EFFECTS OF FLUOXETINE IN JUVENILE

GUPPIES , *POECILIA RETICULATA*

By

Marco Pelli

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Chair: Victoria P. Connaughton

Victoria Connaughton, Ph.D.

Stephen MacAvoy, Ph.D.

Elizabeth Malloy, Ph.D.

Dean of the College of Arts and Sciences

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DEDICATION

I fully dedicate my achievements to those that have supported me, Carlos Pelli, Cesar Pelli, Ester Delucchi, and to my grandparents.

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ABSTRACT

This study evaluates the impacts of fluoxetine, used (in great amounts) by humans and discarded in natural aquatic environments (natural concentration ranges from 0.03 µg/l to 0.5 µg/l in most of natural aquatic environments in USA and Europe).
A 4-day (short-term) and a 35-day (long-term) bioassay using natural concentrations of fluoxetine were conducted beginning with 7-day old guppies, *Poecilia reticulata*. The long-term, chronic exposure experiment was conducted to observe and compare all the morphological (length, width, and weight) and behavioral effects of fluoxetine on *P. reticulate* larvae.

Results show no lethality from the short-time experiment, but all values from the long-term experiment show significant differences in morphological measurements between control group and experimental groups, where mean values for weight, notochord length and belly width, were significantly lower within the experimental groups. Further, experimental groups showed greater changes in behavioral patterns and mortality rates (long-term). These findings are consistent with previous studies using the same and similar species, showing the detrimental impacts of prolonged fluoxetine exposure.

ACKNOWLEDGMENTS

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CHAPTER 1

INTRODUCTION

Guppies

The guppy (*Poecilia reticulata*), also known as the millionfish, is one of the most popular freshwater aquarium fish species in the world. It is a small member of the Poeciliidae family (females 4–6 centimeters (1.6–2.4 in) long, males 2.5–3.5 centimeters (1.0–1.4 in) long) and like all other members of the family, is live-bearing. Fertilization is internal and young develop within the mother for 3—4 weeks before they are born. Guppies are native to Antigua and Barbuda, Barbados, Brazil, East Timor, Guyana, Mayotte, Netherlands Antilles, Trinidad and Tobago, the U.S. Virgin Islands, Venezuela, and Vietnam. However, guppies have been introduced into many different countries on all continents, except Antarctica. Sometimes this has occurred accidentally, but most often as a means of mosquito control, the hope being that the guppies would eat the mosquito larvae slowing down the spread of malaria (Webb et al. 2007).

Robert John Lechmere Guppy discovered this tiny fish in Trinidad in 1866, and the fish was named *Girardinus guppii* in his honour by Albert Günther later that year. However, the fish had previously been described in America. Although *Girardinus guppii* is now considered a junior synonym of *Poecilia reticulata*, the common name "guppy" still remains (Gunther 1867).

Guppies are highly prolific livebearers. The gestation period of a guppy is 20–30 days, with a mean value of 28 days, varying according to water temperature. During reproduction, the male will approach a female and flex his gonopodium forward before

thrusting it into her and ejecting balls of spermatozoa. After the female guppy is inseminated, a dark area near the anus, known as the gravid spot, will enlarge and darken. Just before birth, the eyes of fry may be seen through the translucent skin in this area of the female's body. When birth occurs, individual offspring are dropped in sequence over the course of an hour or so (Riehl et al. 1991).

Guppies prefer water temperatures around 26 °C (79 °F) for reproduction. The female guppy births between 2–50 fry per clutch, with typical numbers ranging between 10 and 28. After giving birth, the female is ready for conception again within only a few days. Guppies have the ability to store sperm up to a year, so the females can give birth many times without depending on the presence of a male. From the moment of birth, each fry is fully capable of swimming, eating, and avoiding danger. If not kept separate, the older, mature guppies will eat the fry so the use of a breeder box, net breeder, or a separate tank is recommended. Live plants may be used as hiding places for the fry (Riehl et al., 1991).

Young fry take roughly three or four months to reach maturity. In the aquarium, they are usually fed finely ground flake foods or baby brine shrimp. In addition, they nibble on algae (Encyclopedia Britannica 2007, Kolluru et al. 2006).

Because they are considered to be one of the most important biological control agents against mosquito larvae (Chandra et al., 2008), as noted above, guppies have been used as a animal model for a variety of studies examining changes in environmental parameters, such as temperature (Karayucel et al. 2008), pH (Araujo et al. 2008),

sublethal concentrations of several toxicants (Crandall et al. 1963), and salinity (Shikano et al. 1998).

The guppy is also considered a good model because of its high reproduction rate (livebearers), organs can be clearly studied, and they are sensitive to toxicological studies (Viran et al. 2003; Araujo et al. 2008).

Fluoxetine: General

Fluoxetine (trade name Prozac) also known as *N-methyl-3-phenyl-3-[4-(trifluoromethyl)phenoxy]propan-1-amine,* is an antidepressant of the selective serotonin reuptake inhibitor (SSRI) class. It is approved and used for the treatment of major depression (including pediatric depression), obsessive-compulsive disorder (in both adult and pediatric populations), bulimia nervosa, panic disorder and premenstrual dysphoric disorder (Wong et al. 2005). Despite the availability of newer agents, medical use of fluoxetine remains extremely popular. This chemical compound is water soluble and can be found in kidneys (excretion) and liver (metabolism) of fish in high concentrations when water is contaminated with sewage effluents; fluoxetine also accumulates in skin and gills (Nakamura et al. 2008).

Patients with depression have lower levels of serotonin in their brain. Serotonin is a neurotransmitter that is quickly taken back up into presynaptic nerve terminals once it is released. As a selective serotonin re-uptake inhibitor or SSRI drug, the mechanism of action of fluoxetine is to increase the level of serotonin in the brain by preventing reuptake of serotonin to presynaptic nerve terminals, resulting in higher levels of serotonin (Fig. 1). In this way, fluoxetine eases the symptoms of depression by correcting

the serotonin imbalance in the brain. Patients with increased levels of serotonin were found to usually recover from their symptoms.

