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Behavioral plasticity in the Zebrafish C-start (escape) response

A thesis submitted in partial satisfaction
of the requirements for the degree Master of Science
in Physiological Science

by

William Quy Duong

2012

ABSTRACT OF THE THESIS

Behavioral plasticity in the Zebrafish C-start (escape) response

by

William Quy Duong

Master of Science in Physiological Science

University of California, Los Angeles, 2012

Professor David Glanzman, Chair

Vertebrate models of learning and memory are hampered by massively complex neural circuits. Further, the engrams of memory in many vertebrate systems are highly distributed, increasing the difficulty of their identification. Invertebrate models, which possess much simpler nervous systems, have been used to relate neuronal changes to behavior changes. However, these invertebrate models are phylogenetically distant, and are physiologically and functionally different from mammalian systems. An ideal model to investigate plasticity or memory in vertebrates would entail a simple neural circuit. One such model, the zebrafish, or *danio rerio*, offers a variety of advantages in this regard. In addition to its rapid development, high fecundity, and ease of genetic manipulation, it is transparent at the larval stage, making future experimentation in calcium imaging readily available. Additionally, the zebrafish contains a simple neural circuit that mediates a simple behavior. This behavior is the c-start escape response

(figure 1), which is dependent on large, bilaterally paired Mauthner cells in a well-understood neural circuit. Because of this, the zebrafish model presents an ideal model in the elusive understanding of the cellular mechanism of learning and memory.

The purpose of my thesis is to develop the zebrafish c-startle escape response as a new model of learning and memory to facilitate assignment of neural function and structure to behavioral memory. To achieve this goal, I have pursued numerous avenues, the first being to define many of the basic response properties that influence the escape response, in order to enable efficient and rational design of learning assays. Through experimentation, I've determined some of the factors that influence the escape response, such as volume of water, the zebrafish circadian rhythm, and even certain acoustic properties of the stimulus itself (pulse length, frequency, decibel). Other experiments to discover and describe other factors that may affect the escape response, such as boldness, will also be discussed.

A secondary goal of my thesis is to discover and reveal new types of memories in the escape response of the zebrafish, such as sensitization and classical conditioning. Using an alarm pheromone, H_3NO , we've managed to sensitize the c-start response, and have also elucidated the phototactic preferences of the zebrafish. These results will certainly lead into studies on classical conditioning of the escape response, such as association of a neutral stimulus (amino acid water) with the alarm pheromone.

Ultimately, these projects should elucidate neural function and plasticity at the M-cell synapse and set the stage for more in depth neurophysiological investigation.

The thesis of William Quy Duong is approved.

Dwayne Simmons

Fernando Gomez-Pinilla

David Glanzman, Committee Chair

University of California, Los Angeles

2012

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INTRODUCTION

1.1 THE STUDY OF LEARNING AND MEMORY

The study of learning and memory has been one of the most elusive studies in the field of neurobiology. Years of research have made many advances, but there is still no comprehensive understanding of the molecular mechanism for memory in any species. One of the primary goals in our lab is to find out where the memory engram is, how the memory is etched, and what the physical manifestation of memory is. Being able to find the molecular substrates that facilitate memory or participate in the memory engram will be critical in the ultimate goal of understanding how it is that we learn and remember (Roberts et al., 2011).

Of course, research on learning and memory is done on a variety of animal models, in an attempt to understand it at a simplistic level, before applying it to a more complicated system, made up of over a hundred billion neurons, each with thousands of connections, known as the human brain. Because of this, research on learning and memory in vertebrates is extremely difficult, because not only is the memory everywhere, it is difficult to relate causal changes to behavioral phenomena.

Thus, the obvious answer is to go into a species with a simple neural circuit. Ideally, it would be in a neural circuit that has well-known inputs and outputs, which can provide a perfect system to get causality between physical changes and actual learning.

Thus, an excellent animal model presents itself, *danio rerio* - the zebrafish. Within the zebrafish is the Mauthner cell, a large neuron in its central nervous system that controls its C-start reflex. The Mauthner cell is extremely easy to locate, which means that it is extremely easy

to manipulate. Much research has been done on this single neuron, and has resulted in many advances in neurobiology, synaptic physiology, and more.

1.2 THE MAUTHNER NEURON

Specifically, the Mauthner cells are a pair of large and identifiable neurons found in the reticular formation of anamniotic vertebrates, teleost fishes, and amphibians (Lee and Eaton, 1991). This reticulospinal neuron has a large axon, soma, and dendrite. Its soma is large, over 100 micrometers in diameter, and its two major dendrites are both at least 500 micrometer in length.

The purpose of the Mauthner cell is to propel the fish in a direction that would move it away from a harmful stimulus. By firing, the Mauthner cell acts on certain muscles that produce a C shape in its body, which kicks the fish in a strong, singular motion away from the direction of the stimulus. The C-start escape reflex will be the primary focus on this thesis, as we endeavor to understand the simple forms of learning and memory that is associated with it. In general, this startle response has important implications as an escape reflex, but it has also been shown to be involved in feeding as well (Zottoli and Faber, 2000). At the very least, it is hoped that understanding the Mauthner cell will result in an understanding of the simple system that coordinates motor activity (Fetcho and Liu, 1998)

The Mauthner cell has many inputs. Of particular interest to this thesis is the auditory input onto the Mauthner cell. From here, the Mauthner cell outputs onto motor neurons, with descending interneurons and cross-inhibitory interneurons that are also present and able to

participate in synaptic plasticity. Although this thesis is focused on the auditory input onto the Mauthner cell, it receives input from most, if not all sensory system (Zottoli and Faber, 2000).

Specifically, there are a known set of inhibitory interneurons whose processes terminate on the soma and the proximal dendrites of the Mauthner cell. Their purpose is to regulate the excitability of the Mauthner cell by firing hyperpolarizing potentials at the same time that the Mauthner cell is firing its own action potential. The purpose of these interneurons are to glycinergically and reciprocally inhibit the Mauthner cell so that only one fires at a time in response to a sensory input, and that it only fires one (Zottoli and Faber, 2000).

Thus, the activity of the Mauthner cell acts in this manner: when one Mauthner cell is activated, the other is inhibited by the feedback interneurons. This allows a large excitation of motor neurons on the opposite side of the bottom, and the fish forms a C-shape that indicates a C-start escape response.

Research has indicated that it is the M-cell that is the first to fire in response to an alarm stimulus, and other Mauthner cell homologs exist to allow regulation of the strength of the muscle contractions which might propel the fish away. These other homologs, MiD2cm and MiD3cm are believed to help create different forms of the C-start escape reflex that the zebrafish might use to escape from predators (Liu and Fetcho, 1999). Using laser ablation experiments, Liu and Fetcho determined that the loss of the Mauthner cell and its homologs took away the zebrafish's ability to undergo short latency, high performance escapes from both head and tail directed stimuli. Interestingly, killing off only the Mauthner cell only affected the escapes from the tail-directed stimuli (Liu and Fetcho, 1999), leaving the head-directed stimuli C-starts intact.

While these are the primary neurons that initiate the C-start reflex, there is another set of inhibitory neurons, which are the commissural inhibitory neurons which form a bilateral, feed-forward inhibitory network which regulate the Mauthner cell threshold (Zottoli and Faber, 2000). This class of inhibitory interneurons has also been another area of research that could potentially characterize another area where synaptic plasticity occurs during simple forms of learning.

Thus, the benefits of the Mauthner cell includes it's readily defined networks, and its potential to associate a well-defined behavior (the C-start escape reflex) with some synaptic plasticity at the molecular and cellular level. Additionally, potential mutants with altered development of the Mauthner cell and its homologs make the Mauthner cell a prime target for neurobiological research. So much has already been discovered from the study of this simple neuron, including studies that have challenged accepted convention, when it was discovered that protein synthesis occurs outside of the soma in the axon or that axoplasmic ribosomes exist (Zottoli and Faber, 2000).

1.3 WHY THE ZEBRAFISH IS AN IDEAL CANDIDATE AS A MODEL FOR LEARNING AND MEMORY

The zebrafish has many different characteristics that make it an ideal model for the neurobiology study of learning and memory. The first of these is rapid development, as it only takes 72 hours from the fertilization of the new egg to the larval stage, at which point, it is able to inflate its swim bladder, and begin seeking food and avoiding predators (Kimmel et al., 1995). In this thesis project, much of our data is based upon using zebrafish that are still in the larval stage,

at an age of 5 days, which is why this is of particular importance. Being able to use animals that are quite developed at 5 days allows a much large number of experiments to be done.

Additionally, the zebrafish also have a high level of fecundity. A mature female, at the most, can lay hundreds of eggs every few days. Thus, hundreds of zebrafish larvae are able to be harvested from a breeding pair of adult zebrafish, which allows a large number of experiments to be done without limitation due to animal number, as seen from other animal models, such as *Aplysia*. It is also relatively inexpensive, which lends itself well against other animal models such as rats or mice.

In addition, the zebrafish animal presents itself as an ideal candidate for much future studies involving genetic manipulation. Recent scientific advances have made it possible to rapidly disrupt the normal function of a gene during development, by injecting mRNA, DNA, or even short anti-sense oligomers, creating morpholinos (Hogan et al., 2008).

Another benefit of the zebrafish model is that it is transparent in the larval stage, which makes it a good candidate for optical and optogenetic studies in neurobiology (Roberts et al., 2011). In addition to these optical studies of neuronal activity, it is possible to do noninvasive photoablations of individual neurons (Fetcho and Liu, 1998). In particular, Fetcho and Liu were able to deduce much about the function of the Mauthner cell and its homologs with simple laserablations of these cells.

