Characterization of Selected Single and Convergent Stimuli-Induced Behaviors in Larval Zebrafish

by

Michael Alexander Skuhersky

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ABSTRACT

In this work, we designed and implemented several distinct and combined behavior stimuli experimental setups, which were used to characterize larval zebrafish behavior at various stimuli parameters. Tested stimuli was chosen from the perspective of eventual fluorescent neural imaging, so as to be both compatible with, and aware of the stimulating aspects of, a conventional florescence microscope incorporating an excitation laser. Despite the high variance of typical zebrafish behavioral responses, we were able to draw several conclusions. We characterized some optimal stimuli parameters for eliciting consistent responses, from time between stimuli trials to the speed at which a motion stimuli should be moved. We found that the presence of higher temperatures heavily mediates stimuli response, from startle to food-seeking behavior. We characterized a method of distinguishing between a behavioral movement response in reaction to an externally induced shock stimuli, and a directly-induced muscle contraction from the same stimuli. From an imaging perspective, when performing imaging using a typical, stimulating, florescence microscope laser, it appears that visual stimuli response is mediated, but not the non-visual stimuli of a shock. In the future, observed transitions between behavioral states in response to thresholds of chosen stimuli parameters may be used as tools to explore how decisions are made at these junctures.

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Chapter 1

Introduction

1.1 Introduction

The precise mechanism by which organisms respond to a stimulus is not well understood, and is far from perfectly repeatable. For example, an animal placed in a particular dangerous situation might sometimes run away, but also may lay still or hide. A given neuron in the visual cortex of an animal may respond differently each time an animal views the same visual stimulus.

However, many correlations between neuron response (and its variability) and concurrent behavior have been observed (Orger and Polavieja, 2017). This suggests the existence of overarching neural mechanisms responsible for particular specific behaviors.

Unfortunately, identifying specific neuron-behavior correlations can be difficult. One complicating factor is an often weak and high-variance relationship between any particular neuron and a given observed behavioral response. This suggests that an accurate predictive model of the causal mechanism leading to a specific behavior involves a simultaneous analysis of many neurons, potentially spanning multiple brain regions.

In addition, observations of behavior are inherently influenced by variability in test conditions. Furthermore, even if the test setup, stimulus, and observation method has been designed in a repeatable way, separate organisms, especially those with higher connectome complexity, may react in an inconsistent manner due to their unique past experiences, current mental state, or neuron growth differences.

To explore this topic, the larval zebrafish is one ideal choice to study, and indeed

is often used as a model organism, for several reasons. First, it is easier to breed in a high-throughput way when compared to research mammals such as mice, while exhibiting similar neural spiking. Second, zebrafish are commonly available in genetic variants that express pan-neuronal fluorescent activity reporters (Chen et al., 2013), while exhibiting full transparency through to the brain at up to 7 or 8 days post-fertilization (Lister et al., 1999). Finally, larval zebrafish can easily be headfixed in agar, and exhibit clearly characterized behavioral responses, such as tail movement bouts, or eye movement.

In this work, I attempt to characterize common stimuli modalities, and pairwise convergences of such stimuli, to identify points at which a zebrafish responds to one stimuli rather than another, or responds in an entirely unique way. In the future, I hope to study these decisions in relation to concurrent neural activity.

Chapter 2

Exploration of Stimuli Modalities

2.1 Selection of Larval Zebrafish

The zebrafish used were HuC:GCaMP6f, a stable zebrafish transgenic line established by the HuC gene promoter driving the GCaMP6f protein calcium sensor, at 5-7 days post-fertilization. This line was chosen due to availability, as it is a common choice for our activity imaging, expressing modern protein calcium sensors and near-total transparency until 8dpf or so. Breeding and housing was performed at the MIT zebrafish facility in the Koch Institute for Integrative Cancer Research.

Every experimental recording in this work was performed with a separate unique fish; in total 255 fish contributed to these results, with more than double that unable to be used to do lack of movement, headfixing failures, etc.

2.1.1 Headfixing as a stimuli

Traditionally, vertebrate behavioral experiments that involve concurrent recording of neural activity must either use limited and invasive implantable sensors, or must fix the animal's head in a static position so that more-comprehensive optical or magnetic imaging techniques may be used. Headfixing, for example in the case of zebrafish, easily allows for tracking of the tail, eyes, and other bodily functions as metrics of behavior.

Most existing experiments that test for behaviors in a headfixed zebrafish rely on an assumption that such behaviors are sufficiently similar to those of a fish in its natural environment. However, the fish does not naturally encounter a situation where it is head-fixed, and as a result it is reasonable to posit that there will

be some unknown behavioral differences between a headfixed fish and a free swimming one. Due to this altered state, experiments where a fish is allowed to swim freely is currently viewed as necessary for evaluating most "high-level" behaviors, such as mating, schooling, and food-seeking.

Combined behavior and neural activity imaging experiments within a free-swimming setup, do exist (Kim et al., 2017). However, these methods involve closed-feedback microscopy tracking of the fish combined with software correction, limiting imaging speed, the possibility of single-neuron resolution, and data reliability.

While a zebrafish headfixed in agar is in an inherently unnatural state, this setup was sufficient for the simple behavior I was investigating, as indicated by the conclusions for simple behavior done in works such as Bianco et al., 2011. Despite this, a few experiments in this work were performed on groups of free-swimming fish, and so their stimuli-invoked behavior in relation to that of head-fixed fish can be compared.

To headfix a zebrafish, I used a solution of 1.8% low-melting agarose (A2576 Sigma-Aldrich), heated to just above the melting point at 35C, and then allowed to cool for a bit. Then, a fish was transferred into a drop of this heated agar solution via a glass pipette, upon which it self-oriented to upright. Then, the agar gradually hardened, and I cut away a triangle section of agar for the tail to move while pouring in water.

This procedure was similar to conventional methods used for head-fixing zebrafish, such as those in Bianco et al., 2011 and Pantoja et al., 2016, but with slight differences taken in response to my many attempts. Notably, a slightly lower concentration of agarose was used, in conjunction with adding a bit of water when the fish was added, so as to allow the fish to self-orient rather than orienting manually with tweezers. In addition, after the fish had been fixed in hardened agarose but before space for tail movement had been cut away, slightly more agarose was added on top of the droplet, so as to allow for better adhesion to the plate surface. Adding this much agarose initially would cause the fish to initially swim through the droplet, resulting in undesired final orientations.

To assess whether a fish had not been irreversibly negatively affected by the

headfixing process, fish were checked after headfixing by first confirming the presence of a heartbeat under a microscope, and then checking for organism-initiated tail motion (attempts to swim, escape response to a finger tap vibration, etc). Fish that did not have a response were not used. All fish were humanely euthanized according to approved MIT Committee on Animal Care procedures.

2.1.2 Imaging Laser as a Stimuli

Conceivably, it could be difficult to perform experiments involving visual stimuli methods in conjunction with optical based microscopy which uses a laser of the type commonly used in fluorescence activation imaging. This is because both the intended visual stimuli, and the unintended, visible-wavelength visual stimuli of the imaging laser may be detected by the fish.

Several methods have been developed to mitigate this effect, including sweeping the imaging laser in a precise pattern to avoid the eyes (Vladimirov et al., 2014), but most existing publications that record via fluorescence microscopy in a larval zebrafish illuminate the entire brain with a circular beam profile, which means there is overlap with the eyes (See Figure 2.1 for an example of how this looks in practice). These experiments rely on an assumption that the use of the activation laser does not significantly influence visual-stimulated behavior.

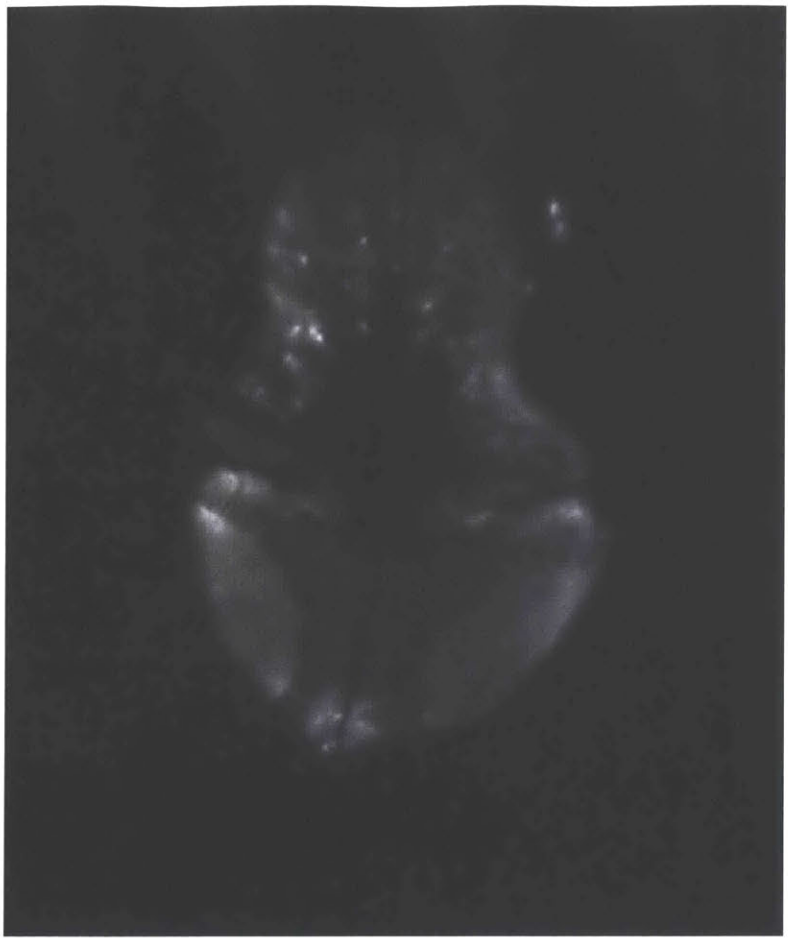


FIGURE 2.1: Example image of larval zebrafish pan-neuronal GCamP6f expression in response to imaging laser illumination.

