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RESEARCH PAPER

Recognition and Response to Native and Novel Predators in the Largespring mosquitofish, *Gambusia geiseri*

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Abstract

The introduction of predator species into new habitats is an increasingly common consequence of human activities, and the persistence of native prey species depends upon their response to these novel predators. In this study, we examined whether the Largespring mosquitofish, *Gambusia geiseri* exhibited antipredator behavior and/or an elevation of circulating stress hormones (cortisol) to visual and chemical cues from a native predator, a novel predator, or a non-predatory control fish. Prey showed the most pronounced antipredator response to the native predator treatment, by moving away from the stimulus, while the prey showed no significant changes in their vertical or horizontal position in response to the novel or non-predator treatments. We also found no significant difference in water-borne cortisol release rates following any of the treatments. Our results suggest the prey did not recognize and exhibit antipredator behavior to the novel predator, and we infer that this predator species could be detrimental if it expands into the range of this prey species. Further, our study demonstrates prey may not respond to an invasive predator that is phylogenetically, behaviorally, and morphologically dissimilar from the prey species' native predators.

Introduction

As human activities lead to the introduction of predator species into new habitats, the ability of prey to recognize and engage in antipredator behavior in response to novel predators becomes increasingly important for the survival of prey species. Novel predators pose a particular threat for native species because prey individuals may not accurately interpret the level of risk and respond appropriately (Lima & Dill 1990; Sih et al. 2010). Failure of a prey individual to respond accurately to a predator may result in immediate consumption, or indirect, non-consumptive effects (Lima & Dill 1990; Salo et al. 2007; Sih et al. 2010). Prey that are hypersensitive to the presence of novel predators may spend a disproportionate amount of time avoiding predators instead of allocating time and energy elsewhere (Ferrari et al. 2007; Davis et al. 2012). Introduced predators can thus decrease prey fitness through indirect non-consumptive effects as well as direct consumption.

In addition to the cost of behavioral trade-offs, non-consumptive effects can occur through physiological mechanisms (Cockrem & Silverin 2002; Fraker et al. 2009). Elevation of glucocorticoid hormones (stress response) such as cortisol (most mammals, fishes) or corticosterone (birds, reptiles, amphibians, rodents) is a common response to the stress of predation threat in vertebrate prey animals (Clinchy et al. 2004; Rödl et al. 2007; Thaker et al. 2009; Archard et al. 2012; Fischer et al. 2014). Elevation of stress hormones can also facilitate an antipredator behavioral response, making responses quicker and more pronounced (Thaker et al. 2009; Hossie et al. 2010). However, naive prey may not show elevated stress hormones in response to a novel predator (Rodl et al. 2007; Anson & Dickman 2013). In fact, it has been suggested that stress response mechanisms may be not be flexible enough to allow organ-

isms to respond optimally to rapid environmental changes resulting from human activity (Angelier & Wingfield 2013).

The effects of a novel predator depend on whether the prey species can recognize it as a threat. Fish exhibit a range of innate and learned antipredator behaviors (Kelley & Magurran 2003). Fish can learn to respond to novel predators via associating chemical alarm or disturbance cues of conspecifics, and in some cases prey may be able to identify unfamiliar predators from kairomones, or chemical signals of the predator alone (Kelley & Magurran 2003; Wisenden 2003). This type of innate recognition is possible when prey species are able to generalize from a native predator to recognize and show antipredator behavior to a closely related non-native predator (Ferrari et al. 2007; Mitchell et al. 2011; Davis et al. 2012). However, generalization is less likely in situations where the introduced predator is dissimilar from native predators in morphology, behavior, and kairomones, an increasingly likely scenario as human activities lengthen accidental dispersal distances (Cox & Lima 2006).

