PREDATOR-PREY INTERACTIONS IN THE SAN MARCOS SALAMANDER

(*EURYCEA NANA*): PREDATOR GENERALIZATION AND STRESS

HORMONES IN RESPONSE TO INTRODUCED PREDATORS

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IN RESPONSE TO INTRODUCED PREDATORS

by

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May 2012

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Predation is a strong, influential force in most ecological communities.

Inappropriately responding to predators typically results in the direct consumption of

prey individuals. In addition to responding appropriately to predators in order to survive

these encounters, prey individuals are under selection to minimize the costs associated

with responding. These costs may reduce the overall fitness of prey individuals as time

spent responding to predators is time not spent increasing fitness through activities such

as foraging or reproducing. As such, prey individuals should optimize their responses in

order to survive encounters with potential predators as well as to minimize the costs associated antipredator behaviors.

The introduction of novel predators into many environments has contributed to the decline in amphibian populations. Often, amphibians lack the ability to recognize these novel predators, and therefore, either do not respond or respond inappropriately to them. One such species which may be negatively affected by introduced predators is the San Marcos salamander (*Eurycea nana*). *Eurycea nana* is a federally threatened, fully aquatic salamander endemic to the headwaters of the San Marcos River. Currently, a diverse assemblage of native and introduced fish predators is present in the San Marcos River. While prior studies have helped to understand some aspects of how these salamanders respond to native and introduced predators, the role of predator generalization has yet to be explored. I found that *E. nana* can generalize its response to novel predators, as long as there are still similarities between these novel predators and predators which they recognize. Additionally, I have examined a stress hormone, corticosterone, to further characterize differences in the way *E. nana* responds to native and introduced fish predators. Differences in the way *E. nana* responds to native and introduced predators suggest that introduced predators may be causing a muted or diminished response. Differences in the temporal variation in risk of predation may be driving these differences, because introduced predators are highly abundant and may be frequently encountered.

CHAPTER I

PREDATOR-PREY INTERACTIONS

Predation is a dominant element of ecological communities (Curio 1976). For prey individuals, encounters with predators may result in injury or death. However, for prey populations, the effects of predators can be varied. Predators can cause local extirpation or global extinctions of prey species (Fritts and Rodda 1998). Predation can decrease prey population densities, which can decrease chances of finding mates or reduce genetic variation within a population (Frankham 1996). Selective predation on certain prey phenotypes or genotypes (Galeotti et al. 2005), sex (Norrdalh and Korpimäki 1998), or size or age classes (Sogard 1997), can change the population structure, and therefore, the viability or reproductive success of the prey population (Taylor 1990; Banks et al. 2000). Additionally, predators have many indirect, or nonlethal, effects on prey individuals. The detection of predators may influence circulating stress hormone levels (Cockrem and Silverin 2002; Figueiredo et al. 2003; Fraker et al. 2009; Sheriff et al. 2009), activity times and habitat use (Taylor 1983; Holomuzki 1986; Sih et al. 1992), group size and vigilance (Altendorf et al. 2001; Peacor et al. 2007), clutch size and fecundity (Magnhagen 1990; Travers et al. 2010), and individual growth rates (Fraser and Gillam 1992; Nakaoka 2000). Preisser et al. (2005) suggested that the resulting indirect

responses to predators can be as strong as direct consumption. Additionally, it is important to consider that the effects of predators do not necessarily cease when predators leave the immediate vicinity, but may persist through time lags (Sih 1992).

<center>Predator Recognition and Generalization</center>

Innate and learned recognition are two primary mechanisms by which prey recognize predators (reviewed by Wisenden 2003). Innate recognition does not require prior encounters with a predator in order for prey to exhibit an antipredator response, but instead this response has a genetic basis. In environments characterized by a static predator community or high-risk predators, innate recognition of predators is expected (Brown and Chivers 2005). Innate predator recognition has been described in numerous aquatic systems including in invertebrates (Turner et al. 2006), fish (Hawkins et al. 2008), and amphibians (Mathis et al. 2003; Epp and Gabor 2008). While prey individuals that exhibit innate recognition are at a selective advantage over individuals that exhibit learned recognition by virtue of not requiring a potentially costly encounter with a predator to respond appropriately, there are environments in which learned recognition may be advantageous for prey. For example, in communities with a dynamic predator community or low-threat predators, learned recognition is advantageous if prey species learn from encounters with novel predator species and subsequently exhibit antipredator responses during future encounters (Brown and Chivers 2005). Recently, the role of embryonic learning in amphibians has been investigated (Ferrari and Chivers 2009; Mathis et al. 2009), and may be important to a wide range of taxa. Strong selection exists

on prey to learn to recognize predators early in life, and receiving information about predators before being directly exposed to them would be advantageous to prey.

Learned predator recognition is often achieved through the association of a predatory stimulus with a stimulus that represents a threat. If learning through association is successful, subsequent exposures to the predator stimulus can elicit an antipredator response (Mathis and Smith 1993; Woody and Mathis 1998; Crane and Mathis 2010). For example, Mathis and Smith (1993) exposed fathead minnows (*Pimephales promelas*), to cues of an unfamiliar fish predator, northern pike (*Esox lucius*), which had either recently eaten conspecific or heterospecific (*Xiphophorus helleri*) fish. Naïve fathead minnows responded to chemical stimuli from northern pike that had recently consumed conspecifics; however, they did not respond to chemical stimuli from northern pike that had recently consumed heterospecifics. These same fathead minnows when later exposed to chemical stimuli from northern pike that had consumed heterospecifics exhibited an antipredator response, demonstrating the ability to learn to recognize the chemical stimuli of a predator regardless of their most recently consumed prey. Similarly, these methods have been used to condition aquatic salamanders to respond to introduced predators before being released as part of a head-starting program (Crane and Mathis 2010). While the costs and benefits of innate and learned recognition differ, both mechanisms are likely important in the detection of predator species by prey (Sih and Kats 1994).

Another important aspect of learned predator recognition is adaptive forgetting (Kraemer and Golding 1997). As time passes, associations between predators and risk may change, and therefore, no longer represent actual risk. Therefore, it would be predicted that prey should 'forget' prior perceived risk to avoid the costs of responding to

predators which no longer represent a threat. Models examining the importance of

present versus past information have suggested that in variable environments, recent

information should contribute more to decisions than older information (McNamara and

Houston 1987). In experimental tests of adaptive forgetting, as predator risk increased,

prey retain predator-related information for longer periods of time (Ferrari et al. 2010a).

Additionally, as uncertainty about predator-related information increases, the duration

prey use this information decreases. Thus, the ability for prey to remember predators may

be strongly influenced by reinforcement and perceived risk (Gonzalo et al. 2009; Ferrari

et al. 2010a).

