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Abstract

In some fish, alarm substances are released from skin cells when they are bitten by a predator, signaling nearby fish in potential danger. Such anti-predator defenses have been studied in the fathead minnow (*Pimephales promelas*), and some have hypothesized that the response to the alarm substance is not instinctual, but rather fish must learn to associate it with a predation cue such as motion. The purpose of this study is to detect an effect of conditioning (associating alarm substance with predation threat) on minnow responses to alarm substance. We tested the prediction that conditioned fish would react more strongly to the alarm substance cue than the unconditioned fish. This study observed this behavior in solitary minnows, since we are only aware of studies that observed groups. Our study revealed no significant differences in behavior between conditioned and unconditioned fish, whether in groups or solitary, when exposed to alarm substance.

Introduction

The evolutionary interaction between predator and prey is often a constant contest of fitness in order for each to be somewhat successful. This contest of sensory systems rewards either contestant when they are able to gain an information advantage over its opponent (Ferrari et al. 2010). Prey must efficiently use time and energy they have available to feed, engage in courtship, defend their territory, and to avoid predators (Ferrari et al. 2005). Prey animals must rely on multiple sources of information to avoid death by predation (Lonnstedt et al. 2012). The fathead minnow (*Pimephales promelas*) is a common prey that must adapt to surrounding predators.

The fathead minnow is a freshwater fish that inhibits lakes, ponds, and slow-moving streams from Canada through the entire continental United States and into Northern Mexico (Hundman et al. 2007). While a fathead minnow's failure to respond to a predator can result in death, responses to a non-threatening predator or other stimulus would be a waste of the minnow's valuable and limited time and energy that could be used for various other activities essential for survival (Ferrari et al. 2005.)

Water-borne cues are the most efficient means for an aquatic organism to detect the appropriate context and timing for maximal effectiveness of anti-predator activity (Wisenden et al. 2001). Water is the universal solvent, allowing a broad range of chemical cues to be dissolved and dispersed in the surrounding waters to be available for detection (Carreau-Green et al. 2008). When certain aquatic organisms, such as a fathead minnows, are able to associate the pairing of a sight or odor of an unknown predator with the odor of a damaged member of its own species, a learned association of the two may take place (Ferrari et al. 2006a, Ferrari et al. 2006b). Fathead minnows that respond to such a pairing may gain a survival benefit by avoiding the predator (Mirza et al. 2003b).

So, how does the fathead minnow become aware of these alarm cues? All Ostariophysan fishes, which account for 72% of all freshwater fishes, are known to possess specialized epidermal club cells (ECCs; Carreau-Green et al. 2008). Predators damage ECCs, causing a release of chemical compounds that are released in no other context. This makes the detection of this specific alarm substance (AS), possibly hypoxanthine-3(N)-oxide (Chivers et al. 1993), an accurate advertisement of the presence of an active and dangerous predator (Ferrari et al. 2010). AS must be fresh or, in experimental conditioning, promptly frozen (Pollock 2005) to be perceived during subsequent trials as a threat to the fathead minnow.

Fathead minnows may rely on AS detection more so than similar species due to their high tolerance for turbid waters (Watanabea et al. 2007) which may limit their visual cues (Carreau-Green

2008). Examples of anti-predator behavior in the fathead minnow includes an increase in shelter use (Pollock et al. 2005), reduced activity, dashing, freezing, increased shoaling (Carreau et al 2008), diving towards the substrate (Wisenden et al. 2001), and escape attempts (van de Nieuwegiessen et al. 2009). A conditioned response of the fathead minnow to AS is witnessed with greater intensity in the laboratory setting, but studies prove the same mechanism is witnessed in nature (Wisenden et al. 2003). Many studies have been done on AS conditioning and anti-predatory responses in large groups (shoals) of fathead minnows, but no conditioning studies have been done focusing on individual fathead minnows.