For this reason, I deemed it important to investigate response to visual stimuli with and without a typical imaging laser. In addition, I deemed it important to investigate other sources of stimuli to compare their effects on behavior with and without the presence of an imaging laser. This is discussed in Chapter 3.

2.2 Visual

2.2.1 Visual - Motion

In conventional visual response experiments, a scrolling sinusoidal grating is often used to induce perception of movement in organisms (Burr, Fiorentini, and Morrone, 1998). When constrained to a small area of the organism's visual field, this scrolling grating can be perceived as an externally moving object, and if the grating

covers the entire field of vision, it may be perceived that the observer itself is moving in the opposite direction of the scrolling.

Rheotaxis behavior, the attempts of an organism to try and correct net movement to zero in response to an externally-induced movement, typically induces a zebrafish to try and swim in same direction of perceived movement (Olive et al., 2016). By observing this behavior or lack thereof, we can try to determine what makes a zebrafish respond in the same way as if it was actually being moved.

FIGURE 2.2: Scrolling stimuli apparatus, showing behavior camera, dual screens with scrolling sinusoidal grating, and sample dish.

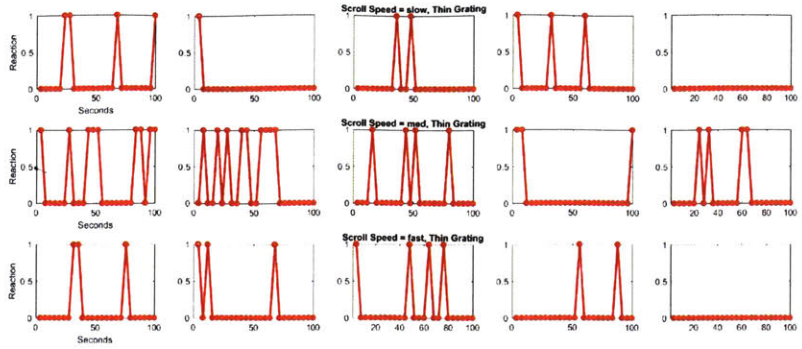
To characterize what it takes to induce a perception of involuntary movement in a headfixed zebrafish, based solely on visual factors, I constructed an experiment consisting of two 5" LCD screens (Elecrow RPA05010R), each normal to the resting state of a zebrafish eye, and an imaging camera (OMAX A3550U3) to record tail movement (Figure 2.2). This side mounted system of visual stimuli allowed for the use of a behavior recording camera mounted under the fish, while allowing for the use of a top-down imaging camera, which was ultimately not used because its construction had not been completed.

This setup resulted in the screen being ~3cm away from each eye at its closest point, and covering the front ~220 degrees from the fish's perspecitive. On the screens, I displayed scrolling, sinusoidal, black-to-white gratings, produced by the GratingStim function of the PsychoPy package, for a period of 100 seconds randomly scrolling either left or right; the presence of a positive reaction (one involving a tail movement in the correct direction) was recorded in 4-second bins. A repsonse was

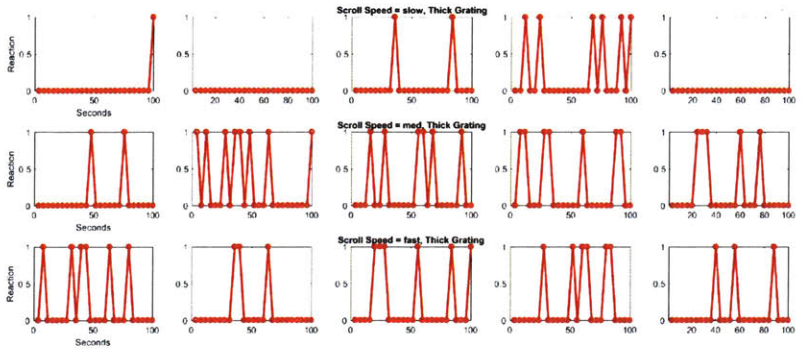
only marked if the tail moved in the correct direction as would be expected to correct movement implied from the direction of the scrolling grating. It was observed that if a fish moved, it would virtually always move in the expected direction. A fish rarely moved more than once within the duration of a binning period; due to this if it did it was counted normally.

Two grating widths were chosen as part of the stimuli parameter space. ~5 degrees per sinusoidal cycle and ~30 degrees per cycle. Three scroll speeds were chosen as part of the stimuli parameter space: ~5 degrees per second, ~40 degrees per second, and ~120 degrees per second.

The experiment was carried out on 30 individual fish: 5 fish each at each combination of one of the aforementioned two grating widths and three scroll speeds (Figure 2.3). The 5-fish averages were plotted in Figure 2.4.



(A) 15 unique fish with thin gratings and one of three scroll speeds.



(B) 15 unique fish with thick gratings and one of three scroll speeds.

FIGURE 2.3: The effects of selected sinusoidal grating stimuli parameters in regards to the reaction of an individual fish (binned in 4 second intervals, where reaction = 1, and no reaction = 0) of the five fish that have been stimulated with the same parameters, for a total of 30 individual fish in the experiment.

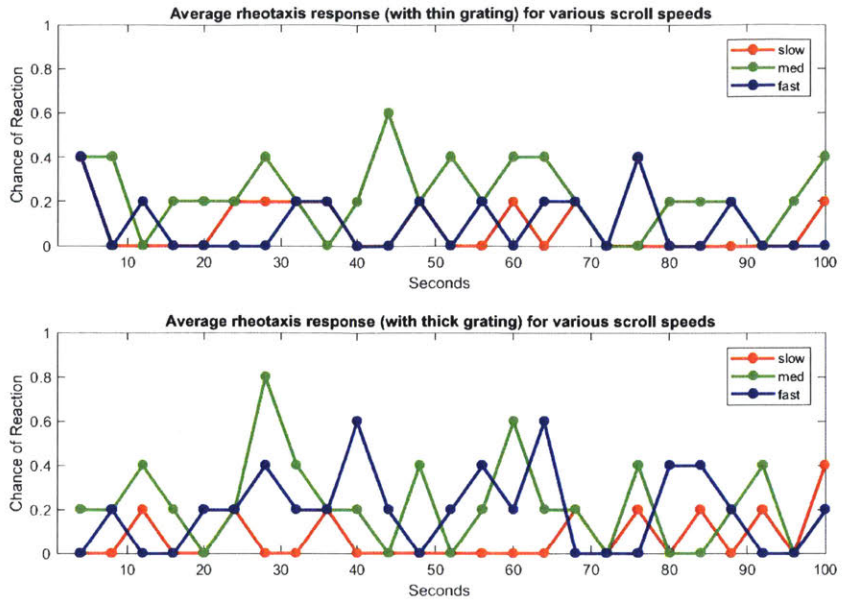


FIGURE 2.4: The effects of selected sinusoidal grating stimuli parameters in regards to the chance of a reaction (binned in 4 second intervals) with each point representing the average of reactions (where reaction = 1, and no reaction = 0) of the five fish that have been stimulated with the same parameters, for a total of 30 individual fish in the experiment.

	Slow Speed	Med Speed	Fast Speed
Thin Gratings	0.08	0.23	0.10
Wide Gratings	0.07	0.22	0.19

TABLE 2.1: Average response chance over the recorded period for the average of reactions (where reaction = 1, and no reaction = 0) of the five fish that have been stimulated with the same sinusoidal grating stimuli parameters.

It is important to note that with such a small sample size, and high variance between individual fish, hard conclusions should not be drawn from the response averages. However, from these averages and the individual traces, we can possibly gain several intuitions: First, because response chance in both the individual and average plots appears to not increase or decrease depending on how long a fish has been exposed to a certain stimuli, it seems that responses are time-invariant at least within our measurement period; over a period of 100 seconds habituation did not seem to occur. Secondly, it seems that fish are more likely to react to thicker gratings

(the overall response average chart values for thicker gratings is higher) , and less likely to react at our chosen fast and slow scroll speeds (the medium speed response averages seems to be slightly higher than both slow and fast response averages in both charts).

