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2890-FISH ON FLUOXETINE: BEFORE DURING AND AFTER BEHAVIORAL ANALYSIS

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Fish on Fluoxetine: Before, During, and After Behavioral Analysis Abstract

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Chemical concentrations of antidepressants have increased over the years steadily building in surface waters globally. These drugs have had various impacts on the animals tested, such as impacts on movement, motivation, and reproduction. In order to further investigate the effects of the antidepressant fluoxetine, male *Betta splendens* were exposed to the drug. These fish are naturally aggressive for reproduction purposes and have easily observable aggressive responses to other male fish. These characteristics make them a good model for studying Fluoxetine's impact on reproduction, motivation, and movement. Males in this study were primed with either a female or empty chamber prior to choice testing trials. Choice testing trials included ten daily trials, five with a mirror to study aggressive responding and another five that served as a timeout condition. Latency and aggressive responding were measured for each trial with video cameras used to record individual behavior. While no significant differences were found for the preference for the mirror vs timeout condition, the fluoxetine did have a significant impact on the fish latency and aggressive responding behavior. Results showed an increase in latency and decrease in aggression when the fish were exposed to the drug. These results show support for the hypothesis that Fluoxetine has an impact on motivation and movement in *Betta splendens*.

Worldwide Contamination

In recent years the scientific community has begun to focus on what happens when pharmaceutical substances are released into the world via wastewater pollutants (Vymazal et al., 2017). The release of these chemicals, or drugs, has earned them the title of pseudo-persistent pollutants due to the constant, continued release of these chemicals into the environment (Vymazal et al., 2017). While the concentration of these compounds may be low, the sheer amount of chemicals mixing together has become a cause for concern, particularly since it is not known if mixing them can cause a synergistic reaction (Vymazal et al., 2017). Furthermore, the half-life of these chemicals varies greatly and not all are biodegradable, meaning they require special circumstances to break down (Vymazal et al., 2017). This is highly concerning for chemicals that are not biodegradable and known to be hazardous, because they are being released directly into the environment in low concentrations and will have to degrade over time (Vymazal et al., 2017).

It is estimated that approximately 20 to 30 percent of the chemicals in sewage from human excretion are not filtered out and released into the world via wastewater (Logarinho et al., 2016). Most chemicals, like psychoactive drugs, are not targeted by the filtration systems currently in place in Water Waste Treatment Plants (WWTP), causing significant amounts of these chemicals to be released back into the waterways (Wu et al., 2015; Borova et al., 2014). Current methods for filtering out pharmaceuticals are not efficient, but the methods required for total chemical removal are expensive and not economical (Koçolu, Bakirdere, and Keyf, 2017). However, new methods are being developed that will help remove or breakdown chemicals at a more economical price, such as using specific nanoparticles to target difficult compounds or special salts that interrupt the ions of the drugs (Koçolu, Bakirdere, and Keyf, 2017; Basheer,

2018). However, until these new methods have been tested and approved chemicals will continue to be released unchecked all over the world (Borova et al., 2014).

Pharmaceuticals are entering waterways all over the world from the most populated cities to the Artic Peninsula creating a global problem that will continue to grow if not addressed (Borova et al., 2014; Mackul'Ak et al., 2016; González-Alonso et al., 2017). One point of entry for a large amount of these chemicals is through hospital WWTP as many drugs pass through the human system and into the sewage of these facilities (De Almeida et al., 2015). This could prove hazardous when considering the large amount of chemicals administered and utilized in hospitals worldwide. However, hospital WWTP's are not the only contributor to the larger problem worldwide. As more people start taking pharmaceuticals to help with illnesses, general population sewage will see an increase in the level of contaminants (Subedi et al., 2013). Areas with high populations are especially at risk for exposure to a high number of contaminants in surface waters (Wu et al., 2015). Wu et al. (2015) found low concentrations of carbamazepine, temazapam, diazepam, oxazepam, and alprazolam in tap water samples in China suggesting surface water contamination of drinking water. However, Subedi et al. (2013) found that water is not the only possible way of encountering the contaminants, as soil tests reveal high levels of soil contamination from water deposits.

While it is encouraging that many researchers have focused on the concentrations and causes behind the wastewater pollution in the environment, little is known about the overall effects these chemicals have on the organisms inhabiting the contaminated water (Subedi et al., 2013). Since water is necessary for life, all living things are affected by the release of chemicals into the waterways, but marine organisms are forced to live in constant exposure to the pollutants (Subedi et al., 2013). The continued release of the high variety of pharmaceuticals has caused

concern for the fate of the marine organisms who live in the polluted environment, particularly the smaller organisms who are necessary for healthy environmental function (Subedi et al., 2013). While research in this department is limited, there are still some particularly striking findings on the effect the polluted wastewater may be having on the marine world.

The amount of chemicals released into the water is increasing, and while methods for extraction are currently in development, if left unchecked, these chemicals will continue to build up in concentration and interact in unpredictable ways (Mackul'Ak et al., 2016). The most common chemicals detected in the water sampled were mood stabilizers, stimulants, and antidepressants (González-Alonso et al., 2017; Huerta-Fontela et al., 2008; Subedi and Kannan, 2015; Wu et al., 2015). Pharmaceuticals are prescribed to help a person recover or manage the symptoms of a disease; however, with the release of these various drugs into the waterways these chemicals are coming in contact with non-target organisms which could have unprecedented effects on the behaviors of these organisms (Subedi et al., 2013). These chemicals can bioconcentrate inside the organism, build up to higher concentrations in animal tissue than surrounding water, and eventually some even achieve bioaccumulation, where the concentration in the animal tissue is 5000 times more than in the surrounding water (Wang and Gardinali, 2013; De Solla et al., 2016). To check fish for chemical bioconcentration fish plasma, bile, and gills are obtained from fish in surrounding waters (Tanoue et al., 2015). Some chemicals have been shown to build up more rapidly, such as carbamazepine and ibuprofen, while others such as caffeine have not been shown to bioconcentrate at all (Wang and Gardinali, 2013; Oetken et al., 2005; Lahti et al., 2011; Ferrari et al., 2003; Garcia et al., 2012). This shows that the chemical concentrations are variable and that some drugs may be more likely to build up in animal tissue over time.

While the buildup of these drugs in the animal system is concerning, the toxic or behavioral effects on the wildlife could have consequences on the population and reproduction of these organisms (Oetken et al., 2005; Lahti et al., 2011; Ferrari et al., 2003; Garcia et al., 2012). Mood stabilizers have been shown to affect reproduction and boldness of fish, which is dangerous for animals that are heavily preyed upon (Oetken et al., 2005; Lahti et al., 2011; Ferrari et al., 2003; Garcia et al., 2012). Exposure to gestagens, birth control compounds, and personal care products also impacts fish reproduction and causes developmental problems in fish sexual characteristics (Fick et al., 2010; Miguel-Queralt and Hammond, 2008; Schebb et al., 2011; Chen et al., 2008). Snails and bivalves are also vulnerable, and exposure to wastewater has been reported to cause immunosuppression and inflammation of their tissues (Fong and Hoy, 2012; Gagné et al., 2006). In addition, antidepressant exposure negatively impacts the snail's ability to attach to soil, which impairs their movement and foraging abilities while leaving them open to predation (Fong and Hoy, 2012; Gagné et al., 2006). These are only a small amount of reported effects faced by aquatic wildlife, and without intervention the risk for more negative consequences remains.

Of concern is the increasing amount of antidepressant and antianxiety medications detected in surface waters, likely due to the rapidly increasing consumption by humans (Mackul'Ak et al., 2016). These drugs have increased in consumption by 400 percent over the last few decades, with the highest spikes in detection occurring in the winter months (Mackul'Ak et al., 2016). There are a variety of drugs used to treat anxiety and depression, and they are classified as beta blockers, SSRIs, anxiolytics, and benzodiazepines (Wick et al., 2009; Mazzitelli et al., 2017). They work primarily with the serotonergic system, and sometimes the dopaminergic system, affecting a variety of receptors in these areas of the brain (Fong and Hoy,

2012). These systems are linked to many key behaviors like reproduction, swimming, and eating in fish, bivalves, and annelids (Subedi et al., 2013). They have also been reported to have a variety of effects related to both drug type, exposed species and drug concentration differences at binding sites, such as γ-aminobutyric acid (GABA) receptors, which are widespread in aquatic life (Chiffre et al., 2016; Brodin et al., 2013). Since these drugs are resistant to photodegradation they are found in the environment and treated wastewater in concentrations between 0.01 to several mg liter^{-1}, creating a wide range of possible effects (Brodin et al., 2013).

The drugs used to treat anxiety and depression have shown a wide range of behavioral impacts tied to both the dose and species, with each drug having various effects even on the same species (Wick et al., 2009; Huggett et al., 2002; Brooks et al., 2005; Brooks et al., 2005; Fong, Huminski, and D'Urso, 1998; Fong and Hoy, 2012; Minguez et al., 2014; Chiffre et al., 2016; Mazzitelli et al., 2017; Brodin et al., 2013). Different drugs used to target the same system can have various effects even at the same dosage which highlights the concerning aspects associated with the drug cocktail mixtures that are released into the environment (Chiffre et al., 2016; Mazzitelli et al., 2017; Brodin et al., 2013). If not removed multiple body systems could be targeted simultaneously and affected by the cocktail mixture of chemicals (Chiffre et al., 2016; Mazzitelli et al., 2017; Brodin et al., 2013). The wide variety of results from drugs targeting just one body system, not even all the possible systems, shows the importance of drug contamination removal (Koçolu, Bakirdere, and Keyf, 2017; Basheer, 2018).

One of the most common antidepressants, fluoxetine, has been found in waterways with various concentrations all over the world (Brooks et al., 2005). This antidepressant has been widely prescribed for humans by physicians and animals by veterinarians (Karagiannis, Burman, and Mills, 2015). Like all anti-depressants this drug has become more common recently with

spikes in consumption (Mackul'Ak et al., 2016). This increase in consumption in the last few decades has made fluoxetine one of the more prevalent drugs in the wastewater effluent (Mackul'Ak et al., 2016). This is causing concern among researchers due to the high potential of fluoxetine to have a negative impact on critical animal behaviors (Clotfelter et al., 2007; Dzieweczynski and Herbert, 2012; Eisenreich and Szalda-Petree, 2015; Eisenreich, Greene, and Szalda-Petree, 2017; Snekser et al., 2006).

Fluoxetine

Fluoxetine is an SSRI prescribed to treat depression and acts on norepinephrine re-uptake receptors, and nicotinergic acetylcholine receptors (Brooks et al., 2003). These receptors are affected by the neurotransmitter serotonin (5-HT), and the drug works by interfering with the reuptake at the presynaptic neuronal membranes (Brooks et al., 2005). After passing through the human body, it is metabolized to norfluoxetine, which is also released into the environment (Brooks et al., 2005). Fluoxetine is resistant to catabolic hydrolysis and photolysis and has a long half-life of 100 or more days before breaking down completely (Mennigen et al., 2011). Of all the SSRIs, fluoxetine is one of the most widely used in research on aquatic animals (Clotfelter et al., 2007; Dzieweczynski and Herbert, 2012; Eisenreich and Szalda-Petree, 2015; Eisenreich, Greene, and Szalda-Petree, 2017; Snekser et al., 2006).

