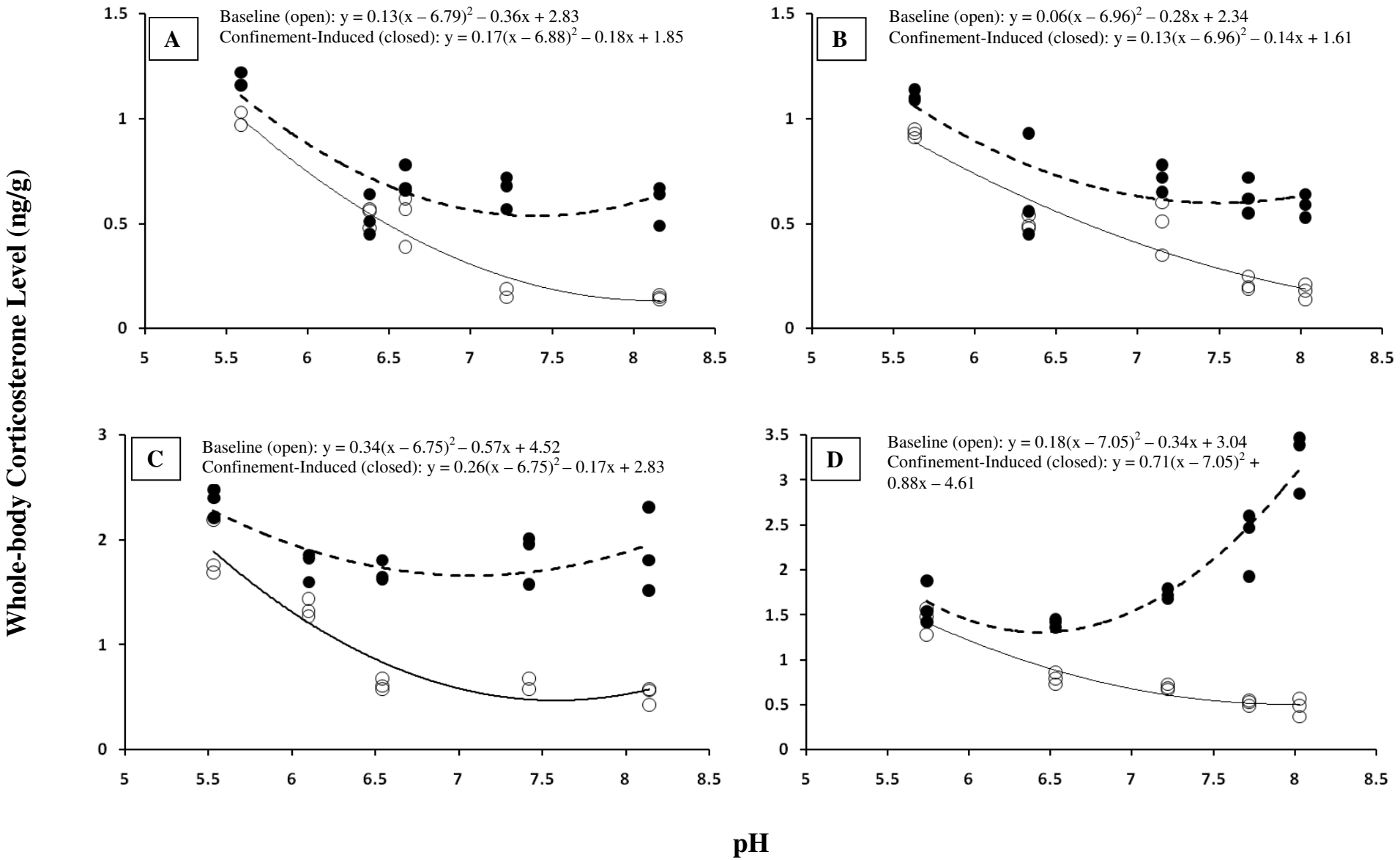
A
Baseline (open): y = 0.13(x – 6.79)^2 – 0.36x + 2.83
Confinement-Induced (closed): y = 0.17(x – 6.88)^2 – 0.18x + 1.85
B
Baseline (open): y = 0.06(x – 6.96)^2 – 0.28x + 2.34
Confinement-Induced (closed): y = 0.13(x – 6.96)^2 – 0.14x + 1.61
C
Baseline (open): y = 0.34(x – 6.75)^2 – 0.57x + 4.52
Confinement-Induced (closed): y = 0.26(x – 6.75)^2 – 0.17x + 2.83
D
Baseline (open): y = 0.18(x – 7.05)^2 – 0.34x + 3.04
Confinement-Induced (closed): y = 0.71(x – 7.05)^2 + 0.88x – 4.61
Whole-body Corticosterone Level (ng/g)
pH

Figure 1: Whole-body corticosterone levels (ng/g) after acidic pH exposure in larval amphibians. Species shown include: *Ambystoma jeffersonianum* (A), *A. maculatum* (B), *Rana sylvatica* (C), and *Hyla versicolor* (D). Open points represent baseline corticosterone levels. Closed points represent confinement-induced corticosterone levels. Each point represents a single individual. Regression equations and lines are shown for baseline (solid line) and confinement-induced (dashed line) corticosterone levels for each species.

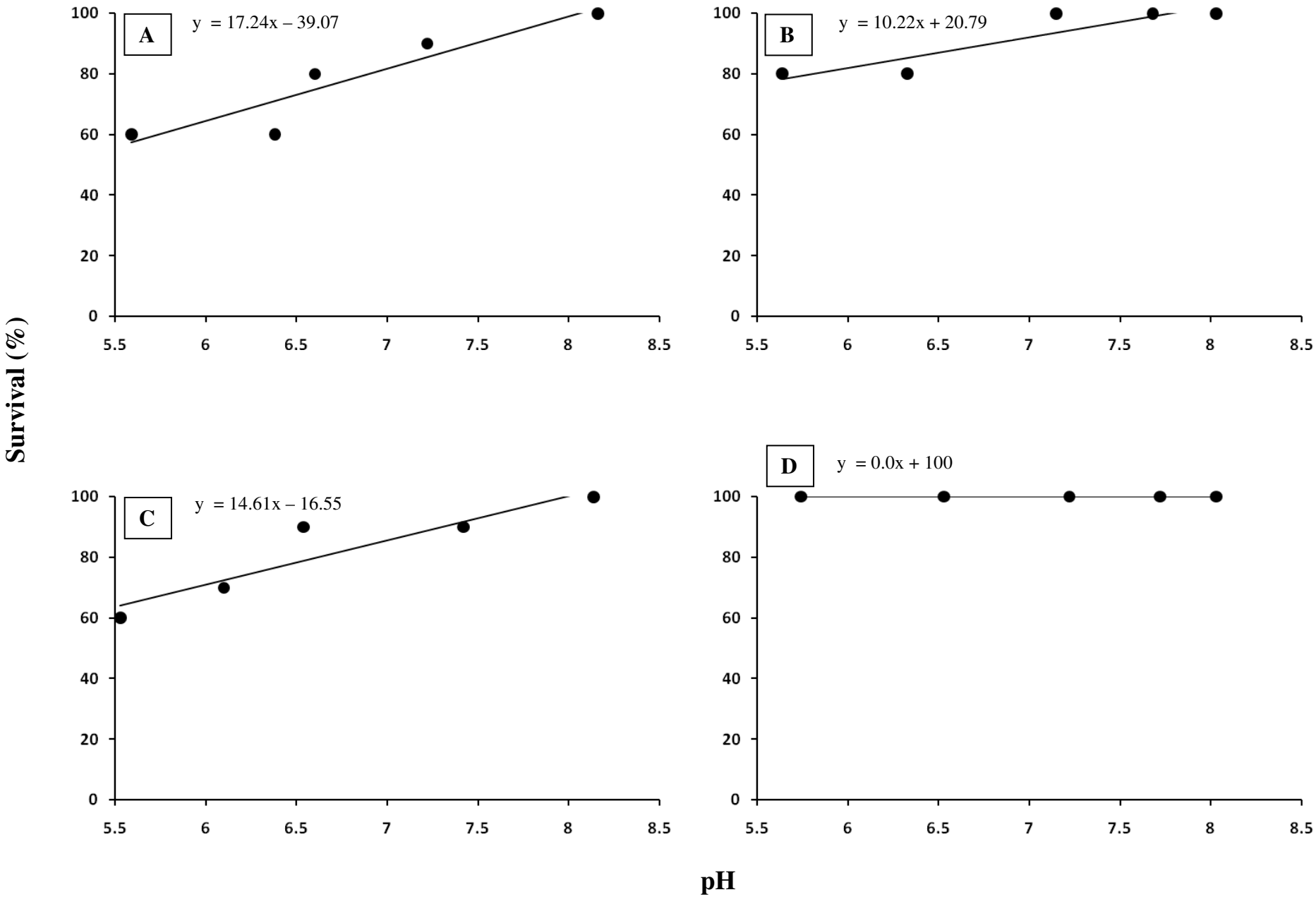
A
y = 17.24x – 39.07
B
y = 10.22x + 20.79
C
y = 14.61x – 16.55
D
y = 0.0x + 100
Survival (%)
pH
0
20
40
60
80
100
5.5
6
6.5
7
7.5
8
8.5