In this study, we measured behavioral and hormonal stress responses of an endemic prey species, the Largespring mosquitofish (*Gambusia geiseri*) to visual and chemical cues (kairomones) of potential predators. We exposed Largespring mosquitofish to cues from native predatory Green sunfish (*Lepomis cyanellus*), novel predatory Gulf killifish (*Fundulus grandis*), and novel, non-predatory, guppies (*Poecilia reticulata*) as a control. Largespring mosquitofish are endemic to the headwaters of springs in central Texas, while the Gulf killifish is native to coastal habitats and has only recently been introduced and established in freshwater rivers in central Texas (Hillis et al. 1980). The Gulf killifish is a member of the order *Cypriniformes,* and unlike the native perciform predators of Largespring mosquitofish in its physical and behavioral characteristics. Thus, we hypothesized that Largespring mosquitofish would not recognize and show antipredator behavior towards this novel species. We predicted that Largespring mosquitofish would respond to native predators with behavioral changes and a correlated stress response, but show less or no change in their behavior or stress hormone levels following exposure to cues from novel predatory or non-predatory fish treatments. This study will not only allow us to assess the capacity of this native species to cope with a potential invasive predator, but also provide insight on the potential role of innate predator recognition during biological invasions.

Methods

Study Species

The Largespring mosquitofish (*Gambusia* geiseri) is a live-bearing poeciliid endemic to the San Marcos and Comal rivers in central Texas. Unlike some of its more widespread congeners (*Gambusia affinis, Gambusia holbrooki*), the Largespring mosquitofish is a specialized spring-adapted species with populations limited to small ranges surrounding headwaters (Page & Burr 1991). The Green sunfish (*Lepomis cyanellus*) is one of several common piscivorous centrarchids native to central Texas, where they share an evolutionary history with Largespring mosquitofish and readily consume them (Blake & Gabor 2014; Hubbs et al. 1991). The Gulf killifish (*Fundulus grandis*) is a member of the topminnow family (Fundulidae), native to fresh and brackish waters along the coasts of Northeastern Florida and the Gulf of Mexico. Gulf killifish consume an omnivorous diet including invertebrates and small fish (Rozas & LaSalle 1900; Hubbs et al. 1991). *Fundulus grandis* can tolerate a range of salinity and has been introduced into many freshwater environments in Texas and New Mexico through bait-bucket releases (Hillis et al. 1980). In its current distribution, *F. grandis* co-occurs with the western mosquitofish (*Gambusia affinis*), but is novel to our focal species, Largespring mosquitofish (Hillis et al. 1980; Thomas et al. 2007). We used guppies (*Poecilia reticulata*) from a laboratory stock population, which were roughly equivalent in size to the focal fish and are also live-bearing poeciliids, as an allopatric, non-predatory control. For the predator treatments, we used juvenile individuals (60–100 mm standard length (SL)) to allow for ease of maintenance in laboratory tanks, but all individuals were large enough to potentially consume focal individuals (20–35 mm SL).

Collection and Laboratory Maintenance

We used wild-caught Largespring mosquitofish collected in Jan. and Feb. 2012 from the headwaters of the San Marcos Spring, Hays County, TX (29.89472°N, −97.930278°W; WGS84). We selected guppies haphazardly from a stock laboratory population of several hundred fish. We maintained mosquitofish and guppies in 38-L aquaria (50 × 25 × 30H cm) on a 14:10-h light cycle and fed flake food (Ocean Star International) *ad libitum* once a day at 1630 h. We collected sympatric Green sunfish from Spring Lake, and collected allopatric Gulf killifish from the Brazos River, Hill County, TX (31.873056°N,

−97.364722°W; WGS84). We maintained predators in single-species 150-L aquaria (91 × 46 × 41H cm) on a 14:10-h light cycle, and fed them pellet food (Purina Aqua Max 200) *ad libitum* once a day. Predator fish were fed at 1630 h daily, and thus did not eat for 16–21 h prior to the start of testing procedures.