2.2.2 Visual - Flash

Typically, a zebrafish may interpret a sudden change in overall lighting as indicative of an approaching environmental danger(Orger and Polavieja, 2017). Here, I use a white light flash to induce a startle response. It seemed important to determine potential habituation response, so as to gain additional insight about how a decision to react is made. What spacing between individual flash events would be required to negate the effects of habituation? Additionally, how does a flash event from complete darkness compare to a flash event in the presence of typical ambient light, in the context of likelihood of a response?

FIGURE 2.5: Flash stimuli apparatus, showing behavior camera, combination white/IR LED, and sample dish.

To characterize what it takes to induce a perception of startling environmental

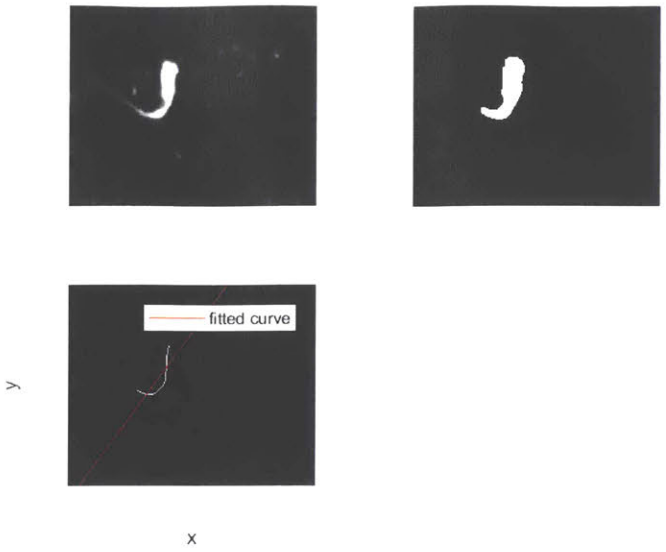
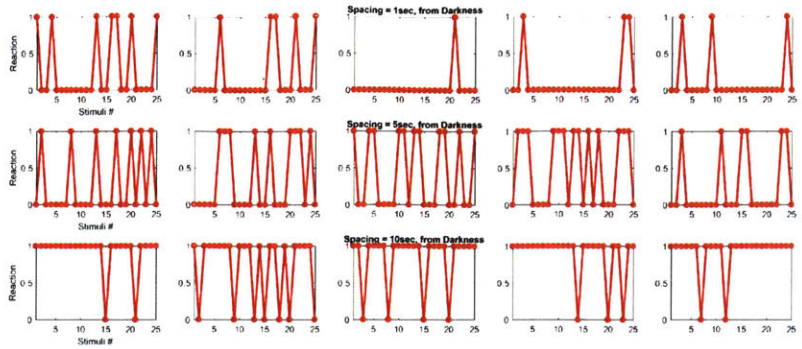


FIGURE 2.6: The process for image analysis of fish tail position: the original image of a zebrafish, blob detection, the skeletonization of the blob, and a resulting straight-line fit, allowing the tail position to be reduced to a single value (the slope).

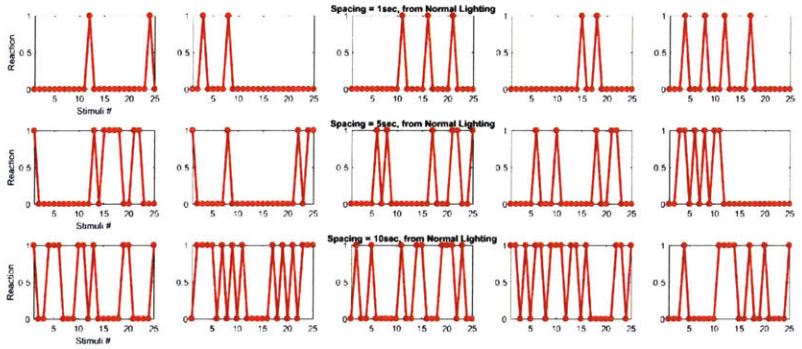
danger in a headfixed zebrafish, based solely on a light flash, I constructed an experiment consisting of a white illuminating LED (Thorlabs WFA1010) mounted overhead 25cm away from the fish eye plane, and an IR behavior imaging camera (FLIR GS3-U3-23S6C-C) to record tail movement (Figure 2.5). This resulted in a flash with average illuminance at the fish eye plane of ~9000 lux. The flash was delivered by a function generator (Agilent 33120A), triggered by TTL output from a DAQ (NI USB-6259). To minimize interference from the effects of visible light, while retaining the ability to image the fish's movement, movement recording was performed in IR, which larval zebrafish are unable to see (Emran, Rihel, and Dowling, 2008). An IR illuminating LED (Thorlabs WFA1020) was used. For the ambient light experiments, room illuminance was calculated at ~761 lux.

From the white LED, I produced a flash lasting 0.5 seconds for 25 trials, at various spacing periods between trials; the presence of a positive reaction (one involving an observed tail motion response within the duration of the stimuli) was recorded for each stimuli. The experiment was carried out on 30 individual fish: 5 fish each at each combination of one of two lighting situations (room lights continually on with flash stimuli, and room darkness with flash stimuli) and 3 stimuli time spacings

(1,5,and 10 seconds apart) (Figure 2.7). The 5-fish averages were plotted in Figure 2.8.



(A) 15 unique fish with flashes from darkness at one of three stimuli spacings.



(B) 15 unique fish with flashes from room lighting at one of three stimuli spacings.

FIGURE 2.7: The effects of selected flash stimuli parameters in regards to the reaction of an individual fish (where reaction = 1, and no reaction = 0) of the five fish that have been stimulated with the same parameters, for a total of 30 individual fish in the experiment.

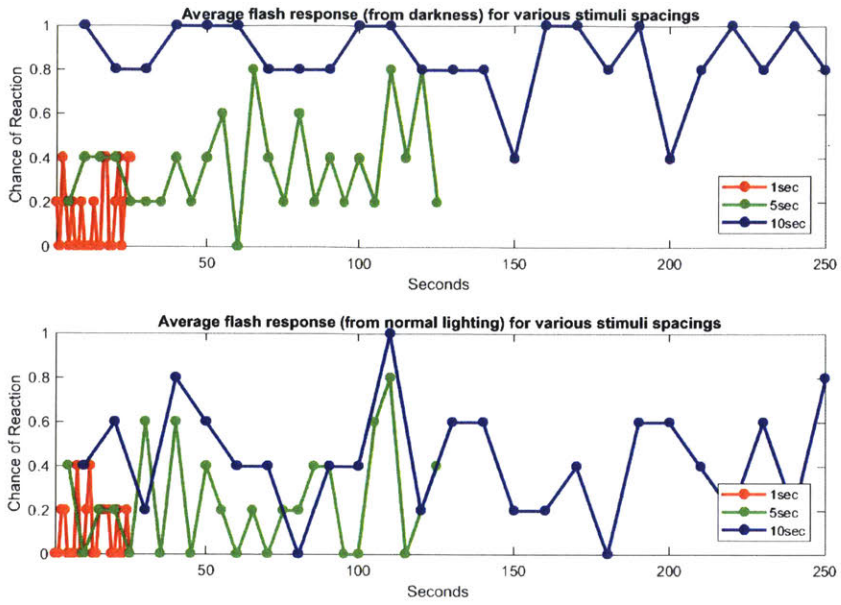


FIGURE 2.8: The effects of selected flash stimuli parameters in regards to the chance of a reaction, with each point representing the average of reactions (where reaction = 1, and no reaction = 0) of the five fish that have been stimulated with the same parameters, for a total of 30 individual fish in the experiment.

	1sec Spacing	5sec Spacing	10sec Spacing
From Darkness	0.15	0.37	0.86
From Room Lighting	0.10	0.24	0.43

TABLE 2.2: Average response chance over the recorded period for the average of reactions (where reaction = 1, and no reaction = 0) of the five fish that have been stimulated with the same flash stimuli parameters.

It is important to note that with such a small sample size, and high variance between individual fish, hard conclusions should not be drawn from my response averages. However, from these averages and the individual traces, we can possibly gain several intuitions: First, as shown by (Figure 2.8), flash chance of response is greatly reduced by short stimuli spacing (as indicated by the obviously lower average response chances at 1sec spacing), while long periods between flashes make it extremely likely for a fish to respond to each one. Secondly, it seems that for all flash stimuli spacings, the probability of response is slightly diminished when the flash occurs in the context of ambient lighting, as indicated by the obviously lower

average response chances at all spacings in the "from normal lighting" chart. Not much habituation was observed for these stimuli parameters, but it is conceivable that we did not make our test durations long enough to observe such effects.

2.3 Shock

Shock is a useful modality due to its relevance to a wide variety of psychiatric questions. In addition, it lacks any interference with optical recording methods of both behavior and neural activity. Here, I attempt to characterize an ideal method of delivering shock stimuli to larval zebrafish.

