

**The Effects of Nitrate Levels on the Response of *Pimephales promelas* to  
Conspecific Chemical Alarm Cues**

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Total number of words in text: \_\_\_\_\_

**Abstract**

I investigated the effects of nitrate levels on the chemical alarm cue response of fathead minnows (*Pimephales promelas*) under laboratory conditions. I acclimated single fatheads in aquaria with 0, 5, 10, 75, and 300 parts per million of nitrate from calcium nitrate tetrahydrate ( $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ ) for a period of no less than 48 hours and no more than 72 hours. I recorded the experiment on VHS for in-depth analysis of the reactions. The specimens were recorded for eight minutes, subjected to fathead minnow chemical alarm cue and recorded for eight more minutes. Only 26 of the 80 specimens survived long enough to be tested. Of the 26, only three did what past studies have shown they should do. The only antipredator response exhibited was retreating to hide under cover. Using a repeated measures analysis, I determined a significance of 0.295. Thus, there is no significant relationship between nitrate level and the chemical alarm cue response of fathead minnows for the present study.

## Introduction

Chemical alarm cues in aquatic environments elicit varying and diverse responses from the receivers. A variety of fishes produce or react to alarm cues. Fish in the superorder Ostariophysi, which represents 64% of all freshwater fish species (Wisenden and Thiel 2002), produce the chemical alarm cue named Schreckstoff (von Frisch 1938, 1942). Northern redbelly dace produce and respond to chemical alarm cues (Dupuch et al. 2004). Some species in the superorder Acanthopterygii produce an alarm cue, such as reticulate sculpin (Chivers et al. 2000), slimy sculpin (Bryer et al. 2001), yellow perch (Mirza et al. 2003), and green sunfish (Brown and Brennan 2000). Convict cichlids display antipredator behavior when subjected to conspecific alarm cues (Alemadi and Wisenden 2002, Brown et al. 2004a). Rainbow trout demonstrate antipredator reactions when subjected to conspecific skin extracts (Brown and Smith 1997, Mirza and Chivers 2003b). Although most of these experiments have taken place under laboratory conditions, Wisenden et al. (2004) have shown that chemical alarm cues do elicit an antipredator response in the wild.

Alarm cues are very useful tools for fish that possess them because their purpose is twofold. They attract predators to the site of release (Mathis et al. 1995; Wisenden and Thiel 2002) and they warn other prey of danger. The fish cannot voluntarily release these alarm cues. They are contained in cells of the skin that have no openings (von Frisch 1938, 1942). This means the skin has to undergo mechanical damage, such as attack, for the alarm cue to be released into the water (Mathis et al. 1995). The fact that chemical alarm cues attract other predators is a direct benefit to the prey because of interference from secondary predators that are drawn in disrupting the primary predation event and thus allowing a greater chance for prey to escape (Chivers et al. 1996). Other possible prey respond to alarm cues by exhibiting antipredator responses such as freezing, shoaling (Chivers et al. 1995) and dorsal fin erection. The antipredator benefit of shoaling is enhanced by the presence of an alarm cue (Wisenden et al. 2003). Fish can learn to recognize only the visual cue of a predator after just one occasion where they both see a larger fish and sense an alarm cue (Chivers and Smith 1994). Fathead minnows are able to transfer knowledge about dangerous habitats amongst each other even to predator naïve conspecifics (Chivers and Smith 1995). Chemical alarm cues do not just warn conspecifics, members of the same species; fish can learn to respond to heterospecific alarm cues (Mathis et al. 1996, Mirza and Chivers 2001c, Chivers et al 2002, Pollock et al. 2003). Although,

Pollock and Chivers (2004) showed that this heterospecific alarm cue learning is dependent upon the density of the heterospecifics. The complexity of the habitat that contains various prey species can influence the ability of prey fishes to recognize and respond to a heterospecific alarm cue (Pollock and Chivers 2003). Prey can choose not to respond to a heterospecific alarm cue because they do not perceive a threat based on their size in relation to the heterospecific, but will show an antipredator response if in the presence of conspecific alarm cue (Golub and Brown 2003). Naïve prey can also learn to recognize the odor of predators when it is coupled with a conspecific alarm cue (Gazdewich and Chivers 2002) or just by being in a shoal with predator-conditioned prey (Mathis et al. 1996).