Also, as described in the previous section, despite the fact that *danio rerio* is a complex vertebrate, it has certain simple behavioral phenomena that are controlled by seemingly simple neural circuitry, its Mauthner cell. From above, it is possible to do many neurophysiological experiments on these circuits to fast-track a study of learning and memory. One such simple

behavioral phenomenon is the C-start escape reflex, which is mediated by a well-known set of reticulospinal neurons (the Mauthner cell). Although there are other hindbrain neurons and Mauthner cell homologs that control the C-startle behavior, it is primarily initiated by the Mauthner cell. It is also well known that this circuit is highly plastic (Roberts et al., 2011).

1.4 THE C-START REFLEX

Interestingly, C-startle behavior undergoes many simple forms of learning and memory. One of our goals in this lab has been to create robust protocols that will elicit these simple forms of learning and memory, and then utilize the modern tools of calcium imaging and electrophysiology to study them. For example, habituation is one non-associative form of learning that the C-startle reflex undergoes. During habituation, an animal is given stimuli repeatedly, and over time, it decreases its responsiveness to the stimuli. For example, auditory pips given at 1 Hz for 2 minutes will show a decrease from an 80% response rate to almost 0% response rate in the zebrafish (Rankin et al., 2009). The same goes for the ability for humans to ignore stimuli presented by wearing clothing and accessories.

Sensitization is another simple form of learning and memory. In this case, sensitization is the increased responsiveness of an animal due to a stimulus that may be harmful or be cause for alarm. For example, a gun-shot going off in a room would increase an individual's responsiveness to future stimuli, which would indicate sensitization. In zebrafish, successful sensitization of the animal would result in an increased responsiveness of the animal. Our lab will show that it is possible to elicit sensitization of the C-start behavior by the use of an alarm pheromone as the sensitizing stimulus.

Finally, an ultimate goal in the lab will be to discover a robust protocol that will show classical condition of the C-start behavior. At the moment, no one has discovered such a protocol, but this thesis will describe our current work and attempts in this endeavor. Being able to describe the behavior of classical conditioning in the zebrafish, by teaching it to pair one conditioned response with an unconditioned response will be a huge step forward in our attempts to understand learning and memory.

1.5 HABITUATION OF THE C-START REFLEX

One of the primary goals of this thesis project was progress and continue the experimentation done on “rapid-term” and “short-term” habituation in the laboratory. Because a robust protocol had already been established for these habituation experiments, it is necessary to study the underlying factors that may affect the efficiency and efficacy of these protocols. Factors such as volume of the well, diameter of the well, pulse-duration, as well as the effects of the time of the experiment with studies on the circadian rhythm of the zebrafish were examined and studied in order to fine-tune a habituation protocol. Of course, these findings would be useful in experiments in sensitization and classical conditioning as well. Studies in the boldness and aggressiveness of the fish, as well as the developing environment of the zebrafish were also initiated, as these may have an effect on the responsiveness of the fish and influence its behavior in the subsequent memory and learning experiments.

Before discussing the experiments and results from this thesis project, it is necessary to go into the literature and examine the recent findings in this area and how this project plays a necessary role. One particularly relevant study is one involved the existence of multiple

mechanisms of habituation in zebrafish. Roberts et al. found that there were at least two forms of short-lived habituation of the C-startle reflex. Using auditory stimuli, Roberts was able to induce a “rapid form” habituation which lasted greater than 1 minute but less than 15 minutes (Roberts et al., 2011). He was able to elicit this “rapid form” habituation with 120 pulses of auditory pips delivered at 1 Hz over the course of 120 seconds. Additionally, Roberts was able to discover a more extended form, a “short term” habituation that lasted greater than 25 minutes but less than 60 minutes. This required a more complex spaced training, which consisted of 10 blocks of auditory pulses delivered at 1 Hz (5 min interblock interval., 900 pulses per block), using the same equipment setup as before (Roberts et al., 2011). Using pharmacological studies, Roberts determined that these were actually two different mechanisms since their characterization was blocked by different drugs.

In particular, the “short term” form of habituation depends on N-methyl-D-aspartate (NMDA) receptors, while the “rapid form” did not. Using APV, a common selective antagonist of NMDA receptors, Roberts showed that APV does not disrupt the “rapid form” habituation. The response rate during a 1 minute post-test was not significantly different from the controls. However, using APV during the spaced training described above, there was a significant difference between the control trained and the APV trained, as the habituation index was much closer to zero (Roberts et al., 2011).

Currently, our lab is attempting to find a “long-term” habituation protocol that will elicit a memory that lasts longer than 1 hour, ideally over 24 hours. As a result, it is necessary to describe the effects of certain variables in the experiment. How the fish respond over the course of a day is extremely important, because in a 24 hour memory, they will be tested at many

different points in one day. Additionally, even short-term habituation experiments are often run throughout the day, and it is important to understand if there are any differences in response rate.

1.6 CIRCADIAN RHYTHM IN THE ZEBRAFISH

One of the major concerns throughout our endeavors to discover a “long-term” memory which lasts over 24 hours has been that the tests and trainings interfere or are affected by the Circadian rhythm of the fish. Perhaps they are more responsive at certain times in the day and less responsive at other times of the day. Additionally, short-term experiments are run throughout the day – it is important to know if the time of day affects their behavioral responses.

Contextually, the fish are housed in aquariums with a light/dark schedule but are removed into a completely dark incubator prior to their training and testing. The question of circadian rhythms in zebrafish arose when we came across recent studies that have shown that zebrafish have definitive levels of hypocretin/orexin (Hcrt), which is a common mammalian sleep/wake regulator. Loss of this regulator in mammals often leads to sleep disorders such as narcolepsy (Prober et al., 2006). With regards to development, zebrafish larvae already characterize strong locomotive sleep/wake behaviors as early as the day 5 in their development (Prober et al., 2006). In Prober’s experiments, he went on to overexpress Hcrt in order to determine its effects, using an adenovirus carrying hcart with an enhanced green fluorescent protein gene. He found that overexpression of the Hcrt actually promotes and consolidates wakefulness and inhibits rest (Prober et al., 2006).

Because of the implications of these results, it was important for us to at least determine how the responses of the zebrafish to the auditory pips change throughout the day. Experiments

involving response rate in relation to the time of day were done in order to elucidate this concept. There were also concerns that being placed in a well throughout the entire day could affect the response rate of the fish as well, and experiments to answer the first question would indubitably indirectly answer the second question.

1.7 PHOTOTAXIS IN ZEBRAFISH

Another primary goal of this thesis project was to examine phenomena that could ultimately contribute to a protocol for classical conditioning. This included a study of the “death scent” pheromone, the alarm pheromone, as well as phototaxis.

Our endeavors to find an unconditioned response to use in classical condition have led to attempts to study the phenomenon of phototaxis in zebrafish. Generically, phototaxis is the orientation of the animal away or towards a light, such as that found in mice, to stay hidden. A recent scientific study found that phototaxis was also found in zebrafish, that there is a differential use of the retinal ON- and OFF- pathways which allow them to undergo phototactic movements (Mueller and Neuhauss, 2010).

Burgess’ experiments found that there were two retinal pathways for zebrafish visual information. The first is the ON- pathway, which is activated by an increase in light intensity, and the second is the OFF- pathway, which is activated by a decrease in light intensity (Mueller and Neuhauss, 2010). Elegantly using a mutant line of zebrafish with disrupted ON- pathways, they were able to study each of these retinal pathways independently (Burgess et al., 2010).

Thus, during positive phototaxis, there were no additional scoots (routine turns and slow swims) for the ON- pathway mutants, since the increase in light intensity was not picked up by the fish. Because there was also no additional scoots during negative phototaxis, Burgess concluded that it is probably the ON- pathway that regulates the approach of the fish by triggering these scoots (Burgess et al, 2010). Burgess then showed that the purpose of the OFF- pathway was necessary for the regulation of the turns. Using laserablation of the optic tectum, he was able to present that the OFF-pathway triggers turns when there is a decrease in the amount of light. If the eye on the left perceived less intensity, the fish would turn away from that eye.

This interesting discovery showed that zebrafish were indeed capable of phototactic movements, and one endeavor of this thesis project was also to determine a protocol to elicit this phenomenon and then potentially apply it towards classical conditioning as the unconditioned response.

1.8 ZEBRAFISH BOLDNESS AND ITS EFFECT ON ITS SOCIAL STATUS

Another variable in these experiments were the boldness of the zebrafish. Generically, the average response rate to an auditory pip of 27 dB was around 80%. However, due to sample size, it was sometimes possible to gather an average response rate of 60%, and requiring large sample sizes to ensure that an accurate and stable value could be represented. One of our hypotheses was that it could be affected by the boldness of the zebrafish. Recent studies have shown that there are in fact differences in boldness among zebrafish of the same clutch, and there were actual pecking orders which could categorize these zebrafish.

In fact, a recent study proposed that boldness of a zebrafish, measured by a variety of factors, could be used to predict social status (Dahlbom et al., 2011). Using a variety of different environments, including an unfamiliar open field, an unfamiliar roofed field, or a familiar field with an unfamiliar object, Dahlbom observed and recorded their behavior, giving them a relative boldness score, based on how long they were near the unfamiliar object or in the unfamiliar zone. Then, placing them together with other fish, he was able to determine where they stood on the social ladder, by looking for where they were swimming (who was swimming higher), or who ate first, or who attacked who.

As a result, the study found that in general, males were bolder than female, and that boldness was related to social status (Dahlbom et al., 2011). The fact that this boldness exists could contribute to the variability of the response rate and another goal of this thesis project was to determine if boldness could be easily determined and then fishes segregated for more robust average response rates, without requiring such large sample sizes.

