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Response to Conspecific Alarm Cues by Spotted Salamander (*Ambystoma maculatum*) Larvae

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**RESPONSE TO CONSPECIFIC ALARM CUES BY SPOTTED SALAMANDER
(*AMBYSTOMA MACULATUM*) LARVAE**

A Master's Thesis

Presented to

The Graduate College of
Missouri State University

In Partial Fulfillment

Of the Requirements for the Degree

Master of Science, Biology

By

Katlyn Marie Gardner

May 2019

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(*AMBYSTOMA MACULATUM*) LARVAE**

Biology

Missouri State University, May 2019

Master of Science

Katlyn Marie Gardner

ABSTRACT

Detection of predators early in a predation sequence may allow prey to increase their probability of survival by taking evasive action. For aquatic species in ephemeral ponds visibility is often limited, so predation risk assessment via chemical cues can be useful. Most mole salamanders of the genus *Ambystoma* breed in vernal ponds, and larvae suffer high mortality rates due in part to high levels of predation. I tested whether larvae can assess predation risk by detecting chemicals (alarm cues) released from the skin of damaged conspecifics, and, if so, what factors influence the response to this alarm cue. Field-caught spotted salamander larvae (*Ambystoma maculatum*) responded with fright to the chemical cue of damaged conspecifics, but not to control cues. To investigate the ubiquity of this response throughout ontogeny, I conducted further experiments to examine effects of age and experience of cue donors and receivers on alarm responses. In a second experiment, I measured responses of neonate larvae to alarm substance from two different age classes of conspecifics, using heartrate as indicator of fright. The neonate larvae showed increased heartrate (fright) to the cue from older conspecifics, but not the cue from conspecifics from their own age class. My third experiment tested whether larval experience is required for response to the alarm cue. In this experiment, lab-reared individuals did not distinguish between conspecific alarm cues and the control stimuli. My study provides the first evidence for the production of chemical alarm cues by larval salamanders and indicates that alarm responses may vary due across ontogenetic development.

KEYWORDS: *Ambystoma*, chemical communication, alarm cue, ontogeny, heartrate

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In the interest of academic freedom and the principle of free speech, approval of this thesis indicates the format is acceptable and meets the academic criteria for the discipline as determined by the faculty that constitute the thesis committee. The content and views expressed in this thesis are those of the student-scholar and are not endorsed by Missouri State University, its Graduate College, or its employees.

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I dedicate this thesis to my parents, just as they have dedicated so much of their lives to helping me succeed, and to my younger sister. Without your love, encouragement, and support, none of this would be possible. No matter how far I travel, you are always near to my heart. I love you.

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INTRODUCTION

Interactions between an actively foraging predator and its prey are a part of the predation sequence: prey encounter, detection, pursuit, capture, handling, and consumption (Endler 1986). For a predator to secure a meal, it must outperform prey in each step of the sequence. However, if a prey individual can evade or escape the predator at any one of these steps, it may live a while longer. This inequality of outcomes—death vs. a lost meal—leads to what is known as the “life-dinner principle” (Dawkins and Krebs 1979): the prey’s higher cost of losing (death) should result in antipredator strategies being under stronger selection pressure than predators’ hunting strategies. Therefore, prey should remain a step ahead in the predator-prey arms race.

The earlier in a predation sequence that a prey individual can detect a predator, the greater its odds of surviving the sequence (Lima and Dill 1990). A predatory encounter begins when one individual enters the potential detection field of the other. The best way for prey to survive an encounter is to avoid a physical interaction altogether by avoiding detection by the predator (Fuiman and Magurran 1994). Some prey species rely on morphological anti-predator tactics, such as camouflage, to avoid detection (Ydenberg and Dill 1986). Cryptic individuals can enhance the efficiency of their camouflage by becoming immobile or decreasing activity (Azevdo-Ramos et al. 1992). If detection and identification by the predator is inevitable, a prey individual may be able to use other tactics, such as avoiding capture by fleeing (Samia et al. 2016), subjugation by fighting the predator (Silva et al. 2018), or consumption by being armoured (Stankowich and Campbell 2016) or distasteful (Halpin and Rowe 2016). In addition to

the benefits of avoiding physical interactions with prey, escaping detection through camouflage, freezing or hiding is generally less energetically costly than later-stage defenses like fleeing or fighting.

Virtually all antipredator defenses require individuals to cease other activities (e.g., foraging, searching for mates) during the encounter (Lima and Dill 1990).

Therefore, fitness may be maximized by employing antipredator responses only when the risk level is high. Early detection of predators allows increased time for threat assessment.

Predator detection can occur via a variety of cues, including visual, acoustic, tactile, or chemical cues. In addition to being alerted to the presence of potential predators, prey can often use these different sensory modalities to assess the level of risk by determining predator proximity and whether the predator is actively hunting (Ferrari et al. 2006). Prey that can glean detailed information about predators early in the predation sequence should gain some advantage and make better-informed decisions about how to allocate their time (antipredator vs. other behaviors). The effectiveness of different cue modalities can depend on a variety of factors, including habitat. For example, in a low visibility habitat, visual cues may be less reliable than they are in a clear habitat or at close range (Endler 1993).

