Anti-predator Behavior in Response to Conspecific Visual, Olfactory, and Damage Cues in Three-spined Sticklebacks

UNIVERSITY HONORS PROGRAM
UNIVERSITY of DAYTON

Honors Thesis

Claire VanMeter

Department: Biology

Advisor: Karolyn Hansen, Ph.D.

April 2024

Anti-predator Behavior in Response to Conspecific Visual, Olfactory, and Damage Cues in Three-spined Sticklebacks

Honors Thesis

Claire VanMeter

Department: Biology

Advisor: Karolyn Hansen, Ph.D.

April 2024

Abstract

Predation is a major source of mortality for prey, which creates a selective pressure to avoid predators whenever possible. By using alarm cues produced by conspecifics, individuals can be alerted of nearby predators without coming into direct contact with them. However, whether individuals can distinguish between different conspecific cues or whether some cues might indicate a more severe predation threat than others is not known. Studied was the three-spined stickleback's ability to distinguish between environmental cues by exposing conspecifics to four different treatments in a randomized order: 1) Control (control olfactory cues with an unexposed demonstrator), 2) Visual Only (control olfactory cues with an exposed demonstrator), 3) Visual and Stress (stress cues with an exposed demonstrator), and 4) Visual, Stress, and Damage (stress and damage cues with an exposed demonstrator). For five minutes before and after exposure to the cues, I watched for four key defensive behaviors: hiding in plants, hiding in the gravel at the bottom of the tank, thigmotaxis, and shoaling. Directly after each assay, the focal subject was placed in 200 ml of RO water in a 600 ml beaker for one hour to collect waterborne cortisol. Observer hiding behavior increased when observers were exposed to visual cues of stressed conspecifics paired with either olfactory cues of stressed conspecifics or conspecific stress and damage cues. Observers also shoaled less when exposed to visual cues paired with olfactory stress cues, but not with visual cues alone, or visual cues paired with stress and damage cues. Males shoaled more than females but hid less. No change in behavior was observed for demonstrators, regardless of what cues were added. Also, no differences in waterborne cortisol levels were measured for each of the treatments.

Acknowledgements

I would like to thank Dr. Jenn Hellmann and the Hellmann lab, the University of Dayton Biology department headed by Dr. Karolyn Hansen, and the University of Dayton Honors department.

University *of* Dayton

Table of Contents

Introduction

Predation is a major source of mortality for prey, so it is evolutionarily advantageous for prey to avoid predators without having to come into direct contact with them. This has selected for the evolution of different types of cues, which convey information to conspecifics about their experiences without having to contact the stressor directly. Individuals can use visual cues by observing the behavior of others. For example, zebrafish will behave as if a predator is present when a conspecific (a demonstrator fish) behaves defensively, even without direct visual confirmation of a predator being present (da Silva et al., 2019). Olfactory systems can also sense chemical cues which convey information about the environment. For aquatic organisms, this mechanism is key for successfully navigating, finding a mate, avoiding predation, and finding food. Some olfactory cues are released when individuals are stressed. For example, the Iowa darter is able to voluntarily release disturbance pheromones when exposed to a stressor to alert conspecifics of danger (Wisenden, Chivers & Smith, 1995). Other olfactory cues come from the damaged bodies of conspecifics. Brown et al. (2000) also found that fathead minnows show increased antipredator behavior when exposed to the chemicals that are released from conspecific skin when damaged, as it is a sign that a predator might be in the area. Most animals use a combination of these kinds of cues to communicate with both conspecifics and other organisms in their environment.

Conspecifics use these cues to gauge predation, yet it is unknown as to how conspecifics respond according to the severity of these cues. Stress cues indicate a conspecific is in potential danger, while damage cues alert that a conspecific has been harmed within close proximity to the individual. This creates a difference in potential perceived danger based on the information each cue provides. Performing antipredator behaviors is costly because they are both energetically intensive and reduce time for other activities. Using the minimum level of defensive behaviors needed to maintain safety so an organism's time and energy can be spent on foraging and reproduction rather than defense (Tollrian et al., 2015) is adaptive. If individuals can distinguish between the severity of a conspecific cue, they can alter their behavior accordingly and spend less time performing unnecessary defensive mechanisms. By exposing conspecifics to cues of varying degrees of severity, their ability to respond according to the severity of the cues can be measured.

