

2016

Chemically-mediated prey responses: invasive brook stickleback induced changes in behavior and life history of larval long-toed salamanders

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CHEMICALLY-MEDIATED PREY RESPONSES:
INVASIVE BROOK STICKLEBACK INDUCED CHANGES IN
BEHAVIOR AND LIFE HISTORY OF LARVAL LONG-TOED SALAMANDERS

A Thesis

Presented To

Eastern Washington University

Cheney, Washington

In Partial Fulfillment of the Requirements

for the Degree

Master of Science in Biology

By

Rena K. Reed

Spring 2016

THESIS OF RENAE REED APPROVED BY

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ABSTRACT

The purpose of this research was to assess the predator-induced responses of a larval amphibian to its natural predators and to an unfamiliar fish. Amphibians express chemically-mediated antipredator defenses in behavior, morphology and life history, and are currently threatened with predation by invasive fish. To investigate this issue, we first initiated a behavioral assay to test the null hypotheses that predator type and diet have no effect on long-toed salamander (*Ambystoma macrodactylum*) behavior. We exposed individual replicate *A. macrodactylum* to chemical cues (kairomones) from garter snakes (*Thamnophis elegans*), tiger salamanders (*A. tigrinum*) and brook stickleback (*Culaea inconstans*), which had been fed a larvae-diet of *A. macrodactylum* or a null-diet of earthworms (*Lumbricus terrestris*), and measured subsequent changes in *A. macrodactylum* behavior. *A. macrodactylum* decreased activity in response to their native predators, and to a greater degree if the predators were fed *A. macrodactylum*. Larvae increased activity in response to null-diet fish, but decreased activity in response to larvae-diet fish, indicating the use of a diet cue to identify a potential threat. We then conducted a conditioning experiment to test the null hypothesis that repeated exposure to *C. inconstans* kairomones with larvae-diet cues would not affect *A. macrodactylum* behavior when later exposed to the predator kairomones alone. *A. macrodactylum* were repeatedly exposed to kairomones from larvae-diet *C. inconstans* and later tested for a response to null-diet *C. inconstans*. Conditioned *A. macrodactylum* decreased their activity in response to fish kairomones alone, indicating they were able to learn adaptively through the use of the diet cue. We believe this is the first example of diet-dependent learning in an amphibian-fish model. During the conditioning experiment, we also measured for change in morphology, growth and development towards metamorphosis, an important life history event, as indicators of other predator-induced plastic responses. While we did not detect a significant difference in morphology or growth, conditioned *A. macrodactylum* reached the final stage of metamorphosis at an accelerated rate. We suggest *A. macrodactylum* is able to use a diet cue for predator labelling and learning, and to make potentially beneficial adjustments to its life history.

ACKNOWLEDGEMENTS

I would like to thank Ross Black, my thesis advisor, who served as a constant inspiration in research and teaching. Gratitude goes to committee members Charlie Herr and John Buchanan for their sage advice.

Heartfelt thanks go to my partner in life and lab, Adam Gilles, without whom I would still be searching for the salamanders...literally and figuratively.

Logan and Samantha Cook, Skylar Mayfield and Megan Nelson provided invaluable help in the field, which would not have even been possible without the cooperation of Jason Lowe of the Bureau of Land Management. I also thank fellow graduate student Jarrett Cellini for comments on this manuscript, as well as his commiserations on life and graduate school in general. Thanks also go to the biology department's John Shields, David French and Lisa Williams for their friendship and help along the way.

And, of course, to my father, Kenneth Sprott, my mother, Annie Reed, and my brother, Bryan Reed, whose patience, love and support made this project possible.

Collections were obtained with U.S. Fish and Wildlife permission (# 15-230).

Experiments were conducted with approval from the International Animal Care and Use Committee of Eastern Washington University (# 2015-04.02). Financial support was provided by an Eastern Washington University Department of Biology grant.

Thank you all,

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INTRODUCTION

The purpose of this study was to explore the chemically-mediated interactions between a larval amphibian and its natural predators, and to test if predator-induced defenses extend to an unfamiliar invasive fish. In a world without language, chemicals are often a primary mode of communication within and between species. Intraspecific interactions are mediated by pheromones used for territory and trail marking, alarm signaling, aggregation and mate attraction (Nordlund et al. 1981). Interspecific interactions are dictated by allelochemicals: kairomones infer a benefit upon the receiver of the signal, and are often used by predators to find prey or by prey to avoid predators; allomones benefit the sender, such as defense secretions and repellants; synomones are beneficial to both sender and receiver, exemplified by some plant-insect interactions (Nordlund & Lewis 1976).

Kairomones have been the subject of intense study, as they define chemically-mediated predator-prey interactions and induce phenotypic plasticity in prey species. Phenotypic plasticity is the ability of a single genotype to express different phenotypes in response to environmental changes (Bradshaw 1965; Stearns 1989), allowing a single genotype to occupy a greater breadth of environmental conditions successfully (Stearns 1992). Predator-induced phenotypic plasticity is a phenomenon occurring across many taxa (Gilbert 1966; Baldwin et al. 1990; Benard 2004), and has been well-studied in terrestrial plants and aquatic organisms. Prey may express plastic responses in behavior (Larsson & Dodson 1993), morphology (Dodson 1974) and life history (Black 1993). These predator-induced defenses reduce predation risk (Harvell 1990) and have arisen as

adaptations in heterogeneous environments, as prey experience predation risk that is unpredictable across space and time (Havel 1987).

Water is the ideal solvent for the transmission of chemical cues (Wisenden 2003). Hence, predator-induced phenotypic plasticities have been discovered in a wide range of aquatic taxa living under heterogeneous predation regimes (Gilbert 1966; Harvell 1984; Laurila et al. 1998). For a predator-induced phenotypic plasticity to evolve, a genotype must successfully detect, interpret and respond phenotypically to changes in predation risk (Stearns 1992). In aquatic systems, detection occurs through the utilization of kairomones released into the water by predators (Gilbert 1966). This informs prey species on the abundance and proximity of a predator before a potentially lethal encounter, allowing it time to express an advantageous plastic response. A well-studied example is that of the water flea (*Daphnia pulex*), which expresses a small spine when reared in the presence of phantom midge larvae (*Chaoborus americanus*; Krueger & Dodson 1981). This plastic morphological trait increases escape potential when *D. pulex* is attacked (Krueger & Dodson 1981). Predator-induced phenotypic plasticities are found in vertebrate taxa as well. The crucian carp (*Carassius carassius*) expresses a deep-bodied morphology that reduces its vulnerability to gape-limited predation by pike (*Esox lucius*; Brönmark & Miner 1992), while fathead minnows (*Pimephales promelas*) respond to predator kairomones with a variety of antipredator behaviors, including shoaling and decreased activity (Chivers & Smith 1998).