Collection of Chemical Cues

We maintained all stimulus fish on a diet of pellet food while they were in the laboratory. Although commercially prepared pellet food may contain fish products, feeding predators a dry, non-poeciliid diet ensured that our chemical cues contained kairomones of the treatment species without alarm or diet cues of our focal prey species. After determining the volume of each stimulus animal through displacement, we filled the cue collection tank with 230 ml of water per 1 ml of water displaced by the stimulus animal to maintain a consistent concentration of chemical cues for all treatments. For the guppy treatment, we collected cues from a large shoal of fish in a single collection tank to reach an adequate volume, while for the two predator species we collected cues from each individual predator in separate tanks (n = 3 individuals per predator treatment). We placed stimulus animals into separate tanks containing the appropriate volume of aerated, dechlorinated tap water for 24 h. After removing the stimulus fish from the collection tanks, we mixed equal proportions of water from the different predator individuals and stored 50-ml aliquots at −20°C. We thawed samples immediately prior to testing following prior methods (Mathis et al. 2003; Epp & Gabor 2008; Davis et al. 2012).

Testing Procedure

We tested the behavioral and stress hormone (cortisol) responses of individual Largespring mosquitofish (n = 20) to visual and chemical stimuli from each of three predator treatments: (1) native sunfish (*L. cyanellus*), (2) novel killifish (*F. grandis*), and (3) non-predatory guppy (*P. reticulata*). Each focal individual received each predator treatment in a random order over three consecutive days of testing. Our setup consisted of two adjacent tanks, one 38 l (50 × 25 × 30 cm) and one 19 l (40 × 20 × 25 cm). The larger tank contained the focal individual and two shoal mates, chosen haphazardly from a laboratory stock tank of around 20 individuals, while the smaller tank contained an individual *L. cyanellus*, *F. grandis*, or *P. reticulata*. The long side of the stimulus tank was positioned against the short side of the focal fish tank such that the stimulus individual was always within 20 cm of the focal fish tank. We covered the front of the focal tank with one-way tinting and the other exposed sides of the tanks in opaque plastic to reduce visual disturbance during observation. We hung a fluorescent light directly above the tanks to light the fish but left the testing room dark. We placed an opaque barrier between the focal and stimulus tanks until the introduction of the predator stimulus during the trial. To introduce chemical stimuli, we injected cues through airline tubing attached with a suction cup 10 cm under the surface of the water, on the side of the focal tank nearest to the stimulus tank. Cues were injected via two 60-ml syringes connected to the tubing with a T-joint, one containing a chemical stimulus treatment and the other plain water to flush the tubing. We used hydrogen peroxide and water to clean tanks and syringes between trials.

Prior to testing, we measured SL from tip of the snout to the end of the last vertebra, and marked focal mosquitofish with elastomer (Northwest Marine Technology) to distinguish them from their shoal mates. To initiate the testing procedure, we placed a marked focal individual into the testing tank with two conspecifics to allow natural shoaling behavior. After spending 14–18 h in the testing tank overnight, we captured the focal fish and placed them in a 150-ml beaker with 60 ml of water to obtain the pre-stimulus water-borne hormone samples from the focal fish for 30 min, following the methods of Gabor & Contreras (2012). We then returned the focal fish to the testing tank, and after 5 min acclimation, we recorded pre-stimulus behavior for 5 min. A single observer (JEG) measured behavior by recording the vertical position of the focal fish in one of three equal (7 cm) zones and the horizontal position of the focal fish in one of ten equal (5 cm) zones, every 30 s using the event recorder JWatcher (Blumstein & Daniel 2007). A focal fish in horizontal zone one was within 5 cm of the stimulus tank, whereas a fish in zone 10 was 45–50 cm from the stimulus tank. We then introduced the stimulus by removing the visual barrier between the focal tank and the stimulus tank containing a single individual of the stimulus species. At the same time, we injected 50 ml of chemical stimulus, followed by 50 ml of water flush at a rate of approximately 2 ml per second. Following the introduction of the stimulus, we again recorded vertical and horizontal position of the focal fish every 30 s for 5 min. After the conclusion of behavioral observations, we removed the focal fish to collect a post-stimulus

water-borne hormone sample for 30 min (following the same methods as above). We then returned the focal individual to the testing tank and repeated this process on subsequent days (≥14 h later) for all three treatments. All testing was performed between 0800 and 1300 h.

