1. Introduction

Predators can influence prey fitness either directly, through the consumption of individuals, or indirectly, through costs accrued from antipredator behavior [1]. In aquatic systems, predator recognition may result in prey species forming groups [2], increasing refugia use [3,4], or reducing overall activity levels [5,6]. Although these behaviors may decrease the immediate threat of predation, predators can have lingering, nonlethal effects on prey species, which may persist through time [7]. Nonlethal effects are important considerations for the fitness of species, even though they may not be as apparent as direct predation upon individuals. One such example includes the predatory influence on circulating stress hormone levels in prey species that may be involved in escape behaviors [8]. Additionally, stress hormones may also be important for responding appropriately in subsequent encounters with predators, as stress hormones may play a role in learned predator recognition [9-11].

Most vertebrates respond to stressors with a rapid elevation of glucocorticoid (GC) hormones, where the intensity of the stressor can affect the degree of the GC response [12]. Elevated GC levels trigger the metabolism of lipids, proteins, and carbohydrates, enhancing functions necessary for immediate survival of an individual [13,14]. However, over longer periods of time, chronically elevated GC levels can directly suppress immune responses, reproduction, growth, and decrease expression of androgen-mediated mating behaviors [13,15]. Acute stress responses are likely beneficial, but chronic activation of the stress
system to prolonged predation pressure may have fitness consequences. Prior studies have assumed that as exposure to stressors increases, so do baseline levels of corticosterone (CORT; a major GC), and chronically higher levels of CORT are associated with reduced relative fitness of individuals or populations, also known as the Predation Stress Hypothesis [16] or the CORT-Fitness Hypothesis [17].

Direct predator exposure or indirect exposure through chemical cues (kairomones) can cause immediate increases in circulating GC levels in prey [18,19] but not always [16]. Elevated CORT levels can enhance antipredator response [11,20] and may modulate subsequent behavioral and morphological responses to predators. Hossie et al. [21] experimentally demonstrated that Lithobates (Rana) pipiens tadpoles, when exposed to a CORT receptor inhibitor, showed decreased behavioral and morphological responses to predators when compared to control groups. A second study by Middlemis Maher et al. [22] found that long-term exposure to CORT might enhance survivorship of Lithobates sylvaticus (Rana sylvatica) tadpoles through the induction of morphological changes in tail shape. Both Fraker et al. [23] and Middlemis Maher et al. [22] found lower levels of CORT immediately following predator exposure in L. sylvaticus tadpoles. This decrease in CORT levels is contrary to what has previously been seen in other vertebrate groups, even though the antipredator response (freezing behavior) is similar [24]. These differences across vertebrate groups suggest that the expression of CORT and the subsequent modulation of behaviors vary across taxa.

Another factor in antipredator (and possibly CORT response) to predators may be perceived risk levels. Lima and Bednekoff [25] developed the Risk Allocation Hypothesis (RAH) that suggests that prey foraging under temporal variation in risk of predation face problems in how to optimally display antipredator behavior. For example, if predators are encountered infrequently and periods of risk are brief, then foraging prey should exhibit heightened antipredator behavior; any costs to foraging or mating 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. At the same time, chronic CORT levels can have negative fitness consequences and therefore, blunted CORT responses may be expected when prey are exposed to common, abundant predators.

We examined the antipredator and CORT responses of the San Marcos salamander, Eurycea nana, to temporal variation in risk of predation by fish predators. Eurycea nana is a federally threatened, IUCN redlisted, neotenic (fully aquatic) salamander endemic to the headwaters of the San Marcos River, Hays County, Texas [26]. Previous studies have demonstrated the use of chemical stimuli in the detection of both conspecifics [27] and fish predators [6,28]. Eurycea nana shows the antipredator behavior of freezing in response to chemical cues emitted by a variety of fish predators (Lepomis cyanellus, Lepomis auritus, Micropterus salmoides, Herichthys cyanoguttatus) [6,28] including the alltopatic species Lepomis gibbosus indicating a generalized antipredator response [6]. In this closed system, both M. salmoides and L. auritus are the most abundant species and they have significantly increased in abundance compared to other large, predatory fish species over the past three decades [29]. Because E. nana shows a generalized antipredator response to Lepomis, we propose that this species will be perceived as a higher temporal risk than M. salmoides. We examine the antipredator and CORT responses of wild-caught (predator experienced) E. nana to M. salmoides and L. auritus, and predict that E. nana will show antipredator and CORT responses to both species but will have a muted antipredator and CORT response to the temporally abundant L. auritus.