Aquatic organisms often use chemical cues released by prey to associate greater risk with predator kairomones (Larsson & Dodson 1993). This associative pairing of cues is termed predator labelling and is conducted with chemicals released by a frightened or

injured conspecific (alarm cues; Chivers & Smith 1998) or prey chemicals released by the predator post-digestion (diet cues; Chivers & Mirza 2001). For example, Stirling (1995) found *D. galeata mendotae* did not respond behaviorally to kairomones from an unfamiliar fish unless a diet cue was included.

The occurrence of unfamiliar predators in aquatic environments has reached unprecedented rates as invasive species have become a global issue (Mack et al. 2000; Mooney & Cleland 2001). Invasive predators are particularly troublesome as their impacts may be compounded by native prey's inability to detect or interpret kairomones from a functionally novel predator (Salo et al. 2007; Sih et al. 2010). Ineffective antipredator behavior by prey can facilitate and increase the impacts of an invasive predator (Cox & Lima 2006), and plastic defenses geared towards a specific threat may be wholly ineffective for an invasive predator (MacDonald & Harrington 2003). A New Zealand mud snail, while able to detect kairomones from an invasive crayfish, responds by burrowing into the substrate – an effective response for its native fish predators, but not for crayfish – and suffers from predation despite its plastic response (Sih et al. 2010).

Predation by invasive fish is a major contributor to the current amphibian decline (Blaustein et al. 2011; Bucciarelli et al. 2014). The aquatic life stages of amphibians leave them vulnerable as eggs and larvae, as both are consumed readily by invasive fish, from salmonids introduced as game species (Tyler et al. 1998; Pilliod et al. 2010) to small-bodied fish released by accident or by intention (Monello & Wright 2001; Leu et al. 2009).

Amphibians are model organisms for the study of predator-induced phenotypic plasticity, as they represent an independent taxon with chemically-mediated antipredator

abilities. Mortality rates from oviposition to metamorphosis are an average of 87% due to heterogeneous pressures from competition, pond desiccation and predation during larval stages (Wells 2007). In response, amphibians have evolved plastic defenses in behavior, morphology and life history (Relyea 2007). Like other aquatic organisms, amphibians utilize chemical cues to detect and interpret predation risk, including predator labelling (Chivers & Smith 1998). Some amphibians have shown an ability to retain learned information, later identifying the predator cue as a threat without the reinforcement of a conspecific cue (Suboski 1990). This has been demonstrated primarily through the use of alarm cues in lab-based experiments across many amphibian taxa, including frogs, newts and salamanders (reviewed by Chivers & Smith 1998, Wisenden 2003 and Ferrari et al. 2010).

Upon cue detection, amphibians typically respond immediately with plastic antipredator behavior, reducing activity and/or seeking refuge in vegetation or substrate (Wisenden 2003). Amphibians may also express plastic morphologies when under a continued threat of predation (Smith & Van Buskirk 1995; Van Buskirk et al. 1997). The common responses of altered tailfin and body depth, as well as shorter tails and bodies, may lead to improved escape abilities (Doherty et al. 1998; Landberg & Azizi 2010). Predator-induced defenses in behavior and morphology often result in altered growth and development, which may impact the timing of metamorphosis and the size of the amphibian when this significant life history event occurs (Werner & Anholt 1996; Relyea & Werner 1999; Relyea 2001).

Given the widespread predator-induced defenses of aquatic organisms, we assessed the plastic antipredator abilities of larval salamanders in response to natural

predators and an invasive fish. We expected larvae to detect, interpret and respond behaviorally to kairomones from their natural predators, but to require a conspecific diet cue to correctly interpret kairomones from an unfamiliar fish. We further expected that after repeated exposure to fish kairomones with conspecific diet cues, larvae would be able to learn to recognize the fish kairomones alone. We anticipated plastic morphologies would be induced, leading to changes in the length and depth of the head and body, as well as a life history shift expressed as an altered rate of metamorphosis.

METHODS

Study species

The long-toed salamander (*Ambystoma macrodactylum*) has five recognized subspecies defined by coloring and distribution across the Pacific Northwest (Ferguson 1961). The central long-toed salamander (*A.m. columbianum*, referred to as *A. macrodactylum*) ranges from southeast Alaska through British Columbia, across eastern Washington and northern Idaho, to north-central Oregon and east-central Idaho (Stebbins 1985), inhabiting wet temperate forests, mountain lakes and arid shrub-steppe habitats across its wide range. Fossorial adults emerge in late winter for breeding in natatorial ponds (Pilliod & Fronzuto 2005), eggs hatch in early spring and larvae are aquatic until metamorphosis in mid to late summer (Corkran & Thoms 2006).

We used dipnets and funnel traps to collect *A. macrodactylum* larvae (n = 115; 1.1-4.2 g) from fishless ponds in Spokane and Lincoln counties, Wash., USA from June 16-22, 2015. Animals were housed at 13-14 °C with a 14:10 light:dark photoperiod. Larvae were housed in 150 x 150 x 50 mm plastic tubs with 350 ml of water and an

aquarium plant to provide refuge. Water was changed every other day, the day after the feeding of mixed assemblages of zooplankton.

Predator species and collection

We chose the blotched tiger salamander (*A. tigrinum melanostictum*, referred to as *A. tigrinum*) and the wandering garter snake (*Thamnophis elegans vagrans*, referred to as *T. elegans*) to serve as native predators and the brook stickleback (*Culaea inconstans*) as an invasive predator. *A. tigrinum* exists sympatrically with *A. macrodactylum* across the eastern Washington part of its patchy western range (Stebbins 1985). Due to significant temporal and spatial aquatic habitat overlap, the larger *A. tigrinum* preys upon *A. macrodactylum* (Fronzuto & Verrell 2007). We found the two species of larvae co-located in the majority of ponds from which we collected. *T. elegans* is generally aquatic, foraging in streams and ponds (Drummond 1983; Drummond & Burghardt 1983). The snake is a frequent predator of larval amphibians, including *A. macrodactylum* (Nussbaum et al. 1983). *C. inconstans* is not native to eastern Washington; it was first documented in the Turnbull National Wildlife Refuge of Cheney, Wash., USA in 1999 (McLellan 2000; Scholz et al. 2003). This small-bodied fish consumes zooplankton, aquatic insects and amphibian larvae either by gulping or gang-eating, depending on the size of the prey item (Reisman & Cade 1967). *C. inconstans* has been attributed to larval amphibian injury and decline from direct predation (USFWS 2008) as well as the decline of migratory waterfowl due to competition for food sources (Bridges 2011) on the Turnbull National Wildlife Refuge.

