Predator Generalization Decreases the Effect of Introduced Predators in the San Marcos Salamander, *Eurycea nana*

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Abstract

The introduction of novel predators into an environment can have detrimental consequences on prey species, especially if these species lack the ability to recognize these predators. One such species that may be negatively affected by introduced predators is the federally threatened San Marcos salamander (*Eurycea nana*). Previous research found that predator-naïve (captive-hatched) salamanders showed decreased activity in response to the chemical cues of both a native fish predator (*Micropterus salmoides*) and an introduced fish predator (*Lepomis auritus*), but not to a non-predatory fish (*Gambusia geiseri*). We tested the hypothesis that *E. nana* recognized the introduced *Lepomis* (and other non-native *Lepomis*) because they share chemical cues with other native congeneric *Lepomis* predators in the San Marcos River. We examined the antipredator response of predator-naïve *E. nana* to chemical cues from (1) a sympatric native sunfish (*Lepomis cyanellus*; Perciformes: Centrarchidae); (2) a sympatric introduced sunfish (*L. auritus*); (3) an allopatric sunfish (*Lepomis gibbosus*); (4) a sympatric non-native, non-centrarchid cichlid (*Herichthys cyanoguttatum*; Perciformes: Cichlidae); and (5) a blank water control to determine whether individuals make generalizations about novel predators within a genus and across a family. Exposure to chemical cues from all fish predator treatments caused a reduction in salamander activity (antipredator response). Additionally, there were no differences in the antipredator responses to each predatory fish treatment. The similar responses to all sunfish treatments indicate that *E. nana* shows predator generalization in response to novel predators that are similar to recognized predators. Additionally, the antipredator response to *H. cyanoguttatum* indicates that predator generalization can occur among perciform families.

Introduction

Declines and losses of amphibian populations are a global problem (Lawler et al. 2006; Wake & Vredenburg 2008) with complex local causes. Anthropogenic causes include diseases, predation, ultraviolet radiation, environmental toxicants, habitat modification and loss, and climate change (reviewed by Alford & Richards 1999; Sodhi et al. 2008; Wake & Vredenburg 2008). Amphibians with small geographic ranges are also more susceptible to decline, and in such cases, multi-foci management is necessary for conservation. Habitat protection and studies of ecological conditions, such as the effects of predators, are important management issues (Kiesecker 2003; Sodhi et al. 2008).

Chemically mediated predator detection is widespread in aquatic environments and among amphibians (Kats & Dill 1998; Mathis 2003; Ferrari et al. 2010). The use of chemical stimuli to detect predators can be particularly important in aquatic habitats because turbidity, vegetation, or low light levels can impair visual or tactile stimuli (Abrahams & Kattenfeld 1997). Both innate (Sih & Kats 1994; Mathis et al. 2003; Epp & Gabor 2008) and learned (Woody & Mathis 1998; Ferrari et al. 2009) predator recognition mechanisms have been observed in amphibians.

Often, learned recognition of predators is achieved through the association of a predatory stimulus with a stimulus that represents a threat (e.g. alarm or diet cues). Alarm cues are chemical compounds released by the skin cells of prey species upon predation or injury, which, when detected, can alert nearby conspecifics of predatory threats (Wilson & Lefcort 1993; Schoeppner & Relyea 2009). Diet cues are associated with recent foraging of predators and are complex mixtures of both stimuli from digested prey items and metabolic wastes, and these cues can facilitate learned recognition of predators (Mathis & Smith 1993; Chivers et al. 1996). If learning is successful, subsequent encounters with that predator should elicit an anti-predator response (Mathis & Smith 1993; Woody & Mathis 1998; Crane & Mathis 2010).

While both alarm and diet cues seem to be important in achieving learned recognition of predators, many prey that exhibit innate predator recognition can detect predator kairomones. Kairomones are stimuli produced by one species that are then detected by another species (Brown et al. 1970). These cues are believed to be complex mixtures of metabolic wastes and hormones and relatively little is known about their exact constituents in amphibians (Mathis et al. 2003). These stimuli may serve as chemical signals intraspecifically (pheromones), but when detected by heterospecifics, are classified as kairomones. Detection of kairomones can aid in the location of heterospecifics, and as a result, in predator-prey interactions, the production of kairomones is often beneficial for the receiver and costly for the emitter (Brown et al. 1970).