It is important to recognize that innate and learned predator recognition likely fall

at two ends of a continuum. Ferrari et al. (2007) introduced the Predator Recognition

Continuum Hypothesis (PRCH), in which they suggested that prey species may be able to

make generalizations about novel predator species based on similarities to known

predator species. Prey that can generalize the recognition of a specific predator species to

closely related but novel predators should be at an advantage over those which cannot

generalize (Ferrari et al. 2007). The ability to generalize across predators would benefit

prey species in the detection of new predators, provided there are similarities between the

predator species. For example, Ferrari et al. (2007) examined the response of fathead

minnows to a suite of predator species. Fathead minnows were initially trained to

recognize chemical cues from predatory trout (genus *Salvelinus;* Salmoniformes:

Salmonidae). After this conditioning, fathead minnows, when presented with chemical

cues of a related predatory trout (genus *Oncorhynchus*; Salmoniformes: Salmonidae),

exhibited antipredator responses but failed to respond to cues of the distantly related

predatory pike (Esociformes: Esocidae). Predator generalization has been documented in numerous taxa and systems (Griffin et al. 2001; Ferrari et al. 2007, 2008, 2009a; Ferrari and Chivers 2009; Mathis et al. 2009; Brown et al. 2011); however, it has only been demonstrated in prey species which rely on learned predator recognition. To date, no study on predator generalization has centered around prey that exhibit innate predator recognition.

<div align="center">Detection of Predators in Aquatic Environments</div>

Sensory modalities used for predator recognition in aquatic systems include visual, chemical, tactile, and electroreception (Roth 1987; Kats and Dill 1998; Collin and Whitehead 2004; Preisser et al. 2005). Many predatory fish detect potential prey through visual cues (Mussi et al. 2005; Meager et al. 2010). Both chemical and tactile stimuli can also be important in predator-prey interactions in aquatic systems that are highly turbid, have low light levels, or are densely vegetated (Wilkens et al. 1996; Abrahams and Kattenfeld 1997). Chemical cues have repeatedly been shown to be more important than visual cues alone in both prey and predator detection in fish (Kusch et al. 2004; Ferrari et al. 2006) and amphibians (Kats and Dill 1998; Woody and Mathis 1998; Sullivan et al. 2000; Mathis 2003; Hickman et al. 2004; Ferrari et al. 2010b). Studies examining response to electric stimuli have been conducted with species of weakly electric fish and salamanders, and suggest electroreception may be more important in mediating predator-prey interactions than what is currently known (Schlegel 1997; Collin and Whitehead 2004).

For our purposes, we define three primary categories of chemical cues used by prey to detect predators. These chemical cues are kairomones, alarm cues, and diet cues. Kairomones are any signal produced by a species, which is detected by another species (Brown et al. 1970). Often, these chemical compounds serve as pheromones within conspecifics, but when detected by heterospecifics, are considered kairomones. In predator-prey interactions, detection of kairomones can allow predators to locate prey, or for prey to detect nearby predators, and as a result, the production of kairomones is maladaptive for the emitter and beneficial for receiving species. 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). Detecting kairomones of predators allows prey to respond to nearby predators. Kairomones of the predatory eastern newt, *Notophthalmus viridescens*, can be detected by larval ringed salamanders, *Ambystoma annulatum*, and trigger the antipredator response of a reduction in activity levels (Mathis et al. 2003). By recognizing predator kairomones, prey species can decrease the probability of predation, thus increasing their fitness.

Other forms of chemical cues involved in predator-prey interactions include alarm cues and diet cues. Alarm cues are released by specialized skin cells of prey species upon predation or injury, which can alert nearby conspecifics (Wilson and Lefcort 1993) or heterospecifics (Mathis and Smith 1993; Schoeppner and Relyea 2009) to the threat of predators. Additionally, prey can detect predator diet cues, which are associated with recent foraging activity by the predator along with waste products of digestion. The combination of kairomones from a predator and alarm cues from prey has been shown to label unrecognized species as predators (Crowl and Covich 1990; Mathis and Smith

1993). The fact that prey species can respond differently to chemical cues derived from predators that have fed on conspecifics compared to those that have fed on other prey species suggests tradeoffs in responding to diet cues from heterospecific prey (Lefcourt and Blaustein 1995; Chivers and Mirza 2001; Sullivan et al. 2003). Both alarm and diet cues require a prior predation event, while the detection of predator kairomones does not rely on prior predation events. For example, when predators are generalists, species of prey relying solely on alarm or diet cues may be unable to detect them if the predators have recently consumed heterospecifics. As such, prey species which detect kairomones of predators have the ability to recognize predators regardless of recently consumed prey items.

A number of studies of antipredator responses have focused on alarm cues generated by prey and diet cues generated by predators (Mathis and Smith 1993; Wilson and Lefcort 1993; Sullivan et al. 2003; Schoeppner and Reylea 2005), including some studies of how cues are shared across a suite of related prey species (Dalesman et al. 2007). Dalesman et al. (2007) found that the response of a species of snail (*Lymnaea stagnalis*) to alarm cues produced by heterospecific snails decreased as genetic distance between it and heterospecific species increased. Additionally, the response of the target species to the alarm cues of sympatric snail species was greater compared to allopatric snail species suggesting an influence of intraguild members on predator avoidance responses. However, little research has examined the response of a single prey species to a suite of related predator species (Ferrari et al. 2007, 2009; Brown et al. 2011).

Predation Risk and Stress Hormones

Upon recognition of predators, many aquatic prey species form schools (Rödel and Linsenmair 1997), alter activity times and locations (Taylor 1983; Holomuzki 1986), increase use of refuge habitats (Sih et al. 1992), and reduce overall activity levels (Semlitsch 1987; Wildy and Blaustein 2001; Epp and Gabor 2008). These behaviors generally diminish the direct threat of predation, but predators can also have additional effects on prey individuals. These nonlethal effects are important considerations for the fitness of species, even though these effects may not be as obvious as direct predation. One such nonlethal predator effect is an increase in circulating stress hormone levels in prey. The presence of predators has been shown to increase circulating stress hormone levels in a wide variety of organisms (Wingfield et al. 1998). While important in the response to immediate threats, stress hormones may also be important in mediating the response to subsequent encounters with predators (Dachir et al. 1993; Mateo 2008). Stress hormones are known to enhance physiological systems associated with the immediate survival of the individual while suppressing those that are not (Cooper and Faisal 1990; von Holst 1998). Over longer periods of time, chronic elevated stress hormone levels can directly suppress reproduction through effects on several important regulatory glands and can indirectly reduce survival and reproduction through effects on the immune and digestive systems (Moore and Miller 1984; Sheriff et al. 2009).

Glucocorticoids are the main vertebrate stress hormones and, for amphibians, are released with stress-induced activation of the hypothalamus-pituitary-interrenal (HPI) axis (Sapolsky et al. 2000; Nelson 2005). Glucocorticoids can suppress activity of the hypothalamus-pituitary-gonadal (HPG) axis, resulting in decreased secretion of gonadal

hormones and decreased expression of androgen-mediated mating behavior (Adkins-Regan 2005; Nelson 2005). Upon release, glucocorticoids trigger the metabolism of lipids, proteins, and carbohydrates, enhancing functions necessary for immediate survival of an individual (Moore and Jessop 2003). Over longer time periods and chronic exposure to the stressor, the above responses can result in decreased growth, delayed development, and immunosuppression (Greenberg and Wingfield 1987; Sapolsky et al. 2000; Adkins-Regan 2005). One such glucocorticoid, corticosterone (CORT), is frequently used as a physiological index of the relative heath of individuals or populations (McEwen and Wingfield 2003). As a result, the CORT-Fitness hypothesis suggests that higher baseline levels of CORT indicate reduced relative fitness in individuals or populations (Bonier et al. 2009). Traditional methods of measuring circulating hormone concentration in aquatic vertebrates involve assaying blood plasma (Idler et al. 1964). However, recent methods of extracting water-borne hormones have been established and do not require bleeding subjects, which can be problematic in small subjects or species of conservation concern (Ellis et al. 2004; Scott and Ellis 2007).