Lentic habitats such as wetlands or ephemeral pools are areas in which visual cues may be less efficient in detecting risk due to turbid water and high vegetation densities possibly obstructing the prey's view. Aquatic prey often rely heavily on chemical cues that pass through or around obstructions and persist in the water column (Brönmark and Hansson 2000). In addition, chemical cues can allow the receiver access to information at

further distances or more accurately than permitted by visual and tactile cues (Hickman 2004). Acoustic cues are an uncommon source of information in these habitats, as many of a pond's inhabitants are unable to vocalize. For example, only one larval amphibian has been shown to vocalize (Natale et al. 2011). For these reasons, chemical cues are used by many aquatic species and have developed into sophisticated sources of information (e.g. Ferrari and Chivers 2009).

Predators can be detected either directly via cues given from the predator (Garvey et al. 2016) or indirectly, via cues from conspecific (James and McClintock 2017) or heterospecific (Crane et al. 2013) individuals that have detected the predator (Wisenden 2014). Indirect cues are typically categorized as either "alarm" cues, which are released after damage to the individual, usually from an attack by a predator, or "disturbance" cues which do not require damage for release (Chivers et al. 2012). Damaged-released cues are a reliable cue particularly because they not only indicate that a predator is nearby but also that it is actively hunting.

Vertebrate chemical alarm cues were first documented in ostariophysan fishes (von Frisch 1938; reviewed by Smith 1992) and toad tadpoles (Kulzer 1954), with subsequent decades of research revealing that alarm substances are present in a wide range of non-ostariophysan fishes and larval anurans (reviews: Mathis 2009; Chivers and Smith 1998; Ferrari et al. 2010b). Generally, aquatic amphibians have received less attention than fishes, and, among amphibians, anurans have received considerably more attention than salamanders. In reviews covering chemical alarm substances in aquatic species, only three publications investigate salamanders, two of which are adult newts in the family Salamandridae (Marvin and Hutchison 1995; Woody and Mathis 1998). In one

additional study of larval long-toed salamanders, *Ambystoma macrodactylum*, the authors (Wildy and Blaustein 2001) suggest that chemical alarm cues may be present, but their methodology did not allow them to distinguish between alarm cues per se and other diet-based cues. Thus, there have been no studies definitively indicating the presence or response to conspecific alarm cues in larval salamanders to date.

The site of alarm cue production is thought to be related to the skin in most species that have been studied (Ferrari et al. 2010b). For the best-studied group, the ostariophysan fishes, the alarm substance is produced in club cells of the epidermis (Smith 1992). These cells have no ducts connecting them to the surface of the skin, indicating that alarm cues can only be released upon damage to the epidermis. In other species, including amphibians, the specific site of alarm cue production is not known. Adult amphibians have numerous epidermal glands (Fox 1994) that are potential sites of alarm cue production. Prior to metamorphosis, the amphibian epidermis undergoes numerous changes in cellular composition during the transition from an aquatic to terrestrial life stage (e.g. Regueira et al. 2016; Warburg et al. 1994). For example, in salamanders, metamorphosis has been shown to cause a loss of certain larval epidermal cells, such as Leydig cells and accessory cells, while mitochondria-rich cells become indistinguishable (Perrotta et al. 2012). Changes in cell abundance and type are not isolated to the metamorphic period but occur throughout ontogeny until adulthood (Lewinson et al. 1983; Pederzoli et al. 2002). Ontogenetic shifts have been shown to affect the production or response to alarm cues in a variety of organisms but have been most widely studied in fishes (e.g. sculpin: Gall and Mathis 2011; chromis: Mitchell and

McCormick 2013; damselfish: Lönnstedt and McCormick 2011; minnow: Mirza and Chivers 2002).

Response to alarm cues may also vary across ontogeny (Harvey and Brown 2004; Gall and Mathis 2011; Xia et al. 2017). Ontogenetic variation in the presence or intensity of alarm responses could be due to experience. Increased experience with risk can lead to enhanced function of antipredator behaviors (Fraker 2009; Mirza and Chivers 2003). Consequently, older individuals may be better at responding to antipredator cues than younger individuals (Putman et al. 2015). The influence of experience on antipredator behavior can also be less intuitive. Two common risk factors are predation and starvation, and individuals that can balance these two risks should be more successful in terms of fitness. Individuals that experience a high background level of predation risk would risk starvation if they responded with increased antipredator behavior, and consequently lower levels of foraging, every time a predator was nearby. Thus, the “risk allocation hypothesis” (Lima and Bednekoff 1999) leads to the prediction that animals in high-predation populations should exhibit relatively low-level responses to the immediate threat of predation. In contrast, individuals that experience low background levels of predation risk should respond strongly to relatively rare immediate predatory threats because doing so does not appreciably increase the risk of starvation. Mitchell et al. (2016) found that high-risk *Lithobates sylvaticus* tadpoles did not reduce activity as much as low-risk tadpoles when exposed to conspecific alarm cues but exhibited neophobia in response to novel cues whereas tadpoles in the low-risk treatment did not. Prey have even been shown to “forget” (cease responding to) innate knowledge of certain predators if the risk associated with that predator is not reinforced (Ferrari et al. 2010a).

