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TAILS AND TOXINS: EXPLORING LIFE HISTORY TRAITS AND PREDATOR-INDUCED DEFENSES IN EASTERN RED-SPOTTED NEWTS (*NOTOPHTHALMUS VIRIDESCENS VIRIDESCENS*)

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by

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ABSTRACT

Environments can be dynamic due to fluctuations in localized biotic and abiotic factors. Some environmental changes occur rapidly, which result in organisms having traits that are mismatched with current conditions, incurring costs. To cope with changing conditions, many organisms exhibit phenotypic flexibility, or the ability to alter phenotypes given the same genotype. In doing so, organisms benefit by altering their phenotype to keep up with their environment. Such flexibility of traits helps to ameliorate costs associated with maintaining or producing traits that are not optimal to current conditions.

Environmental variation can be predictable, such as changes that occur seasonally. Animals that utilize different habitats during the breeding and non-breeding season, for example, need to adjust to predictable changes in their environment. Trait flexibility can help these organisms improve their performance in these different environments. If traits carry-over between environments, however, traits providing benefits in one setting might impose costs in the other setting.

Other environmental factors can vary unpredictably over short time scales, such as predation threat; an important selective pressure that can shape individuals, populations, and communities. Inducing predator defenses (e.g. chemical or morphological defenses) only when a predator is present can minimize costs of production and maintenance for these traits. Behavioral defenses, such as refuge seeking and alarm signaling, are often driven by an organism's physiological response to stress. The role of "stress" (including the production of glucocorticoid stress hormones) in driving morphological and chemical responses to predators is poorly understood, and will provide important insight into predator-prey interactions.

Here, I explored the flexibility of traits in Eastern Red-spotted Newts (*Notophthalmus viridescens viridescens*). In Chapter 1, I examine whether tail size in adult newts carries-over

from the aquatic (breeding) to the terrestrial (non-breeding) phase, and whether this imposes costs to locomotor performance across environments. I found that tail size did carry-over – newts with larger tails in the aquatic phase also had larger tails in the terrestrial phase. Larger tails were beneficial in the aquatic phase as they increased swim speed, but did not impede locomotion in the terrestrial phase.

In Chapter 2 and 3, I investigate predator-induced morphological (tail size) and chemical defenses (tetrodotoxin, TTX) in larvae and adult Eastern Red-spotted Newts. Larval newts increased tail size in the presence of a predator cue, but did not alter chemical defenses. By contrast, adults showed evidence of chemical but not morphological defense flexibility during the course of the trials. To evaluate the relationships between the stress hormone, corticosterone (CORT), and the production of defensive traits, I validated a water-borne hormone collection technique for adults. A positive relationship between CORT levels and TTX was found, but no relationship was identified between CORT and tail size.

Overall, the results of this research reveal the flexibility of traits in shifting environments. My work has revealed that flexible traits may carry-over between habitats, and further research examining potential costs of this would be informative for understanding the evolution and maintenance of these traits. I found that an organism's morphological and chemical defenses can be modified, and the degree of flexibility can vary seasonally and across developmental stage. Flexibility of traits can be triggered by predator presence (morphological traits) and may be linked to stress hormone concentrations (chemical defenses). Further exploration into the factors regulating flexible traits will shed light on the evolution of complex life histories and anti-predator defenses.

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Chapter 1

AQUATIC TAIL SIZE CARRIES OVER TO THE TERRESTRIAL PHASE WITHOUT IMPAIRING LOCOMOTION IN ADULT EASTERN RED-SPOTTED NEWTS (*NOTOPHTHALMUS VIRIDESCENS VIRIDESCENS*)

Summary

Many species have evolved phenotypic flexibility to adjust to seasonal changes in their environment, including seasonal breeding phenotypes that increase reproductive success. If there are limits to this flexibility, such that traits carry over across seasons, there may be costs incurred as a result of trade-offs in optimal performance. Male and female Eastern Red-spotted Newts (*Notophthalmus viridescens viridescens* (Rafinesque, 1820)) increase tail size for the aquatic breeding season, and reduce their tail size as they return to the terrestrial environment after reproducing. I tested whether large aquatic tails (which should increase swim performance) carry over to become larger tails in the terrestrial phase (relative to body size), and whether this incurs a cost of decreased walking speed on land. I found a strong correlation between tail size in both phases, suggesting that this trait does carry-over between seasons and environments. Tail size was positively related to locomotor speed in the aquatic phase, but I found no evidence of a locomotor trade-off associated with tail size in the terrestrial phase. Further research that tests for alternative costs of developing large aquatic tails that are then carried over to the terrestrial environment would help to clarify the evolution of this life-cycle staging trait.

Introduction

Phenotypic traits can vary between seasons, as shifts in the phenotypic optima are often correlated with changes in an organism's abiotic and biotic environment (Piersma and Drent 2003; Crozier et al. 2008; Fraser et al. 2011). For instance, many species undergo periods of mating, migration, and (or) hibernation, each with unique corresponding phenotypic optima. The arctic fox's (*Vulpes lagopus* (L., 1758)) cryptic brown summer coat would strongly contrast with a snowy background in the winter (Audet et al. 2002), and the conspicuous plumage of birds such as the breeding male Rock Ptarmigan (*Lagopus muta* (Montin, 1781)) exposes them to greater predation risk when they are not competing for mates (Hamilton and Barth 1962; Montgomerie et al. 2001). To cope with the problem of seasonally fluctuating phenotypic optima, organisms can exhibit seasonal phenotypes, adjusting their physical traits in accordance with predictable changes in the environment; organisms with reversible traits of this kind are said to have phenotypic flexibility (Piersma and Drent 2003; Condon et al. 2010; Schiesari et al. 2011). This creates a potential conflict in the direction of phenotypic optima across seasonal life-history phases; that is, phenotypes favored during one phase may be selected against in another. Life-cycle staging, a subcategory of phenotypic flexibility, describes how individuals regulate expression of traits based on cyclic environmental cues (Jacobs and Wingfield 2000).

Nevertheless, there are limits to phenotypic flexibility (DeWitt et al. 1998). Plasticity may be constrained such that traits carry over across time and situations (Auld et al. 2010). There is a great deal of empirical research that describes how phenotypically flexible traits that affect resource acquisition ability (Broderick et al. 2001; Perryman et al. 2002; Sorensen et al. 2009), habitat selection or quality (Gill et al. 2001; Bearhop et al. 2004; Norris 2005), or body condition (Cook et al. 2004; Bregnballe et al. 2006) in one life-history phase can carry-over and impact an individual's reproduction in subsequent phases (Harrison et al. 2011). Some species with flexible

Figure 1-1. A typical shape and size of tails of adult Eastern Red-spotted Newts (*Notophthalmus viridescens viridescens*) during the aquatic (breeding; A) and terrestrial (nonbreeding; B) phases.

flexible breeding traits have been examined (e.g., badge coloration of lizards and birds: Vinegar 1972; Slagsvold and Lifjeld 1988; tail size of newts: Verrell 1983; Able 1999; presence of antlers in male deer: Lincoln 1992). However, few studies address how traits that are associated with reproduction may carry-over to later life-history phases (Harrison et al. 2011), and if residual presence of these traits out of the breeding season is costly. Furthermore, even if a trait can approach the optimal phenotype in both life phases, there may be significant transitional periods during which the trait will exhibit a suboptimal value (DeWitt et al. 1998; Piersma and Drent 2003).

The Eastern Red-spotted Newt (*Notophthalmus viridescens viridescens* (Rafinesque, 1820)) has reproductive traits that change with breeding status and may carry-over across seasons, having residual effects. Native to eastern North America, populations of this amphibian typically utilize two distinct habitats: they breed in ponds and other bodies of water during the spring, and transition to land as ponds dry or oxygen levels are depleted (Hurlbert 1969). When newts move into the water to breed, both sexes undergo a transformation that includes the tail becoming paddle-like—taller and longer—for the duration of the reproductive period (Hurlbert

1969). This is caused by the tail fin, not the musculature, becoming larger (Walters and Greenwald 1977; Gill 1978), and enables newts to better propel themselves through water (Able 1999; see Fig. 1-1 for a photograph of this tail transition). This increased locomotor performance improves the ability to capture prey, escape predators, and obtain mates in scramble competition during this aquatic phase (Able 1999; Jones et al. 2002). Newts undergo a potentially energetically expensive reduction in tail size before returning to their terrestrial habitat (Berner and Puckett 2010), suggesting that these large aquatic tails are disadvantageous on land.

I hypothesize that tail size carries over between the breeding and nonbreeding phase within an individual (Harrison et al. 2011), and has associated carry-over effects on terrestrial locomotion. I first tested whether tail size in the aquatic phase was positively correlated with tail size in the terrestrial phase, which would indicate that life-cycle staging flexibility in tail size is somewhat constrained. Alternatively, if no relationship in tail size exists between these phases, this would imply that tail size exhibits enough flexibility in life-cycle staging to decouple the two seasonal phenotypes. Since large aquatic tails have been shown to enhance aquatic locomotion (see above), I predicted that large terrestrial tails would impair terrestrial locomotion (e.g., reduced walking speed owing to increased drag), generating a conflict in phenotypic optima between the phases. Our goal was to provide insight into how limits to life-cycle staging traits may generate trade-offs between habitats and life phases.

Materials/Methods

I collected 49 adult newts (29 males and 20 females) from three ponds in State Game Lands 176 (Centre County, Pennsylvania, USA; 40°46=N, 78°0=W) in May 2010, during their aquatic breeding season. I used dip nets to collect newts from these ponds (Beaver 1, n = 10 males; Twin, n = 16 females and 14 males; Greenbriar 1, n = 4 females and 5 males), with pairwise distances

between these ponds ranging from approximately 400 to 1800 m. Most newts initially arrive at breeding ponds in this area in early spring (March), and mating activity may last into early autumn if water is still present in the ponds (Gill 1978; B.E. Carlson, personal observation). Captures from drift fences and field observations in this area reveal that migration to ponds occurs over a short time period and at similar times for all ponds, suggesting that the breeding phenology is fairly similar among these subpopulations (B.E. Carlson, personal observation). I transported newts to the Pennsylvania State University where they were housed in plastic enclosures (45 cm × 30 cm × 30 cm, length × width × height) filled with 10 cm of dechlorinated water. Each enclosure housed two to seven newts of the same sex and pond origin. I assigned newts an individual identification code based on their unique markings (Gill 1978). Room lights were set on a 10 h light : 14 h dark photoperiod, with a mean room temperature of 24.4 °C (SE = ±0.09 °C); this is comparable with conditions these animals experience in the field during the summer months (Schlegel and Butch 1980). Complete water changes were conducted once a week and newts were fed wingless fruit flies (*Drosophila melanogaster* Meigen, 1830) and red worms (*Eisenia fetida* (Savigny, 1826)) ad libitum.

