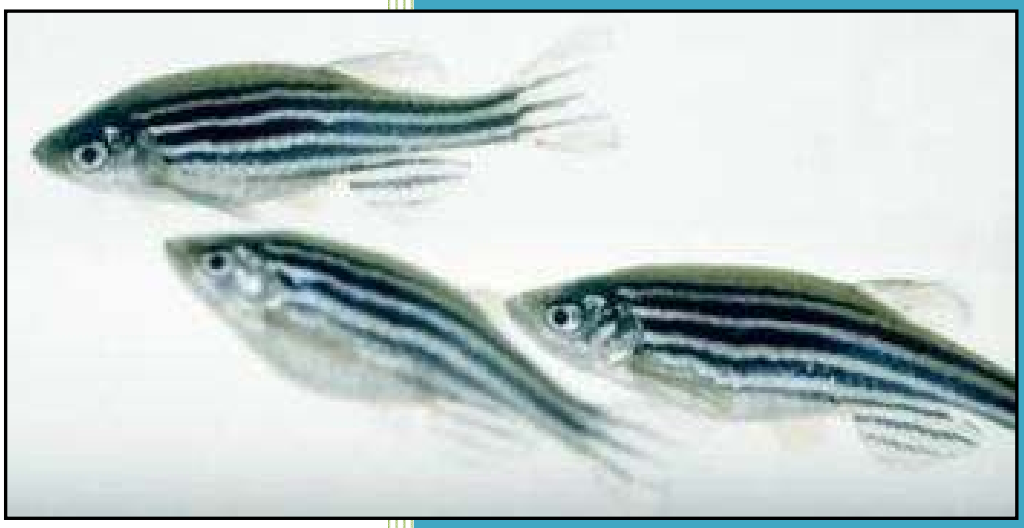
2009

Zebrafish Stress Responses to Alarm Pheromone



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[5/8/2009]

Introduction

Zebrafish (*Danio rerio*) have served as animal models in behavioral neuroscience and behavior genetics for the past three decades. The zebrafish has all the neurotransmitters that humans possess (Shin and Fishman, 2002). Their robust physiological responses to stress are due to their well-developed corticosteroid stress axis (Alsop and Vijayan, 2009). Although zebrafish have been studied extensively, their emotionality responses such as fear and anxiety are still unclear (Bass and Gerlai, 2008) and alarm pheromone response is displayed by many fish species.

Alarm pheromone is also known as antipredator behavior a chemical that triggers fear (Jesuthasan and Mathuru, 2008). The olfactory sensory system plays a role in triggering the alarm pheromone responses. When a fish is injured, it releases a substance producing specific avoidance responses in zebrafish. Alarm pheromone is important to fish because when one fish releases this chemical, it alerts the other fish to escape. Generally, the fish cluster in a group, dart in different directions (Whitlock, 2006), jump out of the water, swim to the bottom, or freeze when they sense the alarm pheromone (Jesuthasan and Mathuru, 2008). In response to alarm pheromone zebrafish normally increase their shoal cohesion, swim faster with spontaneous swift turns (erratic movements), increase occurrence and duration of freezing bouts, decreased aggression, and clearly increase bottom dwelling (Rehnberg, 1988). These responses are associated with the plasma glucose and cortisol – stress hormone – levels (Jesuthasan and Mathuru, 2008).

Although alarm pheromones are present in many fish species, it takes approximately forty-eight to fifty-two days, after hatching, for the naïve zebrafish to response to pheromone. It takes approximately seventeen days before the fish can produce their own alarm pheromones (Bosio, 1994). Alarm pheromone reaction changes as a fish mature. As a fish mature, they only keep a portion of their original pheromone (Bosio, 1994).

Strong anxiety responses in zebrafish are exaggerated when they are exposed to novelty. The novel tank test can allow the experimenter to measure and collect behavioral parameters – latency to enter the upper-half, transitions into the upper half of the tank, erratic movements and freezing bouts – to evaluate anxiety.

Methods and Materials

Animals and Housing

Adult zebrafish of both sexes were obtained from a local commercial distributer (5O Fathoms, Metaire, LA). All fish were placed in groups of forty fish in a 40-liter tank for at least ten days to be familiar with the laboratory environment. The temperatures of 25-27°C were maintained in the room and water. Lighting was provided by fluorescent light tubes on the room ceiling. These lights are turned on at 9:00 AM and off at 9:00 PM. All of the subjects were experimentally naive, and fed with mixtures of ground flake food (Tetramin Tropical Flakes; Tetra USA, Blacksburg, VA).