Some species respond to the chemical alarm substances of heterospecifics, forming an information-sharing species assemblage known as a prey guild. Prey guilds typically form when species are syntopic and share a common predator (Chivers et al. 1997). Prey guilds should be advantageous to prey cohabitating in high densities, increasing the amount of information at their disposal and increasing the chances of early predator detection. Prey guilds have been shown to function in a variety of taxa, such as fishes (Mirza et al. 2003), salamanders (Chivers et al. 1997), and herbivorous mammals (Pays et al. 2014; Schmitt et al. 2016), and even across widely different taxonomic groups (e.g., fish and salamanders: Anderson and Mathis 2016; birds and mammals: Lea et al. 2008). Response to signals by individuals other than the intended (usually conspecific) receiver is referred to as “interceptive eavesdropping” (McGregor and Horn 2014).

Larvae of the study species, the spotted salamander (*Ambystoma maculatum*), occupy low-visibility and predator-dense temporary ponds throughout most of the eastern United States and into Canada (Zamudio and Savage 2003). Although larval spotted salamanders are not considered social, they are often found at high densities (Stenhouse 1985; Shoop 1974) in ponds suggesting that nearest-neighbor distances are sufficient for detection of alarm cues released by conspecifics. Additionally, terrestrial juvenile spotted salamanders may possess an alarm cue contained within the skin (Hunt 2011). Spotted salamander larvae co-exist with high densities of other prey with which they share common predators. The close proximity to other species in the same prey guild provides spotted salamander larvae with the opportunity to eavesdrop on heterospecific alarm cues, such as those produced by American toad tadpoles (*Anaxyrus americanus*).

I hypothesized that larval spotted salamanders produce a cutaneous, damage-released alarm substance that conspecifics respond to with antipredator behavior. I also hypothesized that the salamanders eavesdrop on alarm substances from syntopic prey in the same prey guild. In my first experiment, I investigated whether field-caught, free-swimming spotted salamander larvae would respond with fright behavior (i.e. reduced movement, a freezing response) to (1) putative alarm substance from conspecifics and (2) the known alarm substance of American toad tadpoles (*Anaxyrus americanus*). Field-caught larvae likely have experience with predators and with alarm cues.

Building upon the results of the first experiment, my second experiment tested whether developmental stage affected (1) the presence of the alarm cue in the donor and (2) the ability to detect and respond to alarm cue by conspecific receivers. Focal larvae were lab-reared neonates, and cue donors were from two developmental stages (1-day-old and 1-month-old). Because the focal larvae were not free-swimming at this early stage of development, I measured heart rate to assess the presence of a fright response; *Ambystoma* respond to predator cues with increased heart rates (Shelton 2016). I hypothesized that the larvae would respond with an increased heart rate to the putative alarm substance from both the early-stage and late-stage conspecifics.

In the third experiment I tested whether free-swimming, lab-reared larvae exhibited a fright response to conspecific alarm cues even though they had no experience with predators during the rearing period. As in the first experiment, I tested whether the lab-reared free-swimming larvae would respond with fright behavior (i.e. reduced movement, a freezing response).

METHODS

Experiment 1: Field-Caught Larvae

Spotted salamander larvae were collected from a pond in Compton Hollow Conservation Area (Webster CO., MO) in May 2016 and 2017 (n = 72, 84). American toad tadpoles (n = 100) were also collected each year from the same location. For a novel control stimulus, we used skin extracts from Ozark zigzag salamanders (*Plethodon angusticlavius*) collected from Drury-Mincy Conservation Area (Taney CO., MO) in November 2016 and November 2017 (n = 3, 3). In the lab, salamander larvae (total length (TL) 28.42 ± 0.614 mm) were housed individually in small plastic boxes ($8 \times 8 \times 8$ cm) containing water from their natal pond and were fed blackworms (*Lumbriculus variegatus*) every other day. American toad tadpoles (TL approx. 21 mm) were housed together in a 5.7-L plastic box ($35.6 \times 20.3 \times 12.4$ cm) and fed algae wafers every three days. Ozark zigzag salamanders (n=3, 2; SVL = $36.8 \pm .86$ mm) were housed in individual Petri dishes (13 cm diameter \times 1.5 cm height) on moistened filter paper and kept in an environmental chamber at $15^\circ \pm 2^\circ$ C. Ozark zigzag salamanders were fed 10 *Drosophila hydei* once a week. Salamander larvae and toad tadpoles were kept at $20^\circ \pm 2^\circ$ C. All animals were kept at a 12:12 light cycle.