Smaller aquatic animals are particularly vulnerable to even small increases in drug concentrations as these smaller organisms can experience large effects on their behavior or even succumb to toxicity (Brooks et al., 2003). This follows the bottom up approach, suggesting that smaller organisms will be affected first and then the effects will spread up the food chain to larger organisms (Brooks et al., 2003). Basically, if the algae are experiencing toxic effects

causing a decrease in population, the organisms who eat the algae will be the first ones affected, and the problem will continue to spread from there (Brooks et al., 2003).

Brooks et al. (2003) examined sensitivity to fluoxetine across a variety of aquatic organisms. For this study they used, algae (*Pseudokirchneriella subcapitata*), Cladoceras (*Ceriodaphnia dubia* and *Daphnia magna*), fish (*Pimephales promelas* and *Oryias latipes*), an amphipod crustacean (*Hyalella azteca*), and a type of midge (*Chironomus tentans*) (Brooks et al., 2003). The crustacean and midge were used as a way to test sediment toxicity, and all other organisms were used for water toxicity (Brooks et al., 2003). At concentrations between 0.756 to 2.65 μM *Ceriodaphnia dubia*, *Daphnia magna*, and *Pimephales promelas* succumbed to toxicity, and concentrations of 15.2 mg/kg proved toxic for *Chironomus tentans* (Brooks et al., 2003). Fortunately for *Oryias latipes* and *Hyalella azteca,* no toxicity effects were found, though the exact reason they were unaffected is unknown (Brooks et al., 2003). It was noted that the effect on algae varied by age, with younger specimens of algae showing toxicity effects at lower concentrations than older specimens (Brooks et al., 2003). This shows the importance of considering both the species and age of the organism exposed to the drug, as each individual species is different and reacts differently to drug exposure (Brooks et al., 2003).

Brooks et al. (2003) shows the importance of considering the drug's effect on smaller aquatic species like algae, but other small aquatic species are also vulnerable. Other popular species used to study the effects of fluoxetine include clams, which are easy to obtain and maintain in a laboratory setting (Fong, Huminski, and D'Urso, 1998). While algae are an important prey species, clams fill a different role in the ecosystem, namely filtration (Fong, Huminski, and D'Urso, 1998). Clams and snails have an interesting niche in waterways as they filter the sediment, meaning they are exposed to chemicals through both the water and soil (Fong

and Hoy, 2012; Gagné et al., 2006). Antidepressants have shown a wide effect on clams and snails, impairing movement and reproduction thus putting these species at risk (Fong, Huminski, and D'Urso, 1998; Fong and Hoy, 2012).

While fluoxetine is toxic to algae, a different reaction to antidepressants has been recorded for clams (Clotfelter et al., 2007; Fong, Huminski, and D'Urso, 1998). Fong, Huminski, and D'Urso (1998) tested the SSRIs fluvoxamine, fluoxetine, and paroxetine on the spawning behavior of fingernail clams (*Sphaerium striatinum*). These drugs have been shown to trigger birthing in clams by blocking the re-uptake transmitters at serotonergic synapses, causing increased serotonin neurotransmissions (Fong, Huminski, and D'Urso, 1998). This has been commonly used by laboratories to boost fingernail clam populations for research, but Fong, Huminski, and D'Urso (1998) were interested in finding the minimum concentration to create this effect. They found that concentrations as low as 100 pM to 100 μM for fluvoxamine, and 10 μM for paroxetine induced birthing in the clams, but these concentrations of fluoxetine had no effect (Fong, Huminski, and D'Urso, 1998). These concentrations are much lower than those typically used and have the ability to trigger birthing in clams (Fong, Huminski, and D'Urso, 1998). While antidepressants have been shown to decrease reproduction in fish, the clams show the opposite effect, suggesting a wide range of effects from these drugs (Fong, Huminski, and D'Urso, 1998).

Antidepressants clearly have effects on algae, snails, and clams which are important small species in the ecosystem (Brooks et al., 2003; Fong, Huminski, and D'Urso, 1998; Fong and Hoy, 2012). These species fill important roles in the environment and if disrupted this could have large impacts on the ecosystem (Brooks et al., 2003; Fong, Huminski, and D'Urso, 1998; Fong and Hoy, 2012). However, all aquatic species inhabiting the same water space are exposed

to the same concentration of chemicals (Mackul'Ak et al., 2016). While it's true that this problem is following a bottom-up structure where smaller animals are likely to see the largest impacts, animals of all species are at risk of chemical exposure (Mackul'Ak et al., 2016). This makes work with fish both large and small important, especially since the fish will be getting exposure through the surrounding water and food consumption (Mackul'Ak et al., 2016; Mennigen et al., 2011).

Fish have also been shown to have more conservative binding sites in their hypothalamus and telencephalon than mammals, which could lead to different ways of up-taking and disposing of the drug (Mennigen et al., 2011). This makes fish an interesting model to consider for drug research, which has revealed a variety of effects of Fluoxetine such as decreased aggression, reproduction, and swimming behavior in some species, while simultaneously having the opposite effect on other species (Eisenreich, Greene, and Szalda-Petree, 2017; Fong, Huminski, and D'Urso, 1998; Mennigen et al., 2011). With the current amount of drug being dispensed into the water, this makes research with marine life important to monitor the environmental impact long-term (Mackul'Ak et al., 2016).

Brooks et al. (2005) took to the field and collected specimens from a nearby stream to examine the effects of fluoxetine and sertraline found in the environment. The researchers collected three species of fish: bluegill (*Lepomis macrochirus*), channel catfish (*Ictalurus punctatus*), and black crappie (*Pomoxis nigromaculatus*) from two separate streams in Texas, one used to release wastewater and the other serving as a control stream (Brooks et al., 2005). After dissection of the subjects to obtain brain, liver, and muscle tissues the researchers used gas chromatography to check the concentration levels in each type of tissue (Brooks et al., 2005). The highest concentrations were found in the brain tissues at 1.58 to 4.27 ng/g, and liver tissues

at 1.34 to 3.59 ng/g of the fish caught in the contaminated stream, with lower concentrations in the muscle tissues between 0.11 to 0.34 ng/g (Brooks et al., 2005). Overall, concentrations of Sertraline were found to be higher than the Fluoxetine concentrations (Brooks et al., 2005). However, the most disturbing finding is that the concentrations in the brain and liver tissues exceeded the concentrations found in the surrounding waters, suggesting a slow buildup of the drug over time, also known as bioconcentration (Brooks et al., 2005). Bioconcentration occurs when the concentration of the drug is higher in a species tissues than in the surrounding waters, and the amount of bioconcentration is measured by dividing the species concentration by the concentration of the surrounding area (Brooks et al., 2005). This method has been used in fish species to measure the buildup of drugs in the system over time, just like the results of Brooks et al. (2005). Therefore, the researchers concluded that the fish had a higher bioconcentration than the surrounding waters, and that the drug was accumulating in the fish systems faster than the animals could metabolize it out (Brooks et al., 2005). This could be problematic for species that live in water with concentrations close to their toxicity range, as buildup could push them past that level (Brooks et al., 2003).

Another influential study conducted by Nakamura et al. (2008) on marine life using the drug Fluoxetine focused on bioconcentration in relation to pH. The researchers reported the highest concentrations of Fluoxetine at the time of the study were 0.012 lg 1^{-1} in streams of the United States and were 0.099 lg 1^{-1} when wastewater was directly sampled (Nakamura et al., 2008). While the drug is easily transported by soil and water it is possible that absorption of the drug by dissolved organic matter would increase the speed of transport, and thus increase bioaccumulation in the surrounding marine life (Nakamura et al., 2008).

Nakamura et al. (2008) used Japanese medaka to test the effect changes in pH would have on Fluoxetine bioconcentrations in the fish. As noted earlier, pH affects the ionization of drugs, and most are positive above a pH of 2.5, meaning a change in pH affects the stability of most drugs (Borova et al., 2014). The impact on the ionization of the drug is important because for ionization to occur an active transport system needs to present inside the animal for the drug to move across the membrane (Borova et al., 2014; Nakamura et al., 2008). If the stability of the drug is impacted, then the animal's system may not be able to pass it through the membrane (Borova et al., 2014; Nakamura et al., 2008).

To determine how bioconcentration levels were affected the fish were exposed to 300 lg 1^{-1} of fluoxetine and placed in one of three pH conditions: 7.2, 8.1, and 8.9 (Nakamura et al., 2008). After the exposure period the fish were dissected and bioconcentrations were measured, with results showing higher bioconcentrations at the higher pH levels (Nakamura et al., 2008). The researchers concluded that the level of pH in the water affected the ionization of the drug, and thus affected the bioconcentration in the fish (Nakamura et al., 2008). This result is similar to other results found by Mennigen et al. (2011), showing long half-lives of fluoxetine even at varying pH levels. This shows that fluoxetine is highly sensitive to changes in pH, and this knowledge could prove useful in finding more ways to break it down or reduce the bioavailability before it enters the environment (Nakamura et al., 2008; Mennigen et al., 2011).

Clearly fluoxetine's long half-life poses a problem for smaller species when considering bioconcentration (Mennigen et al., 2011; Brooks et al., 2005). As the drug concentration increases in the surface water, the potential increases for bioconcentration turning into bioaccumulation, where the drug buildup in the animal's tissue may exceed 5000 times the surrounding water concentration (Nakamura et al., 2008). With the drug building up in the

animal's system it is possible to reach toxicity levels and result in death for certain species (Brooks et al., 2005). However, as the drug accumulates in the animal's system it has been shown to cause behavioral changes in these species, which could also contribute to a loss of life (Margiotta-Casaluci et al., 2014).

Fluoxetine has been shown to have behavioral effects in fathead minnows (Clotfelter et al., 2007; Fong, Huminski, and D'Urso, 1998; Margiotta-Casaluci et al., 2014). Margiotta-Casaluci et al. (2014) studied the effect of fluoxetine on male fathead minnow behavior at six concentrations: 0.1, 1.0, 8.0, 16, 32, and 64 mg/L. They found that fish exposed to higher concentrations of the drug displayed bolder behavior, such as more exploration at the top of the tank than the controls (Margiotta-Casaluci et al., 2014). Furthermore, once the experiment was over the fish were dissected and norfluoxetine, the major metabolite of fluoxetine, was found in the fish plasma, meaning the fish were able to metabolize fluoxetine (Margiotta-Casaluci et al., 2014). This research shows that fish are able to process the drug and that they display bolder behavior, which could be problematic for predation in the wild (Margiotta-Casaluci et al., 2014). In addition to showing bolder behavior other research has revealed movement effects (Eisenreich, Greene, and Szalda-Petree, 2017). If the prey animals are showing bolder behavior and decreased movement, they could be easily picked off by predators, which could have a major impact on the prey's population (Margiotta-Casaluci et al., 2014; Eisenreich, Greene, and Szalda-Petree, 2017).