Novel Tank Diving Test

Individual zebrafish were placed in a 1.5-L trapezoidal tank, approximately 15.2cm H x 27.9cm TW, and completely filled with aquarium treated water. The tank was divided into two equal horizontal halves. The upper and lower halves were marked by a black sharpie on the outside of the tank. In a minimal disruption environment, the tanks were situated on a level and stable surface. With careful contemplation to condense the net-stress, the Zebrafish were relocated from home-tanks to novel tanks with fish nets. Observations were made immediately after the fish was in the novel tank for the time span of six minutes. This experiment can be repeated many times for

more precise data. Moreover, the experimenter changed the water each time an individual fish was finished testing so the next fish can have the same starting environment.

The following endpoints were quantified by human observations over a six minute period: latency to reach the upper portion of the tank, time spent in the upper portion of the tank, number of entries into the upper portion of the tank, erratic movements, freezing bouts, and freezing duration. The total absence of movement, with the exception of gills and eyes, were known as freezing. The sharp changes in direction and velocity, and repeated rapid darting behaviors were known as erratic movements. A good indicator of high-anxiety phenotypes can be determined when there is a significant decrease in exploration – longer latency to reach the top, fewer entries to the top, longer freezing duration, or elevated erratic movements.

Alarm pheromone was extracted from the epidermal cells of zebrafish. The fish was euthanized and dried off before their epidermal cells were damage within the range of ten to fifteen slight incisions without drawing blood. Shallow cuts were made with a clean razor blade on one side of the fish body. After the incisions, the fish was washed for five minutes with the damaged side down in the ten milliliter Petri dish filled with distilled water. The Petri dish was immediately placed on ice and shaked for one minute to preserve the extracted pheromone. The Petri dish was in a resting position for four minutes after the one minute shaking. Afterward, the fish was removed from the Petri dish after the four minute resting time, dried, and the protocol was repeated for the opposite side of the fish. The extracted alarm pheromone was stored in a collected tube, and seven milliliters of the collected solution were added to the fresh water in the novel tank prior placing the fish into the tank for observations. This protocol can be repeated for other fish.

Results

Zebrafish shows different behaviors when they were exposed to alarm pheromone for an acute period of time and a chronic period of time. The novel tank test was used to measure this difference.