All the following cue collection and experimental procedures were approved by the Missouri State University IACUC (Protocol #17-012). For our novel control stimulus (terrestrial salamander), we prepared extracts using skin tissue that contained a small amount of adhering muscle tissue. The salamanders were euthanized (pithed and decapitated) before we removed the skin from the neck to the base of the tail. The

resulting tissue was macerated with a mortar and pestle. Stimuli from salamander larvae and toad tadpoles was obtained via whole body maceration after chilling donor animals in a freezer for approximately 15 minutes at $-14^{\circ} \pm 2^{\circ}$ C. Following maceration, stimuli was filtered through glass wool and diluted to a concentration of 0.0015g of tissue/1mL, as in Woody and Mathis (1998), to control stimulus strength across the species. The cue was frozen in 2.5-mL aliquots until the day of testing. Dechlorinated water was also frozen into 2.5 mL aliquots to serve as a blank control cue.

Trials were conducted in the focal larva's home chamber which was placed over a 2.5×2.5 cm grid. The day before testing, pond water was replaced with 250 mL of dechlorinated water to eliminate any extraneous chemical cues present in the container. Focal larvae were allowed 24 h to acclimate in the dechlorinated water before testing. Larvae were randomly assigned to one of the four treatment groups (n=30 per treatment). Larval movements were recorded as lines crossed for 4 min (PRE). Then, randomly-selected stimulus aliquots were added to the chamber and allowed to disperse for 30 s, and movements were recorded for an additional 4 min (POST). The observer was blind to the stimulus identity.

All statistical analyses were performed using Minitab 17.1.0 statistical software. I calculated a response index as the change in number of moves (POST – PRE) and compared the treatments with a Kruskal-Wallis nonparametric ANOVA. Post-hoc comparisons were conducted using Mann-Whitney U tests with α adjusted to 0.025 using a Bonferroni correction to control for compounding experiment-wise error (Holm 1979). To determine whether size influenced responses, I used a Pearson correlation analysis to test for correlations between total length and number of moves for data from 2017.

Experiment 2: Neonate Heartrate

Spotted salamander egg masses ($n = 6$), were collected on 26 April 2018 from a pond in Compton Hollow Conservation Area (Webster CO., MO). Spotted salamander egg masses were housed in 5.7-L plastic shoebox containers ($35.6 \times 20.3 \times 12.4$ cm) in pond water collected from their natal pond. Larvae hatched from 26 April – 6 May 2018 in the lab. Upon hatching, larvae were individually housed in freezer boxes containing pond water and stored in an environmental chamber, with a 12:12 light/dark cycle and at $15 \pm 2^\circ$ C. Neonates do not require food for several days because they feed on post-hatching yolk. Neonates were tested 30 April 2018 and 6 May 2018, 1–4 days post-hatching, at Harrison developmental stage 39–42 (Harrison 1969).

We used terrestrial Southern Red-backed salamanders, *Plethodon serratus*, ($n = 3$, 40 ± 1.53 mm) as cue donors for the novel odor control treatment. These salamanders were collected from Reis Biological Station (Crawford Co., MO) in May 2017 and maintained in the lab in the same way as the *P. angusticlavius* in experiment 1. Control extracts were prepared as in experiment 1.

Spotted salamander alarm cue was prepared from two age classes. Neonates ($n = 46$) from six clutches were sacrificed on the day of hatching (26 April 2018) to provide the “young” larvae alarm cue. Larvae were chilled for approximately 15 min in a freezer at $-14 \pm 2^\circ$ C before being macerated via mortar and pestle. For the “old” larvae alarm cue, lab-reared 1-mo-old larvae ($n=29$) from different clutches (total clutches = 12) were also chilled, pithed, and macerated with a mortar and pestle. Macerated tissues were diluted to a 1:100 g/mL cue strength (stronger than in experiment 1, see: Shelton 2016),

filtered with glass wool to remove particulate matter, and frozen in 1 mL aliquots to be defrosted prior to each trial.

Trials were conducted with neonate larvae placed individually in small dishes (19 mm radius and 9 mm height) in 1 mL of well water. Each neonate (n=20 per treatment) was allowed 20 min to acclimate in the dish before being placed under a dissecting microscope for observation. In neonates, the blood pulses through gill capillaries simultaneously with heart beats (Shelton 2016). Gill pulses were counted for 1 min, a 1-mL aliquot of randomly-selected stimulus was added to the dish, and gill pulses were then immediately counted for an additional minute. Larvae that moved during any point of the trial were omitted from data analysis because physical activity can affect heart rate. Data were analyzed as in experiment 1, although no Pearson correlation was run on length data due to small size of larvae and lack of variation in developmental stage.

