THE EFFECTS OF FLUOXETINE ON AGGRESSIVE BEHAVIORS IN SIAMESE FIGHTING FISH (*BETTA SPLENDENS*)

A Report of a Senior Study

by

Ivy Gale Parsons

Major: Biology

Maryville College

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Date Approved_____, by_____

Faculty Supervisor

Date Approved_____, by_____

Editor

ABSTRACT

Pharmaceuticals, household chemicals, biogenic hormones, and other consumables are released directly into the environment after passing through wastewater treatment processes, which are often not designed to remove these chemicals from the effluent. Approximately 50 million people today are taking fluoxetine, normally recognized under the trade names Prozac®, Prozac Weekly®, and Sarafem®. Norfluoxetine is the only known active metabolite, which in the blockade of serotoin reuptake appears to be as effective as its parent. Since fluoxetine effects the serotonin levels in the body, it has also been speculated that fluoxetine could also effect the reproduction rate in aquatic animals. There have been very few studies that have tested the relationship between aggression in aquatic animals and serotonin. The purpose of this study was to determine if realistic environmental concentrations of fluoxetine can alter the behavior of *Betta splendens.* Five fish were given 0.54μg of fluoxetine and four fish were used as controls. After the study concluded, the experimental fish did reduce their aggressiveness significantly (*p*-value = .002). This reduced aggression could be reducing the reproductive output of male *Betta splendens*.

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CHAPTER I

INTRODUCTION

Pharmaceutical drugs in water supplies

Pharmaceuticals, household chemicals, biogenic hormones, and other consumables are released directly into the environment after passing through wastewater treatment processes, which are often not designed to remove these chemicals from the effluent. Many of these OWCs are in streams throughout the United States and include antibiotics, various prescription drugs, nonprescription drugs, steroids, reproductive hormones, personal care products, products of oil use and combustion, and other extensively used chemicals (Kolpin, Furlong, Meyer, Thurman, Zaugg, Barber, Buxton, 2002). Thus, the extent of pharmaceutical and personal care products (PPCPs) in the aquatic environment and their consequences are beginning to be monitored by some scientists (e.g., Potera, 2000). The forefront of PPCP monitoring has been in Germany, because water quality issues are of primary importance in Europe.

Table 1 lists the prescription drugs that were tested for in streams by U.S. Geological Survey personnel and includes the data measured for each drug in a total of 139 stream sites (Kolpin et al., 2002). This list contains drugs that are used for a variety of different conditions in the human body. Albuterol is used to help people with

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

Chemical	N	RL	Freq (%)	Max	Median
Albuterol	84	0.029	0	ND	ND
Cimetidine	84	0.007	9.5	0.58	0.074
Codeine	84	0.1	10.4	1	0.2
Digoxin	46	0.26	0	ND	ND
Dilitiazem	84	0.012	13.1	0.049	0.021
Fluoxetine	84	0.018	1.2	0.012	0.012
Gemfibrozil	84	0.015	3.6	0.79	0.048
Metformin	84	0.003	4.8	0.15	0.11
Paroxetine HCL	84	0.26	0	ND	ND
Rantidine	84	0.01	1.2	0.01	0.01
Warfarin	84	0.001	0	ND	ND

breathing troubles such as asthma, cimetidine is used for those with antacid problems, codeine is used to help relieve pain, fluoxetine is an antidepressant, metformin helps type-2 diabetics control their blood glucose levels, and warfarin is used to thin the blood of a human. Antibiotics were not placed in this list because thirty-one antibiotics were tested and were categorized under veterinary and human antibiotics, without distinguishing which antibiotic is used for which species. However, numerous antibiotics are found in high concentrations in water supplies, primarily as a result of farm usage (Kolpin et al., 2002). According to Christian Daughton, chief of the Environmental Chemistry Branch of the U.S. Environmental Protection Agency (EPA) Environmental Sciences Division in Las Vegas, Nevada, researchers worldwide have discovered more than sixty different PPCPs in water sources (Potera, 2000).