Figure 2: Percent survival after acidic pH exposure in larval amphibians. Species shown include: *Ambystoma jeffersonianum* (A), *A. maculatum* (B), *Rana sylvatica* (C), and *Hyla versicolor* (D). Regression equations and lines are shown for each species. Regression equations and lines are shown for each species.

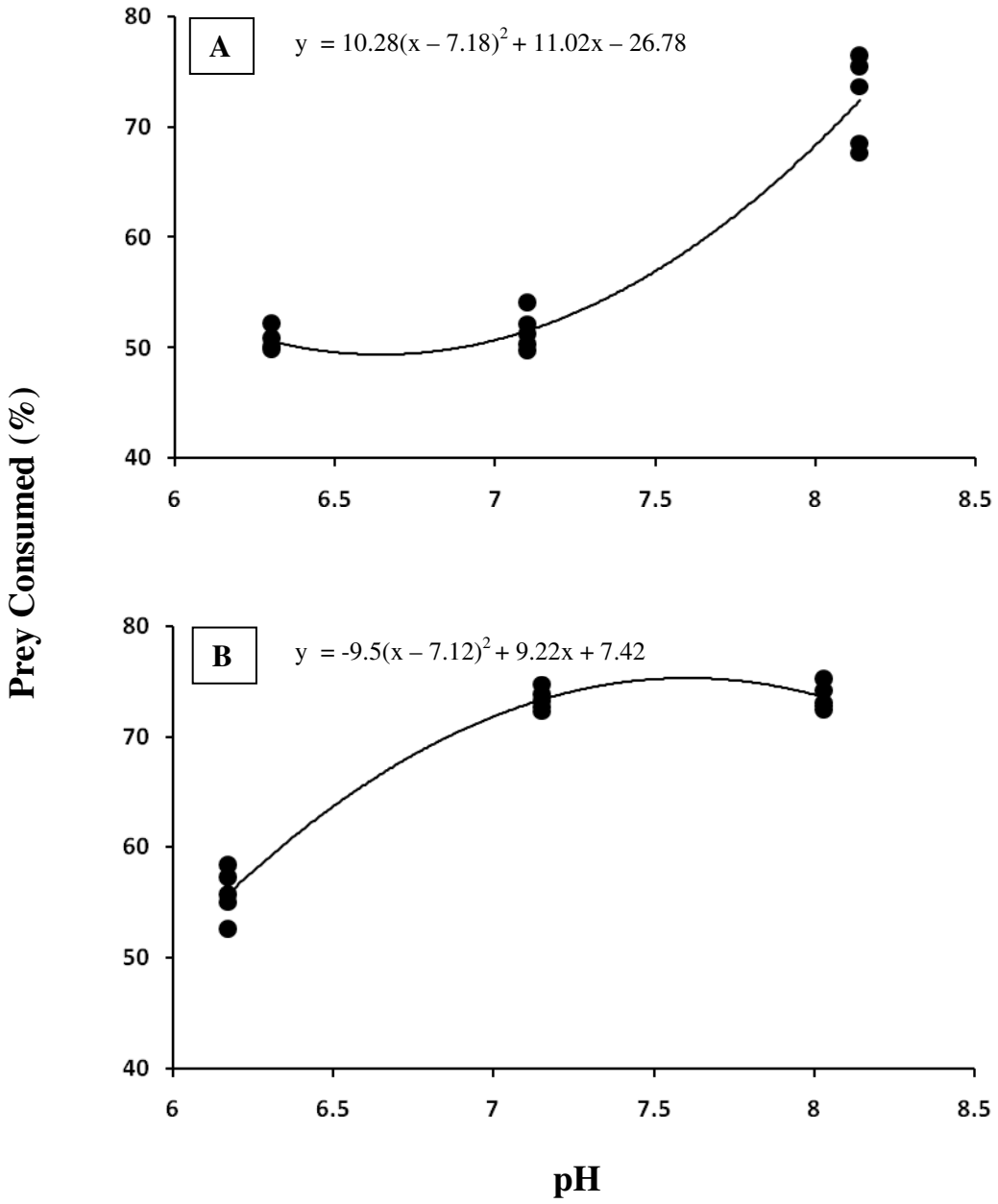
A
y = 10.28(x – 7.18)² + 11.02x – 26.78
B
y = -9.5(x – 7.12)² + 9.22x + 7.42
Prey Consumed (%)
pH
80
70
60
50
40
6
6.5
7
7.5
8
8.5

Figure 3: Percent prey consumed after acidic pH exposure in *Ambystoma jeffersonianum* (A) and *A. maculatum* (B). Each point represents a single individual. Regression equations and lines are shown for each species.

Increased conductivity as a physiological stressor of larval amphibians

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Abstract

Exposure to altered conductivity can negatively impact many freshwater system inhabitants including bacteria, plants, invertebrates, and vertebrates. Amphibians may be particularly sensitive to freshwater quality alterations. Several studies have shown increased conductivity to affect amphibian behavior and ecology. Comparatively fewer studies have taken a physiological approach to understanding the effects of conductivity in amphibians. Our study used laboratory experiments to assess conductivity effects on stress hormone (corticosterone) levels in Jefferson salamander (*Ambystoma jeffersonianum*), wood frog (*Rana sylvatica*), and grey treefrog (*Hyla versicolor*) larvae. We also examined prey consumption (only in *A. jeffersonianum*) and survival (in all species) after exposure to increased conductivity (0, 2000, 4000, 8000 ppb). There was a significant positive relationship between exposure to increased conductivity and baseline corticosterone levels in *A. jeffersonianum* and *R. sylvatica* after one week exposure. Exposure to increased conductivity did not influence baseline corticosterone levels in *H. versicolor* or confinement-induced corticosterone levels in all three species. Prey consumption in *A. jeffersonianum* was significantly negatively associated with increased conductivity (4000 and 8000 ppb). No mortality occurred in any species as a result of exposure to increased conductivity. Our results suggest that while exposure to increased conductivity may have species-specific effects on corticosterone levels in amphibians, it can still be a powerful environmental stressor.

