

Activity Level and Predation Risk in the Least Killifish, *Heterandria formosa*

Samantha T. Levell¹ and Joseph Travis²

While organisms are capable of detecting predators via chemical cues, how well these detection abilities are matched to different historical predation regimes remains poorly understood. The Least Killifish, *Heterandria formosa*, is a small live-bearing fish whose predominant predators differ among habitats. We performed two experiments to see how *H. formosa* from different populations respond to a chemical cue from either a familiar predator (abundant in that population's habitat) or novel predator (absent from its habitat but abundant in another habitat inhabited by a different population of *H. formosa*). Our first experiment compared fish from two populations exposed to a familiar predator, a novel predator, and water from each habitat. Fish from the population with lower historical predation risk were more active regardless of treatment but were especially active when cues from a novel predator were offered. Our second experiment compared fish from four populations exposed to either a familiar or novel predator at the same time. Large fish were more active than small fish and, as in the first experiment, fish from the population with the lowest historical predation risk were more active regardless of the identity of the predator. These results suggest that predator recognition is not specific to individual populations but that historical levels of predation risk have selected for different characteristic levels of activity in different populations.

PREDATORS shape the evolution of many characteristics of their prey, including coloration (Godin and McDonough, 2003; Husak et al., 2006; Gordon et al., 2011), morphology (McPeck, 1996; Spitze and Sadler, 1996; Langerhans et al., 2005; Heinen-Kay and Langerhans, 2013), and life history (Endler, 1992; Godin, 1997; Richardson et al., 2006; Reznick et al., 2012; Agrawal et al., 2013). When predator regimes vary among populations, populations often diverge in these traits (Godin, 1997; Richardson et al., 2006; Reznick et al., 2012) but also should be expected to diverge in their abilities to recognize predators (Seghers, 1974a, 1974b; Cressler et al., 2010; Creel, 2011).

Organisms use a variety of sensory cues to detect predators in their attempts to avoid predation (Sih, 1986; Templeton and Shriver, 2004; Mortensen and Richardson, 2008; Ferrari et al., 2010). Visual cues are frequently used, especially in terrestrial environments (Apfelbach et al., 2015). In aquatic environments, where chemical signals are readily transmitted, chemical cues play a significant role, especially in fishes (Kotrschal, 1991, 2000; Chivers et al., 2013). Chemosensory detection has been observed in a variety of fishes (Lehtiniemi, 2005; Dixon et al., 2012; Nelson et al., 2013; Mitchell and McCormick, 2013; Atherton and McCormick, 2015). Chemical detection of predation risk can be induced through conditioning, either through detection of prey alarm cues or when the predator has been fed prey conspecifics (Giaquinto and Hoffmann, 2010). There is also evidence that prey can detect predators based solely on kairomone cues even when the predator has not been consuming conspecific prey (Schoeppner and Relyea, 2009; Sih et al., 2010).

Both conditioning and kairomone cues have been implicated in the responses of prey to novel predators but with mixed results. Blake et al. (2015) showed that mosquitofish responded to chemical cues from a familiar predator but not a novel predator when both predators were fed the same diet of fish pellets. Alternatively, prey can exhibit conditioned responses to a novel predator if that novel predator has been fed conspecifics (Mathis et al., 1995; Magurran et al., 1996; Rosella et al., 2013).

These contrasting results raise the question of how specifically prey will respond to predators. If most prey

responses are based on conditioning, then prey will respond to novel predators once those predators have killed and consumed conspecifics. On the other hand, we might expect that prey with a long ecological and evolutionary history of coexisting with a particular predator would be under selection to evolve the ability to detect a threat from that predator. Prey should respond to the potential threat even if the predator had not consumed conspecifics recently. There are few tests of this hypothesis that employ prey populations with known histories of co-occurrence with different predator species.