Among the common adverse effects associated with fluoxetine and listed in the prescribing information, the effects with the greatest difference from placebo are nausea, insomnia, somnolence, anorexia, anxiety, nervousness, asthenia and tremor. Those that most often resulted in interruption of the treatment were anxiety, insomnia, and nervousness (1-2% each), and in pediatric trials—mania (2%) (Eli Lilly 2008, MacKay et al. 1997). Similarly to other SSRIs, sexual side effects are common with fluoxetine; they include anorgasmia and reduced libido (Feighner et al. 1991, Zajecka et al. 1991, Herman et al. 1990). In addition, rash or urticaria, sometimes serious, was observed in 7% patients in clinical trials; one-third of these cases resulted in discontinuation of the treatment. Postmarketing reports note several cases of complications developed in patients with rash. The symptoms included vasculitis and lupus-like syndrome. Death has been reported to occur in association with these systemic events (Eli Lilly 2008).

Fluoxetine: Impact on Other Pathways

As noted above, fluoxetine (Flx) is selective for serotonin reuptake. However, Eli Lilly® researchers found that there are two different enantiomeres of fluoxetine (R-fluoxetine and S-fluoxetine). Both S-Flx and R-Flx have high affinity to serotonin, however, a single injection of a rat with a large dose of R-Flx (4 to 6 mg) also results in a significant increase of brain concentrations of norepinephrine and dopamine (Perry et al. 1997, Bymaster et al. 2002, Koch et al. 2002). This effect may be mediated by specific serotonin receptors (5HT2a or 5HT2c), both of which are inhibited by higher

concentrations of R-Flx. As an alternative, the ability of fluoxetine to interact with dopamine and norepinephrine pathways may be due to the poor selectivity of fluoxetine for its receptors. There is a 10-fold difference in binding affinity between its first and secondary neural targets (i.e., the serotonin and norepinephrine uptake pumps, respectively) and anything greater than a 10-fold difference in affinity results in significant activation of the secondary neuronal targets (Sheldon 1999). It has been suggested that fluoxetine's effects on dopamine and norepinephrine may contribute to the antidepressant action of fluoxetine (Koch et al. 2002) though in the opinion of other researchers, the magnitude of this effect is unclear (Henry et al. 2005). A racemic mixture of both S-Flx and R-Flx is being distributed and prescribed to patients.

The function of two enzymes, Monoamine Oxidase (MAO) and Catechol-*O*-methyltransferase (COMT), are also associated with depression and serotonin (Fig. 1). Monoamine oxidases catalyze the oxidative deamination of monoamines, such as serotonin. Oxygen is used to remove an amine group from a molecule, resulting in the corresponding aldehyde and ammonia. This occurs inside the presynaptic nerve terminals and results in the inactivation of neurotransmitters, reducing the amount available for synaptic transmission. As a result, MAO dysfunction is thought to be responsible for a number of psychiatric and neurological disorders such as depression (Meyer et al. 2006), schizophrenia, substance abuse, attention deficit disorder, migraines, and irregular sexual maturation (Domino et al. 1976, Schildkraut et al. 1976). Monoamine oxidase inhibitors (MAOIs) are one of the major classes of drug prescribed for the treatment of depression, although they are last-line treatment due to risk of the drug's interaction with diet or other

drugs. In fact, MAO-A inhibitors act as antidepressant and antianxiety agents, whereas MAO-B inhibitors are used alone or in combination to treat Alzheimer's and Parkinson's diseases (Riederer et al. 2004).

Catechol-*O*-methyltransferase (COMT) is an intracellular enzyme located in the presynaptic neuron. Any compound having a catechol structure, like catecholestrogens and catechol-containing flavonoids, are substrates of COMT. In humans, catechol-*O*-methyltransferase protein is encoded by the COMT gene (Grossman et al. 1992).

Fluoxetine Bioaccumulation

As a result of the way fluoxetine is metabolized, the bioavailability of fluoxetine in humans is relatively high (72%), and peak plasma concentrations are reached in 6 to 8 hours. It is highly bound to plasma proteins, mostly albumin (RxList.com. 2007). Fluoxetine is metabolized in the liver by isoenzymes of the cytochrome P450 system, including CYP2D6. Only one metabolite of fluoxetine, norfluoxetine (N-demethylated fluoxetine), is biologically active. The slow elimination of fluoxetine and its active metabolite norfluoxetine from the body distinguishes it from other antidepressants. With time, fluoxetine and norfluoxetine inhibit their own metabolism, so fluoxetine elimination half-life decreases, as well as the half-life of norfluoxetine after long-term use (Burke et al. 2000). Therefore, the concentration of the drug and its active metabolite in the blood continues to augment through the first few weeks, and their steady concentration in the blood is achieved only after few weeks (Pérez et al. 2001).

Few data on environmental fluoxetine exposure and hazard to aquatic life are currently available in the literature. Only a small number of studies have tested impacts

of fluoxetine in different species, such as decrease in ability to capture prey in hybrid striped bass (Gaworecki et al., 2008), the potential to affect sex hormones and modulate genes involved in reproductive function and behavior in the brain of female goldfish (Mennigen et al. 2008), inhibition of egg production in zebrafish (Lister et al. 2009), and hyperglycemia and significant increase in the circulating levels of CHH in *O. limosus* and *Chasmagnathus granulata* (Santos et al. 2001). These animals are exposed to fluoxetine that enters habitats via sewage effluent.

Medical use of fluoxetine is prevalent. Over 22.2 million prescriptions for generic formulations of fluoxetine were filled in the United States in 2007 (Verispan 2008) and even more the following years, making it the third most prescribed antidepressant. It has also been prescribed significantly in Trinidad (Moore et al. 2002), one of the countries where guppies originate.

Fluoxetine is discharged from municipal wastewater treatment plant effluents to surface waters so it is important to be able to evaluate the potential impact of pharmaceuticals and personal care products (PPCPs) on aquatic life and have an approach for determining protective levels for aquatic organisms. Environmental fluoxetine concentration is reported to be 0.03 μg/L in US streams and can reach as high as 0.45 μg/L in effluents of sewage treatment plants (Nakamura et al. 2008).

Purpose and Objectives

To determine the effects of fluoxetine on growth, development, and behavior in fish, larval/juvenile guppies were exposed to two different concentrations of fluoxetine. The specific objectives of this study are:

(1): To determine the impacts of fluoxetine (Prozac, an antidepressant of the selective serotonin reuptake inhibitor (SSRI) class) on the development and behavior of juvenile Guppies (*Poecilia recticulata*).

(2): To relate these results to potential ecological impacts.

(3): To obtain a more complete understanding of fluoxetine toxicity.