CORT is the primary glucocorticoid in amphibians, birds, reptiles, and non-primate mammals (Herman 1992). In amphibians, like in other vertebrates, CORT increases blood glucose levels, inhibits growth and reproduction, and alters behavior (Sapolsky et al. 2000). However, some studies show that the effects of CORT on reproduction in amphibians appear to be context dependent (Moore and Jessop 2003; Moore et al. 2005). Increased CORT has been found to suppress reproductive behaviors in some caudate amphibians (*Taricha granulosa*; Moore and Miller 1984), but not in others (*Triturus carnifex*; Zerani and Gobbetti 1993). For rough-skinned newts (*Taricha*

granulosa), when levels of CORT were increased through intraperitonial injection, the amount of time males spent exhibiting reproductive behaviors and engaging in courtship decreased significantly (Moore and Miller 1984). Benner and Woodley (2007) found that in the Allegheny dusky salamander, *Desmognathus ochrophaeus*, CORT was positively correlated with testosterone and that elevated CORT levels did not decrease reproductive activity. These differing results of the role of CORT in reproductive behaviors in amphibians suggest that the action varies between taxa and needs to be independently examined in different taxa.

While predators have an important influence on stress hormones in CORT - producing animals, there has been little evaluation of this in amphibians (Sapolsky et al. 2000; Romero 2004). Studies examining CORT in amphibians have focused on the effects of handling stress and isolation (Belden et al. 2010; Ricciardella et al. 2010). In a study examining the effects of acute stress from handling (perhaps analogous to the effects of predation risk), male *D. ochrophaeus* increased CORT levels, but only during the nonbreeding season (Ricciardella et al. 2010). In contrast, Fraker et al. (2009) examined CORT levels in *Rana sylvatica* tadpoles following exposure to either high- or low-risk predator diet cues, and found lower levels of CORT following exposure to high-risk cues. This decrease in CORT levels is contrary to what has previously been seen in rodents even though the behavioral response is similar (i.e., freezing behavior) (Apfelbach et al. 2005). These results suggest that there may be differences in the how stress cues influence CORT production and inhibition across taxa. Additionally, little information is known about field versus laboratory levels of stress hormones in most species of amphibians. Houck et al. (1996) found that lab and field levels of CORT were

similar for *Ambystoma opacum*. However for *A. maculatum* and *Hyla regilla*, lower

CORT levels were reported from wild individuals compared to laboratory animals

(Cooperman et al. 2004; Belden et al. 2005). While the CORT response of prey species in

response to the presence of predators may be species dependent, it may also differ

depending on attributes of the predator. High-risk or native predators may elicit a

different stress hormone response in prey than low-risk or introduced predators (Lima

and Bednekoff 1999; Chivers et al. 2001). By examining CORT levels in the presence of

potential predators, a more complete understanding of how various classes (native,

introduced, high-risk, low-risk) of potential predators differentially influence amphibian

prey species may be gained.

<u>Costs of Antipredator Behaviors and Risk Allocation</u>

While the benefits to responding to predators are apparent when enhanced

survival of individuals is documented, there are also costs associated with responding.

For example, time spent avoiding predators comes at the cost of foraging or reproducing

(Lima and Dill 1990). Moreover, prey that make overly inclusive generalizations about

their predators or are hyper-sensitive to predator cues compared to conspecifics may

spend a disproportionate amount of time avoiding predators instead of increasing their

fitness through foraging or reproducing (Ferrari et al. 2007). Thus, prey species benefit

from responding differently to high- and low-risk predators (Kats and Dill 1998). The

ability of prey to match the intensity of their antipredator behaviors to the level of

predator threat is known as threat-sensitive predator avoidance (Helfman 1989; Chivers

et al. 2001).

In addition to threat-sensitive predator avoidance, temporal variation in the risk of predation may also influence the antipredator responses seen in many prey species (Lima and Bednekoff 1999). Lima and Bednekoff (1999) developed the "risk allocation hypothesis" (RAH) which explains how temporal variation in predation pressure affects allocation of antipredator behavior and foraging effort across different risks of predation. This hypothesis states that a prey individual's response to a predator during one time period should be influenced by the risk experiences at other times. Therefore, antipredator responses to predators which are frequently encountered should be less intense than responses to predators which are infrequently encountered. For predators that are frequently encountered, the costs of elevated levels of antipredator behavior are high and time when prey do not experience these predators is minimal. Therefore, prey should exhibit reduced antipredator behavior, and should continue to forage while these predators are present. Alternatively, if predators are infrequent and periods of risk of predation are brief, then foraging prey should exhibit heightened antipredator behavior; any foraging time lost can be regained during periods of low or no risk. To accurately assess whether temporal variation in the risk of predation may be influencing antipredator behaviors detailed information on encounter frequencies and likelihood of encounter survival for different predators is needed. Although mixed support for the RAH has been found, differences in methodology or failure to meet the assumptions of may account for studies that had results that did not match the predicted outcomes (Ferrari et al. 2009b). Numerous studies have indeed tested the predictions of this hypothesis and found that prey exhibited decreased antipredator behaviors when encounter frequencies with

predators were high, compared to prey whose encounter frequencies with predators were lower (Sih and McCarthy 2002; Brown et al. 2006; Mirza et al. 2006).

Study System

Central Texas is home to a diverse group of plethodontid (Caudata: Plethodontidae) salamanders in the genus *Eurycea* (Chippendale et al. 2000). These salamanders are neotenic and fully aquatic, inhabiting spring and aquifer systems found along and throughout the Edwards Plateau. Many of these spring sites are considered to be thermostable, with temperatures being relatively constant, in turn contributing to year-round activity and reproduction (Tupa and Davis 1976). Spring species live among cobble substrates and feed upon a wide range of aquatic invertebrates (USFWS 2006).

Eurycea nana is a federally threatened species restricted to the headwaters of the San Marcos River, Hays County, Texas (Nelson 1993). Within the headwaters, salamanders are confined to areas in the immediate vicinity of spring vents along the original river channel and immediately downstream of the Spring Lake dam. Within these habitats, *E. nana* can be found in cobble substrates and is associated with *Amblystegium* moss (Diaz 2010). The species is primarily nocturnal (USDI 1980). The presence of gravid females and small larvae year-round as well as highly acyclic oviposition suggest that *E. nana* breeds and reproduces throughout the year (Tupa and Davis 1976). Prior studies have demonstrated the use of chemical cues rather than visual cues for conspecific associations (Thaker et al. 2006). A captive population of *E. nana* is maintained at the San Marcos National Fish Hatchery and Technology Center, which includes both field collected and captive bred salamanders.