Analysis of Hormone Samples

We stored all water samples at −20°C until ready to be thawed for extraction (Ellis et al., 2004). To obtain total cortisol release rates, we extracted hormones from water using C18 solid phase extraction columns (Waters Inc., Milford, MA, USA) on a vacuum manifold. We eluted hormones via the columns with methanol (following Gabor & Contreras 2012). The collected methanol was evaporated using nitrogen gas. We resuspended the residue in a 350 μl solution of 5% ethanol and 95% enzyme-immunoassay (EIA) buffer (Cayman Chemicals Inc., Ann Arbor, MI, USA). Cortisol release rates were measured in duplicate for all samples using a cortisol EIA kit (Cayman Chemicals Inc.) on a spectrophotometer plate reader (BioTek Powerwave XS).

Time Release of Cortisol into Water

We examined the release rates of cortisol in water for Largespring mosquitofish by obtaining water-borne hormone samples from non-stressed Largespring mosquitofish (n = 8/treatment) repeatedly over four 30-min periods for a total of 120 min. Individual fish were placed in a 150-ml beaker with 60 ml of water. We weighed (g) and measured SL (mm) each fish after the four samples were obtained. These data were collected in May 2012. We did not find a significant difference in the cortisol release rates of the fish over time (Rm ANOVA: $F_{3,3} = 2.97$, p = 0.198). Based on these results, we only obtained a 30 min sample because the cortisol level did not change over time and repeated measures.

Water-borne Hormone Validation

To validate the use of the cortisol EIA kits (Cayman Chemical: www.caymanchem.com) for Largespring mosquitofish, we obtained 10 non-experimental fish and collected water-borne hormones from each following the collection and extraction methods described above. The suspended samples were pooled and the serially diluted from 1:1 to 1:64. We compared the slopes of the standard curve and serial dilutions curve and found them to be parallel to each other ($t = -1.64$, d.f. = 11, p = 0.98). The quantitative recovery of the water-extracted hormones was obtained by comparing a pooled sample spiked with each of the eight standards to an unmanipulated pooled control sample. Expected recovery was calculated from know unmanipulated samples. Minimum recovery was 91.9%. The slope of the observed vs. expected curve was 0.98, suggesting a linear relationship between observed and expected water-borne hormone levels ($F_{1,7} = 385.63$, $r^2 = 0.98$; $p < 0.0001$). We used five plates for the experiment. The overall interplate variation was 12.83%, and the intraplate variation was 7.81%, 7.93%, 5.19%, 2.61%, and 8.95%.

Statistical Method

We calculated behavioral responses of Largespring mosquitofish as the difference in the focal individual's mean position by subtracting the focal fish's average zone during the pre-stimulus from its average zone during post-stimulus observation. Thus a positive value for horizontal response represents a move away from the stimulus, while a positive value in vertical response indicates a higher position in the water column post-stimulus. For hormone responses, we first multiplied cortisol concentrations (pg/ml) by the amount we diluted the sample then divided by the SL of each individual, and then multiplied by 2 (pg/SL/h). We normalized our data by taking the natural log of these values. Finally, we calculated cortisol response as the ratio of post-stimulus to the pre-stimulus cortisol. We used a crossover ANOVA to compare means of cortisol responses between the three treatments while testing for fixed effects and possible interactions of sequence, order, and subject with treatment group (Table 1). We used the same crossover ANOVA method to analyze both horizontal and