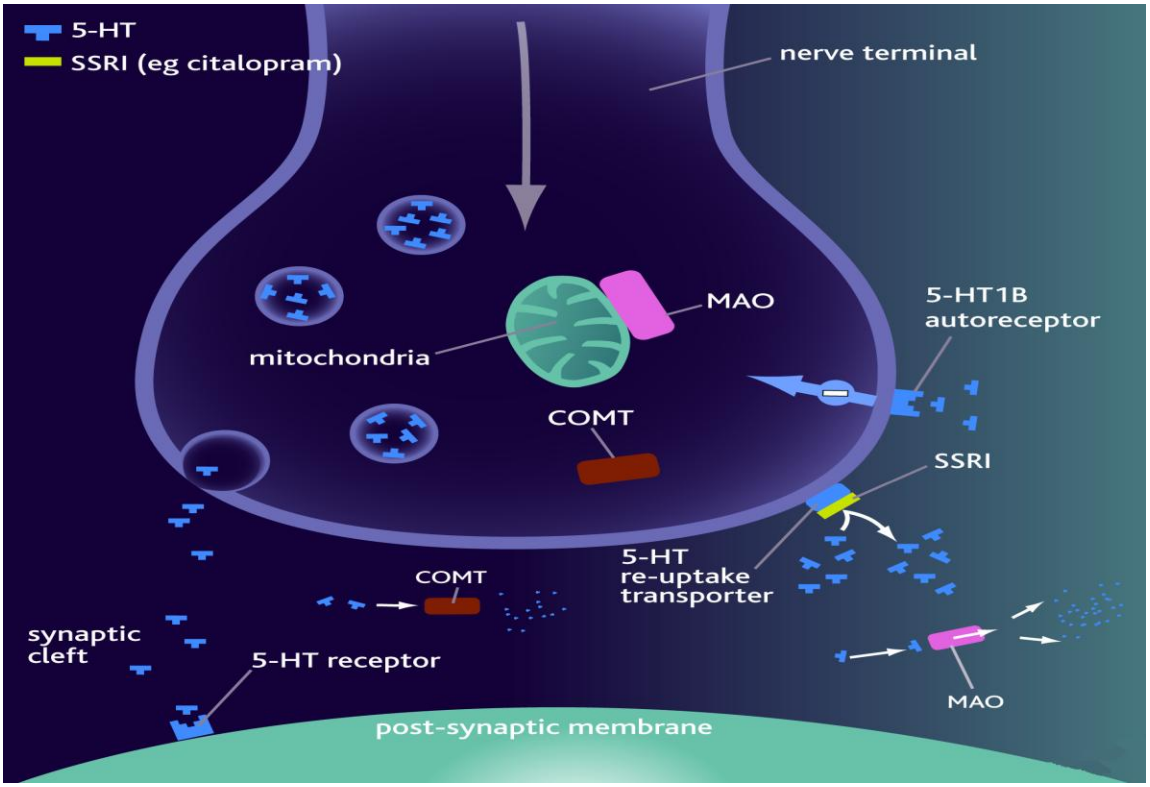

Figure 1: Mechanism of Action of Fluoxetine (as a Serotonin Reuptake Inhibitor, or SSRI). Here, fluoxetine (yellow rectangle) binds to serotonin (5-HT) reuptake transporters blocking reuptake of serotonin. This results in increased levels of serotonin in the synaptic cleft that are available to bind to 5-HT receptors on presynaptic nerve cells. [MAO: Monoamine Oxidase is an enzyme that can lead to reduced neurotransmitter concentration in presynaptic terminals. MAO inhibition is another treatment for depression. Catechol-*O*-methyltransferase (COMT) is involved in the inactivation of the catecholamine neurotransmitters (dopamine, epinephrine, and norepinephrine). The enzyme introduces a methyl group to the catecholamine, which is donated by S-adenosyl methionine (SAM). COMT is an intracellular enzyme located in the presynaptic neuron (Grossman et al. 1992)]. Figure extracted from Rang et al. 2001.

CHAPTER 2

STUDY DESIGN

Adult guppies (*Poecilia reticulata)* were purchased from a commercial vendor (LiveAquaria.com®) and maintained in the lab until needed. Guppy larvae/juveniles used in experiments were obtained from in house mating of these adults.

Once delivered, adult guppies were maintained in 10 gallon tanks in the laboratory (Hurst Hall, Rm. 4) on a 14hr light:10hr dark photoperiod at 25-26^{o}C. Adults were fed daily Tetramin flake food and allowed to acclimate for 2 weeks. During the first week, the males and females have been kept separate. Beginning with the second week, experimental mating was conducted by placing 3 females in an aquarium at ~28^{o}C with a known male and allowing them to interact freely for at least 1 week before removing the male.

Larvae were obtained by isolating single pregnant females in breeding compartments, allowing them to give birth and then collecting their young ones.
A population of 120 juvenile *Poecilia recticulata* was allowed to acclimate to lab conditions for 1 week prior to the start of experiments. Juveniles were maintained in 6L tanks (10 fish per tank) filled with Deer Park water.

Two different experiments have been designed to answer the main question of this study, the first one is a short-term experiment with the purpose of calculating the LC_{50} (for sub-lethal concentrations) for this compound, and the second one is a long-term experiment with the purpose of measuring the behavioral and physiological impacts of sub-lethal concentrations of this compound.

Short Term Experiment/LC_{50} Determination

In toxicological studies, lethality of a given compound is determined by the LC_{50}, or lowest concentration at which 50% mortality is observed, and experiments are typically run over a short period (48-96hrs) using a range of concentrations (Stephan 1977). To determine the LC_{50} for juvenile guppies exposed to fluoxetine the following experiment was proposed.

Groups of 10 juvenile fish were randomly assigned to one of 3 treatments: negative control (0µg/L fluoxetine), 0.03 µg/L, and 0.50 µg/L. These concentrations had been chosen because they reflect the range of concentrations observed in natural aquatic environments (Brooks et al. 2003).

Twelve 6 L aquaria were prepared (i.e.: filled, positioned in the lab, etc.) prior to assignment of fish or treatment. Aquaria were assigned a treatment randomly with use of a random number generator. Juveniles were individually segregated into each container, and then randomly assigned to aquaria such that each contains 10 juveniles. All treatments and negative control were performed with 4 replicates. Random numbers were generated using "Randomizer" software. Fish had been fed once every 12h with Tropical flakes (Tetra®). Experimental containers had been monitored daily and overall survival noted after 24hr , 48hr, and 96hr (modified from Henry et al 2008).