In this experiment, I sought to first condition predator-naïve fathead minnows in a laboratory setting by introducing the AS (broken ECC from a fellow minnow), combined with motion to represent a predation event. After conditioning, I placed each individual conditioned minnow in their own beaker, and expose them to AS without motion. If the minnows were successfully conditioned, and anti-predatory responses are evident on an individual level, I predicted I would be able to witness an increase in anti-predatory behavior in individual minnows when exposed to AS alone in anticipation of a predatory attack. I predicted anti-predatory behavior would be more frequent during individual assessment trials for conditioned minnows compared to the control minnows.

Materials and Methods

Collection and Maintenance of Minnows

I purchased 75 fathead minnows from a commercial supplier 28 days prior to the first trial focusing on individual behavior. The minnows were from a manmade pond that did not contain predatory fish. The minnows were placed in a 76-L aquarium filled with dechlorinated tap water. This tank was maintained at approximately 20^{o}C, contained two airstones, and a filtration system. The tank

was floored with commercial aquarium gravel. The minnows were fed daily with commercial fish pellets. All of the minnows were kept in this aquarium for 9 days before being placed in the conditioning tanks.

The Conditioning Fish Tanks

Two 38-L aquaria were used as the conditioning tanks. The tanks were identical; all containing the same dechlorinated tap water, two airstones, a filtration system, and the bottom covered with the same aquarium gravel. Before introducing the fish into the conditioning aquaria, an apparatus to provide a moving vibrating stimulus (a bottle cap attached to a wand) was added. I was able to use an object dissimilar in shape to the minnow's natural predator since Wisenden (2001) proved motion facilitates predation risk in fathead minnows instead of shape. Both aquaria were maintained at 20^{o}C. Each aquarium had two plastic shelters. Minnows were placed in each of the two aquaria (n=15 each) and allowed to acclimate for 3 days prior to conditioning.

Preparation of Stimuli

A single healthy minnow was selected to be sacrificed for this experiment. The skin was removed from both sides of the body producing 4.45 cm^2 of skin. The strips of skin from each side were cut into 5 smaller segments, and added to small plastic 2-mL vials and filled with 1-mL of distilled water. I then used a glass rod to crush the skin cells thoroughly, ensuring a release of AS. The liquid from each vial was collected and measured. The liquid was then diluted to a final volume of 44.5-mL (dilution: 1cm^2 of skin to 10-mL distilled water). The substance was placed into individual vials to be used as an additive to the experimental 38- L aquarium (a volume of 2-mLs). For use in tests of individual minnows in 400-mL beakers, exactly 0.0212-mLs were added to the vials. This amount of alarm substance maintained the same concentration of alarm substance in proportion to aquarium water in the 38-L aquarium. Distilled water was measured out in the same frequency and volume as the AS, and added to the vials as well to use as the control. All vials were then stored at -15^{o}C until immediately prior to use.

Conditioning Trials

One of the two 38-L aquaria was established as the control, with the other being exposed to AS. Before the conditioning trials, a 2-mL vial of frozen alarm substance and a 2-mL vial full of frozen distilled water were thawed in warm water. After being motionless by the aquarium for 2-min, I recorded minnow behavior for 8-min prior to the addition of AS using an ethogram (Table 1).

Table 1: The various behaviors displayed prior to the addition of each stimulus, displayed in an ethogram.

Behavior	Description
Shoaling behaviors	
SSB - Shoal on bottom back	Grouped in the bottom back of the aquarium
SS - Shoal on side	Grouped on the side of aquarium
SFB - Shoal front bottom	Grouped on the front and bottom of the aquarium
Individual behaviors	
SU - Swimming upwards	Swimming towards the top of the aquarium
TT - Top of tank	At the top of the aquarium
SUD - Swimming up/down tank	Vertically swimming up and down the aquarium
PR - Picking at rocks	Picking through the aquarium gravel
BS - Behind Shelter	Behind the shelter
PAF - Picking at filter	Picking at the suction part of filter device in the aquarium
C - Chasing	The minnow was chasing another minnow

After the 8-min observation period, AS was introduced directly into the fresh stream of water from the filtration system. I was sure to prevent the fish from detecting my motion through this process. Immediately upon this addition, the bottle cap was rapidly moved up and down in the aquarium for 2-min, and the fish were chased using a long glass rod for an increased predation effect. I observed the behavior of the minnows for an additional 8 minutes after simulated predator motion. An ethogram was also used during this time frame observe the anti-predatory actions. For the control aquarium I followed the same procedure as that used for the AS treatment. I continued this conditioning procedure for 1-wk prior to individual analysis. I did a second trial of both the group and individual trials.

