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Behavioral Responses of Ringed and Spotted Salamanders to Diet-Related Cues from Predators

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BEHAVIORAL RESPONSES OF RINGED AND SPOTTED SALAMANDERS TO

DIET-RELATED CUES FROM PREDATORS

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In Partial Fulfillment

Of the Requirements for the Degree

Master of Science, Biology

By

Kayla Marie Shelton

August 2016

BEHAVIORAL RESPONSES OF RINGED AND SPOTTED SALAMANDERS TO

DIET-RELATED CUES FROM PREDATORS

 Biology

Missouri State University, August 2016

Master of Science

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ABSTRACT

Multiple species of *Ambystoma* can sometimes coexist in ponds, often forming a prey guild. In the Ozarks, ringed salamanders (*Ambystoma annulatum*) can coexist with marbled salamanders (*A. opacum*) and spotted salamanders (*A. maculatum*). Ringed and marbled salamanders lay their eggs in ponds in the fall and overwinter as larvae, whereas spotted salamanders breed in the spring. Ringed and marbled salamander larvae can be cannibalistic and can prey upon larvae of the other two species. Larvae should experience increased survival if they can discriminate between predators that have recently consumed another salamander (high risk) versus those that have not (low risk). I exposed two life stages of ringed and spotted salamanders to chemical cues from marbled salamanders that had consumed different diets and quantified their responses. Embryos of spotted salamanders showed increased heart rate in response to cues from predators that had consumed congeneric *Ambystoma* than predators that had consumed worms. Larval ringed salamander showed increased movements in response to predators that had consumed a conspecific versus a worm. Therefore, predator diet influences responses of both embryonic and larval ambystomatid salamanders.

KEYWORDS: diet effects, prey guild, predator recognition, chemical cues, *Ambystoma*

This abstract is approved as to form and content

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Alicia Mathis, PhD
Chairperson, Advisory Committee
Missouri State University

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By

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August 2016

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INTRODUCTION

Predator recognition is vital to survival during a predation event. Individuals that are able to rapidly detect threats and engage in the appropriate antipredator behavior should have a higher probability of survival (Lima and Dill 1990). While increased survival is an obvious benefit of employing antipredator behaviors, there are also costs associated with them, such as missed foraging and mating opportunities. For example, if an individual spends most of its time being alert and vigilant, it will have a decreased risk of predation but will have less time to consume food or search for potential mates. Individuals must therefore balance the costs and benefits of antipredator behaviors to maximize their lifetime fitness. One strategy by which individuals can improve the balance between predation risk and risk of loss of time for other activities is to make fine-scale discriminations between stimuli that likely represent a high level of danger and those that are likely less dangerous.

The primary sensory modes used for predator recognition are visual, auditory, and chemical. The strength and availability of a cue depends on a number of characteristics, including the cue's duration, the capacity of the cue to move past obstacles, the degree to which the cue is localizable and the energetic cost of producing the cue (Krebs and Davies 1993). Visual cues are useful in predator recognition because they can readily indicate the location of the predator and sometimes whether the predator is actively hunting (Chivers et al. 2001; Hemmi 2005). Auditory cues can be useful in habitats where visual cues are not available (Wisenden et al. 2008), but many predators are quiet, particularly when hunting. Chemical cues may be present in habitats with low visibility,

when predators are visually cryptic, and when the focal individual is not facing the predator. Unlike visual and auditory cues, chemical cues can have a relatively long duration which can serve to inform prey of not only immediate predator presence but past predator presence as well (Van Buskirk et al. 2014). Chemical cues can also move past barriers that would otherwise prevent predator detection by the individual until it is too late to perform and antipredator response. The early warning provided by chemical cues can have important consequences because the earlier an individual engages in antipredatory behavior during a predation attempt the better are its chances of survival (Lima and Dill 1990).

In aquatic habitats, predators are frequently silent and visually cryptic, habitats often have low light due to depth and turbidity, and visual disruptions such as vegetation are common. Consequently, chemical cues may be especially important for aquatic species (review: Chivers and Mirza 2001a). In some cases, chemical cues have an additional benefit of providing information about the predator's recent diet (reviews: Chivers and Mirza 2001a; Ferrari et al. 2010b). For example, fathead minnows (*Pimephales promelas*) exhibit a fright response when exposed to chemicals from northern pike (*Esox lucius*) that had eaten conspecific minnows, but not to chemicals from pike that had eaten other species of prey (Mathis and Smith 1993). Even when individuals exhibit fright responses to predators regardless of diet, the response may be a heightened when exposed to cues from predators that had consumed conspecifics (reviews: Chivers and Mirza 2001a; Ferrari et al. 2010b). For example, wood frog (*Rana sylvaticus*) tadpoles exhibited fright responses when exposed to predatory fish (*Perca flavescens*) regardless of diet. However, their fright responses significantly intensified

when exposed to cues from a fish that had recently consumed a conspecific (Chivers and Mirza 2001b).

