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# Antipredator Adaptations of Spotted Salamander Larvae across a Geographic Gauntlet of Predation Risk

Jessica M. Rack

*University of Connecticut*, [jessie.rack@gmail.com](mailto:jessie.rack@gmail.com)

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# Antipredator Adaptations of Spotted Salamander Larvae across a Geographic Gauntlet of Predation Risk

Jessica Marie Rack, PhD

University of Connecticut, 2016

Community interactions often differ quite strikingly across natural landscapes. Environmental differences do not explain all of this spatial variation in community patterns — evolutionary dynamics might often play an important role. Interacting populations can evolve different responses to each other based on divergent natural selection regimes or past evolutionary histories, which in turn affect their ecological interactions. Aquatic spotted salamander larvae (*Ambystoma maculatum*) are preyed upon by many species, but, like many amphibians in aquatic environments, have evolved the ability to use chemical cues to detect predator presence. In this dissertation, I investigate the limits and specificity of this species' ability to differentiate between predator chemical cues, and examine the broader picture of selective forces that may drive trait responses and ultimately affect survival in spotted salamanders. Each inquiry has a geographic component, dealing with salamander populations from three sites across New England. From south to north, these sites vary in predator composition, and in each study, I found idiosyncratic responses of salamander larvae at each location. With a better understanding of the ways in which antagonistic species interact, we can predict the outcomes of novel interactions more accurately, and will have insight into how predator-prey interactions could be altered by a changing world.

Antipredator Adaptations of Spotted Salamander Larvae across a Geographic Gauntlet of  
Predation Risk

Jessica Marie Rack

B.A., West Virginia University, **2005**

B.S., Slippery Rock University, **2010**

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at the

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APPROVAL PAGE

Doctor of Philosophy Dissertation

Antipredator Adaptations of Spotted Salamander Larvae across a Geographic Gauntlet of  
Predation Risk

Presented by

Jessica Marie Rack, B.A., B.S.

Major Advisor

---

Mark C. Urban

Associate Advisor

---

Carl D. Schlichting

Associate Advisor

---

Kentwood D. Wells

Associate Advisor

---

Eric T. Schultz

Associate Advisor

---

Rick A. Relyea

University of Connecticut  
2016

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## Introduction

Across a species' range, geographic variation in biotic and abiotic factors can lead to genetic and phenotypic differentiation between populations. This spatial variation means that the outcomes of interspecific interactions may differ among communities (Thompson, 1999a; Johnson & Stinchcombe, 2007). In predator-prey interactions, for instance, some populations of a prey species may be extremely effective at avoiding predation, and will therefore persist at high abundances, while other populations under the same risk will lack the traits that provide an advantage against predation, and may be extirpated (Abrams, 2000). Differential interspecific interactions across a landscape can result in a selection mosaic, in which there is variation between locations in how natural selection operates on interacting populations of predators and prey (Thompson, 1999a, 1999b; Brodie et al., 2002; Thompson & Cunningham, 2002). Additionally, the genetic landscape of the interacting species is constantly changing through gene flow and drift, adding another dimension to differentiation between populations (Thompson, 1999a, 1999b). Ecologists must try to understand the effects of the ecological and evolutionary forces that work to shape species interactions across geographic space. Studies like these may allow us to predict the outcome of novel species interactions, an essential consideration in the face of global climate change, species introductions, and other anthropogenic disturbances (Parmesan & Yohe, 2003; Parmesan, 2006; Gilman, Urban, Tewksbury, Gilchrist, & Holt, 2010).

In this dissertation, I examine geographic variation in the responses of spotted salamander larvae (*Ambystoma maculatum*) to three predators. Many of these interactions are chemically mediated, since animals in aquatic environments often rely on chemical cues to provide information about food, conspecific location, and predation risk (Kats & Dill, 1998;

Tollrian & Harvell, 1999; Brönmark & Hansson, 2000). Here, I examine the ways in which this species can adapt to different local predator regimes, and how investing in adaptations for predators might mean a tradeoff in competitive ability.

The spotted salamander, *Ambystoma maculatum*, is a terrestrial salamander that occurs throughout much of eastern North America. Each spring, adults emerge from underground and migrate to temporary ponds to breed. Aquatic larvae hatch after about eight to ten weeks, undergoing metamorphosis and leaving the ponds by late summer when the ponds begin to dry or temperatures cool (Petranka, 1998; Urban, 2007a). Larvae of the spotted salamander are subject to predation risk from a variety of predators across its range, including marbled salamander larvae, adult Eastern newts, and diving beetle larvae (Urban, 2007a, 2008a). Each of these predators differ in predation behavior, risk, and densities across the region (Urban, 2008a). Previous studies have shown that spotted salamander larvae respond uniquely to chemical cues from different predator species (Urban, 2007b, 2008a, 2008b; Yurewicz, 2004), and some of this variation provides an escape advantage relative to the identity of the predator (Urban, 2010). Additionally, these larvae adapt on a microgeographic scale (Richardson, Urban, Bolnick, & Skelly, 2014), altering the degree of plasticity in response to varying predator densities, even over short geographic distances (Urban, 2007b).

**In Chapter 1, I ask whether spotted salamander larvae can differentiate between the predator population co-occurring in the same pond and predator populations from geographically distant ponds.** When conditions differ among habitats, populations can become locally adapted if they evolve traits that convey higher fitness to the population in its local environment compared with other populations (Hereford, 2009; Kawecki & Ebert, 2004; Richardson et al., 2014). Also, genetic drift could lead to differentiation among populations. I

predicted that if predators diverge in their chemical cues and spotted salamander larvae can differentiate between predator chemical cues based on geographic origin of the predator, then larvae should respond to local predator chemical cues with divergent behaviors.

**In Chapter 2, I test whether spotted salamander larvae can recognize predator chemical cues based on the geographic origin of the predator's diet.** In particular, I test if behavioral responses to chemical cues from predators consuming local conspecifics differ from responses to cues from predators consuming conspecifics from foreign populations. If spotted salamander larvae can recognize geographic origin of predator diet, I predicted that they would respond differently to predators that have been eating local conspecifics. This study was designed to obtain more information about the nature of the chemical cue and how finely prey can discriminate and use information on intraspecific diet cues.

**Finally, in Chapter 3, I look more broadly at the ways in which selective factors across the landscape interact to affect prey traits, and how this affects survival in the presence of a predator.** I measured behavioral, morphological, and performance traits from 24 populations of spotted salamanders from three geographically distinct sites to examine how these traits change in response to different selection pressures. I predicted different suites of traits to dominate under each of three selection regimes. If home ponds contain high densities of gape-limited marbled salamander larvae, I predict that prey larvae should develop bigger bodies, larger tailfins, wider heads, and should swim quickly. If home ponds contain high densities of gape-unconstrained *Dytiscus* larvae, I predict that spotted salamander larvae should develop large tailfins, narrow heads, and should swim quickly. If, instead, the selection regime in the home pond is dominated by conspecific density, I predicted that larval spotted salamander larvae should forage more, grow more slowly, and swim more slowly. A tradeoff between competitive

ability and antipredator adaptations may result in antagonistic selection, providing the mechanism for local adaptation across a shifting landscape of predator-prey interactions.

This dissertation explores prey adaptations to variable predation risk across a geographic landscape. A better understanding of the scale at which local versus foreign cues can be discriminated as well as how adaptation to local selection regimes can affect suites of traits will provide insight into how forces like selection and coevolution can affect predator-prey interactions across the landscape. Selection for particular antipredator traits may depend on type and density of both predators and competitors in the community, as well as on abiotic factors such as canopy cover or temperature of the pond. By gaining a better understanding of the breadth and drivers of trait changes, we can learn to identify patterns of predator-prey evolution and coevolution, and to predict how those interactions could be affected by changing environmental conditions.

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## **Chapter 1:**

### **Antipredator responses of larval spotted salamanders to Eastern newt chemical cues vary over the landscape**

#### **Abstract**

Multiple selective pressures across a landscape may lead to spatial variation in predator-prey interactions. In some locations, prey animals may become locally adapted to predator presence, able to quickly recognize predators and respond with appropriate antipredator behavior. Here, we tested for the ability of spotted salamander larvae (*Ambystoma maculatum*) to differentiate between predator chemical cues on the basis of predator pond of origin. We quantified behavioral responses of three populations of larval spotted salamanders to newt predator cues from newts collected from geographically distinct ponds. We measured latency to movement, time active, and spatial avoidance. We found that behavioral responses to newt predators varied among populations. Two populations discriminated between predators from near and distant ponds, altering their latency to movement in response to newt origin. The ability of prey to differentiate among newt cues from different populations suggests that predator populations diverge in their chemical cues either through selection or drift. Fine-scaled prey cue discrimination might evolve if prey trade off sensitivity to multiple cue sources.

#### **Introduction**

Community interactions often differ quite strikingly across natural landscapes. For instance, in predator-prey interactions, some prey populations might persist at high abundances despite predator presence, whereas other populations might become extirpated under the same risk (Abrams, 2000). Environmental differences do not explain all of this spatial variation in community patterns — evolutionary dynamics might often play an important role (M. T. J.

Johnson & Stinchcombe, 2007; Thompson, 1999a). Interacting populations can evolve different responses to each other based on divergent natural selection regimes or past evolutionary histories, which in turn, affect their ecological interactions. A suite of recent theories propose that evolution may strongly affect community distributions and dynamics across spatial landscapes (Fussmann, Loreau, & Abrams, 2007; M. T. J. Johnson & Stinchcombe, 2007; Urban et al., 2008). If so, then ecologists need to understand the degree to which the mechanisms that determine the outcome of species interactions vary owing to local adaptive evolution.

Predator-prey interactions can vary across space due to evolution of the predator, the prey, or both. If selection varies across the landscape, then a local population can evolve traits that provide an advantage under local conditions. Such a trait is considered locally adapted if it conveys higher fitness to a local population as compared to genotypes from other populations (Hereford, 2009; Kawecki & Ebert, 2004).

One trait that can vary across space is the ability of prey to recognize and respond to chemical cues specific to predator species. Chemical cues can emerge passively from a predator's skin (kairomones; Brown, Eisner, and Whittaker 1970; Ferrari, Wisenden, and Chivers 2010; Hettyey et al. 2015), or can be released by the physical maceration or digestion of prey (Ferrari et al., 2010; Hettyey et al., 2015; Petranka, Kats, & Sih, 1987). Alternatively, chemical cues can be released by conspecifics, either as a stress response or an alarm pheromone (Fraker et al., 2009; Hettyey et al., 2015; Wisenden, Chivers, & Smith, 1995). Prey responses to predator chemical cues can include modifications in behavior, morphology, or life history traits (Brown et al., 1970; Ferrari et al., 2010; Hettyey et al., 2015; Tollrian & Harvell, 1999). Of these traits, behavioral modifications are the most labile, and may respond quickly to changes in predation risk (West-Eberhard, 2003).



Studies of prey responses to predator chemical cues generally show that prey can recognize and respond to different predator species (Chivers & Mirza, 2001; Chivers, Wildy, & Blaustein, 1997; Murray & Jenkins, 1999; Relyea, 2001; Wilson & Lefcort, 1993) and that different prey species respond differently to the same predator (Lawler, 1989; Relyea, 2001; Werner, 1991). Other studies have shown that different prey populations diverge genetically in their response to chemical cues from predator species, often as a function of predation risk in their natal environment (Carlson & Langkilde, 2014; Relyea, 2002b; Storer & Sih, 1998; Urban, 2008a, 2010). No study has yet assessed if prey populations respond differently to distinct predator populations or if prey can evolve to recognize local predator cues. Such evidence would improve our understanding of the scale at which predator cue recognition evolves.

One way in which prey have been shown to respond to an elevated risk of predation is with alterations in behavior. Behavioral responses to predators have been documented in larval anurans (Werner 1991; Skelly 1994; Relyea 2001), as well as in larval caudates (Semlitsch, 1987; Sih, Kats, & Maurer, 2003; Urban, 2008a). Amphibians often employ two key behavioral strategies when presented with a predator cue. The first strategy is to reduce activity (Skelly and Werner 1990) and is often accompanied by increased refuge use (Van Buskirk and Schmidt 2000). The second strategy is spatial avoidance (Relyea, 2001; Semlitsch, 1987). This method consists of remaining as far from the predator's location as possible. As with other adaptive responses, these plastic responses are not without fitness tradeoffs. Higher activity can reward the individual with more or higher quality resources and enhanced competitive ability, but at the cost of higher predation risk (Skelly 1994). Spatial avoidance could reduce growth if food resources near the predator become unusable. Models of the trade-off between predation risk and acquisition of resources suggest that behavioral decisions made under predation stress are costly

to fitness (Gilliam & Fraser, 1987; Lima & Dill, 1990), and research supports these predictions (Skelly 1992; Relyea 2001).

In this study, we measured the behavioral responses of larval spotted salamanders (*Ambystoma maculatum*) to chemical cues from predatory Eastern newts (*Notophthalmus viridescens*) collected from ponds stratified geographically. Larvae of the spotted salamander are subject to varying predation risks from adult Eastern newts in the study region (Herstoff & Urban, 2014). Spotted salamander larvae respond uniquely to cues from different predators (Urban, 2007b, 2008a, 2008b; Yurewicz, 2004), and some of this variation provides an escape advantage relative to the identity of the predator (Urban, 2010). Additionally, these larvae adapt on a microgeographic scale (Richardson et al., 2014), altering the degree of plasticity in response to varying predator densities even over short geographic distances (Urban, 2007b).

We predicted that spotted salamander larvae would respond more strongly to predatory Eastern newts from local rather than distant ponds, suggesting local adaptation of predator cue detection. Alternatively, predator cues might not differ across the landscape or local populations might not evolve to differentiate these cues. We anticipated that larval spotted salamanders would respond with strong spatial avoidance and reductions in activity to local predator cues. Spatially avoiding a predator is likely to prevent predation, and reduction of movement can prevent attracting the attention of visual predators. Because amphibian larvae raised in the presence of predator chemical cues are known to develop a suite of morphological and behavioral traits that aid survival (Benard, 2006; Laurila, Pakkasmaa, & Merilä, 2006; Urban, 2008a), we predicted that spotted salamander larvae reared since hatching in the presence of newt chemical cue would respond more strongly to predator cues than naïve larvae.

## Methods

### *Natural history and study sites*

The spotted salamander, *Ambystoma maculatum*, is a terrestrial salamander that occurs throughout much of eastern North America. Each spring, adults emerge from underground and migrate to temporary ponds to breed. Aquatic larvae hatch after about eight to ten weeks, undergoing metamorphosis and leaving the ponds by late summer when the ponds begin to dry or temperatures cool. For this study, spotted salamander egg masses were collected in early spring from three populations along a latitudinal cline in southern New England, USA: southern – Northford, CT; intermediate – Union, CT (Yale-Myers Forest); and northern – Winchester, NH (Pisgah State Park; Fig. 1). Two focal ponds, the southern and northern locations, were used in 2012, and we added the intermediate location in 2013 based on pilot data.

The Eastern newt, *Notophthalmus viridescens*, is one of the most important amphibian predators on spotted salamander larvae throughout the study region (Urban, 2007a). Eastern newts have a life cycle in which an aquatic larval stage is followed by a terrestrial juvenile stage (eft) and an aquatic adult stage. Aquatic adults are gape-limited generalist predators, capable of feeding on a variety of smaller items, from worms and insects to amphibian eggs and larvae (Behler & King, 1979; Urban, 2008a).

Studies of Eastern newts have shown that gene flow is quite low among populations, and that populations cluster into geographic units (Gabor & Nice, 2004; Gill, 1978a, 1978b; Grayson, Bailey, & Wilbur, 2011). The density of Eastern newts varies across the area studied, with the highest densities found in northern region, where this species acts as a dominant predator in many temporary ponds (Herstoff & Urban, 2014). Newts also occur at high densities at the intermediate site, and at lower densities at the southern site (Urban, 2007a). At the intermediate

and southern locations, Eastern newts co-occur with another key amphibian predator on spotted salamanders, larvae of the marbled salamander, *Ambystoma opacum*. To estimate densities of the two major predaceous salamander species, we performed annual area-standardized dip net surveys at our three sites over a period from 2002-2014.

### *Animal Collection and Housing*

Ten to fifteen spotted salamander egg masses, depending on natural availability, were collected from each focal pond in each year. Egg masses were split into groups of ten eggs each and hatched in 19-L buckets in an outdoor enclosure at the Spring Hill Research Compound in Storrs, CT. We randomized bucket location with respect to focal pond origin and clutch. After hatching, we thinned larvae to five individuals per bucket, a density within the range of natural larval densities (Urban, 2008a). Larvae were fed live zooplankton *ad libitum* collected from a local pond.

Adult Eastern newts were collected from all three focal ponds in both 2012 and 2013. To assess the spatial scale at which predator recognition occurs, we also conducted a more in-depth study of one of the three focal ponds (southern location) and collected newts from two additional ponds closer to the southern pond (2 and 10 km; intermediate pond is 80km, and northern pond is 160km away). Adult newts were kept in 168-liter containers located at Spring Hill Research Compound. Each container held 2-3 adult newts and contained 20g of leaves. Adult newts were fed five larval spotted salamanders per newt per week.

### *Larval Treatment*

Beginning at hatching, spotted salamander buckets were treated three times weekly with one liter of either blank or newt cues. Blank-treated buckets received water from a container containing no predators (only leaves), but otherwise treated in the same way. Newt-treated

buckets received water from containers of newt predators fed spotted salamander larvae from the same pond as the experimental larvae. In each case, water was first filtered through a 150 $\mu$ m mesh screen to remove leaves or debris. Treatments continued until the end of the experiment.

### *Behavioral Experiment*

We measured behavior of spotted salamander prey from each location in response to the chemical cues of predatory newts from each location. Behavioral assessments were performed indoors. Each experimental arena consisted of a Plexiglas box with dimensions 30.5cm x 50cm x 8.5cm (Fig. 2). Each box was divided into quadrants by wax pencil lines on the bottom. An acclimation chamber/release device was glued to the center of each experimental arena, and was composed of an inverted 120-mL cup that could be twisted to allow the experimental larvae to exit toward either end of the arena. A stimulus delivery system was constructed from a Terumo 60mL syringe (Terumo Medical Corporation, Somerset, NJ) and 0.125" internal diameter PVC tubing attached to a Pasteur Pipette. Each experimental arena was fitted with two pipettes, secured to the walls on either side of the experimental arena.

To prepare predator cue, a newt individual was isolated for 24 hours in a 708-mL container with 3-5 spotted salamander larvae from the same focal pond as the larvae to be tested. After 24 hours, the predator and any uneaten spotted salamander larvae were removed. Cue water was used within 48 hours, a period over which predator cues have been shown to remain effective (Peacor, 2006).

Each behavioral trial lasted five minutes and was videotaped from above. A single larval spotted salamander was placed in the chamber and allowed to acclimate for ten minutes. During this time, two stimuli were prepared, one containing 50mL of newt cue water, and one with 50mL of control water (no newt cue). Side (L or R) was randomized for each trial to control for

any potential side bias. We found no side bias in experimental larvae ( $\chi^2_1 = 0.45$ ,  $P = 0.504$ ). At the start of a trial, the acclimation chamber was slowly turned to release the experimental larvae, and the two stimuli were administered at a rate of  $\sim 1\text{mL/min}$ . This rate, obtained from preliminary dye trials, allows cues to diffuse across the experimental arena within the 5-minute time frame.

Based on past research, we expected age to affect behavior, so *Ambystoma maculatum* larvae from two age classes were tested at two weeks and four weeks post-hatch. Four weeks represents the maximum time it takes for spotted salamanders to reach a size refuge from newts (Urban, 2008a). Behavioral responses generally increase through time as larvae become more active (Urban, 2007b). Each individual was used only once. Five repetitions of each combination of larval focal pond origin versus newt pond of origin were performed for both newt-reared and blank-reared larvae.

#### *Overwintered Predator Experiment*

To control for the possible influence of the home environment on the chemical cues of recently-caught newts, we overwintered six Eastern newts from the 2013 experiment until the following year in two 1100-L cattle tanks. We compared behavioral responses of spotted salamander larvae to overwintered newt chemical cues and to recently-captured newt chemical cues following the same methods as described previously.

#### *Behavioral Metrics*

To determine whether spotted salamander larvae responded differently depending on geographic origin of the predator, we quantified 1) latency, measured as the time before first movement; 2) proportion of time active; and 3) spatial avoidance, measured as the distance of

larvae from cue entry after five minutes. Video recordings of each trial were analyzed using ImageJ (Rasband, 1997-2014).

### *Data Analysis*

All statistical analyses were performed in R, using generalized linear mixed-effects models provided by the lme4 package (Bates et al., 2011; R Core Development Team, 2013). To test for geographic differences, we analyzed the effect of distance from pond of origin, as well as the general effect of home versus away cues. The latter analyses followed standard approaches for detecting local adaptation (Blanquart, Kaltz, Nuismer, & Gandon, 2013): predator population of origin was included as a random effect nested within home, when it matched the prey population of origin, or within away, when the predator and prey origins did not match. Latency and spatial avoidance data were analyzed assuming that data followed a Poisson distribution. We also analyzed these data assuming a binomial distribution with spatial or temporal constraints and arrived at the same results. We used a binomial model to assess the proportion of time active. If a high residual deviance indicated overdispersion in binomial and Poisson tests, we modeled this overdispersion directly using an individual random effect by assigning each individual at each time period a unique identifier, as advocated by Warton and Hui (Warton & Hui, 2010). When there were more than two factors, we analyzed significance using maximum likelihood ratio tests. There were no effects of year of experiment (2012 or 2013) in any analysis, so years were combined.