Animals respond to stress both behaviorally and physiologically, with an increase in stress hormones in the presence of perceived danger. For this study, cortisol levels were measured as a sign of physiological stress. Cortisol provides a key function during times of stress by increasing blood sugar and blood pressure to give an organism the best chance of fighting or escaping. It also decreases sensitivity to pain and suppresses the immune system, which gives the individual the best chance of survival during short, high-danger periods (Ellis et al., 2012). Cortisol is a measurable stress indicator in fish, which has links to behavior and physiological changes. Increased cortisol levels indicate increased stress even when stress-induced behavior is not observed (Ellis et al., 2012). Dellinger et al. (2018) found that male sticklebacks use water-borne cortisol to assess the quality of a female based on her past experiences with predators. However, this distinction was only temporary as the response to female cues decreased over time.

Here, three-spined sticklebacks, *Gasterosteus aculeatus*, were used to understand how individuals alter their anti-predator behavior in response to visual and olfactory conspecific cues. Sticklebacks display a variety of well-studied anti-predator behaviors (Landeira-Dabarca et al., 2019), making them an ideal subject for analyzing how conspecifics respond to varying degrees of conspecific cues. A range of cue severity was created through the use of a demonstrator (who was in the line of sight of a predator while the observer remained predator-naive) and conspecific olfactory cues. These cues included conspecific stress cues, which are naturally secreted by conspecifics who perceive danger, and conspecific damage cues, which are the pieces of damaged conspecifics that enter the water when one is damaged or eaten. The range started with no negative cues (control), followed by only a visual cue, then visual and stress cues, and finally, visual, stress, and damage cues. The observer fish was observed both before and after the addition of conspecific cues for defensive behaviors, and waterborne cortisol was collected after the assay. Cortisol is naturally secreted under times of stress, so individuals under higher stress should secrete more than those with fewer stress-inducing cues (Ellis et al., 2012). I hypothesize that sticklebacks will be able to distinguish between the severity of conspecific cues and that individuals will react significantly more defensively in response to olfactory and visual cues than visual cues alone. Conspecifics will react the most defensively when in the presence of all three danger cues (visual, stress olfactory, and damage olfactory). I also hypothesize that as the degree of severity of the cues increases, secreted cortisol levels will also increase, resulting in very low levels for the control, and very high levels in the presence of all three danger cues.

Methods

Housing Conditions

Adult three-spined sticklebacks *(Gasterosteus aculeatus)* were collected from Putah Creek (CA, USA) in June 2021 and shipped to the University of Dayton (OH, USA). Prior to the experiment, they were housed on a recirculating system in groups of 10-12 per tank to mimic shoaling conditions in the wild. The fish were fed twice a day ad libitum with a mixture of previously frozen bloodworms *(Chironomus spp.)*, brine shrimp *(Artemia spp.)*, Mysis shrimp, and calanus. Individuals were housed in the lab in the absence of predators.

Generating Conspecific Cues

Focal subjects were given three different conspecific olfactory cues depending on the trial: control conspecific cues, stress conspecific cues, and conspecific damage cues. For the control conspecific stress cues, five sticklebacks were placed in a clean 10-gallon tank, where they sat for one hour, undisturbed. Then the fish were removed and the water was frozen in 400 ml aliquots until the date of the assay when it was needed. This exposed the focal fish to the scent of unstressed conspecifics, indicating that conspecifics are present but not in danger (Brown &

Godin, 1997). For the conspecific stress cues, a 6-inch clay model trout chased the fish for 90 seconds, and the sticklebacks remained in the tank for one hour. This caused the conspecifics to excrete stress hormones, which collected in the water (Brown & Godin, 1997). 400 ml of these cues were used for the Visual and Stress, and Visual, Stress, and Damage groups.

Conspecific damage cues were generated by removing the head and organs of adult sticklebacks, grinding the body into fine particles, and allowing the paste to sit in 75 ml of water per stickleback for five minutes. When the 5-minute period was over, the large particles were removed through a fine strainer, and the liquid was frozen until the date of the assay. This procedure is a modified version of the procedures done by Brown & Godin (1997), and Mathis & Smith (1993).

Procedure

During June-July of 2022, 40 adult sticklebacks were the focal subjects, with each stickleback going through four different treatments in a randomized order: 1) Control (control olfactory cues with an unexposed demonstrator), 2) Visual Only (control olfactory cues with an exposed demonstrator), 3) Visual and Stress (stress cues with an exposed demonstrator), and 4) Visual, Stress, and Damage (stress and damage cues with an exposed demonstrator). Each treatment group increases the severity of predation risk for the focal fish, creating different levels of potentially perceived danger.