Introduction

Human disturbances have permeated all levels of most environments (Walker et al. 2005). Unfortunately, increasing rates of these disturbances are predicted to contribute to the extinction of nearly one-half of all living species by the end of this century (Myers and Knoll 2001; Sala et al. 2000). One of the highest extinction rates in the world is found in freshwater systems (Riccardi and Rasmussen 1999). Several anthropogenic disturbances are thought to be responsible for these extinctions, including habitat modification and xenobiotic contamination (Riccardi and Rasmussen 1999). One common anthropogenically-based disturbance to freshwater systems is increased conductivity.

Conductivity is a measure of electrical conductance (Wetzel and Likens 2000). In freshwater systems, conductivity is influenced by the presence of ions in the water column (Wetzel and Likens 2000). There are several anthropogenic-based mechanisms believed to be responsible for altering conductivity in freshwater systems. One such mechanism is the application of chemicals, such as de-icing agents, to roads (Collins and Russell 2009; Sanzo and Hecnar 2006). In North America, an estimated 14 million tons of salt-based compounds are applied to roads annually (Environment Canada 2001). During spring rains and snow melts, these salt-based compounds can runoff into surrounding freshwater systems. In addition, agricultural practices, such as the construction of irrigation canals and the removal of perennials in favor of annual crops, can dramatically degrade soil, thus altering conductivity (Smith et al. 2007).

Exposure to altered conductivity can have profound impacts on several taxa including bacteria, invertebrates, reptiles, fish, birds, and plants (Cheong and Yun 2007; Grosell et al. 2007; Hart et al. 1991). Some of these impacts can include decreased size, decreased activity,

decreased species diversity, altered habitat preference, and increased mortality (Allen et al. 1996; Sanzo and Hecnar 2006; Smith et al. 2007; Warnock et al. 2002). Because of their permeable skin, amphibians are constantly transporting ions (mainly Na+ and Cl-) across their skin to maintain water and ion balance (Ultsch 1999). As a result, exposure to increased conductivity could have drastic effects on amphibian water and ion balance. Other studies have shown exposure to increased conductivity to negatively affect amphibian behavior (Haramura 2007; Karraker et al. 2008), growth and development (Gomez-Mestre and Tejedo 2002; Snodgrass et al. 2008), and survival (Sanzo and Hecnar 2006).

Environmental alterations can act as physiological stressors (Wikelski and Cooke 2006). Vertebrates physiologically respond to stressors by releasing glucocorticoids from the adrenal cortex after hypothalamic-pituitary-adrenal axis activation (McEwen and Wingfield 2003). Physiological effects of glucocorticoids could involve energy mobilization, along with suppression of growth and reproduction (Moore and Jessop 2003). In general, studies that examine corticosterone, the main glucocorticoid in amphibians, measure both baseline and stress-induced corticosterone levels (Romero and Reed 2008). Baseline corticosterone levels reflect an individual's physiological state at a specific moment, while stress-induced corticosterone levels reflect the functionality of the stress axis (Gendron et al. 1997). Prolonged exposure to elevated corticosterone levels may have profound impacts on an individual's fitness. Studies examining corticosterone levels in amphibians have correlated increased corticosterone levels to altered behavior (Moore and Miller 1984), growth and development (Belden et al. 2005; Glennemeier and Denver 2002), and immune system components (Ducoroy et al. 1999; Tournefier 1982).

In this study, we examined the effects of conductivity on baseline and stress-induced corticosterone levels under laboratory conditions in larvae of three amphibian species: Jefferson salamander (*Ambystoma jeffersonianum*), wood frog (*Rana sylvatica*), and grey treefrog (*Hyla versicolor*). Some of our previous work has shown a potential link between increased chloride levels and baseline corticosterone levels in free-living *A. jeffersonianum* larvae, and also between decreased conductivity and increased baseline corticosterone levels (Chapter III). These conflicting results warrant further investigation as to the effect of increased ion content, thus increased conductivity, on corticosterone levels in larval amphibians. We hypothesized that exposure to increased conductivity would significantly increase baseline corticosterone levels in our focal species under laboratory conditions. In addition, we examined the influence of increased conductivity on prey consumption (*A. jeffersonianum* only) and survival (all tested species). We hypothesized that increased conductivity would significantly decrease prey consumption in *A. jeffersonianum* and survival in all our amphibian species.

Materials and Methods

Amphibian egg mass collection and rearing

Recently deposited egg masses of *R. sylvatica* (*N*=parts of 12 masses) were collected from Montgomery County, Virginia. Once collected, egg masses were transported to a 1000 L mesocosm stock tank at an outdoor research facility owned by Virginia Tech. Prior to egg mass addition, the stock tank was filled with well water and contained approximately 250 g deciduous leaf litter and wild-caught zooplankton (screened from 1 L water). For *H. versicolor* egg mass collection, we set out an array of 100 L plastic wading pools at the same outdoor research facility as our 1000 L mesocosm stock tanks. Each 100 L pool was filled with well water and contained

approximately 100 g deciduous leaf litter. *Hyla versicolor* typically breed and deposit their egg masses in these wading pools under their own accord. After *R. sylvatica* and *H. versicolor* egg masses were in their respective stock tanks, we covered each tank with a shadecloth lid to prevent colonization by other species. We collected free-living *A. jeffersonianum* larvae from the same pond as the *R. sylvatica* egg masses for experimentation. At the time we collected individuals for experimentation, we collected *N*=10 additional larvae to verify starting stage (Watson and Russell (2000) for *Ambystoma*; Gosner (1960) for *Rana* and *Hyla*) and mass homogeneity. Mean larval mass (g) (±SD) and developmental stage (±SD) for each species prior to experimentation were, respectively: 1.07 ± 0.09 g and 17.7 ± 0.67 (*A. jeffersonianum*), 0.664 ± 0.08 g and 31.3 ± 1.42 (*R. sylvatica*), and 0.225 ± 0.04 g and 28.6 ± 1.26 (*H. versicolor*).

Conductivity treatment exposure

Conductivity experiments were designed using a regression approach with *N*=5 treatments. The five treatments (0 (control), 2000, 4000, 6000, and 8000 ppb) represented how much ion content we added to the experimental tubs (described below). Conductivity was increased by using Amphibian Ringer's Solution (1 L H_20, 6.6 g NaCl, 0.15 g KCl, 0.15 g $CaCl_2$, 0.2 g $NaHCO_3$; Wright and Whitaker 2001) to get the desired ion concentration (ppb). After making our stock solution (7.1 x 10^6 ppb), we used dilution techniques based on the volume of our treatment containers to achieve desired ppb concentration. We used 6 L rectangular plastic bins filled with 3.5 L dechlorinated water for each conductivity treatment. All bins were placed in a temperature (~ 22°C) and photoperiod (12 h light: 12 h dark) controlled room. For each species, *N*=10 larvae were placed in each tub. After one week exposure to conductivity treatment, *N*=4 larvae were removed from each bin and immediately (within 3 min) frozen in a

dry ice/ethanol slurry for baseline corticosterone level measurement. Frozen samples were then stored in a -80°C freezer until radioimmunoassay (RIA) could be performed. Confinement-induced corticosterone levels were also sampled for by taking *N*=4 larvae from each bin and individually placing them in 120 mL cups filled with 80 mL dechlorinated water for 30 min. Cups were gently agitated every three min. After 30 min, larvae were frozen and stored in the identical manner as previously described until RIA could be performed.

Prey consumption trials

Prey consumption trials were conducted with *A. jeffersonianum* larvae following five days of exposure to increased conductivity. Three experimental treatments (0, 4000, and 8000 ppb) each contained *N*=5 larvae. Conductivity treatments were prepared in the identical manner previously described. Larvae were individually housed in opaque white cups (approximately 946 mL) filled with approximately 800 mL in the same temperature/light controlled room previously described. Larvae were not fed during the duration of this experiment. After five days, larvae were removed from respective conductivity treatments and placed in another cup with dechlorinated water to minimize the effects of conductivity on prey. Each *A. jeffersonianum* larvae was given 1.5 g live black worms and allowed to feed for 24 hr. Following this allotted time, all remaining black worms from each cup were removed and weighed for wet mass, and prey consumption (%) was calculated. As much water as possible was removed from the weighing dish using a suction bulb prior to pre- and post-pH exposure weighing of live black worms.