Environmental engineer Glen Boyd and his students at Tulane University performed one of the first studies in the United States on the occurrence of drugs in drinking water (Potera, 2000). They obtained water samples from the Mississippi River, a local lake, and the city tap water. Varying concentrations of the pain reliever naproxen, the sex hormone estrone, and clofibric acid (a bioactive metabolite from anti-cholesterol drugs) were detectable in most water

sources. It should be noted that these three drugs were targeted for this experiment due to the potential of inferring with normal reproduction and development in fish living downstream from sewage plants. Little information is known about the effects of environmental occurrence, transport, and ultimate fate of many pharmaceuticals that are designed to stimulate a physiological response in humans and animals (Kolpin et al., 2002). A major unaddressed issue is the long-term outcome of humans ingesting subtherapeutic doses of many drugs as well as any dose at all of substances not meant to be ingested (Potera, 2000).

Naproxen and ibuprofen, both mild pain relievers/anti-inflammatory drugs, have been detected in raw sewage (Boyd, Palmri, Zhang, & Grimm, 2004). In samples collected from the two stormwater canals that were tested, naproxen was detected at concentrations ranging from 1.6 to 145 ng/l. In about half of the canal water samples, ibuprofen was detected with concentrations up to 674 ng/l. The data that Boyd et al. collected showed that increased rainfall increases contaminant concentrations. All three sites where samples were collected naproxen was detected. Only in the stormwater canals ibuprofen was detected.

Effects of fluoxetine on humans

Approximately 50 million people (www.Prozac.com) today are taking fluoxetine, normally recognized under the trade names Prozac®, Prozac Weekly®, and Sarafem®. Fluoxetine is typically prescribed to patients having symptoms of depression, and fluoxetine is the most widely used antidepressant drug in the world (Fuller, 1996).

This antidepressant belongs to the selective serotonin reuptake inhibitors (SSRIs). The SSRIs are structurally distinct from other classes of antidepressants such as monoamine oxidase inhibitors and trycyclics (Clinical Pharmacology, 2004). Fluoxetine shows the longest half-life

of all SSRIs and was also the first SSRI approved in the United States. In October 1999 fluoxetine was specifically approved for use in geriatric depression.

It is thought that the most important effect of fluoxetine is the enhancement of the actions of serotonin due to highly specific serotonin reuptake blockade at the neuronal membrane, although the exact action of SSRIs is not completely understood (Clinical Pharmacology, 2004). The dopamine transporter is weakly inhibited by fluoxetine. Due to dramatically decreased binding to receptors of histamine, acetylcholine, and norepinephrine the SSRIs have less anticholinergic, sedative, and cardiovascular effects than do the tricyclic antidepressant drugs. In fact, anticholinergic activity is almost completely absent. When serotonin levels are lower in vertebrates, the males are less aggressive (Perreault, Semsar, & Godwin, 2003).

Fluoxetine is take orally, in the form of tablets or capsules, and is absorbed from the GI tract (Clinical Pharmacology, 2004). From regular capsules and tablets, the peak plasma concentrations occur in six to eight hours. Fluoxetine's principal active metabolite norfluoxetine and steady-state plasma concentrations of fluoxetine occur in two to four weeks. Fluoxetine gets distributed well in the body, and it can readily cross the blood-brain barrier and assumingly the placenta. Breast-feeding mothers are advised against taking fluoxetine as it is distributed through breast-milk .

In the liver fluoxetine is demethylated to several metabolites (Clinical Pharmacology, 2004). Norfluoxetine is the only known active metabolite, which in the blockade of serotoin reuptake appears to be as effective as its parent. After chronic fluoxetine administration the elimination half-life is four to six days and sixteen days for norfluoxetine. Within twenty-eight days about 12% of an oral dose is excreted through the feces and within thirty-five days about

60% is excreted through the urine (Clinical Pharmacology, 2004). Therefore approximately 72% of a fluoxetine dose is released into the sewage system and eventually the water supply.