Epp and Gabor (2008) found that both predator-experienced (field collected) and predator-naïve (captive bred) *E. nana* show decreased activity in response to the chemical cues of the native fish predator (*Micropterus salmoides*). Since many predatory fish are visually oriented predators, by decreasing activity levels, risk of predation may be lessened (Kats et al. 1988; Skelly 1994). However, when presented with chemical cues of a non-native fish predator (*Lepomis auritus*), only predator-naïve individuals showed a significant decrease in activity (Epp and Gabor 2008). Since both predator-experienced and predator-naïve individuals responded to predators, it is likely that predator recognition is an innate response. The variable response to the non-native fish predator of predator-experienced and predator-naïve individuals raises a question regarding the basis of predator recognition. Predator-naïve individuals never exposed to the chemical cues of the non-native fish predator exhibited an antipredator response, yet predator-experienced individuals failed to show an antipredator response. Since there are native congeneric predators (*Lepomis* sp.) in Spring Lake and the San Marcos River, it is possible that there is a shared chemical cue among *Lepomis*, which is generalized and triggers the antipredator response of naïve salamanders. These results suggest both an innate and learned component of the response of *E. nana* to fish predators. Epp and Gabor (2008) hypothesized that the lack of response by predator-experienced salamanders to *L. auritus* may be explained by these fish being lower risk predators than the native species, *M. salmoides*, rather than the inability to recognize these predators.

To further explore these results, Epp (2010) examined the effect that predation risk had on prey response. In experiments examining the response of predator-experienced salamanders to diet cues from low-risk (fed a neutral diet) and high-risk (fed

adult *E. nana*) *M. salmoides* and *L. auritus*, it was found that the behavioral response of

salamanders exposed to low- and high-risk diet cues from *M. salmoides* and low-risk diet

cues from *L. auritus* did not differ from that of the blank water control. However, activity

scores for high-risk diet cues from *L. auritus* were significantly different than the blank

control as well as significantly lower than low-risk diet cues from *L. auritus*. Epp (2010)

attributed the differences seen between studies to the lack of a controlled predator diet

prior to kairomone collection or to digestive differences. Additionally, Epp (2010) tested

the RAH in *E. nana*, finding no effects of encounter frequency or risk intensity on

antipredator behaviors.

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CHAPTER II

PREDATOR GENERALIZATION DECREASES THE EFFECT OF INTRODUCED
PREDATORS ON THE SAN MARCOS SALAMANDER, *EURYCEA NANA*[1]

Abstract

The introduction of novel predators into an environment can have detrimental
consequences on prey species, especially if prey lack the ability to respond to 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*). 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 (*L. cyanellus;* Perciformes: Centrarchidae), (2) a sympatric
introduced sunfish (*L. auritus*), (3) an allopatric sunfish (*L. gibbosus*), (4) a sympatric
non-native, non-centrarchid cichlid (*Herichthys cyanoguttatum*: Perciformes: Cichlidae),
and (5) a blank water control to determine if individuals make generalizations about

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[1] In preparation for submission to *Ethology*

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 may 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 & Vrendenburg 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 & Vrendenburg 2008). Amphibians also are more susceptible to decline when they have small geographic ranges, 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 the effects of turbidity and 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. Often, these threat stimuli are either 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 antipredator 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.

It is important to realize that innate and learned recognition are two ends of a continuum by which prey recognize predators (reviewed by Wisenden 2003; Ferrari et al. 2007). 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 were presented with the chemical cues of brook trout, rainbow trout, and northern pike. Both brook trout (*Salvelinus fontinalis*; Salmoniformes: Salmonidae) and rainbow trout (*Oncorhynchus mykiss*; Salmoniformes: Salmonidae) are closely related to lake trout, while northern pike (*Esox lucius*; Esociformes: Esocidae) are more distantly related. Fathead minnows responded to all trout treatments, but failed to show an antipredator response to cues of the northern pike 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.

Eurycea nana is a federally threatened (USDI 1980) and IUCN red-listed (IUCN 2011), neotenic salamander endemic to the headwaters of the San Marcos River, Hays Co., Texas (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 and Gabor (2008) found that *E. nana* shows innate predator recognition by decreasing activity levels after exposure to kairomones of predatory fish. Predator-naïve individuals 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. We tested the hypothesis that *E. nana* recognize *L. auritus* due to predator generalization by examining the response of predator-naïve *E. nana* to kairomones of three species of *Lepomis*; (1) a

sympatric native sunfish (*L. cyanellus*), (2) a sympatric introduced sunfish (*L. auritus*), and (3) an allopatric sunfish (*L. gibbosus*). Additionally, because most native predatory fish are centrarchids (Perciformes: Centrarchidae), we also tested the response of *E. nana* to (4) a more distantly related introduced cichlid (*Herichthys cyanoguttatum*; Perciformes: Cichlidae). By including a non-centrarchid predator, we were able to examine if *E. nana* has the ability to show generalized antipredator responses to a more distantly related perciform predator.

Materials and Methods

Predator Species

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: *L. cyanellus*, *L. gulosus*, *L. macrochirus*, and *L. microlophus*. An additional species, *L. auritus*, was introduced in the early 1950's (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 *Eurycea nana* (Tupa & Davis 1976; Epp & Gabor 2008). We used *L. gibbosus* as an allopatric non-native predator. *Lepomis 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 1930's after accidental release from the U. S. Fish and Wildlife Service Station in San Marcos, Texas (Brown 1953).

Experimental Protocol

We tested predator-naïve (captive-hatched), adult *Eurycea 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 (n = 15 salamanders/treatment): (1) a sympatric native sunfish (*Lepomis 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). 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 though 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.