The research on the SSRI fluoxetine has shown many different behavioral effects, but these effects are species dependent with some species showing increases in certain behaviors and decreases in others (Fong, Huminski, and D'Urso, 1998). The potential range of effects is concerning when considering the spike in prescriptions used to treat anxiety and depression,

which will only add more pollutants to the environment (Mackul'Ak et al., 2016). The research on the effect of fluoxetine is likely to increase, providing more information on behavioral effects and the potential environmental consequences.

Fluoxetine and *Betta splendens*

Since fluoxetine has been shown to impact aggression, movement, and reproduction in fish, the Betta fish or Siamese fighting fish (*Betta splendens*) is a useful model to use in studying the effects of fluoxetine on behavior (Abate, 2005; Bronstein, 1989; Hollis et al., 1984; Hogan, 1967; Eisenreich, Greene, and Szalda-Petree, 2017; Goldstein, 1975). Male bettas are highly aggressive and are responsible for building the nest, attracting the female, obtaining the territory, and raising the young so their aggressive ability is necessary for reproduction (Hogan, 1967; Hollis et al., 1984; Dzieweczynski et al., 2005). This makes them and ideal candidate to study the effect of fluoxetine on movement and aggression.

Early work with *Betta splendens* focused on defining the aggressive response of the fish with a focus on the relationship with associative conditioning processes (Hogan, 1967; Hollis et al., 1984). This research identified specific behaviors associated with courting and fighting among male fish (Hogan, 1967; Goldstein, 1975; Hollis et al., 1984; Bronstein, 1989). The male fish are territorial and will attack any male who enters their territory (Hogan, 1967; Goldstein, 1975; Hollis et al., 1984; Bronstein, 1989). This territorial response stems from the reproductive responsibility of the male fish (Hogan, 1967; Goldstein, 1975; Hollis et al., 1984; Bronstein, 1989). Male fish are responsible for obtaining a territory, building a nest, attracting a female, and raising the young alone (Hogan, 1967; Goldstein, 1975; Hollis et al., 1984; Bronstein, 1989). This places selective pressure on the males of the species to have a strong aggressive reaction to other male fish, and occasionally females if the male is occupied raising fry (Hogan, 1967;

Goldstein, 1975; Hollis et al., 1984; Bronstein, 1989). In addition to live conspecifics, the male fish will also respond to a mirror suggesting that the fish do not demonstrate self-recognition (Hogan, 1967). This fighting response is so strong learning research with males yielded results revealing that these encounters could be used to reinforce a male fish (Hogan, 1967; Goldstein, 1975; Hollis et al., 1984; Bronstein, 1989). Therefore, the fighting behavior is important to the reproduction of this species (Hogan, 1967; Goldstein, 1975; Hollis et al., 1984; Bronstein, 1989).

The behaviors the fish used for fighting and courting were analyzed, and four primary behaviors were identified: biting, lateral displays, gill flaring, and fin spreading (Hogan, 1967; Goldstein, 1975; Hollis et al., 1984; Bronstein, 1989). These four behaviors make up the primary signaling behavior exuded by males in response to other males or females (Hogan, 1967; Goldstein, 1975; Hollis et al., 1984; Bronstein, 1989).When fighting other males often they start with the low risk behaviors such as lateral displays, gill flaring, and fin spreading to try and intimidate the opponent and scare them off (Matos et al., 2003; Goldstein, 1975). If these behaviors do not scare off the rival then they will move toward higher risk behaviors like biting that could lead to injury or death (Matos et al., 2003; Goldstein, 1975). The length of time it takes for the fish to engage in the first bite in an encounter is largely dependent on the amount of area where the fight takes place (Abate, 2005; Goldstein, 1975). Research on latency to first bite in aggressive encounters with this species found that the time was reduced in smaller tanks when compared to larger tanks (Abate, 2005; Goldstein, 1975). The contextual cues and available space are important in an aggressive encounter between males (Hogan, 1967; Goldstein, 1975; Hollis et al., 1984; Bronstein, 1989).

The earlier work also examined the effect the presence of a female had on aggressive encounters between males (Goldstein, 1975; Bronstein, 1989). These studies found male bettas

tend to scale back aggressive behaviors when a female is present, likely due to the male's larger size and ability to kill the female if the male is too aggressive (Goldstein, 1975; Bronstein, 1989). This allows the female fish to be used as a prime to provide motivation for the male to aggress against the mirror even though the priming effect is short for this species and only lasts about 5 minutes (Bronstein, 1989; Matos et al., 2003). When primed with a female male fish have been found to use more intimidation behaviors like lateral displays, gill flaring, and fin spreading (Bronstein, 1989; Matos et al., 2003). However, when males are primed with another male the latency to the first bite in a subsequent encounter decreases (Bronstein, 1989; Matos et al., 2003). This shows the importance of the sex of the fish used for priming on risk taking when motivating the male to engage in an aggressive encounter with another male.

Due to the large amount of literature detailing their behavior *Betta splendens* have become one of the common models used to study the impacts of drugs like fluoxetine on behavior (Hogan, 1967; Goldstein, 1975; Hollis et al., 1984; Bronstein, 1989; Dzieweczynski and Hebert, 2012; Forsatkar et al., 2014; Eisenreich and Szalda-Petree, 2015; Eisenreich, Greene, and Szalda-Petree, 2017). The large amount of background information detailing the behaviors of betta males is helpful when comparing males exposed to different chemicals.

Research with Betta splendens continues to focus on behavioral learning and drug impacts on learning (Hogan, 1967; Goldstein, 1975; Hollis et al., 1984; Bronstein, 1989; Dzieweczynski and Hebert, 2012; Forsatkar et al., 2014; Eisenreich and Szalda-Petree, 2015; Eisenreich, Greene, and Szalda-Petree, 2017). In addition to learning, research examining behavioral consistency, or predictability, and movement has increased due to the nature of the drug effects on this species. Many studies have revealed negative impacts of fluoxetine on the latency to engage in aggressive responding of *Betta splendens*. In contrast, other studies have

shown positive effects in terms of increases in behavioral consistency. Therefore, exactly what change is occurring and how the drug is affecting the motivation to aggress is not largely explored.

To investigate how the drug affects *Betta splendens* Clotfelter et al, (2007) analyzed the brains of the fish to determine the effect antidepressants have on the amount of serotonin found in fish forebrains. Clotfelter et al. (2007) used mirrors to test the effects of 4.3 nmol fluoxetine on the aggressive display behavior of the fish before dissecting the fish and analyzing their brains for the concentration of the drug. Unlike other studies that have administered the drug to the fish via water absorption, Clotfelter et al. (2007) administered the drug intramuscularly in the fish. While no significant behavioral changes were detected from exposure to the drug, the fish in the group injected with the drug had lower levels of serotonin present in their forebrains when compared to the control subjects (Clotfelter et al., 2007). This suggests that the fish brains are impacted by the drug, and while no behavioral effects were found at this concentration, other studies using different dosing methods have found such effects (Clotfelter et al., 2007; Eisenreich, Greene, and Szalda-Petree, 2017; Eisenreich and Szalda-Petree, 2015).

Past research in our laboratory has explored the effects of the drug on both latency behavior and aggression (Eisenreich, Greene, and Szalda-Petree, 2017; Eisenreich and Szalda-Petree, 2015). In both studies the male fish were exposed to the fluoxetine several hours before being tested in an alleyway maze. The male fish were studied using a Go/No Go task where the latency was measured for the fish to swim from the start box to the goal box containing either a mirror or no mirror. Once the fish reached the goal box for the mirror the aggressive behavior was recorded as either yes, the fish responded aggressively, or no, the fish did not respond

aggressively. Both studies found increased latencies and decreased aggressive responding against a mirror.

While both studies found significant behavioral effects when the fish were exposed to fluoxetine, the concentration used was higher than that used in other studies (Dzieweczynski et al., 2005; Snekser et al., 2006). This higher concentration makes parceling out the behavioral effects of reduced motivation from motor suppression difficult. Therefore, changes were made in an effort to manipulate the males and increase their motivation to aggress.

Such experimental preparations lack a key contextual motivation for aggressive responding – a female betta (Dzieweczynski et al., 2005). Males are aggressive to attract the female and without one present the motivation to act aggressively toward potential rivals could be impacted negatively (Goldstein, 1975). Having a female present would help simulate a more natural context, and potentially increase the motivation of the males to chase off a rival and build a nest (Dzieweczynski and Hebert, 2012;). This increased motivation may result in more intense fighting behavior or increase the length of time the male responds to the mirror. Furthermore, little research has been conducted on the effect of fluoxetine on the interaction between a male and female fish. Past research has found a negative impact of fluoxetine on aggression, so it is likely that the interaction with female fish would also be affected since courting and fighting behaviors are similar. Therefore, determining the effects of both female conspecifics and fluoxetine is important when studying the motivation of male *Betta splendens* aggressive behaviors.

Betta splendens are a useful behavioral model to study the effects of fluoxetine on aggression and movement. While other studies have been instrumental in exploring the impact of the drug on the fish behavior there are still several questions left unanswered. The impact of the

female on male motivation when exposed to the drug is still largely unknown. Could the females increase the motivation of the males to fight and override the effects reported by the drug? Will the drug also impact the male's motivation to court with the female? How long does it take for the subject's behavior to return to baseline, if it is even possible to return to baseline at all? These are some of the unknowns this project hopes to address.

Laboratory Tests for Bioaccumulation

Previous experiments conducted in our laboratory have examined the effects of fluoxetine on male betta aggression measured using the latency to aggress against a male conspecific or mirror. The most recent experiments have introduced a female prime to increase the motivation of the male fish to respond aggressively toward the mirror. While the most recent study produced interesting results, some changes have been included in the present experiment to create a more natural context to study the potential drug effects.

The first experiment with the female prime used a between-subjects design where subjects were assigned to one of four groups: No Female Prime/No Fluoxetine, Female Prime/No Fluoxetine, No Female Prime/Fluoxetine, and Female Prime/Fluoxetine. This experiment used a Go/No Go task with a focus on the drug effects on latency and aggressive responding. The fish were primed for 30 seconds before each trial with either a female or empty container.

The results of the experiment showed an increase in aggressive responding in the males primed with a female when compared with the males that were not primed. In addition, the males in the groups exposed to the drug had decreased aggressive responding when compared with the males not exposed to the drug. No significant results were found for the latency behavior or mirror preference ratio.