2. Materials and methods

2.1. Predator species

To further understand any differences in the effects that predatory fish have on E. nana, we collected chemical cues from two centrarchid (Perciformes: Centrarchidae) fish: the redbreast sunfish (L. auritus) and the largemouth bass (M. salmoides). The diet of L. auritus within the San Marcos River is primarily aquatic invertebrates (Diptera, Ephemeroptera, and Trichoptera), suggesting that this species is a generalist benthic feeder [30]. Examination of the diet of M. salmoides suggests that it too is a generalist feeder, consuming roughly equal proportions of fish and aquatic invertebrates [31], though the specific diet has not been examined for individuals from the San Marcos River. Both species have been observed to consume E. nana [32] and spend large amounts of time foraging among benthic substrates where salamanders are generally found (D.R. Davis, C.R. Gabor, personal observation).

2.2. Stimulus acquisition

We collected adult L. auritus and M. salmoides from the headwaters of the San Marcos River, Hays County, Texas, USA (29.89321°N, 97.93148°W; WGS84). We only used adult fish to reduce any possible ontogenetic effects. Prior to the collection of chemical cues, we fed fish earthworms for 5 d to eliminate any effects of prior diet. We determined the volume of each stimulus animal through displacement. To maintain similar chemical cue concentrations between treatments, we used 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 minimize any individual effects and froze 50-ml aliquots in a −20 °C freezer. This method has been used successfully in previous studies [6,33,34]. Samples were thawed immediately prior to testing.

2.3. Experimental protocol

We used wild-caught adult E. nana (N = 31 females, N = 29 males) collected from the headwaters of the San Marcos River, Hays County, Texas, USA (29.89401°N, 97.92997°W; WGS84) and allowed them to acclimate to laboratory conditions for a minimum of two weeks. All individuals were transported to the San Marcos Aquatic Resources Center (SMARC) and housed in large, 360-l fiberglass flow-through tanks, maintained on a 12:12 h light cycle, and fed blackworms (Lumbriculus variegatus) ad libitum. Following established water-borne hormone collection methods [35], we randomly selected a salamander and placed each individual into separate 250-ml Nalgene container (perforated at the bottom) that fit within a 250-ml glass beaker filled with 100 ml of well water for 1 h. We used well water because it is the same type of water in which salamanders are maintained. After collecting pre-stimulus CORT levels, we then gently lifted out the Nalgene insert (allowing the water to drain through the bottom) and transferred each individual salamander into a separate 9.5-l glass testing aquarium containing 4.5 l of well water. Each aquarium had three sides covered with black plastic to reduce background disturbance. 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 to minimize disturbance from lights. 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 min (following [6]). Active behavior included swimming or walking, but did not include buccal pumping or gill movement that was not accompanied by other movements of the body. These data constitute the pre-stimulus activity level for each individual. Following determination of pre-stimulus activity, we introduced 50 ml of chemical stimuli from one of the following treatments: (1) an introduced redbreast sunfish (L. auritus; N = 20), (2) a native largemouth bass (M. salmoides; N = 20), or (3) only water (a blank control; N = 20). Treatments were tested in random order and coded to control for observer bias.
(all trials conducted by DRD). We introduced cues into the aquarium through a syringe attached to plastic tubing attached to the center of one side of the testing aquarium 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. These methods follow closely those of previous studies [6,28,34]. Immediately after the post-stimulus observation, we transferred the salamander into a clean, 250-ml glass beaker with 100 ml of well water for 1 h. These data constitute the post-stimulus CORT levels. Afterwards, we measured the snout–vent length (SVL) and recorded the sex of each salamander. As a result, each trial yields information on both the behavioral and water-borne CORT levels of individual salamanders to chemical stimuli. Each individual was exposed to a single treatment to eliminate any effects of habituation to stimuli [36]. All testing was performed during the same time range each day to control for circadian variation in CORT levels and gloves were used during water-borne hormone collection. We washed all hormone collection equipment with 95% ethanol and DI water and all testing equipment with 3% hydrogen peroxide between each trial.

2.4. Hormone analysis

Traditional methods of measuring circulating hormone concentration in aquatic vertebrates involve assaying blood plasma. Here, corticosterone (CORT) levels were measured using a recently developed, non-invasive process for measuring water-borne hormone levels [35]. This method allows for repeated sampling of individuals and does not require euthanizing salamanders to collect blood plasma. Gabor et al. [35] validated the use of water-borne hormones from E. nana as they found a significant positive correlation between plasma CORT levels and water-borne CORT release rates. They also demonstrated that serial dilution of CORT from the water-borne hormone collection shows parallelism to the standard curve and that expected recovery concentrations were relative to known amounts of CORT in the standards on enzyme immunoassay (EIA) plates.