1.9 HYPOXANTHINE-3-N-OXIDE, A POTENTIAL SCHRECKSTOFF IN ZEBRAFISH

The idea of a Schreckstoff, or an alarm substance has been around for a long time. However, conflicting past studies has shown that these do not reasonably exist. In fact, an experiment in 1996 passed fish through a skin extract and a control substance, and there was no difference between the behaviors of the treated and the control (Magurran et al., 1996). There was no change in movement, whether it was hiding, darting, or freezing (Magurran et al., 1996).

However, recently, a new study from Gerlai's laboratory showed that there was indeed some sort of alarm pheromone which resulted in certain behavior. Although the quantification

was done by manual observation, Speedie used erratic movement as the most reliable way to determine if a substance could indeed cause alarm (Speedie and Gerlai 2008). In their research, they extracted a pheromone using Waldman's recommendations. With different doses, high, medium, low and control, they exposed the fish to the alarm pheromone and observed and recorded the consequential movements. To facilitate the movement, a predatory fish was placed in the tank as well (Speedie and Gerlai 2008).

From their research, they extracted a chemical substance that shared some similarity to the alarm substance from the species belonging to the order Ostariophysi (Pfeiffer 1977). Specifically, this alarm substance is named hypoxanthine-3-N-oxide, or H3NO (Speedie and Gerlai 2008).

Considering that an alarm substance can be analogous to the gun-shot as it is in humans, our lab theorized that it would be possible to use this alarm pheromone, H3NO, in order to elicit sensitization. The results described later in this thesis will discuss this in further detail. Further experimentation has been started in order to utilize the death scent in classical conditioning, but at the moment, these attempts have proven null.

CHAPTER 2: GENERAL METHODS

2.1 PROCUREMENT AND ANIMAL CARE FOR ZEBRAFISH

Zebrafish animals were obtained from the Zebrafish Core Facility at the University of California, Los Angeles (UCLA). They are classified as Wild-type TL zebrafish. Using standard breeding protocols, zebrafish eggs were collected and placed into an artificial tank water solution of E3. E3 is a saline used to maintain the fish during experiments. It is a mixture which is made up of 5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, 0.33 mM MgSO₄, and 0.00001% (w/v) Methylene Blue, with a pH of 7.2. They are then incubated at 28.5 degrees Celsius to allow for growth and development. The fish were fed with paramecia (Roberts et al., 2011).

2.2 RECORDING AND ANALYSIS OF THE ANIMAL BEHAVIOR

Measuring the zebrafish C-startle reflex was done by using a high speed camera, the TroubleShooter TS100MS (Fastec Imaging, San Diego, CA), and recorded at 1000 frames per second. To provide the visual contrast necessary to see and record the zebrafish C-startle reflex in their wells, a light box had to be used (Gagne Inc., Johnson City NY). The auditory/vibrational (AV) pulse was provided by a speaker that was placed behind the wells. The AV pulse and the camera were connected to a Master 8 box which could coordinate the introduction of the pulse with the start of the camera recording. An LED was also attached to the Master 8 in order to designate visually when the AV pulse was given (Roberts et al., 2011).

2.3 GENERAL EXPERIMENTAL DESIGN

In general, the zebrafish were left to acclimate for 1 hour before each experiment. Specifically, they were collected from the incubator, placed in the wells, and placed in the

experimental room next to the speaker for 1 hour before the experiment in order to allow them to calm down from being physically moved and placed into the well and also to the new environment of the experimental room. Generally, the auditory/vibratory (AV) pulse given was a 1 ms-long (200 Hz ramp wave), 109 dB auditory pulses, produced with a function generator (NS-R2001; Leader, Cypress, CA) and amplifier (Insignia, Richfield) (Roberts et al., 2011). A C-startle response was designated when the animal had the characteristic C-bend within 25 millisecond after the AV pulse was given (Roberts et al., 2011).

2.4 SPECIFIC RAPID-TERM HABITUATION PROTOCOL

For experiments dealing with short-term habituation, zebrafish were first given a 1-acclimation period as stated earlier. Then, habituation training consisted of 120 1-Hz pulses, given at 109 decibels (dB). The success of the training was dependent on a 1 minute post-test, at which a pulse was given 1 minute after the final pulse, to determine whether or not the fish had habituated to the AV pulses. In some experiments, multiple pre-tests and post-tests were taken to establish a more accurate behavioral pattern, for example, taking 5 pre-tests before the training, and 5 post tests after the training, with an inter-stimulus-interval (ISI) of 1 to 5 minutes (Roberts et al., 2011).

2.5 ANALYSIS

To statistically analyze the results, the results were compared using the program INSTAT and the appropriate test. Depending on the circumstances, a two-tailed unpaired t-test, or a one-way ANOVA was used. Manual observation of the C-start was used.

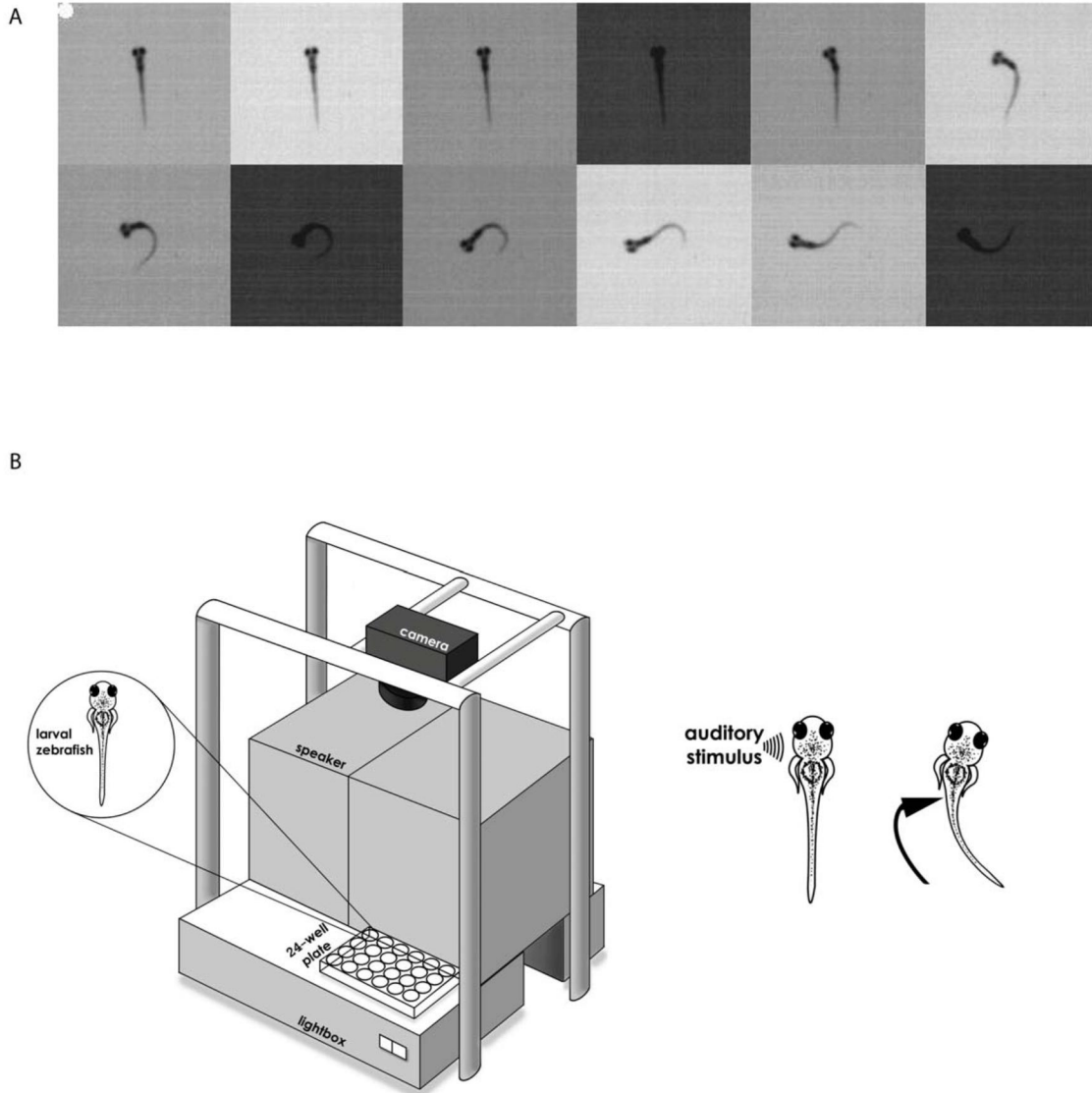


Figure 1. C-start reflex in larval zebrafish and experimental apparatus used to study the behavioral plasticity of that reflex.

(A) A representation of the C-start reflex in a zebrafish larva (Day 5). The white dot in the first frame depicts the instance at which the auditory/vibrational (AV) pulse is given. These were recorded at 1000 frames per second, with every other frame shown for brevity.

(B) A visual representation of the experimental apparatus used to elicit the reflex. A light box allows visualization of the 24-well plate, with a speaker behind the plate to produce the AV pulse. A camera at the very top of the setting allows the camera to begin filming at moment the pulse is given. Each well was filled with 3 mL of E3, and contained one zebrafish larva, allowing filming of 24 zebrafish per session.

CHAPTER 3: BASIC RESPONSE PROPERTIES THAT INFLUENCE THE C-START ESCAPE RESPONSE

3.1 THE EFFECT OF E3 VOLUME IN THE WELL ON RESPONSE RATE

Generically, the response rates of the C-startle reflex of the zebrafish are already relatively variable. One of our primary goals was to establish certain basic response properties that would enable us to obtain the highest and most robust possible response rate. The volume of the E3 in the well, the diameter of the well, pulse length, as well as the effect of the zebrafish circadian rhythm were all properties that we wanted to define. Arbitrarily choosing certain values to do experiments would ignore the fact that there were perhaps values that could give us more vigorous response rates.

