THE INFLUENCE OF STAGGERED EXPOSURE TO FLUOXETINE ON AGGRESSIVE BEHAVIORS IN SIAMESE FIGHTING FISH (*BETTA SPLENDENS*)

A Report of a Senior Study

by

William York McCurdy

Major: Biology

Maryville College

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Date Approved_____, by_____

Faculty Supervisor

Date Approved_____, by_____

Editor

ABSTRACT

The purpose of this study is to examine the effects of environmental concentrations of fluoxetine on aggressive behaviors in *Betta splendens*. It was hypothesized that (1) exposure from day 1-21 would reduce aggressive acts compared to control fish and (2) exposure from day 10-21 would cause rapid aggression reduction compared to control fish. When compared to control fish, it was found that there was no significant reduction in the number of aggressive acts performed by the treated fish. The closest we came to a significant p-value, with regard to the effect of the treatment, was 0.0885 on Day Eight of the experiment. All fish reduced their aggression from the beginning to the end of the study. Thus, the hypothesis that exposure from day 1-21 would reduce aggression was rejected; as was the hypothesis that exposure from day 10-21 would rapidly reduce aggression.

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CHAPTER I

INTRODUCTION

Pharmaceutical Distribution

According to the Centers for Disease Control and Prevention, in 2002 approximately 245 million Americans administered pharmaceutical products to themselves daily (Centers for Disease Control). Although some of these pharmaceuticals and personal care products (PPCPs) are biotransformed in the liver, much is excreted in the original form being sent through sewage treatment plants and ending up in natural water supplies (Derksen, Rijs, & Jongbloed, 2004). In the United States, pharmaceutical products remain in sewage treatment effluent because sewage treatment facilities are not designed to remove trace elements of these potential environmental contaminants. In a study in which a laboratory-scale sewage treatment plant was constructed, the researchers found that almost all of the antibiotics sent through the treatment process came out in the end, the process having little or no effect on them (Junker, Alexy, Knacker, & Kümmerer, 2006).

The largest contribution to the quantity of pharmaceuticals in the environment is excretion from humans by feces or urine (Derksen et al., 2004). Figure 1 shows the pathway that many pharmaceuticals follow after they are excreted or otherwise disposed. A route that has nothing to do with excretion is that of dumping unused PPCPs in the sink or toilet to dispose of them. According to a study in the United Kingdom, this may be a

more prominent route for PPCPs to reach water supplies than previously thought (Bound & Voulvoulis, 2005). Bound and Voulvoulis found that the main effects of the PPCPs were the feminizing effects of endocrine-disrupting compounds (Bound & Voulvoulis, 2005).