Table 2: The various behaviors displayed after the addition of each stimulus, displayed in an ethogram.

Behavior	Description
FS - Frantic shoal	Minnows swam together frantically in a shoal
FSL - Frantic solo	Minnow was swimming around frantically alone
BS - Behind shelter	Minnow was behind one of the two shelters
FB - Frozen at bottom	The minnow was immobile at the bottom of the aquarium
DBP - By "predator"	The minnow remained in close proximity to the motion simulation

Individual Minnow Experimentation

I began the individual treatment observations by thawing the frozen vials. For my first individual minnow analysis trial, I started the testing with the entire control tank. I filled two 1000-mL beakers with water from the control aquarium, and placed 7 fish in each beaker. This was to ensure each capture would take less time and the interval of time each minnow spent in the individual beakers prior to treatment would not be widely variable. A total of 14 beakers with a volume of 400-mL were filled with aquarium water, and a small plastic shelter in each beaker. I placed each minnow in an individual beaker. I then captured the minnows from the large beakers, placing each in their own individual beaker. I placed these beakers in two separate groups of 7, ensuring the minnows could not see the other group, or members of their own group. I allowed the minnows to acclimate in their beakers 8-min prior to stimulus addition.

The responses of each fish for 2-min following the introduction of AS and then water was observed separately. Half of the minnows received water before the AS. Minnow behavior was labeled "anti-predator" if the reaction displayed was included in the list from the introduction (an increase in shelter use (Pollock et al. 2005), reduced activity, dashing, freezing, increased shoaling (Carreau et al 2008), diving towards the substrate (Wisenden et al. 2001), and escape attempts(van de Nieuwegiessen et al. 2009).) If the minnow did not display any of these behaviors, their behavior was labeled "no response." This process was continued until all minnows in the first group were exposed to the

stimulus only, and all minnows in the second group had all been exposed to the distilled water only. I then reversed the process by individually adding the alarm substance to each individual beaker in the second group, and adding the distilled water to each beaker in the first group. I released the minnows back into the tank after that minnow had been exposed to both the alarm substance and the distilled water.

Results

Group Analysis

Aside from the "behind shelter" activity, no anti-predatory behavior was witnessed in either aquaria prior to stimulus addition. Although the "behind shelter" action was witnessed, it contributed to a low overall proportion of activities. Very little activity variability occurred between the two aquaria prior to the addition of either stimulus. Eight min prior to exposure, the minnows spent most of their time shoaling in the bottom back of the aquarium in both conditioning tanks. The minnows in both conditioning aquaria spent slightly more time shoaling on the side of the aquarium, as opposed to shoaling in the center, but overall a change in behavior was not noticeable (Figure 1).