The first variable examined was the volume of each of the wells. Using a 24-well plate, the volume of the well was 3 mL. To determine if volume was influential of response rate, we compared the response rates for zebrafish in wells of volumes 3.5 mL, 3 mL, 2 mL, 1 mL, 0.5 mL, and 0.25 mL. In particular, the 3.5 mL volume created a meniscus that reached over the top of the well. A volume higher than 3.5 mL would have made visualizing the C-startle reflex of each zebrafish difficult.

The reason that this could be important is the oxygen content of the wells. Some experiments may involve training the zebrafish for hours, and it is possible that even after 1 hour of acclimation, the oxygen content becomes insufficient for healthy functioning of the animal.

From these wild-type TL zebrafish, we determine results that proved that after a certain volume, volume was no longer significant. However, low volumes of water certainly affected the C-startle reflex response rates of the zebrafish, which is logical since lower volumes of water provides less oxygen content, as well as less space to even respond with the C-start. Incomplete

submerging of the zebrafish larvae could have also played a role in discomfiture of the larvae and decreased response rate.

The protocol utilized was to place the Day 5 TL wild-type zebrafish into wells of differing volumes of E3, 3.5 mL, 3 mL, 2 mL, 1 mL, 0.5 mL, and 0.25 mL. They were then allowed to acclimate for an hour, before taking 5 post-tests of AV pulses with 5 minute inter-stimulus intervals. The results given are an average of those 5 post-tests (i.e., if the animal responded all 5 times, then it would be scored 100%, 4 times out of 5 scored 80%, and so on).

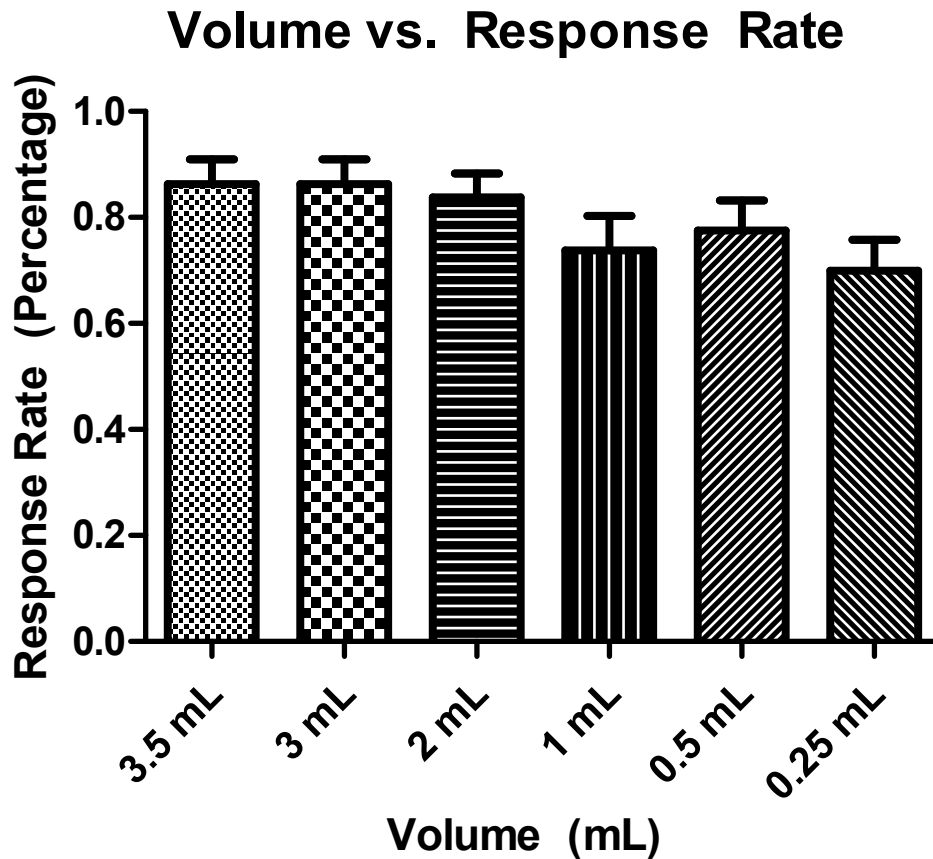


Figure 2. Volume vs. Response Rate

(A) The graph depicts the different volumes of the zebrafish wells used, 3.5 mL, 3 mL, 2 mL, 1 mL, 0.5 mL, and 0.25 mL. The wells were filled with E3. The average response rate for them was 0.8625, 0.8625, 0.8375, 0.7375, 0.7750, and 0.7700 respectively. These are the averages of

each well for 5 post tests with an ISI of 1 minute. The P value is 0.1603, using a one-way analysis of variance (ANOVA), and is considered not significant. However, it is our belief that these differences will be significant with increased sample sizes.

As seen from the data, it appears that a volume of 3 mL is ideal for a response rate of 86.5% from the zebrafish. Any further, include adding E3 above the level of the well, did not significantly change the response rate. This finding was logical because given enough O₂, and room to move was important to obtain a robust response rate. However, the question arises about the diameter of the well. Using smaller wells (i.e. a 48 well plate) would allow us to do a greater volume of experiments, but would require smaller volumes and diameters. Does the diameter also play a role in producing a robust response rate of the C-startle reflex?

3.2 THE EFFECT OF WELL DIAMETER ON RESPONSE RATE

Another variable that could potentially affect response rate was the diameter of the well itself. In theory, a well that could not comfortably fit the zebrafish would affect its ability to initiate and complete a C-startle reflex. However, the smaller the diameter of the well, the greater the quantity of experiments that could be done in a single session. Thus, it was a matter of efficiency in terms of discovering an ideal diameter, and whether or not diameter would affect the robustness of the results.

Using 3 different plates, a 24-well plate, a 48-well plate, and a 96-well plate, we wanted to determine what the effect of diameter was on response rate. In order to keep the volumes constant, a volume of 0.4 mL was utilized in order to keep the volumes constant, since 0.4 mL

was the largest that the 96-well plate could comfortably contain. Of note is that this volume of 0.4 mL was significantly smaller than that of the 3 mL or greater result determined by the previous experiment.

The protocol utilized was to use 0.4 mL volume of E3 in each plate. 24 animals were placed in the 24-well plate, while 30 animals were placed in the 48-well plate and the 96-well plate, in order to keep sample size constant. The 24-well plate had a diameter of 1.5 cm, the 48-well plate had a diameter of 1 cm, and the 96-well plate had a diameter of 0.75 cm. After allowing the animals to acclimate for 60 minutes, 5 post-tests were given and recorded with 5 minute inter-stimulus intervals, and an average response rate was obtained for each well. The experiment was done with day 5 old wild type TL zebrafish.

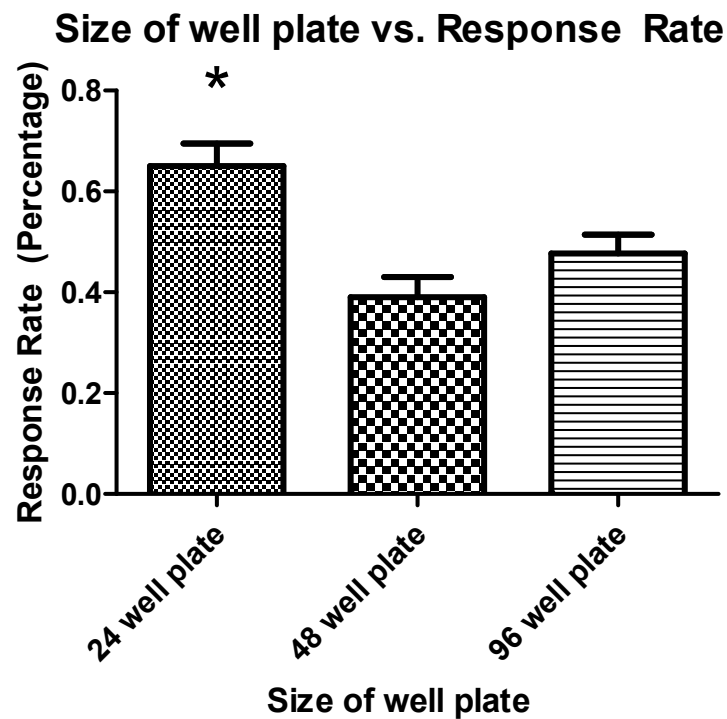


Figure 3. Diameter vs. Response Rate

(A) The results determined that the 24 well plate had an average response rate of 65%, while the 48-well plate had an average response rate of 39%, while the 96-well plate had an average response rate of 48%. There was a sample size of 48, 60, and 60 respectively. Using a one-way ANOVA test, the p value was determined to be less than 0.0001, and considered extremely significant. The Tukey-Kramer Multiple Comparisons Test was also utilized and it also determined that the p value was less than 0.05, and the results were significant.

As seen in the results, the 24 well plate had the largest response rate, although it was only at 65%. There are a few things that can be determined from this, the first of which is that volume appears to be more important than well-plate diameter. As long as the zebrafish can comfortably initiate and complete the C-startle reflex, volume becomes the most important factor. As noted, with a 0.4 mL diameter, there was only a 65% response rate for the 24-well plate, while the 3.0 mL volume for the same well plate in the previous experiment showed that there was an 86.5% response rate. The smaller diameter well plates could have also had inhibitory processes due to the constraint. Moving forward, an ideal experimental setup would be to use a 3.0 mL volume along with a 24-well plate.