The present experiment was designed to increase statistical power and create a more natural motivational context to study the aggressive behavior of *Betta splendens*. The first major change is in the experimental design itself. A within-subjects repeated-measures ABA design was used in place of the previously used between-subjects design. The ABA design, also called a reversal design, is typically more statistically powerful than the between-subjects design because subjects serve as their own control. In the ABA design the return to baseline allows for casual conclusions to be drawn about the effects of fluoxetine on aggressive responding.

The second major change was a modification of the female prime procedure. The male betta was allowed to view a female betta each day before the start of daily trials for 3 minutes rather than 30 seconds before each trial. Compared to the previous experiments, this allows more time for the exposure to the female (female prime) while potentially decreasing potential distraction during the daily trials.

The last major change was to include detailed behavioral observations of typical aggressive behaviors in the mirror encounters. Previous experiments from our laboratory have focused on the negative impacts of fluoxetine on movement and aggressive responding. However, these experiments lack a detailed analysis of fighting behavior that videos provide. The proposed experiment used digital recording methods to allow for more detailed analysis of individual fighting and to reduce potential experimenter bias. The use of videos allowed the results to analyze the potential negative effects on different aspects of the fighting behavior and discern if some behaviors experienced a higher impact.

The present experiment examined the effects of fluoxetine and the presence of a female betta (female prime) on male betta aggressive responding and the latency to aggress using a Go/No-Go task. It is hypothesized that:

Latency Effects

1. All fish will have a higher preference ratio for the mirror compared to the non-mirror presentation regardless of group or condition.
2. The female primed group will have lower latencies to enter the goal box with the mirror compared to the non-primed group.
3. Subjects will have longer latencies to enter the goal box with the mirror under the fluoxetine condition compared to the non-fluoxetine conditions.
4. There will be a Prime X Condition interaction such that the female prime group will show a smaller increase in mirror latency than the non-primed group when exposed to fluoxetine.

Behavioral Effects

1. The female primed group will exhibit more fighting behavior toward the mirror compared to the non-primed group.
2. Subjects will exhibit less fighting behavior toward the mirror under the fluoxetine condition compared to the non-fluoxetine conditions.
3. There will be an increase in the total amount of bites, and time lateral displaying, gill flaring, and fin spreading between the female primed and non-primed group.
4. There will be a decrease in the total amount of bites, and time lateral displaying, gill flaring, and fin spreading between the fluoxetine condition and non-fluoxetine conditions.

Methods

Participants

Twenty-six male and nine female *Betta splendens* were obtained from Live Aquaria and were approximately five cm long with red and blue coloration. All fish were housed individually and maintained on a 12 hr dark/light cycle with tank water temperatures maintained at 76-82 ^{o}F.

Material/Apparatus

Individual tanks were approximately 67 cm x 40 cm x 18 cm (L x W x H), containing approximately 20 liters of water treated with water conditioner to remove chlorine. Each tank also contained brown gravel, a T-maze, a heater, a thermometer, and a bubbler hooked up to an airstone. The alleyway maze, in which the fish lived, measured approximately 53 cm x 20 cm x 10 cm (L x W x H). The fish were maintained on a diet of Tetra min betta pellets and received about eight pellets a day.

A stock solution of 0.5 mmol was created using the SSRI fluoxetine, obtained from TCI America or Sigma Aldrich. The fish were exposed to the stock solution by mixing 4 ml of the stock solution with 196 ml of the tank water from each individual fish tank in a separate dosing chamber, resulting in a 10 μmol concentration of fluoxetine.

Procedure

Male subjects were screened for aggression and matched on latency to aggress toward a mirror. Matched sets of fish were randomly assigned to two groups: Female Prime (FP) and No-Female Prime (NFP). FP subjects were exposed to a female prior to the start of trials each day, while NFP subjects were exposed to an empty chamber before the start of trials each day. The experiment featured three conditions: baseline, fluoxetine exposure, and return to baseline. Each

condition continued until the fish showed consistent responding over five consecutive days but did not exceed 25 days.

The daily testing consisted of 10 trials, with five trials involving the mirror condition and the other five involving the timeout condition. During the drug condition fish were exposed to fluoxetine three hours before the beginning of each daily testing session with fish in the non-drug condition using the same procedure without the drug. The fluoxetine exposure consisted of placing the fish in a dosing container with 196 ml of their own tank water and 4 ml of the fluoxetine solution for thirty minutes, resulting in a 10 μmol concentration of fluoxetine. In the FP group a female conspecific was placed into a clear holding chamber directly adjacent to the start box before the start of daily trials for three minutes (see Figure 1). In the NFP group fish were exposed to an empty holding chamber for three minutes before trials started. Once the three-minute exposure elapsed the view of the holding chamber was blocked with a white wall and trials began. A unique discriminative stimulus pattern was associated with each reward condition (mirror or timeout). The mirror or timeout conditions were counterbalanced across days such that the mirror condition is presented first every other day.

The format for the trials consisted of placing the fish into the start box and then raising the dividing door between the start box and the holding box for three minutes (see Figure 1). The courting behavior exhibited by the fish was recorded as either courting occurred or courting did not occur. After three minutes the dividing door between the start box and the holding box was lowered and the door between the start box and alleyway was raised. The latency for the fish to swim into the goal box of the alleyway was recorded. The fighting behavior of the fish was recorded as either aggressive responding was present or aggressive responding was not present.

After the 30 seconds of exposure to the mirror had elapsed, the fish were moved back to and locked in the start box for 30 seconds, after which the next latency trial began.

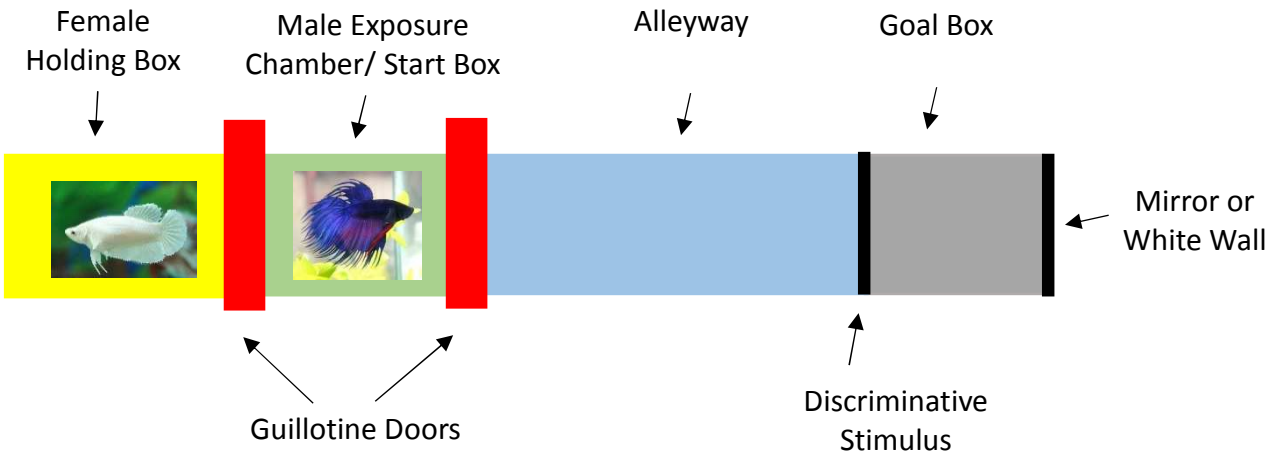

Figure 1. Diagram of the testing apparatus depicting the alleyway maze.

Behavioral observation

During the last five days of data collection in each condition the fish were randomly assigned a day and their mirror trials were recorded with a camera. Video recordings were coded using four separate behaviors: fin spreading, lateral displaying, gill flaring, and biting. Fin spreading was defined as the fish spreading their fins outward from the body. Lateral Displaying was defined as the fish facing parallel to the mirror with their nose facing the wall and their body presented to the mirror in either the left or right direction. Gill flaring was defined as spreading the gills out from the body along the face. Finally, biting was defined as mouth contact with the mirror with each time the mouth contacts the mirror as one bite. Coders were trained to code these behaviors until 80 percent interrater reliability was reached.

Analysis

Tests of normality conducted on the data revealed several violations of normality and skewed distributions. Histograms are presented below in Figure 2 for the three parametric tests

that had the highest reports of abnormality. Nonparametric tests (Friedman's ANOVA and Mann-Whitney U) were conducted in parallel to parametric tests. The hypothesis decisions from the nonparametric tests did not differ from the parametric tests. As such, parametric tests are reported for the experiment.

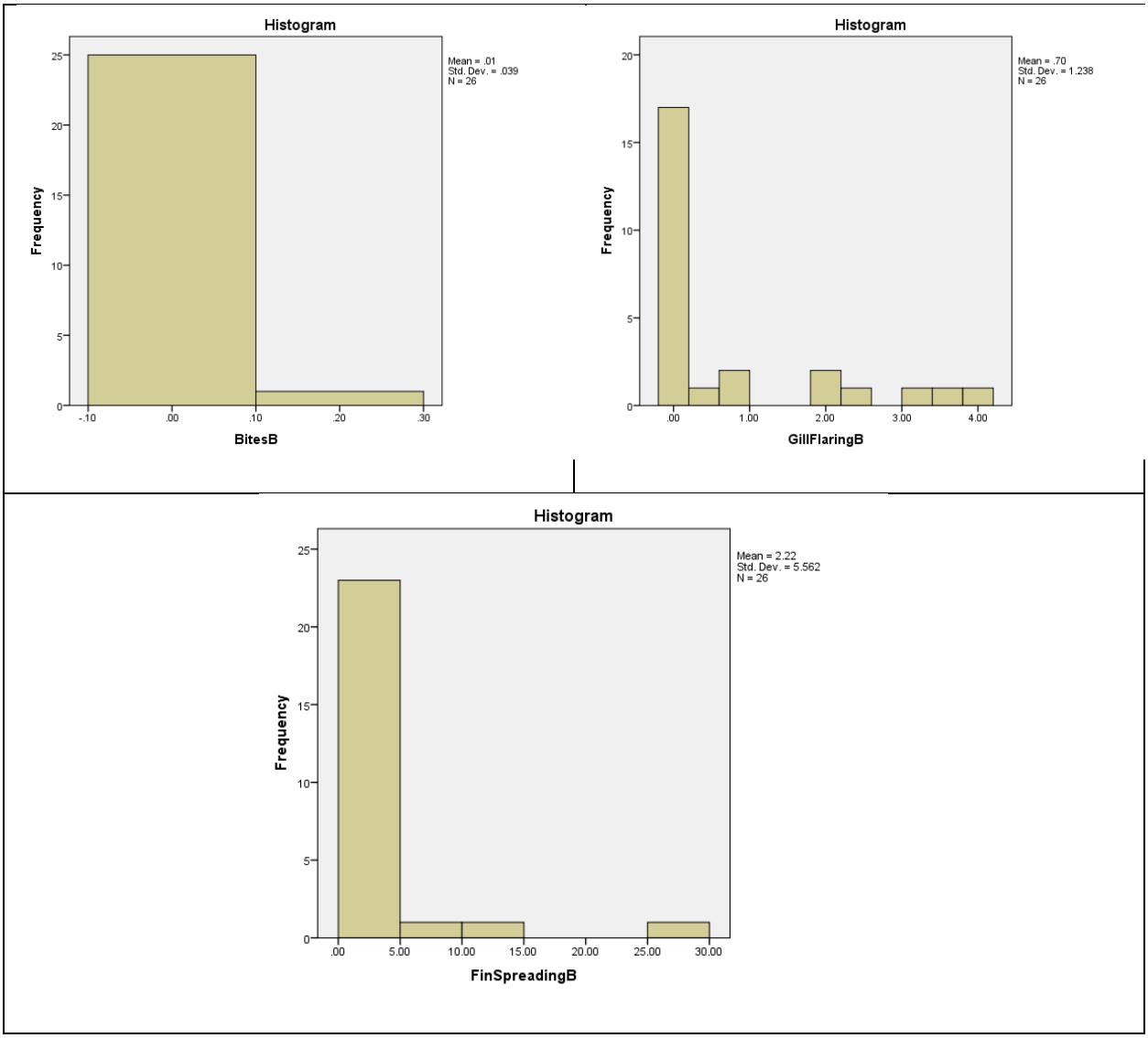

Figure 2. This histogram represents the abnormal biting, gill flaring, and fin spreading data distribution for the drug (B) phase of the experiment. Tests of normality for Kolmogorov-