Long Term Experiment

Twelve 6 L- Aquaria were set up under the conditions described above. Fluoxetine concentration for the different treatment groups were the same as the previous experiment (Control group, 0.03 µg/L, and 0.5 µg/L). As was done in the short term experiments, aquaria preparation preceded random assignment of the treatment group,

which preceded the random assignment of 10 juvenile fish to each aquarium. Diseased fish were recorded and removed on observation.

Experimental containers were monitored and fish fed twice a day. Every other day, 50% of the water was changed to remove debris and ensure drug concentrations remain constant throughout the duration of the experiment. Lethality was observed daily, and the fish within the containers videotaped to determine behavioral changes every other day, for a period of 5 weeks, based on the schedule shown in figure 2.

Behavior patterns were videotaped and categorized on a scale from 1-5 where

1. Normal behavior (looking for food).
2. Scratching against rocks or leaves.
3. Abnormal swimming.
4. Hypoxia.
5. Death

These 5 categories reflect reported changes in guppy behavior in response to altered environmental conditions (Kramer et al., 1981). As noted above, every other day, all tanks were monitored for behavior and the proportion of fish expressing the different behavior patterns (i.e., the number of fish experiencing each behavior pattern for each tank) was noted. At the end of the experiment, average values for each group were calculated.

Physiological Analysis

At the end of the long term experiment, the larvae/juveniles were anesthetized in a tricaine methane sulfonate solution (0.02% solution). The larvae remained in this solution for 10min after body movement has stopped, as gill movements are not

discernible due to the small size of the animals. Following euthanasia, larvae were fixed in 4% formaldehyde and transferred to 70% ethanol prior to microscope analysis.

Week 1	Day 1 Start expt	2 behavior	3 50% water change	4 behavior	5 50% water change	6 behavior	7 50% water change
Week 2	8 behavior	9 50% water change	10 behavior	11 50% water change	12 behavior	13 50% water change	14 behavior
Week 3	15 50% water change	16 behavior	17 50% water change	18 behavior	19 50% water change	20 behavior	21 50% water change
Week 4	22 behavior	23 50% water change	24 behavior	25 50% water change	26 behavior	27 50% water change	28 behavior
Week 5	29 50% water change	30 behavior	31 50% water change	32 behavior	33 50% water change	34 behavior	35 Expt ends

Figure 2. Schedule for Tasks During the Long Term Experiment.

Changes in growth were determined by measuring differences in length (notochord length as shown in Figure 4), belly width (as shown in Figure 3), and weight (wet weight) of animals within the different treatments. Notochord length and belly width were measured with an ocular micrometer and wet weight determined using a microbalance.

Statistical Analysis

The appropriate first step for statistical analysis is testing if assumptions are met for each data set, and then, if that is the case, proceeding with the proper methodology for further analysis. Main assumptions of these experiments, based on a parametric design,

are: Random sampling, Independence of samples, Normal distribution of samples, and Homoscedasticity. Conducting the appropriate procedures (Q-Q plots, and predicted value vs. studentized residuals, etc.), all these assumptions were met for weight, notochord length, and belly width measurements.

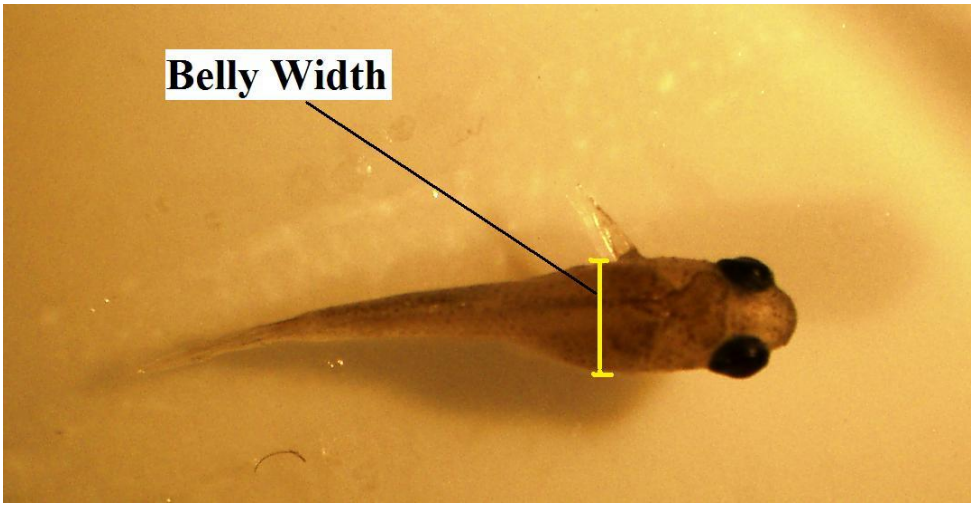

Figure 3. Magnified Digital Picture (Dorsal View) of a Juvenile *P. reticulata*. This shows how Belly width was measured.

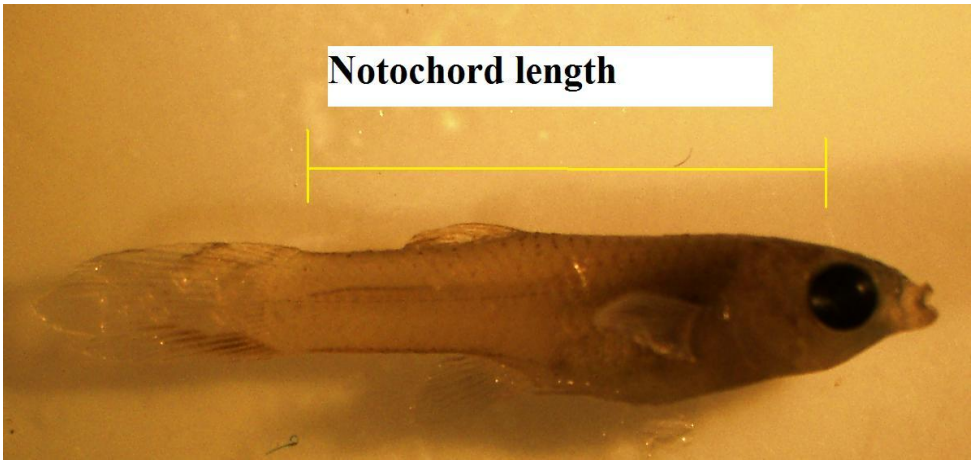

Figure 4. Magnified Digital Picture (Lateral View) of a Juvenile *P. reticulata*. This shows how Notochord length was measured.