<h2 style="text-align:center">Stimulus Acquisition</h2>

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., Texas, 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. Control stimulus consisted of dechlorinated tap water that was also frozen. This method has been used successfully in previous studies (Mathis et al. 2003; Epp & Gabor 2008). 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 afterwards, 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 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 t-test, effect sizes fell within the small and medium classifications designated by Cohen (1988) which suggests high overlap between treatments (Table 1).
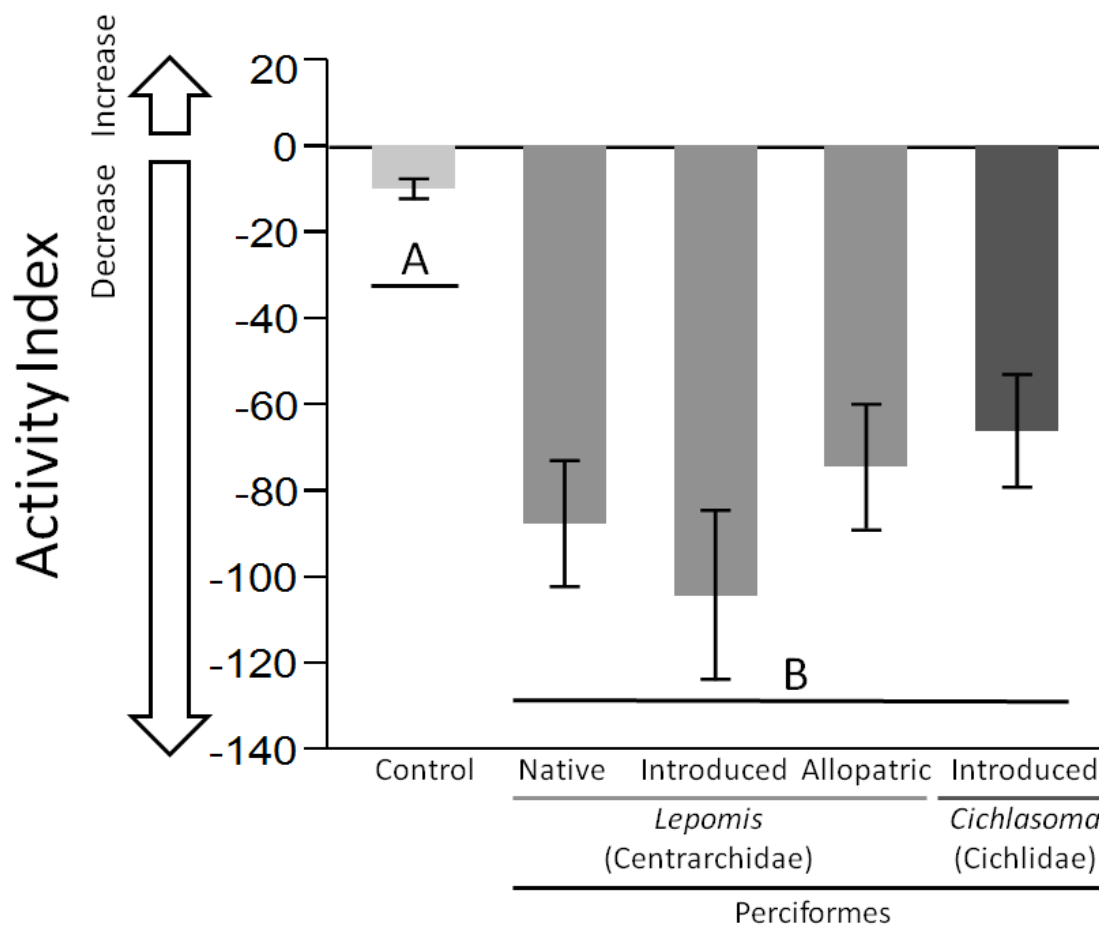
Figure 1. Activity Indices for Predator-Naïve *Eurycea nana* to Native, Introduced, and Allopatric Predators. Means (± SE) of *Eurycea nana* in response to stimuli introduced in five treatments. Letters indicate significant differences between treatments ($\alpha = 0.05$).

Table 1. *P*-values from Comparisons of Predators the Sympatric Native Predator. Numbers in parenthesis represent Cohen's *d* effect size values.

	L. cyanellus
L. auritus	0.749 (0.25)
L. gibbosus	0.261 (0.24)
H. cyanoguttatum	0.141 (0.40)

<u>Discussion</u>

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 *Eurycea 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. Similar to Epp and Gabor (2008), predator-naïve salamanders showed an antipredator 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). We do not think these results are an outcome of salamanders generalizing all fish as potential predators because Epp and Gabor (2008) found that *E. nana* did not show an antipredator response to non-predatory mosquitofish (*Gambusia geiseri*). 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. Since innate predator recognition requires a genetic component, it is likely that species that are innately recognized have shared a coevolutionary history with *E. nana*. Since the introductions of both *L. auritus* and *H. cyanoguttatum* are relatively recent, it is likely that these salamanders innately recognize centrarchid fish, as these are considered native fish predators. 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.

Many species of amphibians are experiencing marked population declines due to a wide range of causes (Stuart et al. 2004), one being the introduction of novel predators (Knapp & Matthews 2000; reviewed by Kats & Ferrer 2003; Kiesecker 2003). Introduced fish predators can decrease survivorship, reduce metamorph size and rate, and alter habitat and foraging behaviors in amphibians (Kats & Ferrer 2003). Juvenile hellbenders, *Cryptobranchus alleganeinsis*, for example, respond strongly to chemical cues from

native fish predators, but only exhibited a weak response to introduced predators (Gall & Mathis 2009). A head-starting program has been implemented for hellbenders, and Crane and 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 hellbenders, and possibly many other salamanders, the results from our study indicate that, for *E. nana*, prior conditioning or training does not appear necessary because these salamanders show predator generalizations toward two 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.

approved by Texas State University-San Marcos IACUC (#11008_0325_07).

Salamander collection and housing permits are held by the San Marcos National Fish

Hatchery and Technology Center Service (TPWD SPR-0390-045; USFWS TE676811-2).

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CHAPTER III

BEHAVIORAL AND PHYSIOLOGICAL STRESS OF AN AQUATIC

SALAMANDER, *EURYCEA NANA*, IN RESPONSE TO NATIVE

AND INTRODUCED PREDATORS[2]

Abstract

Exposure to predatory stimuli typically results in the elevation of circulating glucocorticoid levels in many prey species. While associated with immediate survival, chronic increase in glucocorticoids may have negative effects on individuals through the suppression of digestive, immune, and reproductive systems. Corticosterone (CORT) is the main glucocorticoid in amphibians. The federally threatened San Marcos salamander, *Eurycea nana*, decreases activity in response to both native and introduced predatory fish, however, experience may further influence these interactions. To better understand the effects of introduced fish predators on this salamander, we examined both the behavioral and water-borne CORT response to: 1) a native predator (*Micropterus salmoides*), 2) an introduced predator (*Lepomis auritus*), and 3) a blank water control. Salamanders reduced activity (antipredator response) in response to both predator treatments, but not to the blank water control, and the response to the native predator was significantly stronger that to the introduced predator. The CORT response to both the blank water

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[2] In preparation for submission to *Hormones and Behavior*

control and the introduced predator did not statistically differ, and both were lower than

the response to the native predator. These results suggest weaker responses towards

introduced predators when compared to native predators. Highly abundant introduced

predators may attribute to a greater allostatic load on these salamanders, and therefore,

selection has favored individuals that have decreased stress responses.

Introduction

Predators are known to influence prey fitness either directly, through the

consumption of individuals, or indirectly, through decreasing survival or reproduction, as

a cost of eliciting an antipredator response. In many aquatic systems, predator recognition

may result in prey species forming shoals (Rödel and Linsenmair 1997), increasing use of

refugia (Sih et al. 1992), or reducing overall activity levels (Semlitsch 1987; Wildy and

Blaustein 2001; Epp and Gabor 2008). These behaviors generally decrease the direct

threat of predation, but predators can also have nonlethal effects on prey individuals,

some of which may persist through time lags (Sih 1992). Nonlethal effects are important

considerations for the fitness of species, even though they may not be as obvious as direct

predation upon individuals. One such nonlethal effect of predators is an increase in

circulating stress hormone levels in prey. The presence of predators influences circulating

stress hormone levels in a wide variety of organisms (Wingfield et al. 1998). While

important in the response to immediate threats, stress hormones also may be important in

the response to subsequent encounters with predators and may play a role in learned

predator recognition (Dachir et al. 1993; Mateo 2008).