All hormone analysis methods follow that of Gabor et al. [35]. Water samples containing hormones were maintained at −20 °C until assays were performed. We primed C18 solid phase extraction columns (Sep-Pak, Waters Inc.) with 4 ml of HPLC-grade methanol and 4 ml of millipore water. Water samples were then passed through these primed columns using a vacuum manifold, thus extracting hormones from the water samples. Hormones were then eluted from the columns into borosilicate test tubes using HPLC-grade methanol, placed in a 37 °C water bath, and dried under a stream of low flow nitrogen gas. Prior to assaying samples, we resuspended hormones in a solution of 5% ethanol and 95% EIA buffer for a final resuspension volume of 400 μl. We used commercially available EIA plates (Cayman Chemicals Inc.) to measure CORT levels. We ran samples in duplicate on 96 well plates and read by a fluorescent plate reader set at 415 nm (BioTek Powerwave XS). We used a pooled control that was combined from ten non-experimental E. nana and run in duplicate to measure both intra-assay CV (0.74%, 1.47%, 3.40%, 5.77%, 8.37%, 8.46%) and inter-assay CV (17.59%) for the six plates.

2.5. Statistical analysis

We combined behavioral data into a single activity index for each individual. We calculated the activity index 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. Following Gabor et al. [34], we multiplied CORT release rates (pg/ml) by 0.4 ml (the volume of the resuspension solution) and standardized by dividing by the SVL of each individual, resulting in the CORT release rate units being pg/SVL/h. However, there was no significant relationship between SVL and pre- (N = 51, r = −0.012, p = 0.45) and post-stimulus (N = 51, r = −0.005, p = 0.97) CORT release rates. All CORT data were Ln transformed. We measured CORT response as the ratio of post-stimulus to pre-stimulus CORT release rates, therefore responses greater than one indicate that CORT release rates increased in response to the stimuli. All data met the assumptions of parametric analyses and we analyzed both the behavioral and hormone data using an ANOVA followed by Tukey’s HSD multiple comparisons test (α = 0.05). Additionally, we examined whether there was a difference in pre- and post-stimulus CORT release rates for each treatment using matched pairs t-tests. To explore whether behavior and CORT were related, we examined the relationship between CORT response and activity index and between pre-stimulus and post-stimulus CORT release rates and pre and post-stimulus behavior respectively using Pearson correlations. To aid in visualizing the data, we present non-transformed CORT response values in the figure.

3. Results

There were significant differences in the activity indices among the three treatments (ANOVA: F2,57 = 24.75, p < 0.0001; Fig. 1). The activity index from the blank water control was significantly greater than the mean activity index from both the introduced redbreast sunfish (L. auritus) treatment (Tukey’s HSD: p = 0.0002) and the native largemouth bass (M. salmoides) treatment (p < 0.0001). Additionally, the activity index for the M. salmoides treatment was significantly lower than that of the L auritus treatment (p = 0.028).

There was no significant difference between pre-stimulus CORT release rates (pg/SVL/h) of male (mean pre-stimulus CORT [± 1 S.E.M.]: 5.22 ± 0.78) and female (4.19 ± 0.44) salamanders (Student’s t-test: t=1.70, p = 0.25), and therefore, we combined both males and females in our analyses. We found significant differences in CORT responses among the three treatments (ANOVA: F2,48 = 10.69, p < 0.0001; Fig. 2). The CORT response for the M. salmoides treatment was significantly greater than that of both the L. auritus (Tukey’s HSD: p = 0.002) and the blank water (p = 0.0002) treatments. The CORT responses did not differ between the L. auritus and the blank water treatments (p = 0.764). Additionally, we found that CORT release rates significantly increased after exposure to chemical cues of M. salmoides (matched pairs t-test: t16 = 4.64, p = 0.0003; Fig. 3) and L. auritus (t16 = 2.40, p = 0.029; Fig. 3) but not to the blank water control (t16 = −0.09, p = 0.933; Fig. 3).

![Image](image-url)
There was no significant relationship between pre-stimulus activity and pre-stimulus CORT release rates (Pearson correlation: \( N = 51, r = −0.04, p = 0.78 \)), nor was there a significant relationship between post-stimulus activity and post-stimulus CORT release rates in any of the treatments (control: \( N = 17, r = −0.08, p = 0.76 \); \( L. auritus \): \( N = 17, r = 0.32, p = 0.16 \); \( M. salmoides \): \( N = 17, r = 0.15, p = 0.54 \)). There was no significant relationship between CORT response and activity index in any of the treatments (control: \( N = 17, r = −0.010, p = 0.72 \); \( L. auritus \): \( N = 17, r = 0.31, p = 0.22 \); \( M. salmoides \): \( N = 17, r = 0.01, p = 0.97 \)).