Both the focal subject and demonstrator were gently caught from their home tanks and placed into a divided, 10-gallon tank for 20 minutes prior to the assay for acclimation. The demonstrator was another adult conspecific placed in the line of sight of the focal subject to provide conspecific visual cues to the focal subject. The tank was divided into two, uneven sections, with the focal section three times larger than the demonstrator section. A clear, plastic barrier separated the sections, which allowed the conspecifics to see each other but not smell each other. A 5-gallon tank was placed perpendicular to the 10-gallon, which contained the removable clay model trout. A barrier was placed between the two tanks during acclimation and the first part of the trial. The 10-gallon tank had blackout paper on select walls to ensure only the demonstrator, and not the focal subject, could see the model trout (Figure 1). After the 20-minute acclimation, both the demonstrator and the focal subject were observed for five minutes prior to the addition of any cues, recording four key behaviors that demonstrate stress in three-spined sticklebacks: sitting at the bottom in the gravel, hiding in the plants, thigmotaxis, and shoaling along the barrier (Landeira-Dabarca et al., 2019). Thigmotaxis is a stress behavior where individuals repeatedly swim directly into the walls of the tank. Shoaling is a grouping behavior that was marked by both the focal fish and the demonstrator swimming together within one inch of the barrier.

After the 5-minute observation period, the barrier was pulled to expose the 5-gallon tank (either empty or containing a model trout) while simultaneously adding olfactory cues to both the demonstrator and observer. If the demonstrator was predator-naïve (control treatment), there was no model trout in the central tank, and 105 ml of RO water was added to the tank. If the demonstrator was predator-exposed (other three treatments), the demonstrator was exposed to three types of predator cues: 1) visual predator cues (a model predator in the line of sight of only the demonstrator and not the focal subject), 2) 100 ml of olfactory predator cues and 3) conspecific damage cues. The olfactory predator cues were collected using a slightly modified version of Crane & Ferrari's (2014) method. A 50-70g trout was placed in a clean 37.9L tank, which was filled with clean tap water proportional to the size of the trout (500 ml per gram of fish). The trout was fed one juvenile stickleback daily for four days. After 96 hours, the trout was removed, and the water was collected in 200 ml aliquots.

At the same time the demonstrator received visual and olfactory cues of predation risk, the observer fish received olfactory cues. For the control and visual only treatments, 215 ml of RO was added. For the visual and stress treatment, 200 ml of conspecific stress cue and 15 ml of

RO water were added. The visual, stress, and damage treatment received 200 ml of conspecific stress cue and 15 ml of conspecific damage cues.

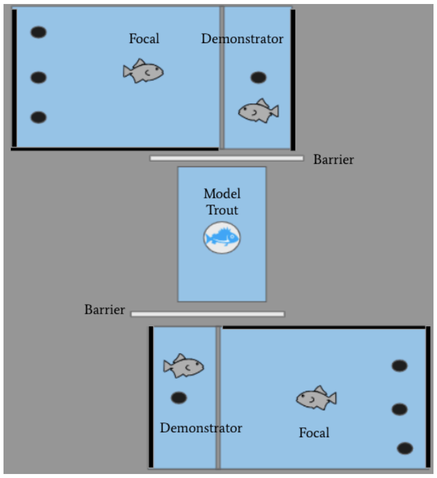


Figure 1. Overhead view of experimental tank set up. Black sides of the tank are covered, and gray sides are clear. The black dots indicate small fake plants where conspecifics can hide.

Waterborne Cortisol

This study investigates two stress responses in three-spined sticklebacks: anti-predator behavior and waterborne cortisol levels. The observed and recorded anti-predator behavior measures the immediate physical response to the stimuli, while waterborne cortisol records the chemical stress response experienced post-assay. The stress hormone is excreted through the gills, urine, and feces and accumulates in the water. The cortisol was collected immediately after each assay by placing the focal subject in a clean, 600 ml beaker with 200 ml of RO water, which was left to sit in a dark environment for one hour. After the one-hour period, the conspecific was removed and collected, and the water was frozen. The week prior to conducting assays, the focal subjects were placed in 600 ml beakers with 200 ml of RO water in a dark environment for one hour for five days. This acclimated the fish to the cortisol collection process and its enclosed space to ensure the stress hormones collected in the water were from the assay and not the enclosed environment.

