Case studies: Using the zebrafish to evaluate neurobehavioral phenotypes

By

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Abstract

The use of zebrafish in behavioral neuroscience is rapidly growing. Zebrafish can be assessed for alterations in multiple behavioral endpoints, creating opportunities to use this powerful model to identify chemicals that alter behavioral phenotypes. To evaluate the utility of zebrafish for neurotoxicity research, we designed custom instrumentation to evaluate numerous embryonic and adult zebrafish behaviors. PRAT or Photomotor Response Analysis Tool was used to analyze the embryonic photomotor response (EPR) behavior in embryonic zebrafish (24 hours post fertilization). Shuttleboxes were used to evaluate learning and active avoidance conditioning and a zebrafish Visual Imaging System (zVIS) was used to measure fear responses. Social behavior was observed using Viewpoint tracking software. Startle responses were also analyzed using taps and Noldus Ethiovision XT tracking software. EPR results showed differential movement activities throughout development of larval zebrafish. Highest movement peaks were seen in 35-37 hours post fertilization fish. Using these custom analysis tools, we also evaluated the impact of Vitamin E deficiency and developmental Benzo[a]pyrene exposure on complex adult behaviors. Generational effects of BaP exposure were also tested. Zebrafish were fed defined-diets that either had sufficient or deficient levels of Vitamin E. The vitamin E deficient zebrafish had a ~30% decrease in learning rate relative to the fish with sufficient levels of Vitamin E. Startle response data showed that vitamin E deficient fish do not get desensitized to tap stimulus. Three exposure groups and generations were reared and spawned for the BaP study (0.1% DMSO controls, 1.25 ppm BaP, 2.5 ppm BaP). The zVIS system consists of an array of 8 tanks with only single side views of video projections on LCD monitors. This allows individual fish to visualize either a group of swimming zebrafish or single predator fish. For the

socialization assay zebrafish were tracked using Viewpoint tracking software. Distances apart from each other were measured and analyzed in BaP exposed fish. For the predator test, zebrafish were expected to move away from the screen. The proximity of the zebrafish is tracked relative to the LCD screen projections. The preliminary results from BaP exposed zebrafish and 0.1% DMSO controls showed the percent of time spent away from the screen during the predator test or fear response assay was in the high 70% range for all fish. The 2.5 ppm BaP fish had on average the highest percentage (65% vs 50%) time spent away from the screen. Although it is uncertain as of now if there are any generational effects because further analysis is needed. Preliminary shoaling data shows that shoaling speed may be affected by DMSO exposure. The use of DMSO controls may not be optimal for this study. Disassociation is seen in both 1.25 ppm BaP and 2.5 ppm BaP exposure groups in the F2 generation. Collectively, these data demonstrate that custom behavioral systems are able to measure complex behavioral phenotypes and suggests that there are enormous opportunities for translation neurotoxicity research using zebrafish.

Introduction

Zebrafish as an Animal Model

Neurobehavioral disorders have become exceptionally hard to study due to their complexity. Thus scientist look towards animal models to study these disorders (Ellis and Soanes). There are many model organisms, such as rats, mice, or daphnia, but the gold standard for animal models is the rat or mouse; the primary reason is that these rodents are mammals (citation). Rodent physiology is similar to humans and they are experimentally versatile enough allowing various assays including many behavior assessments. Although rodents are revered as gold standard, other models can be equally powerful (Iannaccone and Jacob). For example, the use of the zebrafish model is rapidly increasing because they display many favorable characteristics. First

zebrafish are both physiologically and genetically homologous to mammals including humans. The zebrafish genome has been fully sequenced so it is a powerful and tractable system. For example, genes within the zebrafish are easily manipulated making mechanistic research routine. Many genetic tools are available for use in zebrafish. Zebrafish are able to produce abundant supplies of eggs weekly. It takes less than three days for zebrafish to hatch and about 90 days to reach adulthood. Fish embryos develop externally which allows for accessible and well-controlled evaluations outside the mother. The large number of progeny that zebrafish produce allows for high throughput assays and screening. The more animals that are able to be run increases the statistical power of data produced and enables studies simply not feasible in rodents. There are thousands of transgenic and mutant zebrafish strains offering additional advantages. (Stewart et al.). It has been recently accepted that zebrafish model is a validated model to study causes for anxiety disorders. This is in part due to the recognition that zebrafish display complex emotional behavior that are also sensitive to environmental stresses. Finally, zebrafish also display both endocrine and anatomic similarities with human anxiety traits (Stewart et al.).

Embryonic Photomotor Response

The zebrafish model allows for early developmental in vivo assays. In vivo assays, conducted in the whole organism, are useful for examining complex behaviors involving the nervous system. Developing in vivo high-throughput screens would allow the study of possible alterations in complex behavioral changes that result from chemical exposures. One of the earliest complex behavioral phenotypes that the zebrafish displays involves contralateral axial contractions that do not develop until 17 hours post fertilization (hpf) (Saint-Amant, L. and P. Drapeau (1998). When embryos are illuminated with a single pulse of bright light, the frequency of contractions is

greatly increased; this response is termed the embryonic photomotor response (EPR). The complete mechanism of this nonvisual response is still not fully understood, but it is driven by cells located in the hindbrain (Kokel et al.). The response to light stimuli can be divided into two stages. Before the light stimulus is given. Sage 1, the zebrafish spontaneously contract by coiling the body axis. After the first light stimulus is given, stage 2, the zebrafish contract vigorously for ~5-7s. The EPR behavior is a phenotype that allows for behavior altering compound screening (Reif et al.). Using a custom photo motor analysis response tool (PRAT) the EPR assay was further optimized (See methods *Photomotor Response Analysis Tool (PRAT) Assay (Time series)*. A central question I posed was "does the EPR behavior change throughout development time?". In other words, are the EPR behavioral response different at 24, 25, or 26 hours post fertilization (hpf)? If so what development time point would provide the best window for maximal activity with a high signal to noise ratio? To answer these questions a developmental time series was completed for the EPR assay.

Benzo[a]pyrene

The zebrafish is a sensitive model that can be used to identify alterations in multiple behavioral endpoints, which opens the door to use this powerful model to identify chemicals that alter behavioral phenotypes. An example of an important environmental chemical with minimal behavioral toxicity data is Benzo[a]pyrene (BaP). BaP is a polycyclic aromatic hydrocarbon that is produced as a byproduct of combustion wood burning, cigarettes, and coal tar. Because of its chemical properties, BaP is a persistent organic pollutant that is lipophilic which means it can accumulate in tissue. BaP is mutagenic that results in carcinogenicity in many animal species tested. Although it is a known carcinogen, BaP's neurotoxicity remains largely unstudied. Previously published studies revealed that BaP can have a negative impact on human behavior.

For example, occupational exposure of a group of Polish workers had associated neurotoxic symptoms and short-term memory loss. The severity of symptoms were dependent on exposure levels. The children of parents that were exposed to polycyclic aromatic hydrocarbons showed increased risk of neuroectodermal tumors (Shu-qun Cheng et al. 648-658). Based on these studies, there is evidence that BaP is potentially a neurotoxin, but what neurological pathways are affected remains unknown.

There are studies in the literature suggesting that BaP may alter the genome at the epigenetic level. This brings up the possibility that BaP could potentially cause behavioral abnormalities that would be passed onto the next generation. Gene expression can be affected by multiple environmental factors especially in early development. Environmental factors such as stress can cause signals to be transmitted throughout cells. These signals can be picked up by gene regulatory proteins which bind to certain DNA sequences shutting off or turning on genes. When DNA is replicated so are the epigenetic tags and thus epigenetic tags are passed down to daughter cells (Bird). When a new organism is being formed from a sperm and egg cell epigenetic tags are erased through a process called reprogramming. This is needed to create stable gene expression in the newly developing embryo. Although most epigenetic tags are removed during reprogramming some epigenetic tags are still passed down (Rousseaux et al.).

Vitamin E Deficiency

The zebrafish model is a highly versatile model that can be used to not only study chemical insult, but also the impact of micronutrients on embryonic development and function. The Traber and Tanguay groups are interested in better understanding the effects of vitamin E deficiency on development and CNS function (Choi et. al). The main role of vitamin E is to trap free radicals

and thus protect the lipid structures it inhabits. The free radicals interact with those created when unsaturated fatty acids undergo peroxidation. Without antioxidants free radical build up in cells would surely cause tissue damage. There are currently a few vitamin E deficiency studies. One such study demonstrated mice showing an increase in subcellular oxidative damage with decreasing vitamin E intake (Edwin et al.).

Behavioral Assays in Adult Zebrafish

To asses both behavioral and epigenetic effects of BaP exposure, a number of behavioral assays have been constructed. Also three successive generations of zebrafish have been reared and spawned to adulthood, and importantly only the first generation was directly exposed to BaP. Every generation of fish was assessed through the behavioral assays. Fish from the Vitamin E fish were also assessed through two behavioral assays (shuttlebox and startle response).

Shoaling Assay

The first behavioral assay looked at is shoaling behavior. The zebrafish, like humans, is a social organism, and shoaling refers to when zebrafish form close groups and swim together. Zebrafish display this behavior for a variety of reasons such as predator defense and increased mating opportunity. So why is this behavior worth looking into? Humans display a variety of complex social behaviors which can be altered. Many neurodevelopmental conditions including anxiety disorders, depression, and autism attribute to abnormal social behavior. It is yet not understood how these disorders affect social behavior. Zebrafish are well accepted to examine complex behaviors and possibly gain an insight into the mechanisms underlying abnormal social behaviors and their relation to many neurodevelopmental conditions (Buske and Gerlai).

Associated Learning Assay using Shuttlebox

As stated before zebrafish have similar brains and nervous systems to our own. Therefore, alterations in learning and memory in zebrafish can possibly be translated into humans. Zebrafish aslo display a variety of testable cognitive behaviors including habituated learning, active avoidance, conditioned place preference, and associative learning (Bailey, Oliveri, and Levin). The shuttlebox assay examines associative learning in zebrafish (See Methods *Shuttlebox Assay).* Associative learning can be defined as learning that a certain stimulus results in a particular consequence. Associative learning assays (ALA) can be categorized into two groups. The first being positive ALA which usually involve a reward such as food for associative learning. The other is negative ALA which usually involves a punishment like an electric shock for a certain stimulus (Ruhl et al.). The shuttlebox assay in the Tanguay laboratory is a negative ALA since it involves a negative consequence to a certain stimulus (darkness).

Fear Response Assay

Using a custom built zebrafish Visual Imaging System (zVIS) fear responses from zebrafish were analyzed (See Methods *Predator Fear Response/ Startle Response Assays).* Analysis of fear responses serve as a behavioral reaction that may be tied to fitness and cognitive perception. In nature, a fear response to potential dangers such as predators is normal. Fear response analysis holds clinical relevance since some neurobiological disorders can lead to exaggerated fear responses (Ahmed, Seguin, and Gerlai). Analysis of fear responses in zebrafish can help us understand the mechanisms behind many fear response abnormalities. Several behavioral paradigms for fear responses have been developed for zebrafish (jumping and erratic movement) (Ahmed, Seguin, and Gerlai). This behavior can be analyzed using the zVIS instrument that uses

tracking software (Noldus EthioVision XT 10) to track individual fish while a fear response is elicited.

Startle Response Assay

The startle response assay was used to study possible neurological abnormalities (anxiety) caused by chemical exposures or vitamin deficiencies. The startle response assay uses solenoids to generate taps. The startle response can be seen as a reflex invoked by an unexpected stimulus like a tap. This behavior is seen in many species such as humans and is seen as a reaction to possible threats. This startle response behavior is useful in that it can help an organism avoid harm. Startle assays are useful in neurobehavioral studies. Cognitive processing of stimuli is needed to elicit a startle response. This can be helpful in studying cognitive deficits which are presented in many anxiety disorders. Normal startle responses in adult zebrafish can be characterized as high velocity swimming and rapid turns. Habituation of startle stimuli is observed in zebrafish. Habituation of startle stimuli is a cognitive function that is able to be evaluated easily (Pittman and Lott). zVIS is used to analyze this behavior by using tracking software (Noldus EthioVision XT 10) to track fish before and after tap stimulus is given.