For most aquatic species, chemical cues are available across all developmental stages, from embryos in permeable eggs to adults. Although it is rare, embryos of some species can enact effective immediate antipredator behavioral responses (Warkentin 2000), and embryonic exposure to predators can lead to developmental changes in morphology (Laurila et al. 2002). Embryonic exposure to predators can also alter post-hatching behavior, which can lead to increased survival. In ringed salamanders (*Ambystoma annulatum*), embryonic exposure to predator cues resulted in decreased post-hatching activity and increased use of shelter (Mathis et al. 2008). Wood frog embryos can learn to recognize unfamiliar predators through classical conditioning (Mathis et al., 2008) and can retain information on the temporal foraging behavior of predators experienced while in the egg (Ferrari et al. 2010a).

Predators often prey upon multiple species; these target species form a "prey guild", and members sometimes respond to each other's alarm cues (reviews by Brown 2003; Ferrari et al., 2010b) and can learn about predation risk by observing behavior of other species (review: Brown, 2003). Individuals should benefit if they also respond to diet-based cues from predators that have recently consumed other species in their prey guild, although this phenomenon has not been well-studied. In one example, damselfly larvae (*Enallagma* spp.) share a prey guild with fathead minnows. When exposed to cues from a predatory northern pike that had consumed either damselflies or fathead minnows the larvae exhibited fright responses. However, damselfly larvae showed no fright

response when exposed to cues from a pike that had consumed mealworms (Chivers et al. 1996).

Diet cues might be particularly important in species in which there is substantial individual variation in predator diets such that some individuals are dangerous to a particular species and others are not. For example, some amphibian larvae develop a morphology that allows them to feed on conspecifics and congeners, whereas other individuals prey only on heterogeneric prey (Pedersen 1991; Walls et al. 1993). Because the specialized predators are feeding on individuals that are also their competitors, the predatory morphotype has the added advantage of not only satisfying its nutritional and energetic needs but also reducing future competition for prey (reviewed by Polis et al. 1989; Polis and Holt, 1992; Hiltunen et al., 2013). Predation on conspecifics and congeners is common in aquatic ecosystems and has been observed in *Ambystoma* salamanders (Cortwright 1988; Semlitsch and Reichling 1989; Anderson et al. 2013) on both the embryonic and larval stages (Anderson et al. 2013).

Salamanders of the genus *Ambystoma* typically lay eggs in fishless ephemeral ponds (Petranka 1998). These salamanders have a biphasic life cycle in which the embryos and pre-metamorphic larvae are aquatic, and the post-metamorphic larvae and adults are terrestrial. In some instances, several species of *Ambystoma* may coexist, exploiting the same resources within these ponds. When larvae occur in high densities there can be high rates of mortality due to aggression or exploitative competition (Petranka 1989; Semlitsch and Reichling 1989). High densities can also lead to larger larvae becoming cannibals and preying upon smaller conspecifics and congeners (Collins and Cheek 1983).

Three species of *Ambystoma* salamanders can coexist as larvae in ephemeral ponds in the Ozarks region of the United States (e.g., Peterman et al. 2014). In the fall, female ringed salamanders (*Ambystoma annulatum*) lay eggs; larvae then overwinter in the ponds and metamorphosis occurs in early May (Spotila and Beumer 1970; Hutcherson et al. 1989). The marbled salamander (*Amystoma opacum*) is also a fall breeding species. Females lay their eggs on the edges surrounding the ponds (Boone et al. 2002), and the larvae are swept into the ponds with the fall rains. Metamorphosis occurs in April–May (Trauth et al. 2004). Spotted salamanders (*Ambystoma maculatum*) are a spring breeding species, with eggs laid in late February–early March and hatching in 30–40 days. Larvae typically remain in the ponds until late March–June (Trauth et al. 2004).