To measure hormones, the samples were thawed at room temperature in clean 600 ml beakers. When completely thawed, the samples were poured through a funnel with grade 1 filter paper to remove any large organic material in the water. Then, the samples were vacuumed through a C18 solid phase extraction column, which had been primed with 4 ml of ethanol to clear impurities, followed by 4 ml of distilled water. When all 200 ml of the sample had been run through the column, the column was frozen for later extraction.

To extract hormones, the column was thawed and rinsed with 2 ml distilled water. Free (unconjugated) form of cortisol was eluted with 5 ml ethyl acetate in a test tube. These tubes were then put in an evaporator, set at 37 degrees Celsius, under a steady stream of nitrogen gas to ensure no oxidation occurs during the drying process. When the liquid was completely evaporated, the hormone was resuspended in 25 microliters of ethanol and 475 microliters of buffer. The resuspended mixture was then frozen for later use. The ELISA (Enzo Life Sciences, Farmingdale, NY, U.S.A.) kit was used to measure the cortisol levels in the final samples. The standard procedure from the kit was used, and the cortisol levels were measured using a plate reader, set to read 405 (units?). Cortisol levels were measured based on a standardized curve made from the pool, which consisted of 10 microliters of each sample. Samples that did not fall within the 20-80% range were removed from the data set (I forget what this meant totally)
For detailed methodology, reference Dellinger et al. (2018).

Results

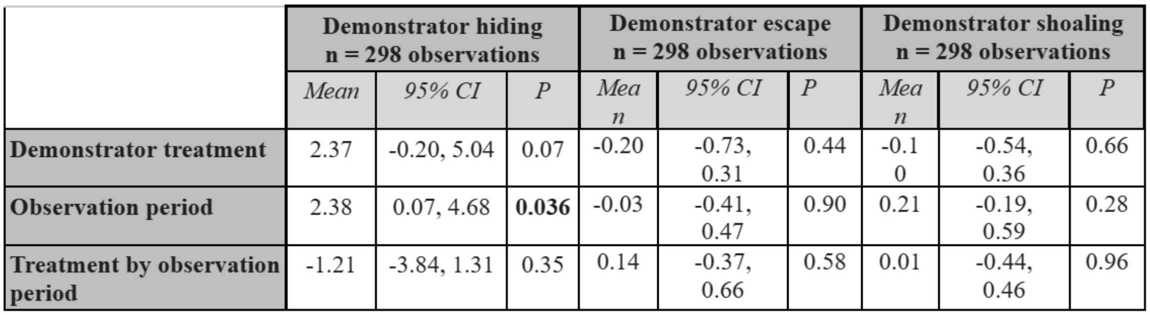
Statistical Analysis

We used the R package MCMCglmm (Hadfield, 2010) to analyze demonstrator and observer behavior (hiding in the gravel, shoaling, and thigmotaxis). Models with a weak prior on the variance (V=1, nu=0.002) were run, as were models for 200,000 iterations, with a burn-in of 3000 iterations and thin = 3. The demonstrator models included fixed effects of demonstrator treatment (control or predator-exposed), observation period (before or during predation exposure), and their interaction, and random effects of assay ID (because the model included two observations per assay: one before the predator exposure and one after). The observer models included fixed effects of observer treatment (control, visual only, visual/stress, or visual/stress/damage), observation period (before or during predation exposure), and their interaction, and a fixed effect of trial order (e.g., first versus fourth trial) and observer sex. We also included random effects of focal ID and assay ID.

Demonstrator behavior

Demonstrator behavior was measured before and after exposure to a combination of predator cues (visual cues of a model trout paired with olfactory cues of a trout and conspecific damage cues; control was an empty tank with RO water). No evidence that demonstrator hiding behavior, shoaling, or escape behavior varied due to predation exposure (Table 1) was found. Demonstrators spent more time hiding in the second observation period compared to the first observation period, regardless of treatment (Table 1).

Table 1: GLMM results for demonstrator behavior.