After allowing the newts a 12 day acclimation period, I measured their aquatic locomotor speeds by timing each newt as it swam the length of a custom-made racetrack (120 cm × 7.5 cm × 6 cm, length × width × height) constructed from vinyl, unpolished gutter pipe. The track was marked in 10 cm increments and filled with water to a depth of 4 cm. A digital video camera (Flip UltraHD U2120 W, Cisco Systems, San Jose, California, USA) was positioned above the racetrack and recorded these trials (at 30 frames/s) as newts individually swam the length of the track (as per Losos et al. 2002). I provided a prodding stimulus to the tail using a paintbrush if newts did not move forward immediately after being placed in the track. Each newt was tested for swim speed in three trials. On the day of the trials, we took photographs of each newt's tail from the lateral view and of the body from the dorsal view, including a metric ruler for scale. From the

photographs, I obtained measures of snout–vent length (SVL) and tail surface area using ImageJ version 1.43u (National Institutes of Health, Bethesda, Maryland, USA). Tail surface area was measured as the area of the right lateral surface of the tail, beginning where the tail joins the body (immediately distal to the cloaca). This measurement thus incorporates differences in tail depth and length, both of which are presumably relevant to aquatic locomotion (Able 1999), and our preliminary analyses indicated that both tail depth and length change during the phase transition. Thus, tail area presents the most holistic and relevant measure of tail size.

Over the following 12 days, newts were stimulated to transition to their terrestrial forms by gradually lowering water in the enclosures and adding a floating piece of polystyrene foam insulation to allow newts to exit the water if desired (Walters and Greenwald 1977). Then enclosures were completely drained, lined with moist paper towels, and furnished with a petri dish of water. At this point, all newts had visibly transitioned to their terrestrial forms, with rough granular skin and reduced tail sizes (Walters and Greenwald 1977). Terrestrial locomotor speeds were obtained in the same manner as the aquatic locomotor speeds; however, the track contained no water. Locomotor trials were run at temperatures consistent with those at which the newts were housed, and each newt was tested three times. Newts were again photographed on the day of these trials (27 days after capture), as described above, to allow direct assessment of the relationship between tail morphology and locomotor performance. All newts were released at their location of capture upon completion of these trials.

Video footage obtained during the aquatic and terrestrial locomotor trials was analyzed using Media Player Classic version 1.3.2121.0 (available from http://sourceforge.net/projects/mpc-hc/, accessed 26 July 2010) to determine the initial burst speed over 10 cm of each newt during the trials (following guidelines of Losos et al. 2002). Aquatic trials were included in analysis if (*i*) the newt was clearly swimming, indicated by the newt holding its limbs against its body and performing a serpentine swimming motion (visibly

distinguishable from aquatic "walking"), and (*ii*) the newt swam in this manner over a complete 10 cm distance marked on the racetrack (n = 45 newts). Terrestrial trials were accepted if the newt traveled a 10 cm distance without stopping (n = 18 newts). I calculated locomotor speeds by counting the number of frames it took for the tip of a newt's snout to move 10 cm, beginning on the frame in which the newt first started to swim or walk, and converting this to centimeters per second. I used the fastest initial burst speeds of the three trials for each individual in both the aquatic and the terrestrial phases in our analyses.

Statistical Analysis

Larger tails are expected to be heavier and indeed may constitute a substantial proportion of a newt's total mass. Because newts with larger tails would also have a larger body mass, I calculated relative tail area by correcting for SVL, and thus avoided conflating these two traits. Tail area and SVL were log-transformed to meet the assumptions of parametric tests, and relative tail area was calculated from the residuals of a regression of log (tail area) against log (SVL), effectively creating a measure of tail area that is independent of body size. This procedure thus allowed us to control for variation in locomotor performance due to general size differences. I first tested the effects of phase (aquatic or terrestrial) on tail area to confirm that tails decreased in size during the experiment, thus demonstrating that the newts had undergone seasonal transition, and concurrently examined the effect of sex and the interaction between sex and phase on tail area. These tests were conducted using a repeated-measures ANOVA with sex as a between subjects effect and stage (aquatic or terrestrial) as a within-subjects effect. I also included pond of origin as a between-subjects effect in the analysis, as the different hydroperiods of each pond may cause differences in the rate or degree of phenotypic change during phase transitions (Lind and Johansson 2007). I included all two- and three-way interactions in the model. I also compared the

proportional rather than absolute change in tail area between the sexes, using a Student's *t* test to compare the percent reduction in tail area of each sex between phases.

To test for any carry-over in relative tail area between phases within individual newts, I evaluated the Pearson product-moment correlation of tail area during the terrestrial phase against that in the aquatic phase. I then tested the relationship between locomotor speed and tail area measured in the aquatic and terrestrial phases in two separate linear regression models. I tested whether there was a relationship between the locomotor speeds of individual newts in each environment using a Pearson correlation of aquatic and terrestrial locomotor speeds. Any trade-off in locomotor speed between phases, whether or not it is due to tail area, would be indicated by a significant negative relationship.

Sex did not affect the relationship between tail area and locomotor performance (linear regression analyses of speed in the different phases, incorporating sex and a sex × tail area interaction term as factors: $p > 0.23$ for these terms), and accounting for sex in ANOVA models provided no significant increase in explained variation (ANOVA comparing full models (with sex and sex × area interactions) with the nested reduced model containing only relative tail area—aquatic: $F_{2,41} = 0.22$, $p = 0.81$; terrestrial: $F_{2,14} = 0.87$, $p = 0.44$). I, therefore, omitted sex from the final analyses involving locomotor performance, pooling male and female newts to preserve our sample size and statistical power to detect tail-area effects. Over half of the newts were prodded once during the aquatic trials, and all newts were prodded a mean of 5 times in the terrestrial trials. The number of times I prodded each newt to stimulate locomotion did not affect locomotion speed ($p > 0.89$ in both aquatic and terrestrial trials), and was, therefore, also

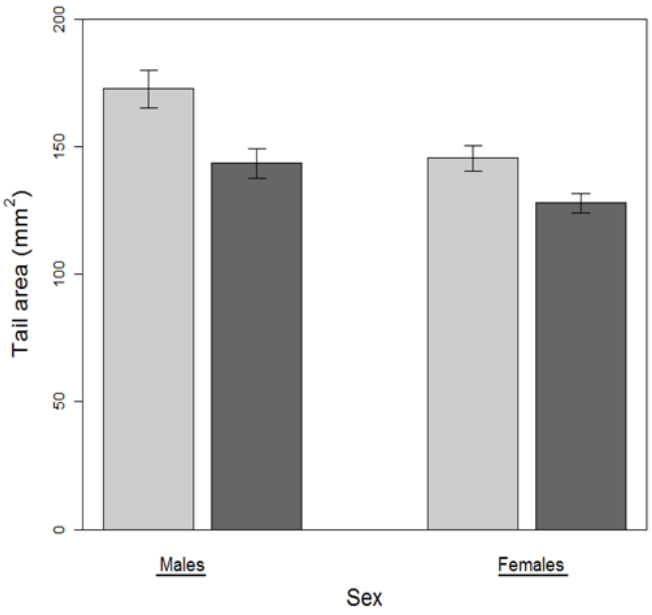

Figure 1-2. Relative tail area decrease over aquatic (light grey) and terrestrial (dark grey) life phases for male and female Eastern Red-spotted Newts (*Notophthalmus viridescens viridescens*). Bars represent ±1 SE (repeated-measures ANOVA: $F_{1,41} = 4.82$, $p = 0.03$).

Table 1-1. Results of repeated-measures ANOVA of the effects of stage (aquatic or terrestrial), sex (male or female), pond of origin, and interactions between these factors on the relative tail area of adult Eastern Red-spotted Newts (*Notophthalmus viridescens viridescens*).

Source	*F*	df	*p*
Stage	231.80	1, 40	<0.01
Sex	6.23	1, 40	0.02
Pond	7.61	2, 40	<0.01
Stage × sex	3.22	1, 40	0.08
Stage × pond	7.80	2, 40	<0.01
Sex × pond	0.012	1, 40	0.91
Stage × sex × pond	1.25	1, 40	0.27

excluded from the final models. All statistical analyses were two-tailed with α levels set at 0.05, and parametric assumptions were met for each analysis. Analyses were performed in R version

2.11.1 (R Development Core Team 2010, R Foundation for Statistical Computing, Vienna, Austria).

Results

Newts had relatively larger tails in the aquatic than the terrestrial phase ($F_{1,40}$ = 231.80, p < 0.01; Fig. 1-2, Table 1-1). Males had significantly larger relative tail areas than did females ($F_{1,40}$ = 6.23, p = 0.02; Fig. 1-2), but there was no effect of sex on the change in tail size between phases (sex × tail area: $F_{1,40}$ = 3.22, p = 0.08) or the proportional change in tail size across phases ($t_{22.56}$ = 1.41, p = 0.17). There was a significant effect of pond of origin on relative tail size ($F_{2,40}$ = 7.61, p < 0.01) and on the change in tail size during phase transitions (pond × phase: $F_{2,40}$ = 7.80, p < 0.01); sex did not affect this relationship (sex × pond: $F_{1,40}$ = 0.012, p = 0.91; sex × pond × phase: $F_{1,42}$ = 1.25, p = 0.27).

The relative tail size of newts in the aquatic and terrestrial phases was significantly positively correlated (r = 0.87, t_{42} = 11.6, p < 0.001; Fig. 1-3). In the aquatic phase, newts that had larger tails swam faster than did newts that had smaller tails (R^2 = 0.20, $F_{1, 43}$ = 10.58, p < 0.01; Fig. 1-4*a*). In contrast, there was no significant relationship between relative tail size and maximum terrestrial locomotor speed during the terrestrial phase (R^2 = 0.007, $F_{1, 16}$ = 0.11, p = 0.75; Fig. 1-4*b*). There was no significant relationship between maximum aquatic speed and maximum terrestrial speed (r = –0.05, t_{16}= –0.20, p = 0.84; Fig. 1-5).

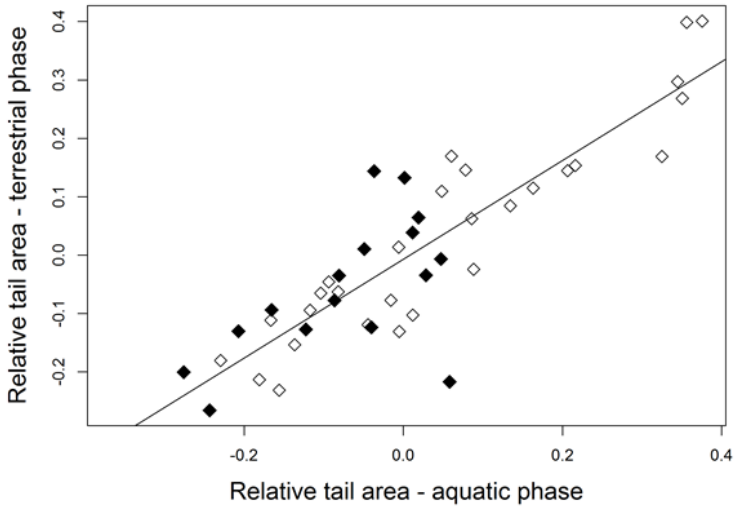

Figure 1-3. Relative tail areas of male (open diamonds) and female (solid diamonds) Eastern Red-spotted Newts (*Notophthalmus viridescens viridescens*) in the aquatic phase versus the terrestrial phase. Line is a best-fit trend line ($p < 0.01$).