## **Results**

### *Latency*

Salamander latency to newt cues varied significantly as an interaction between prey and predator populations of origin ( $\chi^2_{df=2} = 8.05$ ,  $P = 0.018$ ). This interaction occurred because larvae

from the intermediate site responded with significantly higher latency to newts from the same pond as compared to newts from any other pond, while larvae from the northern site tended to respond with the opposite pattern — higher latency to foreign newts (Fig. 3). Latency differences among all prey populations were marginally significant ( $\chi^2_{df=2} = 5.74$ ,  $P = 0.057$ ), and latency varied significantly between the southern and intermediate sites ( $P < 0.01$ ). This difference between sites occurred because intermediate larvae demonstrated lower latency — they moved sooner — than larvae from the southern population, who held still longer in response to predator cues (Fig. 3). Rearing condition was marginally significant ( $P = 0.055$ ), in that larvae reared in the presence of newt chemical cues responded with lower latencies to newt cues in the experiment. Overall larval age was not significant ( $P = 0.16$ ). Younger larvae from the intermediate population had significantly higher latency to movement ( $P < 0.05$ ), and larvae took longer to move when the predator was from their home pond versus foreign ponds (“away,”  $P < 0.05$ ).

#### *Time active*

The proportion of time larvae were active was low overall, and did not differ between focal ponds (southern vs intermediate,  $P = 0.21$ ; southern vs northern,  $P = 0.29$ ), age classes ( $P = 0.15$ ), or rearing conditions ( $P = 0.63$ ). Predator geographic location also did not affect proportion of time active ( $P = 0.40$ ). The interaction between larval age and rearing condition was non-significant ( $P = 0.08$ ).

#### *Spatial avoidance*

Spatial avoidance, the distance a larval salamander was from the source of the predator cue at the end of a trial, varied significantly due to an interaction between larval pond of origin and age ( $\chi^2_2 = 8.07$ ,  $P = 0.018$ ; Fig. 4). This interaction was due to different responses by age of



larvae from the intermediate site: older larvae stayed closer to the source of the newt cue than younger larvae, while the opposite was true of larvae from other sites. Distance from predator cue did not depend on rearing condition ( $P = 0.31$ ) or geographic origin of predator ( $P = 0.99$ ). We found no difference in response between populations from the southern and intermediate locations ( $P = 0.32$ ), nor between the southern and northern locations ( $P = 0.37$ ).

#### *Behavioral Responses and the Spatial Scale of Newt Predators*

For the southern pond, we additionally assessed the spatial scale at which predator recognition occurs. We found that latency varied significantly as a function of predator pond distance from the focal pond ( $P < 0.01$ ). More specifically, spotted salamander larvae responded with higher latency to predatory newts from the most distant pond ( $P = 0.045$ ). Larvae reared in the presence of newt cues had lower latency overall than those reared without cues ( $P = 0.05$ ). We found no significant effect of spatial scale on activity or spatial avoidance.

#### *Behavioral Responses to Overwintered Newts*

When we compared larval behavior in response to overwintered newt predators versus newly captured newts, we found no difference in prey behavioral responses ( $P > 0.2$ ). This suggests that differences in cue responses based on the predator's site of origin did not occur because of short-term cues related to the predator's natural environment.

### **Discussion**

Predator-prey interactions can differ across the landscape, owing to different selection pressures imposed by local conditions (Storfer & Sih, 1998; Urban, 2008a). Predators or prey can become locally adapted if they evolve traits that give them an advantage under local conditions. In an aquatic environment where visual cues are limited, chemical cues provide information to prey animals regarding the location and identity of a predator, allowing prey

animals to change their behavior to limit predation. If these cues change either through predator adaptation or neutral evolutionary processes, then prey populations might need to evolve to recognize local cues. Here we found one population of spotted salamander larvae that altered their latency to movement in response to predatory newts from the same pond compared with newts from foreign ponds.

We predicted that spotted salamander populations would adapt to recognize the chemical cues of predaceous newts in their home pond. What we found, however, was that each of the three focal populations of spotted salamanders behaved idiosyncratically to home versus away predator chemical cues. Since we raised prey populations in a common garden to limit environmental influences, this finding may indicate that prey populations have diverged genetically in their responses to chemical cues. Larvae were raised from eggs collected within a few days of being laid in order to minimize environmental influences. We did not raise multiple generations of spotted salamanders in the lab and therefore cannot reject trans-generational plasticity. However, multiple contributions to trans-generational plasticity have been rejected in this system (Richardson and Urban 2013). The development of different responses to newts from different regions suggests that newt chemical cues might also diverge across landscapes. These findings suggest that predator-prey interactions might become fine-tuned in landscapes as both predator chemical cues and the responses of their prey evolve or even potentially co-evolve.

#### *Activity*

We quantified larval activity in the presence of a predator cue by measuring latency to movement and proportion of time active. We predicted that larval spotted salamanders would respond to local newt predators with higher latency of movement and reduced time spent active.

We found population differences in the latency response, but no difference among populations in proportion of time active, regardless of newt origin.

Salamander larvae from the intermediate population prolonged latency more — they stayed still longer — in response to local newt chemical cues than in response to cues from newts collected from foreign ponds. This result suggests local adaptation at the intermediate pond to newt chemical cues, because previous research has shown that higher activity results in greater predation risk from predatory salamanders (Urban, 2007b). However, further experiments are needed to establish a direct link between latency and prey fitness.

Larvae from the northern population showed a suggestive, but non-significant difference in latency to movement based on newt origin. Larvae from this population tended to move more quickly in response to local newts, and had higher latency in response to foreign newts. This trend is in the opposite direction from our predictions, and could suggest xenophobia, in which larvae were more cautious in response to a novel cue. Alternatively, less caution in the presence of a local predator could be due to a tradeoff between foraging activity and predator avoidance, in which larval salamanders might forage more to grow into a size refuge and escape predation from gape-limited predators.

Finally, larvae from the southern population did not show any difference in latency in response to home versus away predator chemical cues. However, when we included the spatial scale from which predators were collected, we found distance-based differences in latency. Specifically, the southern larvae responded to newts from the most distant locations with higher latency than newts from ponds nearer to the focal pond. This finding also runs counter to our prediction that prey should remain still when they recognize local predators. A foraging tradeoff or xenophobia could serve as possible explanations here, too. More work should be done to

identify the causal factors of the observed behavior. Though our results were not as predicted, this analysis suggests the scale at which newts differentiate in chemical cues. Here, divergent responses to newt populations occurred at distances of 160 km, but not at 2, 10, or 80 km.

### *Spatial Avoidance*

Prey also sometimes spatially avoid chemical cues (Relyea, 2001; Semlitsch, 1987). We predicted that larval spotted salamanders would move farther away from the source of a local predator chemical cue as compared to a predator cue of unknown origin. We found a marginally significant ( $P = 0.06$ ) interaction between focal pond and newt origin. This interaction occurred because salamander larvae from the southern pond more strongly avoided cues from local predators, whereas larvae from the northern population avoided predators from distant ponds and approached local predator cues.

The three populations of spotted salamander larvae considered here diverged in behavioral responses to newt predator chemical cues. The local pond community provides a possible explanation for this observation. Each of the three ponds contains a different suite of predators that feed on spotted salamander larvae. Despite similarities in some predator species, the study ponds differed in the density of different species of predaceous salamanders (Fig. 5). The southern pond, sampled for ten years, contains populations of both Eastern newts (*Notophthalmus viridescens*) and marbled salamander larvae (*Ambystoma opacum*), which occur at similar densities of approximately 0.1 newt per  $\text{m}^2$  and 0.08 marbled salamander larvae per  $\text{m}^2$ . The intermediate pond, sampled for nine years, also contains both Eastern newts and marbled salamander larvae but at higher densities: 0.37 newts per  $\text{m}^2$  and 0.13 marbled salamander larvae per  $\text{m}^2$ . We found that behavioral responses of spotted salamander larvae from this pond diverged the most from responses of larvae from other locations. The northern pond is located

beyond the geographic range of marbled salamanders, so the only salamander predators are Eastern newts, which occur at densities of approximately 0.61 newts per m<sup>2</sup>, based on three years of dip net sampling. We know that spotted salamander larvae respond differently to different species of amphibian predators (Urban, 2008a), so it seems likely that different combinations of predators at different densities could account for behavioral variation among ponds.

### *Newt Evolution*

Adult newts are primarily aquatic, but can live in either aquatic or terrestrial conditions depending on density of the pond community or on physical conditions such as heat stress, pond drying, humidity, and precipitation (Gill, 1978b; Roe & Grayson, 2008). Studies of Eastern newt have shown that gene flow is quite low among populations and that populations cluster into geographic units (Gabor & Nice, 2004; Gill, 1978a, 1978b; Grayson et al., 2011). These factors provide the initial conditions for local adaptation to this species of predator.

In this study, we not only tested whether antipredator behavioral responses varied among larval spotted salamander populations, but we also compared prey responses to populations of newt predators that potentially diverge in chemical cues. Adaptation to local predator cues could occur if newts vary in chemical cues across landscapes, and if recognition of local cues increases prey fitness. The evolution of altered cues in predators requires adequate genetic variation in predator populations. The generality of non-zero heritabilities (Mosseau & Roff, 1987) suggests that at least some predator populations could evolve altered cues.

Eastern newts are an interesting predator to consider in terms of chemical interactions. Juvenile efts and adults contain the chemical tetrodotoxin (TTX) as well as its analogues 6-*epi*TTX and 11-oxoTTX (Yotsu-Yamashita and Mebs 2001; Yotsu-Yamashita and Mebs 2003). Secretion of these chemicals from glands in the skin serves as a defense mechanism against

predators (Brodie, 1968; Hurlbert, 1970). TTX is a common defense chemical in newts and has been shown to be an olfactory cue of intraspecific predation risk in the California newt (*Taricha torosa*; Zimmer et al. 2006), though equivalent studies in Eastern newts are lacking. It seems likely that because TTX is a known intraspecific chemical cue in salamandrids, it could also be used as an interspecific cue, signaling danger to potential prey, such as spotted salamander larvae.

Populations of Eastern newts vary in the amount of TTX individuals contain, though the reason for this variation remains unclear (Yotsu-Yamashita et al. 2012). Other species of newt have increased TTX levels in localities with higher selection pressure imposed by predators (Brodie et al., 2002), so the variation among Eastern newt populations could be due to variation in local predators. Alternatively, difference in TTX levels could be due to neutral divergence. The high lability of TTX levels provides a possible explanation for differences in spotted salamander behavioral responses to different populations of newt predators. Prey animals could be recognizing and responding to TTX, and that recognition could be proportional with level of TTX. Further exploration of this idea is needed, first, by examining how and why TTX in Eastern newts might vary spatially, and then by measuring behavioral responses of spotted salamander larvae to ecologically relevant levels of TTX.

Part of our experiment was designed to test whether prey behavioral responses were due to chemical cues alone or if responses were influenced by remnants of the wild-caught predator's environment. We found that spotted salamander larvae behaved no differently to predator newts that had been kept in captivity for over a year versus recently-caught newts. This suggests that length of time in captivity did not alter the chemical cue and provides further support that newt populations might be diverging genetically in chemical cues.

One difficulty in examining behavioral responses to chemical cues is that the precise identity of the chemical cue causing behaviors is unknown. In this experiment, geographic origin of diet and conspecific cues were held constant, so that the chemical cue presented to the experimental larvae varied only with respect to newt predator population of origin. In nature, chemical cues consist of a suite of chemicals — from the predator’s skin and metabolism, as well as from frightened or chewed conspecifics. A logical next step, therefore, would be to vary geographic origin of other aspects of the cue such as geographic identity of the newts’ diets. This research would allow us to understand which aspect of the cue is the primary driver of observed responses and to test for adaptation to different kinds of local cues.

Understanding the complicated evolutionary relationships of predators and prey may allow us to predict the fate of interacting populations in a changing world. The evolution of populations to have higher fitness in their local environment could mean a disadvantage if novel predators are introduced (Gilman et al., 2010; Parmesan, 2006). Foreign predators could arrive via human-aided dispersal, or could naturally colonize a new environment, perhaps as a result of climate-mediated movement (Gilman et al., 2010; Herstoff & Urban, 2014; Parmesan & Yohe, 2003; Urban, De Meester, Vellend, Stoks, & Vanoverbeke, 2012). The results of this work will add to our understanding of the evolution of predator-prey interactions across multiple spatial scales.

### *Conclusions*

This study provides evidence that prey animals can discriminate and respond differently to predator chemical cues from geographically distinct populations of the same species of predators. Predator-prey relationships in temporary ponds are interesting to consider because varying selection regimes among discrete communities can alter the outcome of interactions.

Selection for particular anti-predator behaviors may depend on type and density of both predators and competitors in the community, as well as on abiotic factors such as canopy cover or biogeochemistry of the pond. By gaining a better understanding of the breadth and drivers of behavioral changes, we can learn to interpret patterns of predator-prey coevolution, and to predict how those interactions could be affected by changing environmental conditions.

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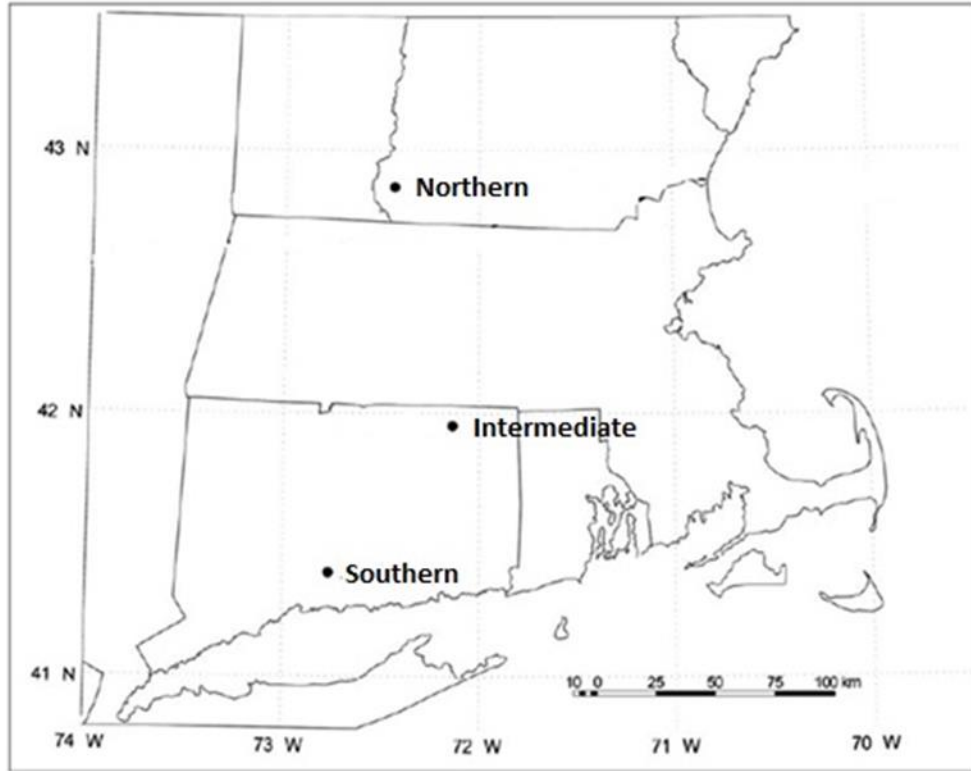


Figure 1: Map of Study Locations (Adapted from Urban 2008).

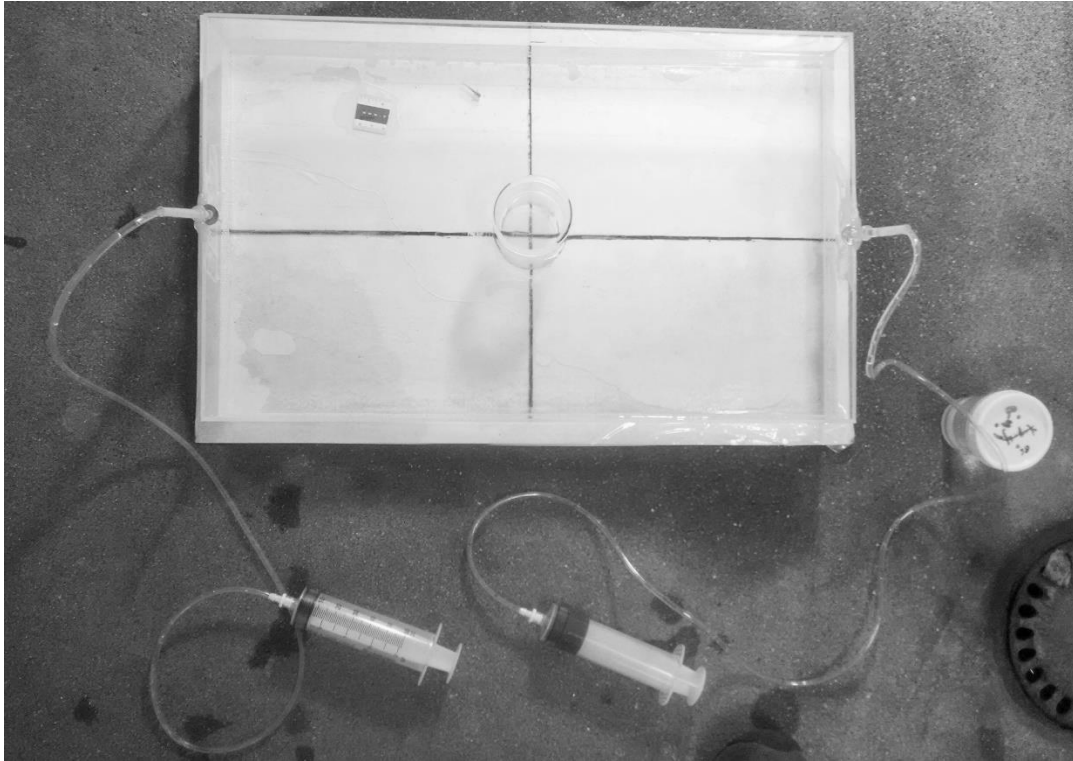


Figure 2: Experimental arena with stimulus delivery system. In each trial, an experimental larva was placed in the chamber in the center, then released as water and chemical cues were slowly injected into the arena through the stimulus delivery system. One syringe held plain water, and the other held chemical cue from a newt predator.

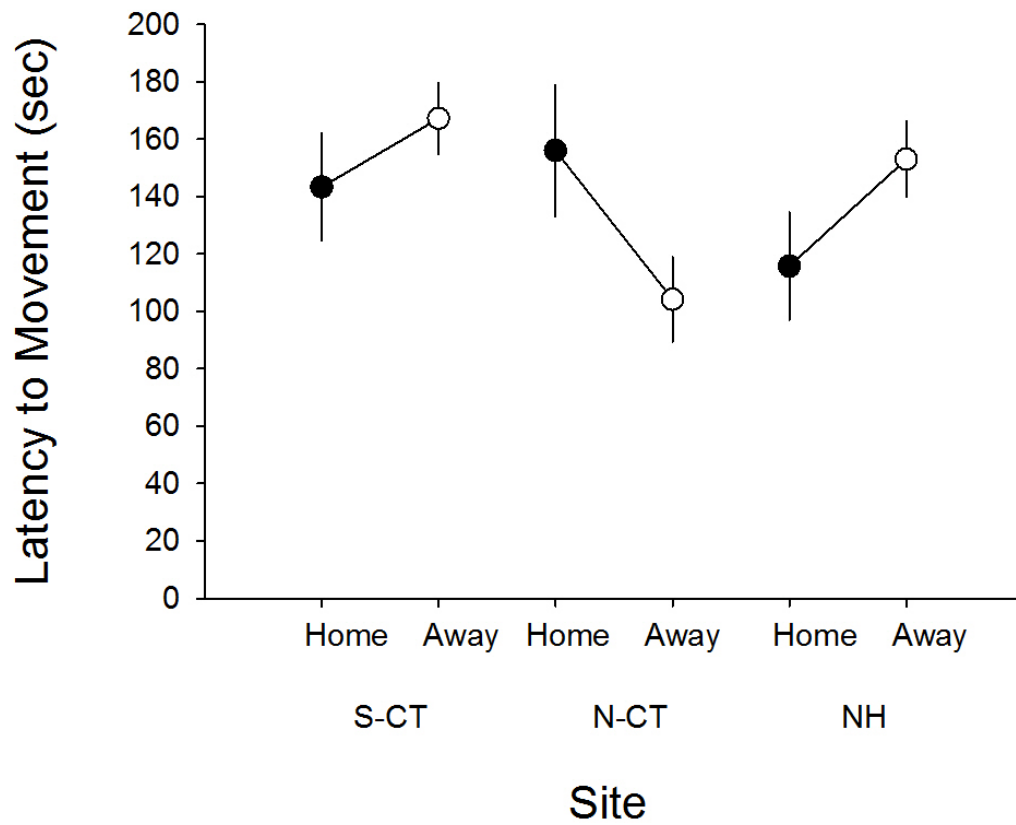


Figure 3: Latency to movement of salamander larvae from the intermediate pond was significantly different than latency responses of salamander from other locations. Filled circles indicate response to newts from the same pond of origin of the prey population. Open circles indicate responses to newts from foreign sites. Symbols indicate mean  $\pm$  SEM.