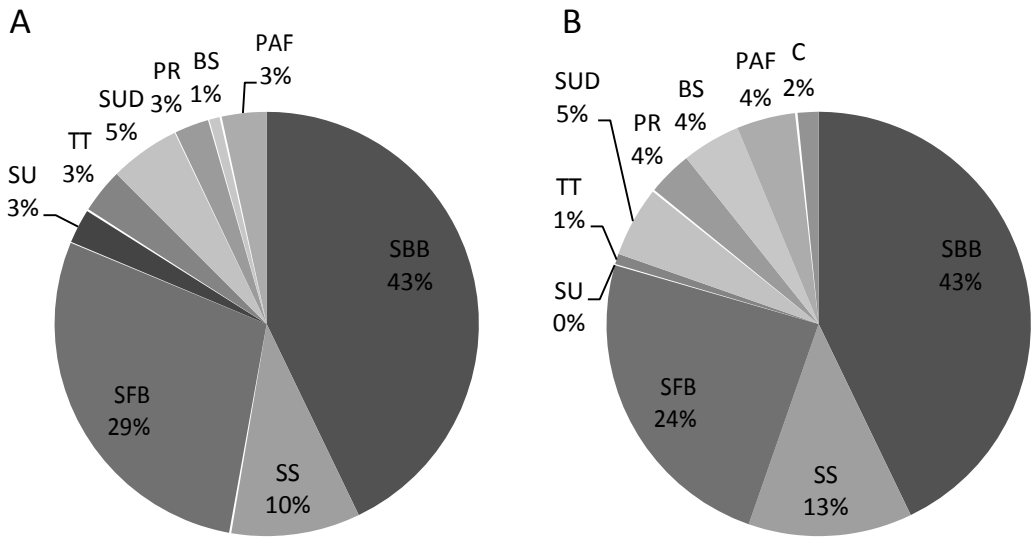
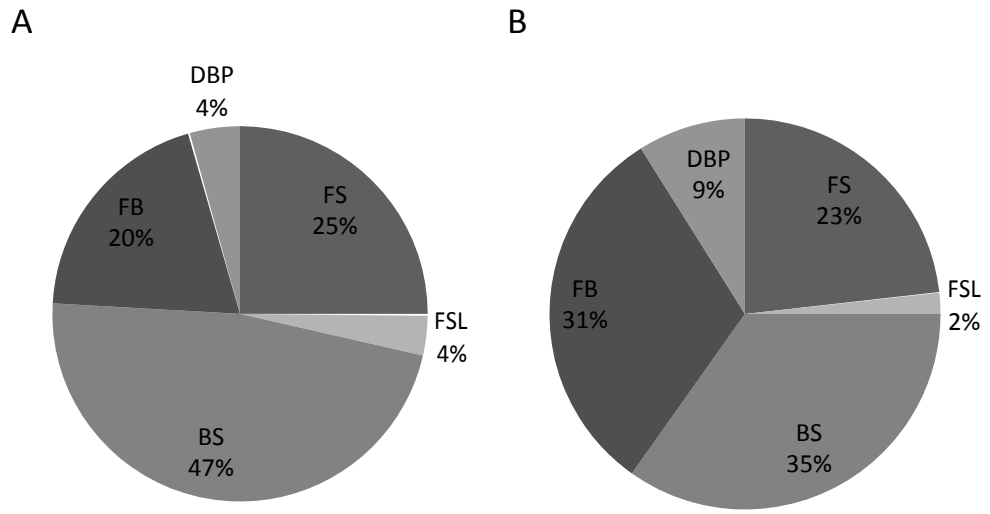


Figure 1. (A):Variation in activity by the group of unconditioned minnows prior to distilled water addition with motion.
(B): Variation in activity by the group of conditioned minnows prior to alarm substance stimulus with motion. This information was collected 8-min prior to the introduction of stimulus.

After each aquarium was exposed to stimulus paired with motion, only anti-predator behavior was witnessed in each aquarium for the entire 8-mins of post observation. This proves my predation event accurately impacted the minnows. Some minnows in each group chose to stay right by the suspended bottle cap immediately after I ceased its motion, which may be the only action not in an anti-predator attempt. Remaining by the “predator” immediately after the “attack” was not a common behavior. The majority of minnow activity in each group was spent taking refuge under shelters.



Figures 2. (A): Variation of activities of the group of unconditioned minnows after distilled water addition with a simulated predation event.
(B): Variation in activities of the conditioned minnows group after AS addition with simulated predation event. This information was collected 8-min after the alarm substance or distilled water was added to the tank with the predation simulation.