Whole-body corticosterone radioimmunoassay

Corticosterone was measured using whole-body RIA procedures of Belden et al. (2003), with the following minor modifications. Larvae homogenization occurred in mass adjusted distilled water (mass of individual x 10 mL + 0.5 mL rinse, with a minimum of 1.5 mL and maximum of 5 mL). After sample extraction, centrifugation at 3000 rpm for 10 min was conducted to break the emulsion. Three standard curves were prepared for each assay. In two instances, sample duplicates did not correspond with each other. As a result, we did not include these two data points in any of our analyses. In total, we conducted two assays (limit of assay one detection was 0.98 ng/g; limit of assay two detection was 1.0 ng/g). Intra-assay variation was 7%, and inter-assay variation was 11%. Based upon individual recoveries, our average extraction efficiency was 54%.

Statistical analyses

We used linear regression to determine the relationship between corticosterone level type (baseline and confinement-induced) and conductivity for each species. We used an analysis of variance (ANOVA) to determine if confinement activated the stress response, thus increasing corticosterone levels compared to baseline levels in all species. Any relationship between prey consumption in *A. jeffersonianum* and survivorship in each species to conductivity was also determined using linear regression. All statistical analyses were conducted using SAS JMP (version 7.0).

Results

Overall, exposure to increased conductivity had mixed effects on corticosterone levels in our larval amphibian species. Specifically, there was a significant positive relationship between conductivity and baseline corticosterone levels in *A. jeffersonianum* (R^2=0.50, P<0.001; Fig. 1A) and *R. sylvatica* (R^2=0.51, P<0.001; Fig. 1B). Conductivity did not appear to influence baseline corticosterone levels in *H. versicolor* (R^2=0.10, P=0.18; Fig 1C) and confinement-induced corticosterone levels in *A. jeffersonianum* (R^2=0.13, P= 0.14; Fig. 1A), *R. sylvatica* (R^2=0.12, P= 0.13; Fig. 1B), and *H. versicolor* (R^2<0.001, P=0.95; Fig. 1C). Overall, confinement significantly increased corticosterone levels above baseline levels for all species combined ($F_{1, 14}$=21.17, P<0.001; Fig. 1). Prey consumption was negatively affected by conductivity in *A. jeffersonianum* (R^2=0.57, P=0.001; Fig. 2). Exposure to conductivity did not result in mortality for any species.

Discussion

Baseline corticosterone levels significantly increased as a result of increased conductivity in *A. jeffersonianum* and *R. sylvatica*. Effects of conductivity on amphibians can greatly vary among species (Snodgrass et al. 2008), with the majority of focus having been placed on behavioral and ecological modifications. Studies have shown amphibians to display altered intra- and interspecies competitive ability, reproductive behaviors, growth/development rates, water retention mechanisms, and survivorship all as a result of exposure to altered conductivity (Gomez-Mestre and Tejedo 2002; Jorgensen 1997; Karraker et al. 2008; Snodgrass et al. 2008; Wallace 1991). Interestingly, we did not find a significant relationship between conductivity and increased baseline corticosterone levels in *H. versicolor*. While this result was unexpected, it was not altogether surprising. The genus *Hyla* is noted to be remarkably tolerant to several

environmental disturbances compared to sympatric heterospecifics. Several studies have shown *Hyla* to be tolerant of nitrate exposure and UV-B radiation (Blaustein and Belden 2003; Shinn et al. 2008). Concerning *H. versicolor* specifically, studies have shown tolerance to acidification, drastic climatic variability, chemical exposure, and UV-B radiation (Chapter IV; Grant and Licht 1995; Otto et al. 2007; Zaga et al. 1998). Our study lends novel physiological data to support the claims of others that *Hyla* are tolerant amphibians to environmental perturbations and challenges.

Increasing baseline corticosterone levels after exposure to increased conductivity could serve several purposes. For instance, additional energy mobilization could be required to maintain/restore ion balance. Water and ion balance in amphibian larvae is primarily centered on Na+ and Cl- regulation, with these two ions constituting up to 80% of the ion content (Ultsch et al. 1999). In addition, amphibian larvae are typically hyperosmotic to their surroundings (Ultsch et al. 1999). Thus, amphibian larvae are constantly allocating energy to replace Na+ and Cl- ions lost to their surrounding environment. However, if the surrounding environment were to become less hypoosmotic compared to the internal fluids of amphibian larvae, then a change in energy economics may result to maintain proper ion balance. Amphibian larvae would have to transition from the uptake of Na+ and Cl- to activating mechanisms to rid the body of excess ions diffusing in from the surrounding environment. If additional energy is required for this task, increasing corticosterone levels may serve to mobilize energy reserves (Romero 2002).

Our data also show interesting confinement-induced corticosterone results. We have shown exposure to increased conductivity to not significantly influence confinement-induced corticosterone levels in any of our focal species. These results can be interpreted in two manners. First, no significant changes in confinement-induced corticosterone levels after increased conductivity exposure suggest that the functionality of the stress axis has not been compromised

in any of the amphibian species. Having the functionality of the stress axis become altered due to prolonged stressor exposure can have profound impacts on an individual. Immune system failure, inhibited reproduction, muscle tissue breakdown, and decreased survival may occur when an individual experiences an alteration of stress axis functionality (Romero et al. 2009). We have previously shown environmental stressors, such as low pH, to lead to a diminished stress response in *H. versicolor* (Chapter IV). There are several mechanisms that can lead to an alteration of stress axis functionality, including seasonal/life history changes, habituation to a stressor, desensitization to a stressor, and physiological exhaustion (Cyr and Romero 2009).

Prey consumption in *A. jeffersonianum* was significantly negatively correlated to increased conductivity. We have previously reported similar results concerning altered prey consumption rates in *A. jeffersonianum* after exposure to another prevalent environmental stressor, acidification (Chapter IV). Another possible explanation for this observation is altered physiological or behavioral processes as a result of having increased corticosterone levels. Increasing corticosterone levels to cope with a stressor can temporarily suppress non-essential processes during stressful events (Moore and Jessop 2003). However, the specific relationship between corticosterone levels and foraging remains unclear. For example, Crespi and Denver (2004) found a positive relationship between increased corticosterone levels and foraging in a larval amphibian, while Angelier et al. (2007) showed a negative relationship in a bird species.

The specific relationship between conductivity and stress in amphibians is still unclear. We have previously shown decreased conductivity to be statistically correlated to increased baseline corticosterone levels in a free-living larval amphibian (Chapter III). Yet, this study reports increased conductivity to be statistically correlated to increased baseline corticosterone levels in laboratory-held larval amphibians. Adding to the uncertain relationship between

conductivity and stress in amphibians, exposure to increased or decreased conductivity does not significantly increase confinement-induced corticosterone levels in free-living or laboratory-held amphibians (Chapter III and this study, respectively). The immediate question then becomes: do amphibians perceive increased conductivity as a physiological stressor? Previous studies have shown increased conductivity exposure to be a stressor that negatively impacts amphibian behavior (Karraker et al. 2008), growth and development (Snodgrass et al. 2008), and survivorship (Sanzo and Hecnar 2006). If amphibians do perceive increased conductivity as a physiological stressor, we would hypothesize that increased conductivity would significantly increase confinement-induced corticosterone levels. An increase in confinement-induced corticosterone levels would be indicative of an individual incurring an allostatic overload after prolonged exposure to a stressor (McEwen and Wingfield 2003). Thus, future studies could examine whether confinement-induced corticosterone levels increase after prolonged exposure to increased conductivity. This study exposed *A. jeffersonianum*, *R. sylvatica*, and *H. versicolor* to increased conductivity for one week. Perhaps exposure to increased conductivity for a longer period of time would deplete an individual's immediate available energy, thus requiring that individual to mount a stress response, subsequently increasing confinement-induced corticosterone levels.