Materials and Methods

General Fish Husbandry

Tropical 5D strain (Danio rerio) zebrafish from the Sinhuber Aquatic Research Laboratory at Oregon State Univesity were used for all studies. The fish water is made of reverse osmosis water containing Instant Ocean^{TM} ,a commercially available salt to maintain salinity was kept at

600 microsiemens and the pH was adjusted to 7.4 by the addition of sodium bicarbonate. Adult zebrafish were kept at 28 °C fish water on a 14h light and 10-hour dark photoperiods (Truong et al.). Embryos were collected from 100 gallon massive embryo production tanks containing 1000 adult zebrafish (Reif et al.).

Embryonic Photomotor Response (EPR) (Developmental Time Series)

At 6 hours post fertilization, fish embryos were placed into 96 well plates, filled with 100 µl of embryo media, using automated embryo placement systems (AEPS). After plates were loaded with embryos they were sealed with parafilm to avoid evaporation, followed by wrapping in aluminum foil to eliminate light exposure. Embryos at this stage of development can adapt to the dark and develop normally. The covered plates were then placed into an incubator at 28 °C. Plates were assessed using the PRAT instrument the next day at every hour starting at 9 am through 10 pm. PRAT was used to observe and analyze zebrafish PMR behavior. A Prosilica GX3300 (Allied Vision, Stadtroda, Germany) is utilized by PRAT. PRAT has a near infrared (NIR) band-pass filter that removes stimulus light. The system contains double telecentric lenses (Navitar, Rochester, New York), which are located underneath the plate holder in an inverted fashion. This is to minimize distortion and interference. Illumination for imaging is achieved with a NIR (850 nm) backlight (Smart Vision Lights, Muskegon, MI). The light stimulus is given by L300 Linear Lights (Smart Vision Lights, Muskegon, MI). Custom hardware controls timing of the high intensity light stimuli and the backlight. Recording begins immediately before the light cycle starts. PRAT captures 850 frames of digital video, and is recorded at 17 frames per second. A light cycle has a 30 second background period (before first light), a 9 second light

pulse is then followed by a second light pulse. After both light pulses there is 10 seconds of darkness (Reif et al.).

Custom MATLAB (Mathworks, Natick, MA) scripts were used to analyze videos. Movement indexes were computed at each frame stamp (the differences in pixels between frames). Custom R scripts (R Core Team 2014) were used to process the MATLAB output. There is lag time between video recording initiation and when the light cycle starts/ends. R scripts thus removed this lag time. From the frame stamp a time stamp was made. This was done by using the first stamp after the video start as time point 0 seconds (Reif et al.).

Adult fish Developmentally Exposed to Benzo[a]pyrene

In the BaP study, there are two exposure groups and a control group. Zebrafish at 6 hours post fertilization (hpf) are placed into individual wells of 96 well plate then exposed to 5 μM (1.25 ppm), 10 μM (2.5 ppm) BaP or 0.1% DMSO (negative control). DMSO had to be used in order to make BaP more water soluble. At 120 hpf, embryos were rinsed and raised in chemical-free water until adulthood (>90days). Exposed parents were then spawned to generate the next generation. In total three successive generations were generated including the original exposed group. These BaP fish were run through shoaling, fear response, and shuttlebox assays.

Adult Fish with Differential Vitamin E Levels

For the vitamin E study, fish were on laboratory diets (a commercial feed) until they were 54 days old. After 54 days, a subset of fish were fed either vitamin E deficient or vitamin E

sufficient food. For the vitamin E study only the shuttlebox, tap response, and fear assays were applied.

Adult Fish Shoaling Assay

To test shoaling behavior, two male and two female zebrafish were placed into tanks. Using viewpoint tracking software individual fish were able to be tracked in the tank. A white backlight was used to improve contrast. Six tanks were placed on a rack and tracked simultaneously. A total of 24 fish were ran at a time, and the fish were tracked for an sixty minutes.

Adult Zebrafish Shuttlebox Assay

Shuttleboxes were used to evaluate learning and active avoidance conditioning in zebrafish. The Shuttlebox design consists of a black acrylic box with dimensions of 200 mm length, 100 mm width, and 90 mm depth. The Shuttlebox has a divider with a 10 mm gap between the floor of the box and the divider. This is to allow the fish to shuttle between sides (avoid shock). A light beam detector located in the 10 mm gap tracked which side the fish was on. The Shuttlebox is filled with about 350 mL of water which ensured that the 10 mm gap was submerged, allowing the fish to swim to both sides. The conditioned stimulus (blue light) was created using LED light bars on the top of the Shuttlebox lid. The unconditioned stimulus was a mild electric shock activated when the fish did not respond to the conditioned stimulus. The whole box is given an electrical charge when the fish is on the wrong side. This electric shock is generated using two stainless steel plates located at each end of the box (Truong et al. 134-142).

Trials are run consecutively and there is a 60 second intermediate trial period before the next starts; the LED white lights are on, in both sides of the box during this time. After the first trial there is an acclimation period (600s) and the trial will start with the 8 second avoidance period and then a 16 shock period. A trial consists of a total of 24 seconds (avoidance and Shock). There is a humane fault out limitation programed into the Shuttlebox in which if a fish fails to be on the non shocked side for 8 consecutive trials then the testing will stop (Knecht 2016). For the BaP study a different Shuttlebox protocol was used. Trials still consisted of 24 seconds total. However, there was a 4 second avoidance period instead of 8s (Knecht 2016).

Adult Predator Fear Response/ Startle Response Assays

Zebrafish Visual Imaging System (zVIS) was used to measure fear responses. The zVIS system consists of an array of 8 tanks with only single side views of video projections on LCD monitors. Each tank was filled with 750 mL of fishwater. This allows individual fish to visualize a single predator fish. For the predator test, zebrafish were expected to move away from the screen. The proximity of the zebrafish is tracked relative to the LCD screen projections using a tracking program called Noldus EthioVision XT 10. Within Noldus arenas are created that cover the rims of the square tanks. The arenas are then subdivided into three zones: close, middle, and far. Each represent the distance the zone is from the LCD screen. The Noldus software tracks the velocity of the fish and the frequency spent in each zone. Data is analyzed by evaluating at the time intervals that each video is played. Within these time intervals time bins of 10 seconds was analyzed. Looking at the data one can see where the fish spent most of its time before, during, and after the video has played. A predator video of 1 minute was played. One minute prior to the predator video was also analyzed. zVIS was also used to analyze tap responses. Underneath the 8

tanks there is a board that has a tapping mechanism to startle the fish. All 8 tanks are able to receive a simultaneous tap. The vitamin E study fish received a total of 3 taps with 5 minute intervals in between. BaP fish received a total of 10 taps with 20 seconds in between.

Consecutive Assays (BaP)

For the BaP exposed fish studies, tests were done consecutively. Each fish was given a number (e.g 1a,1b,1c,1d,2a,2b…6d) to track each fish individually throughout each test. This way data could be collected on individual fish instead of as a group. The sex of the fish was also noted with all "a","c" fish being male and "b","d" fish being female(e.g. 1a=male and 1b=female). All available assays were run for the Bap study and their order is as follows

Shoaling

TANK 6- HIGH BaP	TANK 5- HIGH BaP	TANK 4- LOW BaP	TANK 3- LOW BaP	TANK 2- DMSO	TANK 1- DMSO

Camera 2

5D	5C	5B	5A
6D	6C	6B	6A

Camera 3

3D	3C	3B	3A
4D	4C	4B	4A

Camera 4

1D	1C	1B	1A
2D	2C	2B	2A

Shuttlebox Assay, Run 1

Sh box 14	Sh box 13	Sh box 12 5D	Sh box 11 5C	Sh box 10 5B	Sh box 9 5A	Sh box 8 3D
Sh box 7 3C	Sh box 6 3B	Sh box 5 3A	Sh box 4 1D	Sh box 3 1C	Sh box 2 1B	Sh box 1 1A

Shuttlebox Assay, Run 2

Sh box 14	Sh box 13	Sh box 12 **6D**	Sh box 11 **6C**	Sh box 10 **6B**	Sh box 9 **6A**	Sh box 8 **4D**
Sh box 7 **4C**	Sh box 6 **4B**	Sh box 5 **4A**	Sh box 4 **2D**	Sh box 3 **2C**	Sh box 2 **2B**	Sh box 1 **2A**

Results

Embryonic Photomotor Response Results

Time series results show that time points 10-12 (35-37 hpf) result in the highest movement peaks (**Fig.1)**. Movement peaks are significantly lower during time points 0-2 (25-27 hpf) ranging from 22.89-43.66 (**Table 1**). Time points 3-8 (28-33 hpf) all fall around the 100 range (**Table 1**). After time point 12 (37 hpf) there is a reduction in movement (**Table 1**). B, E, R refer to the PRAT light cycle intervals. B being before the light pulse or background. E being during light pulse (excitation period) and R (Refractory period) is after the second light pulse.

Table 1.

Time Point	Time	Interval	Movement.peak
0	**9:00 AM**	**B**	**24.77**
0	**9:00 AM**	**E**	**43.66**
0	**9:00 AM**	**R**	**3.46**
1	**10:00 AM**	**B**	**19.75**
1	**10:00 AM**	**E**	**22.89**
1	**10:00 AM**	**R**	**1.96**
2	**11:00 AM**	**B**	**17.91**
2	**11:00 AM**	**E**	**39.55**
2	**11:00 AM**	**R**	**2.80**
3	**12:00 PM**	**B**	**19.57**
3	**12:00 PM**	**E**	**105.57**
3	**12:00 PM**	**R**	**1.69**
4	**1:00 PM**	**B**	**18.99**
4	**1:00 PM**	**E**	**121.27**
4	**1:00 PM**	**R**	**0.95**
5	**2:00 PM**	**B**	**21.48**
5	**2:00 PM**	**E**	**136.51**

5	**2:00 PM**	**R**	**2.28**
6	**3:00 PM**	**B**	**18.31**
6	**3:00 PM**	**E**	**105.23**
6	**3:00 PM**	**R**	**5.26**
7	**4:00 PM**	**B**	**23.86**
7	**4:00 PM**	**E**	**141.80**
7	**4:00 PM**	**R**	**0.90**
8	**5:00 PM**	**B**	**18.52**
8	**5:00 PM**	**E**	**152.21**
8	**5:00 PM**	**R**	**3.64**
9	**6:00 PM**	**B**	**13.92**
9	**6:00 PM**	**E**	**202.04**
9	**6:00 PM**	**R**	**2.98**
10	**7:00 PM**	**B**	**10.01**
10	**7:00 PM**	**E**	**264.07**
10	**7:00 PM**	**R**	**2.77**
11	**8:00 PM**	**B**	**10.05**
11	**8:00 PM**	**E**	**295.12**
11	**8:00 PM**	**R**	**3.58**
12	**9:00 PM**	**B**	**3.66**
12	**9:00 PM**	**E**	**255.07**
12	**9:00 PM**	**R**	**1.42**
13	**10:00 PM**	**B**	**2.20**
13	**10:00 PM**	**E**	**132.17**
13	**10:00 PM**	**R**	**2.00**