In addition to innate and learned recognition, prey species may also recognize predators through generalization (Griffin et al. 2001; Ferrari et al. 2007, 2008, 2009; Brown et al. 2011). Ferrari et al. (2007) introduced the Predator Recognition Continuum Hypothesis in which they suggest that prey species may be able to make generalizations about novel predator species based on similarities to recognized predators, such as native species. Prey that can generalize recognition of a specific predator species to closely related, but novel predators should have an advantage over those prey that cannot (Ferrari et al. 2007). As such, generalization may enhance the effective breadth of either innate or learned predator recognition. For example, Ferrari et al. (2007) trained fathead minnows (Pimephales promelas) to recognize chemical cues from lake trout (Salvelinus namaycush; Salmoniformes: Salmonidae). After this conditioning, fathead minnows only responded to the chemical cues of two closely related trout species (Salvelinus fontinalis and Oncorhynchus mykiss; Salmoniformes: Salmonidae) and

not to a more distantly related northern pike (Esox lucius; Esociformes: Esocidae), indicating that prey are capable of making generalizations from familiar predator species to novel predators based on similarities in chemical cues (Ferrari et al. 2007).

With widespread introduction of predators, it is important to study the effects of introduced predators on amphibians with which they have not coevolved. Studies examining the effects of introduced predator species on amphibians have suggested that these introduced predators may be consuming individuals disproportionately more than native predators (Knapp & Matthews 2000; Kats & Ferrer 2003; Crane & Mathis 2010). However, one potential recognition mechanism that may decrease the negative effects of introduced predators on native prey is predator generalization. Herein, we examined whether predator-naïve San Marcos salamanders (Eurycea nana) were capable of making generalizations about novel predators.

Methods

Study System

Eurycea nana is a federally threatened (USDI 1980) and IUCN red-listed (IUCN 2011), neotenic (obligate aquatic) salamander endemic to the headwaters of the San Marcos River, Hays Co., TX (Nelson 1993). Previous studies have illustrated the use of chemical stimuli in conspecific associations (Thaker et al. 2006), and, similar to other amphibians, E. nana decreases activity when exposed to predator stimuli (Epp & Gabor 2008). Epp & Gabor (2008) found that E. nana shows innate predator recognition by decreasing activity levels after exposure to kairomones of predatory fish. The reduction in total activity levels is an appropriate predator avoidance behavior because most fish are visual predators and is a common behavior observed in many other aquatic amphibians (Wildy & Blaustein 2001; Epp & Gabor 2008; Gall & Mathis 2009). Predator-naïve E. nana show antipredator responses to kairomones from both a native predator (largemouth bass, Micropterus salmoides) and an introduced predator (redbreast sunfish, Lepomis auritus), but not to a native non-predatory fish (largespring gambusia, Gambusia geiseri). Both Lepomis and Micropterus are closely related centrarchid genera (Perciformes: Centrarchidae), and other species of Lepomis are native to the habitat of E. nana. Thus, if salamanders recognize predators in either of these genera, then the response to the introduced L. auritus may be the result of predator generalization.

The genus *Lepomis* (Perciformes: Centrarchidae) is a relatively speciose genus of predatory fish, ranging across most of North America (Warren 2009). Four species have native ranges that include the headwaters of the San Marcos River: *Lepomis cyanellus*, *Lepomis gulosus*, *Lepomis macrochirus*, and *Lepomis microlophus*. An additional species, *L. auritus*, was introduced in the early 1950s (Jurgens 1951). We used *L. cyanellus* as our sympatric native predator and *L. auritus* as our sympatric introduced predator. The diets of both of these species primarily consist of benthic macroinvertebrates and small fish (Wallace 1984), and both species have been observed to consume *E. nana* (Tupa & Davis 1976; Epp & Gabor 2008). We used *Lepomis gibbosus* as an allopatric non-native predator. *L. gibbosus* is found in the northern and eastern portions of North America, has not had historic ranges in Central Texas (Scott & Crossman 1973), and is considered to be generalist feeder, consuming gastropods and other invertebrates (Sadzikowski & Wallace 1976). In addition, we tested the antipredator response of *E. nana* to kairomones from a more distantly related sympatric introduced predatory fish, the Rio Grande cichlid (*Herichthys cyanoguttatum*; Perciformes: Cichlidae). These cichlids also are considered generalists, consuming plants, invertebrates, and small fishes (Buchanan 1971) and were likely introduced to the San Marcos River by the 1930s after accidental release from the US Fish and Wildlife Service Station in San Marcos, TX (Brown 1953).