The Least Killifish, *Heterandria formosa*, offers an excellent opportunity to test the specificity of predator detection based on long-term ecological history. The Least Killifish is a small, sexually dimorphic poeciliid found throughout the Lower Coastal Plain of the SE US in the shallow littoral zones of water bodies ranging from rivers to ponds (Baer, 1998; Bagley et al., 2013). Within north Florida, populations occupy habitats along a continuum of historical predation intensity (Leips and Travis, 1999; Richardson et al., 2006; MacRae and Travis, 2014). In addition, the dominant predators differ among populations; the Pirate Perch, *Aphredoderus sayanus*, is the common predator in headwaters of lotic, freshwater springs, whereas the Warmouth Sunfish, *Lepomis gulosus*, is the common predator in lentic, soft water lakes (Leips and Travis, 1999; MacRae and Travis, 2014). Each predator is rare in the habitat in which the other is common, and they are quite distantly related.

If prey evolve to detect a threat from their most commonly encountered predator, then we would expect individual Least Killifish to respond differently to each of those predator species, depending on their population of origin. If conditioning is necessary for predator detection, then we would expect individual Least Killifish to respond similarly to both predators if they have been consuming conspecific prey. The distant phylogenetic relationship of the two predators makes it unlikely that, if prey respond similarly to both predators, that they are responding to a common cue based on phylogenetic affinity.

In previous lab trials, *H. formosa* decreased activity and mating attempts in the presence of a predator with which

¹ Department of Biology, University of California, Riverside, 900 University Ave., Riverside, California 92521; Email: samantha.levell@email.ucr.edu. Send reprint requests to this address.

² Department of Biological Science, Florida State University, 600 W. College Ave., Tallahassee, Florida 32306; Email: travis@bio.fsu.edu. Submitted: 23 October 2017. Accepted: 2 July 2018. Associate Editor: C. Beachy.

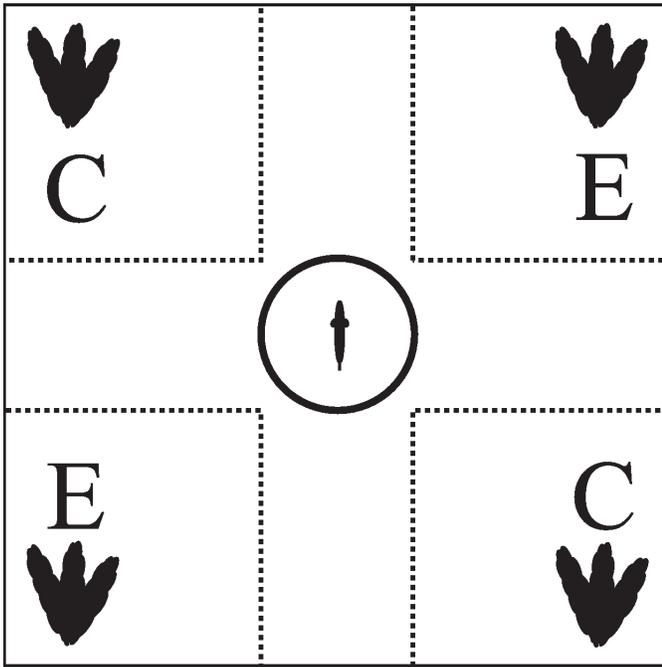


Fig. 1. Diagram of the experimental arena. A fish was placed in a PVC tube in the center of the trial arena and was released when water began dripping into each of the four quadrants. Experimental water (E) was dripped into randomly selected diagonal quadrants (bottom left and upper right in this diagram), while control water (C) was dripped into the other two diagonal quadrants. Plants were placed in all four quadrants and attached to silicone tubing that dripped water into the arena throughout the trial. The unlabeled area represents a “neutral” zone, used for scoring crosses between sections of the arena.

they co-occur (E. Culbreth, unpubl. data). By contrast, Porter-Whitaker et al. (2012) found that *H. formosa* increased their activity level in response to a combination of two invasive cichlid predators but did not alter their activity level from no-predator treatments when either cichlid species was present alone.