Larval ringed and marbled salamanders can be cannibalistic (Jefferson et al. 2014; Walls and Blaustein 1995) and may prey upon each other (fall and spring) and the larvae of spotted salamders (spring) (Walls 1995). Generally, smaller larvae and recent hatchlings are more vulnerable to predation due to reduced swimming ability and being within the predators' gape limitations (Nyman et al. 1993; Anderson et al. 2013). Because *Ambystoma* larvae that have consumed one congener are likely also dangerous to other species, naïve larvae should benefit from discriminating between *Ambystoma* predators that have consumed other *Ambystoma* of any species and those that have consumed non-*Ambystoma* prey. I chose to these three species of *Ambystoma* as my focal species because of the complex interactions that can take place between them. As they are syntopic and share a prey guild, individuals should respond to cues associated with conspecifics and congenerics.

The goals of my research were to: (1) determine whether embryonic ringed and spotted salamanders differentiate between predators (larval marbled salamanders) that had recently consumed ringed salamander larvae versus predators that had only consumed blackworms (*Lumbriculus variegatus*). Because newly hatched ringed salamander larvae exhibit increased heart rates after exposure to cues from predators versus nonpredators (Jones and Mathis, unpublished data), I hypothesized that *Ambystoma* embryos would exhibit faster heart rates when exposed to cues from *Ambystoma*-fed predators. (2) Determine whether larval ringed and spotted salamanders were able to differentiate between predators that had recently consumed ringed larvae versus blackworm prey. Because *Ambystoma* larvae in both lab and field experiments have been shown to exhibit reduced activity when exposed to cues from other predators (Mathis et al. 2003), reduced activity was also considered to be a fright response in my study.

METHODS

Collection and Maintenance of Salamanders

Ringed salamander eggs were collected at Bull Shoals Field Station (Taney County, Missouri) in October 2015. Spotted salamander egg masses were collected at Compton Hollow Conservation Area (Webster County, Missouri) in March 2015. Each clutch (n = 15) of ringed salamander eggs was placed into a separate aerated container with 2 L of 50:50 of pond water:dechlorinated tap water and stored in an environmental chamber at $15 \pm 2°$ C on a 12:12 L:D cycle. Spotted salamander egg masses (n = 10) which support a symbiotic algae (Gilbert 1944), were housed in the laboratory ($20 \pm 2°$ C) in direct sunlight to ensure adequate algal growth. Newly-hatched larvae of both species were moved to the same type of containers as those used for the embryos and contained 50:50 pond water: dechlorinated tap water to allow feeding on the daphnia that were naturally present in the pond water. Fifty percent water changes were conducted weekly.

Larval ringed and marbled salamanders were collected at Bull Shoals Field Station in January 2015. At the time of collection, ringed salamander larvae were smaller ($\bar{x}$ = 3.03 cm, range = 2.5–3.9 cm) than marbled salamanders ($\bar{x}$ = 5.3 cm, range = 4.5–6 mm) and at an earlier developmental stage, having only developed their upper limbs while marbled salamander larvae had fully developed limbs. Larvae collected in the field were placed into individual containers with 0.5 L of pond water, and were slowly transitioned to 100% dechlorinated tap water over a 2–week period. Larvae were fed blackworms to satiation bi-weekly and all containers had a weekly 50% water change.

The Missouri State University Institutional Animal Care and Use Committee approved this project (October 2014, protocol 14-011).

Collection of Chemical Cues

Larval marbled salamanders (n = 8) were randomly designated as either high-risk predators (fed larval ringed salamanders) or low-risk predators (fed blackworms). The four high-risk predators were each fed one ringed salamander larva and the four low-risk predators were each fed 0.5 mL blackworms weekly for 2 wk. All marbled salamander larvae were weighed (0.9-1.9 g) and measured (total length = 4.5–6 cm) 48 h after the prey had been consumed. After prey were consumed, each larva was moved into a container that held a ratio of 1 g salamander to 60 mL of dechlorinated water, to control for concentration of possible chemical cues. After 48 h, the resulting solution was collected and frozen for future use.

Experiment One: Response of Embryonic Salamanders

At the time of testing, embryos of both species were between Harrison developmental stages 36-41 (Harrison 1969), where the gills were visibly developed. Ringed salamander eggs are laid in loose clusters without a jelly coat; individual eggs were separated from their clusters and placed into individual testing containers (2-cm radius) with 4 mL of pond water. Because the eggs of spotted salamanders are embedded in a thick jelly coat (Pinder and Friet 1994), individual eggs were removed from the jelly coat with surgical scissors and placed in the same type of testing containers as the ringed salamander eggs.