Experimental Protocol

We tested predator-naïve (captive-hatched, first-generation offspring from wild-collected salamanders), adult *E. nana* (n = 75) (male SVL > 19 mm, female SVL > 21 mm), which were available from the San Marcos National Fish Hatchery and Technology Center. Salamanders were maintained in flow-through fiberglass tanks on a 12:12 h light/dark cycle and fed blackworms (*Lumbriculus variegatus*) *ad libitum*. We tested salamanders individually in 9.5-l glass aquaria with 4.5 l of well water and covered three sides of the aquarium with black plastic to reduce background disturbances. We tested during peak activity times for *E. nana*, beginning 2 h after sunset and lasting for up to 4 h. We used low-level red light (25 W) during observations. After haphazardly selecting individuals from the housing tanks and placing them in the testing chamber, we allowed individuals to acclimate for at least 20 min. Following acclimation, we recorded the amount of time spent active for 8 min. Active behavior included swimming or walking, but did not include sniffing or gill movement that was not

accompanied by other movements of the body. These data constitute the baseline (pre-stimulus) activity level for each subject. Following determination of baseline activity, we introduced 50 ml of water containing chemical stimuli from one of the following treatments: (1) a sympatric native sunfish (*L. cyanellus*), (2) a sympatric introduced sunfish (*L. auritus*), (3) an allopatric sunfish (*L. gibbosus*), (4) a sympatric introduced, non-centrarchid, cichlid (*H. cyanoguttatum*), or (5) only water (a blank control). We did not include a non-predatory fish control because Epp & Gabor (2008) showed that the response of *E. nana* to chemical cues from non-predatory largespring gambusia (*G. geiseri*) was not significantly different than the response to a blank water control. Each treatment was replicated a total of 15 times. Treatments were tested in random order and coded to control for observer bias.

We introduced cues into the aquarium through a syringe attached to a plastic tube attached to the center of one side of the testing chamber at a rate of 2 ml/s. We positioned the end of the introduction tube approximately 2 cm below the surface of the water to reduce disturbance during treatment introduction. After introduction of the stimulus, we flushed 50 ml of well water through the introduction tube at a rate of 2 ml/s. We recorded the time spent active (post-stimulus) in the subsequent 8 min as an indication of prey responsiveness (antipredator behavior). Each individual was exposed to a single treatment because successive exposures without reinforcement could lead to learning the innocuousness of the predator cue (Hazlett 2003) or habituation. We washed all testing equipment with 3% hydrogen peroxide and fresh well water between each trial to remove any existing chemical stimuli and maintain independence between trials.

Stimulus Acquisition

We collected both the native and introduced sunfish (*L. cyanellus* and *L. auritus*) as well as the introduced cichlid (*H. cyanoguttatum*) from Spring Lake, Hays Co., TX, USA (29°89′N, 97°82′W). The allopatric sunfish (*L. gibbosus*) was purchased from a private fish supplier. We only used adult fish to reduce any possible ontogenetic effects. Prior to the collection of chemical cues, we fed fish earthworms for at least 5 d to minimize the effects of prior diet. We determined the volume of each stimulus animal through displacement. To control for chemical cue concentrations between treatments, we used approximately 230 ml of water per 1 ml of stimulus animal in the collecting chamber. We then placed stimulus animals into separate glass

aquaria containing the appropriate volume of aerated dechlorinated tap water for 24 h. Before acquisition of the chemical cues, we removed the stimulus animals from the tanks and stirred the water. We mixed equal proportions of water from two adult individuals to control for individual effects and froze all samples in a −20°C freezer. These methods have been used successfully in previous studies (Mathis et al. 2003; Epp & Gabor 2008; Brown et al. 2011). Control stimulus consisted of dechlorinated tap water that was also frozen. Samples were thawed immediately prior to testing.