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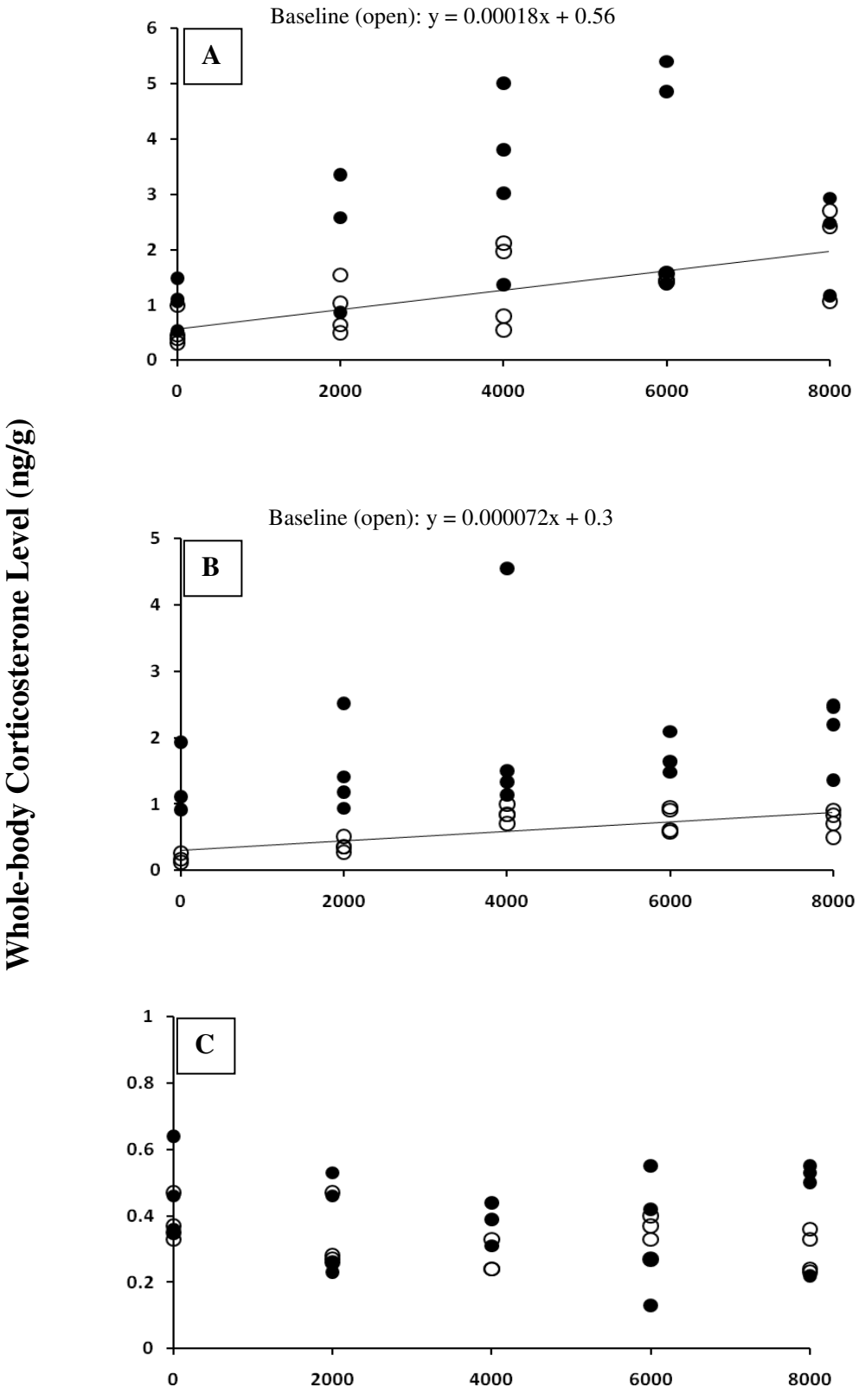
Baseline (open): y = 0.00018x + 0.56
A
Baseline (open): y = 0.000072x + 0.3
B
C
Whole-body Corticosterone Level (ng/g)
Ion Content (ppb)

Figure 1: Whole-body corticosterone level (ng/g) after exposure to increased conductivity in larval amphibians. Species shown include: *Ambystoma jeffersonianum* (A), *Rana sylvatica* (B), and *Hyla versicolor* (C). Open points represent baseline corticosterone levels. Closed points represent confinement-induced corticosterone levels. Each point represents a single individual. Regression equations and lines are shown for baseline (solid line) corticosterone levels for each species. No regression equations and lines are shown for insignificant relationships.

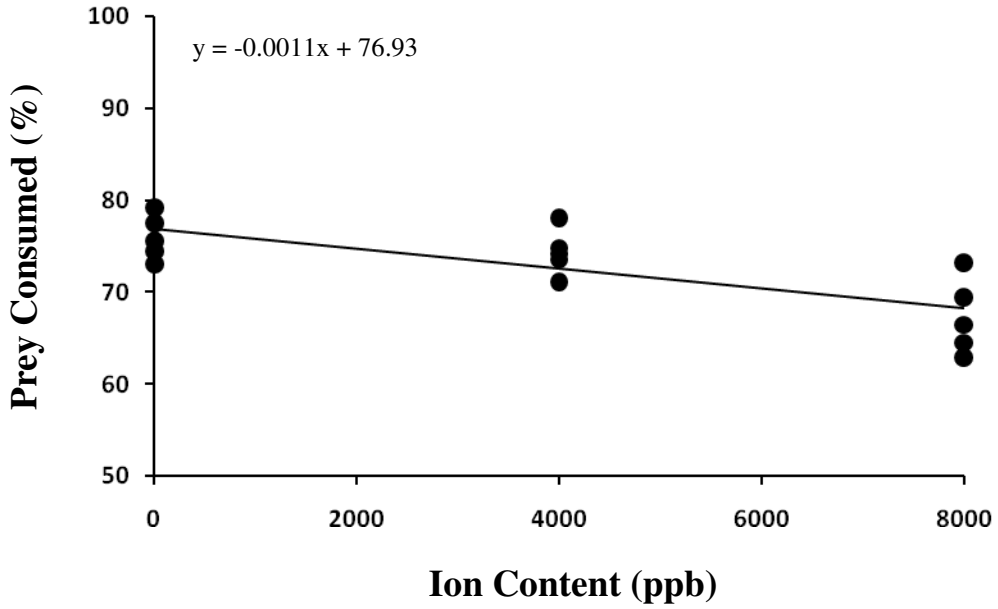

Figure 2: Percent prey consumed after exposure to increased conductivity in *Ambystoma jeffersonianum*. Each point represents a single individual. Regression equation and line are shown.

CHAPTER VI: RESEARCH IMPLICATIONS

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Research Implications

Amphibian Ecology and Physiology

My results show that abiotic factors in freshwater systems are capable of influencing corticosterone levels, particularly baseline levels, in larval amphibians. However, my results indicate that the degree of physiological stress sensitivity varies among larval amphibian species and among abiotic stressors. For example, *H. versicolor* did not increase baseline corticosterone levels in response to increased conductivity levels, while *A. jeffersonianum* and *R. sylvatica* did (Chapter V). Variability in responsiveness to potential stressors in amphibians is not new. No significant corticosterone level increases occurred after increased exposure to other environmental stressors, such as UV-B radiation and coal fly ash, in other amphibian species (Belden et al. 2003; Ward and Mendonca 2006).