When embryos and very young larvae of *Ambystoma* are viewed under a stereoscopic microscope, embryos, blood can be observed to pulse through the capillaries of the gills in synchrony with heart beats (personal observations). After a 30 min habituation period, eggs were placed under a stereoscopic microscope the number of times the blood pulsed through capillaries (hereafter "heart rate") was recorded for 60 s to establish a baseline. After 60 s, 1 mL of a randomly-selected stimulus was poured into the testing container with the embryo: (1) blank control (dechlorinated tap water) (spotted salamanders, n = 15; ringed salamanders, n = 21); (2) water from a low-risk predator (spotted salamanders, n= 15; ringed salamanders, n = 22); (3) water from a high-risk predator (spotted n = 14, ringed n = 23). The stimulus cue was allowed to disperse for 60 s, and then the heart rate was recorded for an additional 60 s. The observer was blind to the chemical cue each embryo received.

Data were included in the statistical analysis as change in heart rate (post-stimulus minus pre-stimulus). The data met the distribution assumptions for parametric statistics, and a one-way ANOVA followed by Tukey-test multiple comparisons (Minitab v. 14) was used to compare the change in heart rate among treatment groups (blank control; low-risk; high-risk). Data for each species were analyzed separately.

Experiment Two: Response of Larval Salamanders

Both species of salamander larvae were randomly exposed to chemical cues from low-risk or high-risk predators (marbled salamanders). Ringed salamander larvae were tested in a 8.5×8.5×10.5cm container with 500-mL of dechlorinated tap water, and the smaller spotted salamander larvae were tested in the same containers with 250-mL of

dechlorinated tap water. Immediately prior to trials, any remaining food was removed from the container, and a 10-min acclimatization period began. After 10 min, the container was carefully placed on a paper grid with 6×6 1.5-cm cells, and either 10 mL (ringed salamanders) or 5 mL (spotted salamanders) or a blank cue (dechlorinated water) was poured directly into the center of the container. After allowing 15 s for cue dispersal, data were collected during a 5-min pre-stimulus period. After 5 min elapsed, the same volume of either low-risk (spotted n = 19, ringed n = 15) or high-risk (spotted n=19, ringed n = 14) predator cue was administered directly into the center of the container. After 15 s, data were collected for a 5-min post-stimulus observation period. Behaviors recorded were: (1) number of moves: number of times the salamander's snout crossed a line on the grid paper; and (2) latency to move: the length of time after the start of the trial until the first movement occurred. Treatments were assigned randomly, and the observer was blind to the treatments.

For statistical analysis, I subtracted the data in the post-stimulus period from the pre-stimulus period to obtain an overall change in activity level or latency to move. Because the data were not normally distributed, I used nonparametric Mann-Whitney U-tests (Minitab v. 14) to compare the changes in number of moves and latency to move between treatment groups (low-risk; high-risk).