Discussion

I found evidence of a carry-over effect in a life-cycle staging trait, tail size, in Eastern Red-spotted Newts. Newts that had relatively larger tails in the aquatic breeding phase also had larger tails in the terrestrial nonbreeding season phase, suggesting that the plasticity of this trait is limited. As predicted, I found that greater tail size is associated with greater locomotor speed during the aquatic phase (Hurlbert 1969; Able 1999); I caution, however, that locomotor performance may be directly affected by differences in muscle mass or energy reserves (manifested in total body mass) that could not be accounted for in this experiment or others like it (e.g., Hoff et al. 1989; Green 1992; Gvoždík and Van Damme 2006). By contrast, I found no evidence that tail size affects locomotor speed in the terrestrial phase. Additionally, I found that

aquatic and terrestrial locomotor speeds are not correlated in this species; newts that moved quickly in the aquatic phase did not necessarily move quickly in the terrestrial phase and vice versa, suggesting that individual newts may be differently adapted to these phases.

The fact that all newts undergo a reduction in tail size upon transitioning to the terrestrial environment (Walters and Greenwald 1977) implies that there is some cost to large tails on land. Our lack of evidence for a cost of tail size on terrestrial locomotor speed conforms to results of an earlier study in female Alpine Newts (genus *Triturus*Rafinesque, 1820) (Gvoždík and Van Damme 2006). Our measure of the costs of large terrestrial tails was conservative, however, and there may be unmeasured costs. I measured locomotion under much simpler conditions than are found in nature where newts navigate over and through more complex substrates of leaf litter and vegetation. Additional environmental complexity may present a greater challenge for newts with larger terrestrial tails, and so large tails in the terrestrial phase may indeed incur a locomotor cost under more natural conditions. Larger tails may incur costs on land other than those associated with locomotor ability. For example, newts with larger tails may move at the same speed but expend greater amounts of energy carrying a heavier tail, potentially reducing endurance over longer periods (Angilletta et al. 2003) and (or) incurring greater energetic costs (Auld et al. 2010) by reduction in foraging behavior, growth, or reproduction (DeWitt et al. 1998). Large tails in the terrestrial environment may incur other physiological costs; for example, the larger surface area could increase the risk of desiccation (Walters and Greenwald 1977). Such potential costs to large terrestrial tails should be explored.

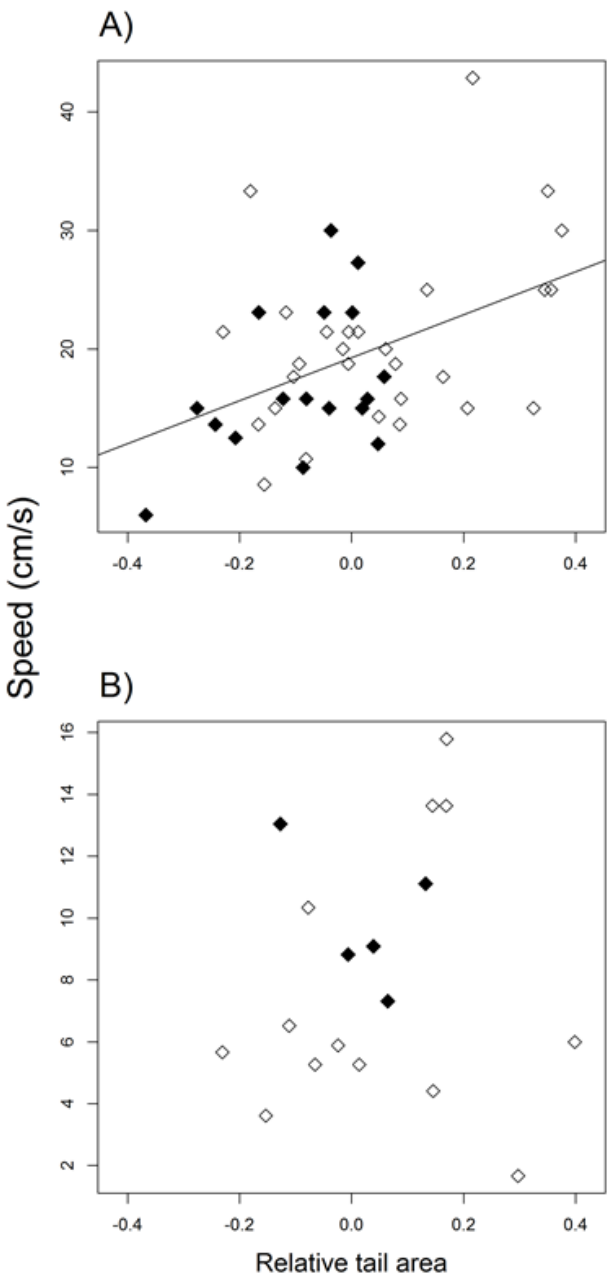

Figure 1-4. Maximum locomotor speed versus relative tail area of male (open diamonds) and female (solid diamonds) Eastern Red-spotted Newts (*Notophthalmus viridescens viridescens*) in the (A) aquatic and (B) terrestrial phases. Line in A is a best-fit trend line ($p < 0.01$); the relationship in B was not significant ($p = 0.75$).

Adult newts spend most of their time in a terrestrial state, typically inactive hiding under leaf litter; however, they have been observed traveling up to 100 m in a single night, usually when it is cool and humid (Gill 1978; Roe and Grayson 2008). Most of the terrestrial phase of this species is, however, spent hiding and they tend to move only when it is cool and humid. This suggests that predation and desiccation are significant threats. Minimizing activity time by moving faster should, therefore, be important. Incidentally, Roe and Grayson (2008) observed that sex and mass do not influence the distances that newts travel, which is consistent with our observations that greater tail size (which should increase mass and will differ between sexes) does not impair terrestrial locomotion. It is important to note, however, that I did not quantify the locomotor cost of aquatic-phase tails on land. Newts begin transitioning before leaving water and, to our knowledge, have never been observed to retain their aquatic tails into the terrestrial phase. Thus, rather than attempting to determine why tail size transition occurs, I aimed to understand possible trade-offs that may be driving all newts to undergo this aquatic–terrestrial transition and may explain the variation in tail sizes among newts in the same phase.

Tail size transitions are believed to be driven by environmental cues (e.g., pond drying) rather than being innately timed (Walters and Greenwald 1977). I found that relative tail size and the change in tail size when transitioning from the aquatic to the terrestrial phase varied among ponds. This pattern suggests a potential role for either different selective pressures on these traits among ponds (Gill 1978; Van Buskirk and Schmidt 2000) or within-lifetime acclimation to variable drying regimes among ponds (Dodd and Cade 1998; Van Buskirk 2009). There are important differences between these ponds: Beaver 1 is a large permanent beaver pond and newts from this pond had relatively smaller tails and more dramatic tail size transitions, whereas Twin and Greenbriar 1 are smaller and dry by the end of summer most years (B.E. Carlson, personal observation). Tail trait differences of newts between ponds more likely reflect plastic responses

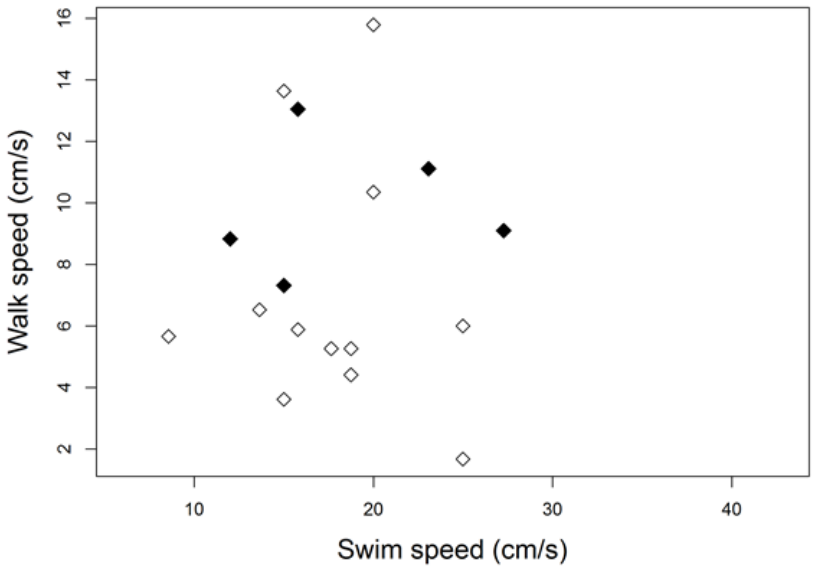

Figure 1-5. Maximum locomotor speed of male (open diamonds) and female (solid diamonds) Eastern Red-spotted Newts (*Notophthalmus viridescens viridescens*) in the aquatic versus terrestrial phases. The correlation between these was not significant ($p = 0.84$).

rather than local adaptation, since the distances between ponds are small relative to the dispersal distances of newts 1 km or more; Gill 1978) and are separated by continuous newt habitat (i.e., without any dispersal barriers; Grayson and Wilbur 2009). The fact that newts from the pond that had the greatest tendency to dry tended to have larger tails and exhibit less plasticity of tail size than the newts from the more permanent source populations is counterintuitive. Newts in ponds with the greatest tendency to dry would be expected to have smaller tails and more plasticity of tail size to adjust to changing environmental conditions. This relationship warrants further investigation.

I know of no other studies that have assessed the impact of conflicting phenotypic optima on flexible traits exhibiting cross-season correlations, despite the ubiquity of phenotypes that vary

within individuals between breeding and nonbreeding phases (Piersma and Drent 2003). These ideas can be extended to other regular phenotypic fluctuations, such as the plumage coloration of breeding birds (Hamilton and Barth 1962; Montgomerie et al. 2001). Phenotypic flexibility, and life-cycle staging in particular, are common strategies used to cope with shifting optima, and limits to plasticity are theoretically and empirically supported (DeWitt et al. 1998; Auld et al. 2010). As a result, I expect that seasonal traits may carry-over in many species in which transitional properties of these traits have not previously been recognized. The importance of carry-over effects and their implications in ecological dynamics have recently attracted attention (Harrison et al. 2011) and are likely to be common in diverse study systems. Future research should continue to examine cross-season costs associated with limited phenotypic flexibility and conflicting optima during different life phases. This will enhance our understanding of how limits to the degree of flexibility may constrain the evolution of seasonal traits or impose trade-offs that favor individual variation in investment in these traits.