Experiment 3: Lab-raised Larvae

Spotted salamander egg masses (n = 12), were collected on 12 March 2018 from a pond in Compton Hollow Conservation Area (Webster CO., MO). Egg masses were kept in 5.7-L plastic boxes (35.6 × 20.3 × 12.4 cm) filled with pond water and kept near a natural light source. Larvae hatched from 20 March – 28 March 2018. Upon hatching, salamander larvae were individually housed in small plastic boxes (8 × 8 × 8 cm) filled with well water and kept at 20 ± 2° C with a 12:12 light cycle. Larvae were initially fed water fleas, *Daphnia* spp., netted from water from their natal pond and then switched to a diet of blackworms (*Lumbriculus variegatus*) three times a week.

American toad tadpoles (n = 14) were collected from Compton Hollow Conservation Area on 21 May 2018. Tadpoles were maintained in a group tank (37.85 L) in lab and fed algae pellets twice a week.

I used the same alarm cue and novel odor cues as were used during Experiment 2, with the addition of American toad tadpole alarm cues (as in Experiment 1), and I diluted them to the strength (0.0015g/1mL) used in Experiment 1 so that the two experiments could be better compared. Test trials were conducted in the same manner as experiment 1 (n=20 per treatment), with movement recorded as lines crossed over a 2.5 × 2.5 cm grid. Data were analyzed as in experiment 1.

RESULTS

Experiment 1: Field-Caught Larvae

Change in number of moves from pre-stimulus to post-stimulus activity differed significantly among treatments ($H = 18.55$, $df = 3$, $P < 0.001$; Figure 1). Larvae showed a significant decrease in movement when exposed to conspecific cues compared to tadpole alarm (post-hoc comparison, $W = 715.5$, $P = 0.0031$), *Plethodon* skin control ($W = 642.5$, $P = 0.0001$) and blank ($W = 1108.5$, $P = 0.0041$) cues. There was no difference among the other treatments ($P > 0.15$). There was no effect of length on activity levels across treatments (Pearson's $r = -0.221$, $P = 0.089$) or within treatment groups ($P > 0.10$).

Experiment 2: Neonate Heartrate

Change in gill pulse rate from pre-stimulus to post-stimulus differed significantly among treatments ($H = 11.92$, $df = 3$, $P = 0.008$; Figure 2). Post-hoc comparisons showed that larvae exposed to the 1-mo old conspecific cue had a significantly higher gill pulse rate than those exposed to the 1-day-old conspecific cue (post-hoc comparison, $W = 472.5$, $P = 0.0092$), the *Plethodon* skin control ($W = 460.5$, $P = 0.0235$) and the blank ($W = 504.5$, $P = 0.0018$) cues. There was no difference in activity response among the other treatments ($P > 0.1$).

Experiment 3: Lab-raised Larvae

There were no significant differences in change in number of moves among stimuli ($H = 7.30$, $df = 4$, $P = 0.121$; Figure 3). There was a significant correlation of

larval length and activity in the blank treatment group (Pearson's $r = -0.530$, $P = 0.020$).

There was no significant effect of larvae length on cross-treatment activity (Pearson's $r = -0.037$, $P = 0.732$) or within the *Plethodon*, toad tadpole, 1-mo-old larvae, or 1-day-old larvae treatments (P 's > 0.05).