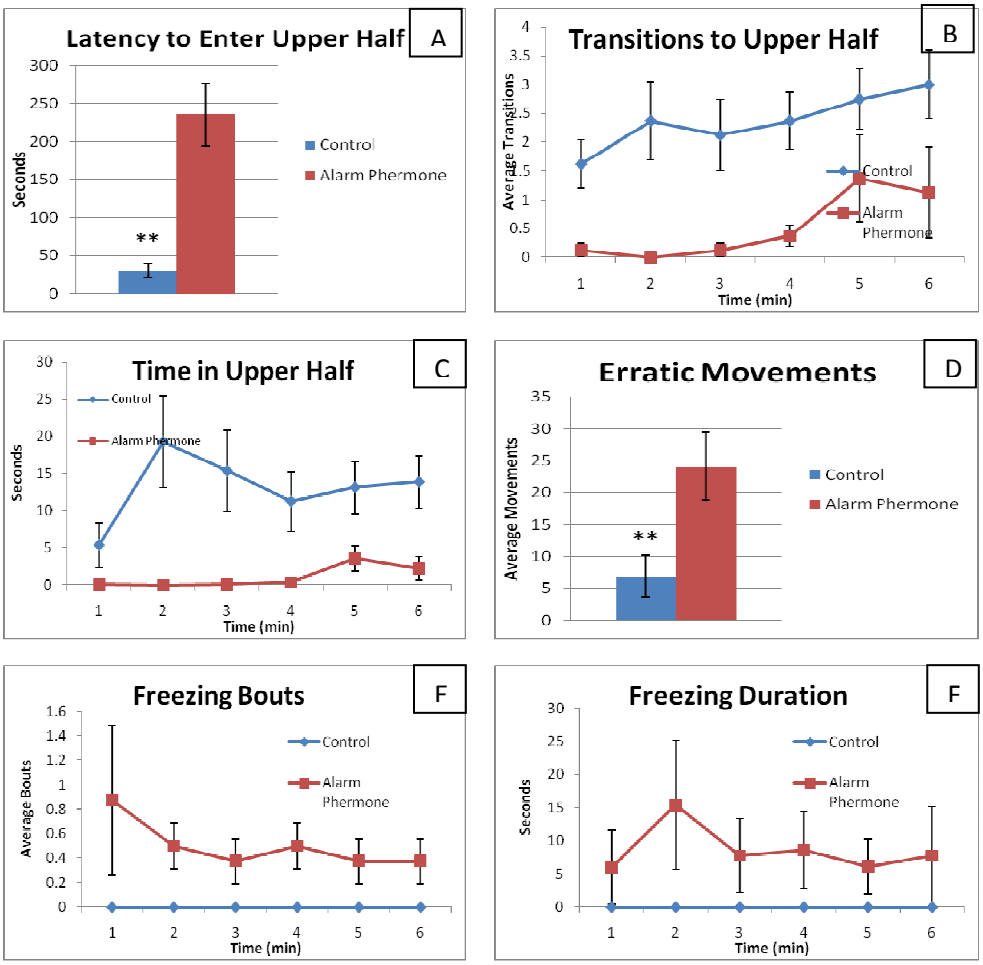
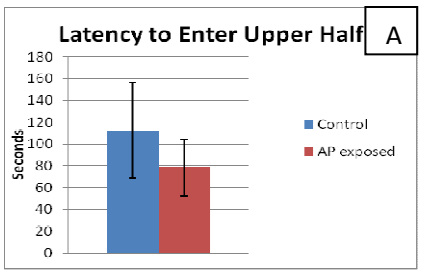
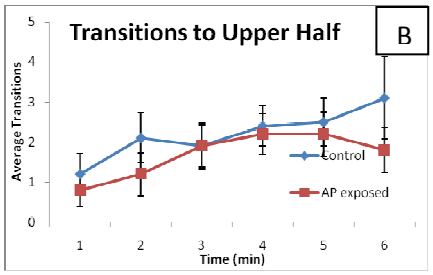


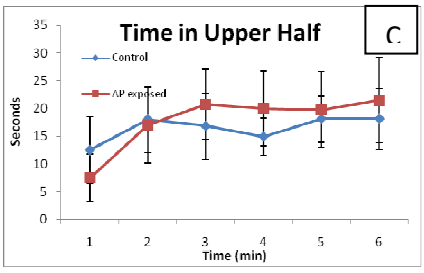
Figure 1 : Acute alarm pheromone effects on zebrafish. A) latency to enter upper half of the novel tank; B) number of times the zebrafish enter the upper half of the novel tank; C) the time the zebrafish spent in the upper half of the novel tank; D) the number of erratic movements; E) the number of freezing bouts; F) the freezing duration.

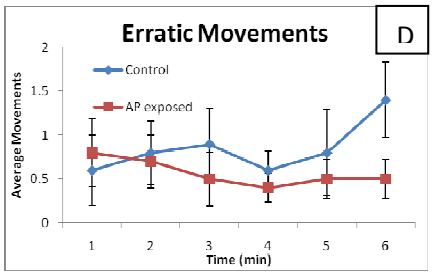
The acute alarm pheromone exposed zebrafish displayed more fear than the unexposed group (Figure 1.A). When the zebrafish sensed the alarm pheromone, they were scared so they were hiding in the corners and bottom of the tank. They hesitated to move because they were afraid that they would run into their predator. The unexposed zebrafish were stress-free and did not sense anything; thus, it takes them a shorter period of time to swim to the top (Figure 1.A). The zebrafish were acutely exposed to the alarm pheromone before testing begins. After the exposure, the zebrafish were placed into the novel

tank for a six minutes observation. From the data, it shows that the exposed zebrafish have less transitions to the upper half of the novel tank (Figure 1.B) as well as the time in the upper half compared to the unexposed zebrafish (Figure 1.C). Usually, when a fish is not stressed, it will naturally explore the upper region. This indicates that the exposed zebrafish was being under a huge amount of stress because it rarely swam to the top. If the exposed zebrafish did swim to the top, they did not stay there longer than five seconds. Stress and the number of erratic movements (Figure 1.D) have a one-to-one relationship. When stress increased, the number of erratic movements increased. When stress decreased, the number of erratic movements decreased. The data shows that amount of the erratic movements of the acute alarm pheromone exposure were roughly three times that of the unexposed group. This evidence signifies that the exposed zebrafish were under a great amount of stress and anxiety. Analyzing the freezing bouts and freezing duration of the exposed and unexposed group of zebrafish indicates an interesting; yet, surprisingly finding. The unexposed group of zebrafish did not show any freezing bout (Figure 1.E) or freezing duration (Figure 1.F) compared to the exposed group of zebrafish. This evidence supports the idea that the stressed fish will show more unusual movements compared to the stress-free fish.