Chapter 2

EASTERN RED-SPOTTED NEWT (*NOTOPHTHALMUS VIRIDESCENS VIRIDESCENS*) LARVAE ALTER MORPHOLOGICAL BUT NOT CHEMICAL DEFENSES IN RESPONSE TO PREDATOR CUES

Summary

Prey traits are often modified in response to exposure to predators, a phenomenon known as predator-induced phenotypic plasticity. Morphological plasticity in response to predator cues is well documented in amphibians; however, predator-induced chemical defenses have received relatively little attention. The Eastern Red-spotted Newt (*Notophthalmus viridescens viridescens*), which possesses tetrodotoxin – a toxin for chemical defense, is most vulnerable to predation during its larval stage. I assessed whether exposing Eastern Red-spotted Newt larvae to predator scent cues (from dragonfly larvae) would elicit change in their morphological and chemical defenses. Newt larvae exposed to scent cues of predatory dragonfly larvae exhibited significantly deeper tail depths, which should enhance predator escape ability by allowing them to swim faster, but did not differ in mass, snout-vent length or tail length. Newt larvae toxin concentrations were not significantly affected by exposure to these predator cues. Larval toxicity may be maternally-derived and inflexible, or induced toxicity may only be detectable later in development. Predator-induced phenotypic plasticity, especially of chemical defenses, warrants greater attention, as potentially important outcomes of species interactions remain unclear.

Introduction

Organisms have traits that increase their chances of avoiding and surviving predation (Via and Lande 1985). Defenses, however, can be energetically costly to produce and maintain, leaving less energy for growth and reproduction (Levins 1968; Harvell 1990; Van Buskirk and Schmidt 2000). Although beneficial in the presence of predators, these traits are often disadvantageous when predation risk is low (Dewitt et al. 1998). One way for organisms to avoid energetic costs of predator defense is to flexibly adjust these defensive traits – for example, growing larger, producing armor, or increasing toxicity only when predators are present (Benard 2004; Black and Dodson 1990; Agrawal et al. 2002). The ability of organisms to appropriately change their traits in response to environmental stimuli is known as phenotypic plasticity (Schlichting and Smith 2002), and can increase survival of the organism and its offspring (i.e. fitness; Agrawal 2001).

Predator-induced phenotypic plasticity occurs when traits change as the result of predation threats (Skelly and Werner 1990), and can include changes in chemical and morphological defenses. Chemical defenses are ubiquitous in plants, and plastic defenses can be initiated by herbivory, including as mechanical damage from a caterpillar to a plant (Mithöfer et al. 2005). Chemical defenses against predators are also common in amphibians (Daly 1995; Marion and Hay 2011). Larval amphibians typically detect predators through the presence of chemical cues from the predators themselves or from injured and/or digested conspecifics (Ferrari et al. 2010; Petranka et al. 1987). The ability to plastically alter the expression of a chemical defense may represent an important strategy since the risk of predation in aquatic systems can be highly variable (Lima and Bednekoff 1999; Kats and Dill 1998). The plasticity of chemical defenses is poorly investigated for amphibians (but see Tsuruda et al. 2002; Benard and Fordyce 2003). Plastic responses more commonly involve changes in morphological traits (Harvell 1990;

Relyea 2001). To reduce predation risk, organisms can increase overall body size when predators are present (Semlitsch 1990) and alter the relative size and shape of morphological features (e.g., development of shorter, wider tails that increase swim speed in larval tiger salamanders, Storfer and White 2004). However, such morphological plasticity is not exhibited when the costs of doing so outweigh potential benefits (Harvell 1990; Dewitt et al. 1998; Relyea 2002a).

I tested for plasticity in chemical and morphological defenses of Eastern Red-spotted Newt (*Notophthalmus viridescens viridescens*) larvae in response to predator cues. The Eastern Red-spotted Newt is a well-studied, toxic salamander (Yotsu-Yamashita and Mebs 2001) that is most vulnerable to predation during its larval stage (Brodie 1968; Mathis and Vincent 2000), which lasts roughly two to five months (Lannoo 2005). Newt larvae are preyed upon by fish, aquatic insects and adult newts (Lannoo 2005; Van Buskirk and Schmidt 2000; Marion and Hay 2011). The presence and abundance of predators of newt larvae varies temporally, due to fluctuations of predator populations within a pond, and spatially as adult newts migrate between, and oviposit in ponds with divergent predator communities (Gill 1978; B. Carlson, unpublished data). This suggests that inducing predator defenses to match rapid changes in environment—rather than evolving fixed defenses—may be beneficial in this species.

Like other newts, adult and juvenile (eft stage) Eastern Red-spotted Newts contain high levels of the potent neurotoxin, tetrodotoxin (TTX), in their skin and internal organs (Mebs et al. 2010). TTX has not been measured in larvae of this species, but is present in larvae of other newt species (Gall et al. 2011). TTX provides an effective defense against predators (Hanifin 2010) and is known to be mostly maternally derived (Hanifin et al. 2003). Even in very small amounts, this toxin causes irritation, muscle paralysis, gasping, and/or death in many newt predators including invertebrates, frogs, turtles, snakes, and other salamanders (Hurlbert 1970; Brodie 1968; Hanifin 2010; Gall et al. 2011). It is unknown if TTX production in amphibians is endogenous or exogenous; endogenous origins would result in energetic costs (Yotsu-Yamishita

et al. 2012). Toxin levels vary among individual adult Eastern Red-spotted Newts (Yotsu-Yamashita and Mebs 2001), which may be explained in part by plastic responses to predation early in development (Yotsu-Yamishita et al. 2012). It seems likely that larval newts would benefit from the ability to alter the strength of their chemical defense. However, if high levels of TTX are too costly to produce or this response is too slow to be useful in the face of an immediate predation threat, plastic increases in TTX production may not be adaptive. Rather, the optimal strategy may be to maintain just enough TTX to be highly distasteful throughout development in order to deter predation.

The ability of Eastern Red-spotted Newt larvae to alter their morphology in the presence of predators is unknown; however, there is evidence of this in ecologically similar newt species. For example, larval *Triturus* newts that are exposed to dragonfly larvae sustain increased tail depth and decreased tail length, which can boost swim speed and should increase survival by aiding escape efforts (Van Buskirk and Schmidt 2000). Thus, I expect larval Eastern Red-spotted Newts to display similar changes in tail morphology in the presence of dragonfly larvae.

Materials/Methods

Study Animals

To test the effect of predator cues on larval Eastern Red-spotted Newt defenses, I used newt larvae hatched from eggs laid in the laboratory as well as larvae collected from the field. Forty-two gravid adult female Eastern Red-spotted Newts (*Notophthalmus viridescens viridescens*) were captured in ponds in several state game lands in Pennsylvania, USA, in April 2011. Only one female (from State Game Lands #100, Centre County, PA, 41˚ 5’ N, 77˚ 58’ W) successfully oviposited in the laboratory, and I collected twenty of her eggs from the enclosure as they were laid. These lab-laid eggs (hereafter “lab-laid”) were placed in groups of four or five in

divided (8.89 cm diameter) Petri dishes containing dechlorinated water until they hatched (about 10 days). An additional 20 newt larvae were collected by dipnet from State Game Lands #176 (Centre County, Pennsylvania, USA, 40˚ 46’ N, 78˚ 0’ W) in July 2011 (hereafter “field-caught”).

I placed all larvae individually in plastic containers (15 x 9.5 x 7 cm, L x W x H) filled with 400 mL of dechlorinated water, each containing half of an oak leaf (*Quercus* sp.) for cover and an airstone to oxygenate the water. Lab-laid larvae were added to containers two days after hatching and field-caught larvae were added the day of capture. I fed all newt larvae a field collected mixture of zooplankton (primarily cladocerans and copepods) *ad libitum* daily. Newt larvae were kept in a temperature-controlled room set at 18.5˚C with a 12:12 hour light: dark cycle.

I collected late-instar aeshnid dragonfly larvae (*Anax junius*, *Aeshna sichensis*, *Gomphaeschna antilope*, and *Aeshna juncea*) from State Game Lands #176 to provide predator cues. Aeshnid larvae are significant predators of larval amphibians, including Eastern Red-spotted Newt larvae, (Buskirk 1988; Storfer and White 2004) and are commonly used to induce plastic responses in amphibians in the laboratory (Relyea 2001; McCollum and Leimberger 1997). The ponds from which I collected dragonfly larvae contained breeding populations of Eastern Red-spotted Newts, so I presume that the dragonflies likely had experience with these newts. I housed dragonflies individually in plastic enclosures (15 x 9.5 x 7cm, L x W x H) containing 600 mL of dechlorinated water and two twigs as perches. Each dragonfly larvae was offered one Eastern Red-spotted Newt larva three times a week from a group of additional newt larvae I collected but did not use in this experiment. Most newt larvae were consumed in less than one minute, preventing prolonged periods of stress. Newt larvae that were not consumed after five minutes were removed and returned to an enclosure with other newt larvae not used in the experiment. Only one dragonfly larva did not readily consume the feeder newt larvae and cues from this individual were not used in this experiment.

Treatments

Lab-laid and field-caught newt larvae were acclimated to the lab conditions within their individual enclosures for 10 days before being assigned to one of two treatments: predator cue or control (n = 10 from each group in each treatment). For larvae assigned to the predator cue treatment, I added 25 mL of fresh dragonfly water from an enclosure where a larval newt was recently eaten (hereafter, predator cue) to their enclosure. Water used for predator cues was only taken from enclosures in which dragonflies had just eaten newt larvae. Newt larvae in the control treatment received 25 mL of dechlorinated water. Respective water additions were made three times per week for 21 days (a total of ten applications). Amphibian larvae are highly responsive to the olfactory cues of predators that have consumed and digested conspecifics, and similar protocols have successfully elicited predator-induced responses in other species of larval amphibians (Mathis and Vincent 2000; McCollum and Leimberger 1997). Both lab-laid and field-caught larvae were at approximately the same larval stage when placed into individual containers (stage 33) and at the end of the experiment (stage 36) according to Epperlein and Junginger's (1982) staging of larval Alpine Newts, *Triturus alpestris*.

Two days after the final water addition, lab-laid and field-caught larvae were euthanized with 70% ethanol. Lab-laid larvae were frozen at -80 ºC for later analysis of TTX concentrations; field-caught larvae were not analyzed for TTX due to logistical constraints. TTX was extracted from each whole lab-laid newt larva using the methods of Hanifin et al. (2002). TTX was quantified using a Competitive Inhibition Enzymatic Immunoassay (as per Stokes et al. 2012). The standard curve fell between 500 and 10 ng/mL, and the minimum level of detection in this assay is 10 ng/mL. Newt samples were not diluted and were run against standards, diluted in 0.1M acetic acid to generate a standard curve. Blackworms (*Lumbriculus variegatus*), which do not contain TTX, were run as negative controls and were extracted and processed in the same

manner as the newt larvae. Samples were run on two plates. The average intra-assay coefficient of variation was 8.16%, and the inter-assay coefficient of variation was 9.68%.

The stored samples of lab-laid and field-caught newt larvae preserved in 70% ethanol were immediately measured after preservation for wet mass and their dorsal and lateral surfaces photographed against a scale bar. Morphological measurements were taken from photographs using ImageJ 1.43u (National Institutes of Health, Bethesda, MD). Specifically, I measured SVL (snout-vent length), tail length, and tail depth at the greatest dimensions (as per Van Buskirk and Schmidt 2000).