My results also show that survivorship varies among larval amphibian species exposed to a variety of abiotic stressors. For instance, survivorship in *H. versicolor* was not influenced by acidic conditions, while *A. jeffersonianum*, *A. maculatum*, and *R. sylvatica* all experienced a significant decrease in survivorship after exposure to acidic conditions (Chapter IV). Neither *A. jeffersonianum*, *R. sylvatica*, nor *H. versicolor* experienced a decrease in survivorship with increased conductivity (Chapter V). As with corticosterone levels and their variability in responsiveness to stressors in amphibians, variability in survivorship after stressor exposure among amphibian species has been previously demonstrated.

Future studies could ask: what effect(s) would exposure to multiple environmental stressors at the same time have on corticosterone levels in larval amphibians? Free-living amphibian populations are rarely exposed to a single environmental stressor at a given time. Rather, exposure to a suite of environmental stressors is more likely (Alford and Richards 1999).

While a small component of my research examined several environmental stressors potentially influencing corticosterone levels among free-living *A. jeffersonianum* populations (Chapter III), none of my laboratory research addressed an interactive effect of these environmental stressors. Such studies could potentially provide more ecologically and physiologically relevant data reflecting conditions typically found where amphibian populations reside.

Allostasis and the Reactive Scope Models

Originally proposed by Sterling and Eyer (1988) for biomedical applications, the term allostasis was applied to ecological concepts by McEwen and Wingfield (2003a) in an attempt to remove some of the ambiguity surrounding the word "stress". Simply put, allostasis is stability through change. All daily and seasonal physiological changes within an individual that maintain optimal set-points for internal processes encompass allostasis (Romero et al. 2009). The cumulative costs of allostasis to an individual are referred to as allostatic load (McEwen and Wingfield 2003a). Assuming sufficient energy income and reserves, the cumulative costs of allostatic load can be overcome without pathological effects upon an individual. However, when unpredictable circumstances arise or when energy demands outweigh energy income and reserves, allostatic load significantly increases and results in allostatic overload (McEwen and Wingfield 2003a). Some types of allostatic overload may prompt an individual to enter into an "emergency life history" phase, and glucocorticoids may be released to promote survival.

Since being published, the Allostasis Model has been somewhat controversial. Several investigators have illustrated shortcomings and weaknesses in the Allostasis Model (Dallman 2003; Romero et al. 2009; Walsberg 2003). Even McEwen and Wingfield acknowledged weaknesses in their own conceptual framework of allostatic overload (McEwen and Wingfield

2003b). One suggested weakness of the Allostasis Model is its reliance on energy intake and output as an index of an individual's energetic stress (Walsberg 2003). The high magnitude variability of energy balance within an individual is poorly understood (Walsberg 2003). This energy balance variability can make it difficult to compare energetic stress among conspecifics, and even more challenging to compare among different taxa (Walsberg 2003). Another suggested weakness of the Allostasis Model is linked to Walsberg's energy balance concerns (Walsberg 2003). Increasing glucocorticoid levels to facilitate energy mobilization is a common assumption among vertebrate endocrinologists. However, as Romero et al. (2009) illustrate, energy mobilization due to glucocorticoid release is primarily based upon studies that used fasted individuals, which is an unlikely circumstance in nature. When using adequately fed birds, corticosterone may not influence glucose levels (Remage-Healey and Romero 2001). Romero et al. (2009) suggest that the insignificant effects of corticosterone on glucose levels can severely weaken the Allostasis Model and its heavy reliance on the glucocorticoid-energy mobilization concept (McEwen and Wingfield 2003b). Aside from suggested conceptual weaknesses of the Allostasis Model, the coinage of the term "allostasis" has not been well received. Dallman (2003) argues that McEwen and Wingfield's attempt to remove some of the ambiguity surrounding the term "stress" has actually only added to the confusion by coining several new terms. Regardless of its shortcomings, the theoretical composition of the Allostasis Model allows investigators to form new hypotheses and predictions concerning normal adaptive responses versus responses requiring additional energetic demands (Romero et al. 2009).

My research provides some supportive examples of the Allostasis Model using larval amphibians. For example, *A. jeffersonianum* larvae exposed to acidic conditions in mesocosms seemingly did not increase stress-induced corticosterone levels in my "mild" acidic treatment

(pH range 6.0 to 6.8) and "strong" acidic treatment (pH range 5.0 to 5.8) as compared to the control treatment (Chapter III). In this instance, I would argue that these *A. jeffersonianum* larvae are incurring an allostatic load. Exposure to acidic conditions are known to have deleterious effects on amphibians. However, there may be sufficient energy income or reserves to cope with the stressor without having to initiate an "emergency life history" phase, thus no immediate need to increase stress-induced corticosterone levels. In addition, since there did not seem to be a major difference in stress-induced corticosterone levels between *A. jeffersonianum* from the "mild" and "strong" acidic treatments compared to the control treatment, *A. jeffersonianum* larvae are likely not incurring an allostatic overload in response to low pH.

Another recent model, the Reactive Scope Model, has been proposed by Romero et al. (2009), and attempts to build upon the Allostasis Model by improving upon its strong points, while removing some weaknesses (previously discussed). The Reactive Scope Model is comprised of four basic phases: predictive homeostasis, reactive homeostasis, homeostatic overload, and homeostatic failure. Romero et al. (2009) use the term "physiological mediator" in defining each stage of the Reactive Scope Model. Romero et al. (2009) define a physiological mediator as any behavioral, central nervous system, cardiovascular, hypothalamic-pituitary-adrenal (HPA) axis, or immune system endpoints. Predictive homeostasis is the phase that encompasses all circadian rhythms of a physiological mediator based on life-history stages and seasonality. Reactive homeostasis is the phase that includes all physiological mediator level increases associated with maintaining homeostasis through an unpredictable event, such as droughts, harsh storms, or anthropogenic disturbances (Wingfield 2008). Homeostatic overload occurs when the physiological mediator, now at its highest levels, starts causing pathological effects to an individual. These pathological effects can greatly vary among species, but can

include immune system suppression, muscle tissue breakdown, reproductive suppression, myocardial infarction, anxiety, fear, depression, and decreased memory capability (see Table 1 of Romero et al. 2009). Homeostatic failure occurs when the physiological mediator levels fall below the range for homeostasis to function.

While the Reactive Scope Model does eliminate some of the weaknesses of the Allostasis Model (previously discussed), it is not without its own shortcomings. I believe that one major weakness of the Reactive Scope Model is in its homeostatic failure phase. The Reactive Scope Model proposes that homeostatic overload is associated with the highest physiological mediator levels while homeostatic failure is associated with the lowest levels. The Reactive Scope Model suggests that death can occur at low physiological mediator levels associated with the homeostatic failure phase (Romero et al. 2009). However, previous work by Romero and colleagues has directly linked increased physiological mediator levels (e.g., stress-induced corticosterone levels) to decreased survival (Romero and Wikelski 2001). This is contradictory to what is proposed in the Reactive Scope Model, where death is proposed to occur at the lowest physiological mediator levels. However, Romero and Wikelski (2001) did not measure corticosterone levels near the time of death, thus it is unknown whether corticosterone levels were high or low at that point.

As Romero et al. (2009) suggest, one of the greatest benefits of the Reactive Scope Model is that it can be modified to fit the needs of other investigators and their specific circumstances. Thus, I propose the following modifications to the Reactive Scope Model based mostly upon my research (see Figure 1). My model, the Homeostasis Battery Model, incorporates components of both the Allostasis and Reactive Scope Models. Glucocorticoid levels will be the focal physiological mediator when discussing the Homeostasis Battery Model

as my research focused specifically on them. Unlike its predecessors who's models are uni-directional in scope (McEwen and Wingfield 2003a; Romero et al. 2009), the Homeostasis Battery Model is bi-directional in regards to the glucocorticoid response intensity (see Figure 1). In other words, the Homeostasis Battery Model incorporates both positive/increased and negative/diminished ends for glucocorticoid responses to a stressor, hence mirroring a battery. Bi-directionality is critical for several reasons. It allows for the inclusion of various mechanisms responsible for a diminished stress response.

