Innate and Learned Predator Recognition Mediated by Chemical Signals in *Eurycea nana*

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Abstract

Effective and efficient predator recognition and avoidance are essential for the persistence of prey populations, especially in habitats where non-native predators have been introduced. Predator recognition studies are commonly couched within a learned or innate dichotomous framework; however, characteristics of some systems or species could favor innate recognition combined with the ability to alter avoidance responses based on experience with predators. *Eurycea nana* is a fully aquatic salamander inhabiting a system with a diverse, yet temporally stable, community of native and non-native opportunistically foraging fish predators. To examine predator recognition, we examined avoidance responses (decreased activity) of predator-naïve (first-generation, captive-reared) and predator-experienced (recently collected) *E. nana* to the chemical cues of a native predator, a non-native predator, a nonpredator, and a blank control. Both predator-naïve and predator-experienced *E. nana* significantly lowered activity in response to the native fish predator when compared with a blank control. Interestingly, predator-naïve *E. nana* decreased activity in response to the non-native fish predator while predator-experienced *E. nana* did not. These results indicate that while there is an innate component to predator recognition in *E. nana*, experience and risk assessment may also be important.

Introduction

Predation affects prey populations through removal of prey and induction of antipredator behaviors (Lima 1998; Werner & Peacor 2003). To persist with predators, prey in diverse systems must be able to recognize and avoid a variety of predatory threats. This can be especially important for native prey in systems where predators have been introduced. Here, we define predator recognition as the detection and identification of predatory stimuli that elicit avoidance responses in prey. Prey may be able to detect and identify a number of predator-related stimuli in aquatic habitats including visual (Brown et al. 1997; Miklosi et al. 1997; Utne-Palm 2001) and chemical (as reviewed by Kats & Dill 1998) cues, although chemical stimuli

appear to be the most commonly used cue by amphibian prey (Kats & Dill 1998; Mathis & Vincent 2000; Ferrer & Zimmer 2007). Recognition of predators may have an innate basis (Griffiths et al. 1998; Laurila 2000) or be learned (Chivers & Smith 1998; reviewed by Wisenden 2003). Prey exhibiting learned predator recognition require experience with predators to identify predatory threats while prey exhibiting innate predator recognition do not.

Innate predator recognition confers different costs and benefits than does learned predator recognition. Innate predator recognition can result from the co-evolution of prey and predator and is advantageous because an encounter with a predator is not required for naïve prey to respond adaptively to predatory threats (Laurila 2000). However, innate

recognition alone can be costly as it may limit the number of recognized predatory species (Wisenden 2003). Prey that must learn to recognize predatory threats typically do so through a potentially costly naïve encounter with predators. Despite this, the ability of prey to acquire recognition of novel predators can be beneficial, especially within diverse or fluctuating predatory communities (Wisenden 2003). Additionally, prey may use experience with predators to assess predation risk and adjust the intensity of their avoidance responses accordingly (Brown 2003; Ferrari et al. 2005; Gonzalo et al. 2007), which reduces the costs associated with predator avoidance such as time allocation trade-offs between avoidance and foraging (Sih 1992; Lima & Bednekoff 1999).

Studies exploring learned and innate predator recognition by vertebrate aquatic prey have found that, in general, fish exhibit learned predator recognition (Brown 2003; Kelley & Magurran 2003) while amphibians rely on innate recognition (Kats & Dill 1998), though notable exceptions exist (Suboski 1992; Miklosi et al. 1997; Woody & Mathis 1998; Wildy & Blaustein 2001; Mandrillon & Saglio 2005). This evidence, in many cases, has led to a conceptual dichotomy when considering how prey recognize predators. However, it is important to consider that some systems may favor prey species that use both methods in concert for more efficient predator recognition and avoidance. Interestingly, while some studies have explored this possibility (Sih & Kats 1994; Laurila et al. 1997; Gallie et al. 2001), we know of only one study which clearly demonstrated experience-mediated, risk-sensitive adjustments to innate avoidance responses of an amphibian species, *Rana perezi* (Gonzalo et al. 2007).