Statistical Analysis

Newt larvae TTX concentrations were compared between predator cue and control treatments using a t-test. One control group newt was excluded from the statistical analysis of TTX due to a clear error in the chemical analysis (i.e. an order of magnitude higher than the next highest measurement). To examine effects of predator cues on larval newt morphology, I first tested for any effect of treatment on overall morphology using multivariate analysis of variance (MANOVA) with predator treatment and group (lab-laid versus field-caught, to control for the variation due to differences in age and prior experience with predators) as factors, and mass, SVL, tail length, and tail depth as the response variables. There were substantial deviations from the assumptions of normality and homogeneity of variances, predominately driven by a few unusually large newts. I therefore log-transformed each morphological variable for all analyses, which substantially improved model fit.

I then evaluated the effect of predator cue treatment on each morphological trait independently using either Analysis of Variance (ANOVA; for mass) or Covariance (ANCOVA; for the other traits, see Wilson et al. 2005); group and treatment were included as factors, and mass was included as a general body size covariate in ANCOVA models (García-Berthou 2001;

Freckleton 2002) to enable us to compare size-corrected morphology. All analyses were performed in R (version 2.13.0, R Development Core Team 2011, Foundation for Statistical Computing, Vienna, Austria) as two-tailed tests with alpha = 0.05.

Results

There were no differences in TTX concentration of lab-laid newt larvae exposed to predator cues versus control (t = 0.20, df = 17, p = 0.85). There was a significant main effect of predator cue exposure on newt larvae morphology (Wilk's lambda = 0.62, $F_{1,36}$ = 4.98, p = 0.003), and this effect differed between lab-laid and field-caught larvae (group x treatment interaction: Wilk's lambda = 0.63, $F_{1,36}$ = 4.89, p = 0.003; group: Wilk's lambda = 0.58, $F_{1,36}$ = 5.95, p = 0.001). I used univariate analyses to elucidate the nature of these morphological differences. Newt larvae that had been exposed to predator cues had relatively deeper tails ($F_{1,35}$= 12.59, p = 0.001; Fig. 2-1A), and this was true for both lab- and field-caught larvae (group x treatment: $F_{1,35}$= 0.46, p = 0.50). Field-caught newt larvae that were exposed to predator cues had greater mass than did field-caught controls, but I found no such effect on lab-laid larvae (treatment: $F_{1,35}$= 1.19, p = 0.28; group x treatment: $F_{1,35}$= 4.69, p = 0.04; Fig. 2-1B). There was no significant effect of treatment (all p > 0.18; Fig. 2-1C, D), or interactive effect of group and treatment (all p > 0.05), on any of the other morphological variables. None of the other morphological traits significantly differed between these groups (all p > 0.06).

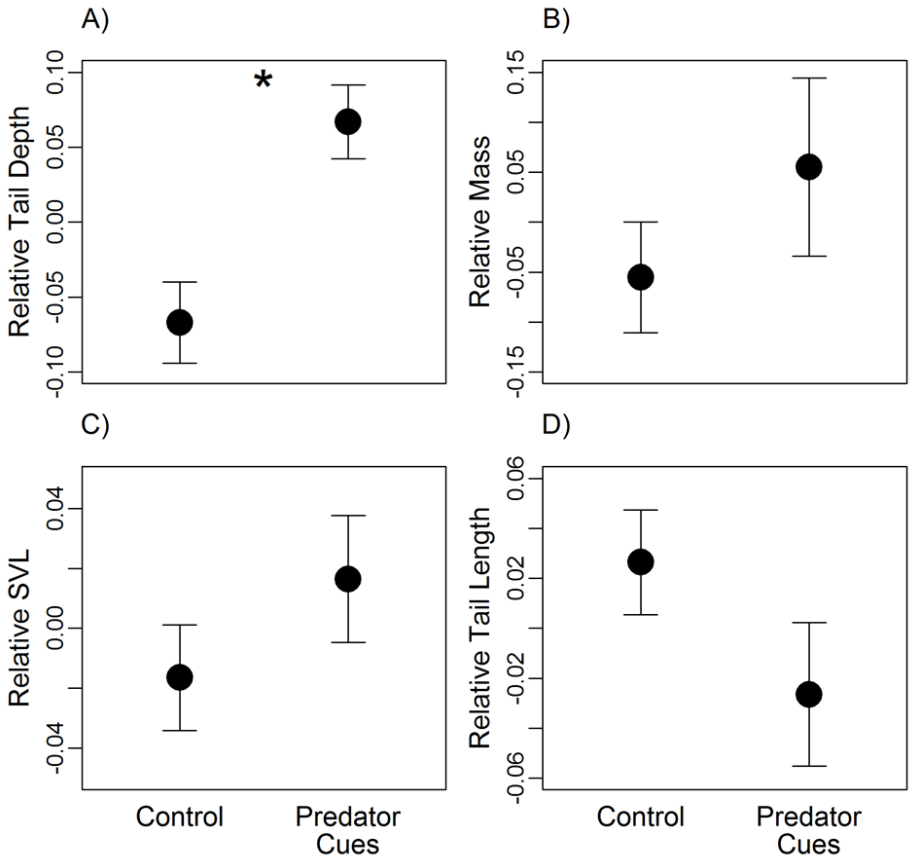

Figure 2-1. The effects of exposure to cues of predatory dragonfly larvae on Eastern Red-spotted Newt (*Notophthalmus viridescens viridescens*) morphology. Values represent residuals of multiple linear regressions of the log-transformed traits against log-mass (except for mass itself) and group (lab-laid or field-caught). Points represent means ± 1 S.E of 20 samples. *Indicates a significant effect of predator cues ($p < 0.05$).

Discussion

This study represents the first measurement of TTX levels and evidence of predator-induced morphological defenses for Eastern Red-spotted Newt larvae. Our results suggest that

Eastern Red-spotted Newt larvae do not exhibit chemical plasticity, but do show morphological plasticity, in response to olfactory cues of odonate predators.

Eastern Red-spotted Newt larvae do not appear to have inducible chemical defenses because no differences in TTX levels were found between predator-exposed and control newt larvae. Our predator cue additions were at similar concentrations to that in other studies, ecologically relevant, and strong enough to produce behavioral responses in *Notophthalmus v. viridescens* and *Triturus pygmaeus* larval newts (Mathis and Vincent 2000; Gonzalo et al. 2012). TTX concentration vary during normal development in a related newt species, *Taricha granulosa* (Gall et al. 2011), therefore TTX concentrations of Eastern Red-spotted Newts should be determined at different developmental stages to find out if they follow a similar pattern. Future studies could then explore the potential plasticity of each stage and whether plasticity is related to initial TTX concentration. Dragonfly larvae can tolerate small quantities of TTX (Gall et al. 2011), but find larger quantities of TTX distasteful and spit out highly toxic larval newts, leaving the larval newt alive but injured (Gall et al. 2011). This suggests that higher TTX concentrations in the presence of dragonfly larvae should be beneficial to larval newts as it would allow them to survive initial predation attempts through unpalatability and allow them to learn to avoid predators. Therefore, I found this lack of TTX plasticity surprising. If future studies show a lack of induced chemical responses, it might indicate that the process is too costly or not rapid enough to prevent lethal injury once a predator arrives, or that an induced chemical response has yet to evolve (DeWitt et al. 1998).

Predator-induced chemical defenses may be initiated or detectable over a longer period of time than the length of this experiment. Western toad tadpoles, *Anaxyrus* [*Bufo*] *boreas*, and cane toad tadpoles, *Bufo marinus*, raised in the presence of predators showed evidence of post-metamorphic predator-induced chemical defenses (Benard and Fordyce 2003; Hagman 2010). Delayed plasticity does not make adaptive sense in the Eastern Red-spotted Newt (or *Bufo*)

system, however, because many predators of larvae pose little or no risk to later developmental stages (Hurlbert 1970; Lannoo 2005; Lawler and Hero 1997). It would be more advantageous for Eastern Red-spotted Newt larvae to rapidly respond to current threats because spatial, temporal, and ontogenetic variation alters their susceptibility to different predators (Gill 1978). Nevertheless, studies examining delayed predator-induced plasticity of chemical defenses across ontogeny may be illuminating.

By contrast to effects on TTX, the predator cues used in our study did induce a morphological response in the Eastern Red-spotted Newt larvae. It is possible that chemical defenses of these newts are less sensitive to predator cues than are morphological traits. TTX levels in this species may plastically respond only to higher concentrations of predator cues. TTX levels may plastically respond to cues from different predator species, such as raccoons (*Procyon lotor*), than those used in this study because mammalian predators are more susceptible to the effects of TTX than invertebrates, reptiles, and other amphibians (Brodie Jr. 1968). Additionally, it is important to note that toxin concentrations were only measured for the lab-laid newt larvae, which were from the same clutch. While this eliminated differences based on maternally derived toxin levels, our results may not necessarily represent all Eastern Red-spotted Newt larvae.

Plasticity of morphological defenses may be more advantageous than that of chemical defenses for Eastern Red-spotted Newt larvae. Morphological plasticity might be more effective than chemical defenses, which may require more time and energy to develop and thus do not match changing predation risks (Holloway et al. 1993; Dodson 1989; Harborne 1988). Eastern Red-spotted Newt larvae raised with predator cues exhibited an increase in tail depth, paralleling morphological predator responses for larvae of other salamanders (Storfer and White 2004; Van Buskirk and Schmidt 2000; Yurewicz 2004) and anuran species (McCollum and Leimberger 1997; Van Buskirk and McCollum 1999; Relyea 2002a). Deeper tails could enhance predator evasion by increasing swim speeds (Storfer and White 2004) or may attract predator strikes away

from the more vulnerable head and torso (Van Buskirk et al. 2003). Thus, this plastic change in tail morphology of Eastern Red-spotted Newt larvae in response to dragonfly larvae predator cues may be adaptive, and more propitious for this species than investing in chemical defenses.

Field-caught larval newts exposed to the predator cue treatment had greater mass; this was not seen in lab-laid larvae. This effect may be due to differences in metabolism, initial size, or diet during initial developmental stages between lab-laid and field-caught newt larvae. Field-caught larvae also likely had previous experience with predators (including and in addition to dragonfly larvae), and increased responsiveness to predators may have been primed by previous exposure to these threats in the ponds (Relyea 2001). Alternatively, this difference in morphological plasticity may simply reflect genetic differences between the sibship raised in the lab and those represented by the field-caught newts.

This study adds to our growing knowledge of the importance and pervasiveness of predator-induced morphological plasticity, and is one of the few studies of inducible chemical defenses in animals (but see Bernard and Fordyce 2003). Increased research efforts in this area are necessary to understand how chemical plasticity within the animal kingdom affects species interactions and toxicology, and may yield information relevant to applications in the field of pharmacology.