Smirnova conducted on the biting behavior revealed a significant violation of normality of data ((26)= 0.539, p> 0.001), the gill flaring behavior revealed a significant violation of normality of data ((26)= 0.368, p> 0.001), and the fin spreading behavior revealed a significant violation of normality of data ((26)= 0.348, p> 0.001).

Data was analyzed using a two (group) X three (condition) Mixed ANOVA. The Greenhouse-Geisser correction was used for all parametric tests reported. The data for analysis included latency to enter the goal box for both mirror and non-mirror trials, preference ratio for the mirror/non-mirror condition, the presence of fighting behavior (Yes or No), and the coding for the four behaviors. Group means were calculated for each condition and averaged across the last five testing days for analysis. To calculate the average for the fight behavior in the latency trial the fish were scored each trial as 0 for fighting behavior not present and 1 for fighting behavior present. The biting, lateral displays, gill flaring, and fin spreading were scored for each individual trial by recording the number of bites and the time spent lateral displaying, gill flaring, and fin spreading. The preference ratio was calculated by dividing the non-mirror latency by the sum of the non-mirror and mirror latencies for that day ($S^{-}/(S^{-}+S^{+})$) which yields a ratio bound between 0 and 1 with a higher ratio indicating a higher preference for the mirror.

Results

Latency Effects

A one-sample *t*-test, using a Bonferroni correction for alpha of 0.0166 (.05/3), conducted on the mirror preference ratio (Latency hypothesis 1) for each condition revealed a non-significant effect for the baseline condition (*t*(25)= 2.042, p= 0.052, d= 0.401), a non-significant effect for the fluoxetine condition (*t*(25)= -2.515, p= 0.019, d= 0.494), and a non-significant effect for return to baseline condition (*t*(25)= 1.236 p= 0.228, d= 0.242). The mirror preference

ratio is calculated by dividing the non-mirror latency by the sum of the non-mirror and mirror latencies for that day (S-/(S-+S+)) which yields a ratio bound between 0 and 1 with a higher ratio indicating a higher preference for the mirror. See Table 1 for descriptive statistics.

Table 1. One-sample t-test descriptive statistics for the mirror preference ratio averaged across the last 5 days of testing.

	Baseline	Drug	Return to Baseline
Mean	0.56	0.48	0.53
SD	0.14	0.04	0.13
N	26	26	26

A two (groups) X three (conditions) Mixed ANOVA conducted on the latency to enter the goal box for the mirror condition (Latency hypotheses 2, 3, and 4) revealed a non-significant main effect for group ($F(1,24)= 0.048$, $p= 0.828$, $\eta_p^2= 0.002$), a significant main effect for condition ($F(1.95, 46.801)= 47.567$, $p< 0.001$, $\eta_p^2= 0.665$), and a non-significant Group X Condition interaction ($F(1.95, 46.801)= 0.994$, $p=0.376$, $\eta_p^2= 0.04$). See Table 2 and Figure 3 for descriptive statistics.

Pairwise comparisons conducted on the three conditions (Latency hypothesis 3) revealed a significant difference between the baseline and drug condition ($t(25)= -9.452$, $p< 0.001$, $d= 1.85$), a significant difference between the drug condition and return to baseline condition ($t(25)= 7.799$, $p< 0.001$, $d= 1.53$), and no significant difference between the baseline and return to baseline condition ($t(25)= -1.174$, $p= 0.251$, $d= 0.23$). The mirror raw latency was significantly longer in the drug condition compared to both baseline conditions.

Table 2. Group X Drug Condition descriptive statistics for the mirror raw latency (secs) data averaged across the last 5 days of testing.

		Baseline	Drug	Return to Baseline
No Female Prime	Mean	51.33	134.05	63.00
	SD	35.00	17.62	48.05
	N	13	13	13
Female Prime	Mean	62.31	123.90	70.42
	SD	48.24	38.59	41.94
	N	13	13	13

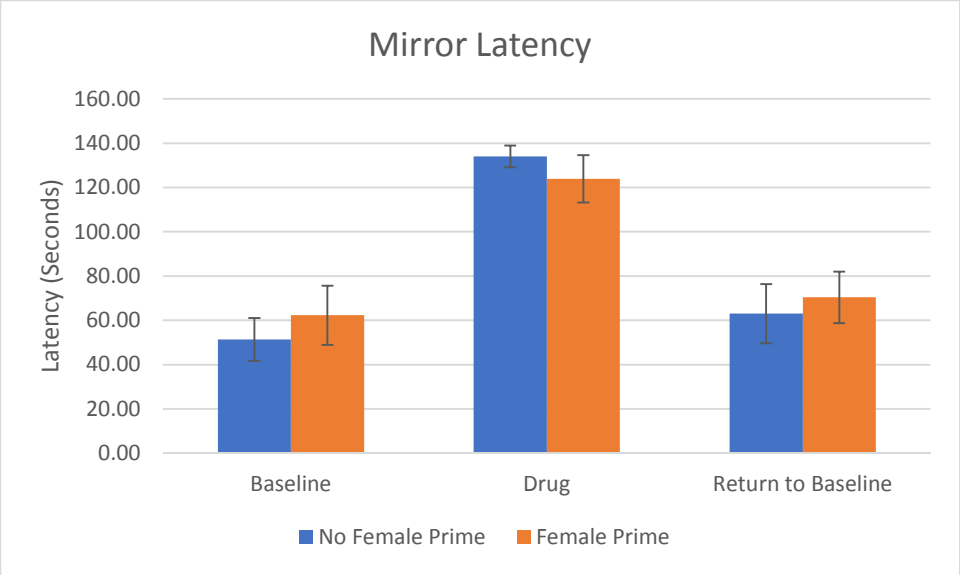

Figure 3. Group X Drug Condition for the mirror raw latency (secs) averaged across the last 5 days of testing.

Behavioral Effects

A two (groups) X three (conditions) Mixed ANOVA conducted on the fight behavior (Behavioral hypotheses 1 and 2) revealed a non-significant main effect for group ($F(1,24)= 0.066$, $p= 0.799$, $\eta_p^2= 0.003$), a significant main effect for condition ($F(1.912, 45.899)= 14.88$, $p< 0.001$, $\eta_p^2= 0.383$), and a non-significant Group X Condition interaction ($F(1.912, 45.899)= 0.436$, $p= 0.64$, $\eta_p^2= 0.018$). See Table 3 and Figure 4 for descriptive statistics.

Pairwise comparisons conducted on the three conditions for the fighting behavior (Behavioral hypothesis 2) revealed a significant difference between the baseline and drug condition ($t(25)= 5.424$, $p< 0.001$, $d= 1.06$), a significant difference between the drug condition and return to baseline condition ($t(25)= -3.754$, $p= 0.001$, $d= 0.74$), and no significant difference between the baseline and return to baseline condition ($t(25)= 1.218$, $p= 0.235$, $d= 0.24$). The fighting behavior was significantly lower in the drug condition compared to both baseline conditions.

Table 3. Group X Drug Condition descriptive statistics for the fight behavior averaged across the last 5 days of testing.

		Baseline	Drug	Return to Baseline
No Female Prime	Mean	0.55	0.12	0.43
	SD	0.36	0.21	0.44
	N	13	13	13
Female Prime	Mean	0.51	0.22	0.47
	SD	0.38	0.32	0.39
	N	13	13	13

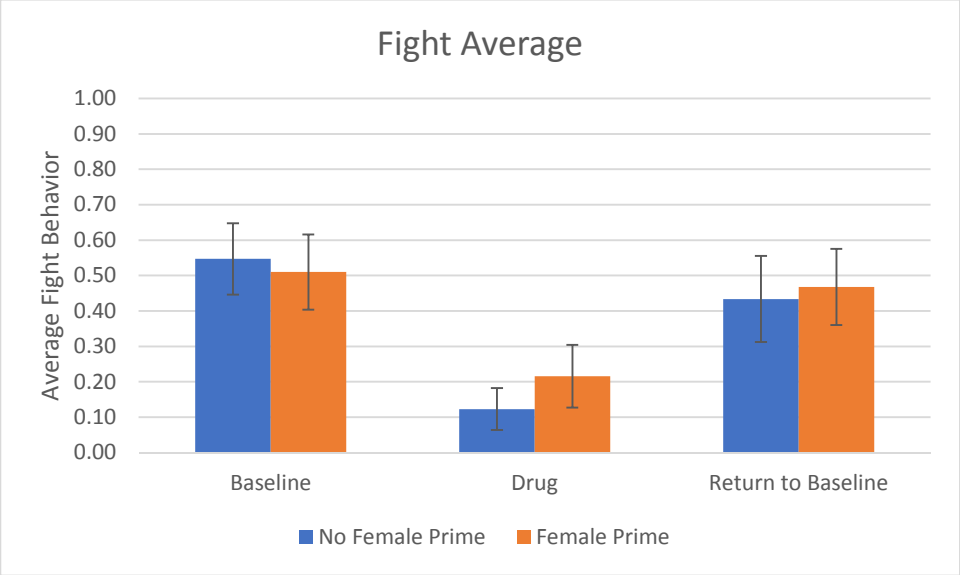

Figure 4. Group X Drug Condition for the fight behavior averaged across the last 5 days of testing.