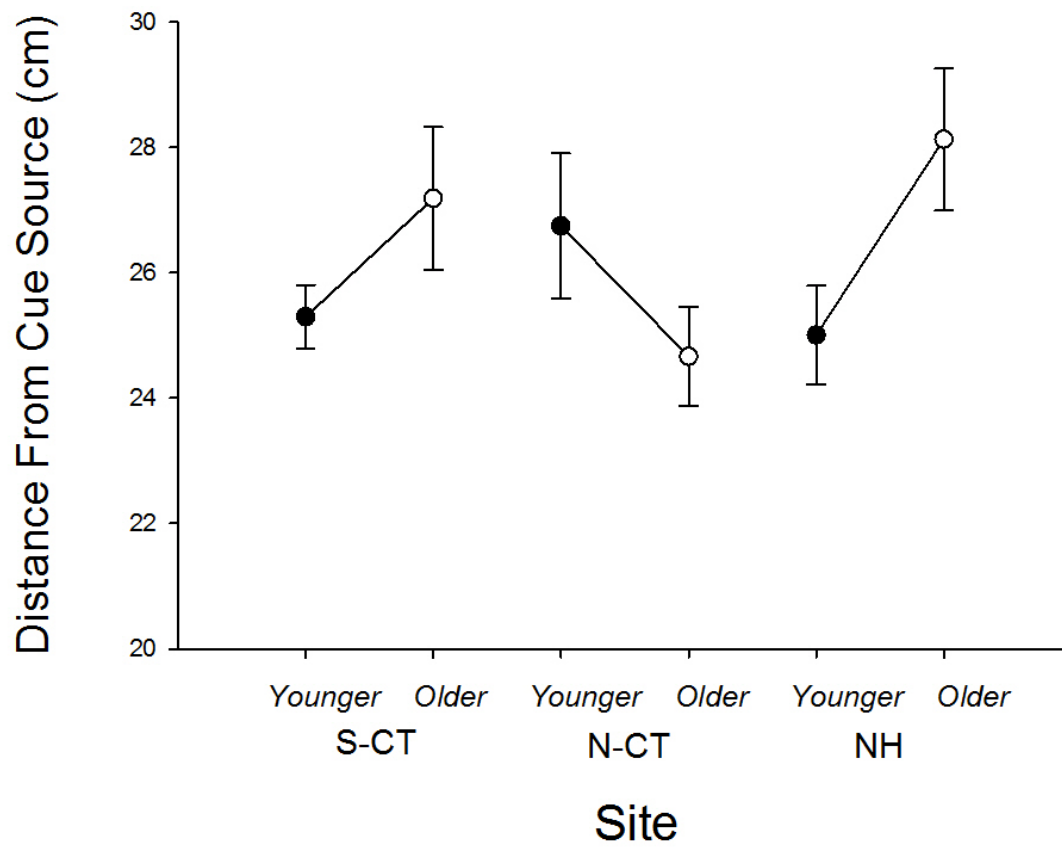


Figure 4: Distance from predator cue source measured spatial avoidance behavior of salamander larvae. There was a significant interaction between larval age and focal pond, due to the tendency of N-CT larvae to place themselves closer to a predator cue than younger larvae. Symbols indicate mean  $\pm$  SEM.

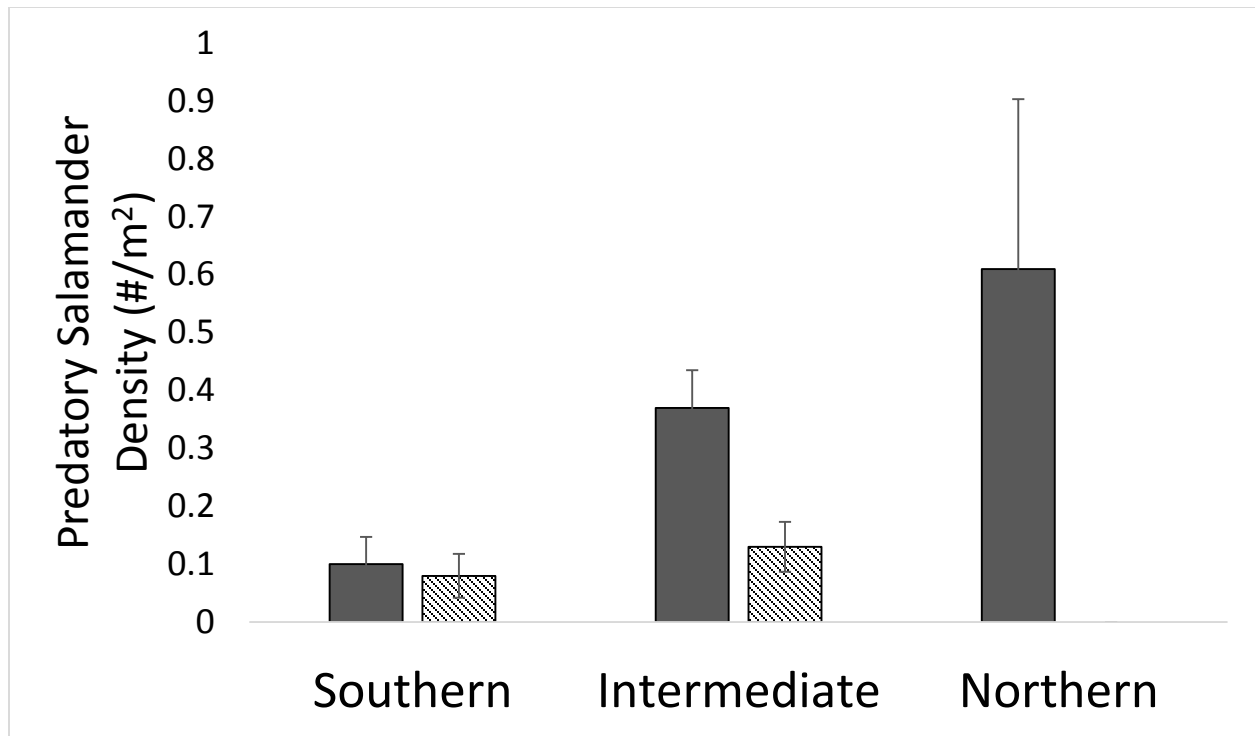


Figure 5: Mean annual densities of predaceous salamanders varied between the three focal ponds. Solid grey bars are densities of adult Eastern newts (*Notophthalmus viridescens*); striped bars are densities of juvenile marbled salamanders (*Ambystoma opacum*). Error bars indicate mean  $\pm$  SEM. The southern pond was sampled for ten years, the intermediate pond for nine years, and the northern pond for three years.

## **Chapter 2:**

### **Larval spotted salamanders can discriminate between predatory newts based on geographic origin of newt diet**

#### **Abstract**

In an aquatic environment where visual cues are limited, amphibian larvae often evolve to recognize and respond to different predator chemical cues. Previous work suggests that spotted salamander larvae can recognize local predators. In this study, we asked whether prey could differentiate between predator chemical cues on the basis of geographic origin of the predator's diet. We collected predaceous newts (*Notophthalmus viridescens*) and their prey, spotted salamander larvae (*Ambystoma maculatum*), from three populations stratified across a geographic landscape, and raised prey in a common garden environment. We fed each population of spotted salamander larvae to newt predators to create chemical cues, then tested for behavioral responses to these cues in each prey population. We matched newt pond of origin to experimental larval pond of origin and varied only the geographic origin of each newt's diet. We measured larval latency to movement, activity, and spatial avoidance in response to chemical cues. Larval spotted salamanders from two of the populations modified spatial avoidance by avoiding cues from predators fed conspecifics from more distant pond. This research suggests that some populations of this species possess a heightened ability to recognize local conspecifics.

#### **Introduction**

Patterns of ecological interactions between and among species vary widely across a natural landscape. Variation within a small geographic area can be partially explained by a combination of local environmental conditions and the evolution of species interactions (Conover, Duffy, & Hice, 2009; Richardson et al., 2014; Thompson, 1999a). When conditions

differ among habitats, populations can become locally adapted if they evolve traits that convey higher fitness to the population in its local environment compared with other populations (Hereford, 2009; Kawecki & Ebert, 2004; Richardson et al., 2014).

Biotic selection is often strong in natural communities, and is associated with local adaptation (Buckley et al., 2010; Kawecki & Ebert, 2004; Thompson, 1999a). Studies have shown that prey animals can adapt to different local predator regimes (Laurila, 2000; Relyea, 2003; Storfer & Sih, 1998). One way in which this can occur is through adaptation to better recognize predator chemical cues in the home environment (Relyea, 2002b). Aquatic organisms that live in temporary ponds often rely on chemical cues to obtain information about food, location of conspecifics, and predation risk (Brönmark & Hansson, 2000b; Kats & Dill, 1998; Tollrian & Harvell, 1999). In particular, the recognition of predator chemical cues is strongly reinforced, because failure to recognize and respond appropriately to the presence of a predator often carries a strong mortality risk (Ferrari et al., 2010; Lima & Dill, 1990; Smith, 1992).

Chemical cues that convey information about predation risk can originate from the predator itself, such as chemicals from the predator's skin or metabolites from its digestion (Brown, Eisner, and Whittaker 1970; Ferrari, Wisenden, and Chivers 2010; Hettyey et al. 2015). Alternatively, chemical cues can originate from damaged or digested prey (Ferrari et al., 2010; Hettyey et al., 2015). Prey responses to cues from a predator's skin depend only on the predator's chemistry, but responses to diet cues could indicate a more sensitive sensory system. Recognition of the complex chemical information contained in a chemical cue could allow the responding prey to not only identify the predator, but to identify recently-consumed prey, allowing for more accurate risk assessment (Schoeppner & Relyea, 2005; Wilson & Lefcort,

1993). This, in turn, would inform behavioral decision-making and might provide a survival advantage to prey.

Many aquatic organisms can recognize conspecific individuals via chemical cues (Gherardi, Tricarico, & Atema, 2005; Johnsson, 1997). In addition, many amphibians respond to digestion cues, and some can differentiate between species eaten by predators (Chivers & Mirza, 2001; Chivers et al., 1997; Laurila, Kujasalo, & Ranta, 1997; Murray & Jenkins, 1999; Schoeppner & Relyea, 2005; Wilson & Lefcort, 1993). Using chemical cues to gather information about predators and predators' diets is especially important in dark, turbid pond environments where many other sensory modalities are dulled. These recognition abilities might be evolutionarily favored, because an organism that can accurately assess predation risk is better equipped to survive and reproduce in an unpredictable environment in which predator composition can change from season to season.

We hypothesize that prey will respond differently to predators that have eaten local rather than foreign prey. For this to be true, local prey populations must differ genetically and phenotypically from each other. These differences could translate into different prey chemistries and thus, digestion cues. If these conditions are met, animals could evolve an ability to recognize individuals in their population, assuming sufficient additive genetic variation exists and selection is strong relative to gene flow. Aquatic amphibian larvae respond more strongly to predators that eat species more phylogenetically similar to themselves (Schoeppner and Relyea 2009), yet no study has determined if this result also applies to locally adapted populations within species.

Our hypothesis may seem counterintuitive, since it would be most beneficial to prey animals to recognize cues from predation on conspecifics from any location. If, however, the chemistries of prey populations differ across the landscape, then adaptation to recognize

predation on local conspecifics may come with the consequence that prey no longer recognize predation on conspecifics from other locations. Alternatively, adaptation to recognize predation on local versus foreign conspecifics could occur if there was selection for kin recognition in prey populations (Hamilton, 1964a, 1964b). In either case, differential responses to predator diet could be maladaptive if prey fail to respond appropriately to predators.

To investigate adaptation of cue recognition, we evaluated the behavior of spotted salamander larvae (*Ambystoma maculatum*) in response to Eastern newts (*Notophthalmus viridescens*) fed spotted salamander larvae from populations across multiple spatial scales and levels of genetic differentiation. We predicted that prey would respond with higher latency (time until movement), lower activity, and greater spatial avoidance to predators fed larvae from local populations compared to foreign populations. These responses would suggest that prey adapt to recognize the chemistries of local individuals. Alternatively, prey might not distinguish between digestion cues based on predator diet if genetic differences among prey populations do not translate into chemical differences in dietary cues; if prey sensory systems are functionally unable to recognize fine distinctions among digestion cues; or if low genetic variation or gene flow prevents an evolutionary response.

## **Methods**

### *Natural History and Study Sites*

Spotted salamanders are large terrestrial amphibians that occur broadly across eastern North America. In early spring, adults migrate to temporary ponds to breed and lay their eggs, which hatch in eight to ten weeks (Petranka, 1998; Urban, 2007a). Fully aquatic larvae undergo metamorphosis and leave the ponds by late summer, when ponds begin to dry and temperatures cool. For this study, we evaluated behavioral responses of spotted salamander larvae from three

populations on a latitudinal cline in southern New England, USA: a southern site (Northford, CT); intermediate site (Yale-Myers Forest in Union, CT), and northern site (Pisgah State Park in Winchester) (Figure 1). Pond communities differ among these regions, and each of the three focal ponds contains a different suite of predators that feed on spotted salamander larvae (Urban, 2007a). Though diversity of invertebrate predatory species is similar across the landscape, the three study ponds differ in the density of two important species of predaceous salamanders: larval marbled salamanders (*Ambystoma opacum*) and adult Eastern newts (Rack and Urban unpublished data).

The Eastern newt, *Notophthalmus viridescens*, is one of the most important amphibian predators of spotted salamander larvae in New England (Urban, 2007a). Eastern newts have a life cycle in which an aquatic larval stage is followed by a terrestrial juvenile (eft) stage and an aquatic adult stage. This species is widely distributed across eastern North America. Adults are gape-limited predators, capable of feeding on prey smaller than their gape size, including worms, insects, small crustaceans, and amphibian eggs and larvae ((Behler & King, 1979; Urban, 2008a).

Predator-prey interactions between adult Eastern newts and larval spotted salamanders occur frequently across the study region (Urban, 2007a). Previous research suggests that spotted salamander prey have evolved adaptive responses to generalized newt cues. Spotted salamander larvae grow more quickly and switch to nocturnal activity patterns in response to the chemical cues of this gape-limited and visual predator (Urban, 2008a). Additionally, these larvae adapt on a microgeographic scale to local predator communities, altering behavior and morphology in response to different predators, even within typical dispersal distances (Richardson & Urban, 2013; Urban, 2007b, 2010). These adaptations occur despite gene flow among breeding

populations (Zamudio & Wieczorek, 2007) because of strong selection barriers against maladapted immigrants (Richardson and Urban 2013). Additional microgeographic adaptations have been demonstrated in this species in response to other selection pressures (Brady, 2012), suggesting that spotted salamanders often adapt to their local environments.

### *Animal Collection and Housing*

In the early spring of 2014, we identified one focal pond at each site that contained both spotted salamanders and Eastern newts. We then collected 15-20 spotted salamander egg masses from each focal pond. After collection, we split spotted salamander eggs into groups of ten to fifteen eggs and placed them in 19-L buckets in an outdoor enclosure at the Spring Hill Research Compound in Storrs, CT. We randomized buckets with respect to egg mass number and focal pond origin. After hatching, larvae were thinned to five larvae per bucket, a density within the range of natural larval densities (Urban, 2008a). All larvae were fed *ad libitum* with zooplankton collected from a local pond.

Adult Eastern newts were collected from all three focal ponds. We collected 21 newts from the southern site, 24 from the intermediate site, and 24 from the northern site. Newts were kept in eighteen 168-L containers in the same outdoor enclosure at Spring Hill Research Compound. Each container held 3-4 adult newts from the same site, and contained 20g of dried leaves. Adult newts were fed once a week, 15 salamander larvae per container. Newts from each focal pond were divided into three groups: one group ate spotted salamander larvae from the same focal pond as the newt; the other two groups ate larvae from each of the other focal ponds. Thus, two containers of southern newts fed on southern larvae; two containers of southern newts fed on intermediate larvae; and two containers of southern newts fed on northern larvae, etc.



### *Larval Treatment*

Chemical cues from local newts were added to the salamander larvae buckets three times weekly. This frequency has been shown to elicit responses from spotted salamanders in previous experiments (Urban, 2008a). Water from bins of predators fed conspecifics from the same focal pond was filtered through a 150- $\mu$ m mesh screen to remove any potential food resources. In each treatment, one liter of cue water was added to each 19-L bucket.

### *Behavioral Experiment*

We used a crossed-treatment design to experimentally measure behavioral responses to geographic origin of newt diet. Spotted salamander larvae from each location were tested with chemical cues from local newts (from the same pond) that had either been fed spotted salamander larvae from the same pond, or that had been fed larvae from one of the other two ponds. Each population of spotted salamander larvae was tested against 3 groups of local newts, each of which had been fed larvae from a different location, for a total of 9 combinations of treatments (experimental larval origin x predator newt origin x predator newt diet; Fig. 2).

Experimental trials were performed indoors in Plexiglas boxes with dimensions 30.5 x 50 x 8.5 cm. At the center of the experimental arena, we placed an inverted 120-mL cup that, when twisted, allowed the experimental larvae to exit toward either the stimulus or control cue (Fig 3). A stimulus delivery system was constructed from a Terumo 60-mL syringe (Terumo Medical Corporation, Somerset, NJ) and 0.125" internal diameter PVC tubing. The end of the tubing was attached to a Pasteur Pipette, secured to the walls of the experimental arena. Each experimental arena was filled with 4 cm of water and fitted with two pipettes on opposite sides. We placed each experimental arena on a background of white plastic to facilitate visibility for video analysis.

To prepare the predator cue, a newt predator was isolated for 24 hours in a 708-mL container with 3-5 spotted salamander larvae from one of the three locations. After 24 hours, the predator and any uneaten larvae were removed. Cue water was considered viable for no more than 48 hours (Peacor, 2006).

For each trial, a larval salamander acclimated in the chamber for ten minutes. Next, two stimuli were prepared: one with 50 mL of control water (no newt cue), and one with 50 mL of newt cue. For this experiment, newt focal pond always matched the focal pond of the experimental larvae. At the beginning of the trial, the acclimation chamber was slowly turned to allow the larva the option to exit, and the two stimuli were administered at a rate of ~1 mL/min. Preliminary trials with dyed water showed that this rate resulted in diffusion across the entire arena during the span of the behavioral assays.

We expected behavioral differences based on age, so two age classes of spotted salamander larvae from each focal pond were tested: at 2-3 weeks and 4-5 weeks post-hatch. Four weeks represents the maximum time it takes for spotted salamanders to reach a size refuge from newts (Urban, 2008a). Behavioral responses generally increase through time as larvae become more active (Urban, 2007b). Stimulus and control sides were flipped randomly to control for side bias. Each individual was used only once. We performed five repetitions of each combination of larval focal pond origin versus newt diet pond of origin.

### *Behavioral Metrics*

To assess larval behavioral responses to newt predator diets, we measured latency, quantified as the time before first movement, and total time active over the 5-minute trial. We also measured spatial avoidance, or how far the salamander had moved from the source of the chemical cue at the end of the trial. Previous studies have shown that amphibians often respond

to predator cues through reduced activity and increased spatial avoidance (Relyea, 2001; Semlitsch, 1987; Skelly & Werner, 1990; Van Buskirk & Schmidt, 2000).

### *Data Analysis*

Video recordings of each trial were analyzed using ImageJ (Rasband, WS 1997-2014). All statistical analyses were performed in R, using mixed-effects generalized linear models provided by the lme4 package (Bates et al. 2014; R Core Development Team, 2013). To test for sensitivity to geographic origin of newt diet, we analyzed the effect of pond of origin, as well as the effect of local versus foreign diet cues (Blanquart et al., 2013). The latter analyses included predator diet population of origin as a random effect, nested in either local, when consumed prey larvae were from the same pond as the experimental larvae, or foreign, when consumed prey larvae were from a different pond than the experimental larvae. Latency and spatial avoidance were analyzed assuming that data followed an overdispersed Poisson distribution. We additionally analyzed these data assuming a binomial distribution with spatial or temporal constraints and arrived at the same result. We used a binomial model to assess the proportion of time active. If a high residual deviance indicated overdispersion in binomial and Poisson tests, we modeled this overdispersion directly using an individual random effect by assigning each individual at each time period a unique identifier, as advocated by Warton and Hui (2010). We analyzed significance using maximum likelihood ratio tests for categorical factors with more than two classes. When we detected an overall significant effect, we evaluated statistical tests for specific comparisons among populations and treatments.

## Results

### *Latency*

Salamander latency did not vary in response to local versus foreign predator diets ( $P = 0.88$ ), or in response to geographic origin of predator diet (southern vs. intermediate,  $P = 0.84$ ; southern vs. northern,  $P = 0.09$ ). However, latency to newt digestion cues did vary significantly as the result of an interaction between age and larval population of origin ( $\chi^2_{df=2} = 8.78$ ,  $P = 0.012$ ) because younger larvae from the intermediate site responded to predator digestion cues with higher latency than older larvae from the same site ( $P < 0.01$ ; Fig 4). This pattern differed from responses of larvae from other populations.