Determining the role of experience in predator recognition is especially important for native prey in habitats where predators have been introduced. If prey exhibiting innate predator recognition are unable to acquire recognition of novel predatory stimuli, they may not respond adaptively to non-native predatory threats (Kiesecker & Blaustein 1997; Pearl et al. 2003; Anthony et al. 2007). This has been described as a primary cause of native amphibian population declines and extinctions in some habitats where non-native predators have been introduced (Knapp & Matthews 2000; Adams et al. 2001; Pilliod & Peterson 2001; Kats & Ferrer 2003). However, prey that are able to recognize some predators at birth and also exhibit the capacity to use experience to acquire recognition of or alter the intensity of avoidance responses to predators would be at a selective advantage in these systems.

It is important to explore the role of experience in predator recognition and avoidance in systems where both innate and acquired responses are expected to be important. We explored the role of experience in recognition of fish predators using the San Marcos salamander, *Eurycea nana*. *Eurycea nana* is a federally threatened (U.S. Dept. of the Interior, 1980) paedomorphic species (obligately aquatic throughout life). The thermostable (21.0–21.5°C, Groeger et al. 1997) habitat of *E. nana* has a diverse and temporally stable predatory community including many native and non-native opportunistically foraging predators (Bowles & Bowles 2001). Because *E. nana* must avoid fish predation as reproductive adults as well as juveniles, the propensity for adverse impacts of predatory fish on the population may be relatively greater, or at least different, than on most amphibian prey that exhibit both aquatic and terrestrial life stages. As with other amphibian species, we predicted that *E. nana* would exhibit innate recognition of native predators and that they might not recognize non-native predators as threats. However, given that *E. nana* face lifelong predation pressures from fish due to being paedomorphic, learning may also be an important factor in this system. To examine this possibility we explored the responses of both predator-experienced (recently collected) and predator-naïve (first generation, captive-reared) adult *E. nana* to the chemical cues of native and non-native syntopic predatory fish species.

Materials and Methods

Study Species

Eurycea nana is endemic to and found only in the headwaters of the San Marcos River, Hays County, Texas (Bishop 1941; Nelson 1993; Chippindale et al. 1998). A captive population is also maintained at the San Marcos National Fish Hatchery and Technology Center (SMNFHTC). In the wild, *E. nana* are typically found along the substrate under refuges such as rocks and vegetation (Tupa & Davis 1976; K. J. Epp, pers. obs.). Gravid females and juveniles of *E. nana* are present throughout the year (Bogart 1967; Tupa & Davis 1976), indicating year-round reproduction and activity for this species. Thaker et al. (2006) showed that *E. nana* relies primarily on chemical rather than visual cues for conspecific association preference.

Stimulus Species Selection

Predators

We used the native species *Micropterus salmoides* (largemouth bass) and the non-native species *Lepomis auritus* (redbreast sunfish) for our predatory stimuli based on available literature (Kelsey 1997) and personal communications (E. Chappel). We used heterogenic species because prey may recognize congeners across species boundaries and respond similarly between native and non-native species (Mirza et al. 2003; Ferrari et al. 2007a). Additionally, these species appear to be among the most abundant predatory species in the San Marcos river headwaters and share similar opportunistic foraging habits (Day 1981; Wallace 1984). Analysis of stomach contents obtained through stomach pumping of the predatory species (*M. salmoides* n = 10; *L. auritus* n = 10) revealed similar digestive remains including benthic organisms which indicates that both species are likely to encounter *E. nana* during regular foraging activity (K. Epp & C. Gabor unpubl. data). Additionally, a diet study of *L. auritus* collected from the San Marcos River 4–6.5 km downstream of the habitat of *E. nana* found that benthic invertebrates composed a substantial portion of the diet (Wallace 1984), indicating that in the headwaters, *L. auritus* are benthic foragers and thus are likely to encounter *E. nana* while foraging. In the headwaters, both species have been observed preying on *E. nana* (K. Epp pers. obs.; E. Chappel pers. comm.) and *Lepomis* spp. and *M. salmoides* are considered predatory threats to *E. nana* in this system (Tupa & Davis 1976; Petranka 1998). We further demonstrated in captivity that both species prey on *E. nana* when given the opportunity, by placing an individual of each predatory species (n = 5) in individual holding tanks with one *E. nana* per tank. After 24 h, presence/absence of *E. nana* was recorded. Absent *E. nana* were assumed to have been consumed, as no alternative escape was available. All individuals of *M. salmoides* and *L. auritus* consumed *E. nana* within 24 h of offering.