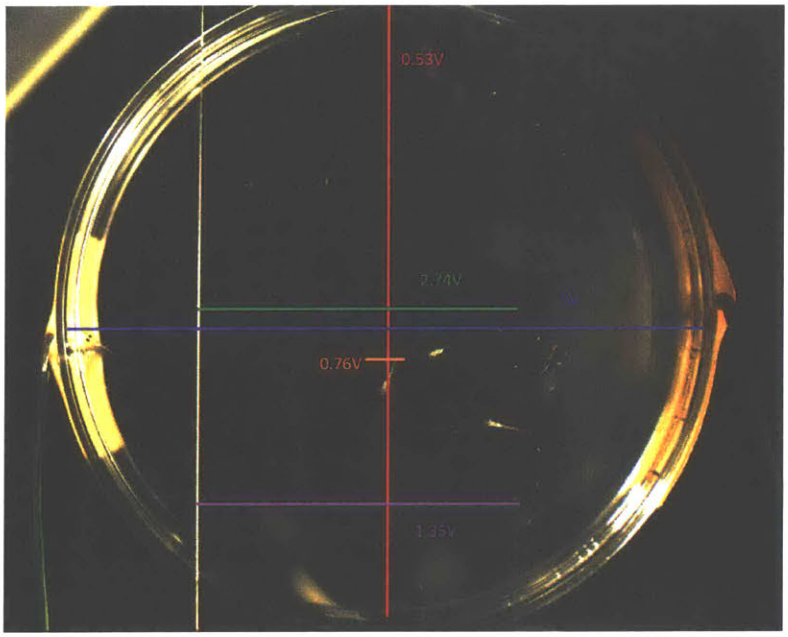


FIGURE 2.9: Sample voltage measurements taken along various lines in a water and zebrafish filled dish, with 5Vpp electrodes across the diameter.

2.3.1 Difficulty of Maintaining Reproducible Effective Shock in Water Environment

It was important to try and characterize an effective voltage that a fish would experience, as a function of its body size and physical position relative to the electrodes. (Figure 2.9) contains various representative sample voltage measurements

taken along various lines in a water and zebrafish filled dish, with 5Vpp electrodes across the diameter.

To see this in action, a recording was taken of how a set of free-swimming larval zebrafish react to this electrode setup. During a period of constant applied 5V voltage, the zebrafish seem to act with a rapid escape bout of swimming occasionally, with the chance of this happening rapidly increasing with the proximity of the zebrafish to the midline formed by the two electrodes. This observation taken in the context of the aformentioned voltage measurements imply that the effective voltage received by a free-swimming zebrafish in this setup would vary wildly depending on such factors as the fish's proximity to an electrode, orientation, and distance from the path of least resistance in the water.

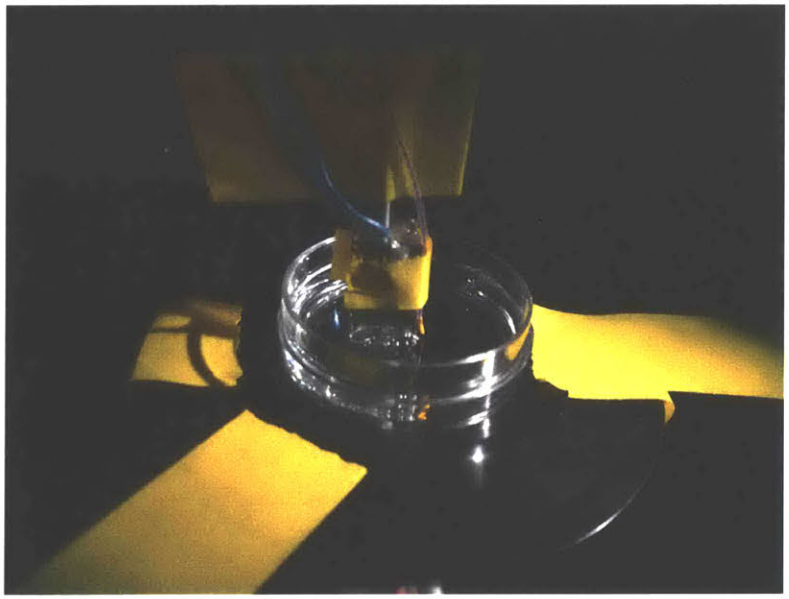


FIGURE 2.10: Shock apparatus, showing positioning arm, parallel electrodes with thermocouple, and specimen dish, with behavior tracking camera below.

This suggested that I create a way to deliver a more consistent effective voltage. Two custom platinum (to prevent corrosion/oxidation, etc) parallel plate electrodes, attached to a custom positioning arm, were mounted in parallel with a distance of 5mm to provide a consistent voltage (Figure 2.10). The positioner arm enabled a higher experimental throughput, as the electrodes could be easily realigned upon the introduction of a new fish to the experiment, with an assumption that the new

fish would be receiving the same level of perceived shock. The shock was delivered by a function generator (Agilent 33120A), triggered by TTL output from a DAQ (NI USB-6259). All shocks consisted of a 0.5 second wide DC pulse (square pulse).

2.3.2 Characterization of Varied Types of Shock Responses

When beginning this route of stimulation, one immediate concern was that an electric shock may induce movement that is not a result of a signal produced by way of the fish itself, but rather that of muscle contractions induced directly by external electrical stimulation. During my experiments, I noticed two types of movements in response to a shock stimuli.

Figure 2.11 shows a comparison between typical sequences of motion for my observed two archetypal induced motion bouts. In A, a lengthy, high-amplitude complex movement is observed. This suggests that the fish is continually reacting to the memory or feeling of shock, even though the shock itself has ceased. In B, a single, slight and almost uniform muscle contraction was observed.

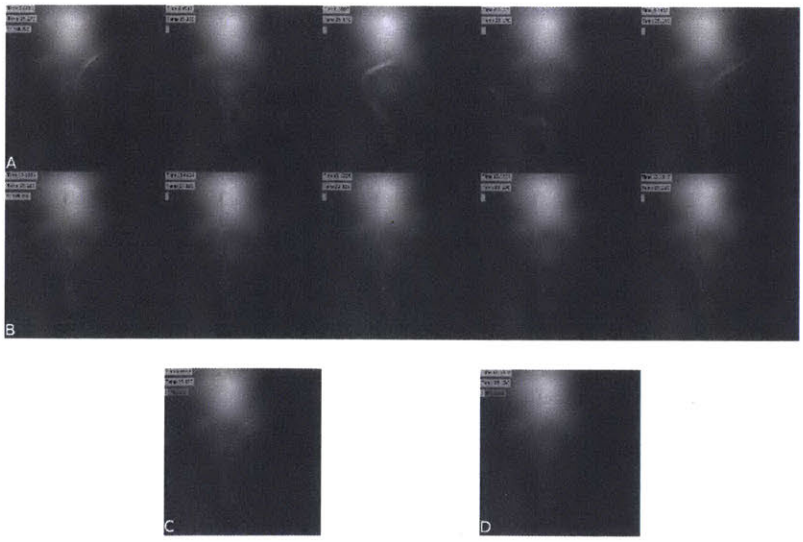


FIGURE 2.11: *Row A:* Sequence of consciously induced motion bout for .1 seconds post-stimuli. *Row B:* Sequence of muscle-contraction induced motion bout for .1 seconds post-stimuli. *C:* First and last images of *A* superimposed. *D:* First and last images of *B* superimposed.

After this, it was observed that when similar shocks were applied to recently deceased fish, a similar movement to type B; instant, low amplitude, and lacking in follow-ups; was occasionally observed. Such muscle contractions as a result of

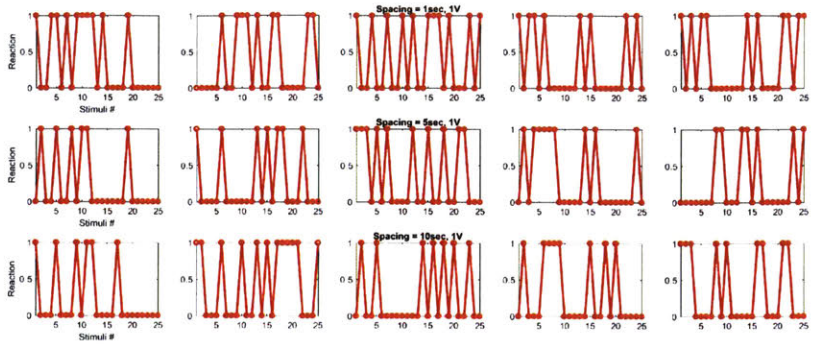
applied shocks, even in the muscles of deceased organisms, have been observed by scientists since near the dawn of the electrical age ("galvanism"). My result seems to support the theory that such movements in live fish may be the result of "involuntary", directly-stimulated electricity-induced muscle contractions.

2.3.3 Characterization of Varied Shock Parameters

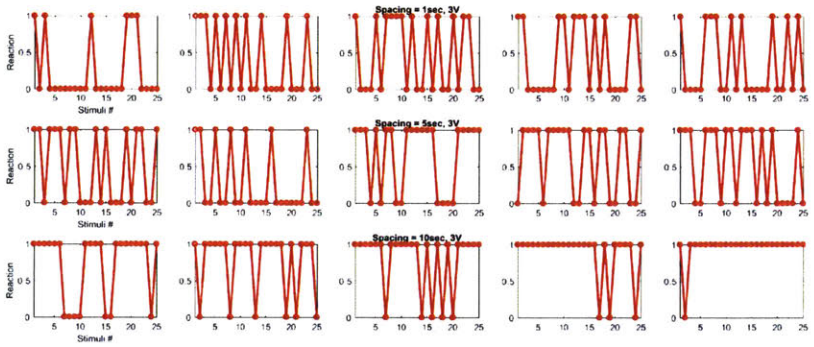
Proceeding under the aforementioned assumption, and ignoring motion that appeared to be an involuntary, direct voltage-induced muscle contraction, I tried to seek understanding of the parameter space of shock stimuli, so as to choose the best parameters to use.