Individual Minnow Analysis

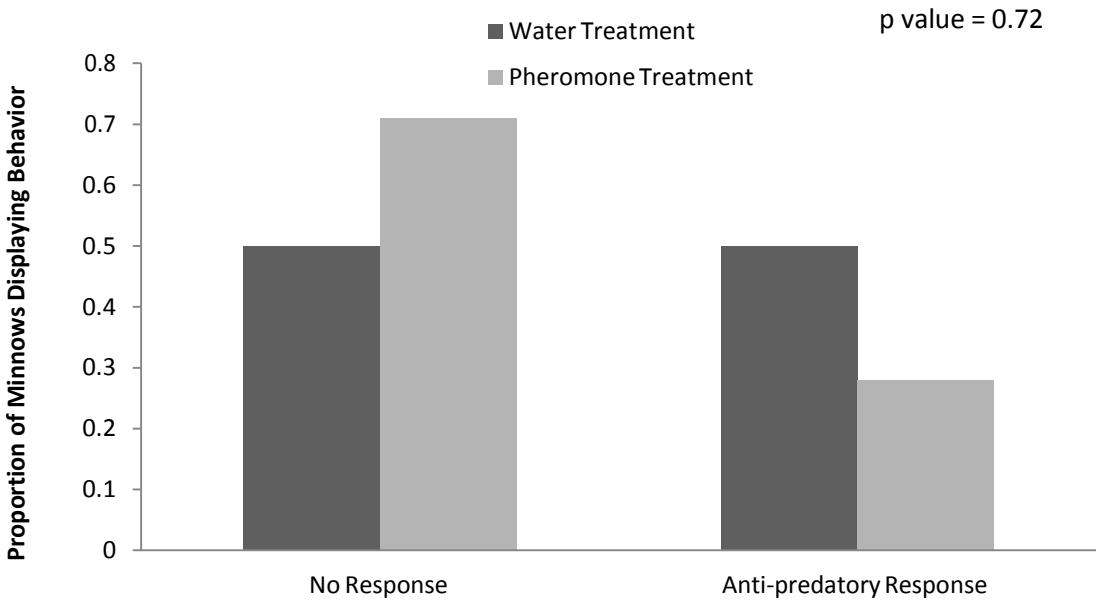


Figure 3: The observed activity of individual minnow response in the unconditioned minnow group after addition of water and alarm substance. Minnow activity witnessed after exposure was either anti-predatory in nature, or no response was given by the minnow.

When the unconditioned minnows were exposed to the water treatment, half of the minnows displayed anti-predatory behavior, while half did not respond to this addition. When AS was added to each individual beaker, most of the minnows did not respond to the stimulus but this was not statistically significant (Chi-square test; P= 0.72).

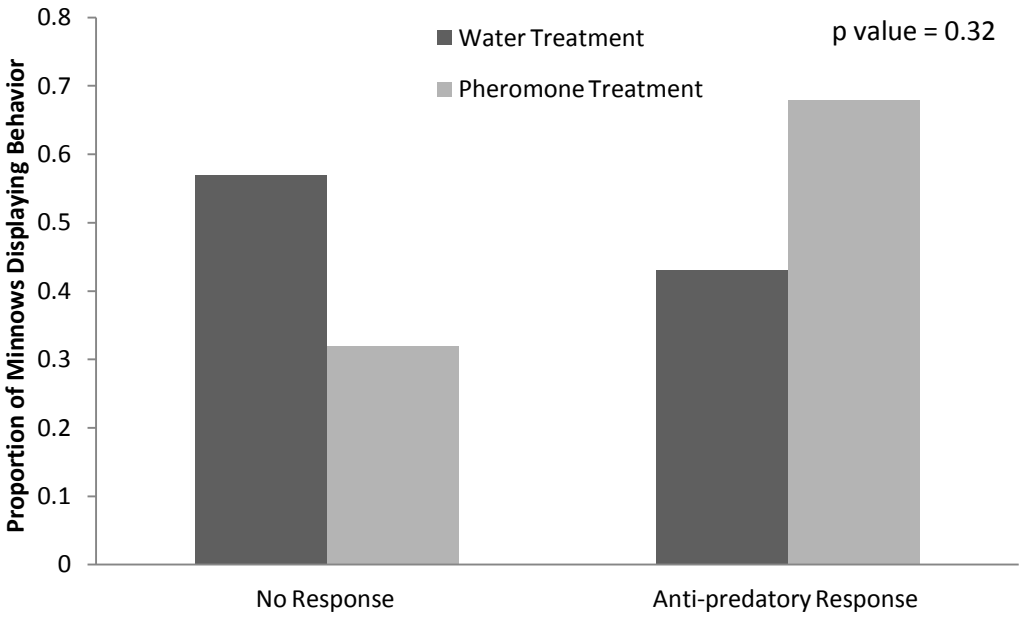


Figure 4: Conditioned minnow response to the addition of distilled water, and AS during individual analysis. Responses to each of the two stimuli were either anti-predatory in nature, or no response was given.