There are various reasons why people take fluoxetine. Table 2 presents indications that fluoxetine is used for and along with some contradictions and precautions. Currently, fluoxetine is the only SSRI approved for the treatment of depression in children; the FDA approved fluoxetine for pediatric use in depression and PCD on January 3, 2003. Supported by much evidence, fluoxetine decreases aggressive behavior in animals (Fuller, 1996).

Table 2. Uses and Precautions/Contradictions with fluoxetine
(Clinical Pharmacology pg. 2 &7)

Indications	Contradictions/Precautions
alcoholism*	Hot flashes*
anorexia nervosa*	obesity*
anxiety*	obsessive compulsive disorder (OCD)
autism*	orthostatic hypotension*
borderline personality disorder*	panic disorder
bulimia nervosa	pasttraumatic stress disorder (PTSD)*
depression	premature ejaculation*
fibromyalgia*	premenstrual dysphoric disorder (PMDD)
abrupt discontinuation	electroconvulsive therapy (ECT)
anorexdia nervosa	hepatic disease
bleeding	hyponatremia
breast-feeding	Infants
cardiac disease	Mania
children	pregnancy
dehydration	renal failure
diabetes mellitus	renal impairment
driving or operating machinery	seizure disorder
elderly	suicidal ideation

*Non-FDA-approved indication

On September 24, 2004, an FDA Advisory Committee concluded that antidepressants, as a class, increase suicidal thinking and behavior in some pediatric patients (Clinical Pharmacology, 2004). The decision to accept or reject the FDA Advisory Committee's recommendations is pending by the FDA as of today's date.

Effects of fluoxetine on aquatic animals

To develop water quality criteria and to monitor whole effluent toxicity in the United States, standardized single species toxicity tests are used to screen for potential hazards of aquatic containments. If an expected environmental introduction concentration (EIC) exceeds 1 µg/l, environmental assessments of pharmaceutical compounds rely on single species responses (Brooks, Foran, Richards, Weston, Turner, Stanley, Solomon, Slattery, LaPoint, 2003). To assess potential effects of fluoxetine on freshwater biota, standardized aquatic toxicity tests have been performed. For aquatic toxicity tests, two cladocerana, *Ceriodaphnia dubia* and *Daphnia magna*, and the fathead minnow, *Pimephales promelas* were all chosen specimens for testing in a study performed by Brooks et al. (2003).

Forty-eight hour acute toxicity tests were performed in reconstituted hard water on *C. dubia*, *D. magna*, and *P. promelas (*Brooks et al., 2003). They performed studies on how much fluoxetine it would take to euthanize the organism. It took an average of 234 µg/l of fluoxetine to euthanize *C. dubia*, whereas the average dose of fluoxetine needed to euthanize *D. magna* and *P. promelas* was 820 µg/l and 705 µg/l respectively. It has been reported that it takes an average amount of 2 mg/l of fluoxetine, over a forty-eight hour time span, to euthanize a rainbow trout (*Oncorhynchus mykiss*).

Effects of fluoxetine on animal reproduction

Since fluoxetine effects the serotonin levels in the body, it has also been speculated that fluoxetine could also effect the reproduction rate in aquatic animals. Also a seven-day static-renewal study was performed on *C. dubia* to observe potential fluoxetine effects on cladoceran reproduction (Brooks et al., 2003). Standard methods were followed for this test; although, an algae-Cerophyll® suspension following daily renewals were fed to the organisms. The lowest

observed effect concentrations and no observed effect concentration (NOEC) were determined at 112 μg/l and 56 μg/l. From the control organisms, there was a statistically difference (α=0.05) at a treatment level of 112 μg/l, although the difference was only a mean of 2.1 neonates per female, so the observed may not be of ecological relevance.