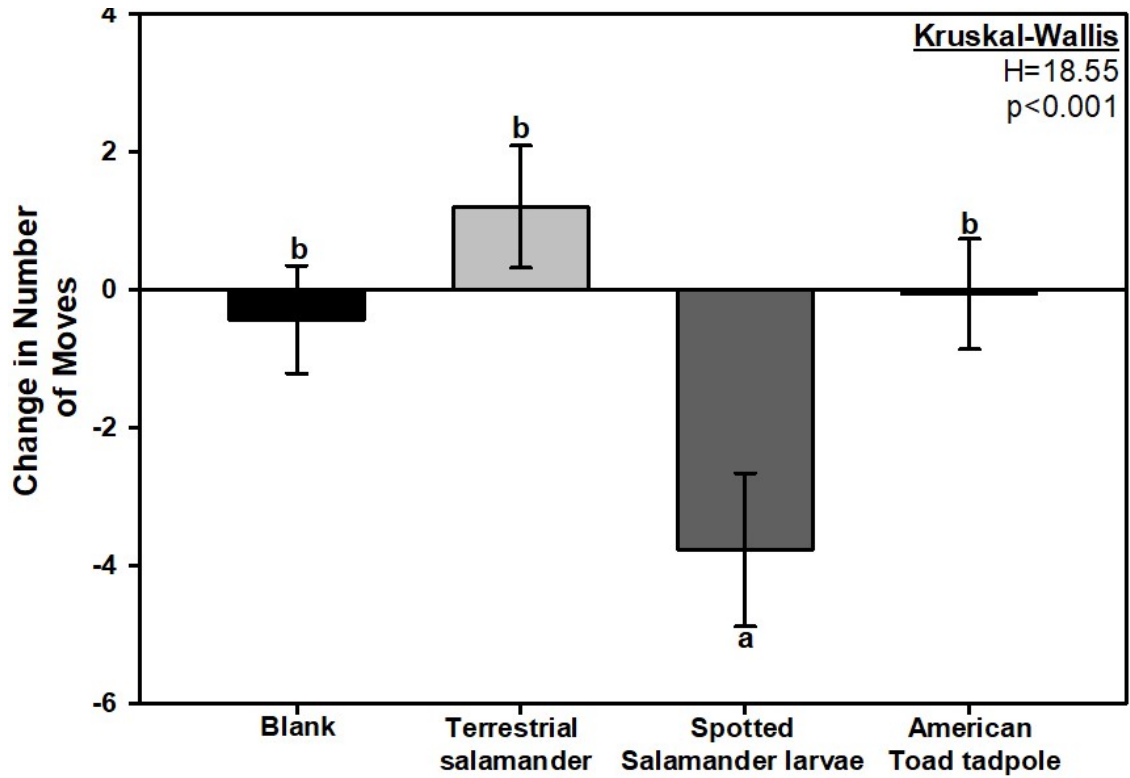


Figure 1. Change in activity from pre-stimulus period to post-stimulus period in Experiment 1, measured as number of moves (mean \pm 1 SE). Treatments were water with no other added stimulus (blank), skin extracts from a terrestrial salamander (*Plethodon angusticlavius*), and whole-body extracts from Spotted Salamander larvae and American toad tadpoles. Significance of post-hoc comparisons is indicated by different letters.

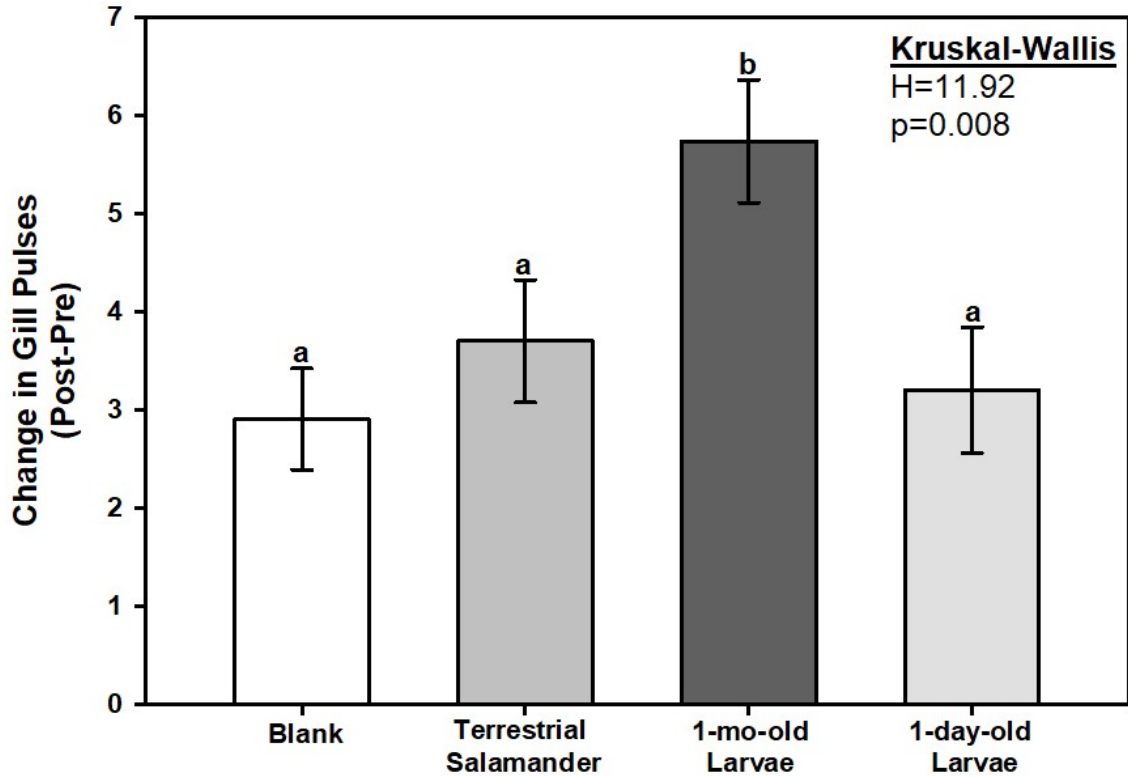


Figure 2. Change in heartrate from pre-stimulus period to post-stimulus period in Experiment 2, measured in gill pulses (mean \pm 1 SE). Treatments were water with no other added stimulus (blank), skin extracts from a terrestrial salamander (*Plethodon serratus*), and whole-body extracts from Spotted Salamander larvae at 1-month and 1-day of age. Significance of post-hoc comparisons is indicated by different letters.