The first parameter was voltage amplitude: at 5mm distance, through a relatively-consistent medium of water and agar, what voltage would be enough to consistently produce an evoked response?

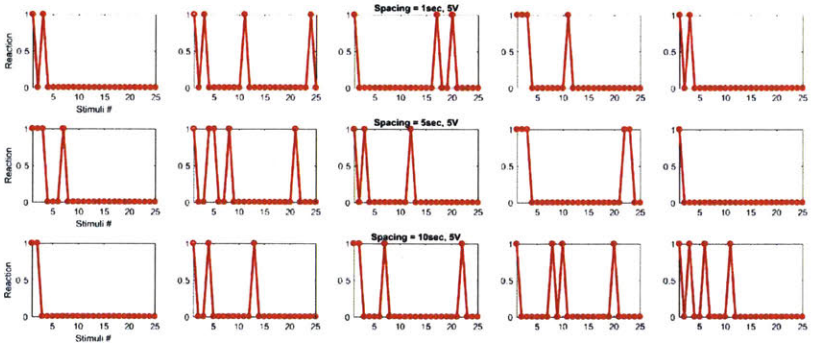
The second parameter had to do with habituation: spacing between voltage stimulations. What spacing between individual shock events would be required to negate the effects of habituation? The experiment was carried out on 45 individual fish: 5 each at each combination of one of 3 shock voltages (1, 3, and 5 Vpp) and 3 spacings (1, 5, and 10 seconds apart)(Figure 2.12). The 5-fish averages were plotted in Figure 2.13.



(A) 15 unique fish with shocks at 1V at one of three stimuli spacings.



(B) 15 unique fish with shocks at 3V at one of three stimuli spacings.



(C) 15 unique fish with shocks at 5Vpp at one of three stimuli spacings.

FIGURE 2.12: The effects of selected shock stimuli parameters in regards to the reaction of an individual fish (where reaction = 1, and no reaction = 0) of the five fish that have been stimulated with the same parameters, for a total of 45 individual fish in the experiment.

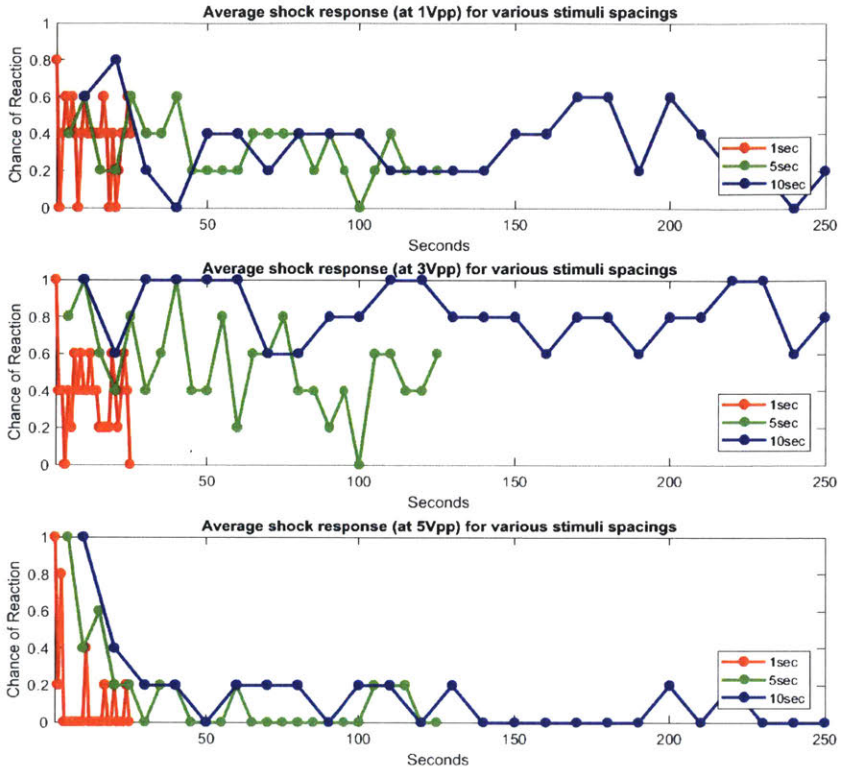


FIGURE 2.13: The effects of selected shock stimuli parameters in regards to the chance of a reaction, with each point representing the average of reactions (where reaction = 1, and no reaction = 0) of the five fish that have been stimulated with the same parameters, for a total of 45 individual fish in the experiment.

	1sec Spacing	5sec Spacing	10sec Spacing
1V	0.38	0.31	0.34
3V	0.38	0.54	0.82
5V	0.12	0.14	0.14

TABLE 2.3: Average response chance over the recorded period for the average of reactions (where reaction = 1, and no reaction = 0) of the five fish that have been stimulated with the same shock stimuli parameters.

It is important to note that with such a small sample size, and high variance between individual fish, hard conclusions should not be drawn from my response averages. However, from these averages and the individual traces, we can possibly gain several intuitions: First, as shown by (Figure 2.13), shock chance of response is

greatly reduced by short stimuli spacing, similar to flash response. Longer spacings also similarly increase chance of response. Another observation was the likelihood of a fish to stop responding permanently at our highest tested voltage, 5Vpp (as seen in many of the 5Vpp individual traces). Not much habituation was observed for these stimuli parameters, but it is conceivable that we did not make our test durations long enough to observe such effects.

2.4 Heat

Conventionally, zebrafish intended for research live and are raised in a temperature of 23C. I modified my shock experiment with a petri-dish heating plate (Warner QE-1HC), and a thermocouple which was attached to both the midpoint of the parallel plate electrodes, and to the side of the plate to measure and maintain the effective fish-experienced temperature as accurately as possible. Temperature was maintained by a temperature controller (Harvard Apparatus TC-324C), with a temperature set by the aformentioned DAQ.

To observe how zebrafish react at temperatures different from that of the standard fish environment, a characterization of differing temperatures was performed: at three different temperatures, 23C, 26C, and 30C, a dish of 10 unrestrained fish was recorded for three minutes. The percentage of time that any fish tail movement was detected in each dish was 4% for the 23C plate, 23% for the 26C plate, and 41% for the 30C plate. This supports the hypothesis that fish try to escape more from higher temperatures.

In the future, it would be interesting to confirm whether or not decreasing the temperature from room temperature, rather than increasing it, affects behavior in a similar way.

2.5 Food

Larval zebrafish at MIT are typically fed live paramecia, from after the period that they have consumed their yolk (5 days-post-fertilization) until the time when they can handle larger food (12 days-post-fertilization).

2.5.1 Food Seeking

As shown in (Figure 2.14) and recorded video, I observed that even headfixed fish attempt to eat the paramecia when a paramecium enters the range of vision. This opened up the possibility of using food seeking as a stimuli modality. This is discussed, in conjunction with how heat mediates this behavior, in Chapter 3.

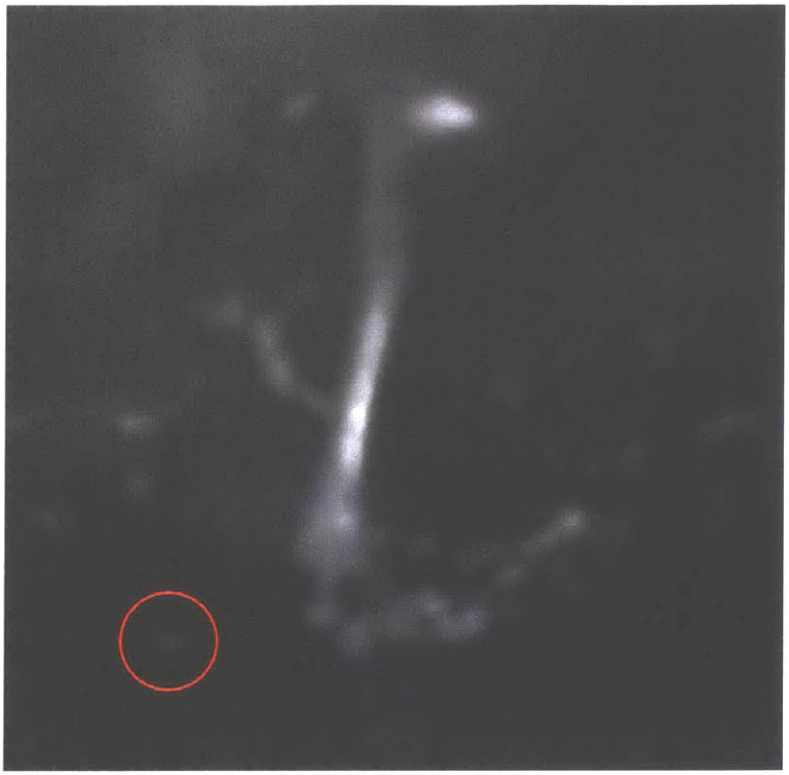


FIGURE 2.14: A head-fixed fish in the process of attempting to catch a paramecium (circled in red), which has entered its visual field.