Statistical Analysis

Time spent moving in each of the pre- and post-stimulus trials was combined into a single activity index for each individual. The activity index is the difference between post-stimulus activity and pre-stimulus activity, such that positive values indicate increases in activity and negative values indicate decreases in activity in response to the stimulus. First, we assessed whether salamanders exhibited responses to fish cues that were different from the blank control by conducting Steel's test ($\alpha = 0.05$), and afterward, we excluded the blank water control treatment. To test the hypothesis that the strength of responses would diminish with increased phylogenetic distance (Ferrari et al. 2007), we compared each predator treatment to the sympatric native sunfish treatment with three independent planned comparisons using one-tailed t-tests. We did not apply Bonferroni corrections so as to avoid over-inflation of Type II error (Nakagawa 2004). Additionally, we examined effect sizes between pairs of treatments of interest using Cohen's d (Cohen 1988). After the blank water control treatment was excluded, variance of the data was homogenous across the remaining treatments. Statistical analyses were conducted using JMP 9.0 (SAS Institute, Cary, NC, USA) software.

Results

Using Steel's test, the activity indices for the sympatric native sunfish ($p = 0.0006$), the sympatric introduced sunfish ($p < 0.0015$), the allopatric sunfish ($p < 0.0001$), and the introduced cichlid ($p = 0.0002$) were significantly lower than the blank water control treatment (Fig. 1). Additionally, there was no difference in activity index between the sympatric native sunfish and the sympatric introduced sunfish ($p = 0.749$), the allopatric sunfish ($p = 0.261$), or the sympatric introduced cichlid ($p = 0.141$). For each
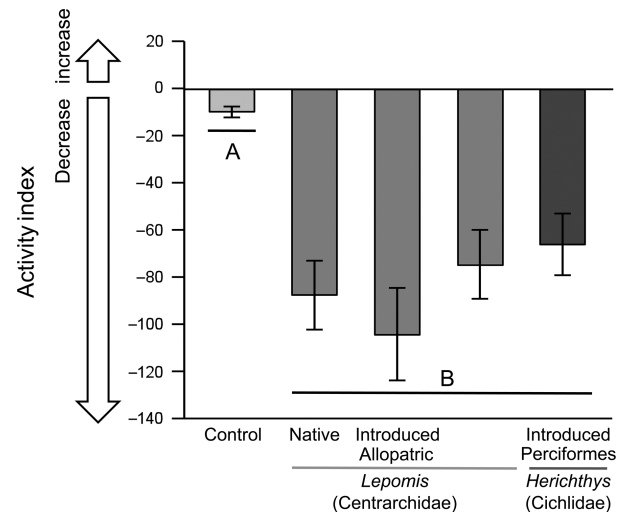
Fig. 1: Mean activity index (±SE) of *Eurycea nana* in response to chemical cues introduced in five treatments. Letters in figure indicate significant differences between treatments ($\alpha = 0.05$).

unpaired t-test, effect sizes fell within the small and medium classifications designated by Cohen (1988), which suggests high overlap between treatments (Table 1).

Discussion

Predator-naïve salamanders significantly reduced activity (antipredator behavior) in response to the chemical cues from sympatric native, sympatric introduced, and allopatric sunfish treatments (*Lepomis*; Perciformes: Centrarchidae) when compared to the blank water treatment. This indicates that *E. nana* is capable of making generalizations about novel predators, possibly based on similarities to recognized predators. To our knowledge, this is the first example of predator generalization in a prey species that does not require prior conditioning or learning. We do not think these results are an outcome of salamanders generalizing all fish as potential predators because Epp & Gabor (2008) found that *E. nana* did not show an antipredator response to non-predatory mosquitofish (*G. geiseri*). Similar to Epp & Gabor (2008), predator-naïve salamanders showed an antipredator

Table 1: p Values from comparisons of predator treatments to the sympatric native predator. Numbers in parenthesis represent Cohen's *d* effect size values

	Lepomis cyanellus
Lepomis auritus	0.749 (0.25)
Lepomis gibbosus	0.261 (0.24)
Herichthys cyanoguttatum	0.141 (0.40)

response to the sympatric introduced *L. auritus*. Kairomones between these three *Lepomis* species may be similar due to their shared ancestry and possibly their shared natural history traits. However, similarity in kairomones among taxa is likely to decrease with increasing genetic differences due to more distantly shared ancestry or differences in natural history traits (Ferrari et al. 2007). Salamanders also showed an antipredator response when exposed to the cichlid treatment (*H. cyanoguttatum*; Perciformes: Cichlidae). In sum, our results indicate that *E. nana* shows predator generalization within a genus and across families of these perciform fish species.