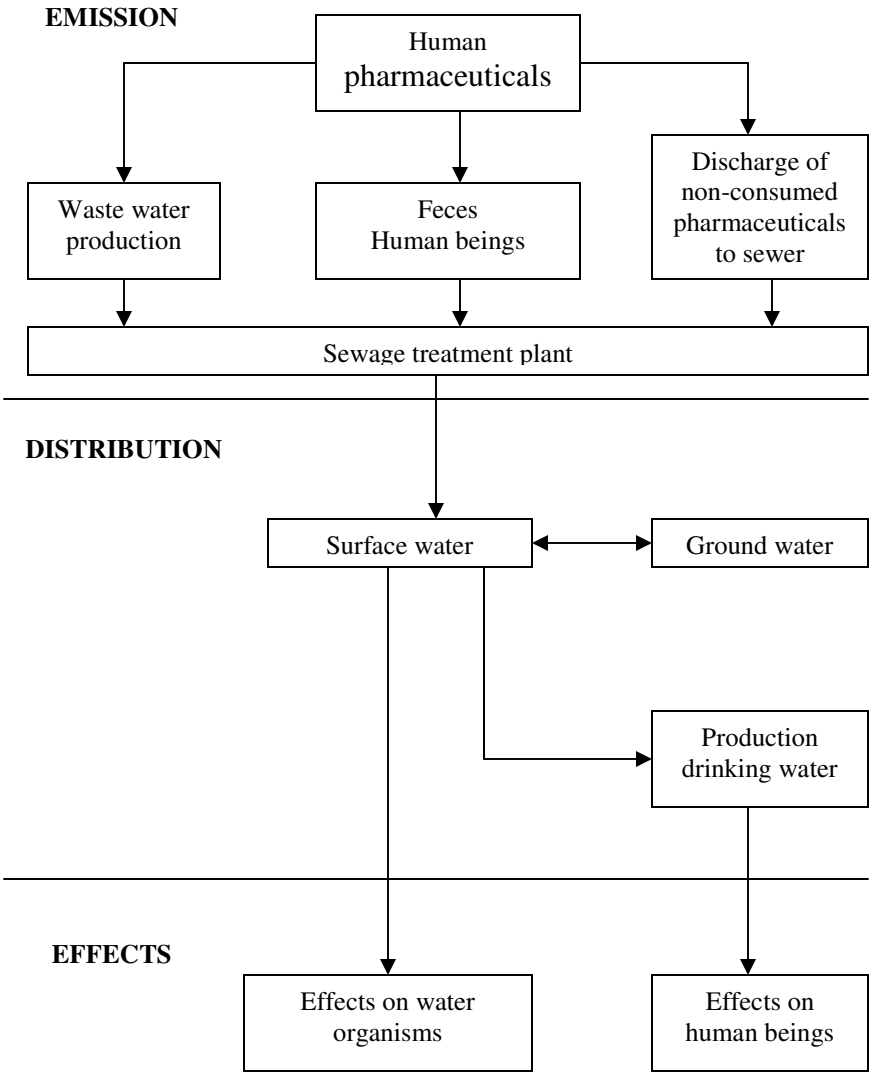

Figure 1. Flow-chart indicating the distribution route of human pharmaceuticals (based on Derksen et al., 2004).

Concentrations of PPCPs in Environment

Because of the vast amount of water on the earth, the concentrations of these PPCPs in water bodies are very low. They usually occur in concentrations of ng/L-µg/L, which is well below the level where they would affect humans (Daughton, 2002). However, studies have shown that exposure even at these concentrations may have an effect on fishes in effluent-dominated streams (Brooks, Chambliss, Stanley, Ramirez, Banks, Johnson, and Lewis, 2005). When, fish tissues were extracted and analyzed using solid-phase extraction and gas chromatography-mass spectrometry, respectively, the antidepressant drugs fluoxetine and sertraline were detected in amounts greater than 0.1 ng/g.

In Germany, a program has been implemented in an attempt to map and monitor the dispersal of pharmaceuticals in surface waters. Pharmaceuticals can enter directly into the surface water after they have been washed out of animal feces and diffused through the soil (Derksen, Rijs, & Jongbloed, 2004).

Table 1 lists a number of prescription drugs that were detected in streams across the United States (Koplin, Furlong, Meyer, Thurman, Zaugg, Barber, and Buxton, 2002). These researchers sampled 139 stream sites in throughout the United States and detected chemicals of a wide variety including veterinary and human antibiotics, prescription and non-perscription drugs, and steroids and hormones (Koplin et al., 2002).

Table 1. List of some prescription drugs found in streams in United States (from Koplin et al., 2002 pg. 1204).

Chemical	N	RL (µg/L)	Freq. (%)	Max (µg/L)	Median (µg/L)	Use
Albuterol	84	0.029	0	ND	ND	Antiasthmatic
Cimetidine	84	0.007	9.5	0.58[d]	0.074[d]	Antacid
Codeine	46	0.24	6.5	0.019	0.012	Analgesic
Digoxin	46	0.26	0	ND[d]	ND[d]	Cardiac stimulant
Fluoxetine	**84**	**0.018**	**1.2**	**0.012[d]**	**0.012[d]**	**antidepressant**
Gemfibrozil	84	0.015	3.6	0.79	0.048	antihyperlipidemic
Metformin	84	0.003	4.8	0.15[d]	0.11[d]	Antidiabetic
Ranitidine	84	0.01	1.2	0.01[d]	0.01[d]	Antacid
Warfarin	84	0.001	0	ND	ND	Anticoagulant

[d] Concentration estimated – average recovery <60%.