A recent review proposes four explanations that could cause a diminished stress response to a repeated stressor (Cyr and Romero 2009). One explanation is that an individual may perceive a stressor differently at different life-history stages or during different seasons (Cyr and Romero 2009). Romero (2002) compiled a thorough review showing stress response variability on a seasonal basis among several vertebrate taxa. In further support of Cyr and Romero's (2009) first explanation, aspects of my research have shown glucocorticoid levels (both baseline and stress-induced) to significantly vary throughout larval amphibian development (Chapter II). In particular, *A. jeffersonianum* larvae and *R. sylvatica* tadpoles appear to have a diminished stress response at early developmental stages perhaps as a result of incomplete development/activation of the HPA axis (Chapter II). Habituation, or the familiarization of a repeated stressor resulting in a diminished stress response, is another possible explanation (Cyr and Romero 2009). The third possible explanation for a diminished stress response proposed by Cyr and Romero (2009) is the desensitization of the stress response. Desensitization without habituation can occur if an individual cannot adapt to a stressor after prolonged exposure, thus perceiving that stressor as noxious (Cyr and Romero 2009). Cyr and Romero's (2009) final explanation of a diminished stress response is physiological exhaustion. Originally proposed by Selye (1946), physiological

exhaustion can occur when an individual is too fatigued to maintain the HPA axis and thus, unable to effectively mount a stress response. One component of my research involving acid exposure provides an example of either habituation, desensitization, or physiological exhaustion. *Hyla versicolor* tadpoles exposed to acidic conditions in the laboratory showed a diminishing stress response as the pH became more acidic (Chapter IV). Regardless of the possible mechanism responsible for a diminished stress response, a few studies do exist that have observed this phenomenon (Dallman and Bhatnagar 2001), and the Homeostasis Battery Model accounts for this possibility.

The Homeostasis Battery Model is composed of four phases: homeostatic equilibrium (H_E), homeostatic tension (H_T), homeostatic fatigue (H_F), and homeostatic buckling (H_B) (see Figure 1). The Homeostasis Battery Model can be explained using an engineering analogy. Steel beams are commonly used in building construction. When under optimal conditions, the steel beam can efficiently perform its support functions for that building. This corresponds to optimal functionality of an individual's internal systems controlled by normal baseline glucocorticoid levels, or the homeostatic equilibrium (H_E) phase. If that steel beam begins to experience unexpected events, such as extraordinarily strong winds or structural stress, the beam experiences tension. However, the steel beam was designed to withstand a certain degree of tension without repercussions to the buildings integrity. This is analogous to an individual experiencing a stressor that is unexpected/unpredictable, yet surmountable with minimum effort. These circumstances are the basis for the homeostatic tension (H_T) phase. If this unexpected stressor persists and tension in the steel beam mounts, fatigue sets in on that steel beam and the building may begin to experience structural damage. This is represented by the homeostatic fatigue (H_F) phase, where an individual may begin to experience deleterious pathological effects

if a stressor persists. If the steel beam is fatigued too long, the beam will buckle and the building may collapse. This corresponds to the homeostatic buckling (H_B) phase, where an individual may experience death as a result of prolonged exposure to a stressor.

Similar to the Allostasis and Reactive Scope Models, the Homeostasis Battery Model accounts for the relative plasticity of the physiological stress response within an individual. This relative plasticity is represented by the width of each of the four model phases (see Figure 1). The homeostatic equilibrium (H_E) phase has the greatest degree of normal plasticity for the physiological stress response. Several studies have demonstrated that glucocorticoid levels may display a high degree of plasticity based upon life-history stage (Chapter II; Wada 2008) and seasonality (Romero 2002; Romero et al. 2008) under unstressed circumstances. The homeostatic tension (H_T) phase has the second greatest degree of normal plasticity for the physiological stress response. Both the Allostasis and Reactive Scope Models suggest that it would be adaptive for an individual to display a certain degree of plasticity to negotiate through stressors rather than mounting a stronger physiological response (McEwen and Wingfield 2003a; Romero et al. 2009). Examples of this phase come from my mesocosm pH manipulations in *A. jeffersonianum* (Chapters III), as previously discussed as a supportive example of allostatic load. When an adaptive response is not sufficient to overcome a stressor, it may be necessary for that individual to mount a stronger response (Romero et al. 2009), thus potentially limiting the normal response plasticity to a stressor. This would render an individual into the homeostatic fatigue (H_F) phase. Also occurring at the homeostatic fatigue (H_F) phase, pathological effects may begin to arise within an individual due to prolonged exposure to a stressor. Physiological stress response plasticity is further reduced at the homeostatic buckling (H_B) phase because of

chronic exposure to a stressor. At this phase, pathological effects are potentially at their extremes, and death will likely result.

Much more work is needed to identify and fully understand the mechanisms by which individuals cope with environmental change (Wingfield 2008). Thus, future studies could attempt to expand or refute the Homeostasis Battery Model. This is because endocrine systems, such as the physiological stress response highlighted by the Homeostasis Battery Model, are primary mediators of physiological, behavioral, and morphological responses to environmental stressors (Wingfield 2008). Much like the Reactive Scope Model, the Homeostasis Battery Model is a broad model that can be custom fitted towards the specific research questions or taxa of an investigator. However, I urge investigators to use amphibians as a focal taxa. Comparatively little is known regarding amphibian stress physiology compared to other taxa, such as birds. In fact, much of the basis for both the Allostasis Model and Reactive Scope Model comes from avian stress physiology studies. Amphibians make for ideal candidates for investigating mechanisms by which individuals cope with environmental change because, among other things, of their trophic importance, research tractability, and environmental sensitivity (Hopkins 2007).

Corticosterone as a Biomarker

Due to increasing rates of anthropogenic disturbances in nearly every habitat type (Walker et al. 2005), understanding how anthropogenic disturbances act as stressors upon system inhabitants is of the upmost importance (Wikelski and Cooke 2006). As a result, investigators have begun using corticosterone levels (both baseline and stress-induced) as a biomarker reflective of a population's health status (Romero 2004). For example, fecal corticosterone levels

in male northern spotted owls (*Strix occidentalis caurina*) were higher in areas with logging-road traffic and where timber harvest activities occurred (Wasser et al. 1997). Snowmobile use can elevate glucocorticoid levels in elk (*Cervus elaphus*) and wolves (*Canis lupus*) (Creel et al. 2002). Comparatively fewer studies have used corticosterone levels in amphibians, particularly larval amphibians, under a conservation physiology context. One study showed that adult male *A. maculatum* migrating across a paved parking lot had significantly higher baseline corticosterone levels compared to conspecifics migrating through undisturbed forest (Homan et al. 2003). Another study showed that exposure to coal combustion waste in southern toads (*Bufo terrestris*) significantly increased baseline corticosterone levels compared to conspecifics at unpolluted sites (Hopkins et al. 1997).

Aspects of my research also lend support to using corticosterone levels as a biomarker. For example, I have shown exposure to acidic pH (pH range of 5.0 to 5.8) to significantly increase baseline corticosterone levels in mesocosm-held *A. jeffersonianum* larvae (Chapter III), and laboratory-held *A. jeffersonianum*, *A. maculatum*, *R. sylvatica*, and *H. versicolor* larvae (Chapter IV; acidic pH level exposure varied among species, but all levels ranged from pH 5.53 to 8.16 (control)). Furthermore, I have shown acidic conditions to significantly decrease both prey consumption (larval *Ambystoma* species) and survival (all species except *H. versicolor* tadpoles) (Chapter IV). Taken together, these results suggest that baseline corticosterone levels can be influenced by an environmental stressor (e.g., acid exposure), and that this environmental stressor is capable of impacting individual fitness, and potentially, population health if extrapolated up to that level. By definition, this is what a biomarker should accomplish; that is, a biomarker should be an indicator (in my research, a physiological indicator) of exposure to an

environmental perturbation that could be linked to adverse individual or population effect(s) (Eason and O'Halloran 2002; Forbes et al. 2006).