Non-predator

To control for response of *E. nana* to predator cues as opposed to fish cues in general, we chose to expose *E. nana* to a native, non-predatory fish species, *Gambusia geiseri*. While *Gambusia* spp. are potential predators of amphibian larvae or eggs in other systems (Hamer et al. 2002; Lane & Mahony 2002; Baber & Babbitt 2003), because of their size, they are not expected to pose a predatory threat to adult *E. nana* used in this study. Additionally, the microhabitat use of *G. geiseri* (Hubbs & Peden 1969) and *E. nana* (Tupa & Davis 1976) differs drastically in this system, making predation by mosquito fish unlikely.

Stimulus Acquisition

Stimulus animals (*M. salmoides* n = 28, *L. auritus* n = 16, *G. geiseri* n = 237) were collected from the San Marcos River headwaters, Hays County, Texas every 2 wk during the same time as testing occurred. They were placed in species-specific aerated tanks for 24 h with 230 ml of de-chlorinated tap water for every 1 cm^3 of stimulus animal by volume. Standard length (SL; $\bar{x} \pm$ SE; *M. salmoides*: 193.68 $\pm$ 12.48 mm; *L. auritus*: 183.44 $\pm$ 9.82 mm) and volume (*M. salmoides*: 498.81 $\pm$ 39.73 cm^3; *L. auritus*: 442.88 $\pm$ 31.98 cm^3) of predatory individuals did not differ between species (unpaired t-test: SL: t = −0.645, d.f. = 41.886, p = 0.5225; volume: t = 1.097, d.f. = 41.886, p = 0.281). To control for the response of *E. nana* to individual fish as opposed to species kairomones, *M. salmoides* tanks contained two individuals per stimulus tank. Because of difficulties with tank size, *L. auritus* were housed individually for 24 h and tank water from two individual tanks was evenly mixed prior to stimulus collection. *Gambusia geiseri* were housed in tanks with 27–33 individuals per collection tank. Each stimulus tank contributed no more than five stimulus samples for use in testing. Tank water was not filtered and stimulus animals were not fed during this time. After 24 h, stimulus animals were released and water from the tanks was stirred, collected in 50-ml plastic falconer tubes, and frozen at −20°C for at least 24 h prior to testing. While freezing may alter the chemical composition of stimuli, this method has been used successfully in many studies (e.g. Woody & Mathis 1998; Mathis et al. 2003; Hickman et al. 2004). No visible particulates (e.g. feces) were included in collected stimulus samples. For control stimuli, falconer tubes were filled with de-chlorinated tap water and then frozen. Stimuli were thawed using a circulating well water bath immediately prior to testing.

Predator Avoidance in Predator-Experienced *E. nana*

We collected adult *E. nana* (n = 62) from the San Marcos River headwaters, Hays County, Texas from Mar. to Jun. 2005. These salamanders were assumed to have had experience with predators in their natural habitat. Individuals with snout–vent lengths

(SVL) greater than 20 mm were considered adults as *E. nana* have been determined to be sexually mature at this size (Tupa & Davis 1976). *Eurycea nana* were housed in flow-through fiberglass tanks at the SMNFHTC on a 12 : 12 h light : dark cycle with 40-W fluorescent lights during daylight hours for at least 2 wk prior to testing. The tanks were filled with well water and maintained at 22–23°C. We fed *E. nana* commercially raised annelids (*Lumbriculus variegatus*) and copepod mixtures ad libitum. Testing occurred from Apr. to Jul. 2005. Thus, all *E. nana* were collected relatively recently from the wild and were likely to have maintained their original predatory responses (Mirza & Chivers 2000). Because decreased activity is a common antipredator behavior in amphibians (Wisenden 2003), we used changes in activity to determine responses of salamanders to predatory stimuli. Salamanders were selected randomly from the housing tanks and placed individually into 9.5-l glass aquaria filled with 4.5 l of well water. A 50-ml syringe attached to air-line tubing was used for stimulus introduction. This was attached to one corner of the testing chamber and extended 5 cm below the surface of the water on the interior and 10 cm below the base of the testing chamber on the exterior. Using established methods (Jaeger 1981; Thaker et al. 2006), *E. nana* were tested under dim 25-W red lighting at night when they are most active (K. J. Epp, pers. obs.). After 15 min of acclimation the amount of time (sec) that *E. nana* spent actively moving was recorded for 10 min (pre-stimulus activity). Individuals of *E. nana* were then exposed to one of four treatments: (1) native predator, largemouth bass, *M. salmoides* (n = 15); (2) non-native predator, redbreast sunfish, *L. auritus* (n = 15); (3) native non-predator, largespring Gambusia, *Gambusia geiseri* (n = 17); or (4) only water (n = 15). Fifty millilitres of one of the four treatments was injected at about 1 ml/s into the testing chamber. Pilot tests using food dye showed that when injected at this rate, stimulus water spread through the entire chamber by the completion of stimulus introduction. Treatment stimuli were randomly assigned and coded and hence the observer was blind to the treatment. After stimulus introduction, the time salamanders spent actively moving (seconds) was recorded for another 10 min (post-stimulus activity). Relative activity scores of *E. nana* to stimuli were calculated as an index ratio of post-stimulus activity to pre-stimulus activity. In this way, the response variable is representative of each individual's change in activity from pre-stimulus (normal) to post-stimulus activity.