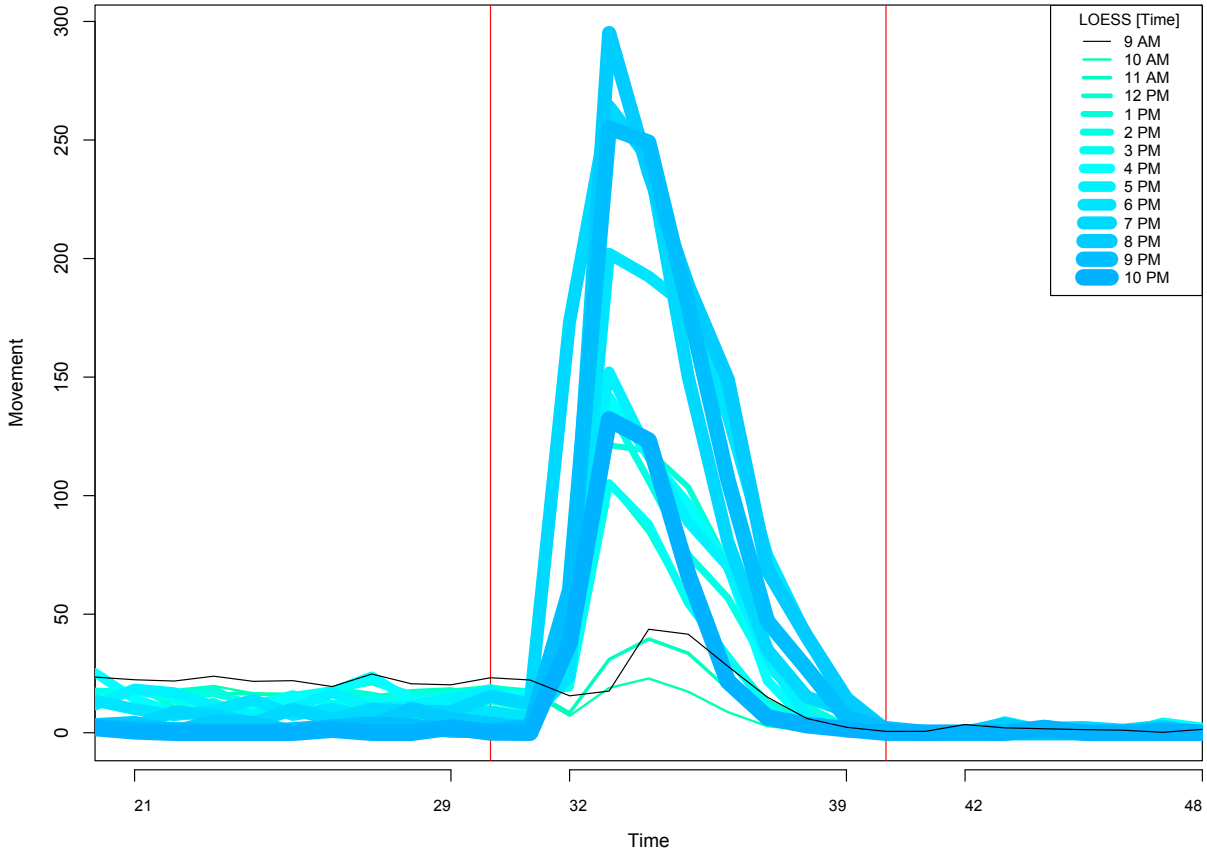

Figure 1. Time series of PMR activity. First red line is the start of the first light pulse. The second light pulse starts at the second red line. Time 21-29 is activity before light pulse. Time 42-48 is after second light pulse.

Vitamin E Study (Startle Response)

Normal swimming activity is shown in **Figure 2**. The startle response data shows slower habituation of vitamin E deficient fish to startle stimulus than E+ fish (**Figure 4**). During the 10-minute acclimation period it can be seen that both groups show similar swimming activity (**Figure 3**). After the first tap was initiated, both groups responded similarly (**Figure 4**). The E- group fish displayed similar magnitudes of swimming activity for all three taps. The E+ group however showed a decrease in swimming activity after each tap.

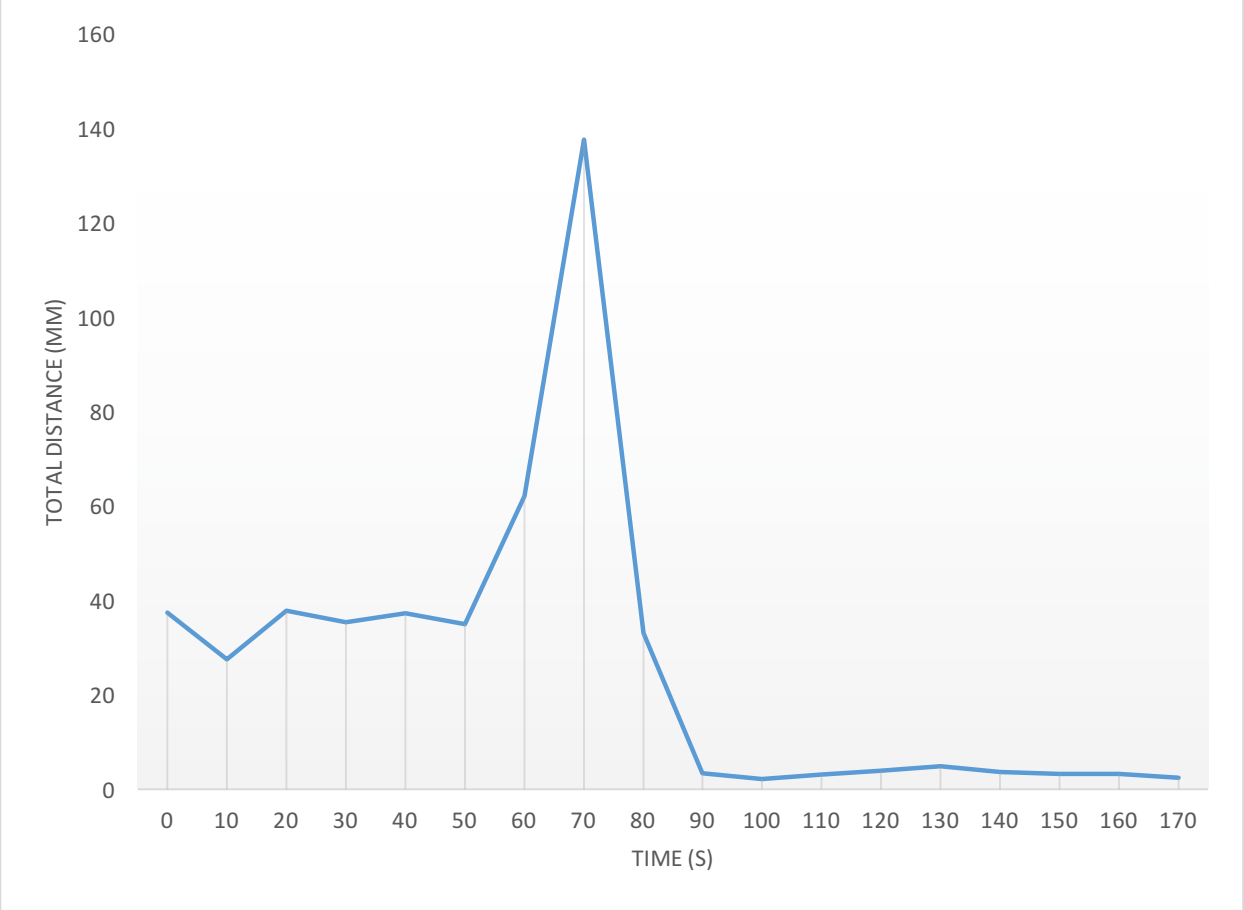

Fig. 2. Typical adult zebrafish swim response to a single-tap startle.

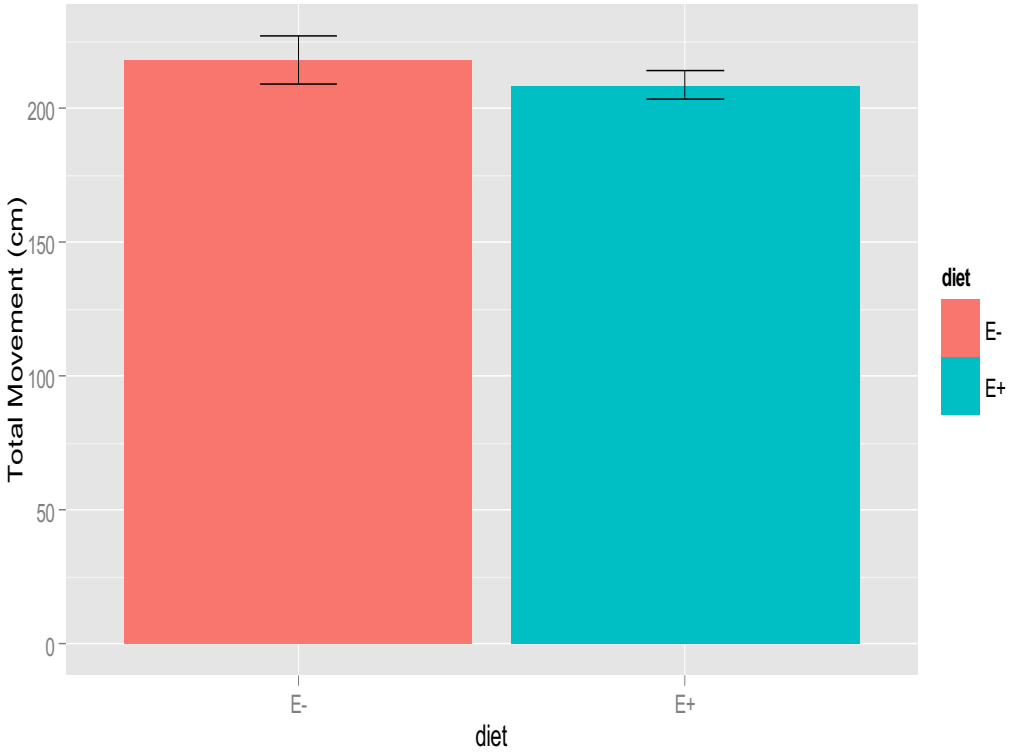

Fig. 3. Total swim distance over the 10 minutes of acclimation prior to the startle. Vitamin E sufficient (blue) and Vitamin E deficient fish (orange).

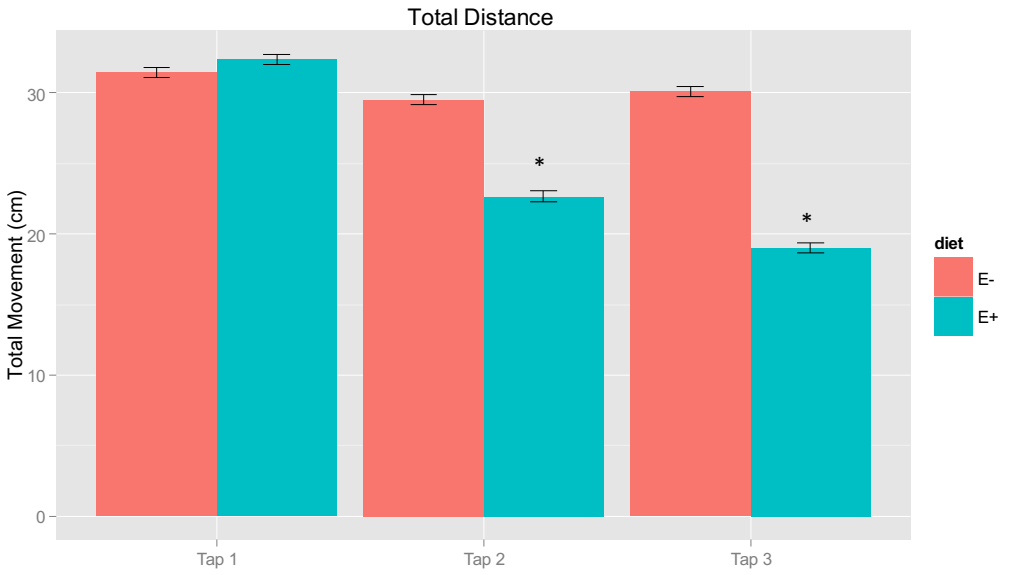

Fig. 4. Swim activity associated with the startle response.
Vitamin E sufficient (blue) and Vitamin E deficient fish (orange). n=64.

Vitamin E Study (Shuttlebox)

The TimetoASide variable was used to access learning in adult zebrafish. TimetoASide refers to the initial time it takes the fish takes to swim to the conditioned stimulus. From the data we see two linear regression trends in both micronutrient groups (**Fig. 5**). The Vitamin E+ fish group trend line is steeper than the Vitamin E- group (**Fig. 5**).