Chapter 3

EVALUATING STRESS MEDIATION OF PREDATOR-INDUCED DEFENSES IN EATSTERN RED-SPOTTED NEWTS (*NOTOPHTHALMUS VIRIDESCENS VIRIDESCENS*)

Summary

To reduce costs of producing and maintaining predator defenses, many species exhibit defensive traits only after being exposed to threats ("predator-induced defenses"). Predator cues can initiate a hormonal cascade via the hypothalamic-pituitary-adrenal axis. This cascade includes the production of glucocorticoid hormones, which indicate the presence of a stressor, return functions to homeostasis, and may induce predator defenses. I used adult Eastern Red-spotted Newts (*Notophthalmus viridescens viridescens*) to determine if predator cues trigger plastic morphological defenses (change in tail size) and chemical defenses (change in toxin concentration), and if changes in these traits are mediated by changes in levels of the stress hormone corticosterone (CORT). Adult newts were exposed to water containing predator cue or adrenocorticotropic hormone (ACTH) injection (to induce a CORT response) and their respective control (distilled water or saline injection). CORT measurements were obtained using a water filtration method, a novel approach I validated for this species. Interestingly, I found that neither morphological nor chemical defenses of adult Eastern Red-spotted Newts responded to predator cues or ACTH injections. Both toxin concentrations and CORT levels, however, increased for all treatments during this study and are positively correlated, suggesting that stress hormones may mediate changes in toxin levels. Further exploration of the mechanisms controlling inducible defenses, including the role of stress hormones, would shed light on this interesting phenomenon.

Introduction

Predation is a ubiquitous feature of populations, communities and ecosystems (Langerhans 2007). This process has selected for mechanisms, including physical (e.g. armor, spines) and chemical (e.g. toxins) defenses (Langerhans 2007) that protect organisms from potential predators (Van Buskirk and Schmidt 2000; Werner and Anholt 1996; Peacor and Werner 2000). Such defensive traits can be costly because they require energy to produce and maintain (Agrawal 2001). Individuals can reduce these costs by inducing defenses in response to cues indicative of predator presence (Agrawal 2001; Auld and Relyea 2011; Relyea 2002b). The benefits of flexible defenses—reversible traits induced during adulthood—should be greatest in dynamic environments where predation threats fluctuate within a generation (Piersma and Drent 2003; Benard 2004; Agrawal 2001).

Environmental cues signaling predator presence can initiate physiological changes, including the production of glucocorticoid (stress) hormones (Scheuerlein et al. 2001; Silverin 1989; Wingfield et al. 1998). These hormones work to restore homeostasis after exposure to a stressor (Crespi and Denver 2005), and can initiate behavioral changes that can increase an individual's chances of survival (Orchinik 1998; Krunk, et al. 2004; Wingfield et al. 1998). The effects of glucocorticoids on other types of predator defenses, such as morphological or chemical defenses, are not well understood. Some studies with amphibians suggest the primary glucocorticoid, corticosterone (CORT) (Idler 1972), plays a role in predator-induced morphological defenses(Hossie et al. 2010; Maher et al. 2013). Wood Frog tadpoles (*Lithobates sylvaticus*) exposed to caged predators or given exogenous CORT generated similar tail morphology, and this morphological response was repressed when CORT biosynthesis was inhibited (Maher et al. 2013). Similarly, predator- induced changes in tail morphology and body size of Leopard Frog tadpoles (*Lithobates pipiens*) were absent when a CORT biosynthesis

inhibitor was applied to the tadpoles (Hossie et al. 2010). These studies suggest a relationship between CORT and the induction of morphological defenses. Relatively little is known about potential relationships between stress hormones and the induction of chemical defenses in amphibians, though chemical defenses can be triggered by the presence of a predator (Benard and Fordyce 2003; Hagman 2010), and there is some evidence of flexible changes in toxicity of a salamander. Toxin concentrations of Rough-skinned Newts, *Taricha granulosa*, increased when they were held in captivity (Hanifin et al. 2002). If stress caused by captivity is similar to that caused by predators, it is possible that predator exposure would similarly induce changes in chemical defenses.

I used adult Eastern Red-spotted Newts (*Notophthalmus viridescens viridescens*) to explore the role of predator-induced stress in the induction of predator defenses. These salamanders breed in ephemeral ponds and are exposed to different levels of predation within a single generation due to changes in habitat use (aquatic and terrestrial) and choice of breeding location (Auld and Relyea 2011; Gill 1978). Eastern Red-spotted Newts hatch in ephemeral ponds and, as adults, transition between water and land each breeding season (Lannoo 2005). Adults have a seasonally changing tail that becomes larger during the aquatic breeding phase, facilitating faster swim speed and making them harder to catch (Walters and Greenwald 1977; Brossman et al. 2013); therefore, predator-induced tail size could act as a morphological defense against some predators. These adults also possess a chemical defense - a distasteful and potentially lethal toxin, tetrodotoxin (TTX) (Hulbert 1969; Mebs et al. 2010). Whether these morphological and chemical defenses of the adult Eastern Red-spotted Newt can be induced by predators is unknown, but natural variation in tail size, toxicity, and predation pressure suggest this possibility (Singhas and Dent 1975; Brossman et al. 2013; Brodie Jr. 1968; Marion and Hay 2011).

I hypothesized that predation threat would elicit a stress response, and that this may lead to the production of flexible morphological and chemical defenses. I predicted that newts exposed to predator cues would have higher CORT levels, and induce morphological (i.e. larger and deeper tails) and chemical (increased TTX concentration) defenses, compared to controls. Secondly, I predicted that if CORT plays a role in inducing these defensive traits, newts injected with a CORT precursor (adrenocorticotropic hormone, ACTH) induce defenses (i.e. larger and deeper tail and higher TTX concentration) compared to newts injected with saline.

Materials/Methods

Study Animals

Eighty adult Eastern Red-spotted Newts (*Notophthalmus v. viridescens*), were collected during the aquatic breeding season from State Game Lands 176 (Centre County, Pennsylvania, USA, 40° 46’ N, 78° 0’ W) in May 2012. Newts were measured for mass and transported to Pennsylvania State University where they were individually housed in plastic enclosures (42.4 x 27.94 x 27.4 cm, L x W x H). Enclosures contained a gravel substrate, *Elodea* plants, and were filled with dechlorinated water to a depth of 10 cm. Partial water changes were conducted once per week. Each enclosure was externally wrapped in brown paper to prevent outward visibility from within the enclosure and reduce disturbance from the investigator in the room. Room lights were set on a 10 h light: 14 hour dark photoperiod and the average room temperature was maintained at 24.4 ºC (± 0.09 SEM), which is comparable to conditions experienced at the sites of origin during the summer months (Schlegel and Butch 1980). Newts were fed black worms (*Lumbriculus variegatus*) *ad libitum*. After final measurements were obtained, newts were released at their point of capture (unless they were used for validation of our hormone methods, described below).

A bullfrog (*Lithobates catesbeianus*) was caught from State Game Lands 176 (Centre County, Pennsylvania, USA, 40° 49' N, 77° 53' W) in May 2012 to generate predator cues. Bullfrogs are natural predators of adult Eastern Red-spotted Newts (Marion and Hay 2011) and these species co-occur at the collection sites. The bullfrog was housed in a plastic enclosure (42.4 x 27.94 x 27.4 cm, L x W x H) filled with 6 cm of dechlorinated water. The enclosure was wrapped externally in brown paper to minimize visual disturbance by the investigator in the room. Water changes occurred once per week. The bullfrog was fed ¼ inch crickets (*Acheta domesticus*) *ad libitum* and was released at the point of capture after two weeks.

Treatments

To assess the effect of predator cues on CORT levels and defenses of adult newts, 40 field-caught adult newts were assigned to one of two treatments 12 days after capture: (1) a "predator cue" group (10 males and 10 females) that received cues from a predator and from injured conspecifics; and (2) a control group (nine males, 11 females) that received dechlorinated water. To generate the predator cues, newts sacrificed for hormone validation (see *Water-borne CORT Collection and Validation* below) were homogenized in 350 mL of distilled water, filtered with glass wool, and immediately frozen at -20 °C in 15 mL aliquots ("conspecific cue"; Rohr et al. 2002a,b). Bullfrog water cues were generated by taking water from the frog's enclosure and immediately freezing it in 6 mL aliquots ("bullfrog cue"). Predator cues given to the treatment group consisted of one aliquot of conspecific cue (15 mL) and one of bullfrog cue (6 mL) for a total addition of 21 mL of predator cue water (Ferrari et al. 2010). The combination of these cues increases the signal of predator presence (Ferrari et al. 2010). Control cues consisted of distilled water frozen in 21 mL aliquots. Cues were thawed immediately before they were added to enclosures, and were administered three times a week for a total of six administrations (Rohr et al. 2002a,b).

ACTH and saline injections were used to determine the direct effect of a CORT precursor on newt defenses. Twenty newts not included in the other treatments or in the hormone validation were divided into two treatment groups: an "ACTH injection" group (seven males and three females) that received 0.1 mL injections of 10 U/mL ACTH (1.3 mg, 80IU/mg, porcine pituitary, Sigma-Aldrich, Inc., St. Louis, MO, USA) (DeRuyter et al. 1986; Rottmann et al. 1991; Tassava 1969) and a "saline injection" (control) group (six males and four females) that received 0.1 mL injections of saline. All injections were given three times a week for a total of seven administrations on the ventral side medially across from the left inferior limb. The mismatch in sample sizes and in the number of trials of water cue versus injection treatments was due to logistical constraints.

Newts were measured for mass, photographed (for tail and body dimensions), stress hormone levels, and toxicity of tail tissue before treatments began and after the final treatment. Newts in the predator cue and control cue treatments had a distal 3 mm of tail tissue removed for measurement of TTX concentration. Tail tissue was stored in a -80 °C freezer until analysis (see below). This tissue sampling method caused minimal injury and stress to the animals; samples were obtained from the thin, seasonally changing tailfin, which newts are able to regenerate (Hanifin et al. 2002). Water samples for hormone analysis were taken for newts in all treatment groups by collecting water from individual glass beakers that contained the newts immediately freezing samples at -20 °C for later analysis (see *Water-borne CORT Collection and Validation*).

Tail and Toxin Measurements

Snout-vent length (SVL), tail depth and tail surface area were measured from photographs using ImageJ (version 1.43u, National Institutes of Health, Bethesda, MD). Tail depth was measured at the greatest dimension, and tail surface area was measured as the area of the right lateral surface of the tail, beginning where the tail joins the body (immediately distal to the cloaca).

To measure toxin concentrations, TTX was extracted from the tail tissue samples using methods similar to those of Hanifin et al. (2002). Tissue was thawed at 4°C overnight and mass was obtained. Tissue samples were ground with a 5 mL glass tissue grinder (Kimble Chase), which was cleaned between samples with 90% ethanol and allowed to air dry. Samples were transferred to 50 mm borosilicate vials and 800 µL of 0.1M acetic acid was added. These vials were vortexed and heated in a boiling water bath for five minutes, followed by an ice bath for two minutes. 1mL extracts were transferred from these vials to microcentrifuge tubes and centrifuged at 13793 rcf for 20 minutes. 0.5 mL of the resulting supernatant was transferred to 0.5 mL Millipore centrifuge filter tubes (Ultrafree-MC, 10000 NMWL filter units) and spun at 13793 rcf for 20 minutes. 20 µL aliquots of TTX extracts were frozen at -80°C until analysis.