Chapter 3

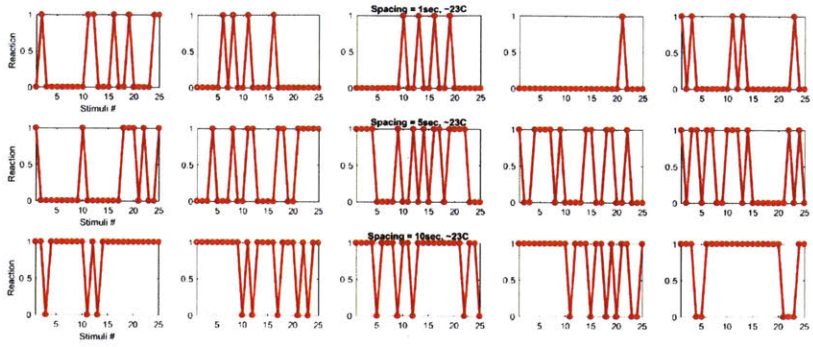
Convergent Stimuli

3.1 Effects of Temperature

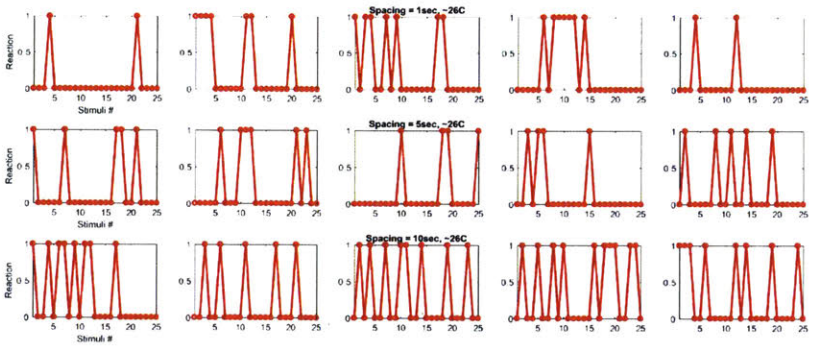
3.1.1 Startle Response

Flash

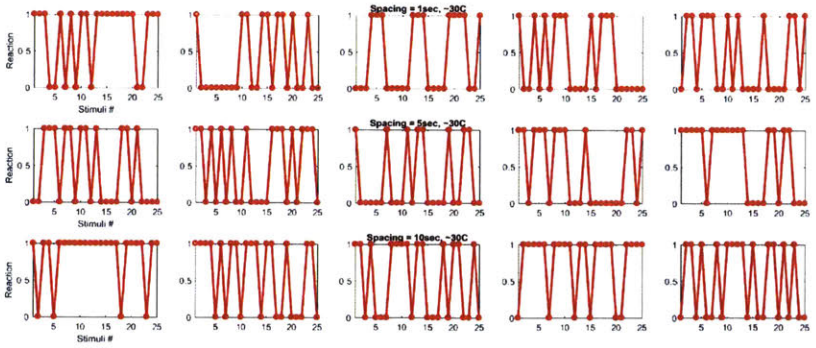
Here, I attempt to characterize how temperature mediates a response to a flash startle stimuli. For this experiment, using the combined heat/flash/shock apparatus shown in Figure 2.10, I selected the flash stimuli type with the highest response rate (flash from complete darkness), and tested it at various stimuli spacings and temperatures. All other parameters were the same as chosen in Section 2.2.2. The individual results are shown in Figure 3.1, and the 5-fish averages are shown in Figure 3.2.



(A) 15 unique fish with flashes from darkness stimuli at 23C at one of three stimuli spacings.



(B) 15 unique fish with flashes from darkness stimuli at 26C at one of three stimuli spacings.



(C) 15 unique fish with flashes from darkness stimuli at 30C at one of three stimuli spacings.

FIGURE 3.1: The effects of selected convergent flash and steady heat stimuli parameters in regards to the reaction of an individual fish (where reaction = 1, and no reaction = 0) of the five fish that have been stimulated with the same parameters, for a total of 45 individual fish in the experiment.

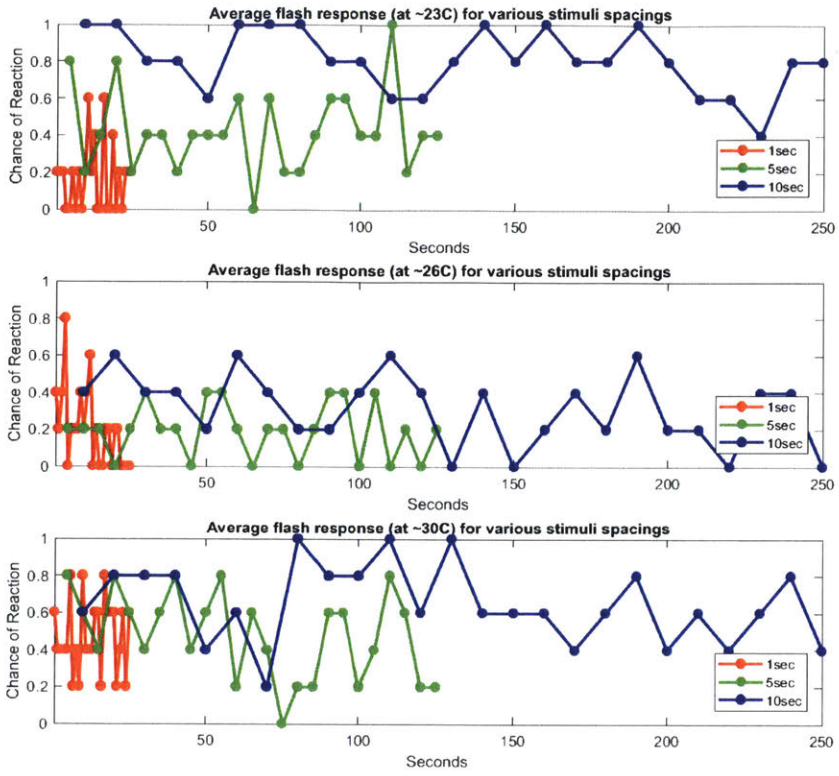


FIGURE 3.2: The effects of selected convergent flash and steady heat stimuli parameters in regards to the chance of a reaction, with each point representing the average of reactions (where reaction = 1, and no reaction = 0) of the five fish that have been stimulated with the same parameters, for a total of 45 individual fish in the experiment.

	1sec Spacing	5sec Spacing	10sec Spacing
23V	0.17	0.42	0.81
26V	0.20	0.19	0.31
30C	0.47	0.48	0.65

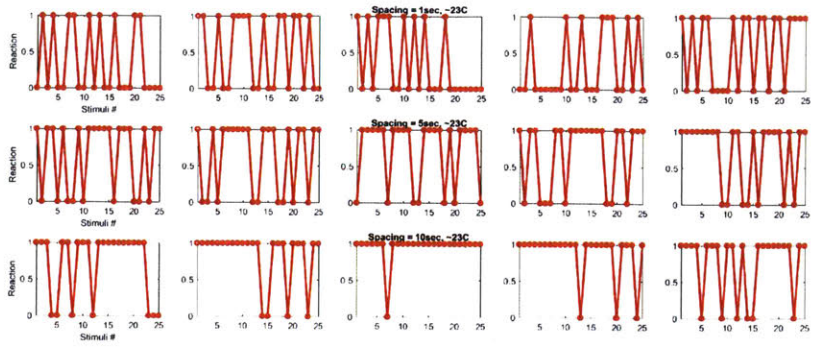
TABLE 3.1: Average response chance over the recorded period for the average of reactions (where reaction = 1, and no reaction = 0) of the five fish that have been stimulated with the same heat/flash parameters.

It is important to note that with such a small sample size, and high variance between individual fish, hard conclusions should not be drawn from my response averages. However, from these averages and the individual traces, we can possibly gain several intuitions. As shown by Figure 3.1 and the data in Section 2.4, at higher

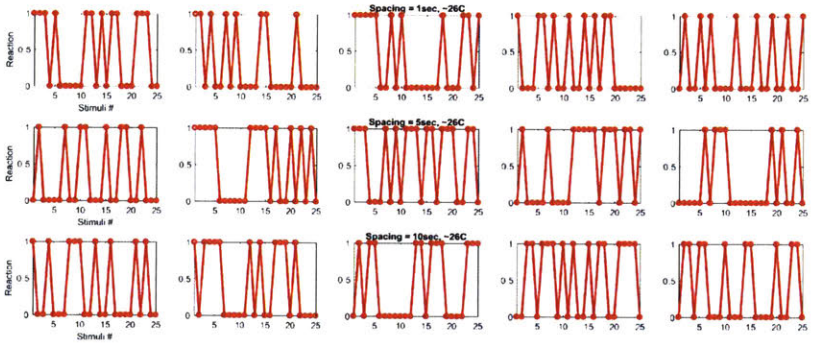
temperatures, a zebrafish continually makes spontaneous attempts to move (higher movement chance). This makes it difficult to identify which movements are heat-induced vs flash-induced. However, we can observe from (Figure 3.2) that for fish at 26C, there seems to be less response chance on average than at 23C or 30C. This seems to indicate that at under these conditions, the increased heat has induced the fish to respond to flashes less, without necessarily making it attempt to escape.