A two (groups) X three (conditions) Mixed ANOVA conducted on the biting behavior (Behavioral hypotheses 3 and 4) revealed a non-significant main effect for group ($F(1,22)= 0.619$, $p= 0.44$, $\eta_p^2= 0.027$), a non-significant main effect for condition ($F(1.678, 36.908)= 0.792$, $p= 0.44$, $\eta_p^2= 0.035$), and a non-significant Group X Condition interaction ($F(1.678, 36.908)= 0.309$, $p= 0.697$, $\eta_p^2= 0.014$). Two outliers were removed from the final analysis. See Table 4 and Figure 5 for descriptive statistics.

Table 4. Group X Drug Condition descriptive statistics for the biting behavior averaged across the last 5 days of testing.

		Baseline	Drug	Return to Baseline
No Female Prime	Mean	0.02	0.00	0.02
	SD	0.06	0.00	0.06
	N	11	11	11
Female Prime	Mean	0.02	0.02	0.05
	SD	0.06	0.06	0.12
	N	13	13	13

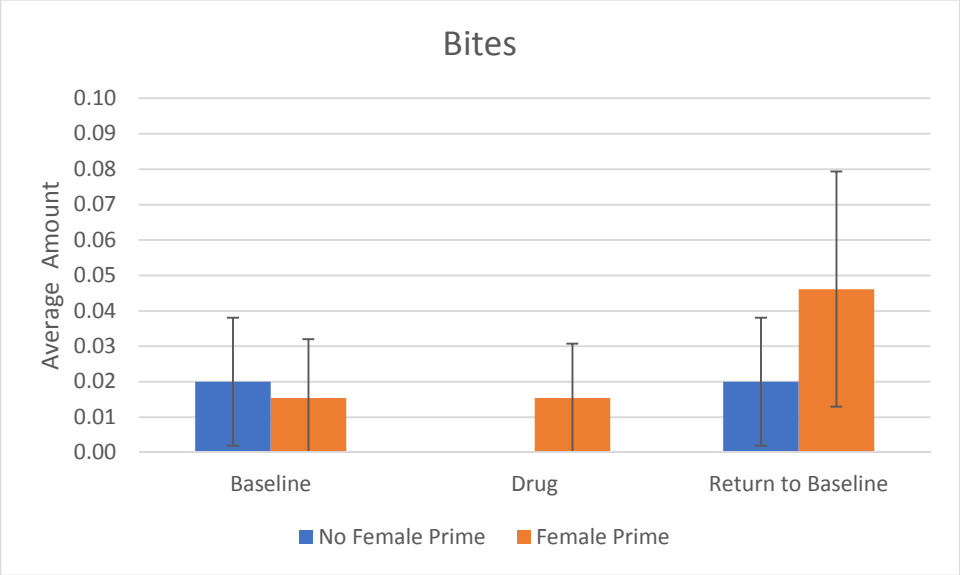

Figure 5. Group X Drug Condition for the biting behavior averaged across the last 5 days of testing.

A two (groups) X three (conditions) Mixed ANOVA conducted on the lateral display behavior (Behavioral hypotheses 3 and 4) revealed a non-significant main effect for group ($F(1,24)= 0.165$, $p= 0.688$, $\eta_p^2= 0.007$), a significant main effect for condition ($F(1.884, 45.213)= 3.874$, $p= 0.03$, $\eta_p^2= 0.139$), and a non-significant Group X Condition interaction ($F(1.884, 45.213)= 0.427$, $p= 0.643$, $\eta_p^2= 0.017$). See Table 5 and Figure 6 for descriptive statistics.

Pairwise comparisons conducted on the three conditions for the lateral display behavior (Behavioral hypothesis 4) revealed a significant difference between the baseline and drug condition ($t(25)= 2.107$, $p= 0.045$, $d= 0.41$), a significant difference between the drug condition and return to baseline condition ($t(25)= -2.452$, $p= 0.022$, $d= 0.48$), and no significant difference between the baseline and return to baseline condition ($t(25)= -0.388$, $p= 0.701$, $d= 0.08$). The lateral display behavior was significantly lower in the drug condition compared to both baseline conditions.

Table 5. Group X Drug Condition descriptive statistics for the lateral display behavior (secs) averaged across the last 5 days of testing.

		Baseline	Drug	Return to Baseline
No Female Prime	Mean	6.35	2.18	7.06
	SD	9.04	2.96	9.59
	N	13	13	13
Female Prime	Mean	6.78	4.62	7.02
	SD	7.94	4.44	6.95
	N	13	13	13

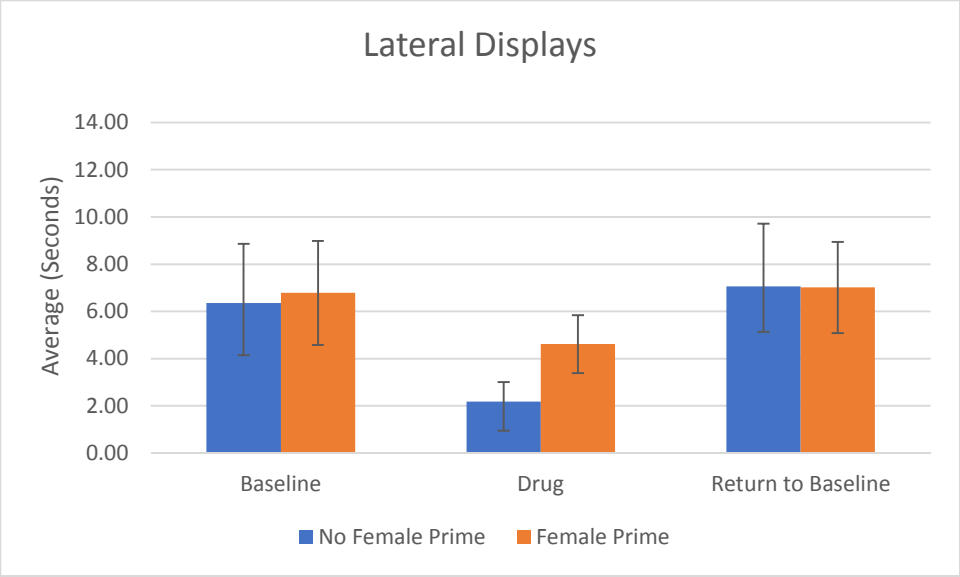

Figure 6. Group X Drug Condition for the lateral display behavior (secs) averaged across the last 5 days of testing.

A two (groups) X three (conditions) Mixed ANOVA conducted on the gill flaring behavior (Behavioral hypotheses 3 and 4) revealed a non-significant main effect for group ($F(1,24)= 0.002$, $p= 0.966$, $\eta_p^2< 0.001$), a significant main effect for condition ($F(1.565, 37.554)= 7.232$, $p= 0.004$, $\eta_p^2= 0.232$), and a non-significant Group X Condition interaction ($F(1.565, 37.554)= 0.005$, $p= 0.985$, $\eta_p^2< 0.001$). See Table 6 and Figure 7 for descriptive statistics.

Pairwise comparisons conducted on the three conditions for the gill flaring behavior (Behavioral hypothesis 4) revealed a significant difference between the baseline and drug condition ($t(25)= 3.384$, $p= 0.002$, $d= 0.66$), a significant difference between the drug condition and return to baseline condition ($t(25)= -2.455$, $p= 0.021$, $d= 0.48$), and no significant difference between the baseline and return to baseline condition ($t(25)= 1.313$, $p= 0.201$, $d= 0.26$). The gill flaring behavior was significantly lower in the drug condition compared to both baseline conditions.

Table 6. Group X Drug Condition descriptive statistics for the gill flaring behavior (secs) averaged across the last 5 days of testing.

		Baseline	Drug	Return to Baseline
No Female Prime	Mean	4.74	0.72	3.69
	SD	4.49	1.35	4.57
	N	13	13	13
Female Prime	Mean	4.82	0.68	3.88
	SD	8.25	1.17	7.88
	N	13	13	13

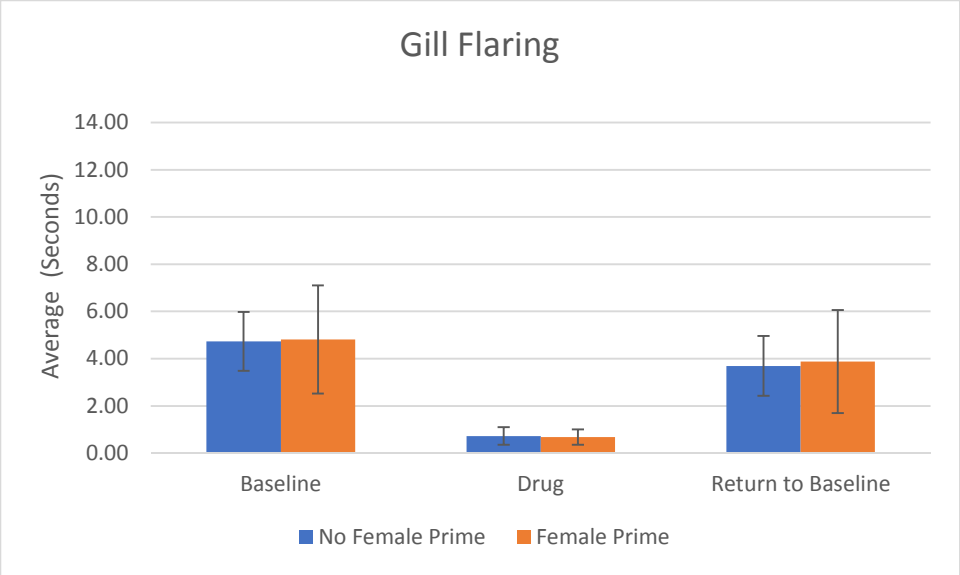

Figure 7. Group X Drug Condition for the gill flaring behavior (secs) averaged across the last 5 days of testing.

A two (groups) X three (conditions) Mixed ANOVA conducted on the fin spreading behavior (Behavioral hypotheses 3 and 4) revealed a non-significant main effect for group ($F(1,24)= 0.006$, $p= 0.941$, $\eta_p^2< 0.001$), a significant main effect for condition ($F(1.746, 41.905)= 5.102$, $p= 0.013$, $\eta_p^2= 0.175$), and a non-significant Group X Condition interaction ($F(1.746, 41.905)= 0.757$, $p= 0.459$, $\eta_p^2= 0.031$). See Table 7 and Figure 8 for descriptive statistics.

Pairwise comparisons conducted on the three conditions for the fin spreading behavior (Behavioral hypothesis 4) revealed a significant difference between the baseline and drug condition ($t(25)= 2.897$, p= 0.008, d= 0.57), a significant difference between the drug condition and return to baseline condition ($t(25)= -2.329$, p= 0.028, d= 0.46), and no significant difference between the baseline and return to baseline condition ($t(25)= 0.258$, p= 0.799, d= 0.05). The fin spreading behavior was significantly lower in the drug condition compared to both baseline conditions.

Table 7. Group X Drug Condition descriptive statistics for the fin spreading behavior (secs) averaged across the last 5 days of testing.