The *C. dubia* fecundity was increased with fluoxetine treatments at 56 μg/l. Fecundity is the number of offspring a female produces at one time. When *D. magna* were exposed to 36 μg/l of fluoxetine (Brooks et al., 2001), they observed a comparable reproductive stimulation of gonadotropin in some fish species. Oogenesis and vitellogenesis is controlled by gonadtropin, and the sex steroid synthesis is also stimulated by gonadtropin. Increased serotonin levels may be the result of the observed stimulation in fecundity.

Foran, Weston, Slattery, Brooks, and Huggett (2003) tested if fluoxetine has potential to disrupt teleost reproductive function in Japanese medaka fish (*Oryzias latipes*). An important neuromodulator of sexual function in vertebrates and invertebrates has been shown to be serotonin, with increased serotonin concentrations causing increased gonadotropin release from the pituitary in many fish. A consequence of this action is an increase in steroidogenesis from the gonads, it is then expected to be seen in the circulating steroid hormone concentrations. Foran et al. exposed the fish to fluoxetine for four weeks and collected data.

Between the control and exposed fish pairs there was no statistical difference in egg production (Foran et al., 2003). The success rate of the eggs hatching did not change with the exposure to fluoxetine; however, fish pairs with the highest fluoxetine exposure had the lowest rate of fertilization. Concerning fecundity, fertility, rate of spawning, or hatching success no change was observed.

Several abnormalities were noted during observations of developing embryos (Foran et al., 2003). The abnormalities included: curved spine, incomplete development (no pectoral fins, reduced eyes), edema, and nonresponsiveness. In the control group fewer abnormalities were observed. It has been found that serotonin can prevent steroid-induced maturation of oocytes in *Fundulus heteroclitus*. A potential disruption of serotonin-mediated physiological processes in exposed wildlife is fluoxetine. Also, different species may react differently on the effects of fluoxetine regarding reproduction. More studies need to be performed to determine if fluoxetine can effect fecundity and fertilization in animals.

Effects of fluoxetine on aggression

There have been very few studies that have tested the relationship between aggression in aquatic animals and serotonin. A study performed by Perreault, Semsar, and Godwin (2003) looked at the effects of serotonin and aggression in the coral reef fish bluehead wrass (*Thalassoma bifasciatum)*. The use of serotonin reuptake inhibitors, e.g. fluoxetine, that prevent serotonin reabsorption at synapticections is one way to increase serotonergic activity. It was found that fluoxetine those males that were less aggressive overall towards an intruder were treated chronically with fluoxetine. These males also had longer latencies to the first chase than the males. The males that were exposed to fluoxetine acutely decreased both the frequency and duration of those who came into contact. Overall, Perreault et al. (2003) found that a decrease in aggression in coral reef fish can be caused by both chronic and acute fluoxetine treatments. More studies need to be performed in the future to determine if fluoxetine can decrease aggression in aquatic organisms.

Betta splendens Background

Siamese fighting fish, *Betta splendens*, are very aggressive towards one another. In pet stores they will be kept in separate tanks from one another. They will fight with one another and can cause a loss of energy that is consumed during the fight, and physical damages may occurr (Ichihashi, Ichikawa, & Matsushima, 2004). These fish are native to Thailand, Cambodia, Malaysia, and Myanmar. The females of this species do not fight as aggressively as the males, the male fights are serious and usually cause physically damage to one another. Females choose their male mates according to the male competitions.

An ideal subject to study aggression in animals is the Siamese fighting fish (Ichihashi et al., 2004). Thus, this study used the Siamese fighting fish as a model species to test the hypothesis that environmental concentrations of fluoxetine can alter the behavior of aquatic species.