We collected *A. tigrinum* larvae (n = 4, 12-16 g) with dipnets and funnel traps, and *T. elegans* (n = 2, 26-30 g) by hand, from fishless ponds in Spokane and Lincoln counties, Wash., USA from June 16-18, 2015 and housed them in separate aquatic or terrestrial aquaria, respectively. We also dipnetted *C. inconstans* (n = 186; .68-1.5 g) from Turnbull National Wildlife Refuge and held them in group housing.

Generation of predator-conditioned water

All predators had food withheld for four days and were then fed either *A. macrodactylum* or bait-shop earthworms (*Lumbricus terrestris*) on the fifth and seventh days. *C. inconstans* were fed chopped-up food items to encourage normal feeding behavior while food was offered live to all other predators. On the eighth day, predators were placed in jars containing 2.5 ml of water per gram of predator body weight. After 24 hours, predators were removed. Water was passed through a blue bonded poly filter pad (Marineland CD-21403) to remove solids, divided into 60 ml aliquots and frozen. Aliquots were brought to room temperature in a water bath on the day of use.

Study design

To assess predator-induced responses of *A. macrodactylum*, we first initiated a behavioral assay during which we tested the null hypotheses that predator type had no effect on *A. macrodactylum* behavior and predator diet had not effect on behavior. We exposed individual replicate *A. macrodactylum* to one of six predator-conditioned water treatments generated from the three predator species, each with or without conspecific

diet cues, and measured subsequent changes in *A. macrodactylum* behavior. We then conducted a conditioning experiment to test the null hypothesis that repeated exposure to *C. inconstans* kairomones with conspecific diet cues would not affect *A. macrodactylum* behavior when later exposed to the predator kairomones alone. *A. macrodactylum* were repeatedly exposed to predator-conditioned water from larvae-diet *C. inconstans* and later tested for a response to null-diet *C. inconstans*. As a control, *A. macrodactylum* were also conditioned to predator-conditioned water from null-diet *C. inconstans*. During this experiment, we also measured for change in morphology, growth and development towards metamorphosis as indicators of other predator-induced plastic defenses.

Part I: Behavioral assay

A. macrodactylum (n = 48) were placed individually into a 4.5 l aquarium with 4 l of degassed tap water. Aquaria were lined with poly bag liners (Uline S-3205) with an aquarium pond plant as an area of refuge in the center. Aquaria were recessed in a 450 mm-deep tub of green plastic. A piece of green cloth-covered Styrofoam with a nested video camera (Sony HDR-CX550) looked down, with another camera looking in from the side.

After a 15-minute acclimation period, we captured 15 minutes of larvae behavior. One 60 ml aliquot of predator-conditioned water, constituting 1.5% of aquarium volume, was then poured into a funnel, which gravitationally flowed through surgical tubing fixed to a random quadrant of the aquarium. 1.5 minutes were given for dispersion, as determined by prior dye tests. We then recorded larvae behavior for 15 minutes.

To prevent cue contamination between trials, bag liners and surgical tubing were changed for each animal, all glassware was autoclaved and plasticware washed with aquarium cleaner (AquaLife, Western Chemical Inc.) between treatments.

Upon video review, point observations of locomotor activity were made for no more than two seconds at pre-determined one-minute intervals, yielding 15 observations pre-cue and 15 observations post-cue introduction. The video reviewer was blind to treatment assignments. Larvae were categorized as active if they were in motion while swimming or walking; otherwise they were deemed inactive. For each replicate individual larvae, we calculated percent change in activity as $([\text{post-cue active observations} - \text{pre-cue active observations}] / \text{pre-cue active observations} * 100)$.

Part II: Conditioning experiment

During the conditioning phase, 5 ml of predator-conditioned water were gently poured down the edge of an individual *A. macrodactylum* (n = 48) housing tub every third day, with water changed three hours later, for a total of three conditioning events. Behavior was tested the day after the third exposure event using the procedures described in Part I. Percent change in activity data was gleaned from video review as described previously.

At the beginning and end of the conditioning phase, we also measured changes in morphology, growth and development between treatments. Larvae were weighed and photographed dorsally and laterally with mounted cameras. Each photograph included a ruler for reference and was analyzed for snout-vent length, head width, body width and

tail muscle width using ImageJ software (Davis et al. 2008). Larval stage was determined using the arbitrary method established by Larras-Regard et al. (1981). By this method, we defined Stage I (larval stage) by long gills and gill filaments, wide tail fin and full dorsal ridge; Stage II (beginning of metamorphosis) by dorsal ridge regression, starting at the anterior end; Stage III (middle of metamorphosis) by advanced dorsal ridge regression, reabsorption of gills and reduction of gill filaments; and Stage IV (end of metamorphosis) by gill stubs or total loss of gills, with complete loss of gill filaments, and reabsorption of tail fin (Figure 1). Change was calculated as (end of conditioning measurement – beginning of conditioning measurement).

Statistical analysis

The response variable percent change in activity was ranked, as the measure and its transformed variants were non-normally distributed. Data then met assumptions of normality (Anderson-Darling tests; v 13.0; Systat Software Inc.). We used ANOVA (Quinn & Keough 2002) to test for effects of predator type and predator diet on *A. macrodactylum* activity in the behavioral assay, and for effects of conditioning and testing cue on *A. macrodactylum* activity in the conditioning experiment.

The frequency of larvae in each stage at the end of the conditioning phase was analyzed with log-linear regression to elucidate the effect of conditioning on *A. macrodactylum* development. Paired t-tests were conducted on the morphometric measures of change in snout-vent length, head width, body width, tail muscle width and

mass taken during the conditioning experiment. These measures met assumptions of normality (Anderson-Darling tests; v 13.0; Systat Software Inc.).