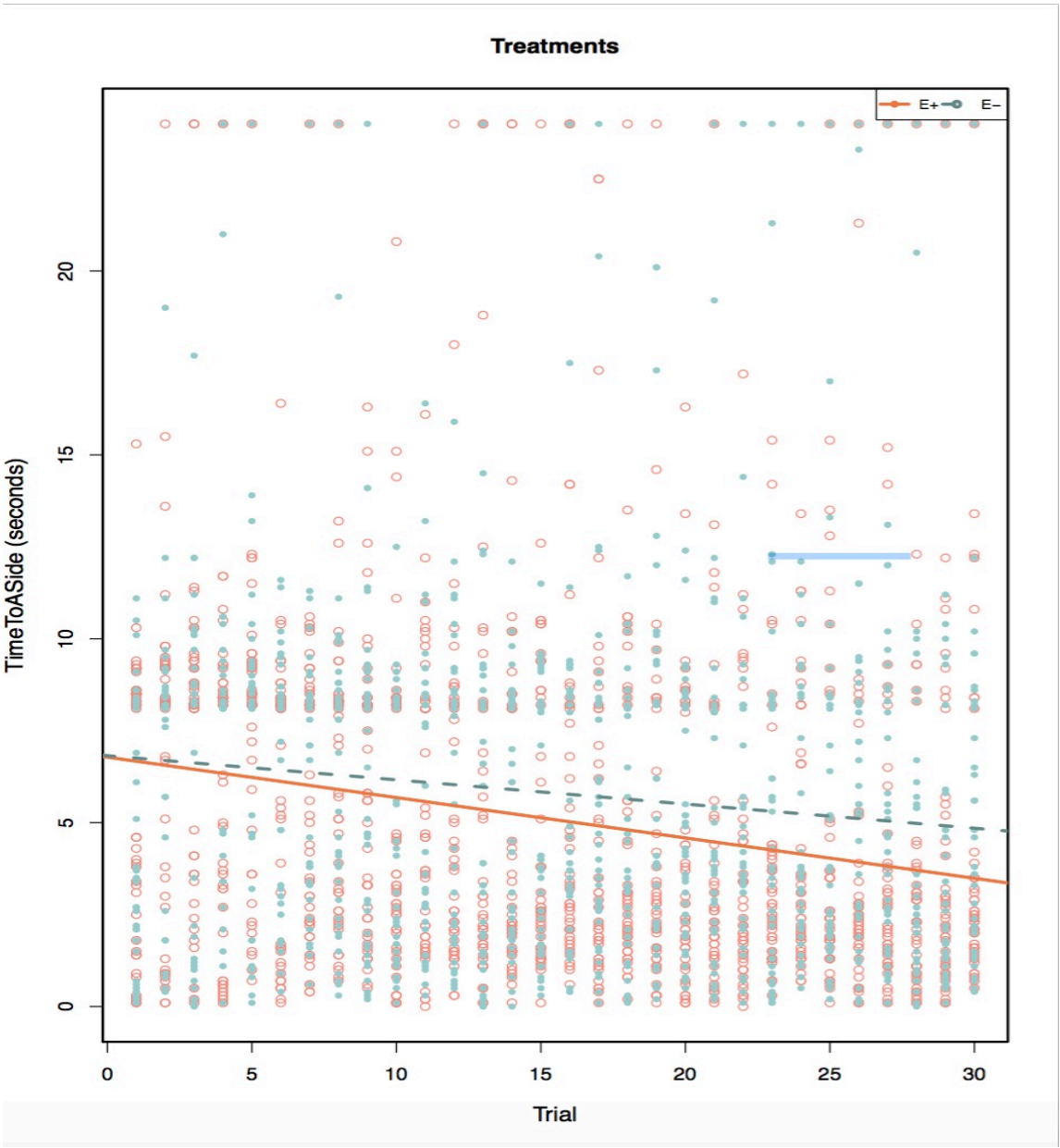

Fig.5. Time it took fish to swim to the conditioned stimuli during avoidance period. Vitamin E sufficient (orange) and Vitamin E deficient fish (blue). n=84

Benzo[a]pyrene Study

Shoaling Assay

In **Fig. 6** we see all F0 generation fish swim slower than the F1 and F2 generations**.** F0 2.5 ppm BaP exposed fish had higher swimming speeds than the rest of the F0 exposure groups (peaks at 5 cm/s) until the 40-minute mark, where the average speed drops to 3 cm/s. F0 1.25 ppm BaP exposed fish swam slower than the 2.5 ppm BaP fish, but faster than the 0.1% DMSO control fish (controls: 4.6 cm/s 1.25 BaP: ~4.7 cm/s). F1 generation fish swim faster than all F0 fish but slower than the F2 fish. We see a similar trend in that 2.5 ppm BaP F1 fish swam the fastest at ~5.9 cm/s, followed by 1.25 ppm BaP F1 fish (~5.5 cm/s) with the 0.1% DMSO F1 control fish being the slowest (~5.3-5.4 cm/s). In the F2 generation, 1.25 ppm BaP fish swam the fastest (~6.3-6.4 cm/s) until the 25-minute mark, when 2.5 ppm BaP fish began swimming at similar speeds to 1.25 BaP fish (~5.7-5.8 cm/s). There are two spikes in the 2.5 ppm BaP F2 group at 49 minutes and 55 minutes where speed reaches 6.3 cm/s.

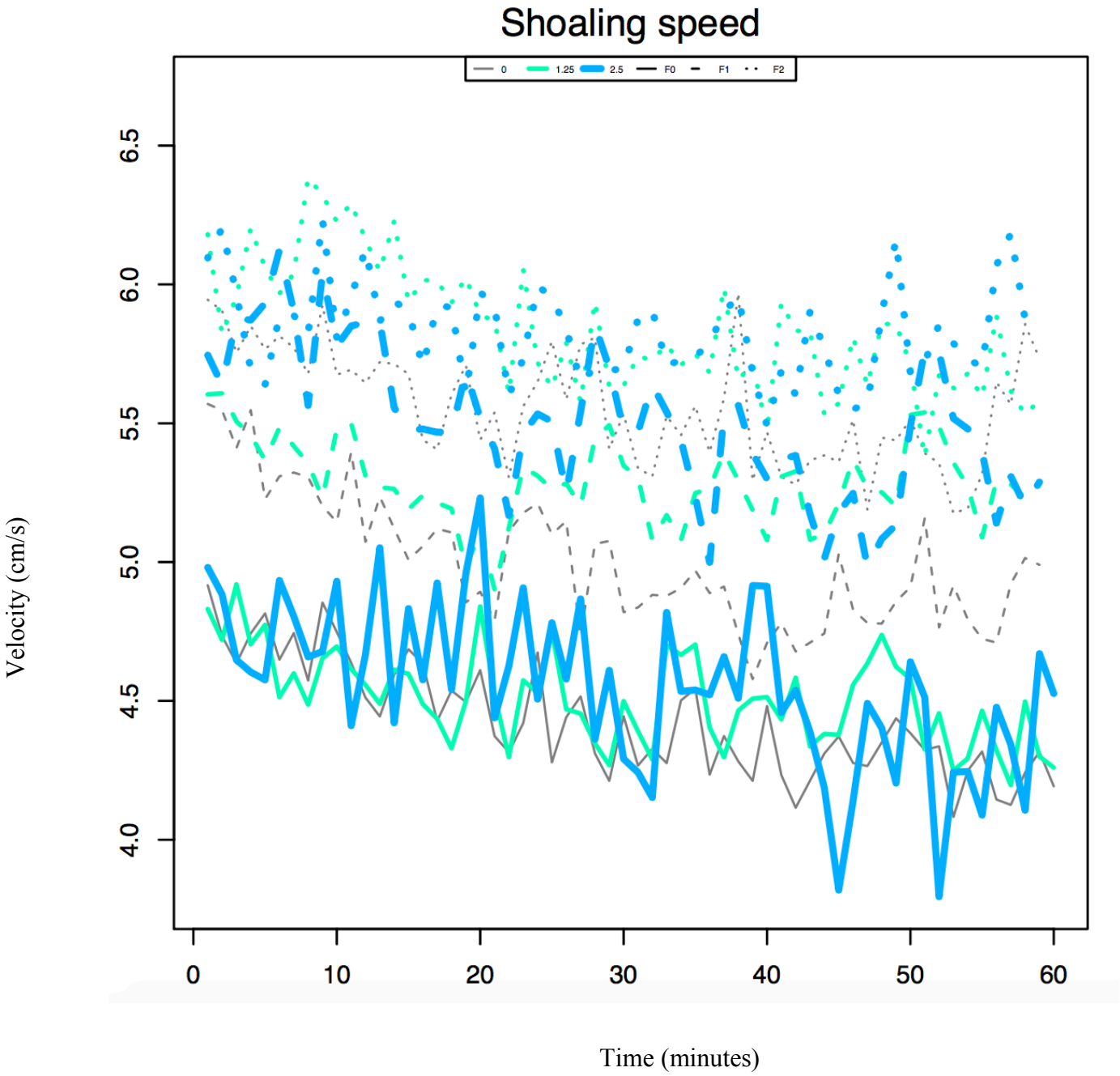

Fig. 6. Average swim speeds during 60-minute time period. Controls (black lines),1.25 ppm BaP treated fish (green lines), and 2.5 ppm BaP treated fish (blue line). F0 (solid lines), F1 (dashed lines), and F2 (smaller dashed lines).

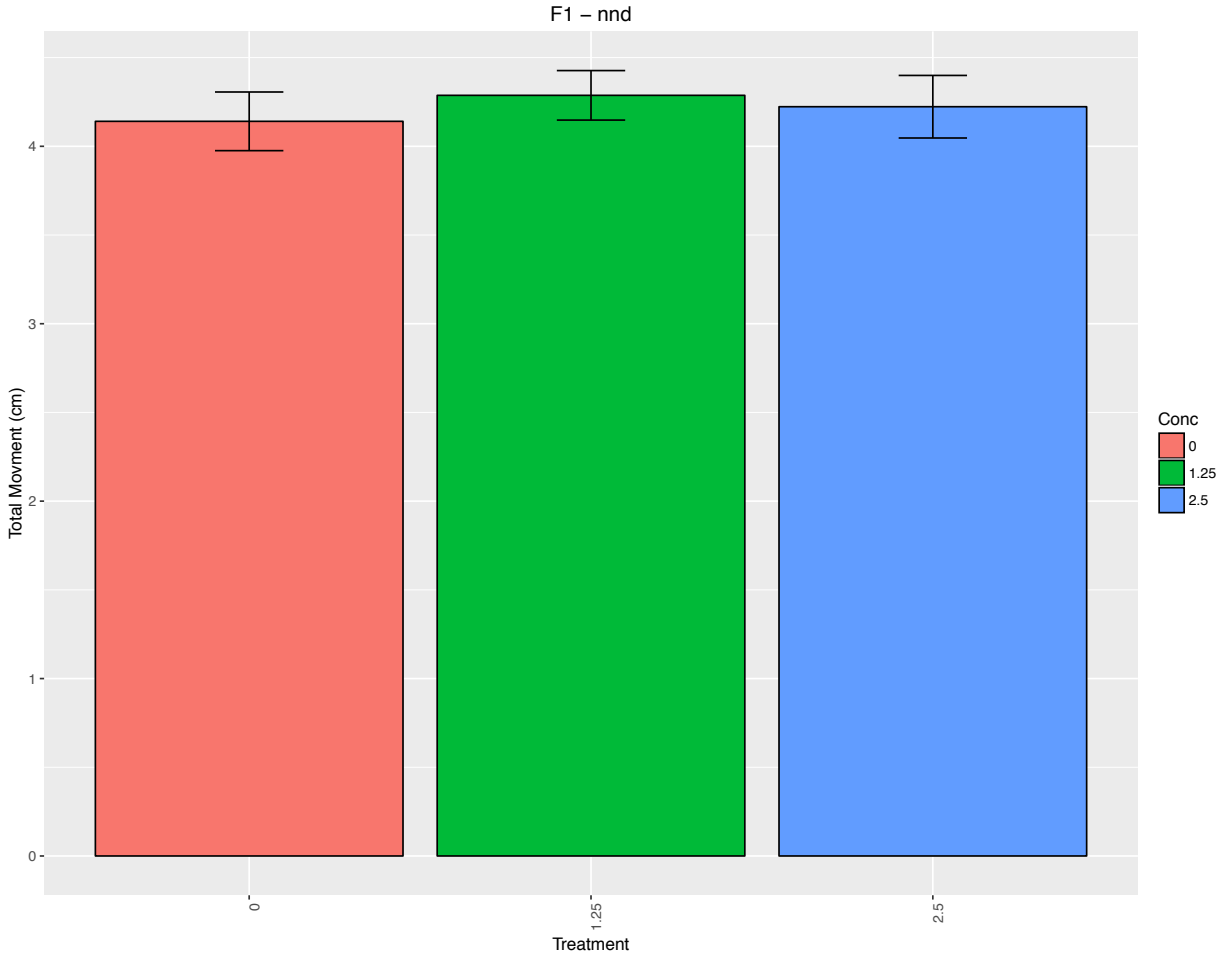

Fig.7. Nearest Neighbor Distance averages for F1 generation fish. 0.1% DMSO controls(orange), 1.25 ppm BaP (green), and 2.5 ppm BaP (blue). n=80