Glucocorticoids are the main vertebrate stress hormones and, within amphibians, are released with stress-induced activation of the hypothalamus-pituitary-interrenal (HPI) axis (Cartensen et al. 1961). Glucocorticoids enhance physiological systems associated with the immediate survival of the individual (von Holst 1998; Sapolsky et al. 2000). Upon release, glucocorticoids trigger the metabolism of lipids, proteins, and carbohydrates, enhancing functions necessary for immediate survival of an individual (Moore and Jessop 2003). Over longer periods of time, chronic elevated glucocorticoid levels can directly suppress reproduction and decrease expression of androgen-mediated mating behaviors (Adkins-Regan 2005; Nelson 2005). Additionally, chronic increases in glucocorticoids can indirectly reduce reproduction and survival through effects on the immune and digestive systems (Moore and Miller 1984; Sheriff et al. 2009). Over longer time periods and chronic exposure to stressors, chronic elevated glucocorticoid levels can result in decreased growth, delayed development, and immunosuppression, which in turn may make individuals more susceptible to infectious diseases or predation (Greenberg and Wingfield 1987; Sapolsky et al. 2000; Adkins-Regan 2005). One such glucocorticoid, corticosterone (CORT), is frequently used as a physiological index of the relative heath of individuals or populations (McEwen and Wingfield 2003). As a result, the CORT-Fitness hypothesis suggests that higher baseline levels of CORT indicate reduced relative fitness in individuals or populations (Bonier et al. 2009).

CORT is the main glucocorticoid in amphibians, birds, reptiles, and non-primate mammals (Herman 1992). Studies examining the effects of CORT on reproduction in amphibians appear to be context dependent (Moore and Jessop 2003; Moore et al. 2005). Increased CORT has been found to suppress reproductive behaviors in some caudates

(*Taricha granulosa*: Moore and Miller 1984), but not in others (*Triturus carnifex*: Zerani and Gobbetti 1993; *Desmognathus ochrophaeus*: Benner and Woodley 2007). These differing results of the role of CORT in reproductive behaviors in amphibians may suggest that the role of CORT is taxon specific.

Activation of the HPI axis and increasing levels of CORT may be critical in surviving encounters with predators (Wingfield et al. 1997; Orchinik 1998) and may influence antipredator responses (Hossie et al. 2010). Hossie et al. (2010) found that CORT may be important in the behavioral and morphological response to predators, because *Rana pipiens* tadpoles exposed to a CORT receptor inhibitor showed decreased responses when compared to control groups. Studies examining CORT levels after exposure to predators have shown increased levels in birds (Travers et al. 2010) and mammals (Apfelbach et al. 2005), but not in amphibians. Fraker et al. (2009) examined CORT levels in *R. sylvatica* tadpoles following exposure to either low- or high-risk predator diet cues, and found lower levels of CORT following exposure to high-risk cues. This decrease in CORT levels is contrary to what has previously been seen in other animals, even though the behavioral response (freezing behavior) is similar.

These differences may again suggest that the expression of CORT varies across taxa. While the role of CORT in response to the presence of predators may be dependent on prey taxa, it may also differ depending on attributes of the predator. High-risk or native predators may elicit a different level of stress response in prey than low-risk or a predator that the prey species have not coevolved with, such as an introduced predator. Examining changes in CORT response in the presence of potential predators will provide

a perspective on the physiological costs of various classes (native, introduced, high-risk, low-risk) of potential predators.

Eurycea nana, is a federally threatened and IUCN red-listed (IUCN 2011), neotenic salamander endemic to the headwaters of the San Marcos River, Hays Co., Texas (Nelson 1993). The antipredator response of predator-experienced *E. nana* has been inconsistent across experiments (Epp and Gabor 2008; Epp 2010). Epp and Gabor (2008) showed that predator-experienced salamanders respond to kairomones from a native fish predator, *Micropterus salmoides*, but not to an introduced fish predator, *Lepomis auritus*. However, predator-naïve salamander responded to both native and introduced predators, suggesting that they can recognize these introduced predators, but perhaps experience with predators can modify these responses. While the behavioral responses of *E. nana* to predators have been examined, we do not know if or how native and introduced predators are creating physiological stress in these salamanders. Herein, we examined both the behavioral and CORT response of San Marcos salamanders to chemical stimuli from three different treatments: (1) an introduced fish predator (*L. auritus*), (2) a native fish predator (*M. salmoides*), and (3) only water (blank control) to better understand how nonlethal effects of predators may be influencing this species.

Materials and Methods

Predator Species

To understand any differences in the way *Eurycea nana* responds to native and introduced predators, we used two centrarchid fish predators (Perciformes: Centrarchidae). We used *Lepomis auritus* as our introduced predator and *Micropterus*

salmoides as our native predator. Both predators are abundant in Spring Lake and the headwaters of the San Marcos River (Perkin and Bonner 2011). *Lepomis auritus* was introduced into Spring Lake and the San Marcos River in the early 1950's, where it has since become established (Jurgens 1951). The diet of *L. auritus* within the San Marcos River is primarily aquatic invertebrates (Diptera, Ephemeroptera, and Trichoptera), suggesting that this species is a benthic feeder (Wallace 1984). Examination of the diet of *M. salmoides* suggests that it too is a generalist feeder consuming both limnetic and benthic prey species (Matthews et al. 1992). Both species have been observed to consume *E. nana* (Tupa and Davis 1976; Petranka 1998; Epp and Gabor 2008).

Experimental Protocol

Traditional methods of measuring circulating hormone concentration in aquatic vertebrates involve assaying blood plasma. Here, CORT levels were measured using a recently developed, non-invasive process for measuring water-borne hormones (Ellis et al. 2004) that is advantageous for repeated sampling or when species of conservation concern are used. We collected adult *E. nana* (n=60) (male SVL > 19 mm, female SVL > 21 mm) from the headwaters of the San Marcos River, Hays Co., Texas, USA (29° 54' 36" N, 97° 55' 53" W) and allowed them to acclimate to laboratory conditions for a minimum of two weeks, where they were housed in large flow-through fiberglass tanks, maintained on a 12:12 h light cycle, and fed blackworms (*Lumbriculus variegatus*) ad libitum. Following established methods (Gabor and Grober 2010; Gabor et al. in prep), we randomly selected a salamander and placed each individual in a 250-ml glass beaker containing 100 ml of well water for 1 h. These data represent baseline (pre-stimulus) CORT levels. We then moved salamanders into 9.5-l glass aquaria containing 4.5 l of

well water. We covered three sides of the aquaria with black plastic to reduce background disturbances. We tested during peak activity times for *E. nana*, beginning 2 h after dark and lasting for up to 4 h. We used low-level red light (25 W) during observations. After placement in the testing chamber, individuals were acclimated for 20 min. Following acclimation, we recorded the amount of time individuals spent moving for 8 mi. 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 individual. Following determination of baseline activity, we introduced 50 ml of chemical stimuli from one of the following treatments: (1) a sympatric introduced predator (*L. auritus*; n=20), (2) a sympatric native predator (*M. salmoides*; n=20), or (3) only water (control; n=20). Treatments were tested in random order and coded to control for observer bias. We introduced cues into the aquarium through a syringe attached to plastic tubing attached to the center of one side of the testing aquaria at a rate of 2 ml/s. The end of the introduction tube was positioned approximately 2 cm below the surface of the water to reduce disturbance during treatment introduction. After introduction of the stimulus, we recorded the activity level of the salamander for another 8 min as an indication of responsiveness. These data constitute the post-stimulus behavioral response for each individual. Immediately after the post-stimulus observation, we transferred the salamander into another 250-ml glass beaker with 100 ml of well water for 1 h. These data constitute the post-stimulus CORT levels. As a result, each trial yields information on both the behavioral and endocrine response of individual salamanders to chemical stimuli. Each individual was exposed to a single treatment to eliminate any effects of habituation to stimuli. We washed all

hormone collection equipment with 95% ethanol and all testing equipment with 3% hydrogen peroxide between each trial.