Significance for all tests was determined at $\alpha = .05$. Analyses were conducted with Systat (v 13.0; Systat Software Inc.) and figures were generated with SigmaPlot (v 11.0; Systat Software Inc.).

RESULTS

Part I: Behavioral assay

A. macrodactylum activity was significantly influenced by both predator type and predator diet ($p = 0.013$ and $p = 0.001$; Table 1). Larvae increased their activity (+42%) in response to *C. inconstans* fed a null diet of earthworms but decreased activity (-11%) in response to *C. inconstans* fed a diet of larval salamanders (Figure 2). An average decrease in activity in response to *T. elegans* and *A. tigrinum* fed a null diet of earthworms (-1% and -15%, respectively) was more pronounced when those predators were fed a diet of *A. macrodactylum* (-22% and -28%; Figure 2).

Part II: Conditioning experiment

There was a significant interaction between conditioning cue and testing cue on *A. macrodactylum* activity ($p = 0.007$; Table 2). Non-conditioned larvae did not respond (+1%) to plain water, but increased activity (+45%) in response to *C. inconstans* cues (Figure 3). Conditioned larvae responded to plain water with an increase (+12%) and to *C. inconstans* cues with a decrease (-20%) in activity (Figure 3).

There was a significant difference in final stage between conditioned and non-conditioned *A. macrodactylum* ($p = 0.021$; Figures 4 and 5), with more than six times as many conditioned larvae reaching Stage IV than non-conditioned (13 larvae vs. 2 larvae). We did not detect significant differences in growth (Figure 5) or morphology, as measured by snout-vent length, head width, body width and tail width, between conditioning treatments (Figure 6).

DISCUSSION

In a heterogeneous environment, prey success is largely dependent on the ability of the prey to accurately detect, interpret and respond to environmental change (Stearns 1992). Prey that can respond quickly to changes in predation regimes by expressing beneficial phenotypic plasticities can avoid complete loss of fitness due to a lack of antipredator defenses and instead tailor responses to specific situations (Levins 1968; Lima & Dill 1990; Brown & Chivers 2005), a trait which would be strongly selected for by natural selection. Our study provides evidence that *A. macrodactylum* exhibit predator-induced plastic defenses in behavior and life history upon detection of water-borne kairomones and conspecific diet cues. Furthermore, we suggest *A. macrodactylum* are able to use these chemical cues to label an unfamiliar predator as dangerous and retain this information for later behavioral decisions.

Empirical studies have demonstrated *A. macrodactylum* larvae exhibit plastic behavioral responses to predation, typically expressed as decreased activity (e.g. Fronzuto 2000). The larvae in this study decreased their activity in response to their native predators, especially when predators were fed a larvae diet (Figure 2). *A.*

macrodactylum did not exhibit antipredator behavior in response to the invasive fish without the aid of a conspecific diet cue. In fact, *A. macrodactylum* responded with increased activity to the fish kairomones alone, which has been interpreted by previous studies as a feeding response (e.g. Wildy & Blaustein 2001). Amphibians frequently do not respond to unfamiliar predator kairomones, even if their native predators are functionally similar (e.g. Gall & Mathis 2010). The failure of *A. macrodactylum* to recognize *C. inconstans* without a conspecific diet cue can be attributed to a lack of shared history on an evolutionary and/or ecological scale (Kats & Ferrer 2003), and emphasizes the impact an invasive predator can have on a naïve prey population (Cox & Lima 2006).

Our salamanders were able to use conspecific diet cues for predator labelling (Figure 2). Conspecific diet cues have been demonstrated to elicit plastic behavior in response to novel predators in many aquatic systems (e.g. Chivers & Mirza 2001). In a caged-predator survey of anuran tadpole species, the majority of species responded with decreased activity and spatial avoidance to the invasive red swamp crayfish (*Procambarus clarkii*) on a tadpole diet and to native dragonfly larvae (*Aeshna* sp.) regardless of diet (Nunes et al. 2013). Diet cues may not always be enough for amphibians to label an unfamiliar predator as dangerous. Polo-Cavia and Gomez-Mestre (2014) tested western spadefoot toad tadpoles (*Pelobates cultripes*) for a response to native dragonfly nymphs (*Anax imperator*) and invasive crayfish (*P. clarkia*), which were either starved or fed tadpoles. While the tadpoles reduced swimming activity in response to both diet treatments of dragonfly, they did not reduce activity in response to crayfish, regardless of diet.

In our second experiment, we repeatedly exposed *A. macrodactylum* to kairomones from *C. inconstans* on a conspecific diet. We saw a significant behavioral and developmental response to conditioning with these chemical cues (Figures 3, 4 and 5). *A. macrodactylum*'s ability to learn (Figure 3) marks one of the few examples of conspecific diet-dependent learning in the literature (see Scherer & Smee 2016), and the first with amphibian prey and an invasive predator. It seems there are only two other examples of amphibian learning through conspecific diet cues. Wildy and Blaustein (2001) first determined that naïve *A. macrodactylum* larvae did not respond behaviorally to cues from injured conspecifics, cannibals fed conspecifics or cannibals fed *Tubifex* worms. However, larvae raised in the presence of cannibals feeding on conspecifics did later respond with decreased activity. Mogali et al. (2012) tested predator-experienced and predator-naïve larval bronze frogs (*Rana temporalis*) for behavioral responses to larval dragonflies (*Pantala flavescens*) fed conspecifics. Tadpoles from both groups responded to the native predator, but experienced tadpoles did so to a greater degree.

The vast majority of empirical studies in the field of diet-dependent labelling and learning have utilized fish species as both predator and prey (Scherer & Smee 2016). Specifically, predator labelling with conspecific diet cues has rarely been conducted with salamanders and is limited to predation by cannibal conspecifics or snakes (Scherer & Smee 2016). There is a need for research with a greater variety of at-risk caudates and their predators, including invasive species. Furthermore, most studies explore predator labelling and learning with the use of alarm cues, pairing predator kairomones with crushed conspecifics (Ferrari et al. 2010). Alarm cues mimic the capture and injury of prey, while conspecific diet cues indicate a successful predation event has occurred and

may facilitate different responses by prey species. Even when utilizing diet cues, the vast majority of studies do so with predators on a single-prey diet. The limited number of studies to examine the effects of a mixed-predator diet have done so with interesting results. For example, Darwish et al. (2005) conditioned predator-naïve glowlight tetras (*Hemigrammus erythrozonus*) to conspecific alarm cues paired with a cocktail of kairomones from predatory and non-predatory fish. Two days later, the tetras displayed antipredator behavior to all cues individually. Clearly, chemically-mediated learning by aquatic animals warrants further exploration with experimental designs incorporating more complex environments.