RESULTS

Response of Embryonic Salamanders

For ringed salamander embryos, all treatments tended to increase post-stimulus heart rates. There was an overall significant difference among treatments ($F_{2,65} = 9.80$, $p < 0.001$), with embryos in both diet treatments exhibiting significantly higher heart rates from the control. (Tukey tests: both p-values < 0.05), but no significant difference between the high-risk and low-risk treatments ($p > 0.97$) (Fig.1a). Embryonic spotted salamanders also tended to increase post-stimulus heart rates in all treatments, with an overall difference among treatments ($F_{2,42} = 9.45$, $p < 0.001$). However, spotted salamanders in the high-risk treatment had significantly faster heart rates than the other two treatments (Tukey tests: ($p < 0.05$) and the low-risk and control treatments did not differ significantly ($p > 0.95$) (Fig.1b).
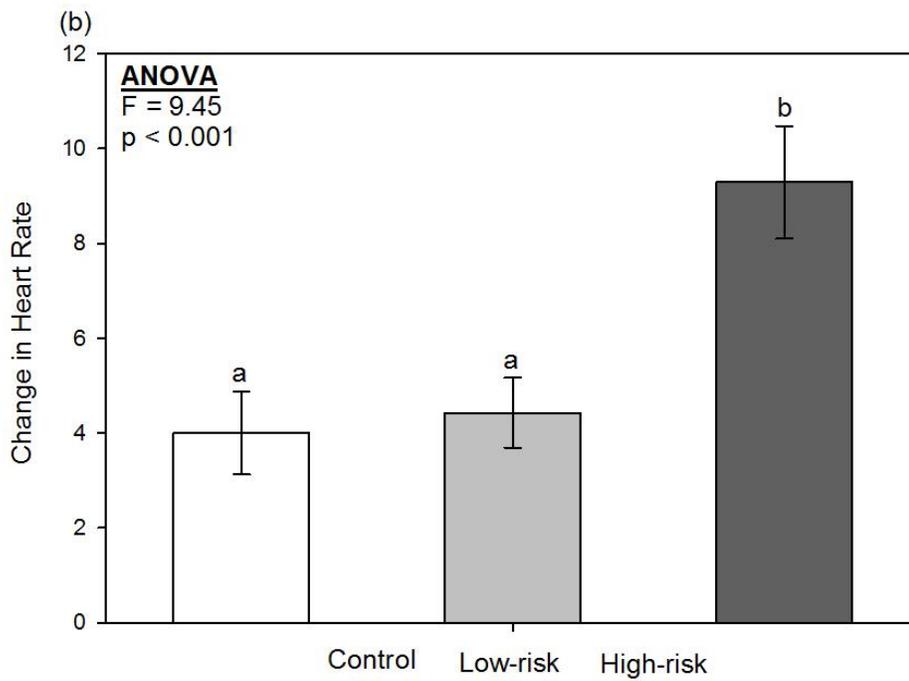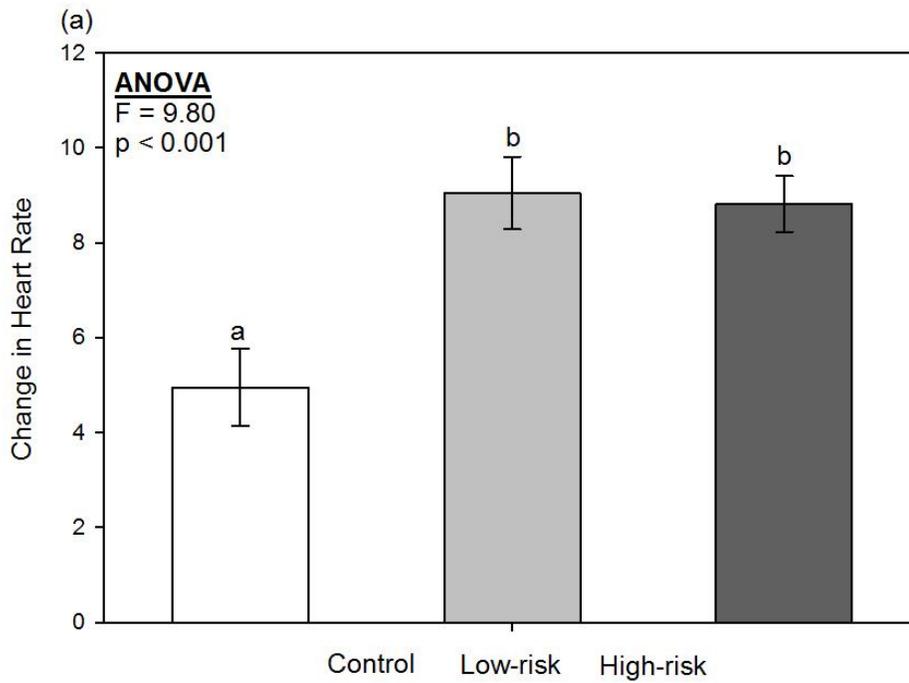
Figure 1: Change in heart rate (post-stimulus – pre-stimulus) for embryonic (a) ringed salamanders and (b) spotted salamanders exposed to a blank control, or to chemical stimuli from a low-risk (fed worms) or high-risk (fed ringed salamanders) marbled salamander predator.

Response of Larval Salamanders

     Movement. On average, ringed salamander larvae in the low-risk diet treatment increased their activity while those in the high-risk diet treatment exhibited decreased activity levels. The diet treatments were significantly different from each other ($W = 259.0$, $p = 0.05$) (Fig. 2a). However, spotted salamander larvae in both low-risk and high-risk diet treatments decreased their number of moves and were not significantly different from each other ($W = 367.0$, $p = 0.93$) (Fig. 2b).

     Latency to Move. For both ringed salamander larvae ($W = 233.0$, $p = 0.49$) (Fig.3a) and spotted salamander larvae ($W = 392.0$, $p = 0.54$) (Fig.3b) the behavior of larvae in the two diet treatments did not differ significantly.