The result obtained from the chronic exposed alarm pheromone zebrafish was very different compared to the acute exposed alarm pheromone zebrafish. The latency to enter the upper half of the novel tank (Figure 2.A) decreased compared to the acute exposure to alarm pheromone.

This amount of decreased suggest that the zebrafish were adapting to the changes in their environment. This theory is supported when both the experimental and control group present a similar number of transition to the upper half of the novel tank (Figure 2.B), as well as the time spent in the upper half of the novel tank (Figure 2.C). Surprisingly, in the chronic exposure experiment, the zebrafish that was not exposed to the alarm pheromone show a higher level of erratic movements than the zebrafish that was exposed to the alarm pheromone (Figure 2.D). They also show a higher level of freezing bouts (Figure 2.E). This verification proved that the zebrafish that was exposed to the alarm pheromone were displaying survival of the fitness by adapting to their environment. Moreover, the closely similar freezing duration further verify this idea by showing that the freezing duration of the exposed and unexposed zebrafish was overlapping (Figure 2.F).

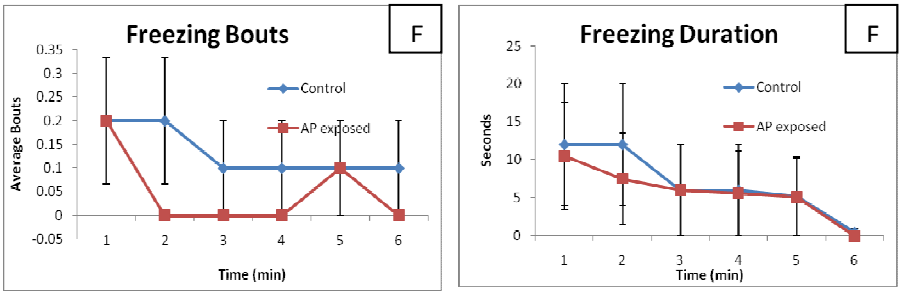


Figure 2 : Chronic alarm pheromone effects on zebrafish. A) latency to enter upper half of the novel tank; B) number of times the zebrafish enter the upper half of the novel tank; C) the time the zebrafish spent in the upper half of the novel tank; D) the number of erratic movements; E) the number of freezing bouts; F) the freezing duration.

Discussion

Base on our results, it was shown that the zebrafish that was acutely exposed to alarm pheromones demonstrated significant anxiety and stress behavior. Their hesitation to explore the novel tank and dramatic erratic movements exhibited great level of stress and anxiety. The zebrafish great increased in the freezing bouts and the freezing duration also support this idea. On the other hand, when the zebrafish were chronically exposed to the alarm pheromone, they did not

show much of a difference when compare to the zebrafish that was not chronically exposed to the alarm pheromone. After being exposed to the alarm pheromone for so long, the zebrafish adapted to it and started to displayed regular behavior again. By having the zebrafish adapting to its environment, it exemplify the concept of survival of the fitness. Overall, this experiment proves that alarm pheromone does have an effect on the zebrafish; however, when the alarm pheromone are being release chronically, the zebrafish will start adapting to it and will not displayed the stress and anxiety behavior.

In conclusion, zebrafish are good models for stress and anxiety studies and have a promising future in the science field. Furthermore, the novel tank test was proven to be a good method to measure the stress and anxiety level of the zebrafish. This method is simple and beneficial in many ways. For example, the fish are being naturally observed in the novel tank for a certain amount of time. The set-up of the novel tank is very fast so a large amount of fish can be observed for data in a short amount of time. This type of observation cannot be done by any other method; thus, the novel tank test is one of the most intriguing method to be use to examine the stress and anxiety level of the zebrafish.

Acknowledgement

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"Zebrafish's Novel Genes Critical In Directing Body Patterns". 2 January 2007. 10 May 2009 <http://www.scienceahead.com/entry/zebrafishs-novel-genes-critical-in-directing-bodys-patterning>.

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