Table 1: Fixed effects on behavioral responses

	Vertical behavioral response		Horizontal behavioral response	
	F	p	*F*	p
Subject	0.08	0.78	0.77	0.38
Treatment	1.30	0.28	5.28	0.01*
Order	0.17	0.68	0.01	0.92
Sequence	0.04	0.84	0.07	0.79
Treatment: order	0.30	0.74	0.33	0.72
Treatment: sequence	0.50	0.61	0.17	0.84
Subject: treatment	0.63	0.53	0.14	0.87

*Indicates significance at $p < 0.05$.

vertical behavioral responses. We used a Tukey's post hoc test to compare means of horizontal behavioral responses among treatment groups. We conducted analyses in R 2.15.0 (www.r-project.org).

Ethical Note

We followed ASB/ABS (2012) guidelines in designing and conducting this experiment. After the conclusion of this experiment, prey and predator individuals were returned to group tanks, and kept at the laboratory for the duration of their lives. Our care and use of the fish in these experiments was approved by the Institutional Animal Care and Use Committee of Texas State University (IACUC # 0515_0612_13).

Results

Behavioral Response

There was a significant difference in horizontal change in position among predator treatments ($F_{2,59}$ = 5.24, p = 0.01), with no significant interactions of order, sequence, or subject with treatment (Table 1). Focal fish showed the greatest change in horizontal position in response to the native sunfish, by increasing their distance from the stimulus (Fig. 1). There was no significant difference in change in vertical position among the three predator treatments ($F_{2,59}$ = 1.18, p = 0.32), with no significant interactions of order, sequence, or subject with treatment (Table 1). The mean vertical response was near zero for all treatments, indicating little difference in vertical position following the introduction of the stimulus (Fig. 2).

Hormones and Behavior

There was no difference in the cortisol response of the focal fish among the three predator treatments, with no significant effects or interactions of testing order, sequence, or subject ($F_{2,59}$ = 0.339, p = 0.715, Fig. 3). Cortisol levels increased slightly for all treatments. There was no relationship between the cortisol response and the horizontal response or vertical response in any of the three treatments (guppy vertical: Pearson = −0.007, p = 0.98; guppy horizontal: Pearson = 0.18, p = 0.58; killifish vertical: Pearson = 0.38, p = 0.09; killifish horizontal: Pearson = 0.10, p = 0.67; sunfish vertical: Pearson = −0.12, p = 0.63; sunfish horizontal: Pearson = 0.12, p = 0.64).

Discussion

Largespring mosquitofish showed a significant behavioral response to the native sunfish predator (*L. cyanellus*) and did not significantly alter their behavior in response to the novel killifish predator (*F. grandis*) or the non-predatory guppy (*P. reticulata*). Prey altered their behavior by moving away from the visual and chemical stimuli of the native predator, but did not change their vertical position in the water column, or show elevated levels of stress hormone. These results

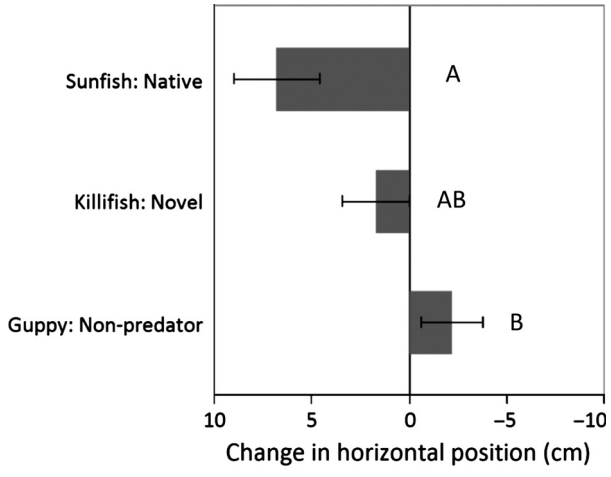

Fig. 1: Behavioral response of Largespring mosquitofish to three stimulus species. Change in horizontal position (post–pre ± SE) differs among treatments ($F_{2,59}$ = 5.24, p = 0.01). Letters indicate significant difference from Tukey's post hoc comparison.