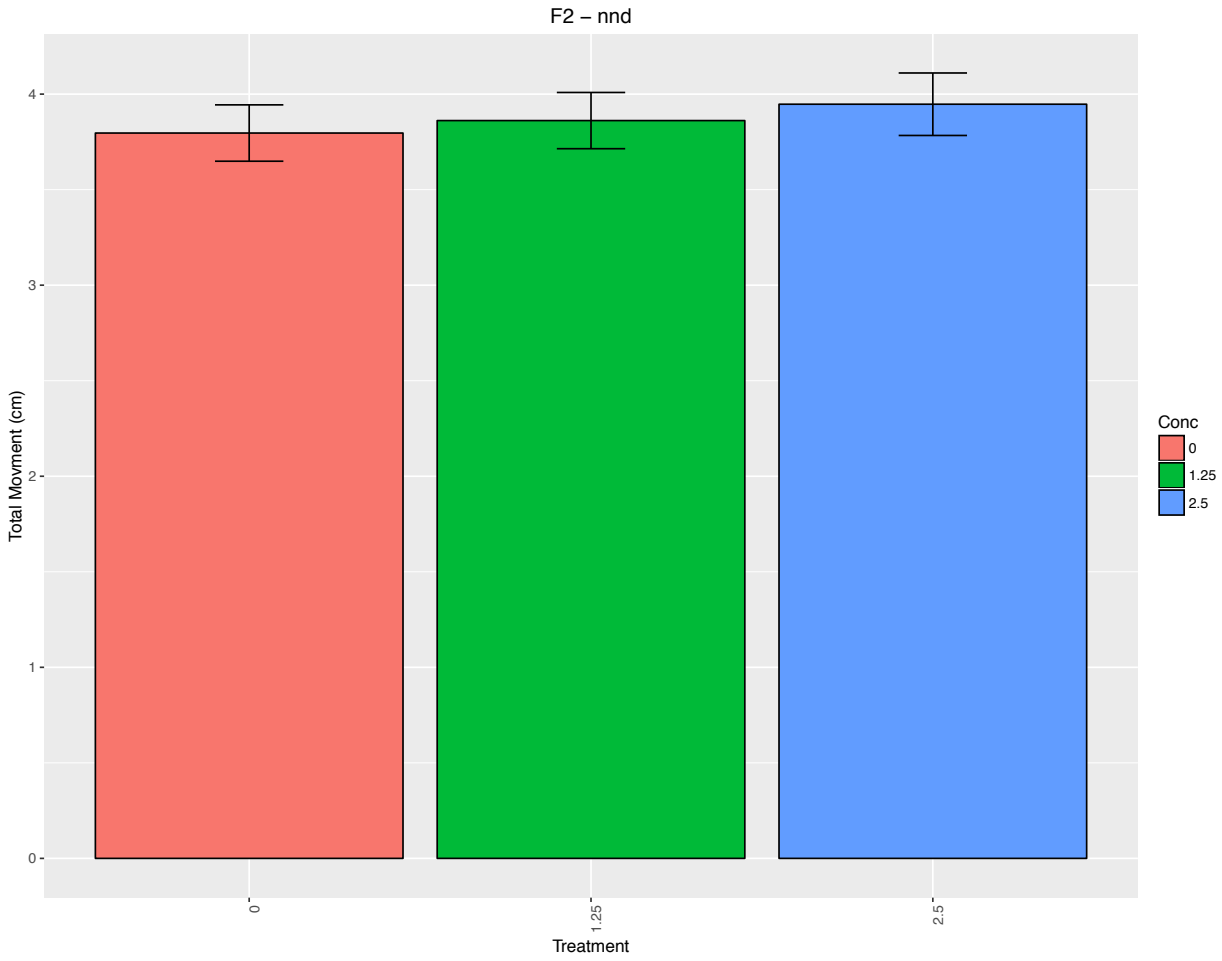

Fig.8. Nearest Neighbor Distance averages for F2 generation fish. 0.1% DMSO controls(orange), 1.25 ppm BaP (green), and 2.5 ppm BaP (blue). n=64

Graphs in **Fig. 7** show Nearest Neighbor Distance (nnd) averages of F1 and **Fig. 8** shows nnd averages for F2 generation fish. The nnd parameter takes averages of the distance between the closest fish in the shoal. Figure 7 shows no significant difference in nnd values between the three exposure groups. They all average around 4-4.4 cm. In the F2 generation we also see no significant difference in nnd values between the three exposed groups (**Fig. 8**). All exposure groups average 3.7-3.9 cm (**Fig. 8**). Between the F1 and F2 generations all values dropped from ~4.5 cm to ~3.8 cm (**Fig. 7 and 8**)

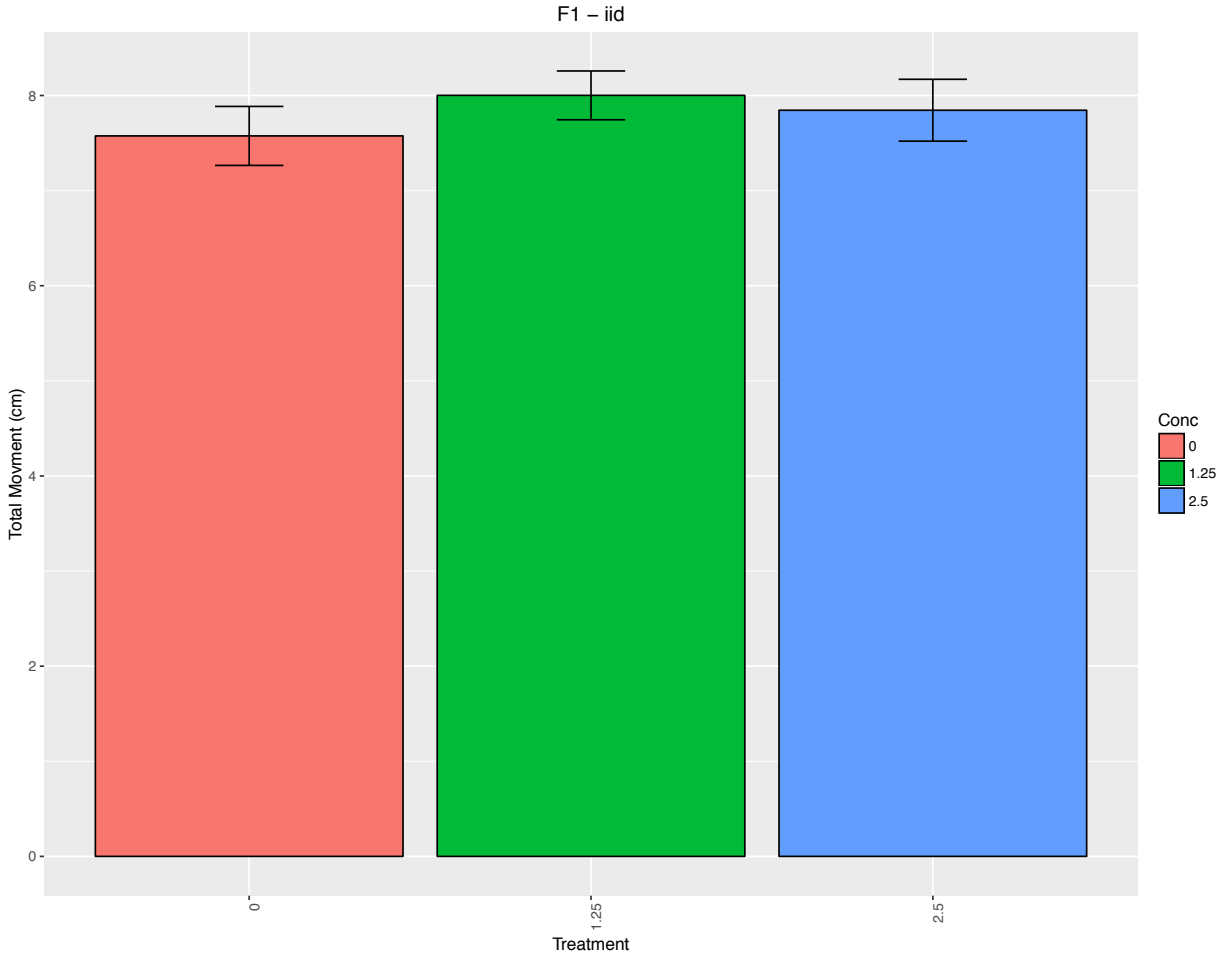

Fig.9. Graph shows Inter-individual Distance (iid) averages of three exposure groups in the F1 generation.0.1% DMSO controls(orange), 1.25 ppm BaP (green), and 2.5 ppm BaP (blue). n=80

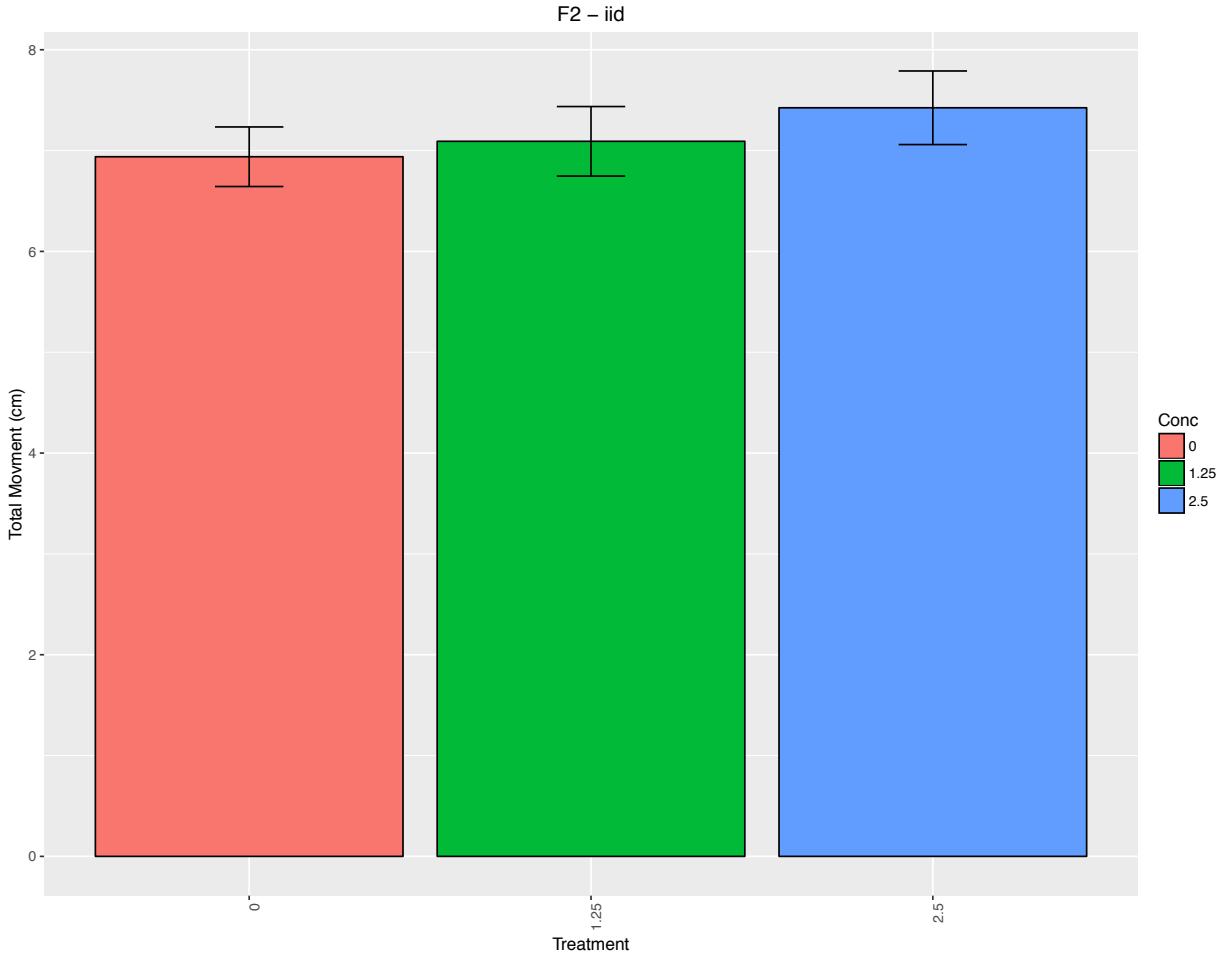

Fig.10. Graph shows Inter-individual Distance (iid) averages of three exposure groups in the F2 generation.0.1% DMSO controls(orange), 1.25 ppm BaP (green), and 2.5 ppm BaP (blue). n=64