4. Discussion

*Eurycea nana* significantly reduced activity (antipredator behavior) in response to chemical cues from both the largemouth bass (*M. salmoides*) and the redbreast sunfish (*L. auritus*) when compared to the blank water treatment, and the response to *M. salmoides* was significantly stronger than the response to *L. auritus*. The CORT response to the blank water treatment and *L. auritus* did not differ statistically; however, the CORT response to *M. salmoides* was significantly greater than both the response to the blank water treatment and *L. auritus*. The differing behavioral response to both predators and the lower CORT response to *L. auritus* may reflect temporal variation in the risk of predation, thus supporting the RAH hypothesis [25]. *Lepomis auritus* is more abundant and frequently encountered compared to *M. salmoides*, and additionally, *E. nana* shows a generalized antipredator response to the chemical cues of the multiple *Lepomis* species found in this system [6]. The high encounter frequency and high abundance of *Lepomis* sp. may account for the muted antipredator and CORT responses of *E. nana* as compared to *M. salmoides*. Alternatively, differences in the way *E. nana* responds to predators may represent a shorter coevolutionary time with this introduced species of *Lepomis* (~60 years) [37]. Further experiments are necessary to tease apart these alternative hypotheses.

Even though *E. nana* clearly shows a behavioral and stress response to predators, when we examined the relationships between CORT release rates and behavior as well as the relationship between CORT response and behavior, we found that CORT did not appear to directly modulate the behavioral response. The Predation Stress Hypothesis predicts a relationship between these two variables and support has been found in birds, rabbits and lizards (review, [16]). Wack et al. [38] found that CORT, however, was not correlated with changes in activity in a terrestrial salamander, *Plethodon shermani*. They suggested that the association between behavior and CORT is not necessarily causative but instead CORT may mediate transitions associated with a stressor. One hypothesis for why salamanders may not show the predicted relationship is because there are high energetic costs associated with increase CORT levels in salamanders [38], yet many plethodontid salamanders such as *Eurycea* are specialized for low energy lifestyles [39].

In contrast to our results, Epp and Gabor [28] found that predator-experienced *E. nana* did not decrease activity in response to *L. auritus*; however, predator-naïve *E. nana* did respond to *L. auritus* (indicating innate predator recognition), and therefore, this apparent lack of response may be muted by experience. Also, Epp and Gabor [28] did not control for diet of the predators and this may have resulted in the behavioral differences observed in our study versus theirs.

Few studies have directly examined the relationship between CORT levels and predation risk in amphibians. Fraker et al. [23] found that *L. sylvaticus* (*R. sylvatica*) tadpoles had significantly lower levels of CORT after exposure to high-risk predator diet-cues. Dahl et al. [40] found that tadpoles of *Rana temporaria* from low-latitude populations exhibited elevated CORT levels after exposure to dragonfly naiads while there was no response from individuals from high-latitude populations. Due to the negative influence of CORT on growth rates, this lack of mounting a CORT response to a common predator was attributed to selection maximizing growth rates in these high-latitude populations, which have short growing seasons [40]. Additionally, Middlemis Maher et al. [22] found that CORT of *L. sylvaticus* decreased immediately after exposure to dragonfly naiads. In tadpoles, increased CORT is associated with metamorphosis [41] and has been shown to increase locomotion and foraging behaviors and therefore, may also increase predation risk [42]. However, we found no relationship between CORT and activity in our study. Metamorphism is driven by CORT in tadpoles and this relationship may drive the differences between the species because *E. nana* does not undergo metamorphosis. Unlike in the above studies where sacrificing the whole individual is required to obtain CORT values, we obtained both pre- and post-stimulus CORT levels for each individual using the non-invasive water-borne hormone collection method. Our method may allow us to gain a closer understanding of how predation exposure affects CORT values at an individual level.

Chronic increases in CORT may have negative fitness consequences including high energetic costs 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. In response to these numerous predators, and to mitigate the costs of responding, salamanders may have muted responses to L. auritus. Alternatively, antipredator behavior in plethodontid salamanders may not be linked to changes in CORT as suggested by our data. Future studies exploring the relationship between CORT levels (both chronically high and muted) and fitness in E. nana are needed to better understand these complex interactions between fish predators and E. nana. Additionally, experimental manipulation of CORT will aid in our understanding of the causal relationship between CORT and antipredator behaviors.

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