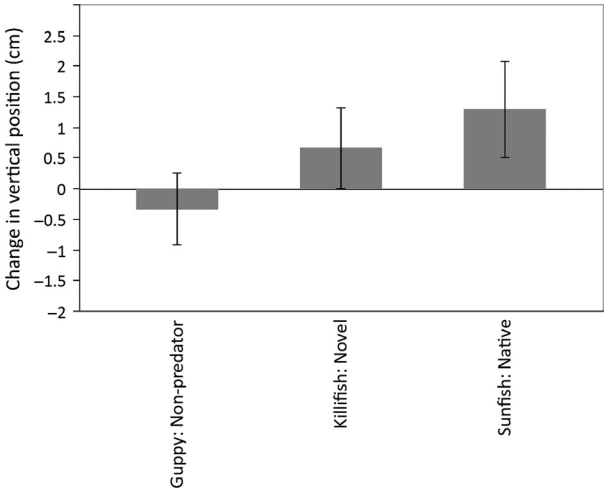

Fig. 2: Behavioral response of Largespring mosquitofish to three stimulus species. Change in vertical position (post–pre ± SE) did not differ among treatments ($F_{2,59}$ = 1.18, p = 0.32).

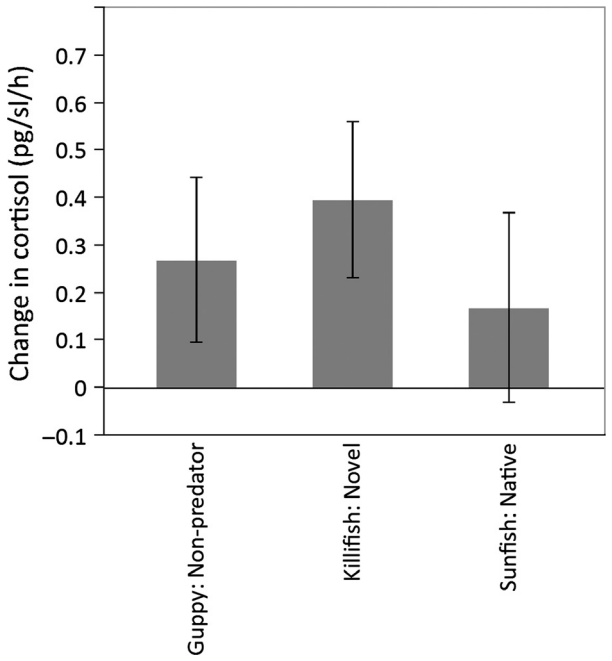

Fig. 3: Hormonal response of Largespring mosquitofish to three stimulus species. Change in natural log of cortisol release rates (post–pre ± SE) did not differ among treatments ($F_{2,59}$ = 0.3391, p = 0.7147).

agree with our prediction that Largespring mosquitofish would show greater antipredator response to a native predator than to a novel predator.

There are several possible reasons why prey showed a significant response to native predators but not the novel predator treatment. One explanation for the lack of significant response to killifish is that the prey did accurately assess the level of predation risk and that killifish in fact pose less risk to Largespring mosquitofish than the native Green sunfish. However, in another study, we found that killifish actually consume Largespring mosquitofish at a faster rate than Green sunfish in single-predator laboratory trials (Blake & Gabor 2014). Although most killifish are not considered predatory, *F. grandis* is the largest of all the killifish and has a varied diet in its native estuarine habitat that includes terrestrial insects, small fish, benthic algae, and crustaceans (Rozas & Lasalle 1990). Little is known about the current diet of the introduced freshwater populations of *F. grandis* in central Texas, but because it is a generalist that feeds throughout the water column, and readily consumes Largespring mosquitofish in the laboratory, it is likely that Gulf killifish would prey on Largespring mosquitofish in the wild if given the opportunity. Thus, we interpret the lack of significant response to the novel predator in this study to be an indication that Largespring mosquitofish failed to accurately assess the predation risk posed by this species.