CHAPTER II

MATERIAL AND METHODS

Ten adult male *Betta* fish were purchased at Ken and Mac's Pet Supply Store (Maryville, TN). Each male was placed in it's own 42oz plastic container filled with dechlorinatedwater. Randomly five fish were determined to be controls and the other five were determined to be the experimental fish. Each container was filled with one liter of clean freshwater that was dechlorinated. Each fish was weighed before the experiment began. Ten milligrams of fluoxetine was purchased from Sigma Chemicals. Two milligrams of fluoxetine was mixed in 500ml of distilled water made the solution of fluoxetine. 135 µl of the solution (0.54µg of fluoxetine) was added to each of the test containers with the freshwater. Previously, Kolpin et al. (2002) found 0.54µg of fluoxetine in freshwater streams and storm water canals (2002). The experimental fish were placed into individual containers that contained the fluoxetine, whereas the control fish were placed into their own container of freshwater with no fluoxetine.

To test the behavior, each fish was taken out of its container and placed into a two and a half gallon tank of freshwater. The fish was allowed one minute to get use to the new surroundings. After one minute a mirror was placed in front of the fish. An ethogram, see Appendix A, was placed beside the tank to help monitor the reactions of the fish to the mirror. The fish was observed for five minutes with the mirror. After observation the fish was removed

from the tank and placed back into its original container. This procedure was repeated until six observations were performed for all fish. After one-week the water was replaced with clean freshwater and 162µg of fluoxetine was mixed in the test containers. Each fish was kept in the same container throughout the experiment.

CHAPTER III

RESULTS

Fluoxetine decreased the aggression in the *Betta* fish signigicantly (*p*-value = .002). Figure 1, shows the mean number of aggressive behaviors (±ISE) on each day of observation in each group of fish. Compared to the control fish, the treated fish had reduced aggressive actions by day four after exposure. After the dose was tripled for the treated fish their aggressive behaviors did decrease slightly. Towards the end of the experiment the control fish began to reduce their aggression.

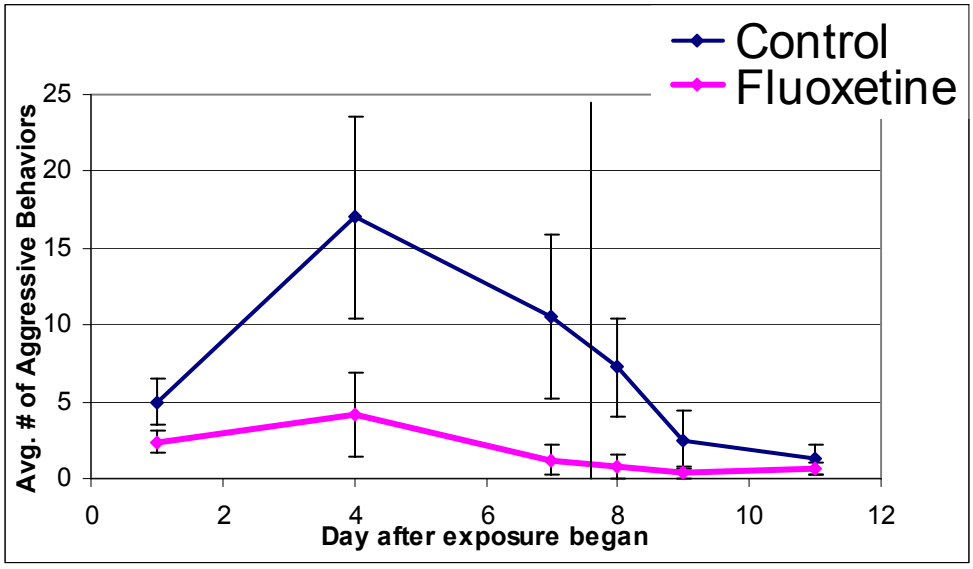

Figure 1. The mean number of aggressive behaviors per day after exposure, line at day 7 indicates tripled dose.

CHAPTER IV

DISCUSSION AND CONCLUSION

Female *Betta* fish choose a male that is the most aggressive because it shows protection for her and eggs. This study shows that realistic environmental concentrations of fluoxetine could be affecting the aggressiveness of the adult male *Betta* fish. This data supports the study by Perrault et al. (2003) who performed fluoxetine treatments on aggressive coral reef fish. Both this study's data and Perrault et al.'s data suggests that acute fluoxetine exposure are affecting aquatic organisms behaviors. This reduced aggression could be influencing the reproductive output for *Betta* males.