Environmental concentrations of SSRIs

Serotonin has been shown to affect the behavior of many vertebrates. Some studies suggest that it may also have an effect on aggression in some invertebrates (Panksepp, Yue, Drerup, & Huber, 2003). Serotonin has been linked to aggressive behavior in humans, as well (Nolen-Hoeksema, 2004). Often aggression is exhibited when an individual has a resource to defend, but some researchers have seen aggressive behavior in test subjects where no resource was present. It is believed that aggression must be affected by other environmental factors and perhaps these environmental factors triggered biological responses that could link serotonin to aggression (Panksepp, Yue, Drerup, & Huber, 2003).

Selective serotonin reuptake inhibitors (SSRIs) are medications that cause serotonin to remain in the synaptic cleft longer than it normally would. This reuptake inhibition causes the effects of serotonin to be prolonged; that is, one 'feels good' for a longer period of time. SSRIs have been found in effluent-dominated streams. In the tissues of fish from an effluent-dominted stream, SSRIs such as fluoxetine and its

metabolite norfluoxetine were detected at concentrations greater than 0.1 ng/g (Brooks et al., 2005). It has recently been suggested that fluoxetine at environmental concentrations can reduce aggression in fish (Parsons, 2005).

Betta fish as a model organism

Members of the species of fish, *Betta splendens*, are known commonly as the Siamese fighting fish. They come from the family Osphronemidae and are the only species of the genus *Betta* (Britz, 2003). Since this is the case, they are often referred to only as 'Betta fish.' These fish are naturally aggressive and will attack intruders (most often males vs. males) who venture too close to their territories (Jaroensutasinee & Jaroensutasinee, 2004). In a study in 1969, researchers paired a neutral stimulus with an individual's sighting of itself in a mirror and received an aggressive response (Braud & Weibel, 1969). After a period of time, the neutral stimulus lost its neutrality and became an inducer of aggression in *Betta splendens*. There is also evidence that aggression may be its own reward for the Siamese fighting fish. The males of this study were conditioned to respond aggressively in order to *receive* an image of another male they could (in their minds) interact with (Thompson, 1963). When an adult male is exposed to an 'audience' outside its tank, a regular response is to become aggressive. If this male is placed with another male, the male that was not primed by the audience will rise to the level of aggression exhibited by its counterpart (Matos, Peake, & McGregor, 2003). However, in a study performed by Halperin, Giri, Elliot, & Dunham (1998), hyper-aggressive fish (those primed by an audience and other stimuli) escalated to biting and tail-whipping early in an encounter with another male. They tired quickly and often lost their fights.

Ichihashi, Ichikawa, and Matsushima (2004) wanted to study the influence of environmental conditions on betta fish aggressive tendencies. This study divided a population of male *Betta splendens* into four groups, each with different conditions of social interaction. The conditions ranged from being reared communally with much social interaction to isolation with no social interaction. Results showed that the isolated fish were much more aggressive than the other three groups and would often continue fighting even after an opponent showed signs of submission. All fish postured in the same way, but only the isolated fish were terribly aggressive. An attempt to reintroduce those isolated fish into social environment failed because they continued to fight with others.

Aggressive acts in *Betta splendens* also depend on the type of intruder the individual detects (Jaroensutasinee & Jaroensutasinee, 2004). One study used three types of betta fish: male, female, and female that had laid her eggs. Gill cover erection, biting and tail beating were the most common aggressive acts toward other males. Females and mated females received fewer and fewest incidents of these acts. The acts most associated with females were attacking and chasing whereas mated females elicited fewer of these responses as well, and males very rarely experienced these types of aggression.

Purpose of this Study

This study has two main objectives: 1- to repeat the findings of Parsons (2005) to determine if her findings that fluoxetine could reduce the aggressive behaviors of *Betta splendens* could be replicated and 2- to study what effect the late introduction of fluoxetine has on a group of Siamese fighting fish that have been in control conditions for some amount of time. It is hypothesized that those fish exposed to fluoxetine for the

duration of the study will have significantly reduced aggressive behaviors and that those fish not exposed to fluoxetine will maintain their aggressive behaviors. It is also hypothesized that the fish kept in control conditions and later exposed to fluoxetine will reduce their aggressive behaviors more rapidly than the control fish after being exposed to flouxetine.