TTX was quantified following the methods of Chen et al. (2011). A TTX standard was made by diluting 1 mg TTX (>98% pure, Abcam, Cambridge, MA, USA) with 2 mM citric acid (Chen et al. 2011). Mass spectra were optimized to obtain tandem mass spectrometry (MS/MS) spectra for the standards. These were used to generate optimal multiple reaction monitoring (MRM) conditions used for liquid chromatography tandem mass spectrometry (LC/MS/MS) methods. TTX from our tissue samples were separated with LC using an Atlantis® HILIC Silica column (100 x 2.1 mm, i.d., 3 µm). Samples were randomized and quantified using a Waters Xveno™ TQ-S. To test for false positives, the sample with the highest TTX concentration was analyzed on a Quadrupole Time-of-Flight tandem mass spectrometer (5600 QTOF). The product ion mass spectrum of this sample was identical to a standard, indicating no false positives. TTX was deactivated by exposing contact materials to a 1.0% hypochlorite solution for 30 minutes before disposal (CDC).

Water-borne CORT Collection and Validation

Twelve days after capture, newts in all treatments (38 males and 52 females; including the newts used for hormone validation) were removed from their enclosures, measured for mass,

and placed in individual 350 mL glass beakers containing 200 mL of distilled water for 40 minutes. This depth of water was adequate to fully submerge the newts. The glass beakers were visually isolated from one another by cardboard dividers and mesh netting was placed on top of the beakers for shading to minimize stress. After 40 minutes, each newt was removed from its beaker with a net, and water from the beakers was poured into 250 mL wide mouth high-density polyethylene bottles. Water samples were frozen at -20˚C for later analysis. Beakers and nets used in the water collection were rinsed with 90% ethanol and air-dried between newts.

Newts used for hormone validation were decapitated immediately after being removed from the beakers and blood samples were collected using microhematocrit tubes (ARMI SOP No. 101, USGS). Blood samples were centrifuged (2040 rcf for 2.5 min) and plasma was pipetted into individual microcentrifuge tubes and frozen at -20 ˚C until analysis.

CORT concentrations were determined from water samples by modifying methods of Earley et al. (2006) and Ellis et al. (2004) for use in this species. Water samples were thawed at 4 °C and filtered (Whatman filter paper, Grade 1, 24 cm). CORT was extracted through pre-primed (2 x 2 mL HPLC-grade MeOH followed by 2 x 2 mL distilled water) solid phase C18 columns (HyperSep, 3 cc/500 mg; Waters, Inc., Milford, MA, USA). Tygon® tubing (Saint Gobain, formulation 2275) was fitted snugly to the C18 column and the other end was placed into the water sample; samples were drawn through the columns, which were fitted to a manifold, by engaging the vacuum. Free (unconjugated) hormones, used for assays, were eluted into 13 x 100 mm borosilicate vials with 2 x 2 mL ethyl acetate (Ellis et al. 2004) and stored at -20 °C. Samples were placed in a 37 °C water bath and dried under a gentle stream of nitrogen gas using an Evap-O-Rac (Cole Parmer). Residues were resuspended in 15 µL ethanol and vortexed for 1 minute. 285 µL of enzyme-immunoassay (EIA) buffer (from the EIA hormone assay kit) was added to the samples and vortexed for 25 minutes, giving a final resuspension volume of 300 µL. 50 µL was

taken from each of the 94 samples, which generated a 4700 µl (4.7 ml) pool that was used to validate the EIA kit.

CORT levels in plasma samples were only taken from newts used for hormone validation. These samples were measured and compared to their water samples for hormone validation, in order to authenticate this method for *N. viridescens*. 4 µL of plasma (or the maximum value, as one sample had only 2 µL and two had only 3.5 µL) was pipetted into individual 16 x 125 mm borosilicate vials and kept on ice. 18 mL of distilled water was added to each vial. Samples were drawn through HyperSep C18 columns in the same way as described above and eluted with 2 x 2 mL ethyl acetate into 13 x 100 mm borosilicate vials, placed in a 37°C water bath, and evaporated with nitrogen via an Evap-O-Rac (Cole Parmer). Samples were resuspended in 25 µL ethanol and vortexed for 1 minute. 475 µL of EIA buffer (from EIA hormone assay kit) was added to an overall resuspension volume of 500 µL, and100 µL from each of the plasma samples was taken to generate a 2100 µL (2.1 mL) plasma pool which would be used to validate the EIA kit and compare with water-borne CORT results.

Enzyme-immunoassay kits (Cayman Chemicals, Inc., Ann Arbor, MI, USA) were used to quantify CORT levels in the prepared plasma and water samples following the kit instructions. Samples were run in duplicate over five 96-well plates. The pooled water-borne hormone control was pipetted in duplicate at the start and end of each plate to calculate intra- and inter-assay coefficients of variation. The intra-assay coefficients of variation were, for plates 1-5, 4.04%, 4.75%, 20.2%, 0.54%, and 3.82%. The inter-assay coefficient of variation was 13.41%. Both the water-borne and plasma pools were y serially diluted from 1:1 to 1:128 using an initial 300 µl. Both serial dilutions were parallel with the standard curve (slope comparisons; water: $t_{12} = 0.42$, $P = 0.68$; plasma: $t_{11} = 0.30$, $P = 0.77$; Zar 1996, p. 355). Extraction efficiency was evaluated with a cold spike. Briefly, 800 µl of the newt water-borne pooled sample (known CORT concentration) was separated into 8, 100 µl aliquots in 16 x 125 mm borosilicate vials followed

by the addition of 100 µl of kit standard (one standard per vial) and 16 ml of distilled water. The sample was then passed through the C18 columns, processed as described above and resuspended in 200 µl of 5% EtOH: 95% EIA buffer prior to assays. The same procedure was used for the newt plasma pool. The slopes of the observed vs. expected regressions were 0.97 (water-borne) and 0.80 (plasma), indicating linear relationships. Minimum recoveries were 85% (water-borne, median = 98.4%) and 74% (plasma; median = 92.6%). Plate development was conducted according to the manufacturer's instructions and previous experience with this CORT kit (RL Earley, unpublished data). The development time with the highest r^2 value (60 min, $r^2 = 0.995 \pm 0.108$) for standard curves and maximum binding (B_o) subtracted values (within range 20-80%) was chosen.

Statistical Analysis

Tail measurements, toxin concentrations, and hormone values were natural log transformed to meet assumptions of parametric tests. Relative tail depth and area measurements were calculated from residuals of a regression of ln (tail measure) against ln (SVL), creating a measurement independent of body size. The effect of time (before vs. after the trials) was tested for relative tail morphology, toxin, and hormone measurements using repeated measures analysis of variance (ANOVA). To test for effects of treatment on relative tail morphology, toxin, and hormone measurements, a one-way ANOVA was performed on the difference between the "before" and "after" treatment measurements. The effect of CORT on tail morphology and TTX concentrations was analyzed using linear regression. All analyses were performed in SPSS version 20.0 (SPSS, Inc. Chicago, IL, USA) with alpha set at 0.05.

Results

Tail and Toxin Measurements

Tetrodotoxin was found in all newts, with concentrations ranging from 25.907 to 2862.069 ng per gram tissue sample. There was no effect of water-borne cue treatment on tail morphology (relative tail depth: $F_{3, 37}$= 1.019, p= 0.397; relative tail area: $F_{3, 37}$= 0.566, p= 0.641), toxicity ($F_{1, 25}$= 0.16, p= 0.689), or hormone levels ($F_{3, 39}$= 0.602, p= 0.618). CORT and TTX increased across the trial (between "before" and "after" treatments; CORT: $F_{1, 35}$ = 61.710, p< 0.001, Fig. 3-1; TTX: $F_{1, 25}$ = 9.166, p = 0.006, Fig. 3-2), whereas relative tail size did not change with time (relative tail depth: $F_{1, 36}$ = 0.006, p = 0.937; relative tail area: $F_{1, 34}$ = 0.001, p= 0.970).

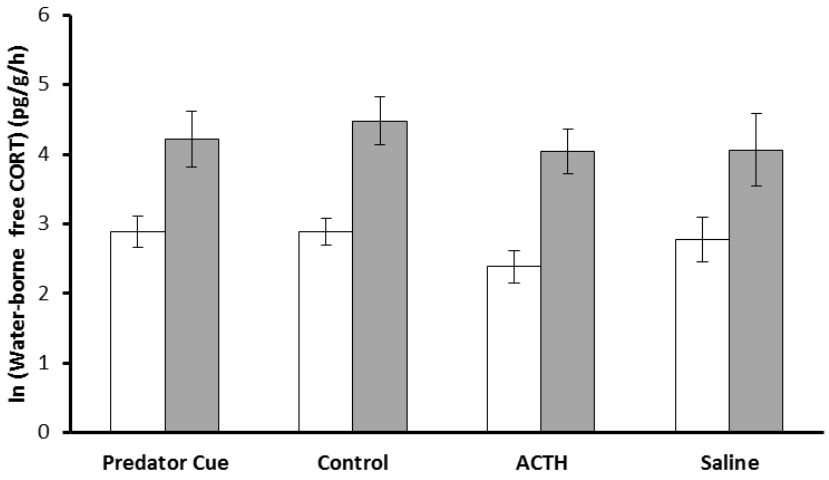

Figure 3-1. CORT significantly increased over the course of the study, between measurements taken before treatments (open bars) and measurements taken after treatments (shaded bars). (Error bars represent ±1 SEM; p= < 0.001).

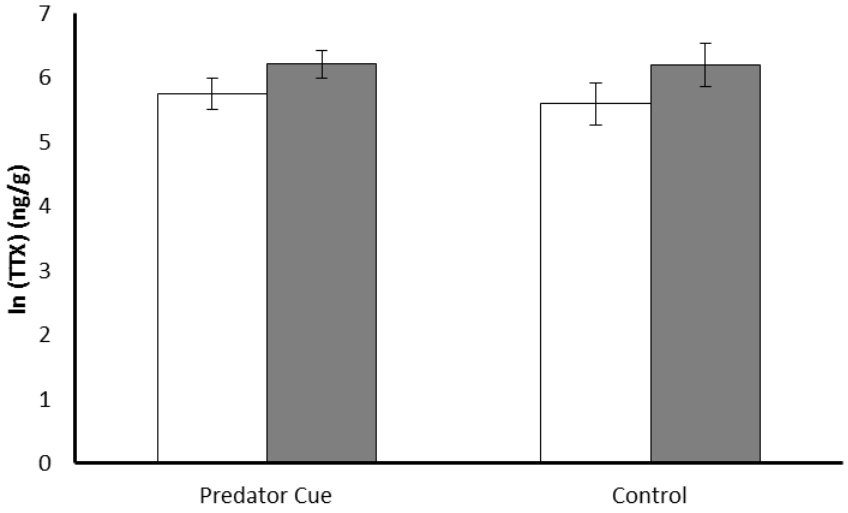

Figure 3-2. TTX significantly increased over the course of the study, between measurements taken before treatments (open bars) and measurements taken after treatments (shaded bars). (Error bars represent ±1 SEM; p= 0.006).