The second part of our study highlighted predator-induced defenses in morphology and life history. Our salamanders exhibited an accelerated advancement to the life history event of metamorphosis, as evidenced by a significant proportion reaching the final stage of metamorphosis before the end of our relatively short study (Figure 4). While certain models predict such a response (Wilbur & Collins 1973), few experimental studies have seen amphibians accelerate metamorphosis in response to a predator. A review by Relyea (2007) determined that the timing of, and size at, metamorphosis were not affected by the presence of predators in the majority of studies using caged and lethal predators fed an amphibian conspecific diet.

However, studies by both Chivers et al. (1999) and Kiesecker et al. (2002) illustrate predator-induced life history shifts in amphibian larvae through the use of diet cues. Chivers et al. (1999) raised western toad (*Bufo boreas*) tadpoles in the presence of conspecific alarm cues, predatory backswimmers (*Notonecta* spp.) on a diet of tadpoles, and non-predatory water boatmen (family Corixidae). Tadpoles in the alarm cue and

predator treatments decreased their time to metamorphosis but did not have a significant difference in size. Kiesecker et al. (2002) found that red-legged frog larvae (*Rana aurora*) tadpoles metamorphosed earlier and at a smaller size when raised in the presence of predatory rough-skinned newts (*Taricha granulosa*) feeding on conspecifics. Evidence also suggests demonstrable life history shifts in response to other environmental cues. Wildy (2000) found that when food levels and larvae densities were held constant, *A. macrodactylum* in fast-drying pond treatments metamorphosed earlier than those in slow-drying treatments, with no difference in size observed.

Predator-induced phenotypic plasticities are typically associated with an energetic cost, or “trade-off,” to the organism. While expressing an alternative phenotype, the animal often diverts energy away from regular growth, reproduction and maintenance (Harvell 1990; Roff 1993), and may avoid normal behaviors such as foraging and mate-seeking (van Duren & Videler 1996). Survivorship and fecundity of the induced morph are typically reduced, resulting in lower growth rate of the induced portion of the population in predator-free environments (Black & Dodson 1990; Riessen & Sprules 1990). Due to the costs of exhibiting induced responses, the prey species only benefits from the defense in the presence of significant predation (Harvell 1990), which is believed to be one of the major selective forces behind the evolution of phenotypic plasticities.

In larval amphibians, the expression of antipredator defenses is typically associated with a smaller size at metamorphosis (Relyea 2007). While smaller size may lead to negative effects in reproduction, food gathering and predator avoidance at maturity (e.g. Berven & Gill 1983; Smith 1987), it may be strategic while still in the

water. Detection by a visual predator such as *C. inconstans* only increases with size, and most fish select for the largest prey items until reaching a practical limit defined by their gape size (Zaret 1980). While we did not see a significant difference in final body size between conditioned and non-conditioned *A. macrodactylum*, both exhibited an overall decrease in body size (Figures 5 and 6). This can be attributed to the cessation of growth and then loss of weight amphibians experience during the latter stages of metamorphosis (Wilbur & Collins 1973). The salamanders in this study were not approaching metamorphosis at a smaller size, but were on their way to an increased time with terrestrial rather than aquatic predators. The threat of predation by terrestrial predators may be minimized by *A. macrodactylum*'s fossorial nature (Pilliod & Fronzuto 2005). When following the mole salamanders (*A. talpoideum*) of a single pond for eight years, Semlitsch et al. (1988) found that neither size at nor time of metamorphosis were correlated with survival to first reproduction.

We found no significant differences between groups in all morphometric measures (Figure 6). Shaffery and Relyea (2015) found disparate responses in five species of larval *Ambystoma* in response to dragonfly larvae (*A. junius*) kairomones in a lab-based study. While larvae, in general, reduced activity in response to kairomones, morphological responses were varied. For example, one species (*A. barbouri*) expressed longer heads while two other species (*A. gracile* and *A. laterale*) developed shorter heads. When considering studies that examined the effect of predator diet on behavior and morphology, Scherer and Smee (2016) found that behavior was affected 70% of the time while morphology was affected 40% of the time. Their review concluded that predator-

induced morphological changes may be rare due to their high cost and often irreversible nature.

Even small invasive fish can be devastating to amphibians (Pearson & Goater 2009), but chemically-mediated learning leading to plastic behavioral responses has been shown to improve fitness when faced with an invasive predator. Polo-Cavia and Gomez-Mestre (2014) conducted live predation trials with toad tadpoles (*P. cultripes*) and invasive crayfish (*P. clarkii*). Tadpoles that had learned to recognize the unfamiliar predator through the use of conspecific alarm cues had a significantly higher survival rate and were often consumed after the naïve tadpoles.

Our study suggests that *A. macrodactylum* were able to use conspecific diet cues to label unfamiliar *C. inconstans* kairomones as an indicator of threat, retain that association for later behavioral responses, and alter the life history event of metamorphosis in response to perceived predation risk. In a heterogeneous environment, there is a selective advantage for adaptive abilities in predator labelling and learning. *A. macrodactylum*'s plastic defenses in behavior and life history, as well as the ability to conduct chemically-mediated learning, may help them adapt to our current high rates of species invasions (Mirza & Chivers 2001).

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TABLES

Table 1. Effect of predator type and predator diet on larval long-toed salamander activity. During a behavioral assay, larvae (n = 8 per treatment) were exposed to cues from invasive fish, native snakes and native salamanders, which had been fed a null diet of earthworms or a diet of long-toed salamanders.

Source	SS	df	MS	F	p
Predator type	1,347.219	2	673.609	4.816	0.013
Predator diet	1,800.750	1	1,800.750	12.873	0.001
Interaction	155.531	2	77.766	0.556	0.578

Table 2. Effect of conditioning and testing cue on long-toed salamander activity. Larvae were conditioned with cues from invasive fish fed a null-diet of earthworms or a larval-diet of long-toed salamanders, and then tested for a response to plain water or null-diet fish.