It is also possible that prey individuals respond differently to killifish predators than to sunfish predators and that our methods did not capture this response. As mentioned previously, killifish differ in their behavior from sunfish in that they spend more time actively swimming and pursuing food than sunfish, which are generally ambush predators. Thus, it is possible that some aspects of effective antipredator behavior could differ for these two types of threats. However, we argue that avoidance is a general behavior that would apply to any predator threat prior to any direct interaction. Further, in a previous study on these same species, Largespring mosquitofish exposed to killifish and sunfish did not differ in the behavioral responses during direct interactions with these predators (e.g., jumping behavior, time spent at bottom) (Blake & Gabor 2014).

Lastly, variation in stimulus fish behavior among trials could have affected the responses we observed from our focal fish. One shortcoming of using live fish as stimuli is that behavior of predator individuals may have differed among trials, which could lead to inconsistent responses from focal prey individuals. Although a standardized stimulus like a video or model of a predator insures that every prey individual receives the same visual cues from the predator within each treatment, this artificially prevents any interaction in the behavior of the prey and predator. We argue that using live stimuli allows for the most natural behavior from prey individuals. Although we did not quantify stimulus fish behavior in this experiment, sunfish and killifish predators both attempted to attack focal fish through the glass during several of the trials. Further, we attempted to control as many variables as possible using all predators of similar size and motivational states (i.e., time since last feeding), and by pairing visual exposure to the predator with standardized chemical cues, which were identical within each treatment. With that said, our sample size should have been large enough to detect significant results even with more variation due to differences in predator behavior.

We propose that our focal individuals did not recognize Gulf killifish as a predator because it is dissimilar in morphology, behavior, and chemical cues from the native predators with which Largespring mosquitofish share an evolutionary history. Previous studies have found that prey species are more likely to have fixed (innate) antipredator responses, and respond to a novel predator if the prey has evolved with a history of multiple predators (Blumstein 2006; Wohlfahrt

et al. 2006). Although Largespring mosquitofish encounter many different species of predatory sunfish (centrarchids) in its native spring habitats, these closely related species represent the same predator archetype, are similar to each other in morphology and behavior, and may even produce molecularly similar chemical cues (Cox & Lima 2006; Ferrari et al. 2007; Davis et al. 2012). Largespring mosquitofish also frequently encounter largemouth bass (*Micropterus salmoides*), which contrast with sunfish in morphology and in some aspects of behavior, but they are still considered ambush predators. In contrast, our observations of Gulf killifish in the laboratory and in introduced wild populations indicate that these relatively small (maximum 18 cm SL), highly active, shoaling fish represent a distinctly different predator archetype from any of the native, comparatively solitary, ambush predators of mosquitofish. Thus, despite the multiple centrarchid predators sympatric with the Largespring mosquitofish, the allopatric Gulf killifish may be too dissimilar from native mosquitofish predators to allow recognition through generalization.

Fish prey species often avoid predators by changing position in the water column. However, Largespring mosquitofish did not show significant changes in their vertical position in the water column for any treatments. Smith & Belk (2001) showed that prey changes in water column use depend on both hunger levels and previous diet of the predator. In the present study, we fed predators commercial pellet food preceding the experiment to obtain kairomones without conspecific diet cues, but using solely kairomones of a predator without conspecific diet cues would likely lower the perceived risk for prey (Smith & Belk 2001). Prey can accurately assess risk level of native predators through a combination of chemical and visual cues that indicates the motivational state of the predator and modify their level of antipredator response accordingly (Licht 1989; Smith & Belk 2001; Chivers & Mirza 2001). Our use of stimulus fish deprived of food for only 16–21 h prior to testing, and chemical cues of predators without accompanying conspecific diet cues could have contributed to the lack of response in water column position. It is possible and at times adaptive for fish to innately respond to predator cues before a conspecific has been attacked, so we argue that kairomones alone could have been sufficient to elicit antipredator behavior. However, it may be Largespring mosquitofish rely instead on the ability to learn from associating conspecific alarm cues with new predators, which could have more adaptive value in environments with frequently varying predator regimes.