A relative activity score ≥1 indicates that individuals were equally or more active after stimulus exposure as before, while relative activity scores <1 indicate reduced activity after stimulus exposure. Each individual was used only once for these studies.

Predator Avoidance in Predator-Naïve *E. nana*

Similar methods were followed for testing and stimulus acquisition in this experiment; however, first-generation captive-born (predator-naïve) adult *E. nana* (SVL ≥ 20 mm; n = 60) were used as test subjects. Testing occurred from Dec. 2005 to May 2006.

Statistical Analyses

Using residual plots and Shapiro–Wilks' tests ($\alpha = 0.05$) we determined that the data met the assumption of normality. We examined residual plots of relative activity scores and determined that the data met the assumption of homoscedasticity. We used a boxplot outlier test to determine statistical outliers (Barnett & Lewis 1984). We determined that one data point in the predator-experienced, native predator treatment was an extreme outlier. When all other data were combined, this point fell 4.5 standard deviations above the mean (n = 121, $\bar{x} = 0.704$, SD = 0.375). Outliers may bias analyses when sample sizes are small (Iglewicz & Hoaglin 1993) and this one was removed from further analyses. We combined predator-experienced and predator-naïve data and performed a two-factor analysis of variance (ANOVA) with predator experience and stimulus treatment as factors. All analyses were performed using JMP® 6.0 (SAS Institute, Cary, NC, USA) software.

Results

Complete Model

Two-factor ANOVA indicated a significant interaction between the factors of stimulus treatment and predator experience ($F_{3,113} = 2.713$, p = 0.048). To elucidate the nature of this interaction we used single-factor ANOVAs with subsequent Fisher's least significant difference (LSD) multiple comparison procedures to compare relative activity scores among treatments within predator experience groups. We then compared responses of predator-experienced and predator-naïve *E. nana* for each treatment using Tukey's honestly significant difference (HSD) multiple comparison procedure ($\alpha = 0.05$).

Predator Recognition in Predator-Experienced *E. nana*

We found significant differences in the relative activity score between stimulus treatments (ANOVA: $F_{3, 57} = 2.880$, $p = 0.044$). Mean relative activity score for the native predator treatment was significantly lower than the control (Fisher's LSD: $p = 0.021$) and the non-native predator treatments (Fisher's LSD: $p = 0.011$), but did not differ from the non-predator treatment (Fisher's LSD: $p = 0.191$). Relative activity scores did not differ between the control and non-predator (Fisher's LSD: $p = 0.258$) and non-native predator ($p = 0.791$) treatments or the non-predator and non-native predator ($p = 0.162$) treatments (Fig. 1a).