A suitable methodology for testing variation among treatment groups is conducting an ANOVA and paired comparisons to detect significant differences. ANOVA tests and post-hoc paired comparisons (Tukey HSD, and LSD) were conducted to detect length and weight differences between treatment groups and controls. The reasons for choosing these two post-hoc contrast methods corresponds to the sampling characteristics for this project, in terms of amount of observations, grouping and how the assumptions for the method are met.

For the long-term behavior experiment, counts/rankings of behaviors were performed. As a result, a Generalized Linear Model (regression) was conducted to identify any differences in guppy behavior patterns between experimental groups.

Finally, hazard ratios were calculated for treatment groups relative to control group, using a Cox regression (Bradburn et al. 2003). This value gives us an idea of the relative risk (death and behavior changes), and provides an approximate scale for the impact that is being measured. For statistical analyses, every result was considered to be significant when the p-value is equal or lower than 0.05 (5% significance). All the analyses were conducted using SPSS® and Microsoft Excel® software. In addition, after concluding these measurements, instantaneous mortality (Z) was calculated for experimental groups:

$$Z = (\ln N_s - \ln N_t) / t$$

Mortality was assumed to be exponential (Cope 1991), where N_s is the number of larvae at the beginning of experiment; N_t is the number of viable larvae at the conclusion of the experiments; and t is the duration of the experiment in days.

CHAPTER 3

RESULTS

After breeding the fish as described in the methodology section, and conducting the detailed experiments, all the measurements were done using the proper procedures and elements mentioned before. Overall the results of these experiments show that fluoxetine exposure significantly alters growth, survival, and behavior in the early development of guppies. These results are explained in detail below.

Short Term Experiment

Following 96 hs of exposure to fluoxetine, regardless of concentration tested, resulted in 0% mortality (100% survival) in all tanks. Thus, the concentrations of this pharmaceutical compound tested (reported at natural aquatic environments close to sewage water disposal locations), are not lethal in a short-term. Consequently, LC50 could not be calculated.

Long Term Experiment

During these experiments, the larvae were recorded with a camera, and individual observations were made for each individual's behavioral patterns. For different reasons, (such as body darkness, length and/or swimming patterns), I was able to identify and track every larvae from each tank. At the end of the experiment (i.e., on day 35), larvae consistently showed only three of the five possible behavioral options: normal behavior (abbreviated as 1 or N), abnormal swimming (3 or AS), and death (5 or D). As a first step, the tank effect (differences between tanks) was tested and, as there was no significant difference between tanks (within groups). Consequently, I pooled the data sets from tanks in each group together in terms of comparing differences between groups-

Results from each tank are shown in appendix (Figures A, B, C and D). Comparisons show a clear difference between the control group and the experimental groups (Figure 5). Hazard ratios were also calculated, using Cox regression, for testing these differences, fitting to a model from SPSS®.

Behavior patterns were significantly different among all groups (Generalized Linear Model, AS $p<0.01$; D $p<0.01$). Post-hoc tests showed the Control group is significantly different from experimental groups (Abnormal swimming $p<0.01$; Dead $p\leq0.01$), but experimental groups were not significantly different (Normal $p=1.00$; Abnormal swimming $p=0.80$; Dead $p=0.879$). The hazard ratios were subsequently calculated for larvae who displayed Abnormal swimming behavior. These models suggest that time to abnormal behavior significantly decreased with increasing concentration of fluoxetine (Figure 6). Further, examination of the results shows that the odds of fish developing abnormal swimming behavior in experimental group 1 (0.03 μg/l) are 12.7 times higher than the odds of fish developing abnormal swimming behavior in control group with a confidence interval 95% (2.7 - 60.0 times). Further, when normal swimming larvae were exposed to 0.5 μg/l, the odds of these fish developing abnormal swimming behavior are 11.4 times greater than the odds of fish developing abnormal swimming behavior in control group with a confidence interval 95% (2.3 – 54.2 times).

In terms of time, it took 3 days for individuals from both experimental groups to start showing abnormal behavior patterns, 5 days for the first dead individual from experimental group 2 (0.5 μg/l), and 23 days for the first dead individual from experimental group 1 (0.03 μg/l). In agreement with these findings, instantaneous

mortality rates are higher for experimental group 2 ($0.0063/d^{-1}$) than for experimental group 1 ($0.0054/d^{-1}$).

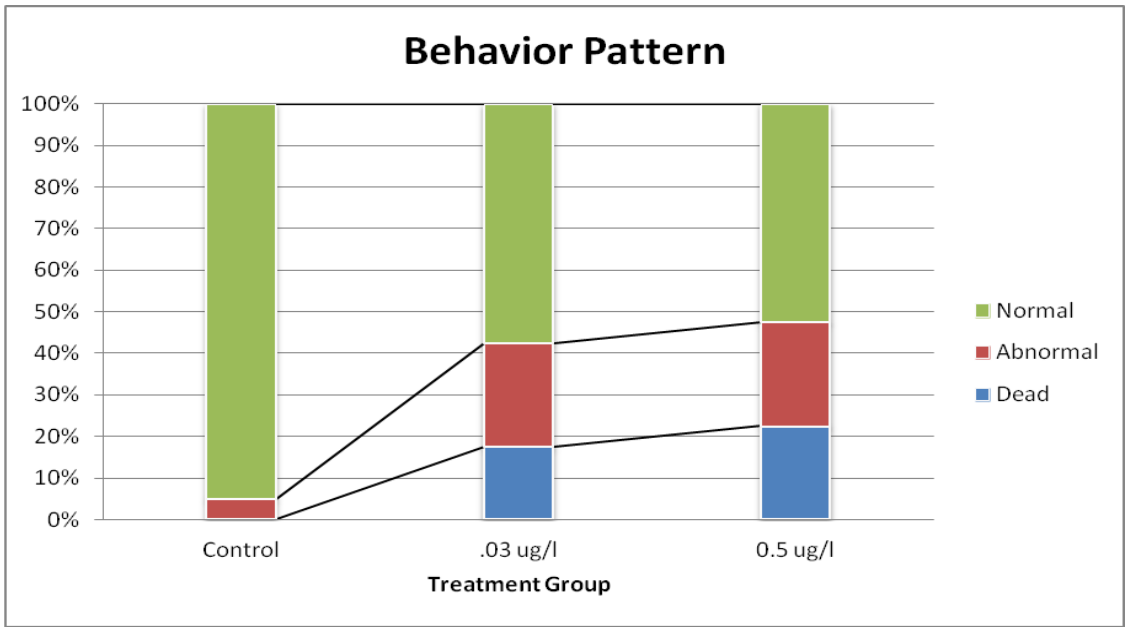

Figure 5. Behavior Patterns for Control Group and Experimental Groups (0.03 and 0.5 µg/l). Percentage represents the average proportion of each behavior category for each group. Data were normalized so all scores in a given treatment equal 100%. Green bars = normal behavior, red bars = abnormal swimming behavior; blue bars = number that did not survive. Where a value of 40 individuals represents 100%.