Graphs in both Figure 9 and Figure 10 show the shoaling parameter Inter-individual Distance (iid). This parameter looks at the average distance the closest fish in the shoal are. Figure 9 shows no real significant difference in iid values between the BaP exposed fish (1.25 ppm BaP and 2.5 ppm BaP). All **Fig. 9** exposure groups averaging ~8 cm. Figure 10 shows no significant difference between the F2 1.25 ppm BaP, 2.5 ppm BaP fish, and 0.1% DMSO controls (~7-7.5 cm). Between the F1 and F2 generations all values dropped from ~8 cm to ~7-7.5 cm (**Fig 9 and 10**).

Fear Response Assay

Close Zone

Figure 11 shows one minute prior to video and during the minute-long video. We see the same trend in all generations and exposure groups. Once the video begins at 60s, the time spent in the close zone sharply drops. What is examined is the time during the predator video. F1 1.25 ppm BaP fish have higher average duration value in the close zone (~10%) than both F1 0.1% DMSO controls (~5%) and F1 2.5 ppm BaP fish (~5%). F2 1.25 ppm BaP (~10%) are seen to again has a higher duration percentage than 2.5 ppm BaP fish (~5%) but has similar values compared to F2 0.1% DMSO controls (~10%).

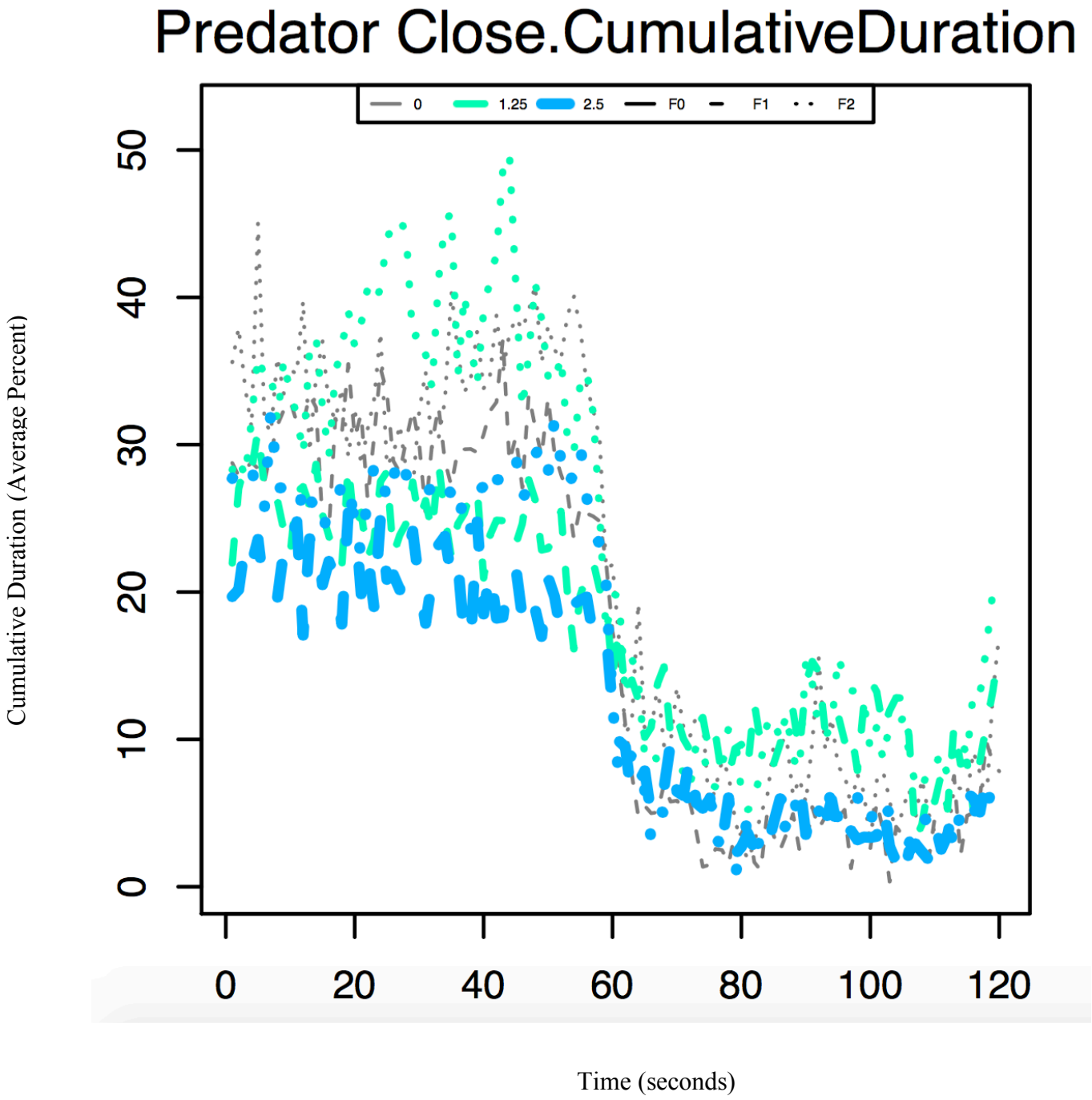

Fig. 11. Graph shows the average cumulative duration as a percent value of each exposure group within a 120 second time period. The Predator Video was played at the 60 second mark. 0.1% DMSO controls (black lines),1.25 ppm BaP treated fish (green lines), and 2.5 ppm BaP treated fish (blue line). F1 (dashed lines), and F2 (smaller dashed lines).

All graphs in **Figure 12** shows the average cumulative duration percentage in the close zone during the predator video. F1 2.5 ppm BaP fish and 0.1% DSMO controls average ~7% while 1.25 ppm BaP fish average ~15%. F2 2.5 ppm BaP fish have the lowest value at 3-5%. F2 0.1% DMSO controls range around 10% and 1.25 ppm BaP F2 fish at ~15%.

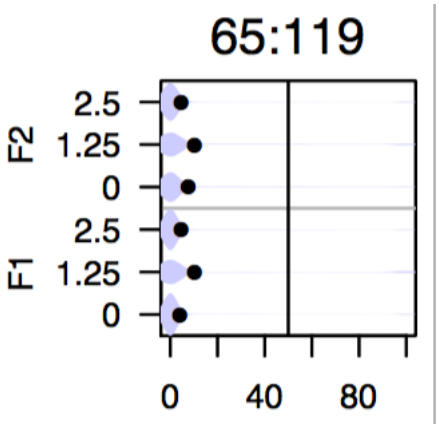

Fig.12. The graph shows data from each generation (F1, F2) and exposure groups (0.1% DMSO control,1.25 ppm BaP, 2.5 ppm BaP). Graph displays the average percentages for the time interval 65-119 seconds.

Middle Zone

All exposure groups and controls throughout each generation seem to display similar cumulative duration percentages over the 120 second time period (**Fig.13**). For the first 60s, duration values were between 30 and 40% for all exposure groups and generations. After 60s, values dropped to between 20 and 30%. There are some low spikes seen in the F2 0.1% DMSO controls, which go as low as 10 percent at 5s and 41s. Also F2 0.1% DMSO controls and F2 1.25 ppm BaP fish at time 60s deviate from the main trend. Values are high in both these groups, rising from 40 to 48-

49%. F2 1.25 ppm BaP fish values start falling at 116s, and at 120s duration drops to 20% in the middle zone.

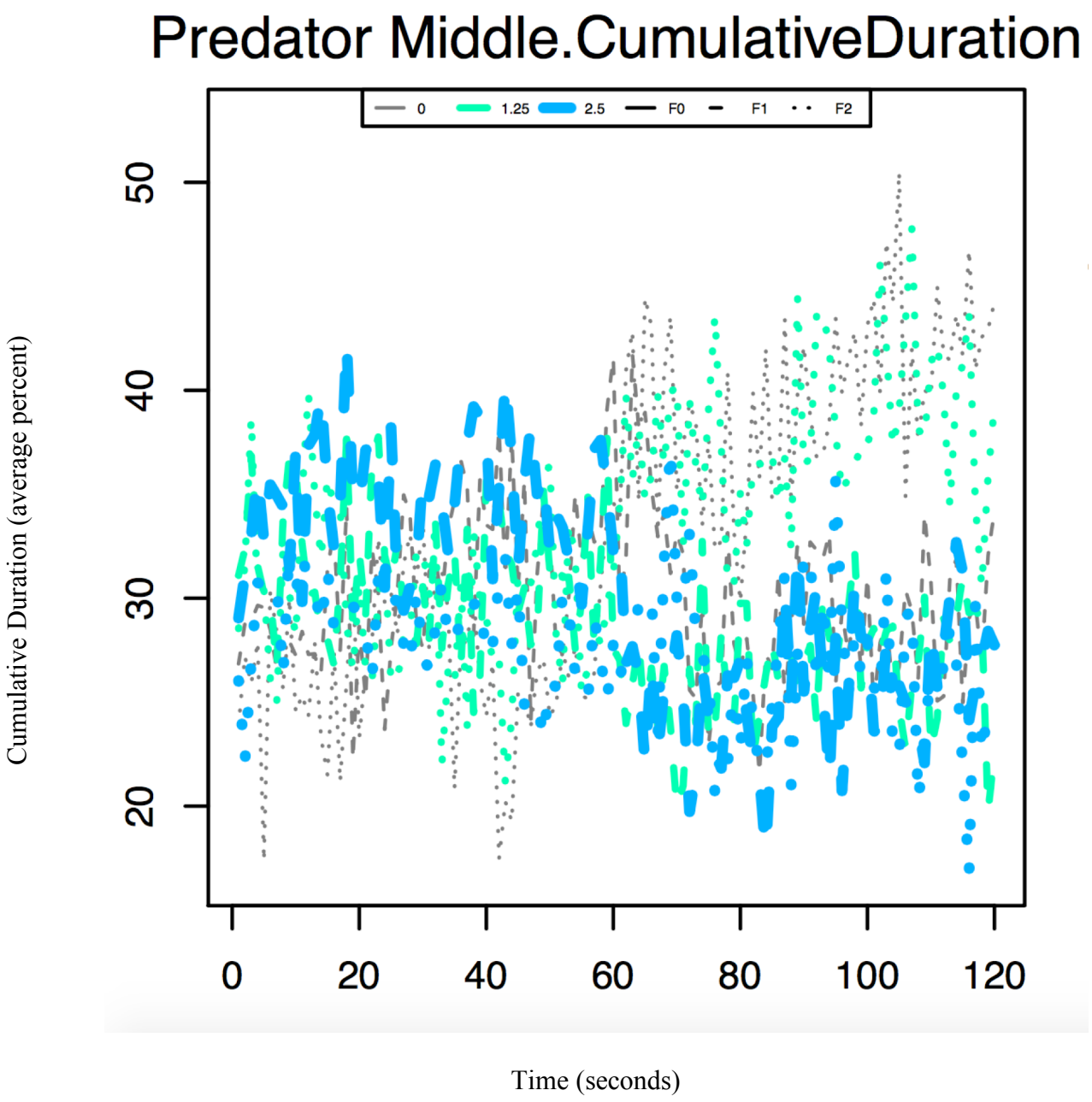

Fig.13. Graph shows cumulative average percent duration in the middle zone. The Predator Video was played at the 60 second mark. 0.1% DMSO controls (black lines),1.25 ppm BaP treated fish (green lines), and 2.5 ppm BaP treated fish (blue line). F1 (dashed lines), and F2 (smaller dashed lines).