Stimulus Acquisition

We collected both the native and introduced predators (*M. salmoides* and *L. auritus*) from Spring Lake, Hays Co., Texas, USA. 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 maintain similar 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. This method has been used successfully in previous studies (Mathis et al. 2003; Epp and Gabor 2008). Samples were thawed immediately prior to testing.

Hormone Analysis

Water samples containing hormones were maintained at -20°C until assays were performed. To extract the hormones from the water samples, C18 solid phase extraction (SPE) columns were used on a vacuum manifold and the hormones were eluted into test tubes using HPLC methyl alcohol. Eluted hormones were then placed in a 37°C water bath and dried using low flow nitrogen gas. Prior to assaying samples, hormones were resuspended in enzyme-immunoassay (EIA) buffer. We used commercially available EIA

kits (from Cayman Chemicals) to measure CORT levels. Samples were run in duplicate on 96 well plates and read by a fluorescent plate reader (BioTek Powerwave XS).

To validate the EIA kits for water-borne CORT, we obtained water-borne hormone samples from 10 adult, captive hatched salamanders (male n=5; female n=5) using identical procedures used during experimental trials. These samples were then eluted, dried, and resuspended, before being combined into a single pooled sample. Using this pooled sample, we assessed the parallelism of the serial dilution curve to the standard curve of CORT and determined the quantitative recovery of water-borne CORT by combining 70 µl of the pooled sample with 70 µl of the eight CORT standards as well as an unmanipulated control. The log-logit transformed dilution curve was constructed using average percent maximum binding and pg/ml concentrations for the six dilution samples (1:1 to 1:32). Dilution curves were parallel to the standard curve for CORT (comparison of slopes: $t_8 = 0.505$, $p = 0.625$). The quantitative recovery of water-borne CORT was determined by combining the pooled sample with eight of the CORT standards as well as a control-pooled sample. Expected recovery concentrations were based on the known amount of CORT in the standards and the control-pooled sample. Minimum recovery was 41.4% and the mean was 80%. The slope of the observed versus expected curve was 1.06, indicating a linear relationship between observed and expected values of CORT ($F_{1,8} = 1438.74$, $r^2 = 0.995$; $p < 0.0001$).

Statistical Analysis

Prior to analysis, behavioral data were combined into a single activity index for each individual. These activity indices were calculated as the difference between post-stimulus activity and pre-stimulus activity. Positive values indicate increases in activity

and negative values indicate decreases in activity in response to the stimulus. CORT response was determined as the ratio of post-stimulus CORT to pre-stimulus CORT, therefore responses greater than one indicate that CORT levels increased in response to the stimuli. Since the both the behavioral and hormone data met the requirements (normality and homoscedasticity) of parametric analyses, we performed an ANOVA followed by subsequent multiple comparisons (Tukey's HSD; $\alpha = 0.05$).

Results

There were significant differences in the activity indices between the three treatments (ANOVA: $F_{2,57} = 24.747$, $p < 0.0001$; Figure 2). The mean activity index for the blank water control was significantly less than that of the introduced predator (Tukey's HSD: $p = 0.0002$) and the native predator ($p < 0.0001$). Additionally, the activity index for the native predator was significantly lower than that of the introduced predator ($p = 0.028$).

Significant differences between CORT responses also were found between the three treatments (Analysis of Variance: $F_{2,57} = 11.765$, $p < 0.0001$; Figure 3). The mean CORT response for the native predator was significantly greater than that of both the introduced predator (Tukey's HSD: $p = 0.004$) and the blank water treatment ($p < 0.0001$). The CORT responses between the introduced predator and the blank water treatment did not differ ($p = 0.385$).
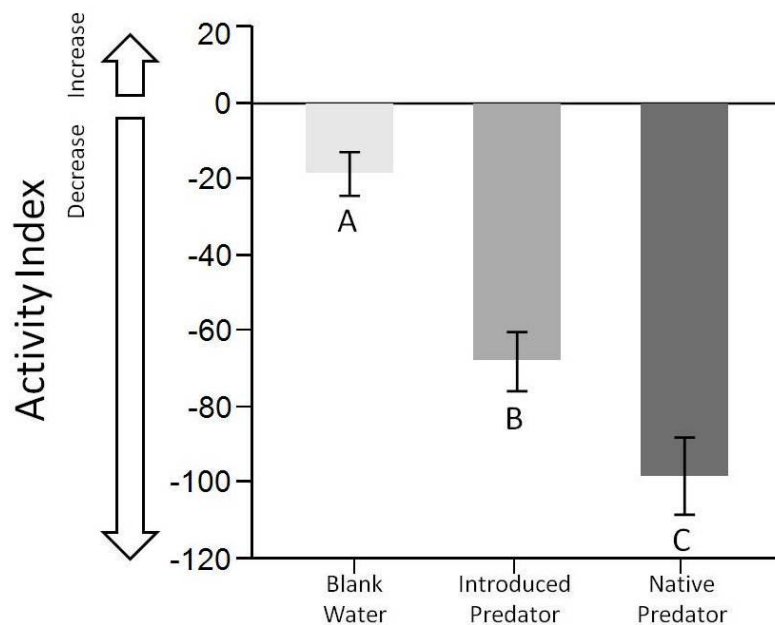
Figure 2. Activity Indices of *Eurycea nana* to Native and Introduced Predators.
Mean activity indices (± SE) of *Eurycea nana* in response to three treatments. Letters in figure indicate groupings from Tukey's HSD means comparisons (α = 0.05).
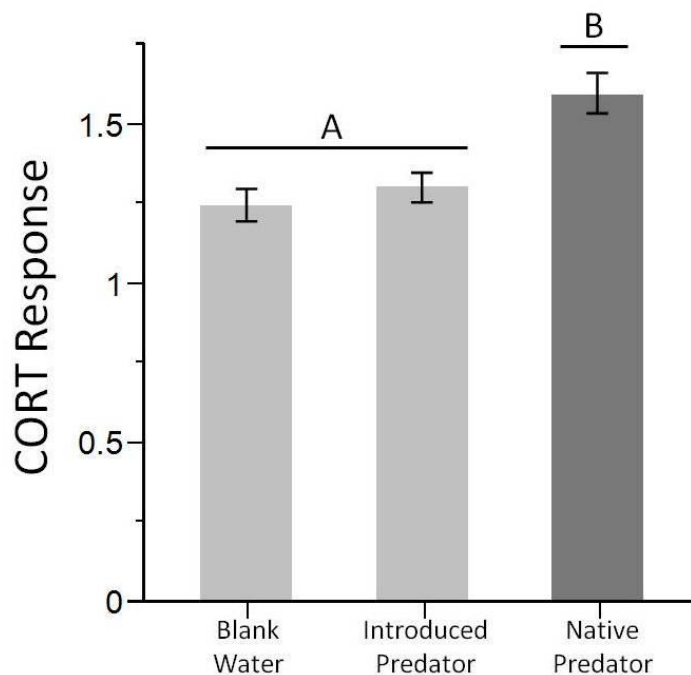

Figure 3. CORT Response of *Eurycea nana* to Native and Introduced Predators.
Mean CORT responses (± SE) of *Eurycea nana* in response to three treatments. Letters in figure indicate groupings from Tukey's HSD means comparisons (α = 0.05).