Morphological measurements of preserved larvae were performed using an electronic balance (weight) and a precise digital camera connected to a computer (length). These results were coherent with the changes in behavioral patterns and mortality rates noted above. As observed with the behavioral data, morphological measurements compared among groups show clear trends (Table 1).

A cursory glance of these values suggest a substantial difference between groups, specifically between the control and experimental groups. To test this, a one-way ANOVA was used, and some outliers were taken out from each group for statistical analyses purposes (4 for control group, 2 for experimental group (0.03µg/l), and 1 for

experimental group 2 (0.5µg/l)). The p-value was significant for the three measurements ($p << 0.01$). Post hoc tests found that, as with the behavioral experiments, measurements from control larvae were significantly different from measurements of treated larvae (Table 2, 3, and 4). However, values from larvae in both of the treated groups were not significantly different (shown in Table 2, 3 and 4).

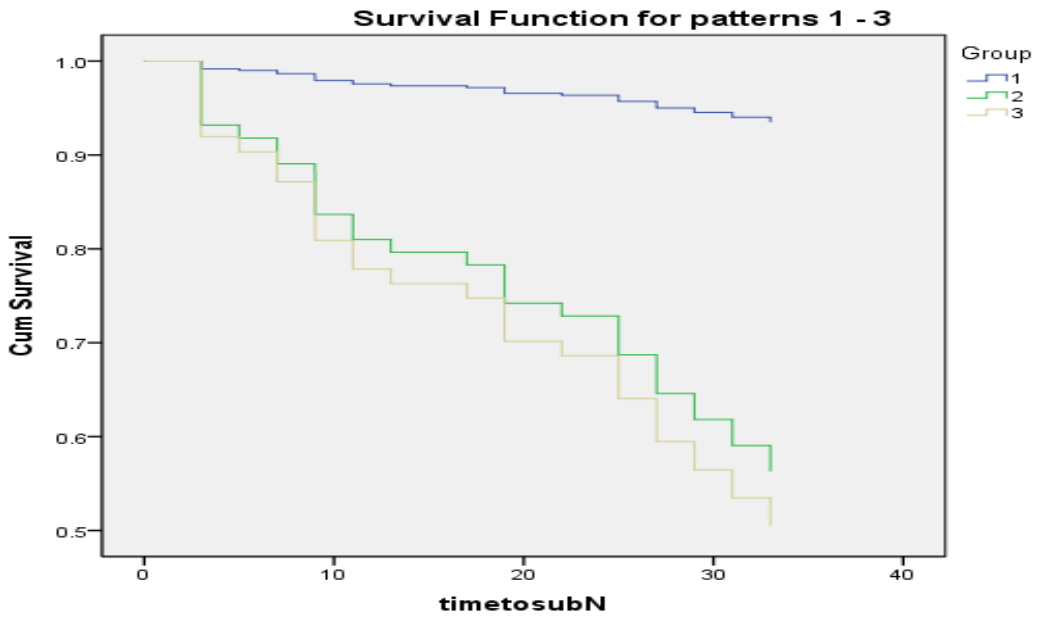

Figure 6. Survival Functions for Larvae Scored as Having Abnormal Swimming Behavior. Time to Abnormal swimming (days) vs. cumulative survival. Significant differences were observed between control (blue line, Group 1) and the 0.03ug/L experimental group (Group 2, green line, $p = 0.004$) and between control and the 0.05ug/L experimental (group 3, tan line; $p = 0.002$). No significant difference between experimental groups was observed (, $p = 0.637$).

Table 1

Mean observed values for weight (mg), Notochord length (mm), and Belly width (mm) for the three experimental groups. Lower and upper bounds (Low and Up) are specified, considering 95% confidence interval.

	Weight (mg)			Notochord Length (mm)			Belly width (mm)		
	Mean	Low	Up	Mean	Low	Up	Mean	Low	Up
Control group (N= 36)	49.2	42.1	56.4	8.3	6.91	12.27	1.63	1.56	1.71
Exp. Group (0.03µg/l) (N=38)	23.5	19.6	26.4	6.2	6.12	10.54	1.31	1.21	1.41
Exp. Group (0.5µg/l) (N=39)	22.5	18.6	26.4	6.1	5.98	10.32	1.25	1.17	1.32

Table 2

Post-hoc Analyzes for Weight. Contrasts between control group and the experimental groups, and between experimental groups. As a reference I used 5% significance.

	Control group		**Exp. Group (0.03µg/l)**	
	Tukey HSD	**LSD**	**Tukey HSD**	**LSD**
Control group	-	-	<<0.01	<<0.01
Exp. Group (0.03µg/l)	<<0.01	<<0.01	-	-
Exp. Group (0.5µg/l)	<<0.01	<<0.01	0.985	0.869

Table 3

Post-hoc Analyzes for Notochord Length. Contrasts between control group and the experimental groups, and between experimental groups. As a reference I used 5% significance.

	Control group		**Exp. Group (0.03µg/l)**	
	Tukey HSD	**LSD**	**Tukey HSD**	**LSD**
Control group	-	-	<<0.01	<<0.01
Exp. Group (0.03µg/l)	<<0.01	<<0.01	-	-
Exp. Group (0.5µg/l)	<<0.01	<<0.01	0.946	0.751

Table 4

Post-hoc Analyzes for Belly Width. Contrasts between control group and the experimental groups, and between experimental groups. As a reference I used 5% significance.

	Control group		**Exp. Group (0.03µg/l)**	
	Tukey HSD	**LSD**	**Tukey HSD**	**LSD**
Control group	-	-	<<0.01	<<0.01
Exp. Group (0.03µg/l)	<<0.01	<<0.01	-	-
Exp. Group (0.5µg/l)	<<0.01	<<0.01	0.539	0.290

CHAPTER 4

DISCUSSION

Results from this study are significant, showing a strong correlation between physiological and behavioral effects of fluoxetine. Individuals from experimental groups showed a significant lower weight, length and width than control group, and they also showed significant changes in behavioral patterns, either turning into an abnormal swimming or dying. Several previous studies show similar results (Gaworecki et al. 2008): fluoxetine affected feeding habits of Hybrid Stripped Bass (exposed individuals had lower feeding rates, weight and length) and fluoxetine reduced weight gain and expression of feeding peptides in the female goldfish brain (Mennigen et al. 2009).