In addition, latency varied significantly among the sites (southern vs. intermediate,  $P < 0.05$ ; southern vs. northern,  $P < 0.01$ ; intermediate vs. northern,  $P < 0.001$ ). Experimental larvae from the northern population had the shortest latency periods, and larvae from the intermediate population had the longest latency periods. The effect of larval age on the latency response was not significant ( $P = 0.50$ ).

### *Time Active*

When we analyzed the proportion of time experimental larvae were active, we found no effect of local versus foreign predator diets ( $P = 0.38$ ) and no effect of geographic origin of predator diet (southern vs. intermediate,  $P = 0.34$ ; southern vs. northern,  $P = 0.17$ ). There was, however, a significant effect of larvae population of origin ( $\chi^2_{df=2} = 18.9$ ,  $P < 0.001$ ), in that the intermediate population had significantly lower activity than the other populations ( $P < 0.001$ ; see Fig 5). Older larvae were more active overall than younger larvae in response to predator digestion cues ( $P < 0.01$ ).

### *Spatial Avoidance*

We found no effect of local versus foreign predator diet cues on overall spatial avoidance ( $P = 0.24$ ), measured as the distance between a salamander larva and the source of the predator digestion cue at the end of a trial. Spatial avoidance varied significantly due to an interaction between focal pond and origin of newt diet ( $\chi^2_4 = 12.82$ ,  $P = 0.012$ ). This result occurred because larvae from the northern population moved significantly farther from predator diet cues when newts ate larvae from foreign ponds ( $\chi^2_2 = 7.30$ ,  $P = 0.025$ ; see Fig 6). This effect meant that northern larvae differentiated between local versus foreign cues ( $P < 0.01$ ). Similarly, larvae from the southern population moved farthest from cues elicited from predators fed northern larvae and stayed near the release point when predators were fed larvae from the southern or intermediate locations ( $\chi^2_2 = 8.51$ ,  $P = 0.014$ ; see Fig 6). The southern population, therefore, also responded differently to local versus foreign cues ( $P < 0.05$ ). Spatial avoidance of the intermediate population did not differ in response to local versus foreign cues ( $P = 0.61$ ) or geographic origin of predator diet ( $P = 0.65$ ; see Fig 6).

We found a marginally significant result whereby experimental larvae from all populations occurred closer to cues elicited from newts fed northern population larvae ( $P = 0.055$ ). Among populations, the magnitude of the spatial avoidance response of the intermediate population did not differ from responses of the southern population ( $P = 0.56$ ), and though larvae from the northern population often occurred farther from the source of the predator cue than other populations, this result was only marginally significant ( $P = 0.055$ ). There was no effect of larval age on spatial avoidance ( $P = 0.54$ ).

## Discussion

In response to the threat of predation, prey animals can evolve behaviors that allow them to avoid or escape a predator. In aquatic systems, chemical cues frequently mediate these behavioral responses, though the specific component of the cue that drives antipredator behaviors remains unknown in most systems. Previously, we have shown that populations of larval spotted salamanders diverge in responses to predatory newt chemical cues, and that some populations have evolved to recognize local predator cues. We designed the current study to investigate the effect of geographic origin of predator diet on the behavior of larval spotted salamanders. This study suggests that two populations of salamander larvae can alter spatial avoidance based on the information contained in predator diet cues.

### *Geographic Origin of Diet Cues*

We predicted that spotted salamander larvae would respond with stronger antipredator behaviors – with higher latency, lower activity, and greater spatial avoidance – to newt predators fed a diet of salamander larvae from the same pond. In making this assumption, we assume that local prey populations diverge in their chemistry, either through evolutionary divergence or environmental factors. We found that salamander larvae from the southern and northern populations spatially avoided cues from predators fed larvae from more distant ponds.

In response to predators eating local larvae, none of the three populations of spotted salamanders moved from the release point in the experimental arena. Larvae from both the northern and southern populations responded differently to cues from predators fed larval conspecifics from distant populations. This suggests that spatial avoidance in these populations could be a response to a novel chemical cue. Instead of remaining in place, as they might have if the predator had consumed a local larval conspecific, larvae moved away from the source of the

predator cue. One possible explanation for this behavior is xenophobia, the fear of something foreign, in which larvae might have responded one way to familiar cues (staying still when predators had consumed local conspecifics), and responded differently to unusual cues (spatially avoiding predators that had consumed foreign conspecifics).

An alternative to the xenophobia explanation is that the observed behavior results from a form of kin recognition, since local spotted salamander conspecifics are more likely to be related individuals. In the theory of kin selection, an individual's fitness is partially determined by the fitness of closely related individuals, with the reasoning that through survivorship of its kin, some of its genes will be passed on to future generations (Hamilton, 1964a, 1964b). Larval ambystomid salamanders have been shown to use chemical cues to identify relatives (Pfennig, Sherman, & Collins, 1994; Walls & Roudebush, 1991), though in these species, kin recognition is most often employed to avoid aggression toward or cannibalism of related individuals (David W. Pfennig et al., 1994; D. W. Pfennig & Collins, 1993; Walls & Blaustein, 1995; Walls & Roudebush, 1991, 1991).

Either explanation fits with the observation that the pattern of spatial avoidance has a geographic component. Here, larvae from the northern population moved farther from predator cues when predators had been fed intermediate and southern larvae; the intermediate population remained in place regardless of diet; and the southern population moved farther from predators fed northern larvae. The greater the distance between populations, the greater the spatial avoidance of a consumed conspecific.

This study shows that spotted salamander larvae can differentiate between cues based on geographic origin of predator diet. All predator newts were collected from the same pond as the salamander population to be tested, so newt kairomones and metabolites were held constant in

each set of trials. As a result, the chemical cues presented to salamander individuals varied only with respect to the population of origin of the newt's diet.

### *Population Differences*

Overall, we found that the magnitude of behavioral responses differed among the three populations. Both latency and time active varied among all populations, though spatial avoidance did not. We found that larvae from the intermediate population had significantly higher latency periods than the other populations, and moved significantly less overall. Larvae from the northern population had the shortest latency periods. One possible explanation for these differences is that each of the three focal ponds contains different densities of amphibian predators. The southern pond contains both Eastern newts and marbled salamander larvae at similar densities; the intermediate pond contains both predator species, but higher newt densities; and the northern pond lacks marbled salamander larvae (Rack and Urban, unpublished data). Antipredator responses to one species may not be appropriate for another, so the different predator regimes may drive differential responses.

### *Larval Age*

Larval age mattered to both latency and activity, in that 4-5 week old larvae tended to move more overall than 2-3 week old larvae. We found that older larvae from the intermediate population exhibited shorter latency than younger larvae from the same population. These results agree with what we would expect from the two age classes of larvae. Salamander larvae at different ontogenetic stages can demonstrate antipredator behavior in proportion to their vulnerability (Mathis, Murray, & Hickman, 2003). Older larvae are often more active, possibly because their larger size means that they are less vulnerable to gape-limited predators (Mathis et al., 2003; Urban, 2008a).



### *Conspecific Recognition*

The ability to differentiate geographic origin of consumed conspecifics could be due to a heightened ability of larvae from the northern and southern populations to recognize conspecifics. Many aquatic organisms are capable of individual conspecific recognition via chemical cues (Gherardi et al., 2005; Johnsson, 1997). In an environment where many other sensory modalities are dulled, the ability to identify what a predator has been eating may be evolutionarily favored, since it could allow prey animals to accurately assess predation risk. Chemical cues from predators' bodies alone are often not enough to induce antipredator defenses in prey; the diet cue is a crucial source of information to larval amphibians (Schoeppner & Relyea, 2009).

In the current experiment, larvae from two populations responded differently to chemical cues depending on the geographic origin of the consumed conspecific prey. Because larvae from the three populations of spotted salamanders were raised in a common garden environment, this result suggests genetic divergence between spotted salamander populations. Larvae were raised from eggs collected within a few days of being laid in order to minimize environmental influences. We did not raise multiple generations of spotted salamanders in the lab and therefore cannot reject trans-generational plasticity. However, multiple contributions to trans-generational plasticity have been rejected in this system for other traits, including habitat choice, maternal care, egg provisioning, and environmental conditioning of adults or eggs (Richardson and Urban 2013).

Across a landscape, selective pressures vary due to local conditions. One possible consequence of this variability is that selection or drift in local populations could cause small changes in the chemical signature of individuals in that population. Population differences in sex

pheromones – another type of chemical cue – have been demonstrated in plethodontid salamanders, as well as in garter snakes and a species of lacertid lizard (LeMaster & Mason, 2003; Martín & López, 2006; Rollmann, Houck, & Feldhoff, 2000). If there was similar chemical variation among populations of spotted salamanders, we might expect larval salamanders to adapt to recognize diet cues from predators eating local conspecifics. Consumed larvae from a foreign population, one in which the chemical signature differed, would not be recognized, since selection would not reinforce recognition of a foreign chemical cue. A fine-tuned ability to differentiate between diet cues could enhance fitness for prey animals if it helped them perform appropriate antipredator behaviors when in the presence of a threat.

Future studies should focus on the mechanisms and scale of conspecific identification, so that we might understand why responses differ among populations and whether this finding holds true among ponds within a metapopulation as well as across a geographic landscape. Additionally, further investigation into the identity of the chemical cue will allow researchers to study if and how these chemicals differ among populations.

### *Conclusions*

This study provides evidence that amphibian prey can extract detailed information about a predator's diet from a predator chemical cue. Between-population differences in antipredator responses to diet cues of predators fed local prey suggest variability in adaptation to local conditions. Understanding the specificity of prey recognition abilities can provide insight into the evolution of receptor systems, and help us understand how forces like selection and coevolution can affect predator-prey interactions over a geographic landscape.

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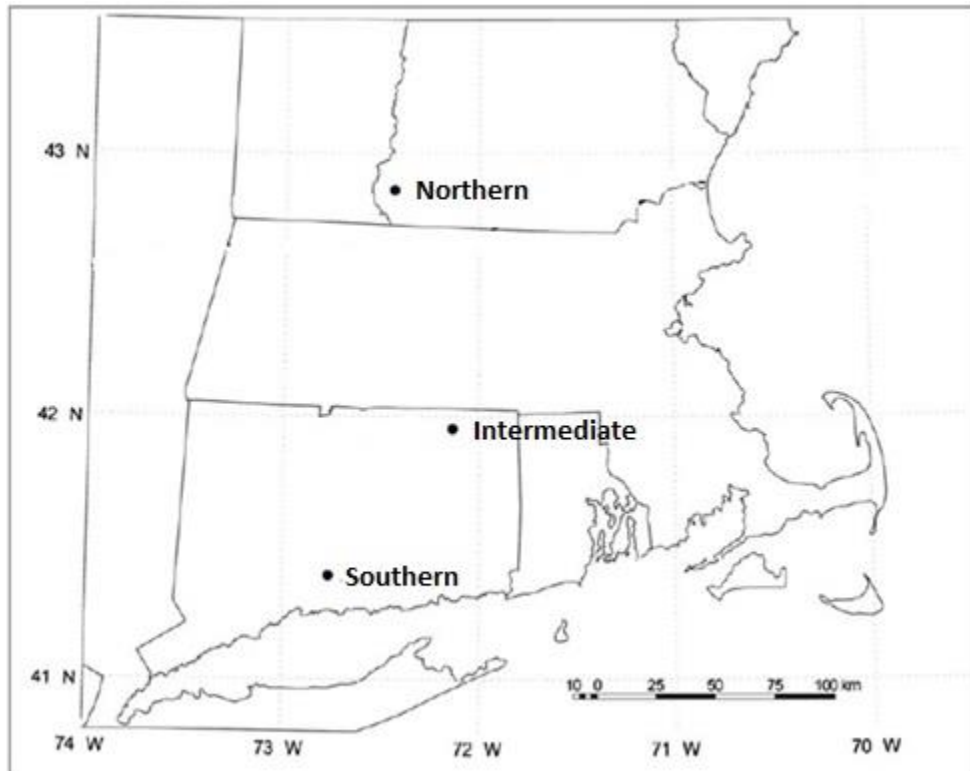


Figure 1: Map of Study Locations (Adapted from Urban 2008).

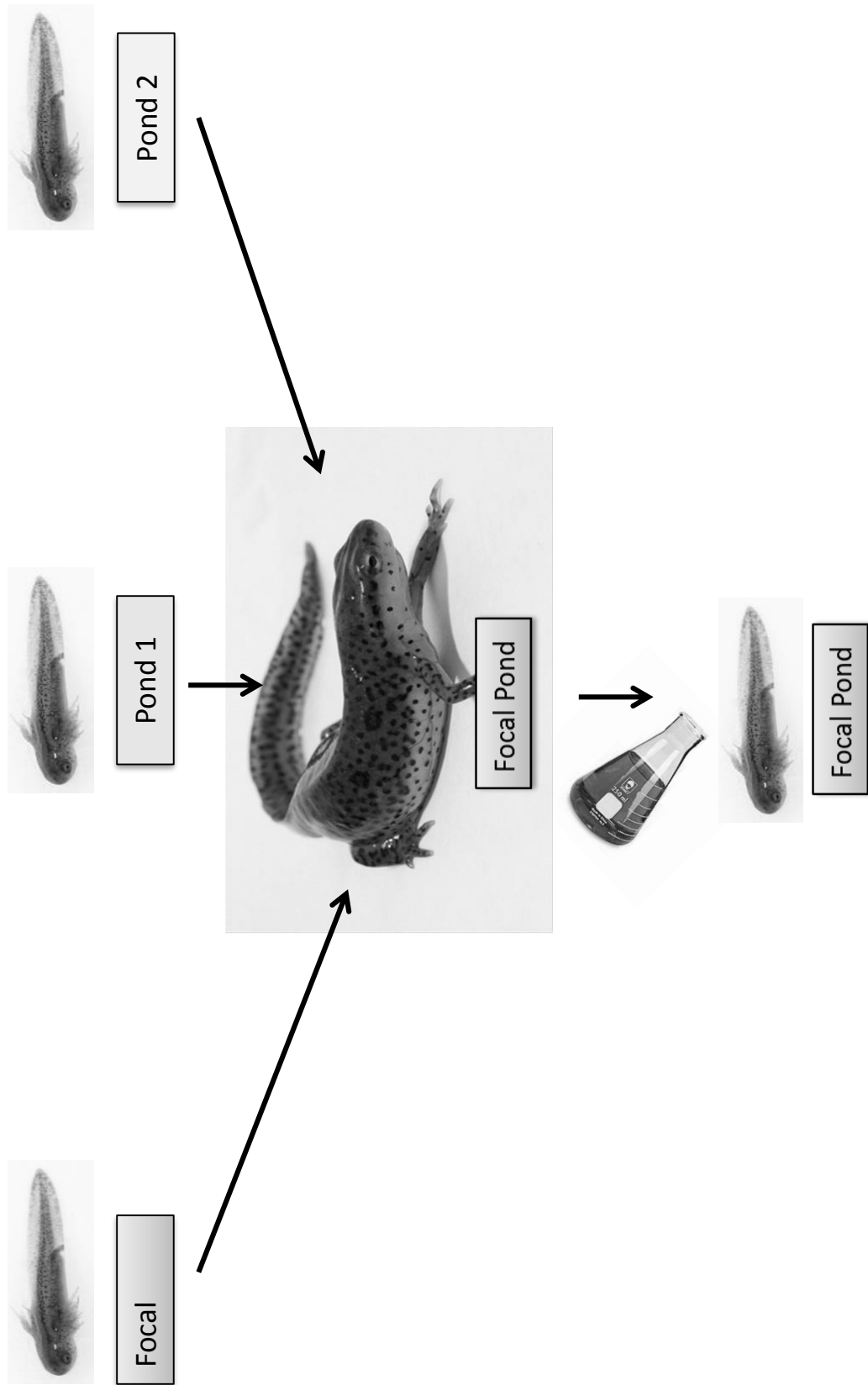




Figure 2: To measure behavioral responses to the geographic origin of newt diet, we used a crossed design, in which experimental larvae from each pond were presented with different predator chemical cues. Each cue was made by feeding a local newt with spotted salamander larvae from either a local or foreign pond.

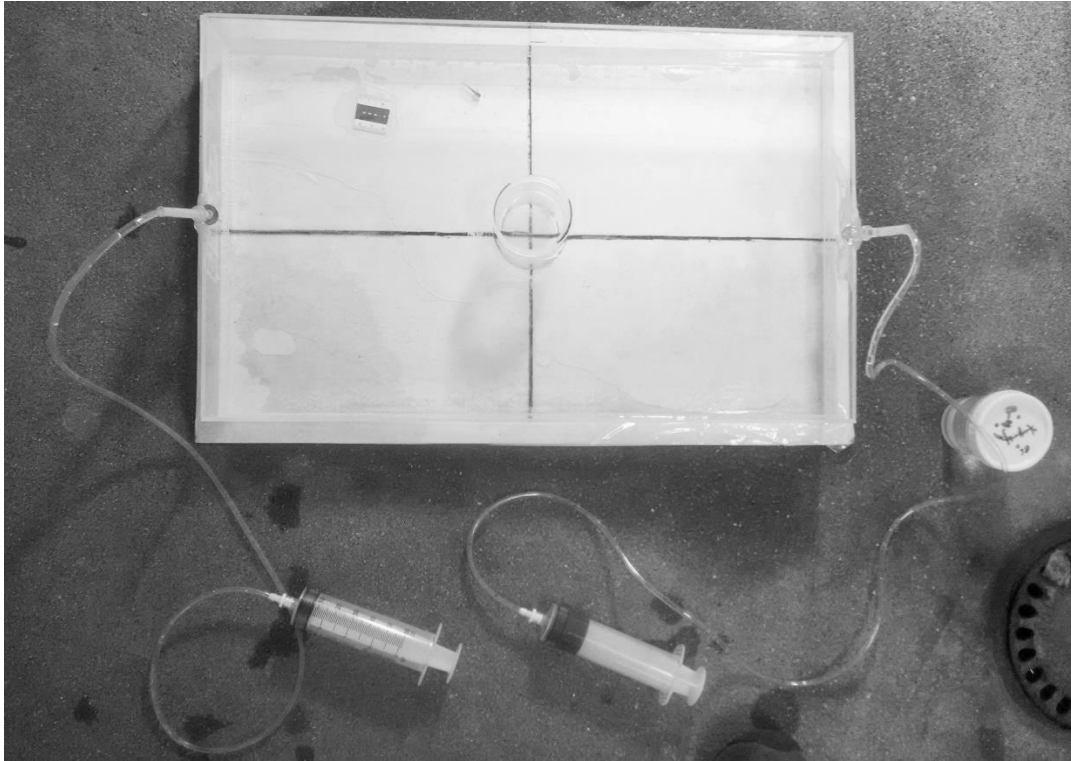


Figure 3: Experimental arena with stimulus delivery system. In each trial, an experimental larva was placed in the center, then released as water and chemical cues were slowly injected into the arena through the stimulus delivery system. One syringe held plain water, and the other held a chemical cue from a newt predator fed a particular diet.

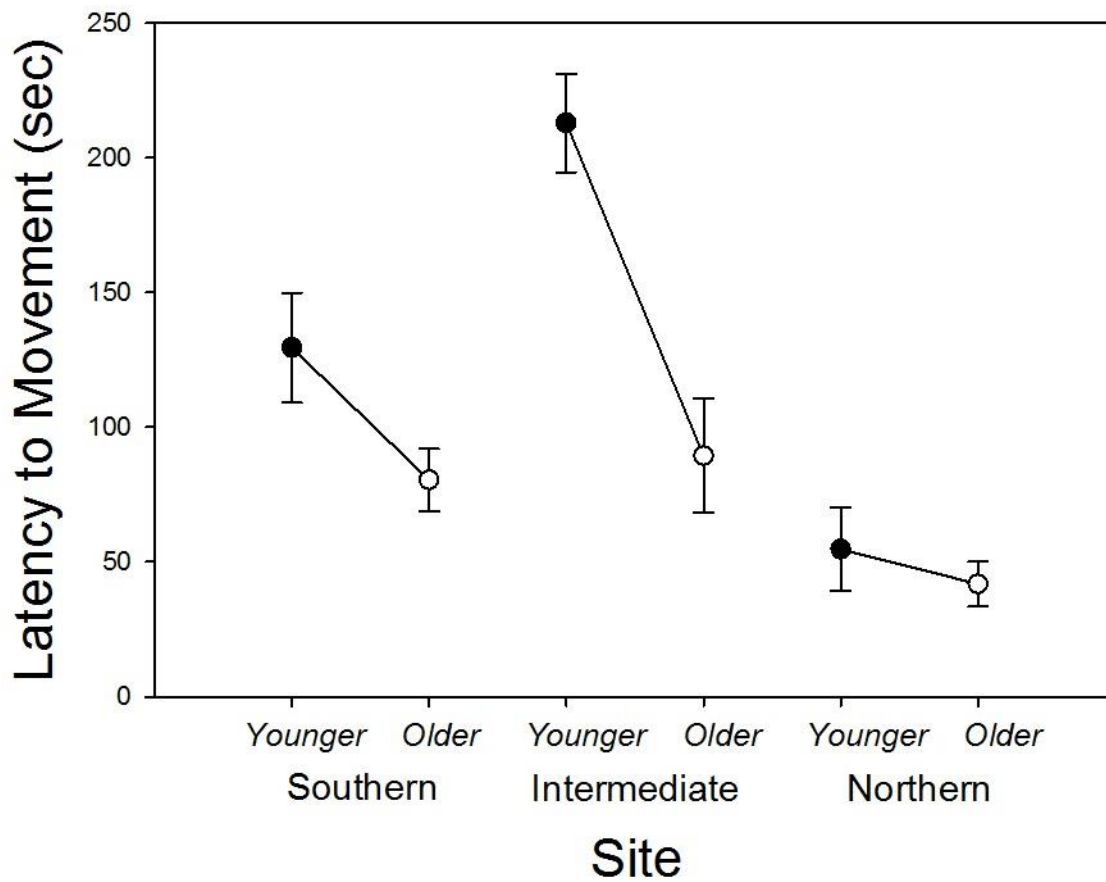


Figure 4: Latency to movement of salamander larvae from the intermediate pond was significantly different than latency responses of salamanders from other locations. Filled circles indicate responses of 2-3 week old larvae. Open circles indicate responses of 4-5 week old larvae. Symbols indicate mean  $\pm$  SEM.