We examined four genetically distinct populations of *H. formosa* for their responses to these two predators. Warmouth Sunfish are the predominant predators in two pond populations, Moore Pond (ML) and Trout Pond (TP) and Pirate Perch are the predominant predators in two spring populations, Wacissa River (WR) and McBride Slough (MS) (Leips and Travis, 1999; Richardson et al., 2006; MacRae and Travis, 2014). From these patterns, we predicted that *H. formosa* would be more likely to respond to familiar than to naïve predators and that larger individuals would be less sensitive to perceived predation risk than smaller individuals because larger individuals are less likely to be consumed by predators (Richardson et al., 2006).

MATERIALS AND METHODS

Collection and husbandry.—Ten male and ten female *H. formosa* were collected from each of four populations near Tallahassee in the fall and spring, Trout Pond (TP), Moore Lake (ML), Wacissa River (WR), and McBride Slough (MS). The predators used in this experiment, Warmouth Sunfish and Pirate Perch were collected from ML and WR, respectively. *Heterandria formosa* were housed in 19 L (40 × 25 × 28 cm l*w*h) aquaria. Each fish was only tested once, to avoid confounding results with an individual’s past experience. Predators were housed in 38 L (36 × 28 × 22 cm l*w*h)

aquaria. Individuals were kept in familiar water; *H. formosa* from TP or ML and Warmouth Sunfish were housed in pond water, while *H. formosa* from MS or WR and Pirate Perch were housed in spring water. All aquaria were fitted with sponge filters and given ample artificial vegetation. Twenty percent of the water in each aquarium was changed weekly, and water quality (pH, ammonia, nitrate, and nitrite levels) was also assessed weekly. Detritus was removed daily. *Heterandria formosa* were fed 10 mg/fish/day of ground TetraMin®. Predators were fed *H. formosa*, frozen brine, or mysid shrimp daily. The temperature in the lab was kept at 78°F with a consistent 14/10-hour light-dark period.

Experimental design.—To test activity-based responses to predator chemical cues, we designed our treatments according to conditions experienced at each population origin of *H. formosa*. In addition to population, fish size in standard length (SL) and sex were also recorded as potential factors influencing activity level. Experiments were filmed using a Kodak PlaySport camera over a plastic tub measuring 44 cm² that was painted dark green on all sides. The tub was gridded on the bottom, and 200 mL of water was dripped into all four quadrants (to be referred to as quadrants) throughout each 180-second trial. The length of the trial was determined in preliminary observations by the time it took dye to intermingle with a neighboring section. Each quadrant had an artificial plant. Experimental water (E) was dripped in two diagonal quadrants while control water (C) was dripped into the other two quadrants (Fig. 1).

We conducted two separate experiments using different sets of wild-caught fish. Because we were interested in whether individuals could detect predators based on long-term ecological co-occurrence, we fed syntopic *H. formosa* to all predators. This ensured that any differential responses were based on perceived predator cues. If *H. formosa* fail to respond differentially to predators based on long-time familiarity, this could be because they respond only through conditioning by prey cues or because they detect all piscine predators via some common cue. In either case, we will reject our hypothesis.

Our experiment was designed to assay three measures of potential predator response. The simplest measure is whether prey avoid regions of the arena where the chemical cue of a predator was being introduced. The other measures are assays of activity level. A very general response of fish to visually hunting predators is to freeze or reduce activity levels substantially to reduce the probability of being detected by the predator (Savino and Stein, 1989; Lehtiniemi, 2005). We measured both the proportion of observation time in which the individual was moving (activity time) and the extent of its movement across regions (crossover movements). These measures allow us to distinguish an individual who was active but only in a region without a predator cue from an individual who was active and moving among regions in apparent obliviousness to all cues. All trials were completed between 1000 and 1200 hr in the months of September and November 2013. During daylight savings time (November–March), the trials only took place during 1000 and 1100 hr to account for the time change. Each trial lasted a total of 15 minutes, including a refractory period, the trial period, and post-trial observations. Tubs were sterilized with ethanol between trials to avoid any cue contamination.