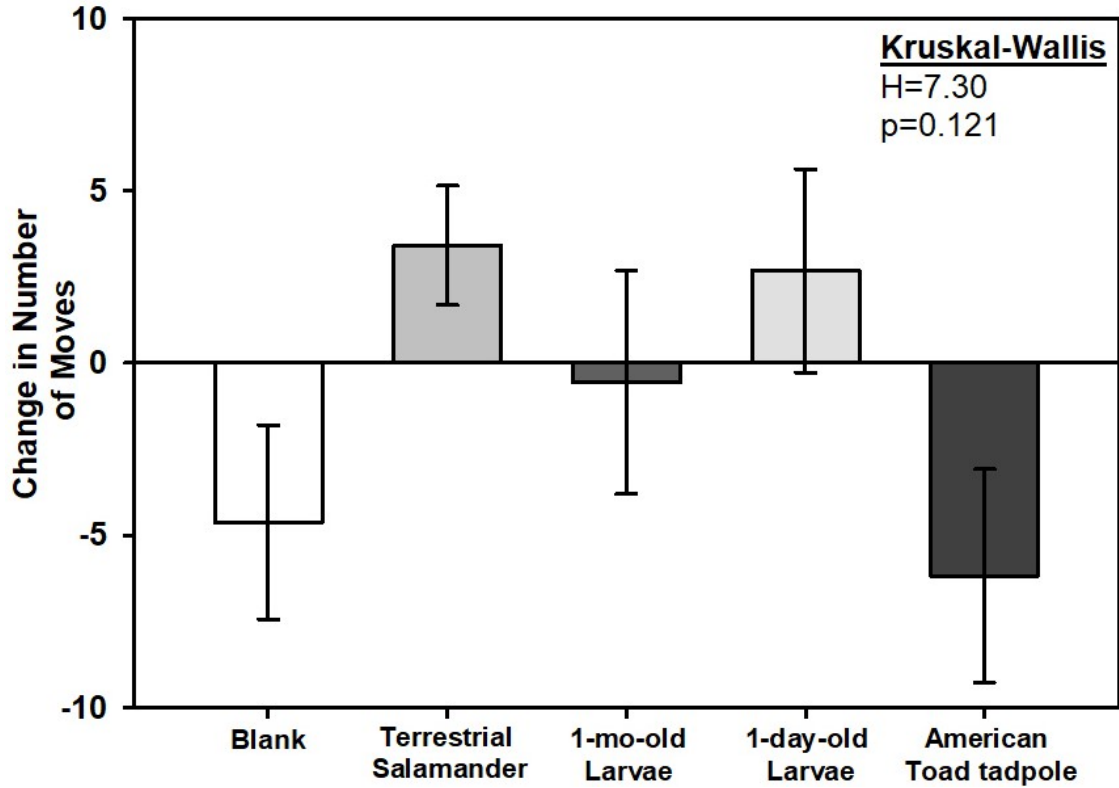


Figure 3. Change in activity from pre-stimulus period to post-stimulus period in Experiment 3, measured as number of moves (mean \pm 1 SE). Treatments were water with no other added stimulus (blank), skin extracts from a terrestrial salamander (*Plethodon serratus*), and whole-body extracts from Spotted Salamander larvae at 1-month and 1-day of age and American toad tadpoles.

DISCUSSION

Spotted salamander larvae decreased activity when exposed to the cue of macerated conspecifics but did not do so upon exposure to other stimuli. A decrease in movement is considered a typical fright response in amphibian prey (e.g. Bourdeau and Johansson 2012; Mathis et al. 2003; Mathis et al. 2008) and has been shown to increase survival of some aquatic amphibians in the presence of predators (Azevedo-Ramos et al. 1992). Because this response was not found in any of our controls (blank, terrestrial salamander, tadpoles), we infer that the response is specific to damaged tissue from conspecifics and not to general disturbance or to tissues (e.g., blood, muscle proteins, etc.) that would also be released due to damage in other species.

My study is the first to show direct evidence that larval salamanders produce chemical alarm cues. The most recent review of alarm cue presence in aquatic taxa lists larvae of one other mole salamander, *Ambystoma macrodactylum*, as having an alarm cue based on the findings of Wildy and Blaustein 2001 (Ferrari et al. 2010b). However, the design of their study did not separate the effects of visual and chemical cues, and the authors had only hypothesized that their results might be due to a chemical alarm cue. Therefore, other cues—the sight or the scent of the predator—cannot be ruled out from possible causes for the fright reaction the authors observed. It is likely that chemical alarm cues play a role in detection of predation risk throughout the life of spotted salamanders. Hunt (2011) reported that post-metamorphic juveniles of this species exhibited avoidance behavior to damaged conspecific skin, indicating that responses to alarm cues persist into the terrestrial phase. In addition, one other member of this family,

Ambystoma macrodactylum, responded to chemical cues from damaged skin as adults (Chivers et al. 1996).