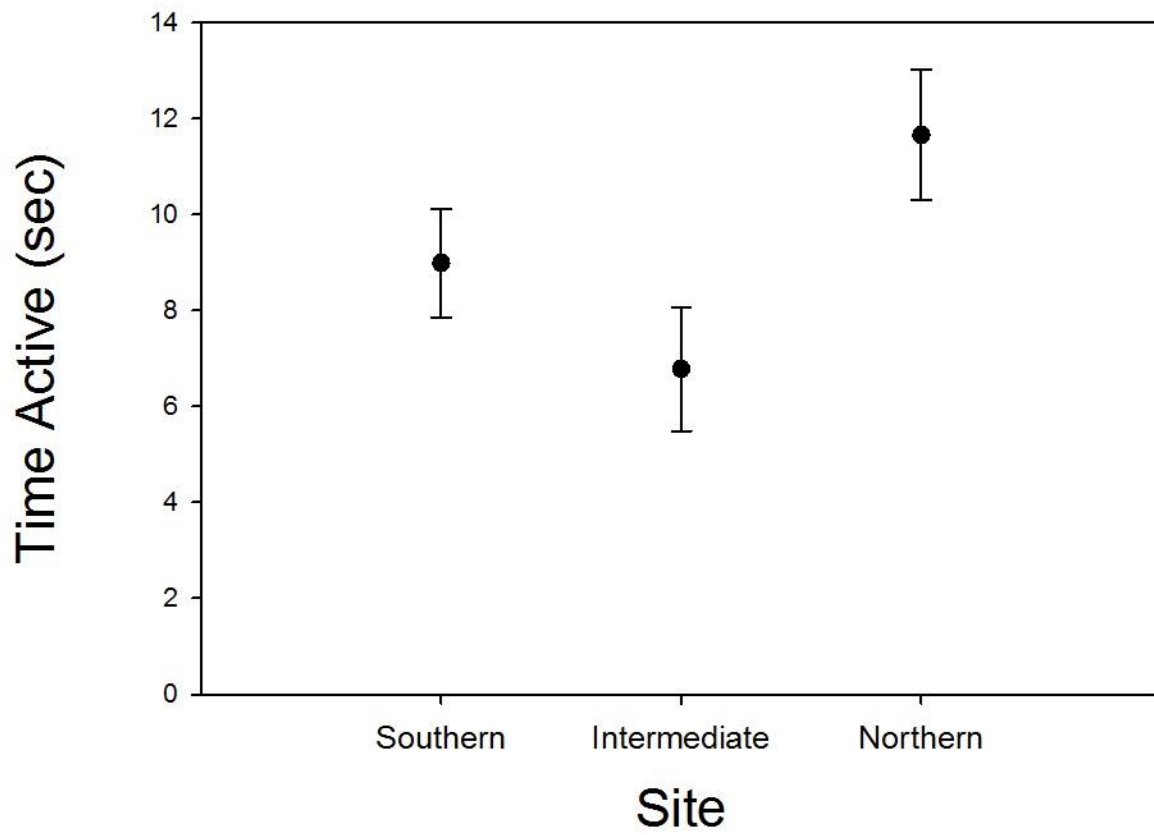


Figure 5: Activity of salamander larvae from the intermediate pond was significantly lower than activity of salamander from other locations. Symbols indicate mean  $\pm$  SEM.

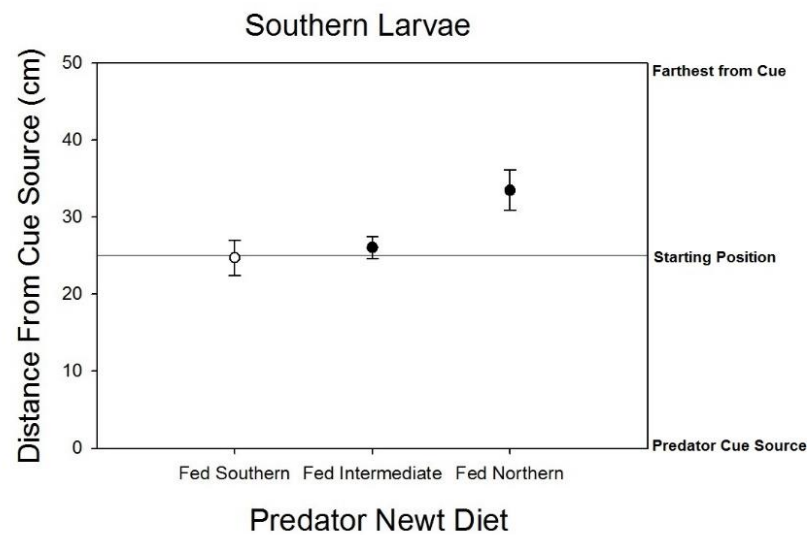
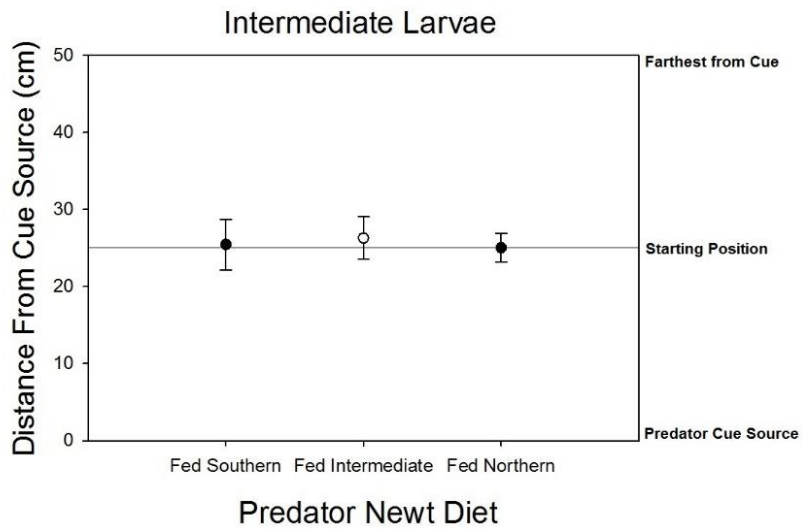
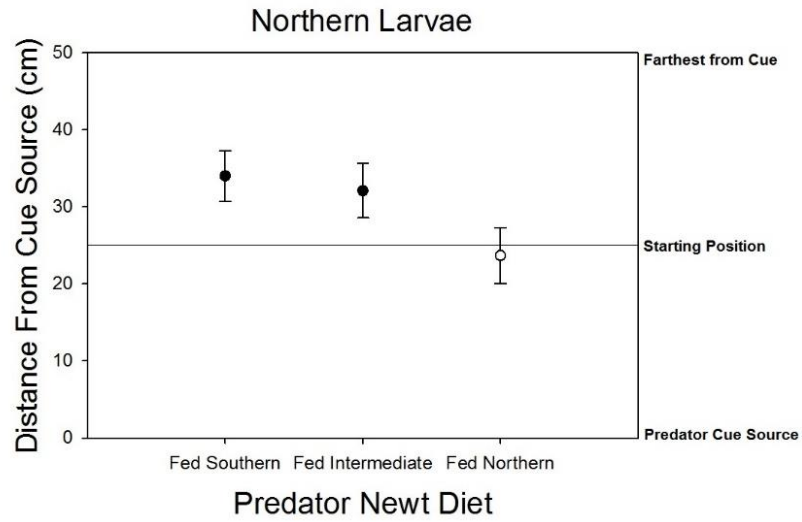


Figure 6: Salamander larvae from the northern pond (top) placed themselves significantly farther from the source of the chemical cue if the newt predator had been fed spotted salamander larvae from a foreign pond (i.e. southern or intermediate). Larvae from the southern pond (bottom) responded similarly, placing themselves farther from newts fed northern larvae. Open circles denote a match between home pond of larvae and origin of predator diet. Symbols indicate mean  $\pm$  SEM.

### Chapter 3:

Tradeoffs between evolved antipredator and competitive traits shape prey responses to predators across a landscape.

#### Abstract

Varying selection across a landscape can sometimes drive divergent trait evolution in prey populations. Traits evolved under specific conditions can influence performance, which ultimately may determine prey fitness. Often, this takes the form of a tradeoff, in which traits that convey an advantage under one set of conditions are a liability under other conditions. We measured behavioral, morphological, and performance traits in a common garden experiment for 24 spotted salamander populations from three latitudes that sample a gradient in predation risk from an important top predator, marbled salamander larvae. Based on previous research and theory, we developed and tested a set of *a priori* hypotheses using structural equation models. We found that the evolution of different suites of prey traits depends on both geographic and regional changes in the strength of selection from predators and intraspecific competition. We also demonstrated that these locally adapted traits affected mortality risk when prey larvae faced predatory marbled salamander larvae. At a southern site, varying predation risk from marbled salamander larvae and diving beetle larvae, as well as intraspecific competition, was linked with local trait adaptations. At an intermediate site, only intraspecific density correlated with genetically divergent traits among populations. At a northern site beyond the range of marbled salamanders, trait divergence was influenced by both diving beetle larvae and intraspecific density. Survival in the presence of marbled salamander larvae decreased on a gradient from south to north. Overall, we find evidence for local and geographic adaptations along a classic tradeoff surface of predation risk and competition.

## Introduction

Across a landscape, prey often face variation in the composition and abundance of predators (Thompson 1999; Richardson et al. 2014). These differences in predator communities can generate antagonistic selection on prey populations co-occurring with them (Abrams, 2000; Endler, 1991). The fitness cost of expressing a maladapted phenotype in the presence of a predator is often high (Gilliam & Fraser, 1987; Skelly, 1994). Assuming sufficient additive genetic variation and low disruptive gene flow, prey populations can evolve traits that convey an escape advantage in response to local predators (Laurila, 2000; Relyea, 2002b; Storfer & Sih, 1998) such that locally adapted phenotypes attain higher fitness in local compared to foreign predator communities.

Researchers commonly assess local adaptation to predators by associating genetically determined trait variation with natural selection regimes (e.g., in common garden experiments), but without testing the fitness of those traits in other selective environments. Alternatively, researchers may evaluate overall fitness differences among populations facing varying natural selection (e.g., in transplant experiments) without evaluating those traits that contribute to fitness differences. These two common approaches miss critical mechanistic linkages between traits and fitness provided by performance traits (Arnold, 1983).

In this study we evaluate connections among genetically determined trait variation, performance, and fitness outcomes under predation risk in a study that spans both local and geographic variation in predator selection regimes. Specifically, we evaluate effects of behavior, growth, and morphology on escape performance and predation survival for larvae from 24 populations of the pond-breeding spotted salamander (*Ambystoma maculatum*) distributed along a latitudinal gradient across southern New England. Prior research demonstrated a decline in



gape-limited marbled salamander larvae (*Ambystoma opacum*) from south to north across three sites (Urban and Richardson 2015). Predation risk from marbled salamander larvae also varies locally within sites (Urban, 2007a). As a result, we expect that regional and local variation in the strength of selection from marbled salamander larvae will determine variation in prey defensive traits; these, in turn, should provide a survival advantage under predation risk.

Based on natural history and previous findings in this system and others, we codify a series of predictions into a series of *a priori* structural equation models (Fig. 1a), find the best fitting model, and explore explanations for patterns of trait divergence among populations. Previous research suggests that higher foraging rate has evolved in response to marbled salamanders in order to enhance fitness by promoting growth into a size refuge (Urban and Richardson 2015). Here, we evaluate if more rapidly foraging larvae get bigger, and if this size difference confers a survival advantage in the presence of gape-limited marbled salamander larvae. Alternatively, rapid foraging could decrease growth rate if the prey spend more energy than they take in (Werner and Anholt 1993), and rapid foraging might increase predation risk by attracting the attention of visual predators (Urban, 2008a). We predicted that marbled salamander predation risk would lead to the evolution of wider heads relative to body size, because spotted salamander larvae would benefit by outgrowing the gape width of these gape-limited predators (Urban, 2007b, 2008a). We predicted that morphometric shape traits related to swimming ability, including tailfin and tail muscle area, would be positively correlated with higher velocity and faster acceleration, because large tails often improve locomotor performance in amphibian larvae (J. B. Johnson, Saenz, Adams, & Hibbitts, 2015; Teplitsky et al., 2005). Finally, we predicted that the performance traits of higher maximum swim velocity and maximum acceleration should

increase survival by allowing a larva to complete a rapid C-turn as the predator attacks, allowing it to escape from this suction-feeding predator.

In addition to the presence of marbled salamander larvae, we included data on other selective agents in our models. Though marbled salamander larvae are the focal predator in these experiments, we also consider selection from predatory, gape-unconstrained diving beetle larvae (*Dytiscus* spp.) and selection from early and late competition from conspecifics, evaluated as egg and larval density, respectively (See Figures 1b and 1c). We predicted that in populations where selection from *Dytiscus* is strong, this predator will have selected for spotted salamander larvae with small bodies, large tailfins, and large tail muscles, that swim and accelerate more quickly (Urban, 2008a, 2010; Van Buskirk & Mccollum, 2000) . In populations with selection from high levels of competition, we predicted that spotted salamander larvae would have bigger bodies overall, but would have small tails, narrow heads, and swim more slowly (Relyea, 2002a, 2004). Tradeoffs in behavior, morphology, and performance traits should affect survival in the presence of marbled salamander larvae.

## **Methods**

### *Natural History and Study Sites*

Spotted salamanders, *Ambystoma maculatum*, are relatively large, terrestrial salamanders that occur across eastern North America. In early spring, spotted salamander adults emerge from underground and migrate to temporary ponds to breed (Petranka 1998). Eggs hatch in four to eight weeks. Aquatic larvae are consumed by a variety of predators, including several predatory salamander species. Larvae undergo metamorphosis and leave the ponds in late summer, when temperatures warm and the ponds begin to dry. For this study, we examined 24 populations of spotted salamanders at three sites across a latitudinal gradient in southern New England, USA in

2010 (Fig. 2). We will refer to the sites as southern (Northford, CT); intermediate (Yale-Myers Forest in Union, CT), and northern (Pisgah State Park in Winchester).

Larvae of another species of salamander, the marbled salamander (*Ambystoma opacum*), are an important predator on spotted salamander larvae when the two species co-occur (Urban 2007c). Unlike spotted salamanders, marbled salamanders breed in the fall, and the larvae hatch and overwinter under the ice. When spotted salamander larvae hatch in the spring, they are preyed upon by the much larger marbled salamander larvae. Predation occurs until spotted salamander larvae reach a size refuge by outgrowing the gape limit of marbled salamander larvae (Urban 2008). The density and occurrence of marbled salamanders in temporary ponds decreases from south to north in New England, and their northern range boundary is at the border between Massachusetts, New Hampshire, and Vermont. Marbled salamanders only rarely occur north of this boundary, and we have never recorded this species at our northern study site in New Hampshire. Within the southern and intermediate sites, ponds vary in the occurrence of marbled salamanders based on the availability of overwintering habitat (Herstoff and Urban 2014).

#### *Common Garden Experiment*

In the spring of 2010, we collected five freshly-laid spotted salamander egg masses from each of the twenty-four ponds and raised them in a common garden to limit environmental effects. After collection, two equal-sized sections were cut from each egg mass with a sterilized scalpel. Each of our three sites was represented by eight ponds, each pond by five families, and each family by two replicates (3 sites x 8 ponds x 5 families x 2 replicates = 240 experimental units). Each egg mass section was placed into an individual 19-L bucket filled with 15 cm (+/- 1 cm) of treated, aged well water. We distributed 19-L buckets into randomly arranged blocks in

an outdoor enclosure at the Spring Hill Research Compound in Storrs, CT. After hatching, spotted salamander larvae were fed wild zooplankton *ad libitum*.

Predator chemical cues were applied to all buckets of spotted salamander larvae three times weekly. Amphibian larvae raised in the presence of predator chemical cues are known to develop a suite of morphological and behavioral traits that aid survival (Benard 2006; Laurila, Pakkasmaa, and Merilä 2006; Urban 2008). To create predator chemical cues, we used marbled salamander larvae collected from two ponds at the Northford, Connecticut site. Marbled salamander predators were raised in five 68-L containers with standardized leaf litter for shelter, six individuals to a container, and were fed spotted salamander larvae *ad libitum* from a mixture of all sites and populations. The water from the marbled salamander containers was then filtered through a 150- $\mu$ m mesh screen to remove any potential food resources. In each treatment, one liter of cue water was added to each experimental unit.

### *Foraging Trials*

When spotted salamander larvae were 2-3 weeks old, we brought them into the laboratory to measure foraging rate as described in previous research (Urban and Richardson 2015). Two larval individuals from each family (10 individuals per population; 80 per site) were housed individually in 18 cm (900-mL volume) glass containers. Larvae were kept in two blocks in a temperature-controlled incubator (Percival Scientific, model I-41). To each container, we added 120 mL of chemical cue from predatory marbled salamander larvae. We then added 240 sets of the four most dominant zooplankton taxa in natural ponds: 10 cyclopoid copepods, 10 large cladocerans (mostly *Scapholeberis mucronata*), and 80 small cladocerans (*Bosmina longostiris* and *Chydorus sphaericus*). After allowing salamander larvae to feed for 24 hours, we collected

uneaten zooplankton with 80- $\mu$ m mesh, and preserved them for counting and identification. Foraging rate was measured as the biomass of prey consumed in 24 hours of salamander feeding.

### *Performance Trials*

We next quantified performance of the 240 laboratory-held larval spotted salamanders by measuring the maximum velocity ( $V_{\max}$ ) and acceleration ( $A_{\max}$ ) of the escape response. Each escape response trial took place in a Plexiglas box with dimensions 30.5 x 50 x 8.5 cm, half-filled with treated water. To elicit escape responses, we used a long probe to deliver a tactile stimulus to the experimental individual. Each larva was lightly touched at least three times. The salamander's responses were filmed from below using high-speed digital cameras (Casio Exilim EX-FH25).

Videos were analyzed using ImageJ (Rasband 2014). We chose three escape responses ("runs") from each individual to comprise a set. For each run, we determined the start frame by finding the initiation of the larva's swim response to the tactile stimulus, and the end frame by when each larva settled to the substrate after its escape swim. Each run was uploaded into ImageJ, converted into binary format, and analyzed using the SpotTracker plugin (Sage et al. 2005).

The maximum magnitudes of velocity and acceleration were calculated from ImageJ outputs of two dimensional position and time using a program we wrote in Matlab. First, we smoothed the x and y data using the loess function, which fits locally-weighted polynomials to sequences of position over time. The smoothing parameter, or span, was set to 27 frames for all analyses. The span for all datasets corresponded to 0.1125 seconds, or just over a tenth of a second, and the extent of smoothing did not vary between trials. After the loess function had smoothed each component of the position vector, we differentiated the position vector with

respect to time twice: first to yield the velocity vector, and second to yield the acceleration vector (Fitzpatrick, Benard, and Fordyce 2003). The maximum absolute magnitude of the velocity and acceleration vectors were recorded for each run, and we used the mean of each individual's three movements as its representative  $V_{\max}$  and  $A_{\max}$ .

We used linear mixed-effects models in R to look for site differences in  $V_{\max}$  and  $A_{\max}$ . We ran models with two nested random effects (family within population; population within site). Since site is a categorical variable with more than two levels, we assessed significance using maximum likelihood ratio tests.

### *Morphology*

After performance trials, we evaluated morphological variation among the three sites. We chose morphological traits that are expected to convey an advantage to amphibian larvae in the presence of predators (Van Buskirk and Schmidt 2000; Fitzpatrick, Benard, and Fordyce 2003; Van Buskirk et al. 2003; Teplitsky et al. 2005; Johnson, Burt, and DeWitt 2008; Urban 2010). These traits included: head width, tailfin area, and tail muscle area; body length; and body size. For body size measurements, we used the lateral centroid, the square root of the sum of squared distances between 16 morphological landmarks and their midpoint (Bookstein 1991). The other morphological traits were corrected for body size by using the residuals of a regression between the trait and body size. Hereafter we will use the term 'relative' to describe these size-corrected shape traits.