3.3 THE EFFECT OF PULSE DURATION ON RESPONSE RATE

The pulse duration experiments determine if there is an effect of pulse duration on the response rate of the zebrafish. That is, the length of time that the auditory pip is delivered to the zebrafish. The theory behind this idea is that certain feed-forward inhibitory mechanisms are often too slow to respond to short pulses. Utilizing longer pulses may allow these feed-forward inhibitory synapses more time to exhibit plasticity.

The protocol for these pulse duration experiments are similar to those described above, but with a few changes. The three pulse durations being measured are 1 millisecond, 10 millisecond, and 50 millisecond. To utilize time efficiently, three separate groups of day 5 TL – WT were used alternately. Specifically, the protocol called for first acclimating the first set of fish for 1 hour. Then 5 post-tests were given with 5 minute inter-stimulus-intervals (ISI) at with a 1 millisecond pulse duration at 27 dB. A 15 minute rest period was given, and then 5 post-tests were given with a 5 minute ISI at the next pulse duration of 10 millisecond. One final 15 minute rest period was given before giving and recording a final 5 post-tests with a 5 minute ISI with a 50 millisecond pulse duration. At this point, the next set of fish replaced the first set and it was allowed to acclimate for an hour before these experiments were repeated for 2 more sets of fish. The volume was also varied, in order to account for weaker response rates. Utilizing the volume of 27 dB may hide the effect of pulse duration so lowering the volume to 15 dB and 0 dB was able to overcome that effect.

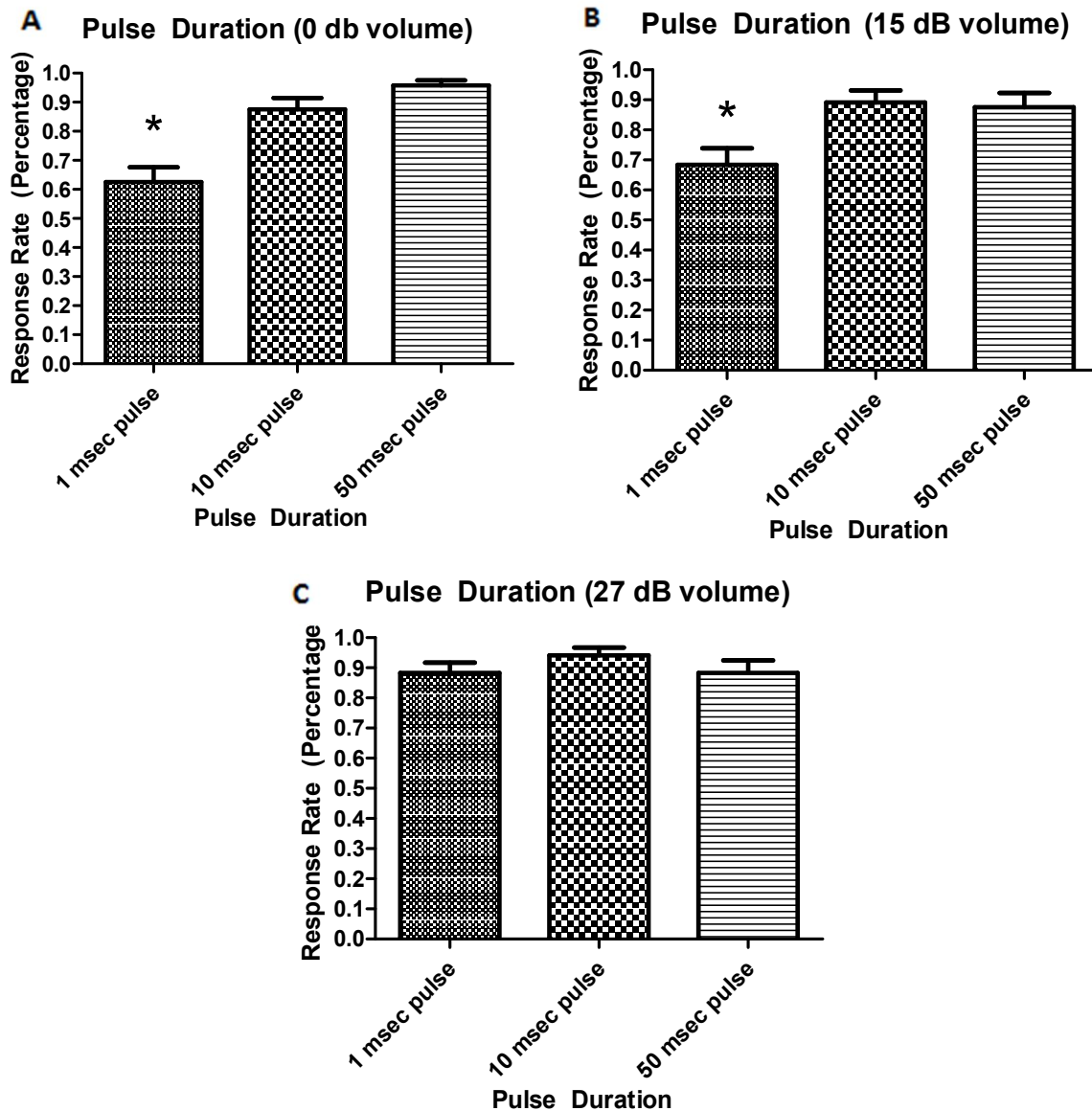


Figure 4. Pulse Duration vs. Response Rate

(A) In A, the pulse duration data was collected at a volume of 0 dB. As seen from the graph, the 1 millisecond pulse was significantly different from the other two, providing a much lower response rate. Using a one way ANOVA, the P value was less than 0.0001, and is considered extremely significant. A consequent Tukey-Kramer Multiple comparisons Test also confirmed that the P value is less than 0.05.

(B) In B, the pulse duration data was collected with a volume of 15 dB. At this volume level, the 1 millisecond pulse was significantly lower from the other two. Using a one way ANOVA, the P value was determined to be 0.0046, and is considered very significant. A consequent Tukey-Kramer Multiple comparisons Test also confirmed that the P value is less than 0.05.

(C) In C, the pulse duration data was collected with a volume of 27 dB. As expected, at this high of a volume, it was enough to hide any effects from pulse duration, as the sound was extraordinarily alarming. In this case, a one way ANOVA test gave a P value of 0.3858, considered not significant.

One of our goals was to see if they tended to respond to just the beginning of the pulse, or if we extended it, would there be an increased in the number of responses due to a response from the end of a long pulse. As mentioned in the introduction, there are many inhibitory neural circuits which hit the Mauthner cell, often with a delay. Thus, longer pulses give inhibition a chance to play a role (Zottoli and Faber, 2000).

3.4 MEASURING THE RESPONSE RATE OVER AN ENTIRE DAY (CIRCADIAN RHYTHM IMPLICATIONS)

Because many of our future experiments trying to find a 24 hour memory required training and testing the zebrafish over the course of an entire day, it was important for us to understand any changes in response rate that might occur, whether due to the circadian rhythm of the fish, or perhaps exhaustion, or even the viability of leaving them in their wells over a 24 hour period.

To test this, our protocol used the same experimental setup as before, with AV pulses delivered from a speaker, at 200 Hz, with a 2 millisecond duration, and a volume of 27 dB. We would allow a set of 24 fish to acclimate for 1 hour, and then giving them 3 tests, with 5 minute ISI. We would then record the C-start averages for that hour. Alternating sets of fish allowed us to allow a 1 hour acclimation time before each set of tests, and we were able to get a range of data over the course of an entire day. The fish used were Day 5, TL-WT fish.

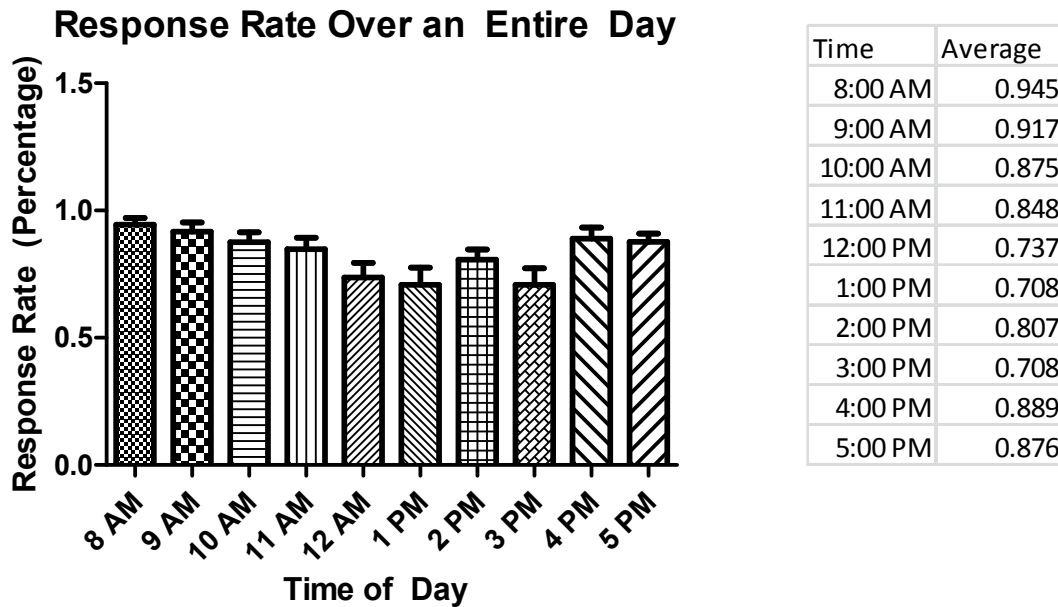


Figure 5. Response Rate Over an Entire Day

(A) As seen in the figure, the response rates over the day are almost bimodal, although these values are not significant. The averages are seen in the table to the right.