CHAPTER II

MATERIALS AND METHODS

Twenty-one adult male *Betta splendens* were purchased from Wal-Mart (Alcoa, TN). Each fish was placed in its own 1.2L plastic container filled with 1000mL dechlorinated water. Seven were chosen at random to be control organisms, seven were chosen to be in the Experimental I (X-I) group, and seven were placed in the Experimental II (X-II) group. Fluoxetine was used from the supply in the Biology Department at Maryville College (Maryville, TN). The fish in the control group were housed in only fresh dechlorinated water for the entirety of the experiment. Group X-I resided in fresh dechlorinated water in which 0.012µg fluoxetine was introduced by use of a micro-pipette at the beginning of the experiment and again when the water was changed at day 10 of the experiment (The fish in Group X-1 were only dosed twice). Group X-II was placed in control conditions for the first half of the experiment and then experienced experimental conditions for days 10-21. At day 10 of the experiment, the water for all fish was changed and fluoxetine was introduced into the bowls of Group X-II for the first time.

When behavior was to be tested, each fish was taken out of its personal container and placed in a large tank with three liters fresh dechlorinated water. After one minute of acclimation time in the test tank, a mirror was placed in front of the fish. The fish's reaction was recorded with the help of an ethogram (See Table 2). Behavior was scored

by a one or a zero. Each twenty second period where the fish exhibited an aggressive act was scored as a one. All twenty second periods where the fish did not exhibit an aggressive act was scored as a zero. See Table 3 for a sample log sheet with twenty second intervals noted. Each fish was allowed five minutes in the tank with the mirror and then replaced in its personal container. This procedure was repeated every two days until each fish had been tested eight times. At the mid-point of the study, Day 10, the water in the fish containers was replaced with fresh dechlorinated water and the test containers were once again infused with 0.012µg fluoxetine. All fish were kept in dedicated containers for the entire experiment.

An ANOVA was used to compare experimental groups. The ANOVA chosen was for aggression dependent on treatment, split by day. Linear regression analysis was used to determine if there was any difference between groups in the rate of decline of their aggressive actions.

Table 2. Ethogram used to determine aggressive and non-aggressive acts.

H	Hover- stationary in water, fins not spread.	0
BR	Bottom Rest.	0
B	Breathing- gulping air at surface.	0
FS	Fin Swim- swimming slowly, fins not spread.	0
SS	Serpentine Swim- rapid swimming from one end of the tank to other, uses whole body.	0
GS	Gill Spread- indicate only if not associated with another aggressive act.	1
FSH	Fins Spread with body horizontal to mirror. May include gill spreading.	1
FSP	Fins Spread with body perpendicular to mirror. May include gill spreading.	1
Ch	Charges mirror- no contact with mirror, may begin as FSP.	1
Ct	Contact with mirror- same as Ch, but with actual contact.	1
TW	Tail Whip- Whips tail against mirror in aggressive way.	1
Bt	Biting mirror- Body perpendicular to mirror, biting at mirror.	1
SB	Swim Behind- attempts and/or succeeds at swimming behind the mirror.	0
L	Leaves- swims away from mirror.	0

Table 3. Sample log sheet on which aggressive and non-aggressive acts were recorded.

Date_____ Group____ Time_____

	Individual	Individual	Individual	Individual	Individual	Individual	Individual
0-20s							
21-40							
41-60							
61-80							
81-100							
101-120							
121-140							
141-160							
161-180							
181-200							
201-220							
221-240							
241-260							
261-280							
281-300							

CHAPTER III

RESULTS

At no point in the study could the reduction in aggression be attributed to the treatment applied. The closest any p-value came close to being significant was on Day Eight (Test Session Four, *p*-value = 0.0885). By the end of the study, all fish in all treatments had reduced their average number of aggressive acts. Figure 2 shows the average number of aggressive acts per test session for each group of fish. This figure shows that all groups of fish reduced their aggression by the end of the study, no matter how insignificantly. Table 4 shows the *p*-values and r-squared values obtained from the linear regression analysis that was used on all groups from test sessions Five through Eight. The group exposed to fluoxetine for the entire experiment (X-I) showed significant correlation between aggressive acts over time ($p = 0.013$, $R^2 = 0.93$). Figure 3 shows the average number of evasive acts per test session for each group, and there was no relationship observed.