To quantify body shape variation among populations, we used a landmark-based geometric morphometrics approach (Bookstein 1991; Zelditch 2004). We took dorsal and lateral-view photographs of each individual using a digital camera attached to a microscope (Leica Microsystems, model DFC420), and placed morphological landmarks on each photograph using

tpsDig v2.22 (Rohlf 2015). Dorsal images were quantified with 14 landmarks, and lateral images with 16 landmarks, after Urban (2010; Fig. 3). Next, we used the Procrustes fit function in MorphoJ v 1.06d (Klingenberg 2011) to generate the consensus shape and remove variation due to scaling, rotation, and translation (Rohlf and Slice 1990; Zelditch 2004). We then used a principal components analysis (PCA) on the covariance matrix produced from the shape data to distinguish the axes of variation in body shape. Finally, we used canonical variate analysis (CVA) in MorphoJ to find shape features that maximized the separation between populations. Our CVA included a permutation test for pairwise distances, for which we ran 10,000 iterations. We visualized body shape variation in the CVA using wireframe graphs in MorphoJ at a scale factor of  $\pm 0.1$ . We used linear mixed-effects models in R to test for an effect of site on shape variables and to determine whether variation in body shape and size affects escape responses.

#### *Predation Trials*

Finally, to assess survival of spotted salamander larvae from each of the three sites, we performed predation trials at the Spring Hill Research Compound. Predation trials took place in 1135-L cattle tanks (Newell Rubbermaid, Atlanta, GA, USA) filled to 32 cm (average pond depth at that time of year) with treated, aged well water and containing a standardized amount of leaf litter to shelter the animals. In each trial, forty spotted salamander larvae from a single site were randomly selected and allowed to interact with four, randomly selected predatory marbled salamander larvae in the cattle tanks for 7 days. Surviving spotted salamanders were photographed, and the photographs were compared with those taken prior to the predation trials to identify surviving individuals using spot pattern (a method verified previously in Urban 2010). In total, we conducted six predation trials: two mesocosm experiments per site.

We evaluated survival differences versus population using a mixed-effects generalized linear model with binomial errors in R using the lme4 package (Bates et al. 2014). We evaluated residual error variance to ensure that models were not overdispersed. We used likelihood ratio tests to evaluate the significance of mixed-effects between models.

### *Structural Equation Modeling*

Structural equation modeling (SEM) provides a method to test multiple relationships among multiple factors simultaneously and use model statistics to choose best fitting models. We used piecewise SEM to test our *a priori* hypotheses about the connections among response variables (Lefcheck 2015). With piecewise SEM methods, we can model data that fails to meet the assumptions of classic SEM analysis, i.e., in our case that all responses shared the same Gaussian error structure. All estimates within our SEM were fitted using linear mixed-effects models with a Gaussian distribution, except for the survival model, where we used a linear mixed-effects model with a binomial distribution. Once again, we ran models with two nested random effects (family within population; population within site). We simplified our models in stepwise fashion, each time removing non-significant pathways until AIC<sub>c</sub> was minimized.

We first attempted to fit a piecewise SEM across all sites that would explain overall relationships between traits. Almost all traits were influenced by geographic site of origin in the best (minimum-AIC) model. However, this model did not meet the criteria of sufficient model fit for piecewise SEM, in that it did not achieve a P-value greater than 0.05. Therefore, we built separate SEMs for each site, and these models achieved good fit, according to the overall model statistics.



## Results

### *Geographic Differences*

Larvae from the three sites differed with respect to all three morphological traits we measured. Relative head width differed significantly among sites ( $\chi^2_{df=2} = 12.82$ ,  $P < 0.05$ ; Fig. 4a). Larvae from the northern sites had significantly smaller heads than larvae from other sites, while larvae from the southern and intermediate sites did not differ in head width (southern vs. northern,  $P < 0.05$ ; intermediate vs. northern,  $P < 0.05$ ; southern vs. intermediate,  $P = 0.19$ ). Relative tailfin area also differed significantly among sites ( $\chi^2_{df=2} = 24.29$ ,  $P < 0.001$ ; Fig. 4b), with larvae from the northern site having the largest tailfins and larvae from the southern site the smallest tailfins (southern vs. intermediate,  $P < 0.001$ ; southern vs. northern,  $P < 0.001$ ; intermediate vs. northern,  $P < 0.001$ ). Finally, relative tail muscle area was significantly different among sites ( $\chi^2_{df=2} = 16.381$ ,  $P < 0.01$ ; Fig. 4c). Larvae from the northern population had tail muscles 21.8% larger than larvae from other sites (southern vs. northern,  $P < 0.001$ ; intermediate vs. northern,  $P < 0.001$ ). The southern and intermediate sites did not differ in relative tail muscle area ( $P = 0.34$ ).

Larval shapes, measured by canonical variate analysis, also differed among the three sites. Lateral CV1, describing depth of the body and lateral breadth of the tail, accounted for 73.9% of the observed variance and differed significantly among the sites ( $\chi^2_{df=2} = 61.71$ ,  $P < 0.001$ ; Fig. 5a). Lateral CV1 scores were highest in the northern population (broad tails and deep bodies; intermediate vs. northern,  $P < 0.01$ ; southern vs. northern,  $P < 0.001$ ) and lowest in the southern population (narrow tails and bodies; southern vs. intermediate,  $P < 0.001$ ). Lateral CV2 scores, accounting for 23% of observed variation, also differed among the three sites ( $\chi^2_{df=2} = 47.32$ ,  $P < 0.001$ ; Fig. 5b). This shape variable appeared to describe curvature of the body, with

larvae from the southern site curved slightly upward in photographs, with head and tail pointed up (southern vs. intermediate,  $P < 0.001$ ; southern vs. northern,  $P < 0.05$ ), while larvae from the intermediate location curved slightly downward (intermediate vs. northern,  $P < 0.01$ ).

Dorsal CV1 accounted for 83.7% of observed variance in dorsal shape, and corresponded with results for relative head width. Higher dorsal CV1 scores described narrower heads that taper to the body, and lower scores described wider heads demarcated from the body (Fig 5c). The three sites all differed in dorsal CV1 scores ( $\chi^2_{df=2} = 68.25$ ,  $P < 0.001$ ). The northern population had the highest dorsal CV1 scores as compared to the other sites (southern vs. northern,  $P < 0.001$ ; intermediate vs. northern,  $P < 0.001$ ), and the southern population had the lowest CV1 scores (southern vs. intermediate,  $P < 0.001$ ).

Dorsal CV2 described head shape as well as a slight curvature of the body and accounted for 16.3% of observed dorsal variance. The three sites were significantly different in dorsal CV2 scores ( $\chi^2_{df=2} = 33.53$ ,  $P < 0.001$ ; Fig. 5d). This difference was driven by the intermediate population, which had lower dorsal CV2 scores as compared to the other sites (southern vs. intermediate,  $P < 0.001$ ; intermediate vs. northern,  $P < 0.001$ ). The southern and northern sites did not differ in dorsal CV2 scores ( $P = 0.75$ ).

Next, we compared performance traits, measured during the larval escape response. Maximum velocity differed among the three sites ( $\chi^2_{df=2} = 39.94$ ,  $P < 0.001$ ; Fig. 6a). Larvae from the southern site swam 26% slower than larvae from the intermediate or northern sites (southern vs. intermediate,  $P < 0.001$ ; southern vs. northern,  $P < 0.001$ ). Velocity did not differ between the intermediate and northern sites ( $P = 0.62$ ). Larvae from the three sites also differed in maximum acceleration, with larvae from the southern site having the lowest  $A_{\max}$ , and those

from the northern site the highest ( $\chi^2_{df=2} = 38.15$ ,  $P < 0.001$ ; southern vs. intermediate,  $P < 0.001$ ; southern vs. northern,  $P < 0.001$ ; intermediate vs. northern,  $P < 0.05$ ; Fig. 6b).

After larvae were exposed to predatory marbled salamanders, 21 southern larvae, 7 intermediate larvae, and 5 northern larvae remained. Larvae from the southern site had significantly higher survival ( $\chi^2_{df=2} = 14.92$ ,  $P < 0.001$ ; southern vs. intermediate,  $P < 0.05$ ; southern vs. northern,  $P < 0.05$ ). Larvae from the intermediate and northern sites did not differ in survival ( $P = 0.51$ ; Fig. 7).

Variation in morphological traits affected performance traits in different ways. Because maximum velocity was affected by a three-way interaction between relative tailfin area, relative tail muscle area and site, we evaluated differences separately for each site. Velocity of larvae from the southern site was higher when they had larger relative tail muscle areas ( $P < 0.05$ ), and was not affected by relative tailfin area ( $P = 0.34$ ). At the intermediate site, maximum velocity was affected by an antagonistic interaction between tailfin area and tail muscle area ( $\chi^2_{df=2} = 12.04$ ,  $P < 0.001$ ). At the northern site, there was no effect of relative tailfin area ( $P = 0.49$ ) or relative tail muscle area ( $P = 0.34$ ) on velocity. Across all sites,  $A_{max}$  was affected only by relative tail muscle area ( $P < 0.05$ ), and not by relative tailfin area ( $P = 0.82$ ).

Next, we tested the effect of foraging rate on morphological and performance traits. Foraging rate had a significant effect on body size ( $P < 0.05$ ), because a higher rate of foraging was correlated with larger size. Lateral CV2 was also significantly affected by foraging rate, in that higher rates of foraging were correlated with higher lateral CV2 scores ( $P < 0.001$ ). We found that dorsal CV2 scores were affected by a significant interaction between foraging rate and site ( $\chi^2_{df=2} = 6.08$ ,  $P < 0.05$ ). Foraging rate did not significantly affect any other morphological or performance traits. See Table 2, column 1.

### *Structural Equation Modeling*

The overall SEM model included measurements of predator and conspecific densities in each home pond as well as trait measurements made in a common garden. Traits included were: velocity, acceleration, body length, relative tailfin area, relative tail muscle area, relative head width, foraging rate, and larval survival in marbled salamander predation trials. The model with the lowest AIC<sub>c</sub> suggested that the greatest influence on morphological and performance traits was geographic site. Site significantly explained differences in relative tailfin area ( $R^2 = 0.23$ ), relative tail muscle area ( $R^2 = 0.10$ ), and relative head width ( $R^2 = 0.14$ ).  $V_{\max}$  was explained by a combination of body length and site ( $R^2 = 0.41$ ); acceleration by a combination of tail muscle area and site ( $R^2 = 0.27$ ); and body length by a combination of foraging rate and site ( $R^2 = 0.33$ ). Neither foraging rate nor survival were dependent on site. Instead, in this model, foraging rate was explained by local marbled salamander occurrence and density of conspecifics ( $R^2 = 0.15$ ), and survival was explained by larval density, acceleration, and body length ( $R^2 = 0.12$ ). Because of the prevalence of significant site effects in this model, and because of its poor overall fit ( $P = 0$ ), we built individual site-specific SEM models.

### *SEMs by Site*

For each site, we chose the structural equation models with the lowest AIC<sub>c</sub> value and an overall  $P$  value greater than 0.05. At the southern site, all three selective factors — marbled salamander occurrence, *Dytiscus* occurrence, and larval/egg density — affected measured traits (Model fit  $P = 0.43$ ; Fig. 8). High incidence of marbled salamanders in the home pond was positively correlated with foraging rate ( $P < 0.05$ ); high incidence of *Dytiscus* in the home pond was positively correlated with relative tailfin area and acceleration ( $P < 0.05$ ); and more competitors in the home pond was positively correlated with body size ( $P < 0.05$ ). The

minimum-AIC<sub>c</sub> model at this site also suggested that foraging rate measured in the common garden experiment was positively correlated with body size ( $P < 0.001$ ); body size and relative tail muscle area were positively correlated with velocity ( $P < 0.01$ ); and relative head width was negatively correlated with acceleration ( $P < 0.05$ ). Body size was negatively correlated with survival in marbled salamander predation trials ( $P < 0.05$ ).

In the best-fitting model at the intermediate site, conspecific larval density and egg density were the only selective factors influencing observed traits (Model fit  $P = 0.11$ ; Fig. 9). These measures of competition were positively correlated with foraging rate ( $P < 0.01$ ), but negatively correlated with velocity ( $P < 0.05$ ), acceleration ( $P = 0.08$ ), and survival in marbled salamander predation trials ( $P = 0.15$ ). Additionally, relative tail muscle area was negatively correlated with acceleration ( $P < 0.01$ ), and relative tailfin area was positively correlated with survival in marbled salamander predation trials ( $P = 0.32$ ).

At the northern site, the best-fitting model included only conspecific larval/egg densities and *Dytiscus* prevalence (Model fit  $P = 0.058$ ; Fig. 10). Marbled salamanders do not occur at this site. Within the northern site, high incidence of *Dytiscus* larvae was negatively correlated with head width ( $P = 0.11$ ) and with survival in marbled salamander predation trials ( $P = 0.22$ ). High *Dytiscus* incidence was positively correlated with relative tail muscle area ( $P < 0.05$ ). High levels of competition were negatively correlated with foraging rate ( $P < 0.01$ ) and with relative tailfin area ( $P = 0.18$ ). The best-fitting model also suggested that foraging rate was positively correlated with body size ( $P < 0.001$ ) and that body size was positively correlated with maximum velocity ( $P < 0.05$ ). Additionally, foraging rate, relative tail muscle area, and maximum acceleration were all negatively correlated with survival in marbled salamander predation trials ( $P = 0.2$ ;  $P = 0.09$ ;  $P = 0.1$ , respectively).

## Discussion

Prey animals face a multitude of selective pressures that could affect their behavior, morphology, and performance in different, sometimes opposing, ways (Sredl and Collins 1992; Abrams 2000; Relyea 2002c). Varying suites of selective pressures across a landscape can result in prey animals adapted to local conditions (Kawecki and Ebert 2004; Hereford 2009). Tradeoffs result when investment in traits that allow survival under one set of conditions impact survival under other conditions (Lima and Dill 1990; Kats and Dill 1998; Anholt and Werner 1995; Tollrian and Harvell 1999; Relyea 2002a; Relyea 2002c; Relyea 2004) .

One such tradeoff is between predation-induced responses and competitor-induced responses. This tradeoff can operate through alteration of activity level. Higher activity may allow prey to forage more, increasing competitiveness, but could make prey more susceptible to predators (Anholt and Werner 1995; Relyea 2002a; Relyea 2002c). Conversely, the presence of predators often results in reduced prey activity, which comes with reduced competitive ability (Werner and Anholt 1993; McNamara and Houston 1994). Investment in antipredator morphologies may allow prey more resistance to predation but may affect competitive ability by slowing growth (Van Buskirk et al., 1997; Van Buskirk & Relyea, 1998; Relyea & Werner, 2000; Relyea, 2002c)

In this study, we evaluated the effect of marbled salamander predator prevalence on behavior, morphology, performance, and survival among 24 populations of spotted salamanders from three sites across New England. We also tested exploratory hypotheses about the effect of another common, though functionally different, predator: the *Dytiscus* diving beetle; and the effect of larval and egg mass density on these traits. However, in this experiment, we can only evaluate non-induced genetic variation among spotted salamander populations, and we miss

variation induced by cues from *Dytiscus* and competitor cues. Since all experimental larvae were reared in the presence of marbled salamander chemical cues, our insights about responses to *Dytiscus* and competitors are limited.

Though we formulated one prediction for all sites, each site differed to such a degree that an overall model did not fit well. Individual site models did meet model fit criteria and therefore we focus on these results. Our findings suggest that traits and their relationships with each other shift appreciably among geographic locations.

### *Site Differences*

At the southern site, all three selective agents influenced some aspects of observed traits. As previously reported, high densities of marbled salamander predators were correlated with higher foraging rate (Urban and Richardson 2015), though only 10% of foraging rate variation among individuals was explained (Fig 8). We predicted that increased marbled salamander prevalence in the natal environment would result in the evolution of increased foraging as prey grew to reach a size refuge from this gape-limited predator (Fig. 1a). This prediction was supported at the southern site, where higher densities of marbled salamanders were associated with increased foraging, which in turn was associated with increases in body size (Urban 2007b). High conspecific densities at this site also contributed to increased body size ( $R^2 = 0.20$ ), presumably related to the need for crowded individuals to reach a larger size and metamorphose quickly (Werner and Anholt 1993). Larger bodies and larger relative tail muscle areas were correlated with faster swimming (higher maximum velocity), as suggested by other studies. Larger relative head width was negatively correlated with maximum acceleration, which might occur if a narrower head reduces drag in the water, allowing for a faster escape response (Fitzpatrick et al., 2003).

Also at the southern site, increased prevalence of gape-unconstrained *Dytiscus* larvae at natal ponds was associated with larger relative tailfin areas in larval spotted salamanders. This trait could be serving two functions against *Dytiscus* predators. Aquatic predators often select for traits that allow prey to improve escape performance (Van Buskirk and Mccollum 2000; Fitzpatrick, Benard, and Fordyce 2003; Teplitsky et al. 2005). Alternatively, increased tailfin area could serve as a target, enticing predators to attack a non-lethal part of the prey's body (Van Buskirk et al. 2003).

*Dytiscus* larvae and marbled salamander larvae differ in modes of predation, which may select for different suites of traits in prey exposed to both predators. To defend against the gape-limited marbled salamander larvae, it is advantageous to be big and fast, with a wide head (Urban, 2007b, 2008). *Dytiscus*, however, is a gape-unconstrained piercer/sucker, so a large body size can actually be a liability to spotted salamander prey. Instead, it helps to have a big, broad, strong tail to speed away from these invertebrate predators (Van Buskirk & Mccollum, 2000; Urban, 2008, 2010). Our results suggest that at the southern site, the interplay between these forces may have influenced larval survival in predation trials, since larvae from this site survived the best in the presence of marbled salamander larvae.

Larvae from the intermediate site had an entirely different suite of traits in response to selective pressures (Fig 9). At this site, we found no significant effect of either predator species. Instead, conspecific larval density and egg mass density (which is an indicator for high larval density immediately after hatching) were correlated with observed trait responses. A previous study (Urban and Richardson 2015) found that the intermediate site is dominated by responses to both competition and marbled salamander predation. At this site, high conspecific larval density decreased both maximum acceleration and maximum velocity. This finding suggests a tradeoff



between investment in the means to escape from predators and investment in traits that make for better foragers. We suggest that the observed effect may be indirect; larval density may have affected some unmeasured trait, which in turn affected performance. The dotted line on our predictions (Fig. 1c) suggests just this relationship, since we do not presume to know all traits that affect performance. We predicted a negative relationship, because speed would not provide larvae with an advantage against competitors. High egg mass density was positively correlated with foraging rate, but was negatively correlated with survival in the presence of marbled salamander larvae. This result suggests a tradeoff in trait responses to high densities of competitors, which may have left these larvae ill-equipped to survive in the presence of marbled salamanders.

At the intermediate site, relative tailfin area was positively correlated with survival in the presence of marbled salamanders. Here, tailfin area was not related to competition, yet having a larger tail may have provided an advantage against predators. This fits with our predictions about performance traits that allow for survival in the presence of predators.

Finally, at the northern site, a combination of *Dytiscus* prevalence and conspecific density suggested an explanation for observed traits (Fig. 10). This site is above the northern range limit of marbled salamanders (Herstoff and Urban 2014), which could account for the extreme body shape and performance differences observed between northern larvae and larvae from other sites. At this site, the prevalence of *Dytiscus* was correlated with larger tail muscles and smaller heads, presumably to increase speed and decrease drag.

High densities of conspecific larvae in the home pond were correlated with decreased foraging rate, which is the opposite of what we observed at the intermediate site. This finding agrees with previous observations of this site (Urban and Richardson 2015). The differences in

foraging rates in response to competitors between sites are likely due to resource availability. As described in Urban and Richardson (2015), theory suggests that foraging rate depends on overall resource environment as well as local abundance of resources (Sih 1984). Variation in resource environments, then, might drive observed foraging behavior at these sites. Larvae from the northern site, perhaps because of adaptation to the presence of invertebrate predators and conspecific densities, had the lowest survival in the presence of marbled salamanders. Also at this site, high egg mass density was negatively correlated with relative head width. This agrees with our prediction that in the presence of high levels of competition, salamander larvae should increase foraging and increase their overall body size, rather than investing in defensive morphological traits (Fig 1c). The negative correlation between head width and egg mass density also fits into a tradeoff scenario, in which wider heads limit predation by gape-limited predators, but require investment which removes resources from adaptations to competitors.

Foraging rate at the northern site was correlated with a number of other traits. Higher foraging rates were correlated with larger bodies and wider heads. Additionally, foraging rates were negatively related to survival, in that animals that foraged more survived less. This result is expected under selection from visually-oriented predators, since increased foraging would only make them more noticeable (Anholt and Werner 1995). The presence of *Dytiscus* in ponds at the northern site was correlated with spotted salamander larvae that had large tail muscles, possibly to equip them for a faster escape response, and smaller heads. Since this predator is gape-unconstrained, a wide head is not an advantage, and will only add drag to an animal trying to make a quick escape (Fitzpatrick et al., 2003). At the northern site, having a larger body was correlated with having higher velocity, and animals with larger tail muscles tended to survive less well, though this relationship was not significant.

### *Tradeoffs*

The interaction between predation and competition is complex, and can shape amphibian populations and communities (Wilbur 1972; Wilbur 1980). Without predators, competition can grow so intense that growth slows, resulting in smaller size at metamorphosis (Brockelman 1969). Competition can lead to the evolution of adaptive plasticity in animals that are better at foraging in low resource conditions, but are susceptible to predators (Anholt and Werner 1995; Welborn, Skelly, and Werner 1996; Relyea 2002c; Relyea 2002a). Conversely, predation can select for morphological and performance changes in amphibian larvae that protect them from predation, but at the cost of competitive ability (Lima and Dill 1990; Kats and Dill 1998; Tollrian and Harvell 1999). Our findings illustrate the population-level consequences of tradeoffs between predation and competition in spotted salamanders.