		Baseline	Drug	Return to Baseline
No Female Prime	Mean	9.40	1.14	7.14
	SD	9.41	2.72	10.33
	N	13	13	13
Female Prime	Mean	6.75	3.31	8.14
	SD	8.49	7.38	9.83
	N	13	13	13

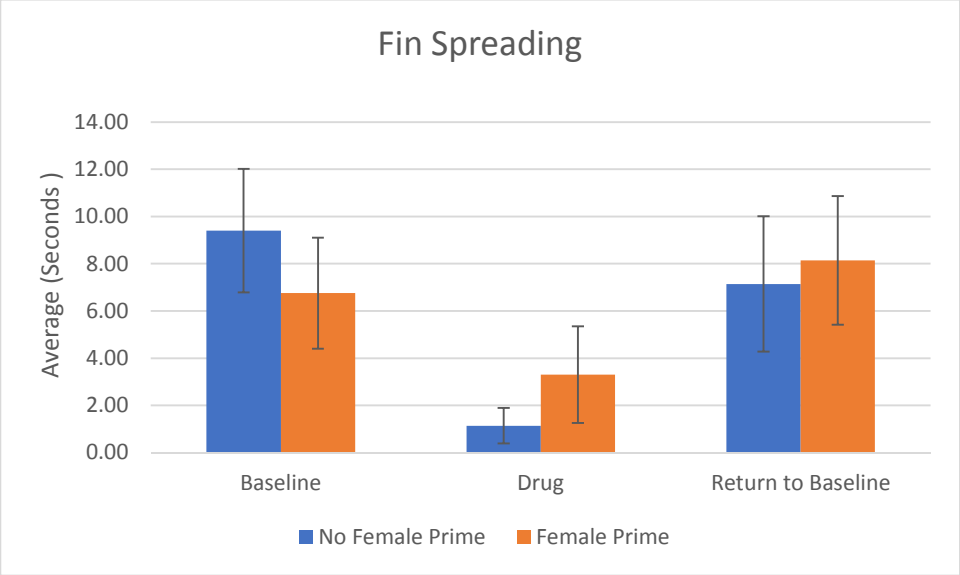

Figure 8. Group X Drug Condition for the fin spreading behavior (secs) averaged across the last 5 days of testing.

Discussion

Of the four latency hypotheses proposed for the latency data only hypothesis three: subjects will have longer latencies to enter the goal box with the mirror under the fluoxetine condition compared to the non-fluoxetine conditions, was supported. Latency hypothesis one: all fish will have a higher preference ratio for the mirror compared to the non-mirror presentation regardless of group or condition, latency hypothesis two: the female primed group will have lower latencies to enter the goal box with the mirror compared to the non-primed group, and latency hypothesis four: there will be a Prime X Condition interaction such that the female prime group will show a smaller increase in mirror latency than the non-primed group when exposed to fluoxetine, were not supported.

Failure to support latency hypothesis one is inconsistent with Eisenreich, Greene, and Szalda-Petree (2017) and Balzarini et al, (2014). Eisenreich, Greene, and Szalda-Petree, (2017) studied the latencies for fish to aggress against either a mirror or another male conspecific. This research showed shorter latencies for the excitatory mirror trials than the timeout condition trials for the fish exposed to a mirror. Fish in the conspecific condition showed longer latencies for the excitatory fighting trials than the timeout condition trials. The results of this experiment suggest that the mirror trails were more motivating for the fish than the time-out condition trials. However, the results of the present experiment do not show a significant preference for the mirror when the mirror preference ratio is calculated, suggesting the mirror was not significantly more motivating than the timeout condition.

The lack of decreased latency for the mirror vs timeout condition could be due to several factors. The first factor could be the length of time over which the experiment was conducted. Each fish was exposed daily to the mirror condition for a total of 75 days, with 5 mirror trials a day yielding a total of 375 mirror trials. The high number of trials completed coupled with the daily schedule could have led to habituation to the mirror, making it less salient and decreasing the fish's motivation to engage in aggressive behavior (Dzieweczynski and Perazio, 2012; Dzieweczynski, Gill, and Perazio, 2012). A Pearson's r correlation conducted on the mirror preference ratio with trial number to test for habituation to the mirror revealed a non-significant correlation ($r(390)= -0.045$, $p= .378$) ruling out habituation as a potential explanation.

In addition to the fish not having a significant preference for the mirror condition, latency hypothesis two: the female primed group will have lower latencies to enter the goal box with the mirror compared to the non-primed group, was not supported. This is inconsistent with Dzieweczynski et al, (2005) and Goldstein (1975), who both found exposure to a female fish led to increased aggressive responding and motivation in male fish. The present experiment found the males primed with a female were not significantly more motivated to respond aggressively toward the mirror when compared with males who were not primed.

Finally, latency hypothesis four: there will be a Prime X Condition interaction such that the female prime group will show a smaller increase in mirror latency than the non-primed group when exposed to fluoxetine, was not supported. This is also inconstant with Dzieweczynski et al, (2005) and Goldstein (1975) who found the males to be more motivated to engage in aggressive responding when primed with a female. The primed males in the present experiment did not show decreased latency to aggress toward the mirror when compared to the males who were not

primed with a female, even when exposed to fluoxetine which impacts the motor and motivational systems of the fish.

The effects of the prime could have contributed to the motivation and aggression in an unexpected way inconsistent with the hypothesis's prediction. The prime lasted a total of three minutes at the very beginning of the experiment with half of the subjects being primed with a female and the other half primed with an empty container. The males in the prime condition were able to court with the females for the full three minutes, while the males who were not primed with a female would not have had anything to respond to. This would lead to the primed males already exerting energy to court with the female, while the other males who were not primed with a female would not have needed to be active. The males who were not primed with a female would therefore be able to dedicate all their energy to fighting off an opponent, but the males in the group primed with a female would have to split their responses between the female and the mirror. This could lead them to become fatigued easier than the males who were not exposed to the female. This would be consistent with Matos et al. (2003) who found that males primed with either another male or a female lost more encounters with conspecifics than males who were not primed.

In addition to losing more encounters with conspecifics other behavioral changes in fighting style and aggressive responding have been reported by Bronstein (1989) and Dzieweczynski et al, (2005). These studies found that males primed with other males showed an increase in risky fighting behaviors such as biting, while males primed with a female decreased risky behaviors such as biting (Bronstein, 1989; Dzieweczynski et al., 2005). This difference shows that fighting style is context dependent and that exposure to the female may have

decreased the aggressive responding in the males primed with a female instead of increasing it as the hypothesis predicted.

The drug effects are clearly illustrated by the latency results of this experiment, showing strong support for latency hypothesis three: subjects will have longer latencies to enter the goal box with the mirror under the fluoxetine condition compared to the non-fluoxetine condition. In addition to longer latencies for the mirror trials, the non-mirror trials also showed a significant increase in latency. A two (groups) X three (conditions) Mixed ANOVA conducted on the latency to enter the goal box for the non-mirror condition revealed a significant main effect for condition ($F(1.540, 36.949)= 60.80$, $p< 0.001$, $\eta_p^2= 0.717$). Pairwise comparisons conducted on the three conditions for the non-mirror raw latency revealed a significant difference between the baseline and drug condition ($t(25)= -8.531$, $p< 0.001$, $d= 1.67$), a significant difference between the drug condition and return to baseline condition ($t(25)= 8.771$, $p< 0.001$, $d= 1.72$), and no significant difference between the baseline and return to baseline condition ($t(25)= -0.781$, $p= 0.442$, $d= 0.15$). The non-mirror raw latency was significantly higher in the drug condition compared to both baseline conditions.

These results support the drug's effect on movement as well as motivation proposed by Eisenreich, Greene, and Szalda-Petree (2017). Eisenreich, Greene, and Szalda-Petree (2017) placed the fish in a small chamber and used a grid system to track the movement of the fish throughout the small chamber. There was significantly less movement when the fish were exposed to the drug when compared to the baseline condition. The effect size reported by Eisenreich, Greene, and Szalda-Petree (2017) for the mirror latency was large, $\omega 2 = 0.4105$, and is reinforced by the results of the current study. Therefore, if the drug is having a negative effect

on the fish's ability to move it would be expected that the fish would struggle with the non-mirror trials as well as the mirror trials.

The latency effects found in this experiment show support for the negative impacts of fluoxetine on the latency to aggress toward a mirror or swim down for the timeout condition. The drug has been reported to negatively impact the behavior of betta fish in other research studies such as Eisenreich and Szalda-Petree (2015), and Eisenreich, Greene, and Szalda-Petree (2017). That effect has been illustrated here using the ABA method of taking a baseline before and after drug exposure. The impact on the latency was significantly higher for the drug condition, providing support for the negative impacts of fluoxetine on both movement and motivation. However, the female prime effects on latency were not significant and did not appear to have an impact on the latency behavior of the fish. Overall, while the latency behavior is clearly impacted by exposure to the drug, the latency predictions for the female prime did not support the hypotheses.

Of the four behavioral hypotheses only hypothesis two: subjects will exhibit less fighting behavior toward the mirror under the fluoxetine condition compared to the non-fluoxetine conditions, and hypothesis four: there will be a difference in the total amount of bites, and time lateral displaying, gill flaring, and fin spreading between the fluoxetine condition and non-fluoxetine conditions, were supported. Behavioral hypothesis one: the female primed group will exhibit more aggressive behavior toward the mirror compared to the non-primed group, and behavioral hypothesis three: there will be a difference in the total amount of bites, and time lateral displaying, gill flaring, and fin spreading between the female primed and non-primed group, were not supported.

Failure to support behavioral hypotheses one and three is inconsistent with Dzieweczynski et al. (2005) who found males were more aggressive when a female was present when compared to males that did not see a female. Dzieweczynski et al. (2005) concluded that the males were more aggressive due to the added motivation of attracting a mate and impressing the female. However, the results of Matos et al. (2003) suggest the opposite to be true; that males primed with a female will exhibit less risky behaviors, but still maintain aggression. Matos et al. (2003) found an increase in biting behavior in fights with males who were not primed with a female, but significantly less biting by males primed with a female. Matos et al. (2003) proposed that this was because the behaviors exhibited for fighting are similar to the behaviors used in courting, but risky behaviors such as biting are used to chase off a rival and are less attractive to a female. Therefore, the males primed with a female may exhibit a different fighting style than males who are not primed with a female. The data from this experiment did not find a significant difference in biting, lateral displays, gill flaring, or fin spreading between the female primed males and males not primed with a female.

While the female prime did not have any significant effect on the aggressive behavior of the males, the drug did show significant results. Behavioral hypotheses two and four were supported by the data, showing a negative impact of the drug on the overall aggressive responding of the fish and on the lateral display, gill flaring, and fin spreading behaviors. A significant impact was found on the aggressive responding behavior of the fish, with far less aggressive responding recorded during the drug condition when compared with the baseline and return to baseline conditions. While the overall aggressive response was lowered, three individual behaviors were significantly impacted by the drug: lateral displays, gill flaring, and fin spreading. Only biting was not statistically significantly impacted by the drug, and the effect size

was smaller. Overall, the drug is impacting certain aspects of the aggressive response, but not others.