Spotted salamander larvae did not react with fright to the alarm cue of American toad tadpoles, contrary to my hypothesis that individuals would respond to alarm cues from other species in the prey guild. This hypothesis was based on their co-occurrence in the same ponds and at least some shared predators (e.g., odonates: Skelly and Werner 1990). Response to alarm cues from heterospecifics in the same prey guild have been reported for several other species (Anderson and Mathis 2016; Mathis and Smith 1993; Crane et al. 2013). Although spotted salamanders and toad tadpoles occupy the same pond, they may not share the same microhabitat. Beiswenger (1977) found that American toads spend most of their active periods in shallow waters near the edge of the pond, and my observations indicate that this is also the case at my study pond. In contrast, spotted salamander larvae tended to occupy dense grasses and floating vegetation located approximately one meter from the shoreline (personal observation). In at least some species (e.g. Pollock et al. 2003), responses to alarm cues from heterospecific prey guild members requires experience. I hypothesize that differences in microhabitat use limits the opportunity for learning in this species pair.

For tadpoles of another amphibian, *Rana clamitans*, the alarm cue is produced by skin cells (Fraker et al. 2009). Because I prepared the alarm cue using whole body macerations of spotted salamander larvae, I am unable to determine the origin of the alarm cue in my study. Few studies outside of ostariophysan fishes have investigated the location of alarm cue production, but research shows the location may not be consistent among taxa (Smith 1979; Meuthen et al. 2018). Moreover, although tissues were

damaged in my study, we do not know whether such damage is required for release of the alarm cue. Although damage is required for alarm cue release in ostariophysan fishes (Smith 1992), alarm cues can be released voluntarily in *R. clamitans* tadpoles and in adult terrestrial salamanders of the genus *Plethodon* (Fraker et al. 2009, Watson et al. 2004).

My second experiment investigated whether response to the alarm cue required experience. Based on my results, larval experience with alarm cues is not required for detection and response to the alarm cue. However, because the eggs were collected from ponds, I cannot rule out whether our results were influenced by their experience as embryos. Spotted salamanders are known explosive breeders, congregating at vernal ponds when temperature and precipitation conditions align in the spring (Semlitsch and Pechmann 1985). Due to the variable weather patterns throughout much of their range, breeding events can occur in two or three separate events throughout the season (Harris 1980). The eggs collected for the second experiment were laid during one of the later breeding events of the season, as I had collected eggs from the first experiment nearly a month prior. This temporal separation would provide the opportunity for embryos to be exposed to the alarm cue of considerably older larvae being preyed upon within the same pond. Thus, I am unable to determine whether the response is innate or a learned response from exposure to alarm cues while still in egg.

The second experiment also tested whether developmental stage of the cue donor (1-day vs. 1-mo old donors) had any effect on the presence of alarm cue. Gill pulse rates of neonates were significantly higher when exposed to the alarm stimulus of 1-month-old larvae in comparison to the controls or to the 1-day-old larvae. Therefore, although the larvae were able to recognize and respond to alarm cues within days of emerging from

their egg, the alarm cue may not be present until larvae are more fully developed. Other studies have compared responses of receivers to conspecific alarm cues from different aged cue donors, but the nature of the results were different from our study, with most test animals responding with fright to the cue from their own age class and ages most similar to their own, and a lack of response to alarm cues from much older or younger individuals (e.g. snails: Ichinose 2002; fishes: Lönnstedt and McCormick 2011, Mitchell and McCormick 2013). Responding only to alarm cues from individuals most similar to their own age/size would be adaptive in cases where predation risk is size-dependent. We see two possible explanations for the lack of response toward 1-day-old conspecific alarm cues by our newly-hatched larvae. First, if the alarm substance is indeed epidermal in origin, perhaps the glands that produce the substance are not fully developed in day-old larvae. The epidermal surface cells in larval salamanders undergo dramatic changes as early as hatching up until metamorphosis, potentially indicating differences between the cue prepared from 1-day-old larvae and 1-month-old larvae (Lewinson et al. 1983). Second, an alarm cue may be present in 1-day-old individuals but is chemically different from the alarm cue in 1-mo-old larvae. If the response to alarm cues is a learned response, then perhaps the 1-day-old larvae lacked the experience with the alarm cue from younger larvae that would be needed to recognize the younger conspecific alarm cue as an indicator of risk.

In contrast to the field-caught larvae in experiment 1, lab-reared individuals in experiment 3 showed no significant difference in activity in response to either of the conspecific alarm cues. This result is surprising given that larvae that emerged from eggs in the lab did exhibit a change in a metabolic response (heart rate as measured by gill

pulse rate) in response to the alarm cue. This result may indicate that the behavioral response to alarm cue requires experience in spotted salamander larvae. If true, the results from our second experiment may be due to embryonic learning that occurred when the eggs were still in the pond, as mentioned previously. In bronze frog tadpoles, behavioral responses to the conspecific alarm cue required experience (Batabyal et al. 2014).

My data confirm the presence of a chemical alarm substance in larval spotted salamanders and suggest that production of the cue may not develop until several days to a month post-hatching. Physiological responses to the alarm cue are either innate or result from embryonic learning. Behavioral responses occurred in field-caught but not in lab-reared larvae, suggesting that experience as larvae may be important. These results provide a foundation for future studies that examine the interactions between genes, development and embryonic and larval experience on responses to predation risk in aquatic species.

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