While some aspects of my research tend to lend support towards using corticosterone levels as a biomarker, my results also suggest that a certain degree of caution must be employed when attempting to use corticosterone levels in biomarker studies. Not only can corticosterone levels significantly change throughout development in a species-specific manner in larval amphibians (Chapter II), but larval amphibians can exhibit varying degrees of tolerance to known environmental stressors (Chapters III, IV, and V). Indeed, Romero (2004) cautioned investigators that higher corticosterone levels do not necessarily suggest that an individual is in poor health. In fact, biomarkers and their use in general under an ecological context have been subject to great debate in recent years. Forbes et al. (2006) outlined a strong and compelling argument against using biomarkers in ecotoxicological research since biomarkers, amongst other things, could yield false warnings of deleterious effects and not be as cost-effective as just directly measuring risk assessment. Forbes et al. (2006) argue that biomarkers should predict some ecologically relevant effect to be justified in use. To date, several studies have been able to use corticosterone levels as a predictor of some ecologically relevant effect. For example, Romero and Wikelski (2001) used increased stress-induced corticosterone levels to predict survival in marine iguanas (*Amblyrhynchus cristatus*) during El Niño events in the Galápagos Islands, and Bonier et al. (2007) used increased maternal corticosteroids to predict female-biased offspring sex ratios in white-crowned sparrows (*Zonotrichia leucophrys*). Thus, using physiological biomarkers, such as corticosterone, could still be a potentially powerful monitoring tool (Wingfield et al. 1997). Romero (2004) suggested that stress physiology studies need to be

highly rigorous in both experimental design and data interpretation. Using that approach, the valid concerns and compelling arguments of Forbes et al. (2006) could be minimized.

Future studies should attempt to establish a more direct link between increased corticosterone levels (baseline or stress-induced) and a reduction to fitness. My research, while providing a link between individual fitness costs (decreased survival and prey consumption rates in some larval amphibians) after exposure to an environmental stressor and a link between exposure to an environmental stressor and an increase in corticosterone levels, does not provide a direct link between corticosterone and a reduction in fitness. Future studies should attempt to identify both short-term and long-term fitness costs associated with increased corticosterone levels after environmental stressor(s) exposure. Such data would only strengthen the argument of using corticosterone levels as a biomarker reflective of a population's health.

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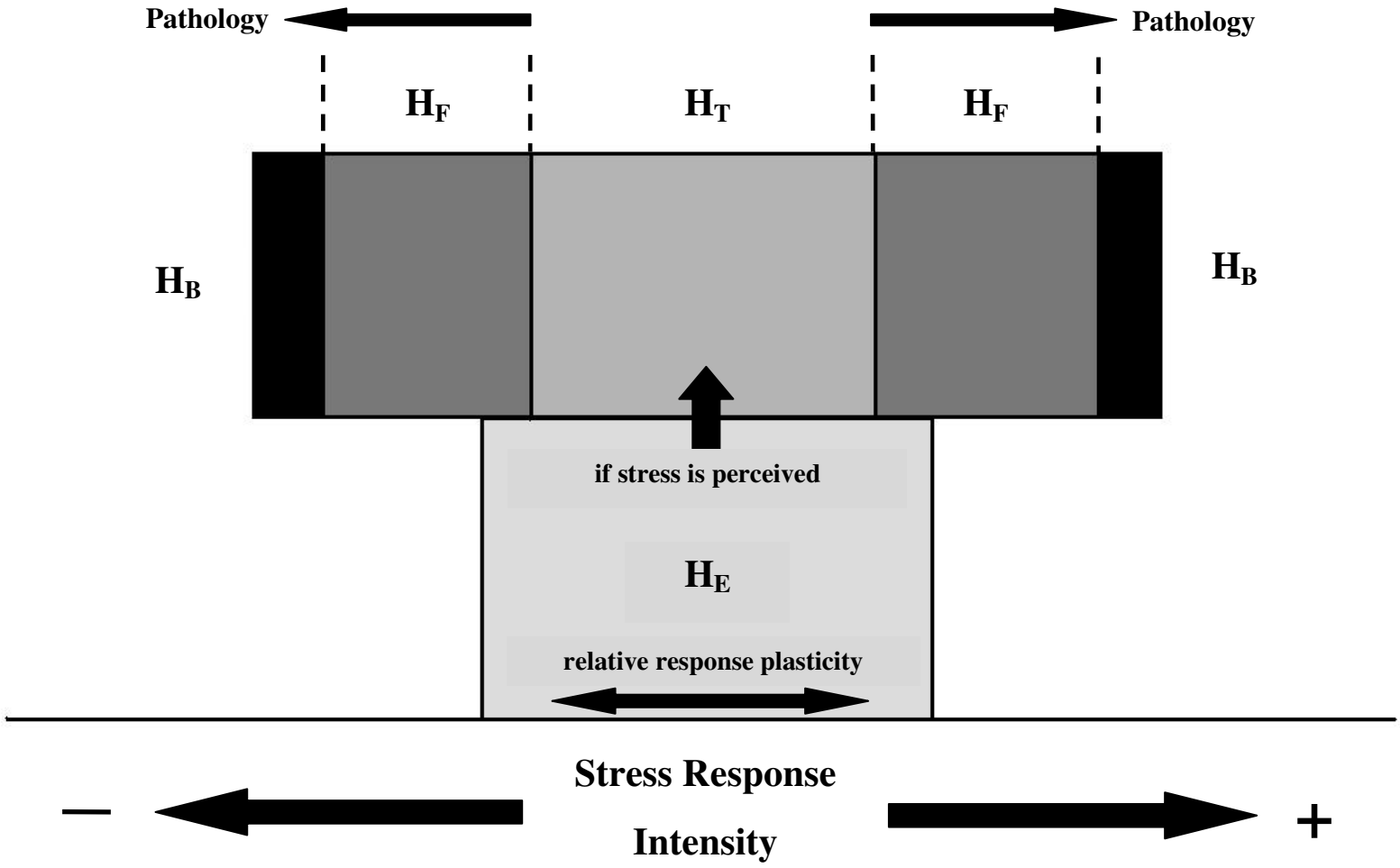

Figure 1: The Homeostasis Battery Model. Four phases are illustrated: (1) homeostatic equilibrium (H_E); (2) homeostatic tension (H_T); (3) homeostatic fatigue (H_F); and (4) homeostatic buckling (H_B). If a stressor is perceived, an individual enters homeostatic tension (H_T). Once in homeostatic tension (H_T), stress response intensity can either increase (illustrated by a plus (+)) or diminish (illustrated by a minus (-)), depending on factors such as specific stressor and/or exposure time to stressor. Relative stress response plasticity is illustrated by the width of each phase. As stress response intensity either increases or diminishes, the degree of normal plasticity of the stress response is diminished. Homeostatic equilibrium (H_E) is associated with a high degree of response plasticity to potential stressors based upon life-history stage or seasonality. Homeostatic tension (H_T) is associated with adaptive responses to negotiate through an unpredictable stressor without the need of a stronger response. Homeostatic fatigue (H_F) is associated with a strong response to a prolonged stressor. Pathological effects can start to manifest themselves at this phase. Failure to respond efficiently and effectively to a chronic stressor is illustrated by homeostatic buckling (H_B). Death may occur at this phase.