Though we do not know the specific mechanism of fluoxetine's actions in these experiments, I hypothesize that fluoxetine interacts with guppies' nervous system and somehow affects their perception and hunger, making them to feed less frequently compared to control, unexposed individuals. This hypothesis is based on published information reporting fluoxetine effects neurotransmitter levels in the nervous system (Rang et al. 2001.). Often, these changes at the neurochemical level are manifested behaviorally as fear, hunger, and libido losses. Decreased feeding can lead to a decrease in weight and overall growth (length and width). In other words, my hypothesis is that fluoxetine interferes with the nervous system, and this affects the individuals' behavior patterns in many ways (direct effect), leading to other physiological effects such as weight loss and decrease in growth rates (indirect effects).

Some other effects of fluoxetine have been observed for a species related to *P. reticulata*, (*Gambusia affinis*). In the mentioned study, delayed sexual maturity in young

males (lethargy) and lethality in the acute toxicity assessed, LC_{50} = 546 µg/l after 96hs of exposure (Henry et al. 2008) were observed. In our experiments we were not able to examine onset of sexual maturity, as the ages examined were too young. However, it is possible that, with continued exposure, a similar response may be observed in guppies.

Also, our concentrations used are much lower than those used by Henry et al.(2008), according to concentration levels reported in aquatic environments by Nakamura et al. (2008), so results from both studies are congruent but, in terms of generalization, our findings are more applicable to circumstances given in natural aquatic environments, and findings from Henry et al. (2008) provide a wider understanding of fluoxetine mechanisms and effects on tropical livebearers fish species (using higher concentration levels).

Guppies have been used in previous studies designed to examine the effects of external zinc exposure. Zinc exposure resulted in significant weight loss and decreases in growth rates (Pierson, 2011), similar to our findings from fluoxetine exposure. The same trends were also observed in guppies exposed to cadmium (Miliou et al. 1998).

CHAPTER 5

CONCLUSION

Our results show a striking and strong coherence among the different experiments and significant differences were clearly evident between control group and experimental groups. Statistical results support the hypothesis that fluoxetine makes an impact on the early life history of *P. reticulata,* decreasing their weight, length and belly width, increasing mortality, and worsening their life style following long term (~1 month) exposure. Thus, it appears that fluoxetine is indirectly harmful for *P. reticulata*. Though the results from the experiments are clear, we cannot make any conclusions about the specific mechanisms on which fluoxetine makes an impact on these fish, though known impacts, such as loss of hunger, libido, and/or fear are possible. All of these side effects make a negative impact on the population, but they are outside the scope of these experiments.

It is also important to note that these experiments have been conducted in a laboratory where all environmental factors were controlled and larvae were not exposed to either predators or other stress factors. Thus, it would be appropriate to validate these results in field, at the natural aquatic environments where *P. reticulata* is exposed to fluoxetine and evaluate other possible interactions.

GLOSSARY

Bioavailability: Is a subcategory of absorption and is used to describe the fraction of an administered dose of unchanged drug that reaches the systemic circulation. It can be affected by some physicochemical characteristics of the medium, such as pH and temperature, among others.

Bioaccumulation: The accumulation of substances, such as pesticides, or other organic chemicals in an organism. Bioaccumulation occurs when an organism absorbs a toxic substance at a rate greater than that at which the substance is lost. Thus, the longer the biological half-life of the substance the greater the risk of chronic poisoning, even if environmental levels of the toxin are not very high.

Cox Regression: Is a class of survival model in statistics. Survival models relate the time that passes before some event occurs to one or more covariates that may be associated with that quantity. In a proportional hazards model, the unique effect of a unit increase in a covariate is multiplicative with respect to the hazard rate.

APPENDIX

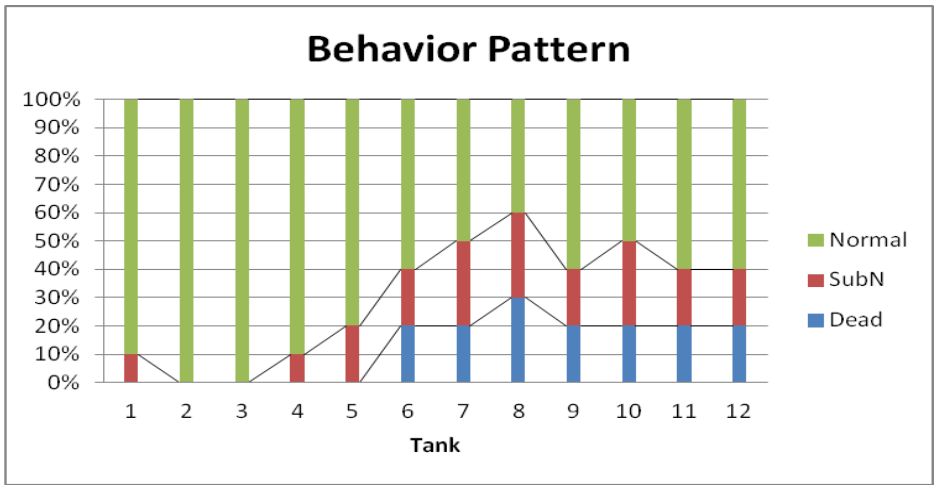

Figure A. Behavior Patterns for Each Tank. Where tanks 1 to 4 are replicates for control group, tanks 5 to 8 are replicates for experimental group 1 (0.03 µg/l), and tanks 9 to 12 are replicates for experimental group 2 (0.5 µg/l). Percentage represents the average proportion of each behavior category for each tank. Data were normalized so all scores in a given treatment equal 100%. Green bars = normal behavior, red bars = abnormal swimming behavior; blue bars = number that did not survive. Where a value of 10 individuals represents 100%. Data are further separated by treatment group in Figures B-D below.