Across a geographic landscape in New England, spotted salamander larvae face many species of predators at different densities, each of which drives selection for particular traits. At the same time, high densities of conspecifics in some populations add different selection pressures. In this experiment, salamanders at the three sites demonstrated possible tradeoffs between adaptations for dealing with predators and dealing with competitors. Different tradeoffs were suggested among sites, yet also among populations within sites, implying great spatial variation at both large and small scales across a landscape.

### *Conclusions*

Here, we looked at how predators and competition shaped trait responses in spotted salamander larvae. On a gradient from south to north across this region, prevalence of marbled salamanders and overall density of gape-limited predators declines (Urban 2007c; Urban 2008). Correspondingly, only our southern site provided evidence consistent with the predicted effects

of selection from gape-limited predators. We found the suggestion of adaptation to intraspecific competition at all sites, but evidence was strongest at the intermediate site. Tradeoffs between competition and predation at each site may ultimately affect survival in the presence of a gape-limited predator.

This study provides a detailed look at how predation and competition may interact across a geographic landscape. To adapt to one set of conditions or another means a tradeoff for prey animals. Studies like these are crucial to understanding and predicting novel interactions as changing conditions affect selective factors and bring new species into contact.

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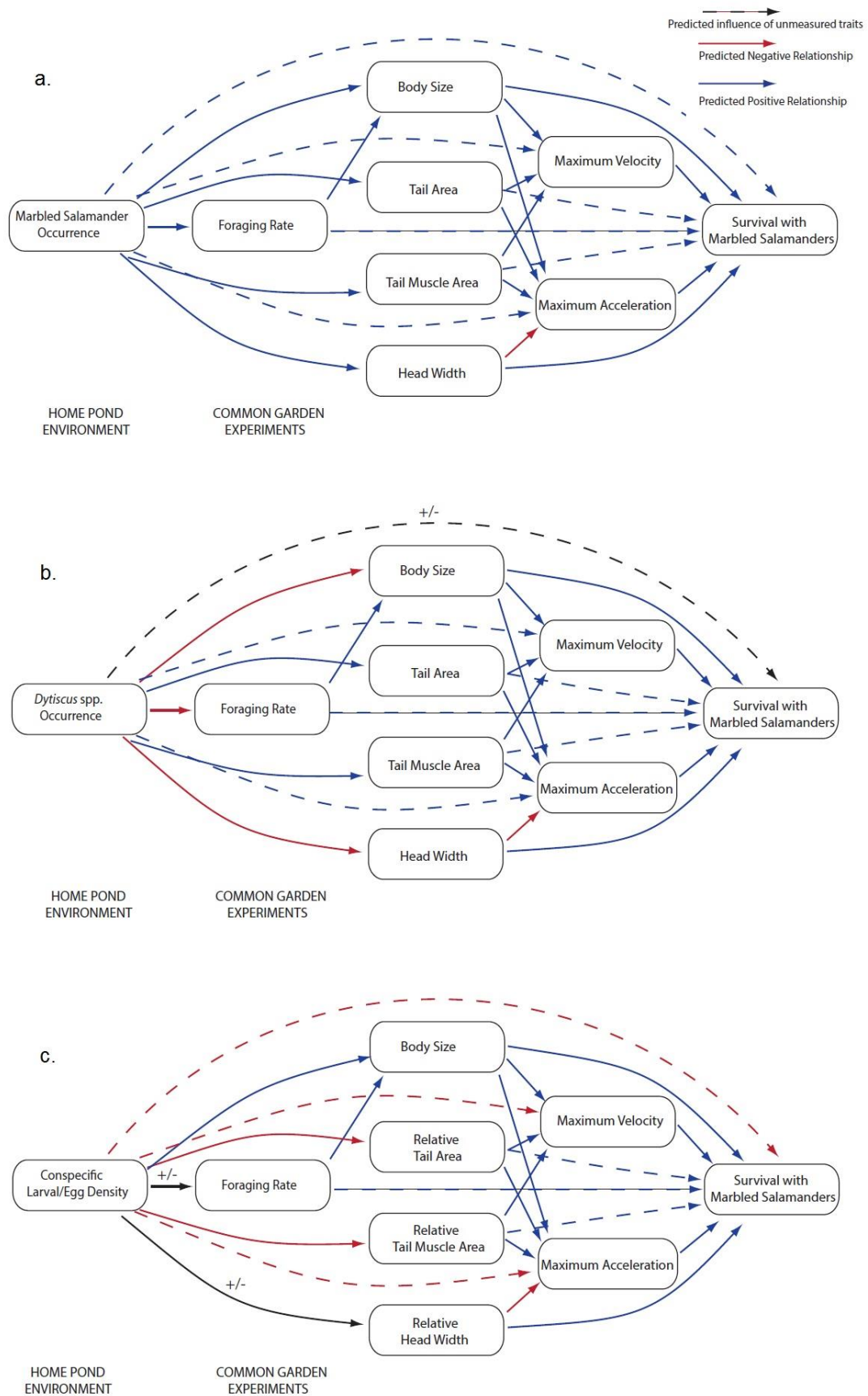


Figure 1: Specific predictions for the evolution of spotted salamander behavioral, morphological, and performance traits under selection from: a) predatory marbled salamander larvae; b) predatory diving beetle larvae, *Dytiscus* spp.; and c) conspecific larval and egg densities. All selective factors were measured in the 24 ponds under study; traits and survival were measured in a common garden experiment. Red lines are predicted negative relationships, blue lines are predicted positive relationships. Dotted lines are the predicted influence of unmeasured traits, meaning that given what we know from the literature and from theory, we predict an effect on those traits, but want to account for the fact that we may not have measured the correct intermediary.

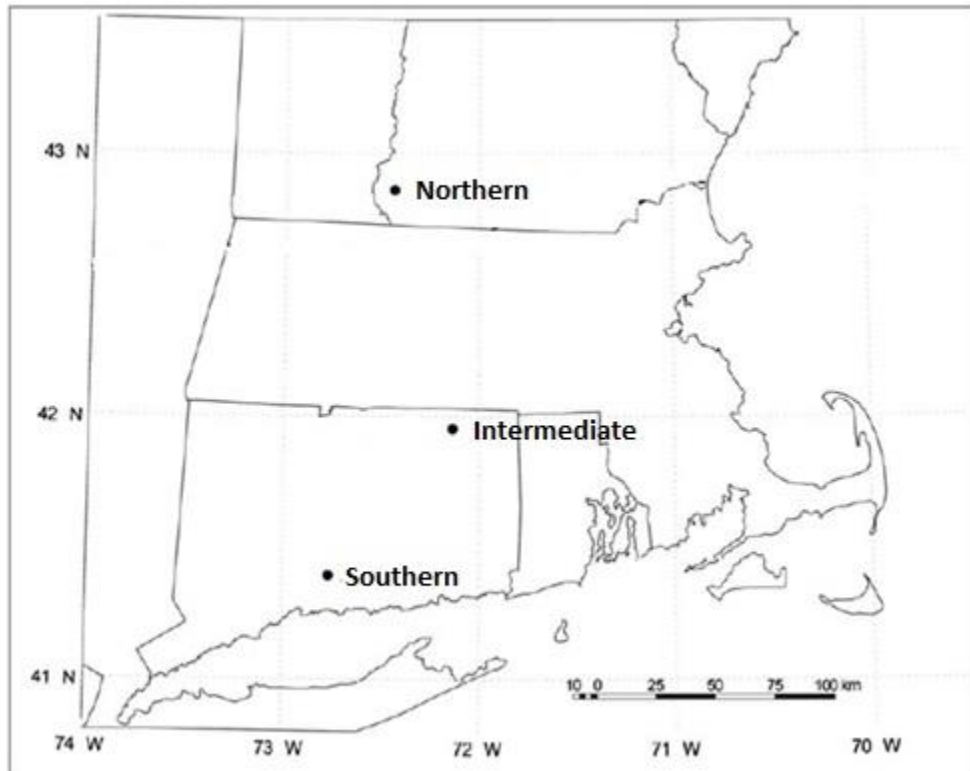


Figure 2: Map of Study Locations (Adapted from Urban 2008).

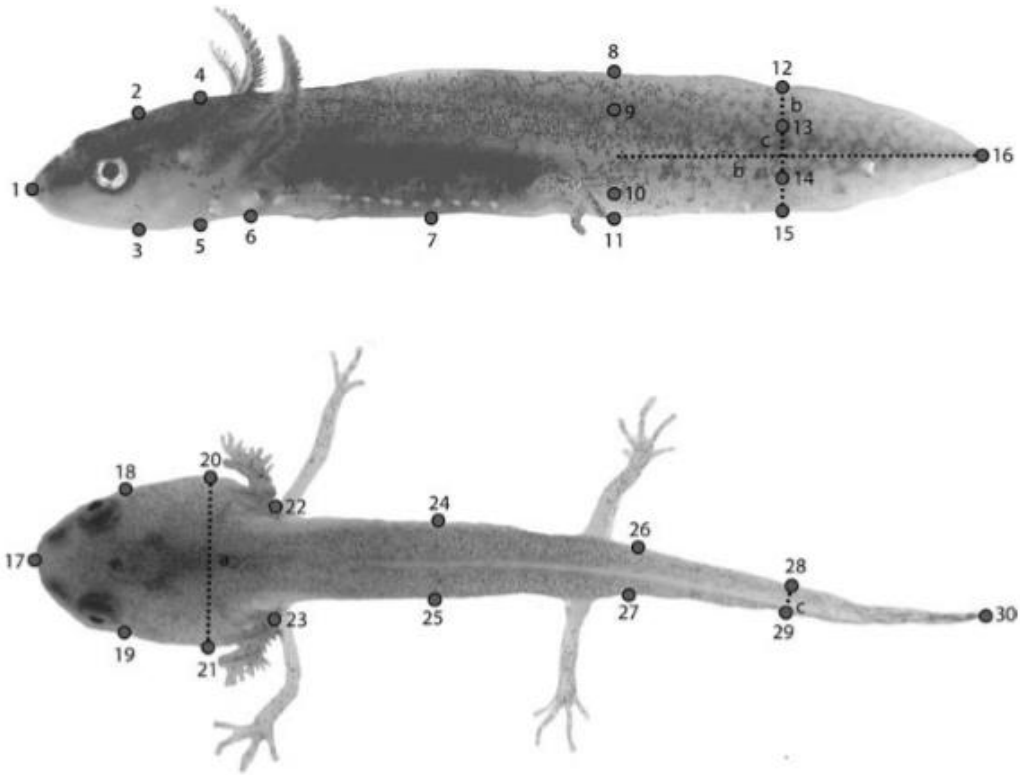
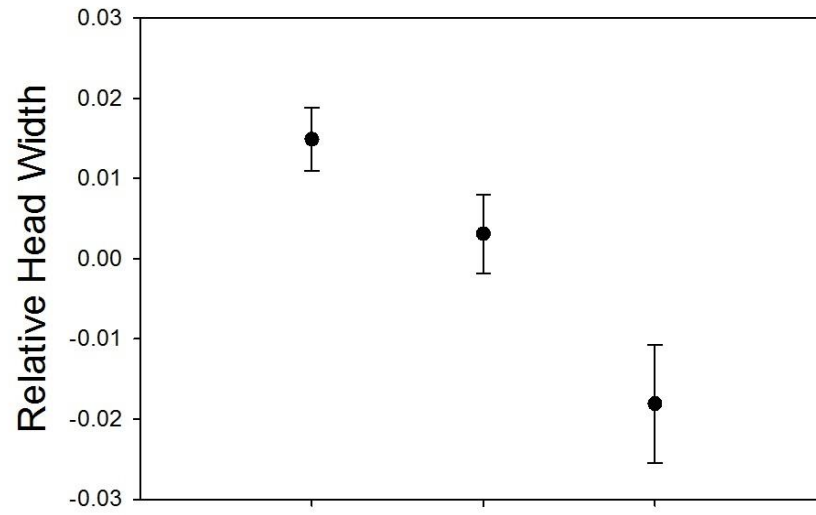
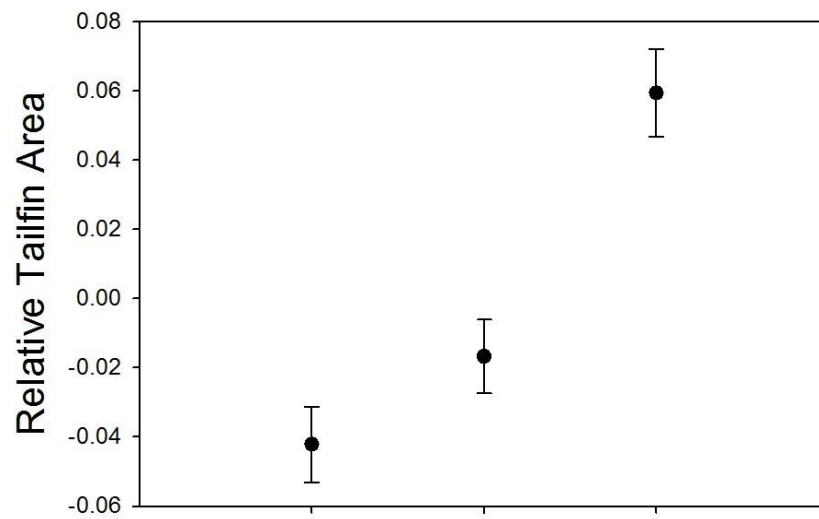


Figure 3: Morphological landmarks used to describe the body shapes of spotted salamander larvae, after Urban, 2010. Numbers indicate landmarks, and lettered lines indicate measurements used to calculate traits used in analyses.

a.)



b.)



c.)

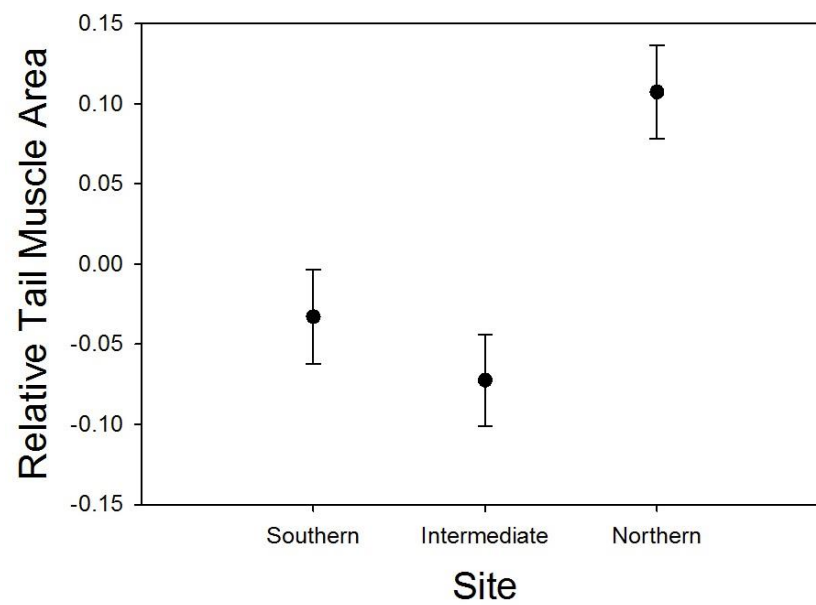


Figure 4: Morphological trait differences among sites. All morphological traits are residuals after correcting for body size. From top to bottom: a.) larvae from the northern site had significantly narrower heads than larvae from the intermediate or southern sites; b.) relative tailfin area differed significantly among all three sites; and c.) larvae from the northern site had significantly larger tail muscle area than larvae from the other sites.

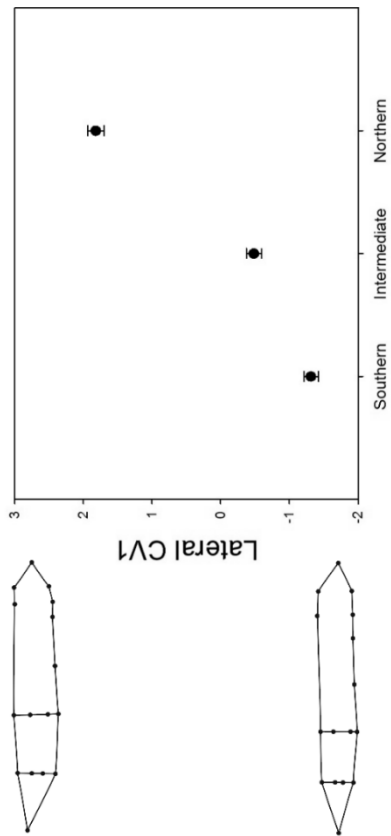
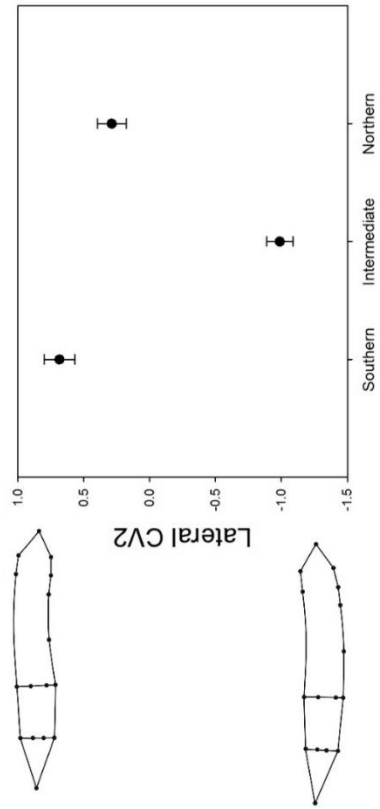
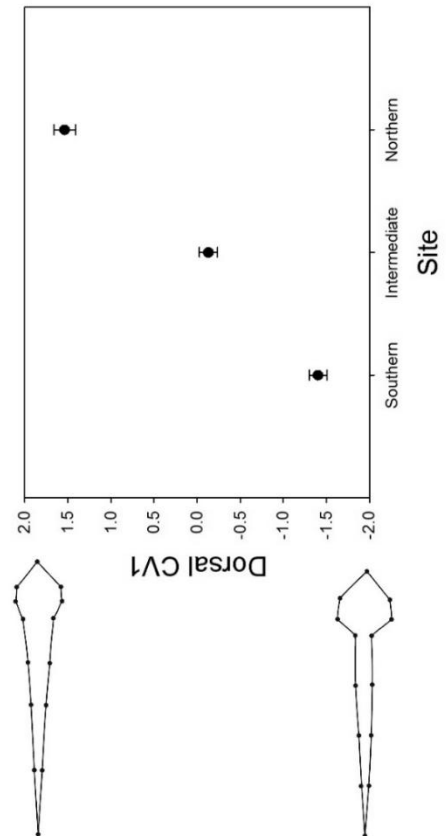
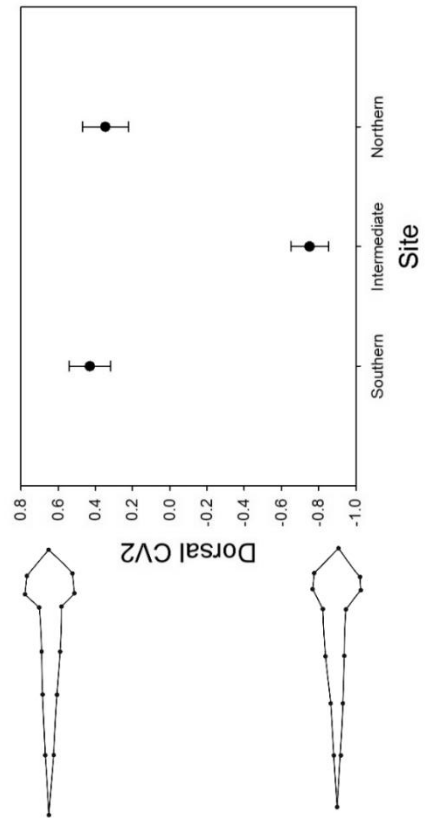
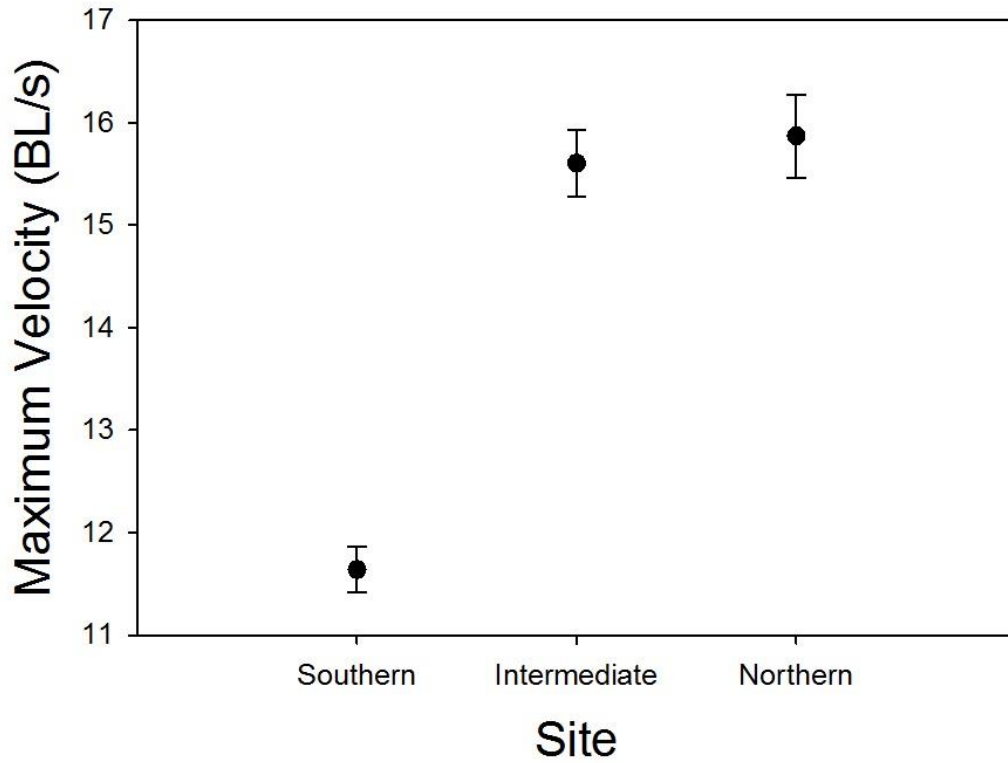


Figure 5: Patterns in body shape divergence among sites. Values are canonical function analysis (CVA) scores for each site, which describe the axis of variation that best distinguished forms among sites. Figures along the y-axis represent extreme cases of shape variation according to the CVA. a.) Lateral CV1 differed among all sites, and explained 73.9% of the observed variance in lateral shape. b.) Lateral CV2 also differed among all sites, and explained 23% of observed variance. c.) Dorsal CV1 differed among all sites, and accounted for 83.7% of observed variance in dorsal shape. (d.). The intermediate site had a significantly lower dorsal CV2 score as compared to the other sites. Dorsal CV2 explained 16.3% of observed dorsal variance.



a.)



b.)