Source	SS	df	MS	F	p
Conditioning	638.021	1	638.021	3.917	0.054
Testing cue	4.688	1	4.688	0.029	0.866
Interaction	1,302.083	1	1,302.083	7.994	0.007

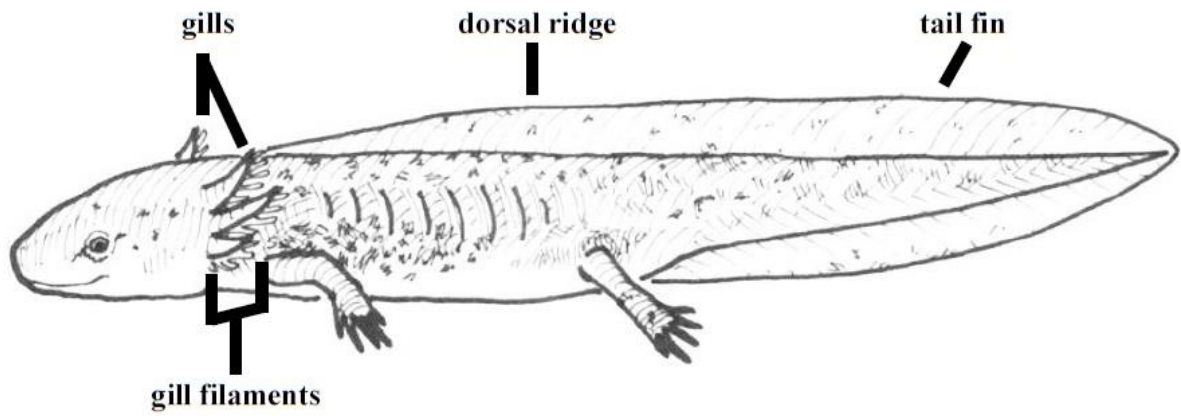
FIGURES

Figure 1. Morphological aspects that are reabsorbed over the course of *Ambystoma* metamorphosis and define the metamorphic stages of larval long-toed salamanders in this study (modified from MacDonald 2004).

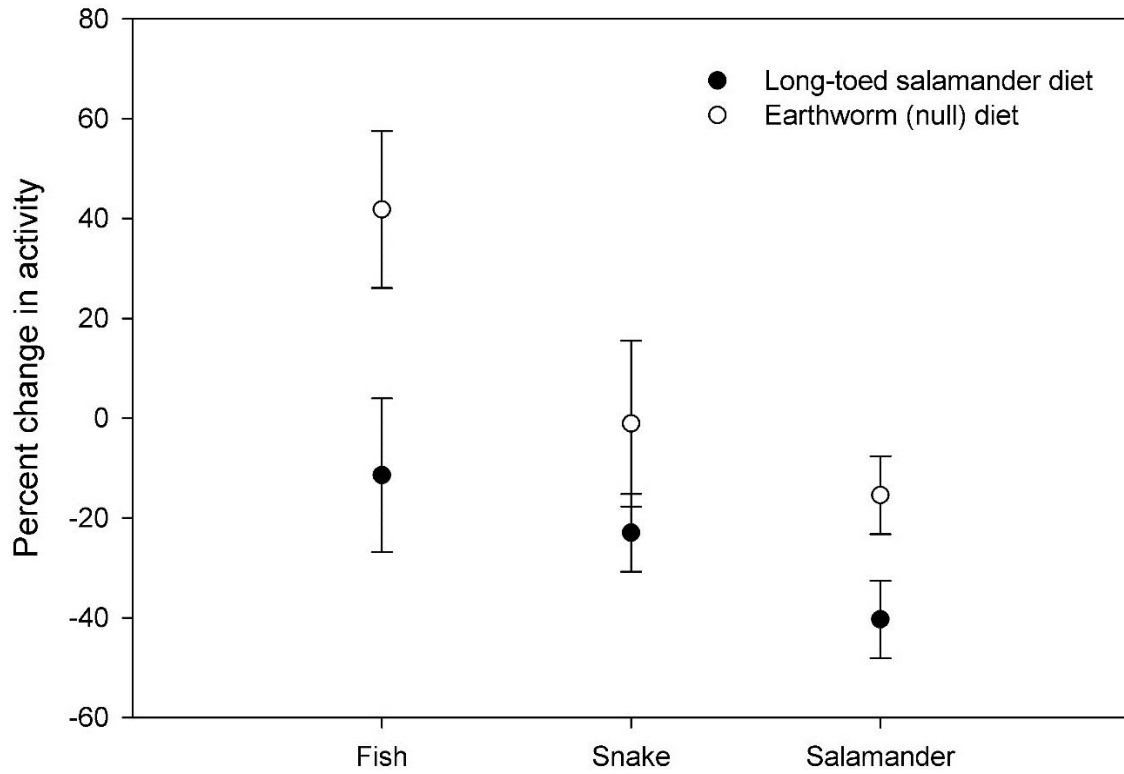


Figure 2. Percent change in activity (mean \pm SEM) of larval long-toed salamanders exposed to water conditioned by potential predators: invasive fish, native snakes and native salamanders fed a diet of larval long-toed salamanders (filled circles) or a null diet of earthworms (open circles).