(a)

(b)

Figure 2: Difference in number of moves (post-stimulus – pre-stimulus) for larval (a) ringed salamanders and (b) spotted salamanders when exposed to chemical cues from low-risk (fed worms) or high-risk (fed ringed salamanders) marbled salamander predators.
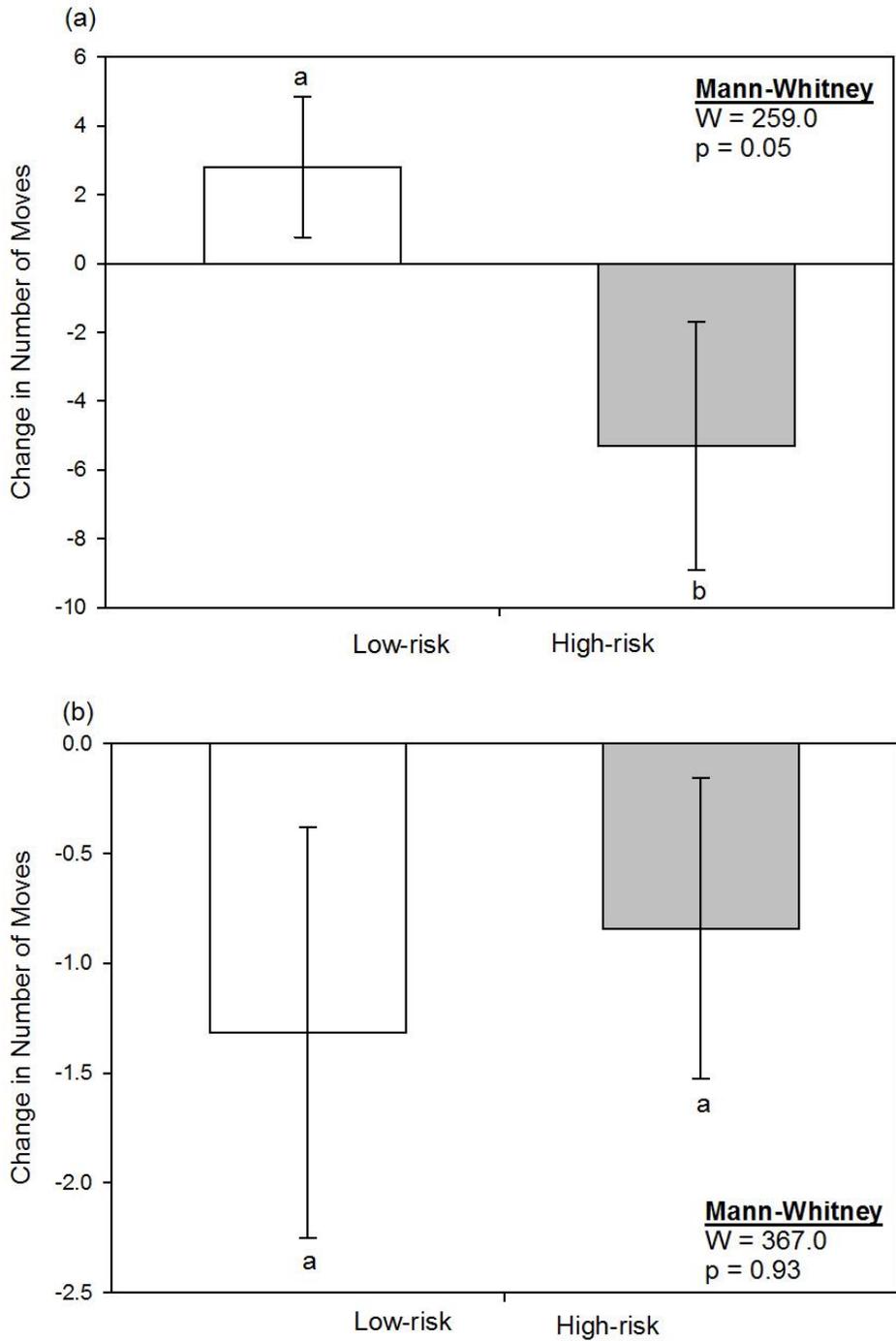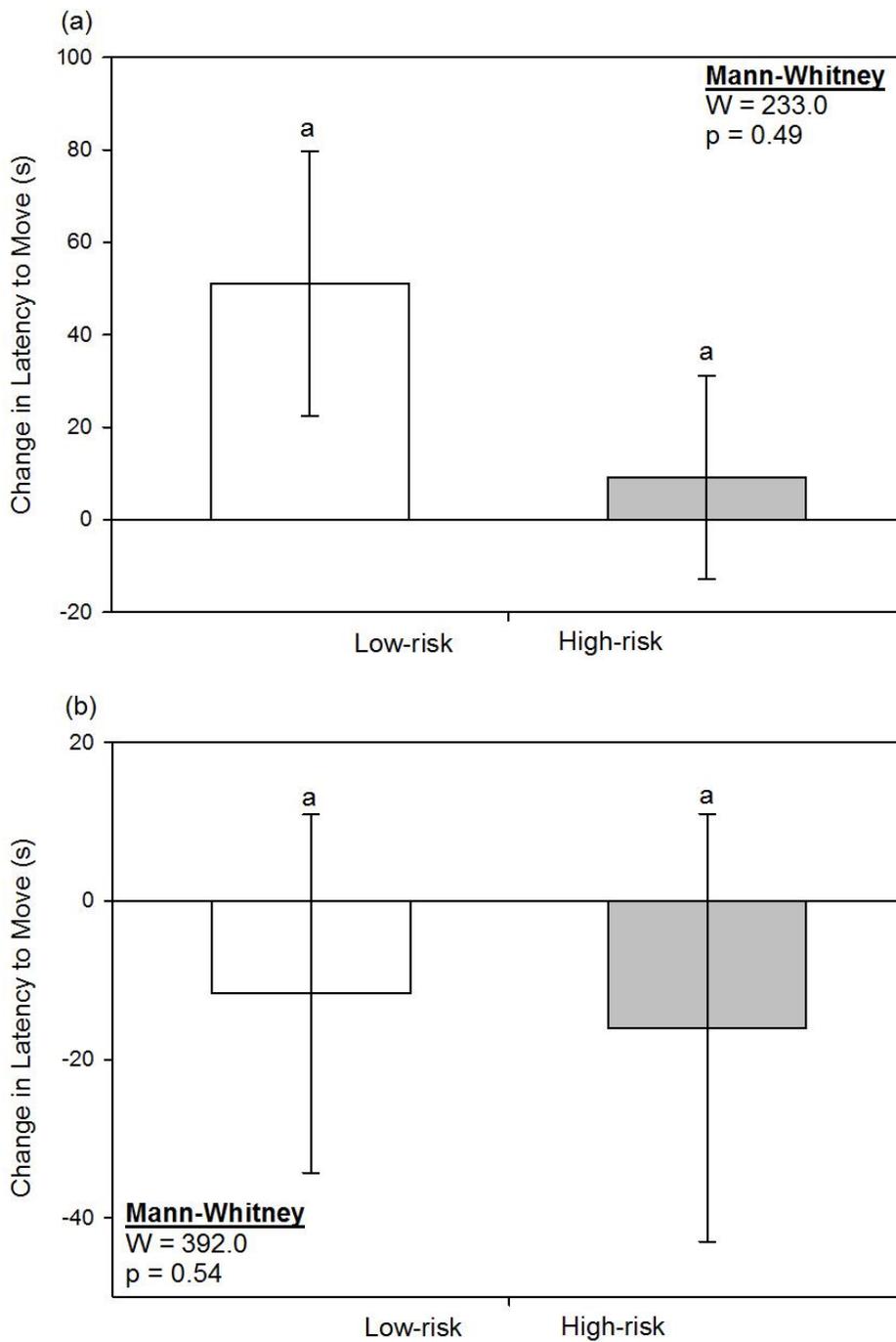
Figure 3: Difference in latency to move (post-stimulus – pre-stimulus) for larval (a) ringed salamanders and (b) spotted salamanders that were exposed to chemical stimuli from low-risk (fed worms) or high-risk (fed ringed salamander) marbled salamander predators.