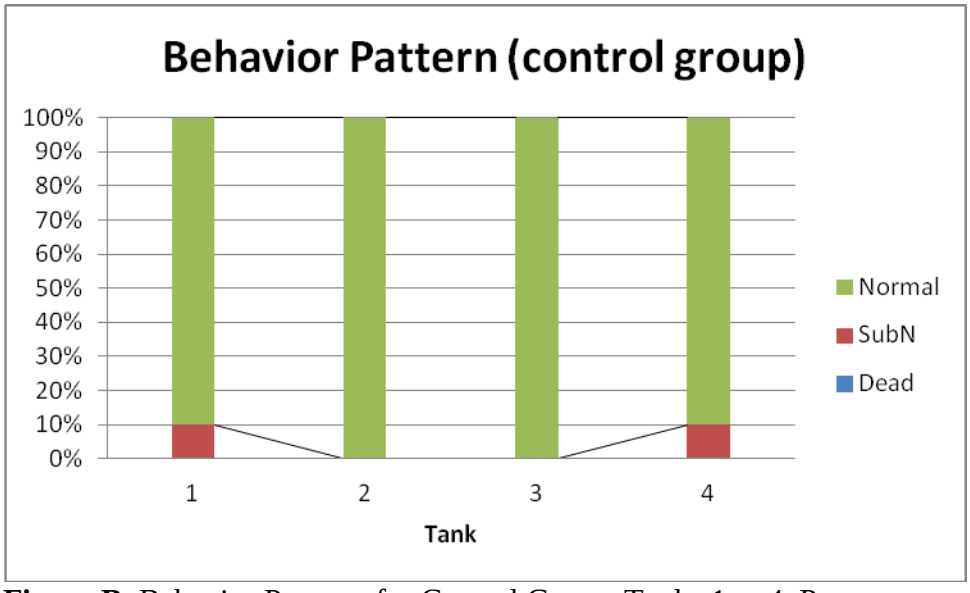

Figure B. Behavior Patterns for Control Group, Tanks 1 to 4. Percentage represents the average proportion of each behavior category for each tank. Data were normalized so all scores in a given treatment equal 100%. Green bars = normal behavior, red bars = abnormal swimming behavior; blue bars = number that did not survive. Where a value of 10 individuals represents 100%.

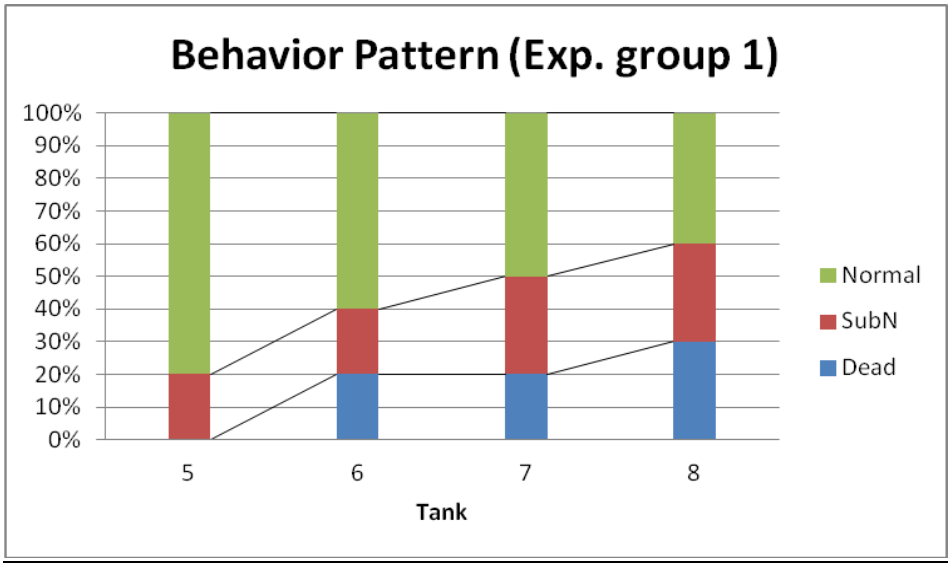

Figure C. Behavior Patterns for Experimental Group 1 (0.03 µg/l), Tanks 5 to 8. Percentage represents the average proportion of each behavior category for each tank. Data were normalized so all scores in a given treatment equal 100%. Green bars = normal behavior, red bars = abnormal swimming behavior; blue bars = number that did not survive. Where a value of 10 individuals represents 100%.

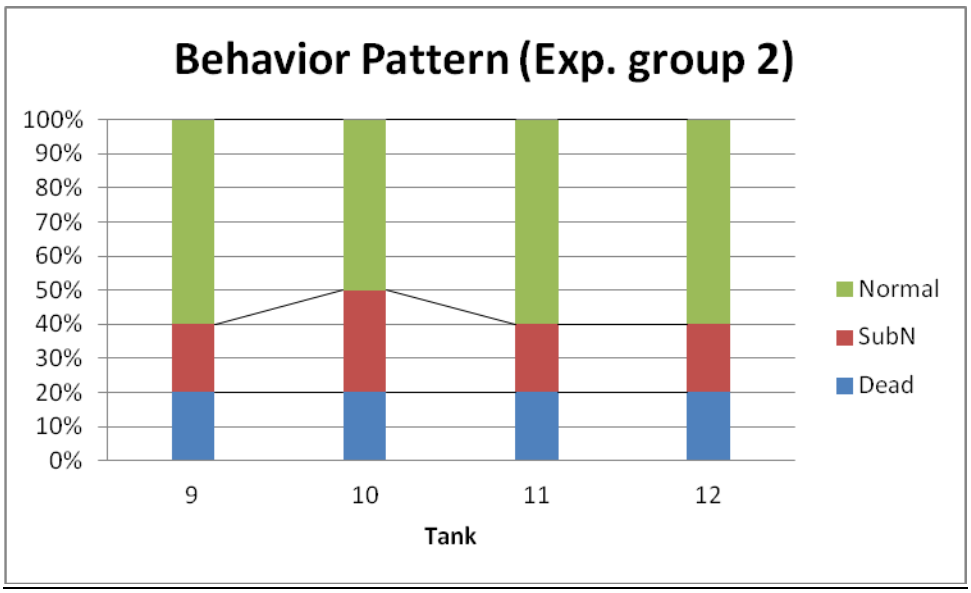

Figure D. Behavior Patterns for Experimental Group 2 (0.5 µg/l), Tanks 9 to 12. Percentage represents the average proportion of each behavior category for each tank. Data were normalized so all scores in a given treatment equal 100%. Green bars = normal behavior, red bars = abnormal swimming behavior; blue bars = number that did not survive. Where a value of 10 individuals represents 100%.

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