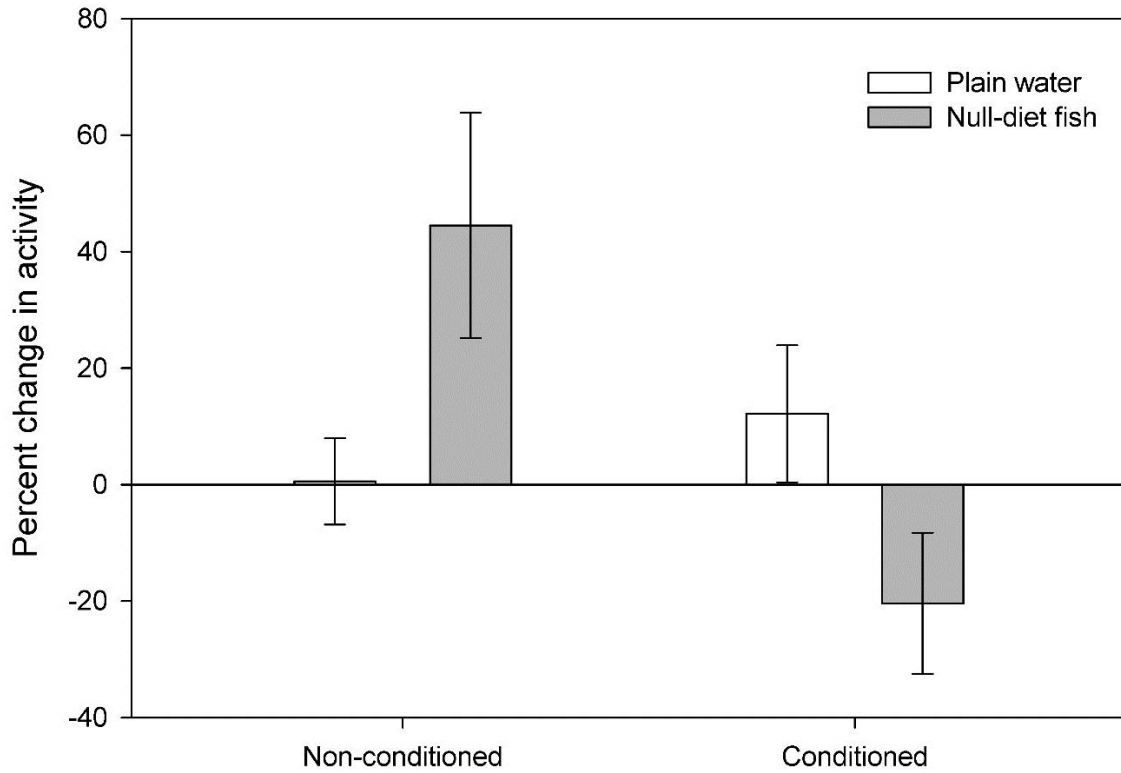


Figure 3. Mean (\pm SEM) percent change in activity of larval long-toed salamanders that were repeatedly exposed to cues from invasive fish fed a null diet of earthworms (non-conditioned) or a diet of long-toed salamanders (conditioned) before testing for a change in activity in response to water (white bars) or null-diet fish (grey bars).

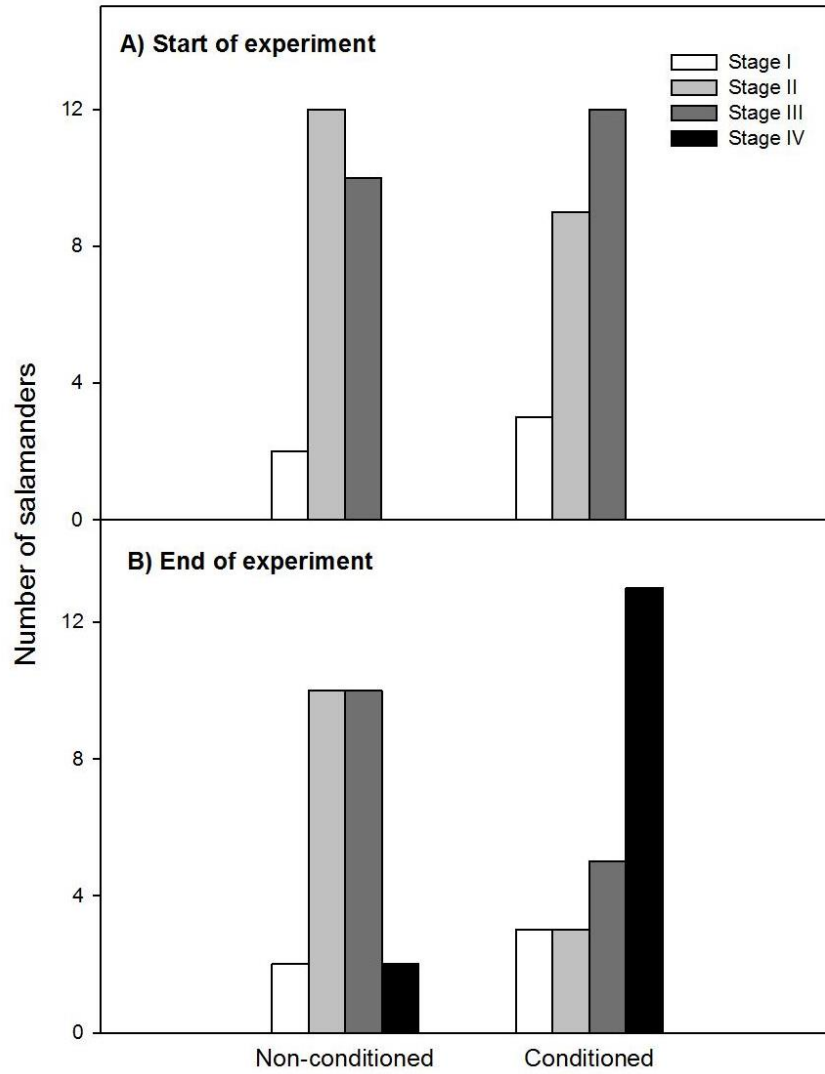


Figure 4. The number of larval long-toed salamanders in each stage of development at the beginning (A) and end (B) of the conditioning experiment. Larvae were either exposed to cues from invasive fish fed a null diet of earthworms (non-conditioned) or cues from invasive fish fed a diet of long-toed salamanders (conditioned).