DISCUSSION

The results of my experiments provide support for the hypotheses that embryonic and larval *Ambystoma* can distinguish between predators that have recently consumed a congeneric larva versus those that have consumed non-salamander prey, although the nature and timing of the response differed between species. While predator diet effects have been observed in some species of aquatic vertebrates (reviews: Chivers and Mirza 2001a; Ferrari et al. 2010b), other studies of *Ambystoma* have failed to find an effect of recent diet of predators on salamander antipredator behavior (Chivers et al. 1997; Hunt 2011). However, some species of terrestrial salamanders (Family Plethodontidae) have been shown to be able to use predator-diet related cues to distinguish between different levels of risk (Murray and Jenkins 1999; Sullivan et al. 2004; Sullivan et al. 2005).

Ringed Salamanders

As embryos, ringed salamanders demonstrated the ability to distinguish between a blank control and chemical cues of predators by significantly increasing their heart rates. However, whether a predator had recently consumed a conspecific had no effect. Although the current study did not include a pungency control (a nonpredator scent), newly-hatched larvae of this species do not increase heart rates when exposed to a nonpredator scent (Jones and Mathis, unpublished data). Exposure of embryonic ringed salamanders to predator cues has been shown to change post-hatching behavior (Mathis et al. 2008). My study suggests that embryos possess the capacity to respond to chemical cues as early as Harrison Stages 36-41 of embryonic development; hatching occurs at

16

stages 39-46 (Harrison 1969; Sih and Morre 1993; Mathis et al., 2008). An increase in

respiration is considered a key component of the vertebrate fight-or-flight response, but it

is questionable as to whether this response could successfully lead to increased survival

in the face of predation for salamander embryos. Vibrational cues produced when an egg

mass is under predator attack can induce early hatching of eggs of red-eyed treefrogs,

Agalychnis callidryas (Warkentin 2005). As part of the fight-or-flight response,

increased heart rate could fuel movements required for early hatching, but early hatching

as an antipredator response has not been studied for salamanders.

The heart rate of embryos of ringed salamanders did not vary between high and

low-risk predators, but discrimination based on dietary cues did occur in ringed

salamander larvae. Therefore, either an inherent variable response to low- versus high-

risk predators develops later or experience is required for discrimination based on

predator diet cues.

At the larval stage, wild-caught ringed salamanders significantly decreased their

activity when exposed to cues from the high-risk predator, which had eaten conspecifics,

in comparison to the low-risk predator, which had eaten black worms. These data

confirm the results of a similar study conducted on lab-reared larvae that were tested with

a conspecific predator on a diet of salamanders vs black worms (Greene, Greene,

Radomski and Mathis, unpublished data). Therefore, the response to predator diet cues

by *A. annulatum* larvae appears to be robust. Because the larvae in the current study

were wild-caught, they may have been exposed to cues from predators consuming

different types of prey prior to testing. The lab-reared larvae in the previous study

(Greene, Greene, Radomski and Mathis, unpublished data) were reared in group tanks

and so also had likely been exposed to cues from cannibals and noncannibals before testing. Whether experience is required for discrimination of diet cues remains untested in this species. However, Wildy and Blaustein (2001) reported that exposure to cannibalism was required for diet-related discrimination by larval long-toed salamanders, *A. macrodactylum*, the only larval salamander that has been tested. In contrast, previous research has shown that inexperienced wood frogs are able to use diet cues to distinguish between predator threats (Chivers and Mirza 2001b).

Spotted Salamanders

As embryos, spotted salamanders were able to distinguish between the low-risk and high-risk predator treatment by significantly increasing their heart rates when exposed to high-risk predators. It is unclear why discrimination based on predator diet cues should develop earlier in in spotted salamanders (embryo stage) and ringed salamanders (larval stage). Ringed and marbled salamander eggs hatch at similar times. At the time of hatching, ringed salamanders are likely not vulnerable to predation from marbled salamanders, which have also just hatched and would not be large enough to consume the young ringed salamander larvae. Early investment in fine-scale discrimination of low- versus high-risk marbled salamander would be of little benefit. In contrast, spotted salamanders hatch in the spring when marbled salamanders (predator) have grown large enough to consume other larvae. At the moment of hatching, spotted salamanders are vulnerable to predation by larval salamanders. Early investment in discrimination between low- and high-risk predators would allow spotted salamanders to

avoid the most dangerous predators while and to not waste valuable foraging time by responding to cues from predators that are low risk.

At the larval stage, spotted salamanders qualitatively decreased their activity when exposed to both predator treatments, but there was no significant difference between diet treatments. Larval long-toed salamanders also failed to distinguish between chemical stimuli from predators based on diet cues (Chivers et al., 1997). Qualitatively, the reduction in activity in both treatments is consistent with a freezing (Semlitsch, 1987; Yurewicz, 2004). However, a loss of discrimination between high-and low-risk predators at a time when it should have the highest benefit is puzzling. Behavior of the spotted salamander larvae in the current study was highly variable, particularly with respect to latency to move. During the period of testing, many spotted salamander larvae exhibited erratic behaviors, including bursts of movement followed by no activity. Mortality for this species was high, with almost all individuals dying within days of testing. Illness may have influenced larval behavior during testing, so interpretation of my results for larvae of this species is ambiguous.

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