F1 fish values are similar between BaP exposure groups and 0.1% DMSO controls **(Fig. 14)**. F1 0.1% DMSO controls average 30%, F1 1.25 ppm BaP fish 28%, and F1 2.5 ppm BaP fish average 27%. F2 0.1% DMSO controls and 1.25 ppm BaP fish have the highest average percent duration at 41%-42%. F2 2.5 ppm BaP fish average ~30% duration in the middle zone.

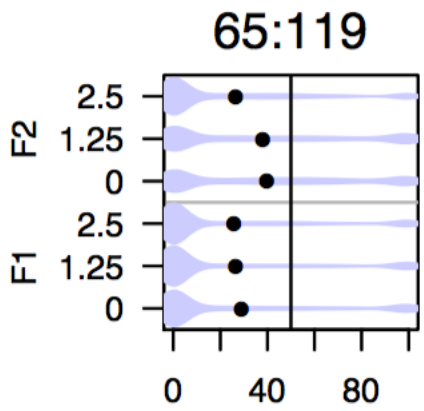

Fig. 14. Graph shows average cumulative duration percentages within the middle zone. Graph shows the averages of the time interval 65-119 seconds.

Far Zone

All fish in **Fig. 15** follow the same pattern of going from low to high values after the 60s time period, which is the time the predator video begins. The 2.5 ppm BaP groups in both the F1 and F2 generation have very similar cumulative duration values. Both generations stay around a duration of 40-45% until the 60s time period. After the 60s time period, duration values spike up and level out at 65-71% at ~80s. Both F1 and F2 controls values stay at 35-40% during the first 60 second time period, with duration values spiking as the soon as the video starts. The F1

controls and 2.5 ppm BaP fish stay at 65-71% from 70-120s time period. F2 controls have values averaging in between 50-60% during the 70-120s time period. F1 1.25 ppm BaP fish averaged a duration of 40-45% before the video, and once the video began, duration spiked to 65% within 5s, and held at 60-65% for the remainder of the second 60s time period. F2 1.25 ppm BaP fish had the lowest duration before the 60s time period have the lowest duration values out of all the fish staying in between 20-35. Value do go up after the video. They reach a value of 60 at ~61s and fall down and stay in between 45-55 for the rest of the time.

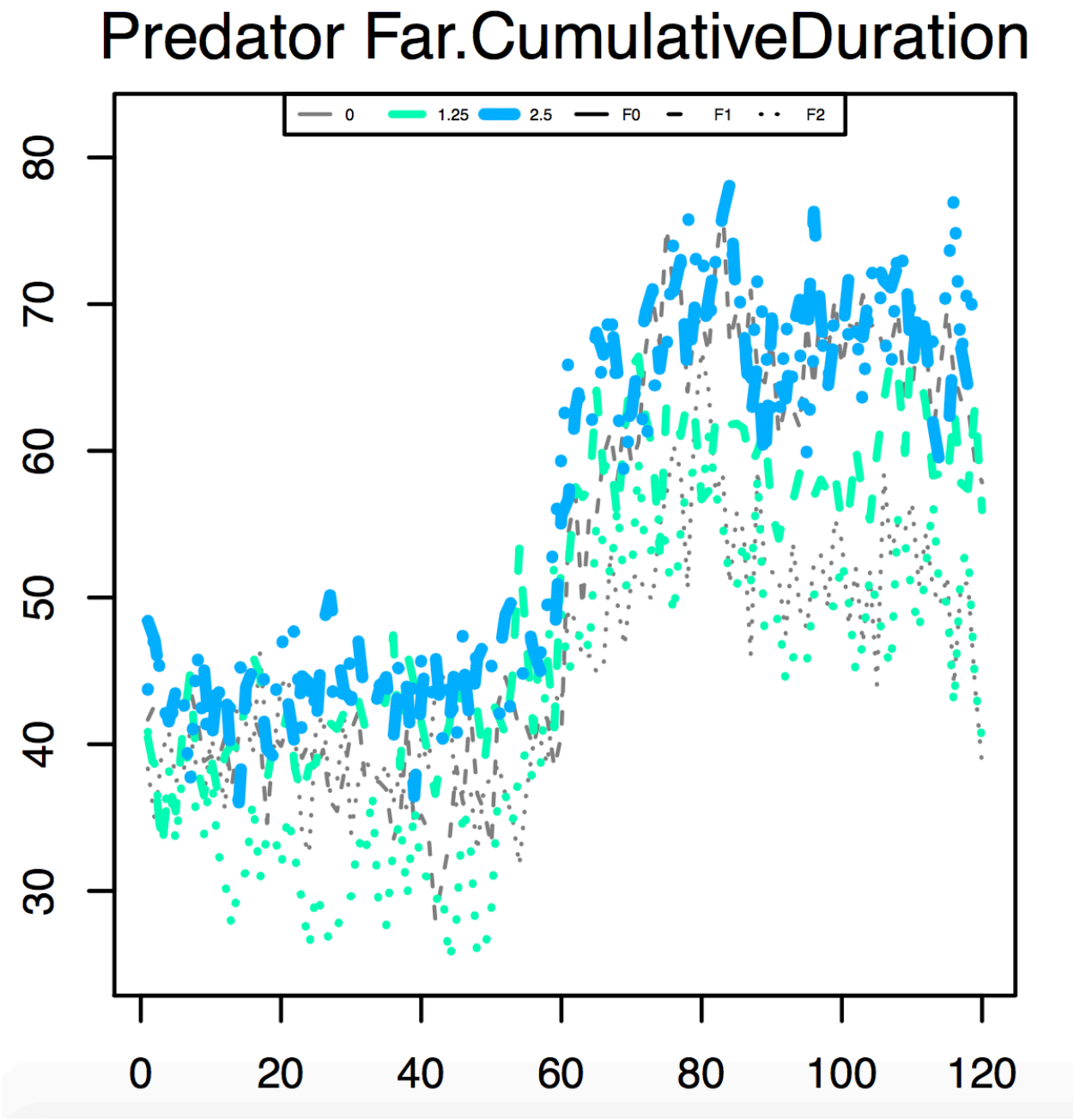

Fig. 15. Graph shows cumulative percent duration spent in the far zone for three generations of 0.1% DMSO controls, 1.25 ppm BaP, and 2.5 ppm BaP exposed fish. The Predator Video was played at the 60 second mark. Controls (black lines),1.25 ppm BaP treated fish (green lines), and 2.5 ppm BaP treated fish (blue line). F1 (dashed lines), and F2 (smaller dashed lines).

F1 1.25 ppm BaP has the lowest duration percentage average at 60%, controls average 65%, and 2.5 ppm BaP fish 67%(**Fig. 16**). F2 0.1% DMSO controls and 1.25 ppm BaP fish average ~50% while 2.5 ppm BaP fish average higher at 65%(**Fig. 14**).

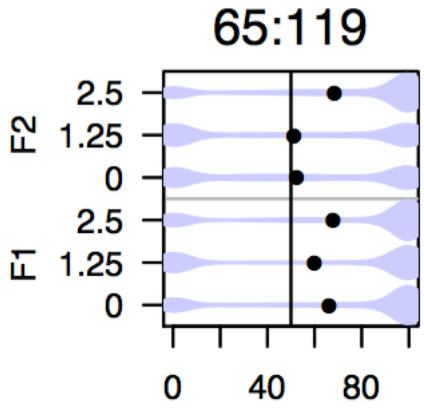

Fig. 16. All graphs show average percent duration in the far zone for three generations of 0.1% DMSO controls, 1.25 ppm BaP, and 2.5 ppm BaP exposed fish. Graph displays the averages during the 65-119 second time interval (while predator video played).

Benzo[a]pyrene Shuttlebox

The TimeToASide variable looks at time it takes fish to swim to the conditioned stimulus at the start of the shuttlebox trial. As shown on **Fig. 17** we see similar trends in all generations/exposed fish groups. All fish after the tenth trial show a dramatically reduced time. Before the tenth trial F0 2.5 ppm BaP exposed fish exhibit the highest time values peaking at ~13 s at trial 3. Time drops to and stays at 4-3 seconds after the 14th trial. F1 and F2 2.5 ppm BaP exposed fish values are roughly the same. Times rises from 8 to 11 seconds by the 5th trial. After the 10th trial time decreases sharply and drops to 4 seconds by the 30th trial. F0 1.25 ppm BaP exposed fish time stays at ~8 seconds up until the 10th trial. Around the 10th trial time values start to drop and reach

~3 seconds by the 30th trial. F1 1.25 ppm BaP time values differ slightly from the F0 time values. F2 1.25 ppm BaP fish also have similar value to both F0 and F1 fish but deviate slightly at the ~18th trial mark. F2 1.25 ppm BaP values are lower then the other generations at ~3 seconds compared to 4-5 seconds. Both F0 and F1 DMSO control fish have closely tied time values throughout the 30 trials. Time values for both groups average around 8-10 seconds up until ~12th trial. Time values begin to drop after the 12th trial. Time values level off at about the 23rd trial. From the 23rd trial to the 30th time values stay at 4-5 seconds. F3 DMSO controls follow a different trend than F1 and F2 controls. Time values start at ~9 seconds and decrease rapidly at ~7th trial. By the 10th trial time values are already at 4 seconds. From the 10th trial on time values stay between 3.5 and 3 seconds.

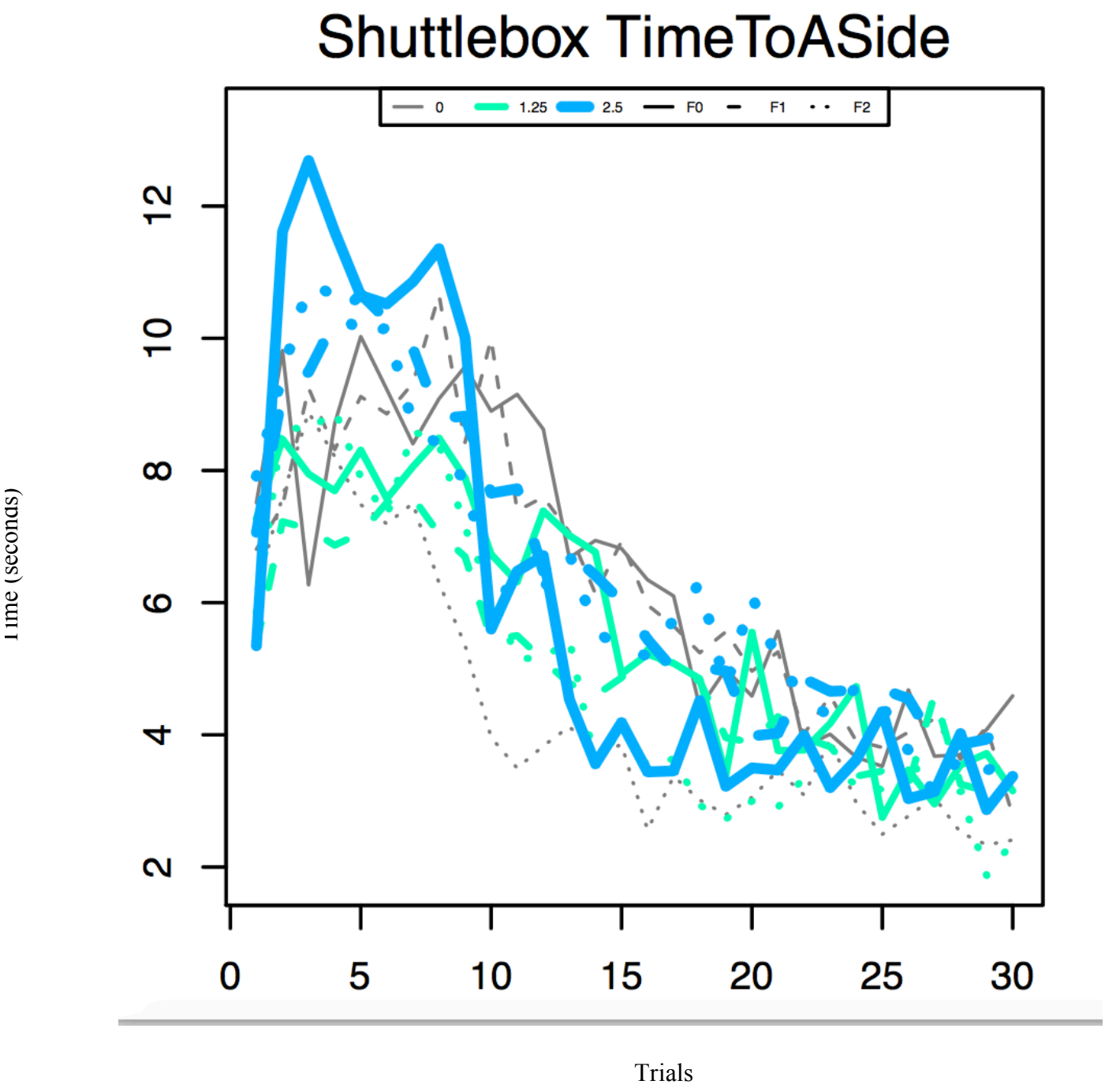

Fig. 17. Time vs Trials is plotted. A total of 30 trials were run. 0.1% DMSO controls (black lines),1.25 ppm BaP treated fish (green lines), and 2.5 ppm BaP treated fish (blue lines). F0 (solid lines), F1 (dashed lines), and F2 (smaller dashed lines).