Shock

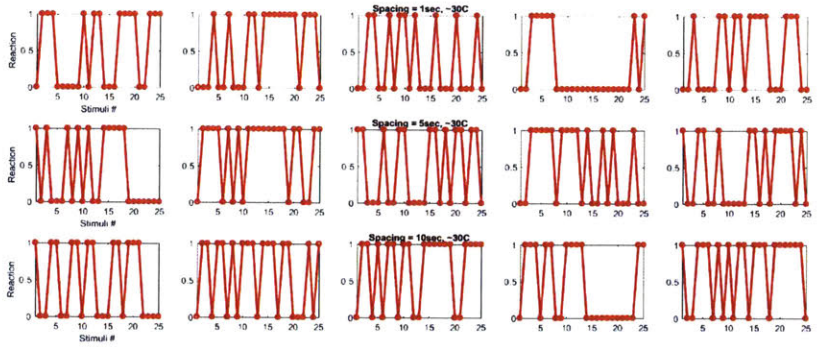
Here, I attempt to characterize how temperature mediates a response to a shock startle stimuli. For this experiment, using the combined apparatus shown in Figure 2.10, I selected the shock stimuli type with the highest response rate (3Vpp), and tested it at various stimuli spacings and temperatures. All other parameters were the same as chosen in Section 2.3. The individual results are shown in Figure 3.3, and the 5-fish averages are shown in Figure 3.4.



(A) 15 unique fish with 3V shock stimuli at 23C at one of three stimuli spacings.



(B) 15 unique fish with 3V shock stimuli at 26C at one of three stimuli spacings.



(C) 15 unique fish with 3V shock stimuli at 30C at one of three stimuli spacings.

FIGURE 3.3: The effects of selected convergent shock and steady heat stimuli parameters in regards to the reaction of an individual fish (where reaction = 1, and no reaction = 0) of the five fish that have been stimulated with the same parameters, for a total of 45 individual fish in the experiment.

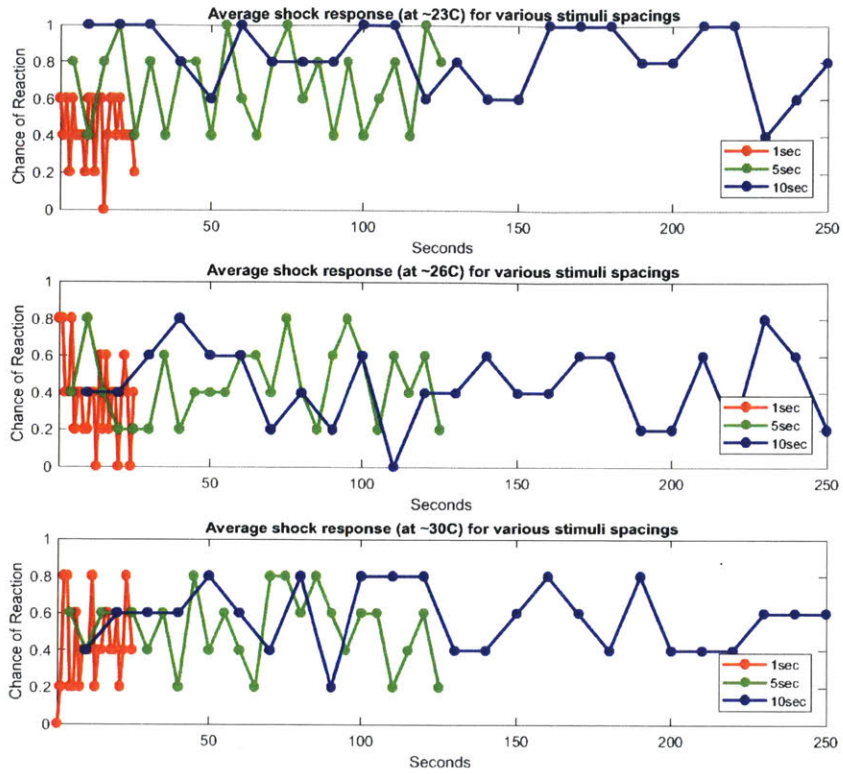


FIGURE 3.4: The effects of selected convergent shock and steady heat stimuli parameters in regards to the chance of a reaction, with each point representing the average of reactions (where reaction = 1, and no reaction = 0) of the five fish that have been stimulated with the same parameters, for a total of 45 individual fish in the experiment.

	1sec Spacing	5sec Spacing	10sec Spacing
23V	0.43	0.68	0.83
26V	0.38	0.45	0.44
30C	0.44	0.52	0.58

TABLE 3.2: Average response chance over the recorded period for the average of reactions (where reaction = 1, and no reaction = 0) of the five fish that have been stimulated with the same heat/shock parameters.

It is important to note that with such a small sample size, and high variance between individual fish, hard conclusions should not be drawn from my response averages. However, from these averages and the individual traces, we can possibly gain several intuitions. As shown by Figure 3.3 and the data in Section 2.4, at higher

temperatures, a zebrafish continually makes spontaneous attempts to move. This makes it difficult to identify which movements are heat-induced vs shock-induced. However, we can observe from (Figure 3.4) that for fish at 26C, there seems to be less response chance than 23C or 30C. This seems to indicate that at under these conditions, the increased heat has induced the fish to respond to shocks less, without necessarily making it attempt to escape.

3.1.2 Food Seeking

At MIT, zebrafish intended for research are fed paramecia after 5dpf. To observe how zebrafish attempt to seek food at temperatures different from that of its typical environment, a characterization of food seeking at differing temperatures was performed: at three different temperatures, 23C, 26C, and 30C, separate dishes of 10 free-swimming fish were recorded for three minutes, before and after the introduction of the same concentration of paramecia (Figure 3.5).

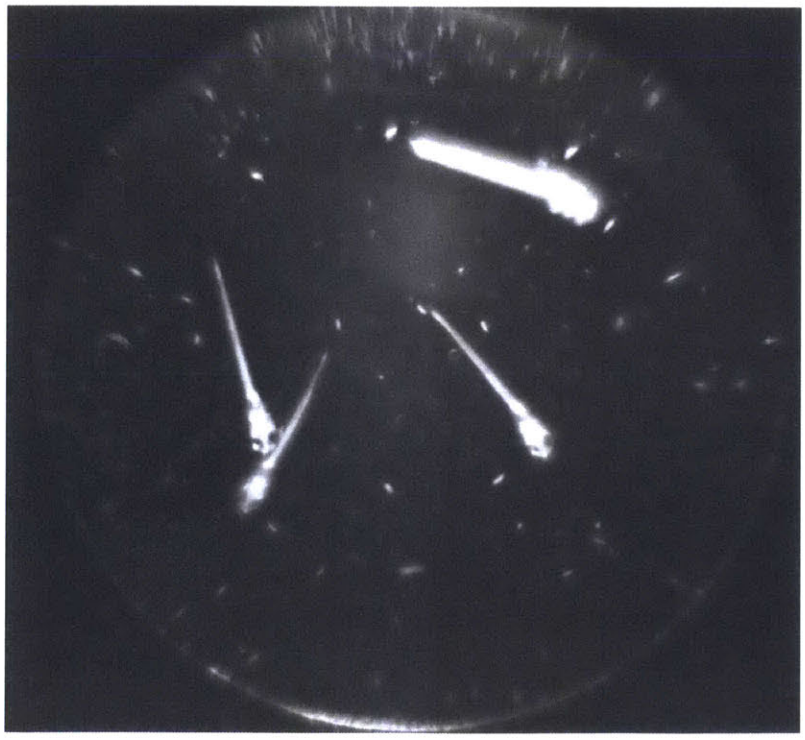


FIGURE 3.5: Free-swimming fish attempting to seek and eat paramecia, while also dealing with increased temperature.

The results of this are shown here:

	23C Dish	26C Dish	30C Dish
Before Paramecium	2.3%	29.8%	45.4%
After Paramecium	28.5%	31.2%	41.1%

TABLE 3.3: Duration of 10 free-swimming fish movements at various temperatures before and after the introduction of paramecia, as a percentage of the total recording time.