From the data, it looks possible that there is almost a bimodal curve in terms of the response rate of the course of the day. The complete story might be told if the experiment was done over 24 hours, rather than just over the course of a working day. One important conclusion to take from these results is that the response rates don't differ that much between the morning, say 11 AM, and the afternoon, at 5 PM. This means that experiments done in the morning and the evening should not differ significantly in terms of the response rates.

3.5 DISCUSSION

From the results of the previous three experiments, we can conclude that there are certain values that can be utilized for each of those variables that might create a more efficient and robust response rate from the zebrafish, especially during experiments that require the elicitation of the C-start escape reflex. In particular, the volume of the well should be at least 3 mL, and despite the fact that there was no significance, it makes sense to have a sufficient amount of medium in order to allow the fish to comfortably initiate and carry out a C-start reflex, as well as provide enough oxygen content over the course of the experiment. In terms of well-diameter, the conclusion goes in line with that of the previous variable, that the larger the well diameter, the more likely occurrence of the C-start. This is quite logical, since at the 96 well size, it is already difficult for the zebrafish larvae to be completely straight.

It is interesting to note that the same volume was used for all three well-diameters. This meant using 0.4 mL in each of the wells. From the previous experiment, we find that ideal volumes should be minimally 3 mL. This is clearly reflected in these sets of experiments, as even with the normal 24-wells, the zebrafish were only responding at a rate of 65%, much lower than the usual response rate of ~80%, as seen in the first set of experiments. Another interesting comparison to make is that when using 0.5 mL in the first experiment, the response rate was still quite high, at around ~78%. When using 0.4 mL in these second set of experiments, the same 24-well size with 0.4 mL reflected a response rate of only 65%. This indicates that for the zebrafish response rate, the more important variable is the diameter.

Finally, the pulse duration experiments concluded that there was a much higher response rate when using the 50 millisecond pulse duration. This effect disappears at 27 dB, but it is

clearly visible and significant at 0 and 15 dB. Although this requires more experimentation involving habituation in order to determine its effects on memory and synaptic plasticity, we can see that it already has an effect on response rate. One possible theory for this simple increase in response rate is actually quite simple – the longer pulse duration makes the sound appear louder, and evokes a greater probability of the animal responding. More likely, however, is that this increase in response rate is due to the fact that you are getting more late responses, so the total number of responses goes up. However, there are so many possible mechanisms that could mediate this, and further research should look into latency. One potentially immediate consequence of this finding is that during habituation, the inhibitory synapses get stronger. So, utilizing a longer pulse, there is a greater chance of the inhibitory synapses playing a role, producing an enhancement of the feed forward inhibition.

Knowing the effect of each of these variables will prove useful in the future when continuing with the same experiments, and choosing how to best train them.

CHAPTER 4: PHOTOTAXIS, DEATH SCENT, SENSITIZATION AND THE PURSUIT OF CLASSICAL CONDITIONING

4.1 PHOTOTAXIS

As discussed earlier in the literature review, phototaxis is the orientation of the animal away or towards a light. A recent scientific study found that the phenomena of phototaxis is present in zebrafish, that there is a differential use of the retinal ON- and OFF- pathways which allow them to have phototactic movements (Mueller and Neuhauss, 2010).

One of our preliminary ideas about phototaxis was that if phototaxis existed in zebrafish, it could be a potential unconditioned response. Since one of the things that we were actively trying to achieve was to classically condition the fish, we were constantly exploring factors and behaviors that we could establish consistently.

Because shoaling is an activity that zebrafish are known to participate in, we felt that their phototactic movements would be affected by the number of fish were around them .In this way, during our phototaxis experiments, we either had them alone, in a group of 10, or in a group of 30.

Much of the procedures described above in the Methods sections were utilized, including using Day 5 TL-WT zebrafish, but we obviously no longer needed an auditory setup. Instead, we placed the fish into a singular compartment that was split into two, a light side, and a dark side. The container was in fact placed on a light box, with thick material covering the container to create a dark side. The light side was then considered the “lit” bottom portion of the rectangular container, while the dark side was the “dark” top portion of the same container. The container

was filled with 30 mL of E3, and a Styrofoam cover was placed over the rectangular container to prevent the zebrafish from being affected by movements around their housing.

Observations were made from the top of the container, and consisted of counting the number of fish in the bottom zone. The control experiments were exactly the same as the regular experiments, only they did not have a dark zone – the thick covering was removed so that both halves got equal amounts of light. Observations were made regularly, once at 1 minute, 2 minutes, 3 minutes, 4 minutes, 5 minutes, 15 minutes, 25 minutes, and 45 minutes. These values are presented in the follow graphs. The average percentage that the fish spent in the bottom zone is also given.

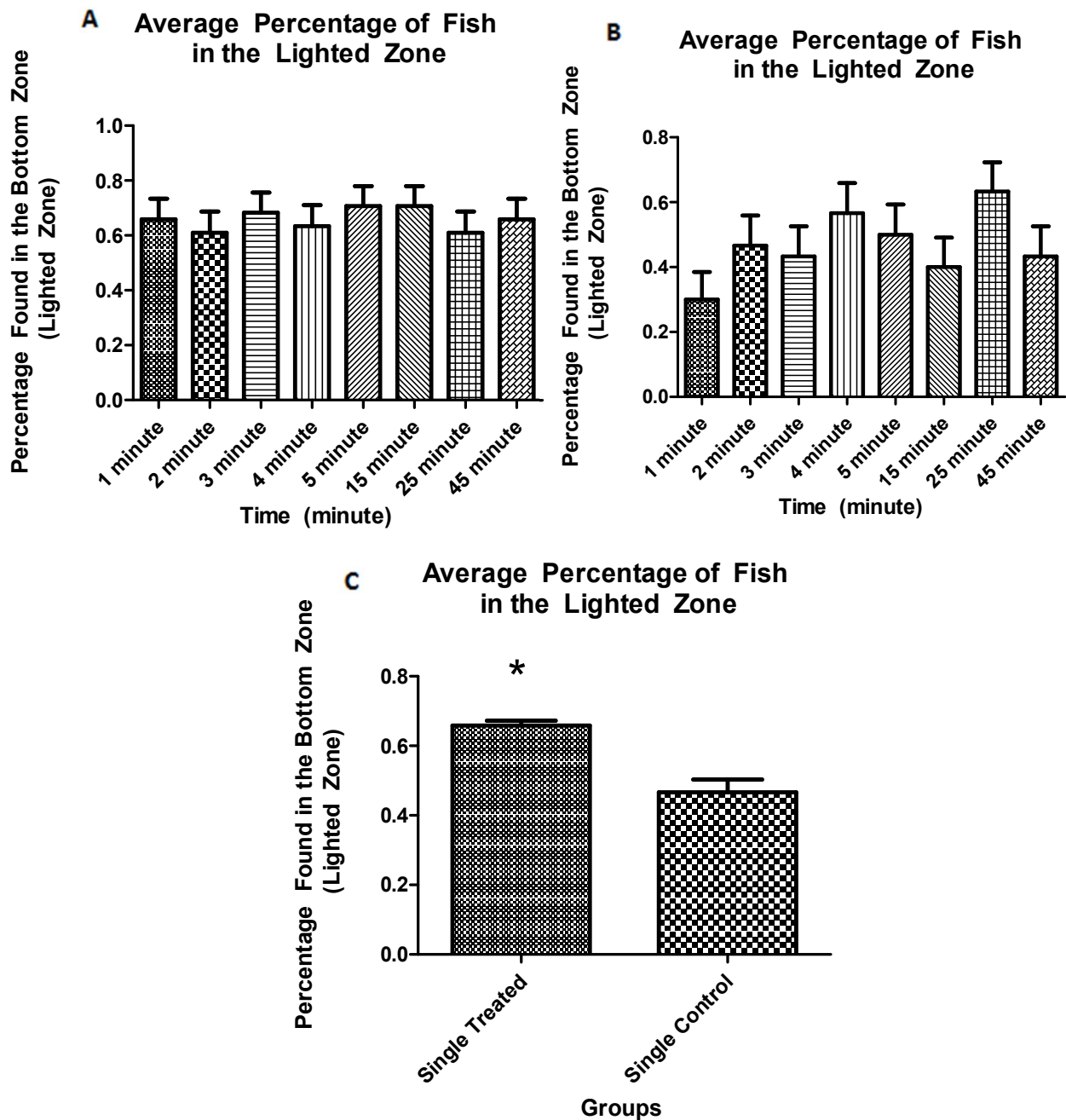


Figure 6. Phototaxis in a single zebrafish

(A) Figure 5 is a series of panels showing the results from phototaxis with a single zebrafish. Panel A is the results from the average percentage of the fish in the “lighted” side of the container in a regular experiment. Over all, the average percentage of the time that the zebrafish was in the “lighted” zone was 65.85%.

- (B) Panel B shows the results from phototaxis with a single zebrafish but from the control experiment. This means that both sides of the container were “lighted”, but measurements were still of the number of fish that were in the bottom, or the normally “lighted” side. The average percentage of time that the zebrafish was in the bottom zone was 46.66%, which is relatively close to 50%, or random.
- (C) Panel C is a compilation of the data, comparing the average values from the normal experiment to the average values in the control experiment, and the results were run through INSTAT for significance. Using an unpaired t test that asks if the means of the Single Treated and the Single Control differ significantly, the two-tailed P value was determined to be 0.0002, which is considered extremely significant.