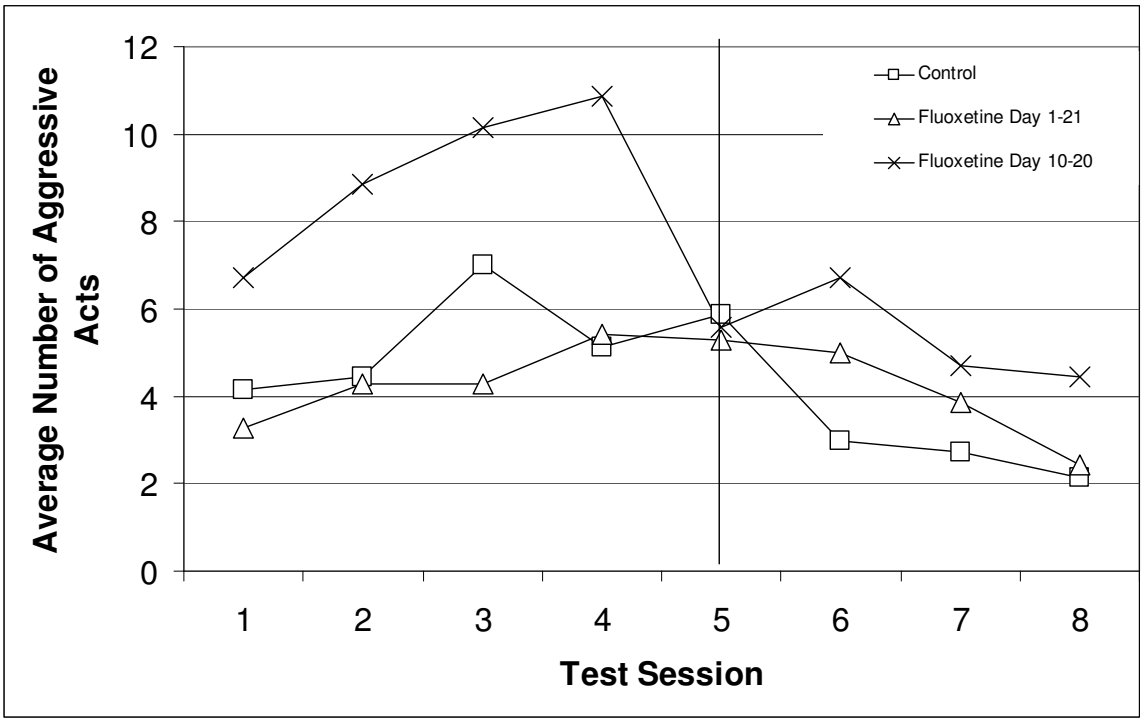

Figure 2. Average number of aggressive acts per test session. Line at Test Session 5 denotes: Water changed and fluoxetine introduced to second experimental group.