No relationship was found between the change in water-borne CORT and the change in tail morphology (relative tail depth: R^2= 0.105, $F_{1, 35}$= 3.98, p= 0.054; relative tail area: R^2= 0.013, $F_{1, 35}$= 0.43, p= 0.515; Fig. 3- 3). There was, however, a positive relationship between the change in water-borne CORT and the change in TTX concentration (R^2= 0.214, $F_{1, 24}$= 6.260, p= 0.020; Fig. 3-4).

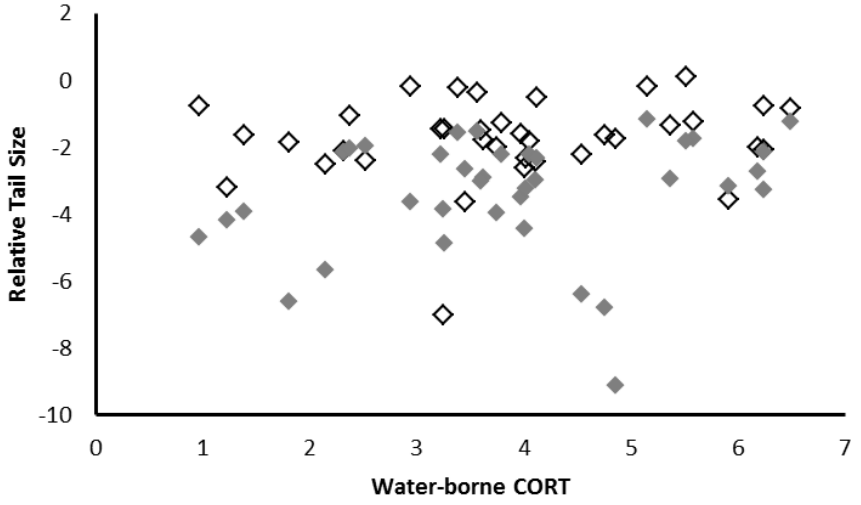

Figure 3-3. There is no significant relationship between relative tail depth (shaded diamonds) or tail area (open diamonds) and free water-borne CORT (All p= >0.05). X-axis represents ln (change in free water-borne CORT) in pg/g/h, while the y-axis represents the ln (change in tail measurement residuals).

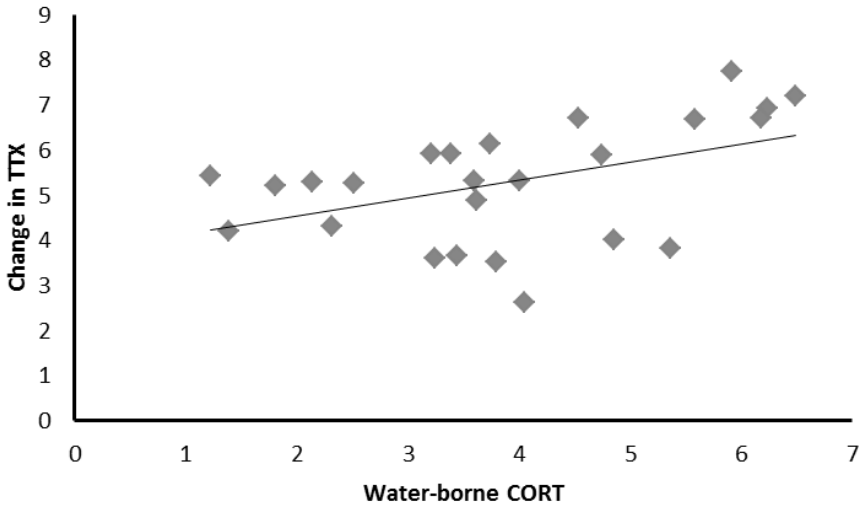

Figure 3-4. Tetrodotoxin is positively related to free water-borne CORT. Line is a best-fit trendline (R^2= 0.214, p= 0.020). X-axis represents ln (change in free water-borne CORT) in pg/g/h, while the y-axis represents the ln (change in TTX) in ng/g tissue sample.

Water-borne CORT Collection and Validation

Water-borne CORT and plasma CORT were significantly positively related (R^2= 0.32, $F_{1, 17}$= 7.54, p= 0.014; see Fig. 3-5).

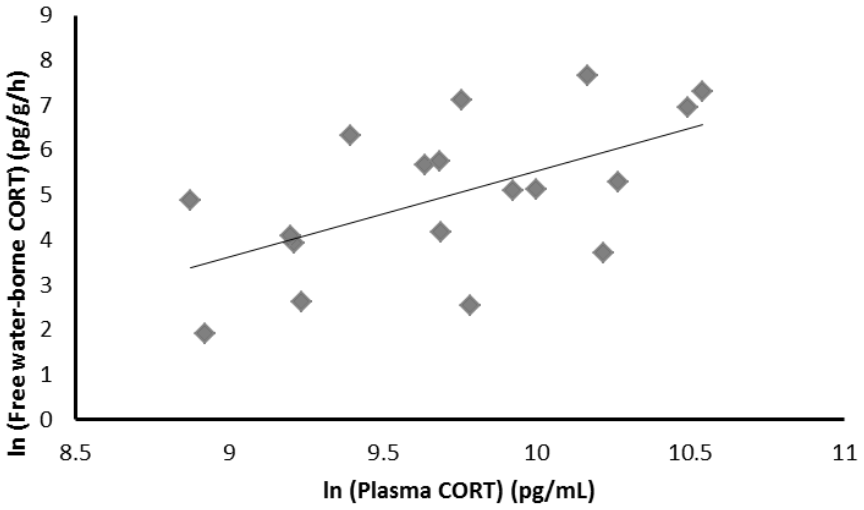

Figure 3-5. Free plasma CORT is positively related to free water-borne CORT. Line is a best-fit trendline (R^2= 0.32, p= 0.014).

Discussion

Our results suggest that administering predator cues or ACTH injections did not induce morphological (tail size) or chemical defenses (TTX concentrations) in adult Eastern Red-spotted Newts. Predator cue and ACTH treatments also did not result in significantly elevated CORT levels compared to the controls (distilled water and saline injection). Together, this suggests that there is either no effect of predator cue addition or ACTH injection on induction of defense, or that some other factor, such as the laboratory environment, predator choice, or seasonal CORT changes, masked the effects of the applied treatments. Future studies should consider the effects of a laboratory setting and attempt to use natural housing, such as mesocosms, to eliminate

possible stressors from the housing environment (Boone and James 2005). These changes in setting would better mimic natural environmental conditions, such as refuge availability or social interactions, and provide insight into natural CORT and TTX responses to predators. Understanding natural variations in CORT and TTX will allow us to determine if the lack of treatment effect is accurate or the result of seasonal changes, like breeding condition. Using alternative predator cues or caged predators, or extending the length of the experiment to capture an entire breeding season, might provide a stronger test of the effect of predator cues on stress and defenses. Alternatively, many species suppress predator responses while in breeding condition (as during these trials), to avoid associated costs to reproduction (sea turtles, red-sided garter snakes, Wingfield and Saposky 2003; southern water skinks, Schwarzkopf and Shine 1992; Magnhagen 1991), and it is possible that this suppression is driving the absence of a CORT response to predator cues and associated morphological and chemical changes in our study.

I revealed a putative connection between CORT and toxicity. Newts exhibited increases in both CORT and tetrodotoxin concentrations over the duration of this study (Fig. 3-1, 2), showing that their chemical defense has the potential to be flexible, and that the magnitude of change in TTX is linked in some way with changes in stress hormones. This study is one of a few to indicate changing tetrodotoxin concentrations within an individual (Rough-skinned newt, *Taricha granulosa*; Hanifin et al. 2002; Cardall et al. 2004), and is the first for this species. Our results support the hypothesis that newts may produce their own TTX (Cardall et al. 2004), rather than this toxin being the product of a symbiotic relationship (Chau et al. 2011). The increasing levels of TTX across time demonstrated by our study could be due to an effect of captivity (possibly stress; Hanifin et al. 2002), natural fluctuations in toxicity, or an internalized cue to begin transitioning to a non-breeding, terrestrial phase (which occurred during our trials). Seasonal changes in TTX have been observed in pufferfish, *Takifugu poecilonotus* (Ikeda et al. 2010), but cyclic variation in newts has not been studied. Understanding the drivers of this

relationship could lead to information about the synthesis or acquisition of TTX in Eastern Red-spotted Newts. The relationship between CORT and TTX indicates that stressors responsible for elevating CORT, including the presence of predators (although not demonstrated by the predator cue used in this study), may result in higher TTX concentrations. Future studies using predators that are known to elicit CORT-responses should investigate their effect on TTX.

Tail size (relative depth and area) remained unchanged over the duration of our trials and was not related to changes in CORT (Fig. 3-5). This is in contrast to studies on tadpoles of other species in which CORT was linked to an increase in tail size (Hossie et al. 2010; Maher et al. 2013). Tail depth and area may require longer periods of stress exposure to induce flexibility of defenses or may not be governed by predator presence in this species, but rather by seasonal changes (Walters and Greenwald 1977; Brossman et al. 2013). Prolactin, a peptide hormone, aids in the maintenance of the aquatic tail fin in male Eastern Red-spotted Newts (Singhas and Dent 1975), and drives the seasonal patterns in tail size of other newts (Italian Crested newt, *Triturus carnifex*; Mosconi et al. 1994). Thus, it may be the naturally decreasing prolactin levels towards the end of the breeding season (around the time of our experiment) and not the increase in CORT that regulated tail size in our study.

I were able to analyze the relationship between changes in chemical and morphological defenses and differences in CORT by obtaining two measurements of stress hormone in the same individuals across this study—before treatments began and after the final treatment. To facilitate this, I validated the water-borne hormone collection technique for this species and showed significant concordance between plasma and water-borne CORT concentrations (Fig. 3-5). This technique is non-lethal, relatively non-invasive, and can be adapted for future research to measure CORT in long-term studies on smaller species, species of concern, or in studies that require repeated sampling (Ellis et al. 2004; Scott et al. 2008; Gabor et al. 2013[ab]).

Further exploration of the intriguing connections between stress responses and prey defenses could shed light on the pathways of induced defense production (see Hossie et al. 2010; Maher et al. 2013) and potentially reveal origins of understudied chemical defenses. Discerning these relationships may be important for management and conservation because environmental stressors, such as climate change, habitat fragmentation, invasive predators, and disease contribute to amphibian decline (Adams et al. 2013). Understanding how environmental stressors affect anti-predator defenses provides insight into how populations, communities, and ecosystems may change when stressors are present.

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