Preparation of the predator chemical cue.—The day before each trial, a predatory fish was removed from its tank and

moved to a 9.5 L tank filled with approximately 3.7 L of either pond (for Warmouth Sunfish) or spring (for Pirate Perch) water. We “soaked” the individual overnight, moved the fish back to the stock tank the next day, and used this water the same day in trials.

Experiment 1.—Two of the four populations (MS and WR) were used in the spring (May 2014) trials to test whether fish from a high predation risk population (MS) and a low predation risk population (WR) change their activity patterns when they receive a predator scent. We selected a fish for each trial haphazardly, so individuals varied in size across trials. We also drew males and females at random because we did not expect the genders to differ in responsiveness. Males are just as likely as females to be preyed upon by *A. sayanus* (Richardson et al., 2006), and although they are less likely than females to be preyed upon by *L. gulosus*, their risk is still substantial (Richardson et al., 2006). Despite being smaller in SL, males mature at a significantly later age than females (Hale and Travis, 2015) so are unlikely to experience a lower cumulative risk because of their life history. The experiment had four treatment levels: water in which Warmouth Sunfish had been housed, water in which Pirate Perch had been housed, spring water in which no predator had been housed, and pond water in which no predator had been housed. When water from either predator was used as the stimulus, that water was dripped into two diagonally opposite quadrants, and water from the habitat (spring or pond) to which the predator was native but in which no predator had been housed was dripped into the other two quadrants. When water from the habitat was used as the stimulus, it was dripped into all four quadrants to gauge fish activity without a predator cue.

Experiment 2.—These trials were performed to test population-specific responses to each of the two predator cues. All four (MS, WR, TP, ML) populations were used in the Fall (November–December 2013) trials, but the fish were not the same individuals as those used in the May 2014 trials. Water was dripped into each of the four quadrants of the experimental arena in the same way as in Experiment 1, but Pirate Perch and Warmouth Sunfish were the only treatments used in this set of trials.

Behavioral data.—From the videos, we recorded movement data in two ways, creating the categories “activity level” and “activity patterns.” Activity level refers to the proportion of time the fish was active or inactive during a trial. Activity patterns refer to where the fish moved throughout the trial, such as crossing from one quadrant into another. The following data were recorded: proportion of time spent in the predator quadrants, proportion of time spent active, and number of crossovers between quadrants.

Statistical analysis.—For Experiment 1, we performed two sets of analyses. For proportion of time active and for the number of crossovers between regions, we analyzed all four treatments together (two predator cue and two water treatments). For proportion of time spent in the predator quadrants, we analyzed only the two predation treatments because habitat water was dripped into all four quadrants to measure activity levels in the absence of any predator cue.

We used a logit transformation (Trexler and Travis, 1993) to normalize the proportional data (proportion time active or proportional time in the predator quadrat). We converted

size data into discrete categories of “large” and “small,” with the small to large cutoff at 18 mm SL for females, and 11 mm SL for males. Females are larger than males and thus absolute size is confounded with sex. This confounding reduces substantially the statistical power to distinguish effects of size from those of gender. By creating categories with different absolute bounds for each gender, our size classes are relative measures with the same interpretation for each gender. For example, a male with SL of 14 mm is large for a male but small for a female. This classification reduces the confounding of size and gender and enhances our statistical power to detect an effect of each predictor in the presence of the other.

We counted the number of times an individual crossed between adjacent quadrants as a measure of the areal extent of that individual's activity level and converted those data for analysis via a square root transform. We analyzed data using a general linear model with three categorical predictors (water treatment, population, size class), first fitting a full model (all main effects, all two-way interactions, and the three-way interactions) and then, working backwards in stepwise fashion, deleting any non-significant effect whose *F*-value was less than 1.0. This criterion is conservative in that it discards only predictors with effect sizes that appear to be very small. For example, a typical criterion in a stepwise model selection process discards a predictor from the model if its Type I probability exceeds 0.15, a level that can cause predictors to be discarded even when their potential effect sizes are substantial. We report *F*-values derived from Type III sums of squares from the final model and present least squares means on the scale of the original data for graphical illustration of results.