An unexpected interesting finding was the control fish decreased their aggression towards the end of the experiment. The controls had decreased their aggression over half by the eleventh day compared to the fourth day from exposure. It is possible the fish were learning or adapting to the larger tank. Learning allows the fish to conserve energy (Johnsson, J.I, & Akerman, A., 1998). During the experiment some of the fish would position themselves horizontally to the mirror and make certain movements to try determine if there was another fish beside of them. It

is also possible they were adapting to the fact when they were placed into the larger tank there was another fish in the tank always in the same spot.

Future research should include replication with a much larger sample size to reduce the chances of learning or adaptation. Also analysis of internal organs, such as gonads and liver, should be researched.

APPENDIX A

Ethogram for Senior Thesis

H	Hover- hangs in water column with fins not spread.
BR	Bottom rest.
B	Breathing (surfacing and gulping air).
FS	Fin Swim- slow swimming using pectoral fins; without fins in spread condition.
SS	Serpentine Swim- rapid, uses entire body and S-shaped movements without fins in spread condition.
S	Shaking- shimmies the body, usually with fins and gills spread.
GS	Gill Spread- only indicate this if the behavior is not done as part of one of the next behaviors.
FSH	Fin Spread with body Horizontal to mirror. This may include periods of gill spreading as part of the action pattern.
FSP	Fin Spreading display with body Perpendicular to the mirror. This may include periods of gill spreading as part of the action pattern.
A	"Arches" body. This may include periods of gill spreading as part of the action pattern.
Ch	Charges mirror- approaches rapidly but does not touch. This may start from FSP. A charging fish may have its fins and gills spread. A movement towards the mirror out of a display makes the behavior a charge.
Ct	Contacts mirror- same as above but with actual contact. Watch approach before designating as either Ch or Ct. Probably a rare behavior for most fish.
L	Leave- swims away from the mirror.

APPENDIX B

MARYVILLE COLLEGE
HUMAN AND ANIMAL PARTICIPANTS REVIEW COMMITTEE
ANIMAL STUDY APPLICATION FORM

1. Student Name: Ivy Parsons

2. Date: August 30, 2005

3. Senior Thesis Advisor: Dr. Crain

4. Pain or Distress Category: A (See listing of Pain or Distress Categories below)

For categories C,D, or E, USDA regulations require that the investigator consider alternative procedures. Please provide a narrative (for instance the end of Chapter 1) describing the methods and sources used to determine that alternatives are not available. If a computer assisted literature search was conducted, provide the names of the database(s) and date(s) of the search.

PAIN OR DISTRESS CATEGORIES

A. ACUTE STUDIES
Studies performed under anesthesia from which the animals are not permitted to regain consciousness, or performed on excised animal tissues collected under anesthesia or following euthanasia.

B. PAIN OR DISTRESS - NONE OR MINOR
Chronic studies that DO NOT involve survival surgery, induction of painful or stressful disease conditions, or pain or distress in excess of that associated with routine injections or blood collection. Included are induction or transplantation of tumors in animals (so long as the tumors do not cause pain and the animals are terminated prior to becoming seriously ill), administration of mildly toxic substances or drugs that cause no significant disease or distress, and antibody production as long as significant disease does not result and antigen booster doses do not include Complete Freund's Adjuvant (CFA).

C. PAINFUL PROCEDURES WITH ANESTHESIA/ANALGESIA
a. Survival surgical procedures.
b. Painful or potentially painful non-surgical procedures; e.g. bone marrow taps, injections into particularly sensitive areas such as foot pads, cardiac punctures, or traumatic procedures such as burns (burns may be category D, depending on severity).