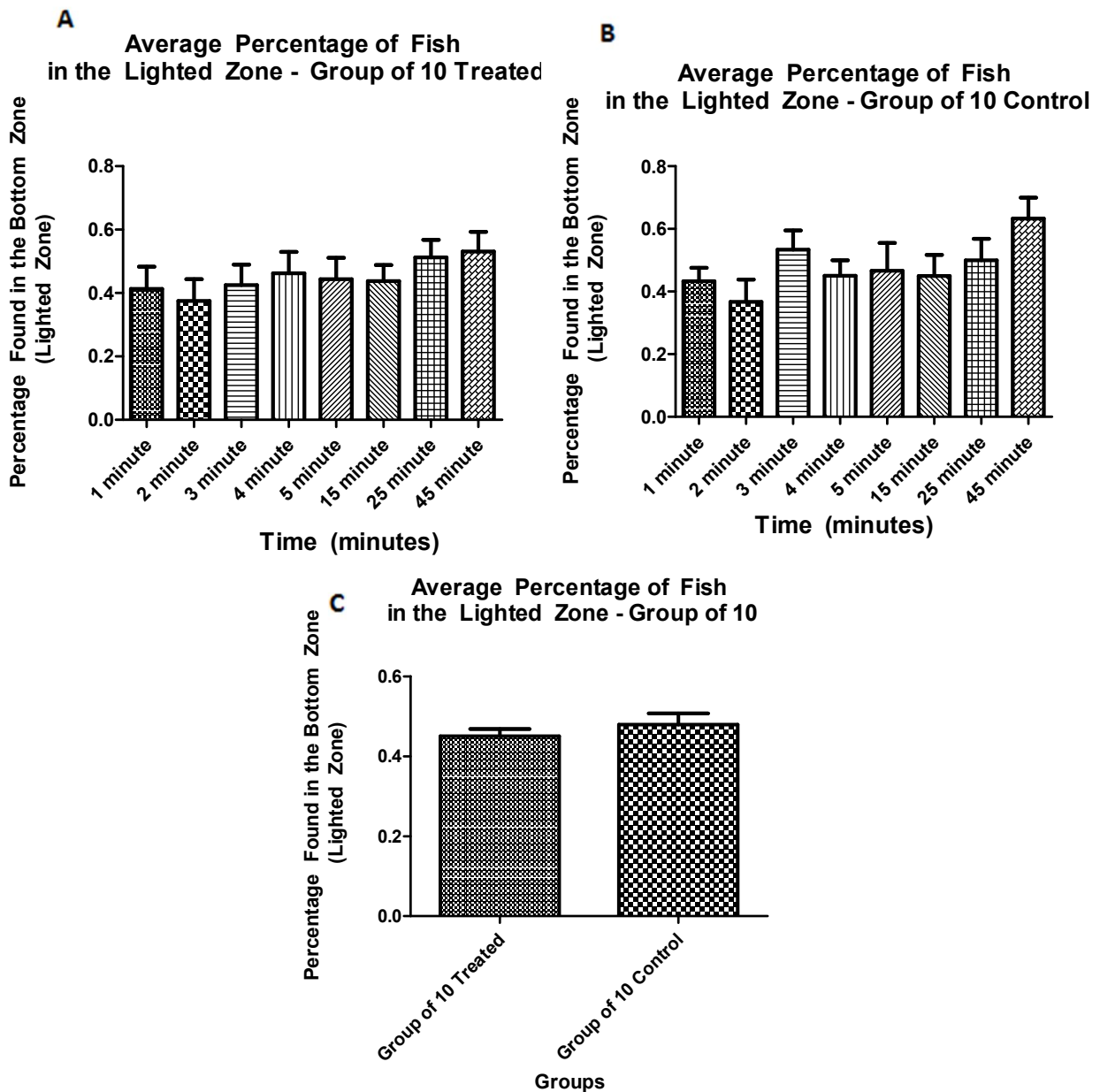


Figure 7. Phototaxis in a Group of 10 Zebrafish

- (A) Figure 6 is a series of panels showing the results from phototaxis in a group of 10 zebrafish. Panel A is the results from the average percentage of the fish in the “lighted” side of the container in a regular experiment. Over all, the average percentage of the time that the zebrafish was in the “lighted” zone was 45.01 %.
- (B) Panel B shows the results from phototaxis with a single zebrafish but from the control experiment. This means that both sides of the container were “lighted”, but measurements were still of the number of fish that were in the bottom, or the normally “lighted” side. The average percentage of time that the zebrafish was in the bottom zone was 47.91%.
- (C) Panel C is a compilation of the data, comparing the average values from the normal experiment to the average values in the control experiment, and the results were run through INSTAT for significance. Using an unpaired t test that asks if the means of the Single Treated and the Single Control differ significantly, the two-tailed P value was 0.3979, considered not significant.

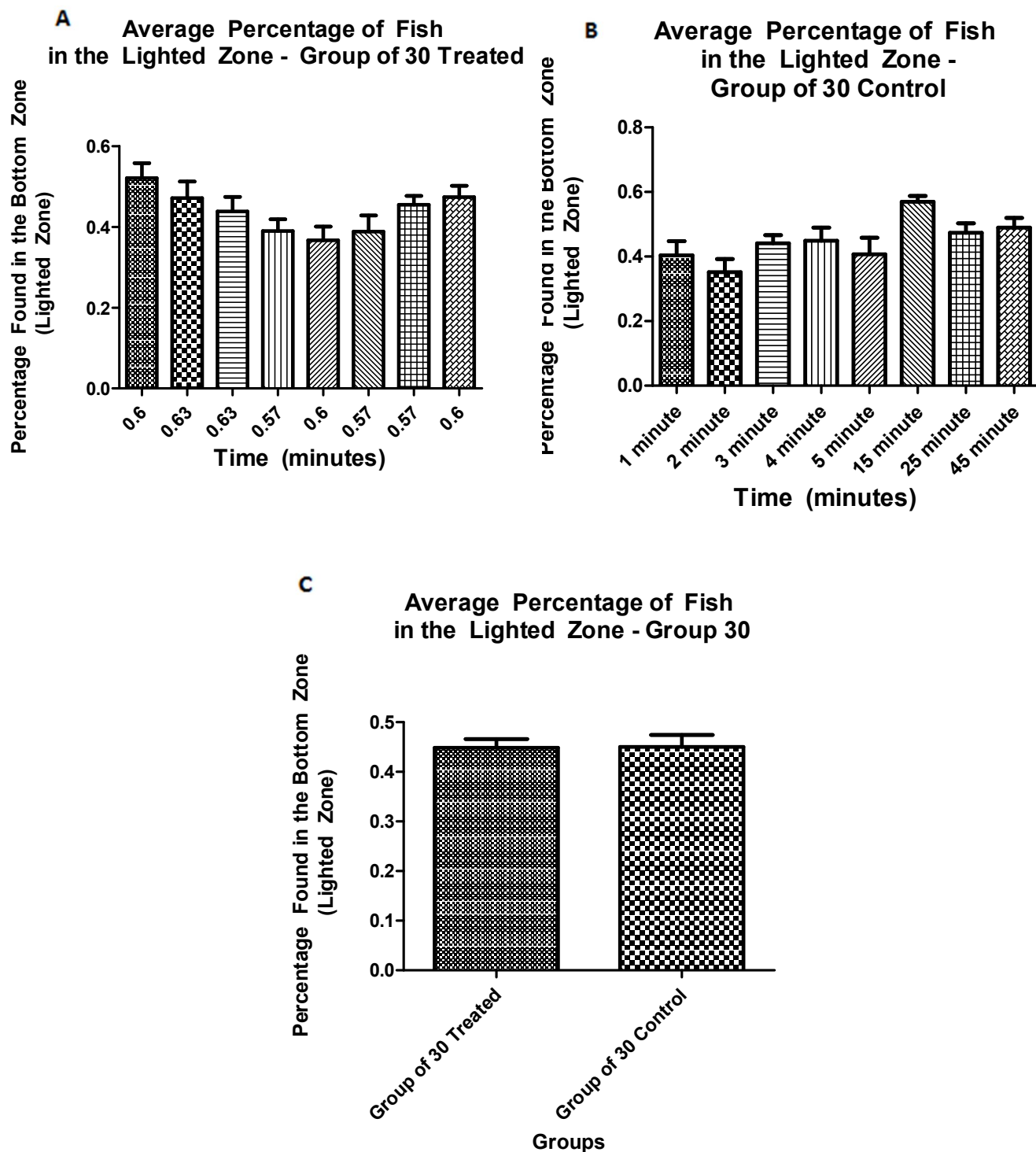


Figure 8. Phototaxis in a Group of 30 Zebrafish

(A) Figure 7 is a series of panels showing the results from phototaxis in a group of 30 zebrafish. Panel A is the results from the average percentage of the fish in the “lighted” side of the container in a regular experiment. Over all, the average percentage of the time that the zebrafish was in the “lighted” zone was 44.86 %.

- (B) Panel B shows the results from phototaxis with a single zebrafish but from the control experiment. This means that both sides of the container were “lighted”, but measurements were still of the number of fish that were in the bottom, or the normally “lighted” side. The average percentage of time that the zebrafish was in the bottom zone was 45.03%.
- (C) Panel C is a compilation of the data, comparing the average values from the normal experiment to the average values in the control experiment, and the results were run through INSTAT for significance. Using an unpaired t test that asks if the means of the Treated and the Control differ significantly, the two-tailed P value was 0.9566, considered not significant.