Graphs 1-4 in **Fig. 18** show the average time for each generation and exposure concentration at certain trials. For example, the first graph averages trials 0 to 29 and the next 0 to 9. Graph 1 F0 and F1 generation fish in all groups time averages range from 5-6 seconds. F2 have slightly lower values of ~4 seconds and 2.5 ppm BaP F2 fish at 5 seconds. Graph 2 F0 2.5 ppm BaP fish has a time average of 6.2 seconds while 1.25 ppm BaP and controls range ~5.9 seconds. F1 1.25 ppm BaP fish have the lowest value at ~5.5 seconds. Control and 2.5 ppm BaP fish average ~5.9 seconds. F2 controls are the lowest at 5.5 seconds, 1.25 ppm BaP fish 5.6 seconds, and 2.5 ppm BaP 6 seconds. Graph 3 F0 2.5 ppm BaP fish and 1.25 ppm BaP fish average 5.2-5.3 seconds while controls average 4.9 seconds. F1 fish average ~5-5.3 seconds. F2 controls average ~4.7 seconds, 1.25 ppm BaP fish ~5 seconds and 2.5 ppm BaP fish average ~5.5 seconds. By the 20-29 trial interval all time values roughly integrate in all generations/concentrations. In the F0 and F1 generation fish have time values at ~4 seconds in the 20-29 interval. In the F2 generation for the same interval values are slightly lower at ~3 seconds.

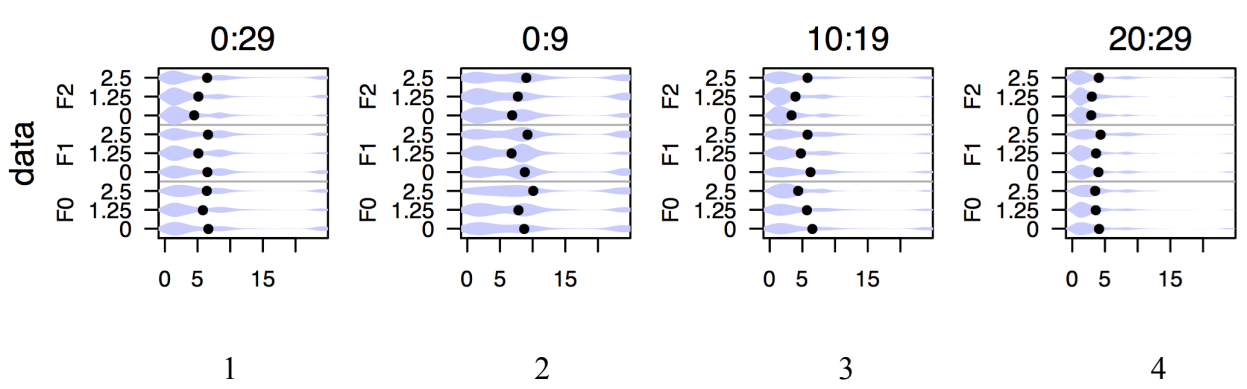

Fig. 18. All graphs show average times over certain trial intervals for three generations of 0.1% DMSO controls, 1.25 ppm BaP, and 2.5 ppm BaP exposed fish. The first graph (graph 1) shows the average times for all 30 trials. Graph 2 shows averages for trials 0 to 9. Graph 3 has averages within trials 10 to 19. Graph 4 shows the average times of trials 20 to 29.

Discussion

Embryonic Photomotor Response

From the results we see that times 9-11 am result in the lowest movement peaks (25-27 hpf). These times are not good to use for creating a baseline as they are too low. In contrast times 7:00 pm-9 pm gives us movement peaks that are too high (35-37 hpf). Movement peak values in between these two would be optimal for creating a baseline. Times 12 pm-5 pm gives us values of ~100 (28-33 hpf). The time point at 12 pm gives us the value closest to 100. This time would be optimal to use for baseline activity.

Vitamin E (Startle response)

Looking at the normal startle response of a fish we see that it does not take long for a fish to respond to a tap. Startle responses to taps are strong and we see peaks in movement distances of fish who have been tapped. Normal swimming activity does not seem to affected by vitamin E deficiency as we see in **Fig. 3.** Normal swimming activity was measured with no stimulus and both groups moved total distances that were roughly the same (Vitamin E+:210 cm,Vitamin E-:220 cm). Micronutrient deficiency does seem to have an effect on habituation. Over the course of three taps vitamin E- fish maintained total movement peaks of ~30 cm. Vitamin E+ fish on the other hand total movement values decreased after each tap meaning that these fish habituated to the startle stimulus. Further optimization of this assay is needed. This assay tested habituation using three taps. To test for anxiety, it may be better to include more taps and shorter interval times. Five-minute interval rest time seems to be too long of an interval to test for anxiety as zebrafish have ample time to recover from the previous tap.

Vitamin E (Shuttlebox)

Looking at the TimeToASide parameter it has been shown that vitamin E sufficient fish learned quicker than vitamin E deficient fish (**Figure 5**). The TimeToASide refers to the time it took fish during the avoidance period to swim to the safe side of the shuttlebox (light side). We would expect this number to decrease after each consecutive trial. This meaning the fish was learning to associate the light stimulus with being safe or non-shocking. In **Figure 5** we see time decreasing greater after each trial in the E+ group than the E- group.

Benzo[a]Pyrene (shoaling)

We see no real significant differences in Inter-individual Distance and Nearest Neighbor Distance among the three exposed groups (0.1% DMSO controls, 1.25 ppm BaP, and 2.5 ppm BaP) in F1 and F2 fish. Between the F1 and F2 generation values for iid and nnd for all exposed groups decreased. Suggesting that DMSO may effect the shoaling parameters since we see a decrease in values in the 0.1% DMSO controls. F0 fish have the lowest swim speeds and after each generation all exposure groups swim speeds increase with F2 having the highest swim speeds. We also see that DMSO controls swim speed increases with each generation. This may suggest that DMSO exposure is a factor in the swimming speed of the zebrafish. The increase in swim speed may be due to recovery after each generation. Non-exposed zebrafish swim speeds have yet to be recorded. By the F2 generation swim speeds increase to about 5.5-6 cm/s in DMSO controls. This could mean that non-exposed fish may have normal swim speeds of about ~6 cm/s. Since DMSO controls seem to affect swim speed, this control may not be optimal for

this assay. For future studies it may be better to include non-exposed controls to account for any effects DMSO may have.

Benzo[a]Pyrene (Fear Response)

The fear response assay tested three things; whether a video projection of a predator would elicit a fear response, whether that fear response could be characterized (using zones), and whether BaP exposure effected the fear response. Optimization of this assay was done on F1 and F2 generations. For the fear response assay it was hypothesized that the fish once introduced to a predator video would remain in the far zone for a large percentage of the video. All fish seemed to display the normal fear response that was expected. All fish once the video began spent on average more time in the far zone than in the close zone. Looking at **Fig. 15** both F1 and F2 1.25 ppm BaP exposed fish had the greatest duration percentages in the far zone while 2.5 ppm BaP and 0.1% DMSO controls had roughly the same values. Middle zone data shows lower duration percentages in both BaP exposed groups relative to the controls with 1.25 ppm BaP having the closest value to the controls. In the middles zone we see lower values roughly in the 30-40% range suggesting this is a transitional zone in which fish cross to move away from the predator. Both generations of 2.5 ppm BaP exposed fish have higher duration percentages in the far zone than controls and 1.25 ppm BaP fish. Although F1 2.5 ppm BaP fish and controls have similar averages at ~65% with 2.5 being only slightly higher. However, in the F2 generation 2.5 ppm BaP fish average duration time exceeds controls and 1.25 ppm BaP fish by ~15%. This could mean that BaP exposure may effect the fear response in eliciting a heightened reaction to a predator video. It also can mean that DMSO exposure lessens the fear response and as generations pass this effect wears off. To test if DMSO has any effect on the fear response non

DMSO fish will have to be tested. Also F0 BaP exposed fish data would be needed to truly asses the affect exposure would have on fear response.

Benzo[a]Pyrene (Shuttlebox)

Two major goals of this assay were to test any effects on learning BaP may have and to see if this effect, if any, could be passed down. F1 and F2 controls show similar linear regression trends and time values are tightly correlated throughout 30 trial period. **Fig. 17** shows that F0 fish average similar values throughout the 30 trials. F2 BaP fish however show average higher times throughout 30 trials than other groups (DMSO,1.25 ppm BaP). However, F3 controls seem to be have a different trend as its trend line is much steeper than the other generation controls. All three generations of 1.25 ppm BaP fish time/trial slopes are concentrated around the same area. It is seen that 1.25 ppm BaP exposed fish (F0, F1, F2) time values appear lower than both F1 and F2 0.1% DMSO controls if only slightly. A greater difference is seen in the F1 2.5 ppm BaP group. Although 2.5 ppm BaP fish time values start of higher than the rest of the fish groups, by trial 12 drop below all other fish expect F2 0.1% DMSO controls. This is up until the 18^{th} trial where all fish groups and generation seem to aggregate to similar values (3-5 seconds). The 1.25 ppm BaP seem to show generational effects as throughout the generations similar trends are seen. F0 2.5 ppm BaP fish have lower values than both F1 and F2 2.5 ppm BaP fish. F1 and F2 2.5 ppm BaP fish show closely related trends. By the 20^{th} trial all generations and exposure groups have similar average TimeToASide times values.

Conclusion

It was found through a developmental time series that EPR is time dependent and the time post fertilization affects movement activity. It was found that at around 28-33 hpf creates movement peaks optimal to use a baseline activity measurement. Through the vitamin E study, it was found that vitamin E deficiency creates habituation to a tap stimulus. Shuttlebox data tells us that vitamin E- fish learn slower than vitamin E+ fish. Shoaling behavior was assessed in Benzo[a]pyrene exposed fish and 0.1% DMSO control fish. It was found that DMSO exposure may effect shoaling behavior and thus may not be optimal for this assay. Fear response to a predator video was also observed. The predator video did elicit the hypothesized response in all the groups of fish (0.1% DMSO, 1.25 ppm BaP, 2.5 ppm BaP). The fish that had the highest fear response as characterized by average duration percentage in the far zone was the 2.5 ppm BaP fish. BaP shuttlebox data shows 2.5 ppm BaP F0 fish learn quicker than the F0 0.1% DMSO controls up until ~25th trial where there are similar time averages.

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