D. MODERATE DISTRESS OR PAIN GENERALLY WITHOUT ANESTHESIA/ ANALGESIA/ TRANQUILIZERS
Induction of moderately distressful or painful disease conditions (examples: arthritis, administration of toxic chemicals, infectious challenges, immunosuppression resulting in infectious disease, peritonitis, severe inflammation, especially of weight bearing surfaces or resulting in external sores), whole body irradiation, stress models, septic shock, hypotensive shock, moderate painful stimuli (examples: low level electrical shock or heat), survival surgical procedures that have the potential to result in long term distressful illness (organ transplants, for example), induction of cardiac ischemia, booster immunizations with CFA, tumor induction or animal cultures that cause significant distress or pain, sight deprivation, restraint for periods longer than 12 hours.

E. INTENSE SUSTAINED OR REPEATED PAIN WITHOUT ANESTHESIA/ANALGESIA

Direct stimulation of CNS pain tracts, nociceptor stimulation by physical or chemical means that causes severe pain (e.g., corneal abrasions), or any category C (see above) procedure if performed without chemical relief of pain

5. Species to be used *Betta splendens*

6. Age of animals Mature Adult Males

7. Number of animals in study 20

8. Duration of study 3 weeks

9. Location of animals during the study (building and room) Sutton Science 114

10. List personnel to call if problems with animals develop:

Name	Daytime Phone	Nighttime Phone	Emergency No.
Ivy Parsons	591-8195	577-3909 or 591-8195	577-7576 or 335-7358
Dr. Crain			

Investigator Assurance

The information provided in this protocol form accurately reflects the intended use of animals for this research activity. Significant changes in procedures will not be undertaken without prior notification and approval of the Human and Animal Subjects Review Committee.

All persons involved in the use of animals on this protocol have been informed of the experimental objectives and methods. Each has received training in the execution of animal-related procedures he/she will perform prior to participation in the protocol, and will participate in any educational or training programs deemed appropriate or necessary by the Human and Animal Subjects Review Committee.

I agree to follow the provisions of the Animal Welfare Act and the guidelines of the National Institutes of Health on the care and use of laboratory animals.

I agree to use anesthesia, analgesia and tranquilization to relieve pain or distress whenever use of these agents will not jeopardize the scientific validity of the data. I have specifically consulted with the Human and Animal Subjects Review Committee regarding any experiments that are classified in pain/distress categories C, D, or E.

I will take appropriate steps to avoid exposure of persons working with these animals to any biohazardous agents used in the study.

State the reasons if you cannot attest to the accuracy of any of these statements:

11. HUSBANDRY REQUIREMENTS: Is anything other than routine care and equipment required?
YES___ No _X__ If "YES", please list below.

12. Is it likely that pain/discomfort will be experienced by animals in this protocol?
YES___ NO__X_____If "YES", describe:

13. What will happen to the animals at the end of the study? If euthanasia is required, state the methods.

Euthanasia: First the fish will be put in a solution of water and MS222. Once the fish are completely unconscious they will be decapitated.

14. Briefly describe your proposed research project (or attach a research proposal). Be sure to include a justification for the species and number.

I will be testing the effects of Fluoxetine (Prozac) on *Bette splendens*, Siamese fighting fish. I will have ten control fish and ten test fish. Twenty fish is a good sample number to get enough consistent data . I will expose the ten test fish to fluoxetine in Gladlock plastic containers where each fish is keep separate from one another. To observe the aggressive behaviors I will remove them from the plastic containers and place them in a tank of clean freshwater to observe their actions when a mirror is placed in front of the fish for five minutes. The control fish will be in plastic containers and will only be observed in the clean freshwater tank for five minutes to observe their behaviors when a mirror is placed in front of the fish. The behaviors between the control fish and the test fish will be compared. After euthanasia, the reproductive organs will be removed from all twenty fish and histology slides will be made to compare the effects of fluoxetine of the test fish to the fish not treated with fluoxetine.

This project has been reviewed by the Maryville College Human and Animal Use Committee.

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