Prey showed no cortisol response to any of the treatments, even when responding to the native predator. Feeding our predators on a diet lacking conspecific cues of our focal species could contribute to the lack of hormonal response recorded in our focal fish, because prey in some species show different levels of stress response to predators fed conspecific vs. heterospecific diets (Fraker et al. 2009). The lack of immediate cortisol response to predator cues in our results mirrors the results found by Fischer et al. (2014) where *P. reticulata* did not differ in their acute cortisol response to the chemical cues of fish predators. Another hypothesis for the lack of a cortisol response is that the San Marcos Spring population of Largespring mosquitofish used in this study may experience high predation in the wild, leading to lower stress responses to encountering a predator (Archard et al. 2012). The lack of a physiological response to novel predators could also indicate that they do not recognize them as predators, as found by Anson & Dickman (2013) with a marsupial, *Pseudocheirus peregrinus*. Alternatively, Largespring mosquitofish may have already been stressed from the handling and collection of pre-stimulus hormone, and thus could not mount a further stress response following the stimulus (Cyr & Romero 2009). We acknowledge, there is some uncertainty in our assessment of hormone levels in this study as we have not showed that plasma and water-born hormone levels collected with the beaker method are correlated for this species. However, plasma levels of hormones, collected without the potential stress of confinement in beakers, were correlated with water-born cortisol levels using the beaker method for another small poeciliid, *Poecilia latipinna* (Gabor & Contreras 2012). Gabor & Contreras (2012) also did not find a significant effect of repeated handling across days or need for habitation for *Poecilia latipinna*. Similarly, there was no habituation to the collection method in the present study as pre-stimulus hormone levels did not significantly differ across the 3 d of testing. Further, there was a high variance in responses, indicating that some but not all individuals did increase their cortisol levels following the trials, suggesting they were not maximally stressed by pre-stimulus hormone collection (Fig. 3).

We also did not find a correlation between behavioral (horizontal or vertical response) and hormonal responses of prey, in contrast to several previous studies on hormones and behavior (Thaker et al. 2009; Hossie et al. 2010). Further, a meta-analysis of glucocorticoid responses to various stressors found a large

amount of variation in the direction of responses among empirical studies (Dickens & Romero 2013). Moreover, Barton (2002) proposed that in fish, the cortisol response may be a tertiary response to the chemical cues of a predator whereas the behavioral responses are direct response to those cues, resulting in no relationship between stress hormones and behavior. Our results add support to the hypothesis that antipredator behavior may not always be paired with a hormonal stress response. Further research is needed to explore the role of cortisol in predator–prey interactions.

Our results indicate that Largespring mosquitofish did not show the same response to novel predators as to native predators, suggesting they may not recognize Gulf killifish as predators. Impacts of invasive predators can be extremely damaging, especially when prey are unable to respond accurately to the novel predator (Goldschmidt et al. 1993; Vermeij 1994; Sih et al. 2010). Furthermore, novel predators can have higher consumptive impacts compared to familiar native predators (Sih et al. 2010). Our findings suggest that further expansion of the introduced Gulf killifish population could have detrimental impacts on naïve inland species. Largespring mosquitofish and its endangered congener *Gambusia nobilis* are endemic species limited to very small ranges in central Texas, so the expansion of an unrecognized novel predator into any of these habitats could quickly threaten the persistence of these species. Further studies should examine the coping ability of other freshwater prey species in response to this novel predator, as well as continue to monitor the introduced populations of Gulf killifish in central Texas.

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