Our prediction about the specificity of predator responses is tested in each experiment by the term for the interaction of treatment and population. Our prediction about the effects of size is tested similarly by the term for the interaction of size and treatment. If these interaction terms are not significant, then our predictions fail.

RESULTS

Experiment 1.—Fish responses were evident only in activity patterns and predominantly based on their population identity. Fish from WR (historically lower predation risk) spent a greater proportion of their time being active than fish from MS (Fig. 2A: $F_{1,36} = 7.76$, $P = 0.02$). There was no main effect of predator identity and water type on the proportion of time being active ($F_{3,36} = 0.35$). While larger fish were more active than smaller fish, this difference was small and not significant ($F_{1,36} = 0.41$). No interactions were even close to the threshold of significance, although both the treatment by population and treatment by size interactions had *F*-values greater than 1 ($F_{3,36} = 1.21$ and 1.68, respectively).

There was a striking interaction between population and treatment in the rates at which fish crossed between quadrants of the test arena (Fig. 3A: $F_{3,36} = 3.69$, $P = 0.02$). In the presence of Warmouth Sunfish, individuals from MS decreased their crossover rates substantially from those in the other three treatments, while fish in WR increased their rates dramatically. The net effect was that fish from WR crossed between quadrants over seven times more often than did fish from MS. Tukey's method for multiple comparisons indicated that this contrast between populations in the presence of Warmouth Sunfish drove the significance of the interaction term (95% confidence interval for this pairwise contrast:

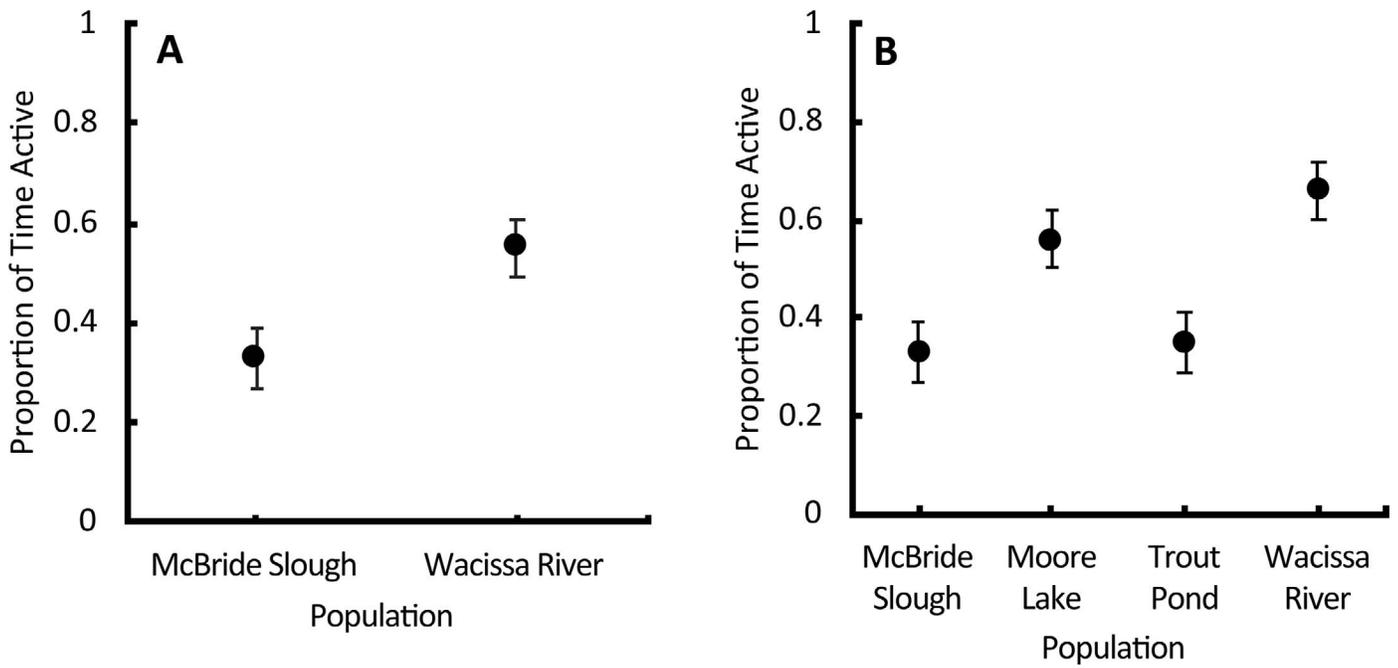


Fig. 2. (A) Least squared means plus or minus one standard error of the proportion of time fish were active during a trial in Experiment 1 as a function of population, regardless of treatment, and (B) least squared means plus or minus one standard error of the proportion of time fish were active during a trial in Experiment 2 as a function of population, regardless of treatment.

–7.43 to –0.46). There was no significant main effect of size ($F_{1,36} = 0.05$) and no significant interaction between size and treatment ($F_{3,36} = 2.20$).

In the predation trials, fish allocated their time among quadrants of the arena randomly, neither avoiding regions in which water from predators was being added nor staying in regions where control habitat water was being added. There were no significant effects of population ($F_{1,20} = 0.21$), treatment ($F_{1,20} = 0.57$), or their interaction ($F_{1,20} = 0.17$) on the proportion of time spent in the treatment regions (as opposed to the control regions). We were unable to include fish size in this model because there were not enough “large” fish in each treatment type.

Experiment 2.—As in Experiment 1, fish responses were evident only in activity patterns and, in this experiment, entirely based on their population identity. Fish from different populations spent different proportions of their time being active, with fish from WR again being the most active and fish from MS and TP being the least active (Fig. 2B: $F_{3,83} = 6.58$, $P < 0.001$). The interaction between population and treatment was not retained in the final model because its F -value was less than one regardless of which other terms were in the model. Larger fish spent a greater proportion of their time active than smaller fish ($F_{1,83} = 4.65$, $P < 0.03$), but the interaction between size and treatment was not retained in the final model.