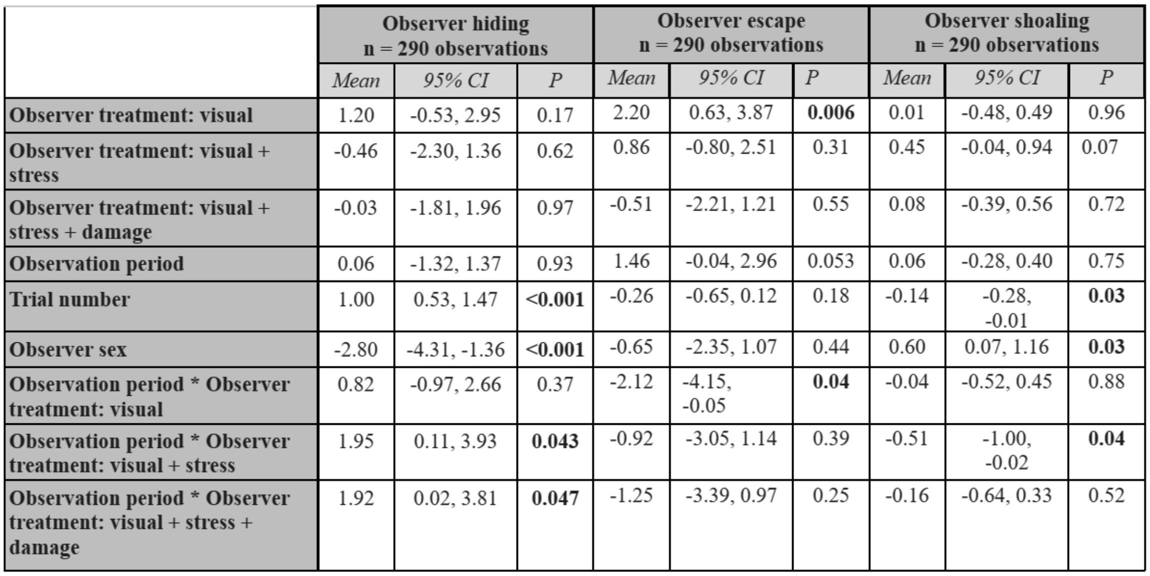
	Demonstrator hiding n = 298 observations			**Demonstrator escape n = 298 observations**			**Demonstrator shoaling n = 298 observations**		
	Mean	*95% CI*	*P*	*Mean*	*95% CI*	*P*	*Mean*	*95% CI*	*P*
Demonstrator treatment	2.37	-0.20, 5.04	0.07	-0.20	-0.73, 0.31	0.44	-0.10	-0.54, 0.36	0.66
Observation period	2.38	0.07, 4.68	**0.036**	-0.03	-0.41, 0.47	0.90	0.21	-0.19, 0.59	0.28
Treatment by observation period	-1.21	-3.84, 1.31	0.35	0.14	-0.37, 0.66	0.58	0.01	-0.44, 0.46	0.96

Observer behavior

Observer behavior was measured before and after exposure to visual and olfactory cues of a predator-exposed conspecific. Compared to the period before the conspecific cues were introduced, observer hiding behavior was not altered when observers were exposed to visual cues of stressed conspecifics alone. However, hiding behavior increased when observers were exposed to visual cues of stressed conspecifics paired with either olfactory cues of stressed conspecifics or conspecific stress and damage cues, (interaction of observation period and treatment; Table 2, Figure 2). Similarly, observers shoaled less with the demonstrator after they encountered visual cues paired with olfactory cues of stressed conspecifics, but not when they were given visual cues alone or visual cues with both stress and damage cues (Table 2, Figure 4). In contrast, observer escape behavior decreased when observers were exposed to visual cues of stressed conspecifics, but not when they were exposed to conspecific stress or damage cues (interaction of observation period and treatment; Table 2, Figure 3).

Males shoaled more than females, but hid less than females (Table 2). Observers in the first trial shoaled more and hid less compared to observers in their fourth trial, regardless of treatment (Table 2).

Table 2: GLMM results for observer behavior.