When AS rather than water was added to each individual beaker of conditioned minnows, a higher rate of anti-predatory behavior occurred in relation with behavior as a result of water addition. This higher rate of anti-predatory behavior was not statistically significant (Chi square test; P = 0.32). When the level of anti-predatory behavior of the conditioned minnows was compared directly to the unconditioned behavior, conditioned minnows displayed more anti-predatory behavior than unconditioned minnows after the addition of pheromone (Figure 5; Chi square test, P = 0.56).

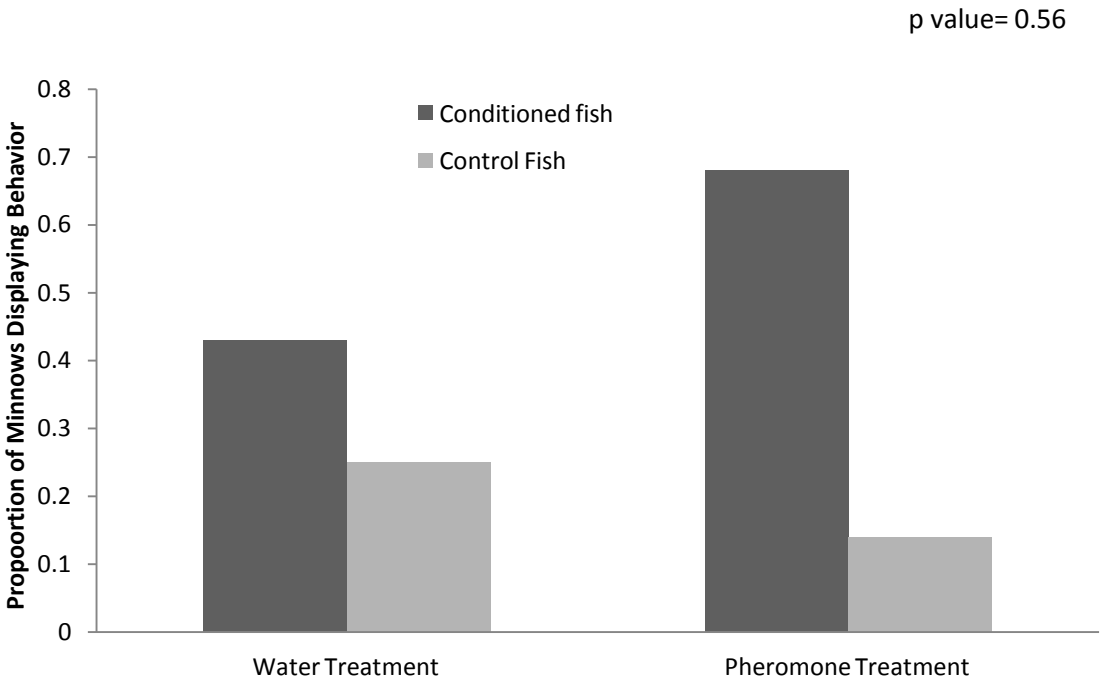


Figure 5: Minnow anti-predatory behavior from the conditioned and unconditioned (control) group were directly compared.

Discussion

During conditioning, minnow behavior was similar before and after the addition of the stimulus (AS in the conditioned aquarium, and distilled water in the control aquarium) when the conditioned and control minnows behavior were compared. The uniform lack of anti-predatory behavior prior to stimulus proves the minnows did not respond to me as they would to a predator during this procedure. This also proves anti-predatory behavior exhibited by the minnows only occurred when they perceived a predator threat, and anti-predatory actions are not naturally exhibited when no predators are observed.