Alarm cues prove to be very useful, but there are times when the prey species do not exhibit antipredator responses while in the presence of a conspecific alarm substance. The antipredator response is dependent upon the hunger level of the prey species (Brown and Smith 1996, Brown and Cowan 2000, Chivers et al. 2000). The prey species may assess the risk of the situation as described by Hartman and Abrahams (2000) and Marcus and Brown (2003), and choose whether or not to respond to the chemical alarm cue depending upon available cover and the turbidity of the environment. If the prey species is subjected to subthreshold concentrations of conspecific alarm cues, they may rely upon secondary cues such as vision to actually elicit an antipredator response (Brown et al. 2004b)

Heterospecific alarm cues can entice an entirely different response from different size classes of the same species of fish (Grant et al. 2001). This change is not only related to length, but also to body depth, which makes the fish shift from antipredator to forager a phenotypically plastic response (Grant et al. 2002).

Alarm cues are not available in the water only when an attack is or has recently taken place. Predatory fish leak their prey's alarm cues from their gut and thus will elicit an antipredator response from the prey species. This can be due to conspecific alarm cues (Mathis and Smith 1993, Mirza and Chivers 2001a,b, Mirza and Chivers 2003c) or learned heterospecific alarm cues (Chivers et al. 2002, Mirza and Chivers 2003a, Mirza and Chivers 2003c, Mirza and Chivers 2003d).

Compounds with a Nitrogen-Oxygen functional group elicit an antipredator response in Ostariophysan fishes (Brown et al. 2003).

The presence of dissolved substances in the water may affect the activity of alarm substances, either by reacting with them, masking them, or increasing tolerance to them (Weirich et al. 1993). Under weakly acidic conditions, pumpkinseed sunfish (Leduc et al. 2003), fathead minnows, and finescale dace (Brown et al. 2002), as well as two salmonids: rainbow trout and brook charr (Leduc et al. 2004) do not show a significant change in antipredator behavior when compared to controls. In this study I tested how various levels of nitrate affected the response of fathead minnows to the conspecific chemical alarm cue, Schreckstoff, which is a nitrogenous-based compound and nitrogen pollution is a major concern affecting many of our nation's waters. No studies have been conducted to see if nitrate pollution is affecting the reception and response of fathead minnows to Schreckstoff.

### **Methods**

I acquired the fathead minnows from a body of water (School Lake, MN) where there are no predatory fish so as to reduce the chance of a conditioned response. They were collected using minnow traps baited with dough balls and dropping them through the ice. The fish were kept in a living stream, where they were fed to satiation daily with commercial goldfish food and kept on a 14:10h light:dark cycle.

For the experiment, 80 adult minnows were sacrificed by putting them in water with Alka-seltzer. Skin fillets were removed from both sides of each fish resulting in 380 cm<sup>2</sup> of skin. This was then placed into 400 ml of distilled water and homogenized. The sample was then filtered through glass wool to remove any particles and diluted with another 400 ml of distilled for a total of 800 ml of extract. This was then divided into 10 ml samples and immediately frozen at -19°C.

One fish was placed in each of five 37 l glass aquaria. Each aquarium had a different level of nitrate: straight well water, 5, 10, 75, and 300 parts per million (ppm). Channel catfish and largemouth bass can withstand nitrate concentrations of up to 400 ppm (Knepp and Arkin 1973). Each tank had a gravel substrate that was thoroughly cleaned and rinsed prior to use and a centrally located shelter object made of a 10 x 20 cm ceramic tile with 5.5cm long cylindrical legs. There was also an airstone in each tank along side which I attached a 2-m long piece of airtube which was used to introduce the test stimuli into each tank. This length of introduction tubing allowed me to inject the stimuli from a distance so as to further not spook the specimen. Each tank was also wrapped in heavy dark plastic so outside distractions would have no effect on

the subjects. On the front of each tank the plastic was formed into a hood that I could fit to a camera and record the test on VHS for future analysis. The fish were allowed to acclimate for no less than 48 hrs and no more than 72 hrs prior to running the experiment.

Prior to each trial 35 ml of water was drawn out of the aquarium and discarded to ensure the cleanliness of the tube. An additional 35 ml was then drawn out, kept, and later used to flush the stimulus into the tank. Each trial consisted of an 8 min pre-stimulus period and an 8 min post-stimulus period where the minnows were watched and their actions recorded. I recorded time spent under shelter, the occurrence of freezing (the cessation of movement, dropping to the substrate and remaining motionless for 30 s), and dashing (rapid and erratic swimming). I did not include test fish that exhibited freezing or dashing in the pre-stimulus period. These three attributes were recorded because they are known antipredator responses in fathead minnows (Smith & Chivers 1998).