	Observer hiding n = 290 observations			**Observer escape n = 290 observations**			**Observer shoaling n = 290 observations**		
	Mean	*95% CI*	*P*	*Mean*	*95% CI*	*P*	*Mean*	*95% CI*	*P*
Observer treatment: visual	1.20	-0.53, 2.95	0.17	2.20	0.63, 3.87	**0.006**	0.01	-0.48, 0.49	0.96
Observer treatment: visual + stress	-0.46	-2.30, 1.36	0.62	0.86	-0.80, 2.51	0.31	0.45	-0.04, 0.94	0.07
Observer treatment: visual + stress + damage	-0.03	-1.81, 1.96	0.97	-0.51	-2.21, 1.21	0.55	0.08	-0.39, 0.56	0.72
Observation period	0.06	-1.32, 1.37	0.93	1.46	-0.04, 2.96	0.053	0.06	-0.28, 0.40	0.75
Trial number	1.00	0.53, 1.47	**<0.001**	-0.26	-0.65, 0.12	0.18	-0.14	-0.28, -0.01	**0.03**
Observer sex	-2.80	-4.31, -1.36	**<0.001**	-0.65	-2.35, 1.07	0.44	0.60	0.07, 1.16	**0.03**
Observation period * Observer treatment: visual	0.82	-0.97, 2.66	0.37	-2.12	-4.15, -0.05	**0.04**	-0.04	-0.52, 0.45	0.88
Observation period * Observer treatment: visual + stress	1.95	0.11, 3.93	**0.043**	-0.92	-3.05, 1.14	0.39	-0.51	-1.00, -0.02	**0.04**
Observation period * Observer treatment: visual + stress + damage	1.92	0.02, 3.81	**0.047**	-1.25	-3.39, 0.97	0.25	-0.16	-0.64, 0.33	0.52

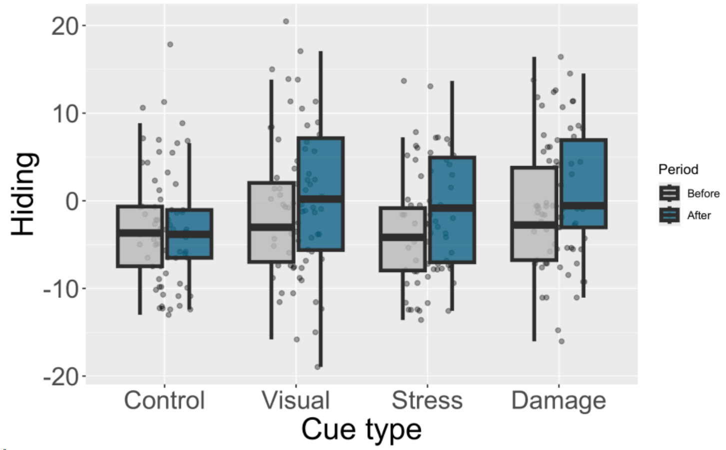


Figure 1. Hiding behavior before and after the addition of cues. Hiding increased under a combination of visual and stress, and visual, stress, and damage cues.

Figure 2. Escape behavior before and after the addition of cues. Escape behavior decreased under visual cues.

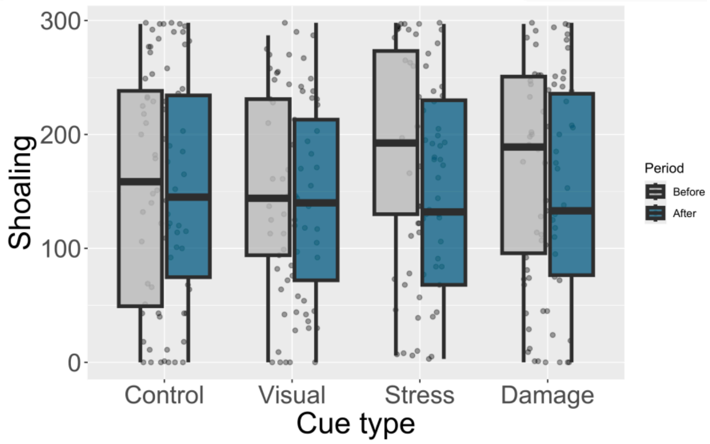


Figure 3. Shoaling behavior before and after the addition of cues. Shoaling decreased under a combination of visual and stress cues.

Cortisol

Water-borne cortisol was collected directly after each assay. There was no significant difference in cortisol levels for any treatment, regardless of trial order (Figure 4)

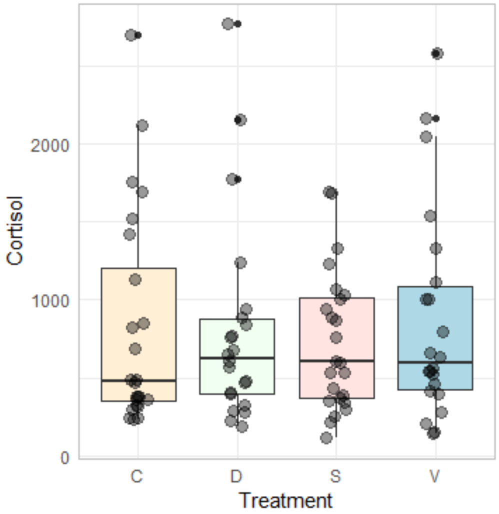


Figure 4. Cortisol levels by treatment collected after each assay. There was no recorded difference in cortisol levels based on treatment type.