Predator Avoidance in Predator-Naïve *E. nana*

We found significant differences in relative activity scores between treatments for predator-naïve *E. nana*
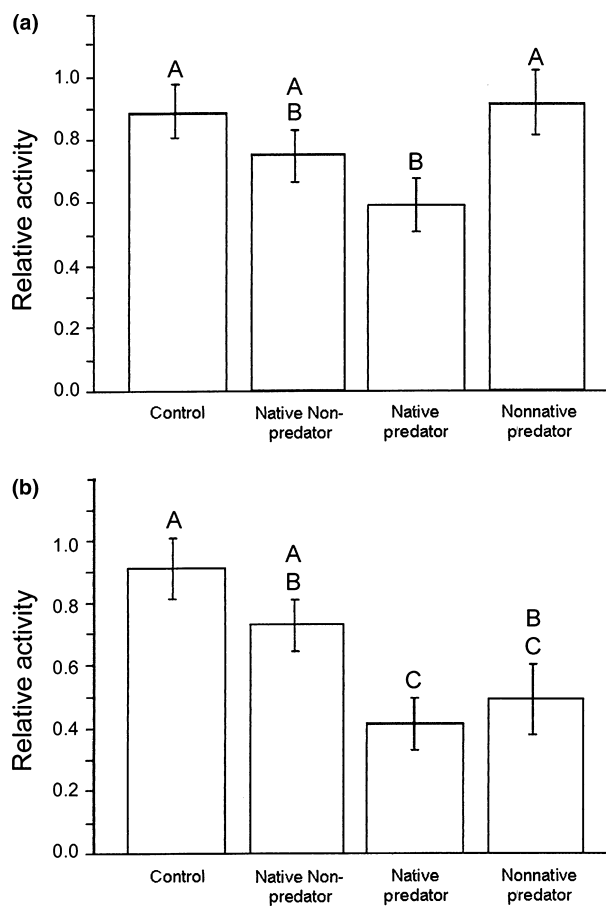
Fig. 1: Mean (±SE) relative activity after exposure to the chemical cues of one of four fish stimuli for: (a) predator-experienced and (b) predator-naïve *Eurycea nana*. Responses not connected by the same letter are significantly different ($\alpha = 0.05$).

(ANOVA: $F_{3,56} = 6.495$, $p < 0.001$). Mean relative activity score for the control significantly differed from the native predator (Fisher's LSD: $p < 0.001$) and non-native predator (Fisher's LSD: $p = 0.002$) treatments and did not differ from the non-predator treatment (Fisher's LSD: $p = 0.147$). Relative activity scores for the non-predator treatment differed significantly from those of the native predator treatment (Fisher's LSD: $p = 0.016$), but did not differ from the non-native predator treatment (Fisher's LSD: $p = 0.069$). Relative activity scores for the native predator and non-native predator treatments were not different (Fisher's LSD: $p = 0.526$; Fig. 1b).

Comparisons of Predator-Naïve vs. Predator-Experienced Responses

Predator-naïve and predator-experienced *E. nana* did not respond differently to the water control, non-predator, and native predator treatments (Tukey's HSD: $p > 0.05$). For the non-native predator treatment, the relative activity score was lower for predator-naïve than predator-experienced *E. nana* (Tukey's HSD: $p < 0.05$).

Discussion

Both predator-naïve and predator-experienced *E. nana* exhibited reductions in activity when presented with the chemical cues of a native fish predator when compared with a blank control. This response is consistent with predator avoidance behaviors exhibited by other amphibian prey species (e.g. Griffiths et al. 1998; Mathis & Vincent 2000; Mathis et al. 2003; Mandrillon & Saglio 2005). Because predator-naïve *E. nana* exhibited avoidance responses when presented with chemical cues of the native predator, there is good evidence that there is an innate component to predator recognition in *E. nana*. Innate predator recognition has been documented for numerous amphibian species (reviewed in Wisenden 2003). Interestingly, predator-experienced *E. nana* did not exhibit significant decreases in activity when presented with the chemical cues of the syntopic non-native predator; however, predator-naïve *E. nana* significantly decreased activity levels when presented with the chemical cues of the non-native fish predator. Because congeners of the non-native predator (e.g. *L. cyanellus*) are native to the study habitat, *E. nana* may innately recognize *Lepomis auritus* as a predatory threat. For example, Ferrari et al. (2007a) demonstrated that prey may recognize congeneric predators across species

boundaries because the kairomones produced are expected to be similar. Experienced *E. nana* exhibited diminished responses, however, when compared with naïve *E. nana*, suggesting that wild-caught *E. nana* may have refined responses to predators given experience.