Discussion

Predator-experienced salamanders significantly reduced activity (antipredator behavior) in response to chemical cues from native (*Micropterus salmoides*) and introduced (*Lepomis auritus*) fish predators when compared to the blank water treatment, and the response to native predator was a significantly stronger than the response to the introduced predator. The CORT response to the blank water treatment and the introduced predator did not differ; however, the CORT response to the native predator was significantly greater than response to the blank water and introduced predator. Since prey should efficiently respond to predators in order minimize costs of antipredator responses (Lima and Dill 1990), the observed differences in the way *E. nana* responds to predators may represent varying levels of perceived threat. Alternatively, differences in the way *E. nana* responds to predators may represent a lack of coevolutionary time with introduced predators. However, it is difficult to discern what may be driving these differences without further testing.

The differing behavioral response to the two predators and the lower CORT response to introduced predators may reflect temporal variation in the risk of predation (Lima and Bednekoff 1999). Lima and Bednekoff (1999) developed the RAH which states that prey foraging under temporal variation in risk of predation face problems in the optimal allocation of antipredator behavior across varying states of risk. For example, if predators are infrequent and periods of risk of predation are brief, then foraging prey should exhibit heightened antipredator behavior; any foraging time lost can be regained during periods of low or no risk. Alternatively, if predators are common and periods of predation risk are prolonged, prey should exhibit reduced antipredator behavior, and

should continue to forage during these high-risk periods. If introduced predators are more abundant and encountered for prolonged durations, this may explain why we observed muted behavioral and physiological responses to stimuli as compared to native predators. Both *L. auritus* and *M. salmoides* are among the most abundant fish in the San Marcos River and additionally, the relative abundances of both species have significantly increased compared to other fish species (Perkin and Bonner 2011). Muted responses to highly abundant predators may be further exacerbated if chemical stimuli among closely related species are similar. Numerous species of *Lepomis* exist in the headwaters of the San Marcos River, and salamanders are known to exhibit predator generalization (Davis and Gabor, in prep). Therefore, the muted responses we saw to the introduced predator may be attributed to the increase in abundance of *L. auritus*, but may also be exacerbated if *E. nana* generalize their antipredator response to all *Lepomis* since multiple species are sympatric with *E. nana*.

In contrast to our results, Epp and Gabor (2008) found that predator-experienced salamanders decreased activity in response to the native predator (*M. salmoides*), but not to the same introduced predator (*L. auritus*). Additionally, Epp (2010) found that predator-experienced salamanders reduced activity to an introduced predator (L. auritus), however, this was not significantly different from the control treatment. Epp and Gabor (2008) found differences in the antipredator behaviors of predator-naïve and predator-experienced salamanders and suggested that experience may influence the responses seen in field-collected salamanders. Since we tested predator-experienced (field-collected) salamanders, it is possible that differences in experience-mediated learning exist between salamanders used in our study. Predators are known to vary spatially and temporally

across landscapes (Laundré et al. 2001) and as such, can contribute differences in experiences, which modify antipredator behaviors.

In an experimental test of the RAH in *E. nana*, Epp (2010) found no effects of encounter risk and frequency on antipredator behaviors. The RAH has been tested in experiments with simulated risk and field studies with variation in real predation risk (Hamilton and Heithaus 2001; Sih and McCarthy 2002; Van Buskirk et al. 2002; Pecor and Hazlett 2003; Sundell et al. 2004; Brown et al. 2006; Mirza et al. 2006; Creel et al. 2008). These studies have shown that prey follow the RAH under some conditions, but not always. Differences in methodology may account for studies that had results that did not match the predicted outcomes of the RAH (Ferrari et al. 2009), and as such, Epp (2010) attributed the lack of effects on antipredator behaviors to differences in methodologies with studies which support the RAH. Salamanders in high frequency predator treatments were exposed to stimuli twice daily unlike in other studies where prey were exposed to predator stimulus three times daily. Additionally, one assumption of the RAH is that prey under high-risk of predation have reduced ability to forage (Lima and Bednekoff 1999). Epp (2010) was unable to limit food availability for these salamanders, and without this, salamanders did not have to change their behaviors to increase time spent foraging. However, Epp (2010) found that prior to stimulus introduction, salamanders in low-risk treatments were more often in shelters than salamanders in high-risk treatments. This suggests that salamanders exposed to high-risk treatments increased their foraging behaviors during safe periods (prior to introduction of stimulus), which follows predictions of the RAH (Lima and Bednekoff 1999).

Our results indicate that native predators elicit stronger physiological responses over an introduced predator. CORT levels significantly increased after exposure to chemical stimuli from a native predator, but not an introduced predator. To date, only a single study has examined CORT levels of amphibians in response to predation. Fraker et al. (2009) found that *Rana sylvatica* tadpoles had significantly lower levels of CORT after exposure to high-risk predator diet-cues. Fraker et al. (2009) suggested that differences in the expression of CORT are attributed to the type of chemical cue influencing the HPI axis. For example, in mammals, chemical stimuli derived from predators triggers the increase in CORT production, while the chemical cues which were responsible for suppressing the HPI axis in *R. sylvatica* tadpoles are derived from prey (conspecifics) (Fraker et al. 2009).

Increases of CORT can negatively affect many different systems. CORT can increase levels of neutrophils and decreases levels of lymphocytes circulating within the blood stream (Gross and Siegel 1983). Davis and Milanovich (2010) found that two color morphs of *Plethodon cinereus* have differences in baseline neutrophil/lymphocyte ratios. These ratios can then be used to estimate CORT levels (Davis et al. 2008). Differences in the predation pressure between color forms of *P. cinereus* are known (Moreno 1989; Venesky and Anthony 2007) and it is this difference in predation pressure that may be creating the differences in CORT and neutrophil-lymphocyte ratios. Chronic increases in CORT may have negative fitness consequences on salamanders, especially if cumulative effects of CORT are increasing due to highly abundant and diverse assemblage of predatory fishes in the San Marcos River. If these salamanders have muted responses to introduced predators, it is possible that costs of responding can be mitigated (Lima and

Bednekoff 1999). Exploration into how CORT may decrease fitness in *E. nana* is needed to further understand if these predators are causing chronic stress in individuals.

Acknowledgements

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