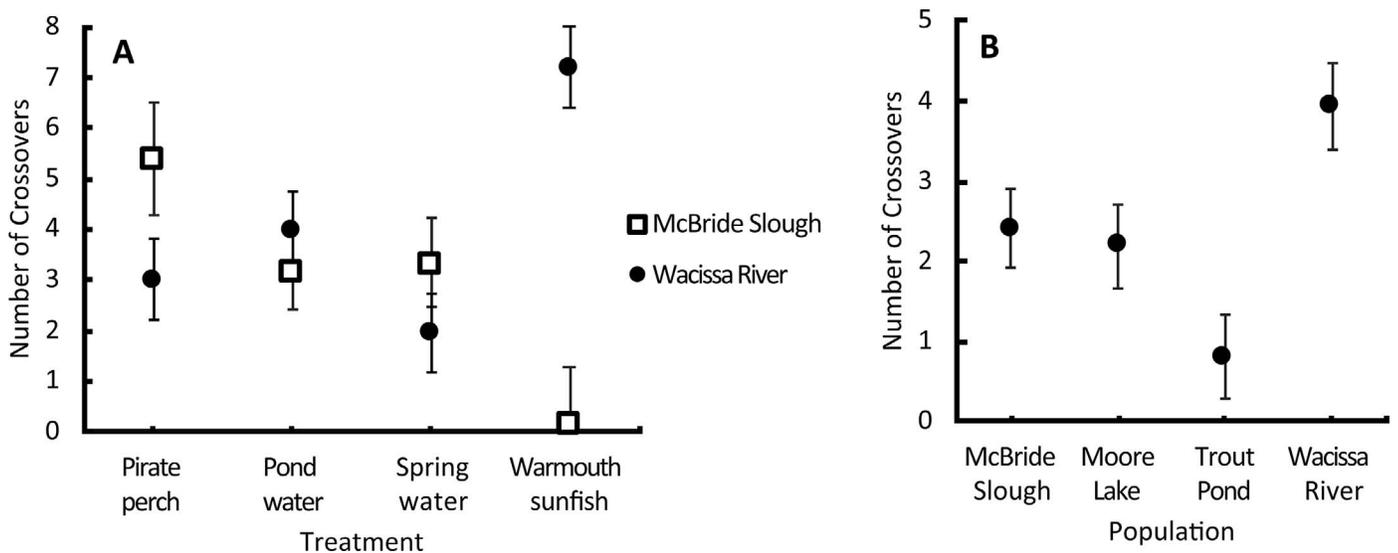


Fig. 3. (A) Least squared means plus or minus one standard error of the number of crossovers between quadrants by fish during a trial in Experiment 1 as a function of water treatment and population, and (B) least squared means plus or minus one standard error of the number of crossovers between quadrants by fish during a trial in Experiment 2 as a function of population.

Fish from WR crossed between quadrants more often than fish from any other population (Fig. 3B: $F_{3,80} = 5.76$, $P < 0.002$). Large fish spent a greater proportion of their time being active than small fish ($F_{1,83} = 4.64$, $P < 0.03$) and were more likely to cross between quadrants than small fish, but this latter difference was not significant ($F_{1,80} = 3.10$, $P = 0.08$). Predator identity did not influence activity rates or movement between quadrants, and the only interaction retained in the model was the non-significant one between treatment and population ($F_{3,80} = 1.57$).

As in Experiment 1, fish allocated their time among quadrants randomly. There was no effect of predator identity on where a fish spent its time ($F_{1,80} = 0.72$), indicating that fish were not distinguishing between familiar and novel predators or even responding to predators at all by avoiding quadrants of the arena in which chemical cues from those predators were present. On average, fish from different locations allocated their time similarly ($F_{3,80} = 0.72$). There was no interaction between predation treatment and location ($F_{3,80} = 1.12$) and no interaction between size and predator treatment ($F_{1,80} = 2.04$).

DISCUSSION

We found no evidence to support our initial predictions. The only significant interaction between treatment and population was in Experiment 1, in which the presence of Warmouth Sunfish was associated with greater movement by WR fish and less movement by MS fish. Unfortunately for our prediction, this predator is uncommon in both populations, so it is not clear how to interpret this result. On the whole, fish from WR spent more time active and moved between quadrants more than fish from the other populations, and larger fish were more active than smaller fish.

Our experiments had little power to detect a weak interaction between predation treatments and population but ample power to detect a strong interaction. In Experiment 1, the largest pairwise difference among the four treatment combinations was 0.77 on the logit scale. This corresponds to an untransformed difference between 0.33 (WR fish exposed to Warmouth Sunfish) and 0.18 (MS fish exposed to Pirate Perch). Our design had little power to detect an effect of this magnitude (~ 0.25), when fish from both populations spent a minority of time in the predator quadrant. However, our design had a power of at least 0.70 to detect a difference of at least 2 on the logit scale, or a difference in proportions of at least 0.38. In other words, had one population spent, say, 0.40 of its time in the predator quadrant and the other, say, 0.78 of its time there, we were likely to have detected a difference of that magnitude. For Experiment 2, the largest pairwise difference in the interaction of predation treatment and location was equal to 2.66 on the logit scale (equivalent to a difference in raw proportions between 0.46 and 0.06). Our experiment had a power of ~ 0.75 to detect an effect of this size. Both experiments had ample power to detect differences in proportions of at least 0.40 so had one population spent a distinct minority of its time in the predator quadrant and another a distinct majority of its time, we could have detected that difference. We had little power to detect more subtle differences.