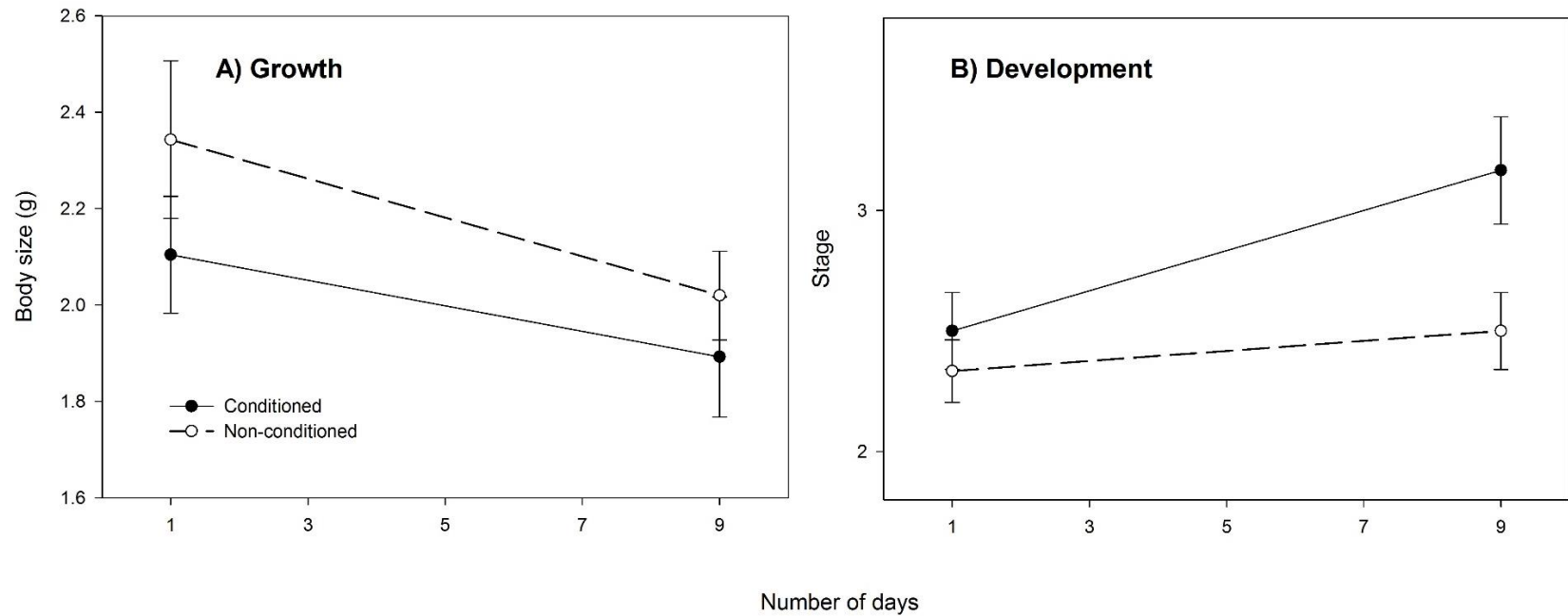


Figure 5. Mean (\pm SEM) of the growth (A; measured as body size in grams) and development (B; measured as metamorphic stage) of larval long-toed salamanders over the nine-day conditioning experiment, during which larvae were exposed to cues from fish fed a diet of larval long-toed salamanders (closed circles, solid lines) or a null diet of earthworms (open circles, dashed lines).

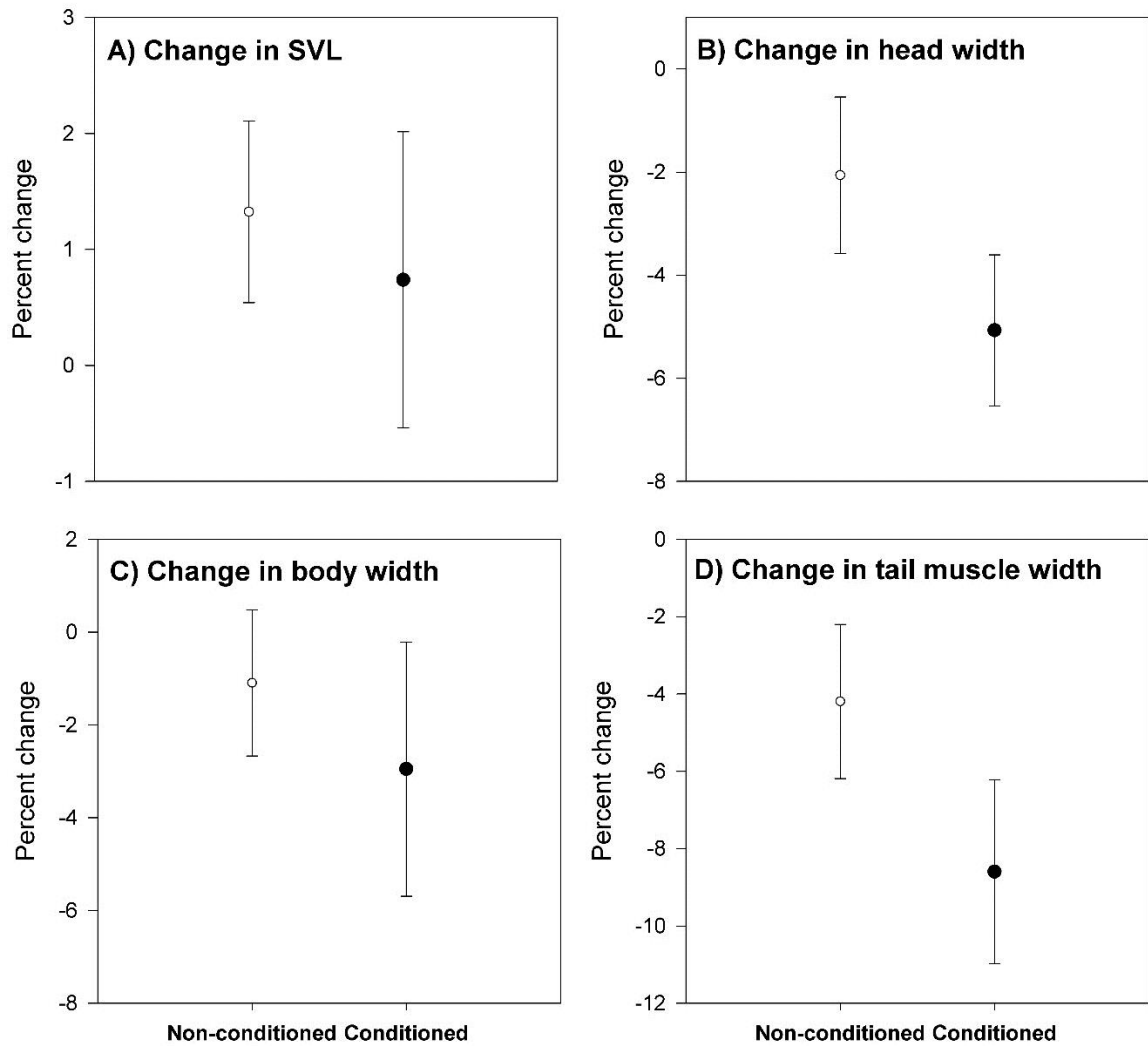


Figure 6. Morphometric measures of change in snout-vent length (A; SVL), head width (B), body width (C) and tail muscle width (D) of larval long-toed salamanders during the conditioning phase. Mean percent changes of non-conditioned (open circles) and conditioned (closed circles) larvae are accompanied by SEM error bars.

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