Then, the same experimental procedure was performed in 5 head-fixed fish at 23C:

	Fish 1	Fish 2	Fish 3	Fish 4	Fish 5	Average	StdDev
Before Paramecium	0.8%	0%	0%	1.7%	0%	0.5%	0.8
After Paramecium	8.6%	12.8%	22.5%	17.4%	6.0%	13.5%	6.7

TABLE 3.4: Duration of individual head-fixed fish movement at 23C before and after the introduction of paramecia, as a percentage of the total recording time.

and 26C:

	Fish 1	Fish 2	Fish 3	Fish 4	Fish 5	Average	StdDev
Before Paramecium	22.3%	6.2%	6.4%	14.0%	16.1%	13.0%	6.8
After Paramecium	23.7%	11.2%	3.1%	16.5%	13.8%	13.7%	7.5

TABLE 3.5: Duration of individual head-fixed fish movement at 26C before and after the introduction of paramecia, as a percentage of the total recording time.

These results are similar to what was observed in Section 3.1.1 and Section 3.1.1: at higher temperatures, the zebrafish exhibits spontaneous movement, making it difficult to determine what movement is a directly a result of momentary stimuli. However, from these results we can posit that headfixing the fish results in less reactions overall (this observation is in line with what is reported in existing literature, such as Pantoja et al., 2016), and that the behavior of a zebrafish may not be affected by the introduction of a food source while under high temperatures.

3.2 Effects of Imaging Laser

3.2.1 Startle Response

Flash

Here, I attempt to characterize how the typical laser used in excitation imaging mediates a response to a flash startle stimuli. For this experiment, I moved my existing apparatus over to a commercial florescence microscope to make use of its 488nm laser (SOLA SE 5-LCR-VB) under typical imaging conditions. Typical imaging conditions in this case were selected to be 15mW at at working distance of 2mm with a 20x objective lens (Nikon S Plan Fluor ELWD 20X), and aimed to just encompass the entire brain region with a gaussian circular beam profile (the eyes also being encompassed), resulting in ~13000lux at the eye of the fish.

I selected the flash stimuli type with the highest response rate (flash from darkness), and tested it at the spacing that had resulted in the highest response rate (10 sec) in conjunction with the steady excitation laser stimuli. All other parameters were the same as chosen in Section 2.3. The individual results are shown in Figure 3.6, and the 5-fish averages are shown in Figure 3.7.

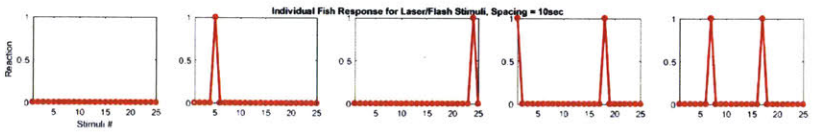


FIGURE 3.6: The effects of selected convergent flash and steady laser stimuli parameters in regards to the reaction of an individual fish (where reaction = 1, and no reaction = 0) of the five fish that have been stimulated with the same parameters.

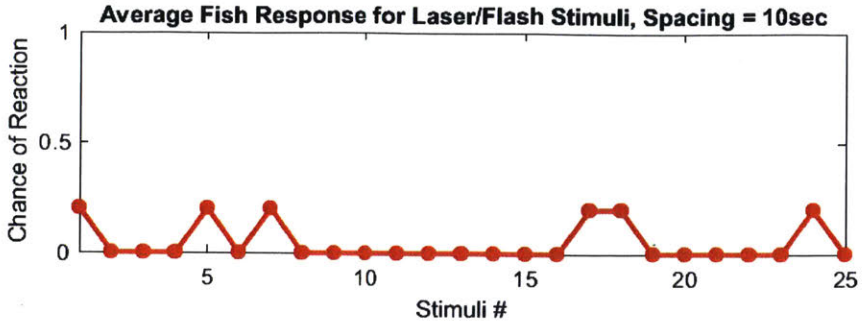


FIGURE 3.7: The effects of selected convergent flash and steady laser stimuli parameters in regards to the chance of a reaction, with each point representing the average of reactions (where reaction = 1, and no reaction = 0) of the five fish that have been stimulated with the same parameters.

Although with a sample size of 5, these results are far from conclusive, we can posit that the presence of a constant excitation laser heavily reduces the chance that a fish may respond to a stimuli flash due to the much lower response averages when compared to those in Section 2.2.2.

Shock

Here, I attempt to characterize how the typical laser used in excitation imaging mediates a response to a shock startle stimuli. For this experiment, I moved my existing apparatus over to a commercial florescence microscope to make use of its 488nm laser under the typical imaging conditions described in the previous subsection.

I selected the shock stimuli type with the highest response rate (3Vpp), and tested it at the spacing that had resulted in the highest response rate (10 sec) in conjunction with the steady excitation laser stimuli. All other parameters were the same as chosen in Section 2.3. The individual results are shown in Figure 3.8, and the 5-fish averages are shown in Figure 3.9.

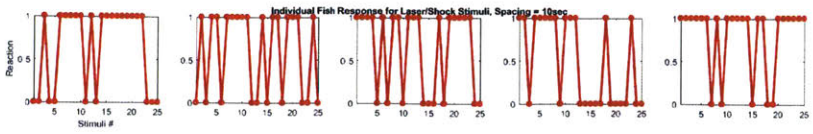


FIGURE 3.8: The effects of selected convergent shock and steady laser stimuli parameters in regards to the reaction of an individual fish (where reaction = 1, and no reaction = 0) of the five fish that have been stimulated with the same parameters.

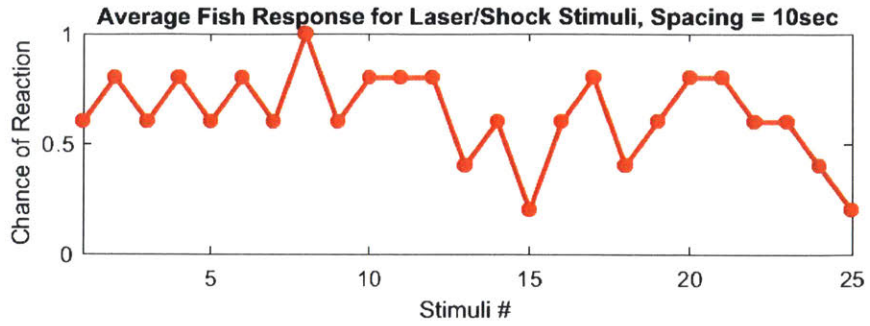


FIGURE 3.9: The effects of selected convergent shock and steady laser stimuli parameters in regards to the chance of a reaction, with each point representing the average of reactions (where reaction = 1, and no reaction = 0) of the five fish that have been stimulated with the same parameters.

Although with a sample size of 5, these results are far from conclusive, we can posit that the presence of a constant excitation laser slightly reduces the chance that a fish may respond to a stimuli shock due to the slightly lower response averages when compared to those in Section 2.3.

3.3 Conclusions

From this limited data we can posit that higher temperatures reduce the likelihood of a zebrafish to respond to stimuli, or to attempt to eat food, as evidenced by observed lower response rates at higher temperatures.

In the presence of a microscope excitation laser, the response to the visual stimuli of a flash was heavily mediated, while the response to shock stimuli was mediated very little. This could suggest that during future experiments that incorporate the recording of neural activity, it would be prudent to not believe visual stimuli can give consistent responses. Shock seems to be a more ideal stimuli for this, but it remains to be seen if a shock can directly induce activity in the brain, in a similar way to how it may induce direct muscle contractions.

Chapter 4

Future Directions

This work attempted to characterize larval zebrafish behavior at various single and convergent stimuli parameters. In the future, it might be prudent to test the same fish with multiple conditions and stimuli types; does a particular fish that happens to react minimally to flash stimuli, for example, also react minimally to a scrolling grating? Does the order in which distinct stimuli are delivered matter? Can the habituation of a zebrafish to one distinct stimuli affect habituation concerning another distinct stimuli? How does a zebrafish make decisions about previously experienced stimuli vs. new stimuli? Can we alter this period of stimuli memory in any way?

Due the breadth of stimuli modalities and parameters examined, and the preparation time required for the many hundreds of fish used, at most only five fish were tested with any particular set of parameters. This limited the degree of quantitative analysis that could be performed, and so in the future, once a subset of stimuli modalities has been chosen as a focus, this analysis should be revisited with the testing of more fish. Ideally, this should remove variance effects caused by such a small sample size, and possibly may allow for noticing patterns such as multimodal distributions.

The experimental procedures were entirely coded in MATLAB, aside from the visual motion stimuli which was done in PsychoPy. In retrospect, MATLAB as implemented was not an ideal environment; parallel processing is poorly supported and is oriented toward executing jobs which neither need to interact nor execute in a time-safe manner (e.g. big data processing jobs). In the experiments of myself and many others, images of behavior must be captured at the same time that a stimuli is being delivered, and the precise time of each of these events must be known and

logged. It is very hard to do this in a single process, particularly in a time-safe manner, because instructions must wait for the previous instructions to complete, and it is impossible to predict how long any instruction may in practice take to complete, due to factors such as operating system overhead, varying calculation sizes, etc.

Obviously the next step would be to connect the observed points at which a fish switches its behavior (in this case, when a stimuli parameter passes one of our observed thresholds), to neural activity. For this, I will build off of existing work in connecting single stimuli response to neural circuitry (e.g. Naumann et al., 2016) and apply these techniques to identifying circuit mechanisms responsible for innate stimuli thresholds.

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