A variation was witnessed when comparing individual anti-predatory behavior in the conditioned and unconditioned (control) minnows as a result of each stimulus addition. The conditioned fathead minnows displayed more anti-predator responses when AS was added to their beakers, whereas

unconditioned fathead minnows seemed to display a higher level of anti-predator behavior during the water addition. This difference was notable, but was not statistically different through Chi-square analysis. The greatest statistical difference was seen in anti-predatory responses in the conditioned group of minnows. The conditioned minnow anti-predator response was greater when exposed to the alarm substance stimuli, in comparison to the control (distilled water) stimuli. Statistically speaking, the minnows did not increase anti-predatory behavior after they were conditioned to the alarm substance. My hypotheses that anti-predatory behavior would be more frequent in the individual assessment trials for conditioned minnows, was disproven by this experiment.

Fathead minnows are increasingly sensitive to alarm substances during their breeding season (early June to late July; Pollock et al. 2006). My study occurred in late February to late March, which suggests their lack of alarm substance sensitivity may be due to this period of time being outside of their breeding season. In addition, other studies suggest male fathead minnows actually lose their club cells during the breeding season, to avoid scaring off potential mates or attracting predators (Halbgewachs et al. 2009). This would mean male fatheads would not be able to produce AS during the breeding season, indicating an increased sensitivity would not occur. I suggest an identical study take place within the breeding season, and the results of this experiment be compared to the results of this study. Comparison of female and male fathead minnow behavior during the breeding season would also be beneficial.

The individual I selected to be sacrificed for this study had a moderate body size. Sensitivity to the alarm substance is said to be size-dependent, with large minnows responding with anti-predatory behavior the strongest when a large minnow has been sacrificed. Small fathead minnows respond to the alarm cues of injured damselflies. This indicates that if two different types of prey are consumed by a similar predator, the alarm cues of one of those species may influence the other specie with the similar

predator (Mirza et al. 2003 a). By selecting a medium sized minnow, I assumed I would be able to notice anti-predator behavior from all the minnows in the conditioned tank, since I assumed all involved minnows would fall under the same predator threshold. My lack of statistical significance may have been caused by greater sensitivity in the sacrificed minnow's size threshold than I originally assumed.

In similar trials, AS was produced when the minnow skin was homogenized using a powered homogenizer (Polytron corporation; Pollock 2006). I did not have access to a Polytron homogenizer. My procedure of manual crushing to the ECC's may not have released as much AS. This may mean the concentration of AS may have been more diluted in this study than similar studies, which may have affected minnow behavior.

My study suggests that anti-predator responses of the fathead minnow may be contingent on being in a shoal formation, since only a small number of conditioned minnows displayed anti-predatory behavior. Perhaps only a small fraction of fathead minnows are particularly sensitive to AS, and are therefore the only ones that can be conditioned. This small fraction of minnows may then react to the AS, and the other minnows in their shoal react to the more sensitive minnows rather than directly to AS. This may explain in part why fathead minnows shoal in their natural environment.

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Sarah Thomson was born in 1990 in Rochester, Minnesota. In 2008, she graduated from Stewartville High School. She graduated from Minnesota State University Mankato in 2012, with a Bachelor of Science degree in Environmental Science. She hopes to pursue her education with a Master of Science degree in hydrology, fish biology, or a similar field.

Sarah has worked at the City of Mankato Wastewater Treatment plant in the laboratory for one year. While pursuing her bachelor's degree she maintained the role of President in the MSU Field Ecology Club for two consecutive years. During her sophomore and junior years of college she was employed as the Radon Director in the WALTER Weather Laboratory on the Minnesota State University campus. Her areas of interest are water quality analysis, aquatic ecology, and environmental concerns in relation to water quality.

Faculty Mentor:

John D. Krenz, a native of Iowa, earned a BS degree in Wildlife Ecology from Oklahoma State University, an MS degree in Biology from University of Minnesota- Duluth, and a Ph.D. in Ecology from the University of Georgia in 1995. His doctoral research on behavior and population genetics of the marbled salamander was conducted at the Savannah River Ecology Laboratory in South Carolina. He worked as a post-doctoral Fellow in the molecular biology program at the University of Missouri on the mating system of the gray tree frog. Dr. Krenz accepted a faculty position at Minnesota State University in 1998 and conducts research on animal behavior, ecology, and evolution.