Discussion

Behavioral assays were performed to determine how three spined sticklebacks behave in the presence of different types of conspecific alarm cues, including visual and olfactory cues. It was hypothesized that individuals would react with more defensive behaviors in response to a combination of cues compared to one or no cues. Cortisol levels were also predicted to increase more as the severity of conspecific cues increased. It was found that individuals do respond differently to different combinations of cues. Thigmotaxis decreased under the visual only treatment, while individuals who received olfactory cues shoaled less and hid more. However, cortisol levels did not change in response to any combination of cues. This demonstrates that individuals respond more defensively when olfactory cues are present. However, damage cues do not increase defensive behaviors relative to stress cues.

Observer Behavior

In the presence of visual stress cues only, thigmotaxis decreased, and hiding and shoaling behavior remained the same as control. While there was no significant change in demonstrator behavior measured, the difference in thigmotaxis behavior under the visual only treatment indicates that there was a difference in demonstrator behavior that was not measured with the recorded behaviors. However, these unrecorded behaviors were still not enough stimuli to warrant hiding or shoaling behavior changes in the focal fish. Thigmotaxis is a general stress response performed when individuals are anxious. Thigmotaxis should have increased with higher perceived predation risk, yet this was not the case. Thigmotaxis decreased in response visual cues only, and remained the same as control for higher risk potential treatments. The decrease under only visual cues could be due to a lack of olfactory cues to confirm the potential danger shown by the visual cues from the demonstrator. Olfactory stress and damage cues confirm danger, as those chemicals are only released under stressful conditions.

Under conspecific visual and olfactory stress cues, hiding increased, shoaling decreased, and thigmotaxis remained the same as control. Olfactory stress cues confirm that there is danger in the environment, but do not specify what type of danger is present. Olfactory stress cues are released under different types of stimuli, like predators, changing environments, and a lack of food resources (Ellis et al. 2012). This lack of information about the type of danger could result in individuals favoring hiding rather than shoaling, as a way to confirm their protection under an unknown threat.

Under a combination of all three types of cues (visual, stress, and damage), hiding increased, while shoaling and thigmotaxis remained the same as control. Conspecific damage cues indicate a conspecific has been damaged or eaten nearby. This confirms the presence of a predator through olfactory cues, so the observer should react as if faced with a predator. However, because the observer was not directly in the line of sight of the predator, they could prefer hiding as a way to stay out of the line of sight of the danger. I hypothesized that all defensive behaviors would increase in response to damage cues, yet this was not the case. Hiding could have been the preferred defensive behavior as the other recorded behaviors would put the observer in a better line of sight for the predator, as they involve moving towards the demonstrator.

Observers shoaled more and hid less in the first trial compared to the last, regardless of the order of the treatments. This could be due to individuals becoming acclimated to the assays and tank set up, where the observers could not reach the demonstrator because of the barrier. If individuals learned that the demonstrator was inaccessible in early trials, they would be less inclined to shoal for protection and would favor a different defensive mechanism like hiding. Dellinger et al. (2018), found that sticklebacks habituate to assays which involve repeated stressors, and decrease their cortisol production over time. This decrease in cortisol over time could be linked to a decrease in defensive behaviors as individuals acclimate to stressful assays. Individuals also received repetitive danger cues in the assay tank, and could have become more fearful as the trials went on, prompting them to hide more and shoal less.

Demonstrator Behavior

Demonstrator behavior did not change in the presence of any of the cues, yet there was still a difference in observer behavior under the visual only treatment. This indicates that there may be differences in demonstrator behavior which were not recorded during the assays. Demonstrators also could have had a lessened response to the predator-treatment due to the presence of a predator-naive conspecific, the observer. Similarly to how a stressed demonstrator can elicit stress behavior in an observer, the unexposed observer could elicit a calming effect on the demonstrator. Demonstrators could have interpreted the behavior of the observer, who was unexposed to the predator, as a sign of safety and lessened their defensive response. Crane et al

(2018), showed this in fish, where individuals decreased stress responses in the presence of calm conspecifics, but only when those calm individuals were repeatedly switched out. Because demonstrators were reused during the week they were assayed, this phenomenon could have occurred as they were repeatedly placed in a tank with different predator-naive individuals.