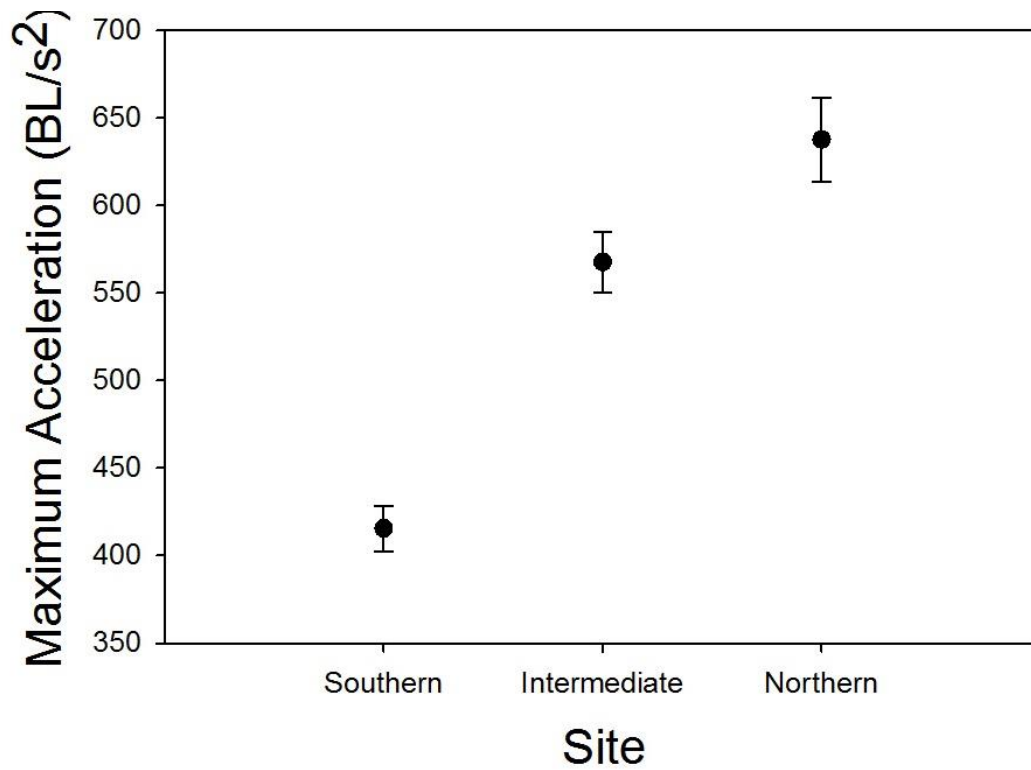


Figure 6: Performance variable differences by site. Larvae from the southern site swam significantly more slowly than those from other sites. Acceleration differed among all three sites.

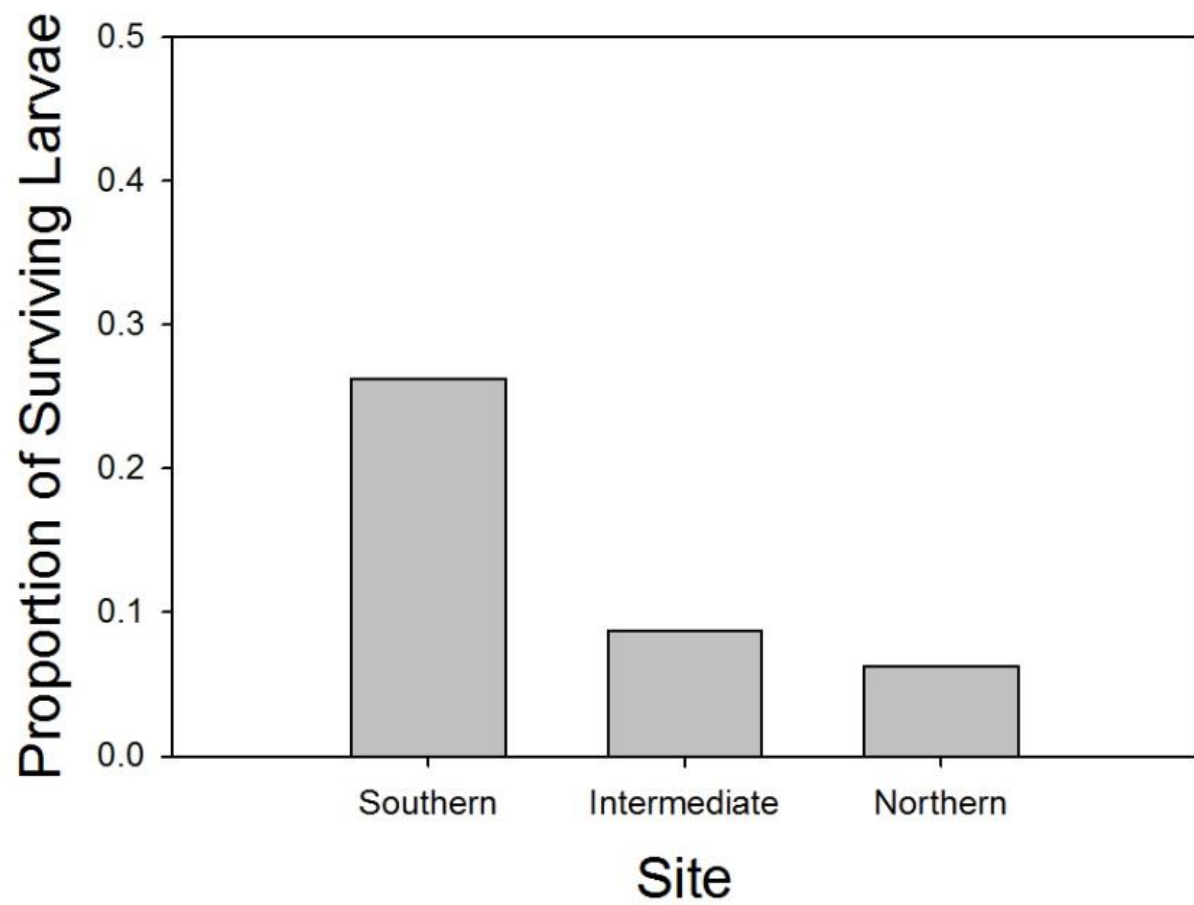


Figure 7: In the presence of marbled salamander predators, spotted salamander larvae from the southern site survived significantly better than larvae from the other sites.

Table 1: The effect of larval foraging rate, local predator incidence, predator density, conspecific density, and egg mass density on spotted salamander morphological traits and shape variables. All numbers are P-values.

	Foraging Rate	Marbled Sal. Density	<i>Dytiscus</i> Incidence	<i>Dytiscus</i> Density	Conspecific Density	Egg Mass Density
$V_{\max}$	0.30	0.77	0.30	0.72	0.38	0.87
$A_{\max}$	0.92	0.94	0.91	0.68	0.81	0.55
Relative Head Width	0.62	0.71	0.23	0.95	0.53	0.19
Relative Tailfin Area	0.26	0.50	0.79	0.18	0.24	0.60
Rel. Tail Muscle A.	0.45	0.67	0.16	0.48	0.69	0.76
Lateral Centroid	*P < 0.05	0.98	0.55	0.85	0.44	0.92
Lateral CV1	0.71	0.56	0.21	0.32	0.99	0.32
Lateral CV2	**P < 0.001	0.47	*Site x <i>Dyt</i> Inc P < 0.05	0.54	0.12	0.55
Dorsal CV1	0.99	0.23	0.44	0.82	0.53	0.44
Dorsal CV2	*Site x foraging P < 0.05	0.20	0.58	0.11	0.47	0.11

## Southern Site SEM Results

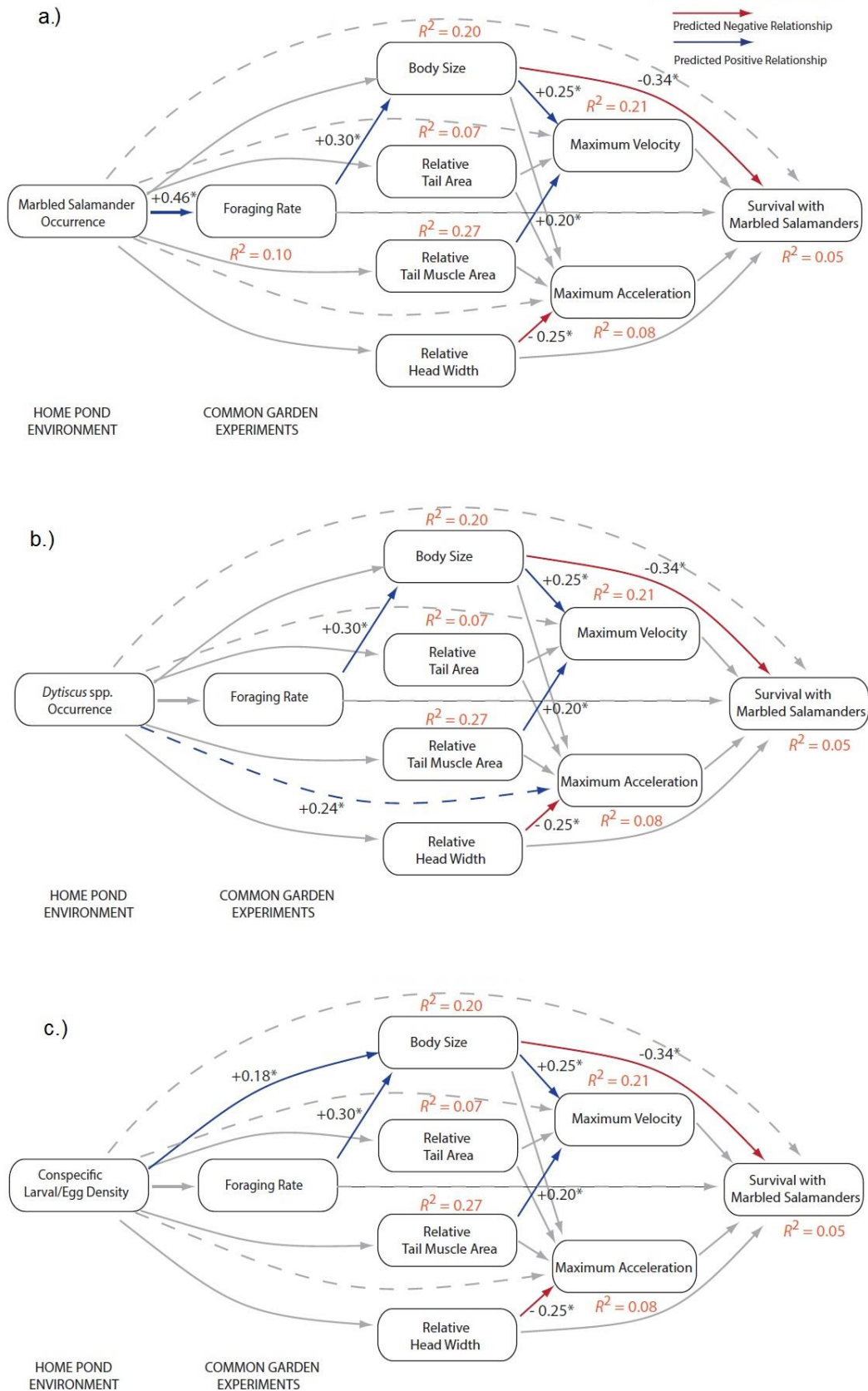


Figure 8: SEM results for the southern site. Traits possessed by larvae from this site were driven by selection from a.) marbled salamander larvae; b.) *Dytiscus* prevalence; and c.) conspecific larval and egg density.

## Intermediate Site SEM Results

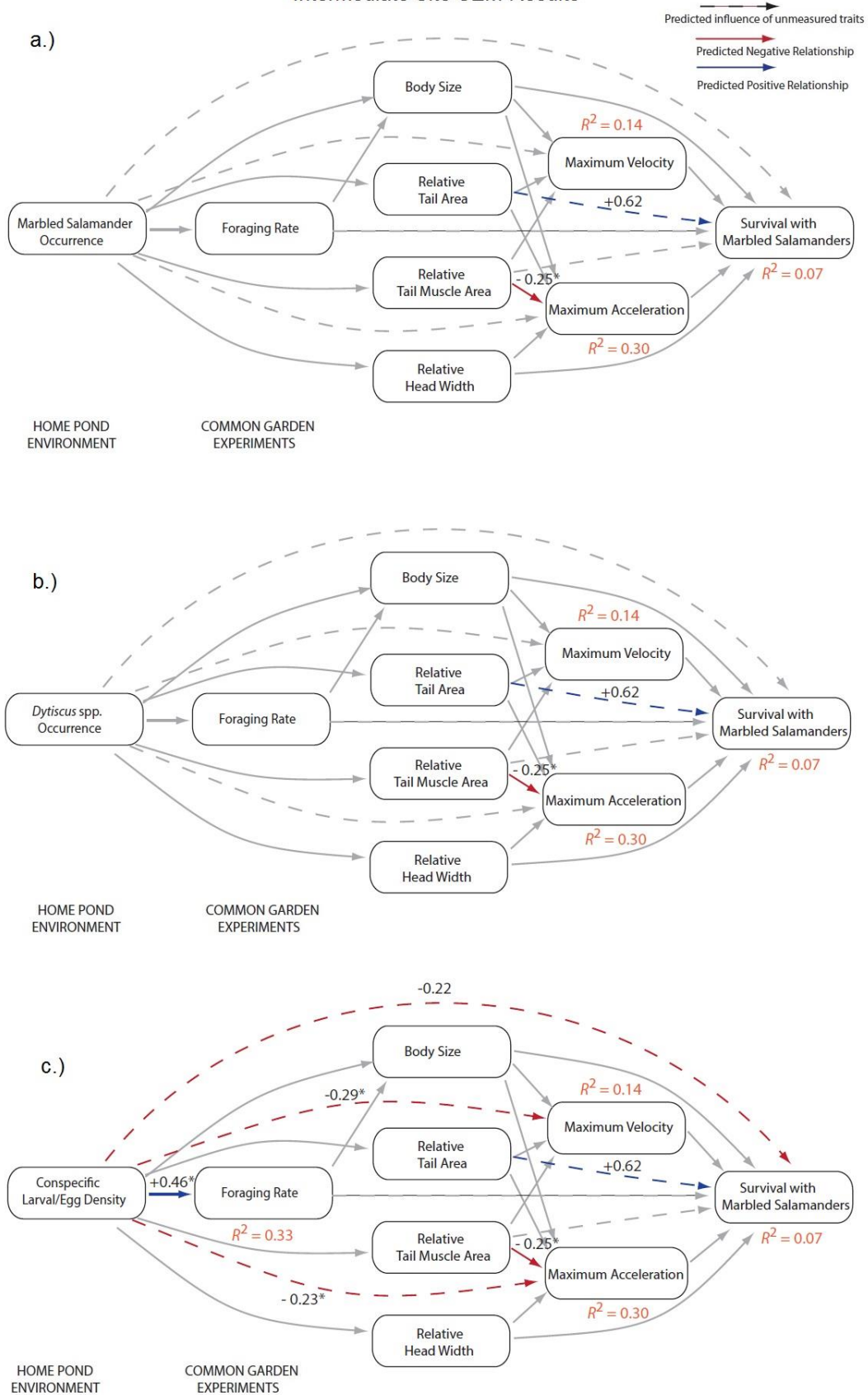


Figure 9: SEM results for the intermediate site showed that traits of larvae from this location were not affected by a.) marbled salamander prevalence or b.) *Dytiscus* occurrence in their home environments, but entirely by c.) conspecific densities.

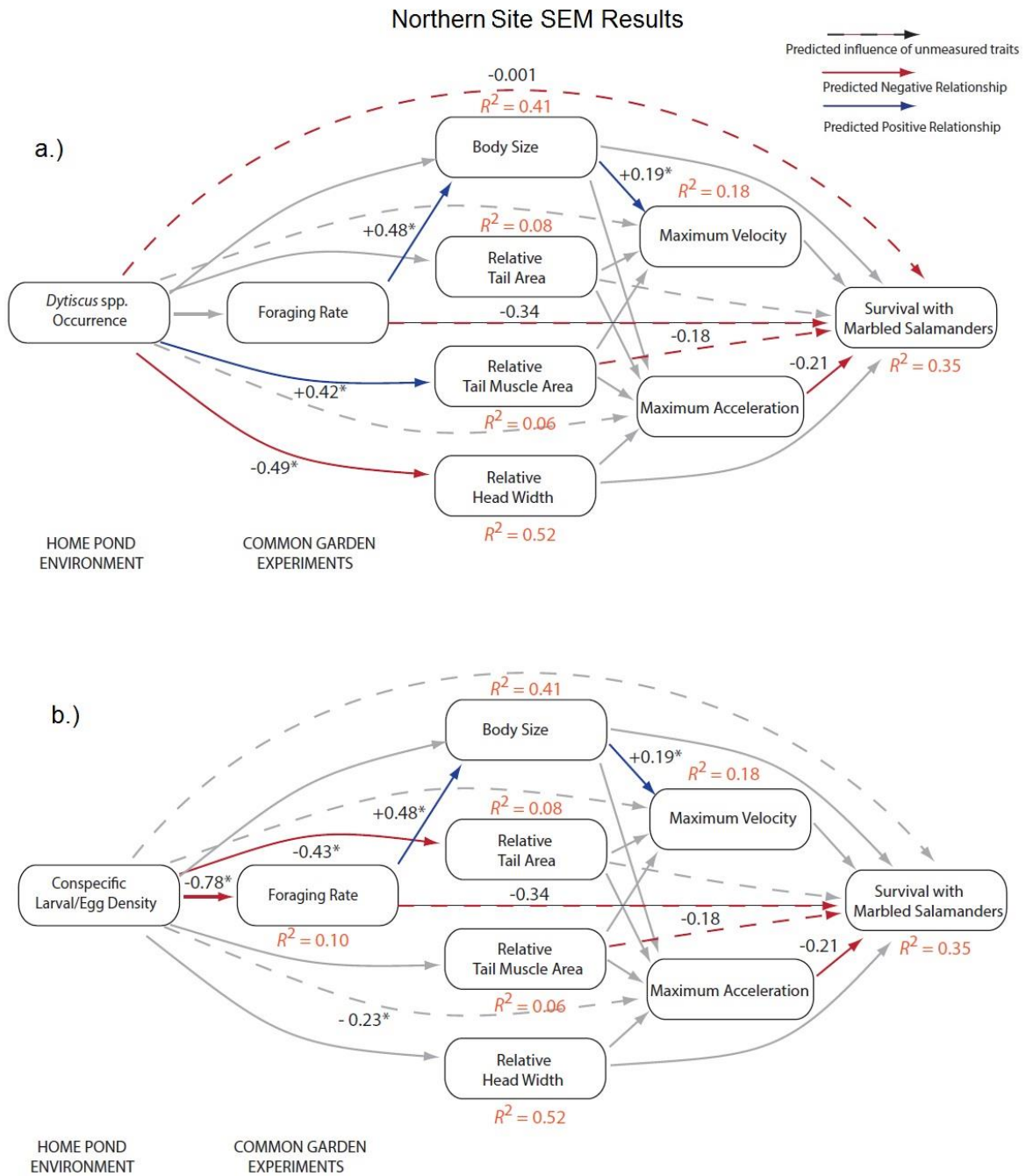


Figure 10: Larvae from the northern site experience no threat from marbled salamander larvae in their home ponds. Selection for traits was due to a.) *Dytiscus* occurrence and b.) conspecific densities.