The lack of evidence for time spent in the predator quadrant of the experimental arena based on treatment could be due to a necessary coupling of visual and chemical cues (Brown et al., 1997). A recent study on the cichlid

Neolamprologus pulcher paired visual and chemical cues, and the strongest reaction was elicited when the cues were combined (Fischer et al., 2017). Other poeciliids respond to visual predator cues (e.g., Mukherjee et al., 2014). Male *H. formosa* change their pattern of mating activity in response to visual cues from a predator and the simulation of a predator attack (E. Humphrey, pers. comm.) so, because predators are always present in the habitat, fish may not respond except when they have perceived an attack to be imminent. However, *H. formosa* increase activity level in response to a combination of invasive predators, but the fish in the study were from vastly different habitats with very different piscivorous predators (Porter-Whitaker et al., 2012). In Experiment 1, neither WR or MS fish responded to the presence of Pirate Perch despite it being the predominant predator in their habitats. This may be because they are accustomed to its presence or because, as reported by Resetarits and Binckley (2013), Pirate Perch have evolved chemical camouflage and are undetectable by *H. formosa*.

The repeatable patterns in activity level are striking. Among the populations, WR exhibited the highest activity levels by far in both experiments, regardless of treatment. Historically, WR is a population with high density and low predation risk. All the other three populations (MS, TP, ML) have higher predation risks and have population densities at least two orders of magnitude lower than seen in WR (MacRae and Travis, 2014). The habitat at the Wacissa River is characterized by high levels of thick vegetation cover, primarily the introduced *Hydrilla verticillata*, which deters predation by Pirate Perch (Richardson et al., 2006). It is possible that high levels of cover, very low predator densities, and high levels of conspecific density have imposed selection on WR fish to be much more active. At TP and ML, there is much less vegetation cover, it fluctuates more, and the predominant predator at these locations, Warmouth Sunfish, is relatively undeterred even by high levels of cover. Fish in these locations may have been selected for lower activity levels. The lower cover levels at MS may have the same force of selection, even though a different predator is predominant.

The significant differences we found in activity patterns of fish belonging to different size classes indicates that size plays a role in response to predator cues. *Heterandria formosa* have an expected lifespan of about 140 days, and therefore rarely survive through two breeding seasons. Size and age are correlated traits (J. Travis, unpubl. data), so we can infer that larger, older fish are exhibiting a reduced response to predator cues. The most common predators of *H. formosa* are size-limited (Richardson et al., 2006), and so individuals may be adjusting their behavior as they grow in response to decreased vulnerability to predators. Ontogenetic shifts in prey response exist in other organisms (Kiesecker et al., 1996; Atherton and McCormick, 2015).

Given that *H. formosa* respond to visual cues from a predator (E. Humphrey, pers. comm.), the failure of our predictions implies either that *H. formosa* are incapable of detecting a risk of predation via chemical cues or that they use conditioning as their principal cue. We cannot distinguish these alternatives purely from our results. The former interpretation is unlikely because it would make *H. formosa* virtually alone in this respect, given that other poeciliids readily perceive chemical cues from predators (McLennan and Ryan, 1997; Blake et al., 2015; Culumber, 2015). The latter interpretation seems more likely. This species has the smallest body size among all fishes in most of its habitats and

feeds very low in its trophic web (Aresco et al., 2015). In addition to the predominant predators we studied, it is preyed on in these areas by small spotted sunfish (*Lepomis punctatus*) and aeshnid dragonfly naiads so predation risk is a major feature of its life history. Populations of *H. formosa* in other parts of Florida have different predator community compositions that may influence activity level differently as well (Porter-Whitaker et al., 2012). If we had looked at other responses like movement to shelter or internal stress responses, we may have had different conclusions.

Finally, it may be that the observed response is shaped by historic predation pressure with populations experiencing low predation pressure evolving substantially higher activity rates. Given how important reduced activity has proven in eluding attack in fish and aquatic invertebrates (Savino and Stein, 1989; McPeck et al., 2001; Lehtiniemi, 2005; Chivers et al., 2013), the elevated activity in WR seems to fit this pattern. This is certainly a pattern in amphibians on a phylogenetic scale (Richardson, 2001), and future work can focus on whether it can be found in poeciliid fishes on either a phylogenetic or microevolutionary scale.

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