Cortisol

We expected to see an increase in cortisol production with the increase in the severity of cues, yet there was no recorded difference in cortisol production for all treatments. This outcome contradicts the findings of Ellis et al. (2012), which recorded increasing cortisol levels under stressful conditions. This could be due to a lack of statistical power as the sample size was only n=92 individuals. The lack of significant results could also be attributed to habituation, as individuals got used to the conditions of the assay over time. This would alter their cortisol production as they got used to the assay conditions. Individuals were habituated to the environment where the cortisol was collected to prevent an increase in stress when placed in the beaker for collection, yet they could have habituated to the assay during the week of trials.

Conclusion

Conspecifics do alter their behavior in response to different combinations of environmental cues, and react according to the amount of information provided by those cues. Olfactory cues are key in eliciting a defensive response, yet the severity of those cues does not influence the amount of defensive behavior. It seems that stress cues elicit the same degree of response as stress and damage cues together. These results indicate that a combination of cues allows individuals to gain the most information to make the appropriate behavioral response. This proves the importance of different types of danger cues in formulating behavioral responses. Going forward, future studies should consider what types of cues are being presented to individuals, and what type of information those cues give to conspecifics. It is key that enough cues are provided to give adequate information to individuals about danger levels so they can behave accordingly.

References

Brown, G. E., Adrian, J. C., Smyth, E., Leet, H., and Brennan, S. (2000). Ostariophysan alarm pheromones: Laboratory and field tests of the functional significance of nitrogen oxides. J. Chem. Ecol. 26, 139–154

Brown, G. E., Godin, J. J. (1997). Anti-predator responses to conspecific and heterspecific skin extracts by threespine sticklebacks: alarm pheromones revisited. *Behavior.* doi: 10.1163/156853997X00098

Crane, A. Ferrari, M. C. O. (2014). Minnows trust conspecifics more than themselves when faced with conflicting information about predation risk. *Animal Behavior, 10,* 184-190. https://doi.org/10.1016/j.anbehav.2014.12.002

da Silva et al., (2019) The complexation of steroid hormones into cyclodextrin alters the toxic effects on the biological parameters of zebrafish (*Danio rerio*). *Chemosphere, 214*, 330-340. https://doi.org/10.1016/j.chemosphere.2018.09.116

Dellinger, M., Zhang, W., Bell, A. B., Hellmann, J. K. (2018). Do male sticklebacks usevisual and/or olfactory cues to assess a potential mate's history with predation risk?. *Animal Behaviour, 145,* 151-159. https://doi.org/10.1016/j.anbehav.2018.09.015

Ellis, T., Yavuzcan, Y., Lopez-Olmeda, J., Spedicato, M. T., Tort, L, Overli, O., Martins, C. I.M., (2012). Cortisol and finfish welfare. *Fish Physiology and Biochemistry, 38,* 163-188. https://doi.org/10.1007/s10695-011-9568-y

Hadfield, J. (2010). MCMC methods for multi-response generalized linear mixed models the MCMglmm R package. *Journal of Statistical Software, 33.* 10.18637/jss.v033.i02

Landeira-Dabarca, A., Naslund, J., Johnsson, J. I., Alvarez, M. (2019). Cue recognition and behavioural responses in the three-spined stickleback (Gasterosteus aculeatus) under risk of fish predation. *Acta Ethologica, 22,*209-221. https://doi.org/10.1007/s10211-019-00324-8

Mathis, A., Smith, R. (1993). Intraspecific and cross-superior responses to chemical alarm signals by brook stickleback. *Ecology, 74,* 2395-2404. https://doi.org/10.2307/1939590

Silva, P. F., de Leahis, C. G., Luchiari, A. C. (2019). Fear contagion in zebrafish: a behavior affected by familiarity. *Animal Behavior, 153,* 95-103. https://doi.org/10.1016/j.anbehav.2019.05.004

Tollrian R, Duggen S, Weiss LC, Laforsch C, Kopp M (2015) Density-dependent adjustment of inducible defenses. Sci Rep 5:12736. https://doi.org/10.1038/srep12736

Wisenden, B. D., Chivers, D. P., Smith, J. R. (1995). Early warning in the predation sequence: a disturbance pheromone in Iowa darters (Etheostoma exile). *J Chem Ecol 21,* 1469-80. doi: 10.1007/BF02035146