I ran each series of trials eight times. Between each series of trials, the tanks were drained and thoroughly cleaned. I then calculated changes in time spent under shelter, freezing, and dashing for each tank. I ran a repeated measures analysis on the data in SPSS to determine the significance of the interactions and variables.

## **Results**

Unfortunately, of the 80 possible test subjects, about 70% died prior to being able to be tested for reasons unknown to me. So, I was only able to test 26 specimens: 4 control specimens (0 ppm), 5 specimens at 5 ppm, 5 specimens at 10 ppm, 6 specimens at 75 ppm, and 6 specimens at 300 ppm. Upon running the experiment, none of the specimens exhibited freezing or dashing during either the pre-stimulus or the post-stimulus times. So, I only looked at and analyzed the time spent under structure for each of the test subjects. For the control specimens, the mean time under structure during the pre-stimulus period was 7.97 minutes, and the mean time under structure during the post-stimulus period was 8.00 minutes. The subjects in 5 ppm spent an average of 5.17 minutes under structure during the pre-stimulus period and 4.78 minutes under structure during the post-stimulus period. The specimens held in 10 ppm had a mean time under structure during the pre-stimulus of 6.25 minutes and a mean time under structure during the post-stimulus period of 7.94 minutes. For the specimens in 75 ppm, they had a mean time spent under structure during the pre-stimulus period of 6.49 minutes and an average of 6.87 minutes

spent under structure during the post-stimulus period. The specimens in 300 ppm had a mean of 7.99 minutes spent under structure for the pre-stimulus period and a mean of 6.82 minutes spent under structure during the post-stimulus period (Fig. 1).

Of the specimens that survived, 11 specimens spent more time under structure during the post-stimulus period versus the pre-stimulus period, 6 specimens spent less time under structure during the post-stimulus versus the pre-stimulus period, and 9 specimens showed no change between the pre-stimulus and the post-stimulus time spent under structure. Only 3 of the 26 or 11% of the specimens did what previous studies have shown they should do. Of these, 2 were in 10 ppm and 1 was in 75 ppm. The effect of nitrate level on the chemical alarm cue response of fathead minnows was not significant in this experiment (Sig. = 0.295).

## **Discussion**

The results that were collected have left more questions than answers. Mostly questions as to why the experiment did not work as previous studies have shown it should. With some studies showing that fish do not exhibit any antipredator response in pH levels as high as 6.3 (Leduc et al. 2004), there is a possibility that adding nitrate from calcium nitrate tetrahydrate could have increased the acidity to a level where a response is undetectable. But that does not answer why specimens in lower or even the control concentrations did not show a significant antipredator response.

Also there could be the effects of naturally occurring elements such as calcium and chloride which have been shown to increase a fishes ability to survive in environments that were previously thought to be fatal (Weirich et al. 1993). Possibly these naturally occurring elements are reacting with the fish and affecting their chemical alarm cue response.

Another variable that I did not look into was the disturbed time. It is possible that when I adjusted the hoods to fit over the camera lens, that I was inadvertently affecting the fish and causing them to exhibit antipredator behavior before they were supposed to. This could be avoided in the future by allowing a 20 or 30 minute acclimation period before recording was started, or by having a way of knowing what the fish was doing prior to the hood adjustment.

Furthermore, beyond the fish that actually survived, future investigations of this caliber should attempt to determine the cause of the many fatalities prior to testing of the specimens. Due to the discrepancies in results between the current study and numerous previous studies,

more research with a better understanding of the testing environment is required to fully determine the effects of various levels of nitrate on the chemical alarm cue response of fathead minnows.

### **Acknowledgements**

I would like to extend my gratitude to Dr. Don Cloutman for his guidance and patience in this project. Thank you to Bemidji State University for providing the equipment needed to run this study. I also appreciate all of the help that my classmates gave in the collection of the study specimens.



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Figure 1: Mean pre-stimulus and post-stimulus minutes spent under structure for each of the five concentrations.

Concentration (ppm)	Pre-stimulus	Post-stimulus
0	7.97	8.00
5	5.17	4.78
10	6.25	7.94
75	6.49	6.87
300	7.99	6.82