4.2 SCHREKSTOFF AND ITS IMPLICATIONS IN SENSITIZATION

Besides habituation, another simple form of learning and memory is sensitization. Sensitization is a learned behavioral enhancement due to an aversive stimulus, such as an electrical shock (Mackey et al., 1987). In the Mackey’s case, an electrical shock delivered to the tail causes a markedly stronger siphon-withdrawal reflex, which would be sensitization. It is possible to then pair the aversive stimulus with a stimulus to the reflex, which would ultimately cause the reflex to undergo classical conditioning. Because one of our goals was to elicit classical conditioning of the C-start escape reflex, we felt that it was necessary to characterize the C-start reflex in zebrafish, a loud gun-shot, so to speak.

Reading about the novel research done on the possibility of an alarm pheromone in zebrafish was exciting because we felt that it was exactly what we needed to cause a behavioral enhancement. Previous attempts to sensitize the fish with extremely loud auditory pips or environmental disturbance proved fruitless, but it seemed possible that if a skin extract could elicit more erratic behavior, it could lead to sensitization (Speedie and Gerlai 2008). Our hypothesis was to use Speedie’s suggestion for the skin extract that was acting as an alarm pheromone, H_3NO , in order to obtain the desired response in our zebrafish.

Originally, our plan was to continue to use larval zebrafish, in order to maintain a level of consistency among our experiments. However, numerous failed experiments indicated that perhaps their ability to sensitize was either underdeveloped (we were using only Day 5 larvae), or that their response to fearful or surprising stimuli was not to sensitize, but to flee and decrease response rate (as seen in mice). Thus, we turned to adult zebrafish in order to conduct our sensitization experiments.

The alarm pheromone in question was hypoxanthine-3-N-oxide, or H3NO. After numerous attempts were completed in order to fine-tune a robust protocol to characterize the sensitization, one was developed. The final protocol consisted of an hour of acclimatization, with 2 fish being recorded at the same time. Each fish was in its own chamber, in 150 mL of tank water. Each fish was shielded from each other, so as to not affect each other's behavior. After the hour of acclimation, a threshold was measured, to determine baseline responses. Starting at 1 dB, and moving up incrementally by 2, auditory pulses were delivered every 5 minutes. At the first volume that a C-start was visualized, that volume was recorded as the threshold.

After measuring threshold, a 15 minute wait time was given before administering either 100 nanomolar of the E3 control, or the H3NO alarm pheromone. Another 5 minutes was given before measuring post-test threshold, using the same method as indicated for the pretest threshold. If sensitization occurred due to the alarm pheromone, the post-test threshold should be lower (the fish would respond to a lower volume in comparison to their original pretest threshold).

Three primary different types of experiments were carried out, in order to both prove the validity of the sensitization, as well as to characterize its behavior.

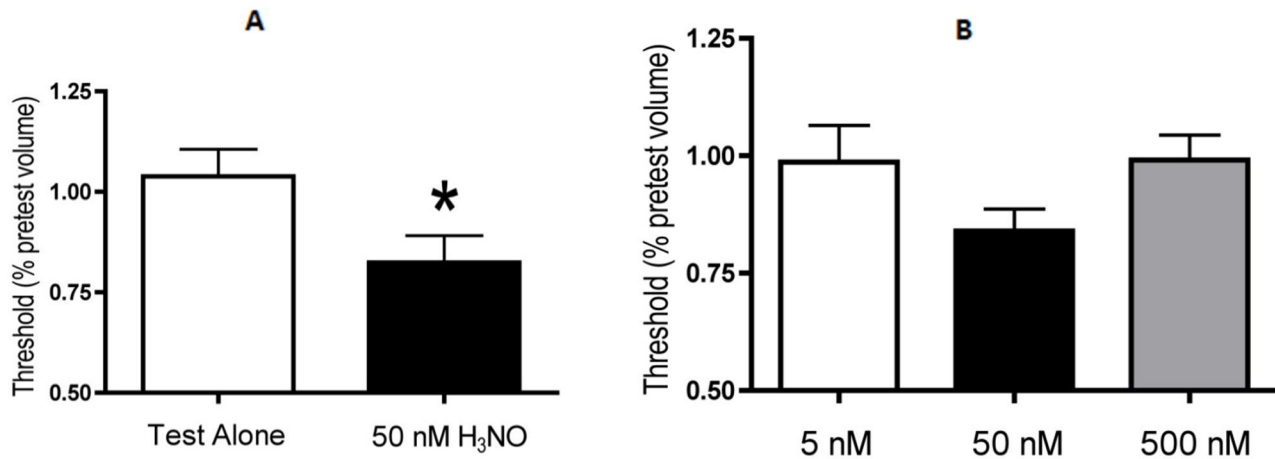


Figure 9. Sensitization of Adult Zebrafish with an Alarm Pheromone

- (A) Exposure of an alarm pheromone (H₃NO) in adult zebrafish induced sensitization of the C-start reflex in response to Auditory/Vibrational (AV) pulses of sound. In panel A, we determined the volume (dB-threshold) required to elicit a C-start escape response before and after application of an alarm pheromone or a control vehicle. Compared to the vehicle-given animals, the zebrafish that were given to H₃NO respond to an AV threshold that was significantly lower than their pretest threshold.
- (B) Panel B displays our experiments in which we varied the concentration of the alarm pheromone, from 5 nanomolar to 50 nanomolar, to 500 nanomolar. In this case, only the 50 nM concentration was able to elicit sensitization of the C-startle reflex, and interestingly, both the 500 nM and 5nM were unable to differ significantly from the control (threshold % at 100).

We also did a third type of experiment which measured the changes in response rate based upon the time differential between the delivery of the alarm pheromone and the time of the posttest threshold (results not shown). In this case, it was shown that there was only a 1-5 minute window in which the alarm pheromone had a significant effect.

4.3 DISCUSSION AND THE PURSUIT OF CLASSICAL CONDITIONING

As seen from panel A in Figure 8, we were able to confirm that delivery of the alarm pheromone was able to successfully sensitize the zebrafish (control zebrafish remained at 100% of the original volume, while H₃NO-fish decreased to 80%). However, it was the second set of results that were the most interesting – only the 50 nM concentration of the alarm pheromone seemed to be able to change the threshold. The lack of a change at 5 nM seemed obvious enough – such low concentrations of the scent was not strong enough for either the zebrafish to pick up, or to generate a response. However, at 500 nM of the peptide – one order of magnitude greater than the working 50 nM concentration - we were unable to obtain sensitization. We came up with two possible explanations for this result, the first of which was that such a large amount of scent probably overrode the sensory receptors of the fish – it is well known that large concentrations of one smell does not smell that same than at more intermediate concentrations. Another possible explanation could be that such a strong amount of alarm pheromone could have elicited such a strong response from the zebrafish that it was more prone to freezing – a phenomenon seen often in mice when they are presented with fearful stimuli.

Our third sets of experiments, which are not shown due to a lack of sufficient sample size, were to determine which time was the best to take the posttest threshold measurement. Measurements of 1 minute, 5 minute, 30 minutes, and 1 hour were taken, with both 1 minute and 5 minute the only time periods capable of a successful out. Although more data is needed before the results become significant, it makes sense that a time period greater than 30 minutes does not elicit sensitization – either the scent has dissipated and broken down, or more likely, the fish has habituated to the scent itself.

Our experiments in phototaxis showed that when alone, the zebrafish were more prone to be in the lighted zone, at 65%. However, when there were more fish together, whether it was 10 or 30, the preference seems to disappear, and their averages for being in the light decreased to random, at around 45% for both the group of 10 and group of 30. There could be a variety of reasons for this, the first being that since zebrafish are a shoaling species of fish, it could be that when they are together, they do not respond as strongly to light stimuli, and are focused more on the movements of their group. Another possible explanation is just the lack of space. With over 30 zebrafish in one container, it is possibly that they are unable to act out their preferences due to space. However, since they were equally likely to be in the lighted zone for both the group of 10 and the group of 30, this explanation seems less likely.

Our successful outcome in this project, both with the phototaxis and alarm pheromone certainly leads appropriately to our attempts to create classical conditioning in the zebrafish. Using the unconditioned stimulus of the alarm pheromone, we will attempt to pair the scent with another one to elicit a conditioned response using a conditioned stimulus. Although we have begun these experiments, acquiring the necessary equipment has taken some time, preventing much progress. Being able efficiently transfer the fish from one scent to another proved a significant problem until we created a setup that could lift the zebrafish from its well consistently and without trouble.

CHAPTER 5: CONCLUSIONS AND FUTURE RESEARCH

In conclusion, all of the basic response property data that has been collected in this thesis will serve useful in future projects that require the same experimental setup. Understanding the best volume, diameter size and pulse duration will be important to produce experiments that provide the most accurate and efficient results. The conclusions that have been drawn from sensitization experiments dealing with phototaxis and the alarm pheromone will also serve as stepping stones for future experiments in classical conditioning. Once we can replicate a behavioral phenomenon consistently, we can then move to understanding its cellular and molecular basis.

Our future projects will include trying to pair the alarm pheromone with a harmless amino acid, and to see if we can classically condition the zebrafish into eliciting a behavioral enhancement with the harmless amino acid. It will take some fine tuning to find the right amount of time to leave the fish with either scent, but at the moment, it appears that the best time frame would be around 3 to 5 minutes.

In addition to attempts to classically condition the fish, we will also be moving forward with our pharmacology experiments in order to elucidate the molecular basis of sensitization of the C-startle reflex. Using drugs that are known to block certain receptors, we can then use occlusion to determine which affects the habituation and sensitization. Drugs such as KN-62 (which antagonizes camKII), okadic acid (which inhibits phosphatase), and PKC inhibitors, are a few of the drugs which will be initially used, with further drugs to be determined in the future based upon the success/failure of these drugs. Future studies in calcium imaging on the Mauthner

cell will also prove useful towards our endeavor to understand simple learning and memory in the zebrafish.

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