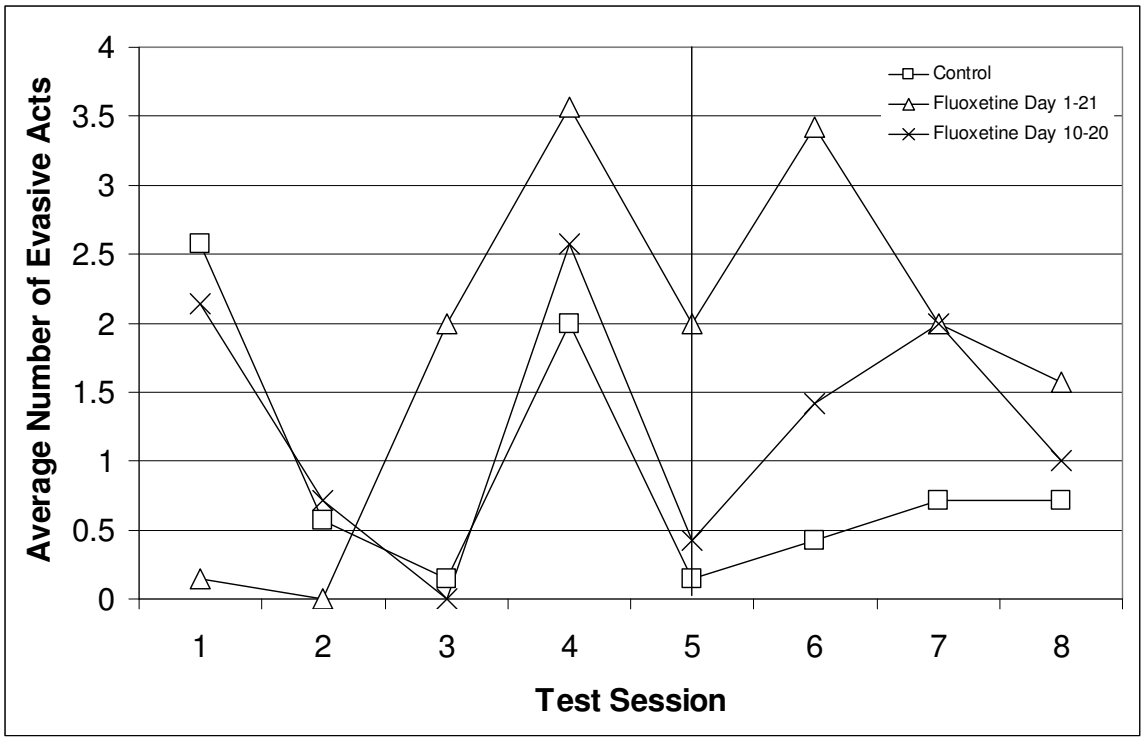

Figure 3. Average number of evasive acts per test session. Line at Test Session 5 denotes: Water changed and fluoxetine introduced to second experimental group.

Table 4. List of p-values and r-squared values of aggression for test sessions Five through Eight obtained by linear regression.

Group	p-value	R-squared value
Control	0.057792	0.792421069
Fluoxetine Day 1-21 (X-1)	0.01356	0.931199453
Fluoxetine Day 10-21 (X-2)	0.081756	0.465715463

CHAPTER IV

DISCUSSION

Although most fish in the study reduced their aggressive behaviors over the course of the study, treatment did not influence them and, thus, the hypothesis that fluoxetine exposure would influence aggression was not supported. Also, the rate of decline in aggression of the second experimental group was not shown to be greater than that of the control group after the introduction of fluoxetine. Many factors may have contributed to this outcome. The most obvious is that the second experimental group (X-2, Fluoxetine Day 10-21) had subjects that were naturally more aggressive than the subjects placed in the other two groups. Also, I did not test the fish to quantify their initial aggression before dividing them into groups. When viewing the data in Figure 2, it is obvious that the second experimental group was more aggressive than the other two groups. They were more aggressive than the other groups at the beginning of the experiment and increased their average aggressive acts throughout the first half of the study.

Also, my results did not support the work of Parsons (2005), which can possibly be attributed to the dosage that was used in our respective studies. Parsons used 0.54µg fluoxetine per liter of water, while I used 0.012µg fluoxetine per liter of water. She showed that the fish exposed to fluoxetine did indeed reduce their aggressive behaviors significantly.

In future studies of aggression in *Betta splendens*, I believe using one fish to incite the aggressive behaviors from others would be more beneficial than using a mirror. Since the dosage of fluoxetine used in this experiment did not significantly reduce the aggression of the subjects, increasing the concentration in future studies would also be advised. To attempt to ensure the equal aggressiveness among the groups, one should run a preliminary test of all fish and rate their aggression. When dividing the fish into groups, equal numbers of more aggressive and less aggressive fish should be place in each group.

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