We do not know what specific compounds trigger an antipredator response in *E. nana* because kairomones are often complex mixtures of metabolic wastes and hormones (Mathis et al. 2003). It is possible that a particular compound is shared among closely related species (Dalesman et al. 2007), and the detection of this compound is how generalizations are made. Within this system, it is possible that certain kairomones among perciform fish are similar enough for *E. nana* to recognize these species as threats. Alternatively, salamanders may recognize chemical compounds that are similar among predators due to convergent similarities in their kairomones. *Eurycea nana* may be responding to cues derived from convergent similarities among large fish in general or among all predatory fish.

It is important to realize that it may be difficult to know how many and which predators *E. nana* may innately recognize. As innate predator recognition requires a genetic component, it is likely that species that are innately recognized have shared a coevolutionary history with *E. nana*. Because the introductions of both *L. auritus* and *H. cyanoguttatum* are relatively recent (1950s and 1930s respectively), it is unlikely that an innate response has evolved. Instead, it is likely that salamanders innately recognize other native fish species, which have co-occurred for much longer periods of time and show generalization based on this recognition. While generalization was seen both within *Lepomis* and across the examined perciform families, if non-perciform fish were introduced, these species may be too distantly related and, therefore, not elicit a generalized response from *E. nana*. Further investigation into the antipredator response for non-perciforms (e.g. Salmoniformes or Esociformes) needs to be conducted to better delineate limits for predator generalization.

For *E. nana*, it is difficult to assess the effects that invasive perciforms may have on population size or stability because historical population estimates do not exist prior to the 1970s (Tupa & Davis 1976). It is also difficult to predict the effects non-native perciform predators may have on populations because empirical evidence provides mixed results. For example, in an artificial pond experiment, the presence of a non-native perciform predator, the bluegill sunfish (*L. macrochirus*), resulted in the exclusion of both American toad (*Bufo americanus*) and spotted salamander (*Ambystoma maculatum*) larvae (Boone et al. 2007); however, the mechanism for this exclusion was not known. Conversely, in a field enclosure experiment, another non-native perciform predator, the smallmouth bass (*Micropterus dolomieu*), had a negligible effect on the growth and development of red-legged frog tadpoles (Kiesecker & Blaustein 1998). Direct examination of the potential effects of non-native perciform predators on *E. nana* would require the use of field enclosures or mesocosms to compare fitness or survival of individuals in the presence and absence of these predators.

Introduced fish predators can decrease survivorship, reduce metamorph size and rate, and alter habitat and foraging behaviors in some amphibians (Kats & Ferrer 2003). Reduced survivorship may result from failed recognition of novel predators by native prey. For example, in another aquatic salamander (hellbenders, *Cryptobranchus alleganeinsis*), juveniles respond strongly to chemical cues from native fish predators, but only exhibit a weak response to introduced predators (Gall & Mathis 2009). A head-starting program has been implemented for hellbenders, and Crane & Mathis (2010) found that they could train individuals to recognize introduced fish predators as part of a captive rearing-release protocol. *Eurycea nana* is also being maintained in a captive breeding program, but, unlike for hellbenders and possibly many other salamanders, our results indicate that prior training before captive release is not necessary because *E. nana* shows predator generalization toward two introduced families of perciform predators.

Our results indicate that *E. nana* can generalize across two perciform families. In future studies, we need to examine the response of *E. nana* to other perciform predators and non-perciform fish as well as examine the effect of predator size. While the introduction of predators can strongly influence native prey species, our results suggest *E. nana* should be able to generalize their response to novel species of perciform fish if they are introduced into the San Marcos River. Additionally, should there be a need for the release of captive-bred salamanders, no prior associative conditioning would be needed for successful recognition of the current predatory community.

Acknowledgements

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