Certain characteristics of both *E. nana* and the predatory community may encourage the development of both innate and experience-mediated avoidance responses. Because *E. nana* inhabits a flowing river system and females lay eggs individually as opposed to in clutches, the opportunities that predator-naïve juveniles have to acquire recognition of predators from conspecifics prior to an attack may be limited. This would promote the development of innate recognition of those predators posing the most significant predatory threats to *E. nana*. However, because *E. nana* experiences fish predation throughout life, the ability to refine responses to predators based on experience may also be important for this species. This is especially true when considering the highly diverse yet temporally stable predator community which preys on *E. nana*. These characteristics imply that experience-mediated modifications to innate responses would be favored in this system.

While *E. nana* may inherently recognize the predatory species used in this experiment as a threat, the intensity of their avoidance responses may be altered based on their perception of predation risk. The ability of prey to alter the intensity of their avoidance responses based on their perception of risk (Ferrer & Zimmer 2007) is beneficial in that it allows prey to minimize the costs associated with predator avoidance (Sih 1992; Anholt et al. 2000). Prey may assess predation risk in many ways including detection of predatory diet cues (reviewed by Chivers & Smith 1998; Wisenden 2003) or through experience with species-specific predator kairomones (reviewed in Kats & Dill 1998). Although primary reliance on predatory diet cues for risk assessment has been shown for some amphibian prey (e.g. Wilson & Lefcort 1993; Lefcort 1996; Chivers et al. 1999; Murray et al. 2004), stimulus individuals in this study were collected using consistent methods across studies and multiple individuals of each species provided stimulus for each study. Thus, we would expect greater variation in responses of *E. nana* within predator treatment groups as well as more similar responses between experienced and naïve *E. nana* than was recorded if predator diet cues alone accounted for the differences observed. Additionally, primary reliance on predator diet cues for identification of risky predators is not expected to be favored in systems composed primarily of opportunistic foragers as opposed to specialist predators because diet cues alone may not be reliable indicators of predatory threat (Ferrari et al. 2007b). Therefore, predation risk assessment based on detection of and experience with species-specific predator kairomones is the most probable explanation for the differences in responses to *L. auritus* observed between predator-experienced and predator-naïve *E. nana*.

If the differences in response between experienced and naïve *E. nana* to the chemical cues of *L. auritus* and *M. salmoides* are reflective of their perception of the risk posed by these species based on their experience, this would indicate that *M. salmoides* poses a more significant predatory threat than the nonnative predator *L. auritus*. This would also suggest that costs associated with avoidance of less risky species are greater than the benefits of avoiding all detected predatory threats in this system. Studies of the foraging behavior of these two species indicate that *M. salmoides* tend to feed on column-dwelling vertebrate prey (e.g. fish) as well as benthic invertebrates (Peterson et al. 2006), while *L. auritus* tend to forage primarily on benthic invertebrate prey (Wallace 1984); however, diet studies with *L. auritus* and *M. salmoides* have not been conducted where vertebrate prey such as *E. nana* are available in the benthos. Thus, to understand the relative risk posed to *E. nana* by these species, diet studies comparing feeding habits of *L. auritus* and *M. salmoides* in this habitat are needed. Through accurate assessment of predation risk, *E. nana* should be able to more efficiently allocate time to other beneficial behaviors such as foraging or mating (Sih 1992; Anholt et al. 2000).

Studies examining innate and learned responses to the chemical cues of predators suggest that amphibians and fish differ in their responses. In general, studies indicate that fishes are more likely to learn which chemical stimuli are dangerous (Brown 2003; Kelley & Magurran 2003) while amphibians more often exhibit innate responses (Kats & Dill 1998). Our data go further, as they indicate that *E. nana* exhibit innate recognition of predators and, with experience, are also able to learn about the predatory threat they pose. While studies demonstrating co-reliance on both innate and learned responses are limited in amphibians, Gonzalo et al. (2007) demonstrated that *R. perezi* also alter innate antipredator responses in a threat-sensitive manner after experiencing predator kairomones in association with damage-released conspecific alarm pheromones. Similar

to *E. nana*, some amphibian species for which associative learning has been demonstrated (e.g. *Notophthalmus viridescens*, Woody & Mathis 1998; *R. perezi*, Gonzalo et al. 2007) have relatively lengthy aquatic stages when compared with other metamorphic amphibians. This may make experience-mediated plasticity in antipredator behavior more useful for these species (Gonzalo et al. 2007). Obligate paedomorphic salamanders pose interesting prey subjects as they must avoid fish predation throughout the entirety of their life cycle and thus, both acquired and innate responses can be beneficial for these species.

Acknowledgements

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