The general effects of the drug on aggressive responding has also been found by Eisenreich and Szalda-Petree (2015), Eisenreich, Greene, and Szalda-Petree (2017), and Dzieweczynski and Hebert (2012). These studies have reported negative impacts of fluoxetine on aggressive behavior and movement of *Betta splendens*. The present experiment supports these findings and dissects the fighting behavior into individual behaviors to examine what behaviors are more susceptible to the drug's effect. While the biting behavior did not show any significant impacts from the drug; lateral displaying, gill flaring, and fin spreading did show significant effects and higher effect sizes. The present findings continue to support the evidence that fluoxetine has a negative impact on movement and motivation in *Betta splendens*.

There was one unexpected finding not hypothesized before data collection; that the male courting behavior was negatively impacted by the drug. The courting behavior was measured as either 1, male courting behavior occurred, or 0, no male courting behavior occurred, similar to the way the fighting behavior was measured. An ANOVA conducted on the courting behavior revealed a significant condition effect ($F(1.379, 16.553)= 16.935$, $p< 0.001$, $\eta_p^2= 0.585$). Pairwise comparisons conducted on the three conditions for the courting behavior revealed a significant difference between the baseline and drug condition ($t(12)= 4.466$, $p= 0.001$, $d= 1.24$), a significant difference between the drug condition and return to baseline condition ($t(12)= -4.170$, $p= 0.001$, $d= 1.16$), and no significant difference between the baseline and return to baseline condition ($t(12)= 1.806$, $p= 0.096$, $d= 0.5$). The courting behavior was significantly lower in the drug condition compared to both baseline conditions.

The courting behavior results parallel those found for the fighting behavior and a Pearson's r conducted on the courting and fighting behaviors for the female primed group revealed a significant positive correlation ($r(39)= 0.652$, $p< 0.001$).The present aggression and courting results support the finding by Dzieweczynski and Hebert (2012) who showed a significant impact on both the fighting and courting behavior of *Betta splendens* when exposed to fluoxetine. This finding provides more support for the impact of fluoxetine on both the motivation and movement systems in the fish as the drug impacted both the courting and fighting behavior. While the behaviors used for fighting and courting are similar, one is used to chase off a rival and the other to attract a mate. However, the male was impacted negatively for both fighting and courting, making their likelihood of breeding success decrease if this concentration of the drug is encountered in the wild. The drug's impact on the motivation to mate would be devastating if it were to reach similar concentrations in the wild and could have unknown consequences on the environment.

A number of potential limitations of the present experiment should be addressed in future studies. The first limitation addresses the amount of motivation and energy a male fish possesses. Matos et al. (2003) found males that were primed lost more aggressive encounters suggesting the males have a limited supply of energy they can use for courting and fighting. Since only one group of males was primed with the female, it is likely that they would have less energy to fight than the group not primed with the female and lose more of their aggressive encounters. In addition to exerting more energy attempting to attract the female, the males in the primed group may have been more distracted by the presence of the female, making them less likely to focus solely on fighting off their opponent. Therefore, the males in the female prime group would have to split their attention between fighting and courting, while the males in the group not primed

with a female would not have to focus on courting. This split of attention could make it more difficult for the female primed males to win their aggressive encounters and increase their latencies to enter the goal box.

Another potential problem with the current study is the method of drug administration. For this experiment the drug was administered to the fish for 30 minutes daily in an isolated chamber. This method has been used in other studies as well, such as Eisenreich and Szalda-Petree (2015), but it is not the only method used for drug exposure. Dzieweczynski and Hebert (2012) added the drug directly into the tank water of the fish which created a varying concentration more reminiscent of fish living in the wild. Even though the studies used different exposure methods for the drug, they found similar results on behavioral effects. What both studies are missing, however, is the amount of the drug absorbed by the fish and the concentration in the animal's body tissue. Both studies were able to measure the concentration in the water the fish lived in but had no data to show how much of the drug was absorbed and metabolized by the subject. This study also does not have data to show overall concentration in the tissue of the fish throughout the experiment. Collecting the tissue of the animal's is difficult, and little is known about the fish's metabolism and ability to uptake the drug. The overall uptake in this experiment has the potential for high variability considering the fish uptake the drug through their skin and gills from the surrounding water, unlike a fixed dose through an injection. The best way to collect the tissue would be to liquify the body of the fish to find the overall concentration in the body, which would have to be done at the end of the drug phase and would not allow for the return to baseline data. This experiment focused on behavioral data and if it was possible for the fish to recover their baseline behavior after the drug has been purged from their

system. Since the return to baseline data was needed, there was no attempt to try and obtain drug concentration data from the subject's tissue.

Despite the potential flaws in this experiment it still has practical implications. The steadily increasing concentration and long half-life of fluoxetine are problematic for the environment when considering the results of this study (Mackul'Ak et al., 2016; Mennigen et al., 2011). The fish in this study had impacts on both courting and fighting behavior, which will make reproduction and territory acquisition difficult in the wild. If the fish are unable to reproduce and maintain their population, this could have impacts on the ecosystem around them. In addition to the effects on courting and fighting the fish also had increased latencies to swim, supporting the hypothesis that fluoxetine negatively effects the movement of the fish. This could make both catching prey and avoiding predation difficult for the fish and further endanger their species. Clearly the drug is causing major life impairments in this smaller species of fish, and this study only explores the impact of the drug over a short time-period, 25 days. Other studies have examined exposure of small fish, clams, and mussels to concentrations of the drug over longer periods of time and found that the bioconcentration, or buildup of the drug in the animal's system due to prolonged exposure, can reach bioaccumulation, where the concentration in the animal's system is 5000 percent higher than the surrounding water (Wang and Gardinali, 2013; De Solla et al., 2016). While the concentration used in the current experiment is higher than reported environmental concentrations, it is not unreasonable for the organism's body to reach this concentration through bioaccumulation. Therefore, the effects seen in this experiment could start to occur in specimens in the wild if the drug concentration continues its steady increase.

If the drug concentration continues to increase, *Betta splendens* will not be the only organisms affected. Other studies have found a wide range of effects on various species, such as

toxic effects in algae, reproductive changes in clams, and changes in feeding behavior in minnows (Minguez et al., 2014; Brooks et al., 2003; Fong, Huminski, and D'Urso, 1998; Margiotta-Casaluci et al., 2014). Smaller species will be the first ones affected, creating an issue that will stem from a bottom-up approach. Due to the aquatic animal's smaller size and constant exposure to the drug through their environment they will be at risk for experiencing the most harmful effects and will likely experience the negative change first. Once the smaller species begin to experience the harmful effects larger species that depend on them will also be affected, through lack of food or change in habitat. In addition, if the smaller species of fish are prey animals and have reached bioaccumulation the predator animal will be ingesting a higher amount of the drug through consumption of the smaller creature. Overall, the impact on the environment could be devasting if nothing is done to prevent chemical pollution from continuing to increase.

While this paints a bleak picture for the future there is hope. If efforts are put forth to combat the increase in chemicals in the waterways, there is a chance to once again allow the animals to recover their normal behavior. As seen with the present experiment the fish recovered their baseline behavior after 25 days once the drug was removed. This suggests that the effects of the drug can be reversible and may not be permanent. However, there currently is not an economically efficient way to remove the chemicals, and current methods using nanoparticles as traps can cause more problems later on (Koçolu, Bakirdere, and Keyf, 2017; Basheer, 2018). The clock is ticking, but the countdown may have already begun.

References

Abate, M. E. (2005). Using a popular pet fish species to study territorial behaviour. *Journal Of Biological Education (Society Of Biology), 39*(2), 81-86.

Basheer, A. (2018). New generation nano-adsorbents for the removal of emerging contaminants in water. *Journal of Molecular Liquids, 261*, 583-593.

Balzarini, V., Taborsky, M., Wanner, S., Koch, F., & Frommen, J. (2014). Mirror, mirror on the wall: the predictive value of mirror tests for measuring aggression in fish. *Behavioral Ecology & Sociobiology, 68*(5), 871-878. doi:10.1007/s00265-014-1698-7.

Borova, V., Maragou, L., Gago-Ferrero, N., Pistos, C., & Thomaidis, P. (2014). Highly sensitive determination of 68 psychoactive pharmaceuticals, illicit drugs, and related human metabolites in wastewater by liquid chromatography–tandem mass spectrometry. *Analytical and Bioanalytical Chemistry, 406*(17), 4273-4285.

Brodin, T., Fick, J., Jonsson, M., & Klaminder, J. (2013). Dilute concentrations of a psychiatric drug alter behavior of fish from natural populations. *Science, 339*, 814−815.

Bronstein, P. M. (1989). The priming and retention of agonistic motivation in male Siamese fighting fish, *Betta splendens*. *Animal Behaviour, 37*(1), 165-166. doi:10.1016/0003-3472(89)90022-5.

Brooks, B. W., Chambliss, C. K., Stanley, J. K., Ramirez, A., Banks, K. E., Johnson, R. D., & Lewis, R. J. (2005). Determination of select antidepressants in fish from an effluent-dominated stream. *Environmental Toxicology and Chemistry, 24*(2), 464-9.

Brooks, B. W., Turner, P. K., Stanley, J. K., Weston, J. J., Glidewell, E. A., Foran, C. M., Slattery, M., La Point, T. W., & Huggett, D. B. (2003). Waterborne and sediment toxicity of Fluoxetine to select organisms. *Chemosphere, 52*(1), 135-142.

Chen, J., Ahn, K. C., Gee, N. A., Ahmed, M. I., Duleba, A. J., Zhao, L., Gee, S. J., Hammock, B. D., & Lasley, B. L. (2008). Triclocarban enhances testosterone action: a new type of endocrine disruptor? *Endocrinology, 149*(3), 1173-1179.

Chiffre, A., Clérandeau, C., Dwoinikoff, C., Bihanic, F., Budzinski, H., Geret, F., & Cachot, J. (2016). Psychotropic drugs in mixture alter swimming behaviour of Japanese medaka (*Oryzias latipes*) larvae above environmental concentrations. *Environmental Science and Pollution Research, 23*(6), 4964-4977.

Clotfelter, E., O'Hare, E., Mcnitt, M., Carpenter, R., & Summers, C. (2007). Serotonin decreases aggression via 5-HT1A receptors in the fighting fish *Betta splendens*. *Pharmacology, Biochemistry, and Behavior, 87*(2), 222-31.

De Almeida, C., Oliveira, M., Mallmann, C., & Martins, A. (2015). Determination of the psychoactive drugs Carbamazepine and Diazepam in hospital effluent and identification of their metabolites. *Environmental Science and Pollution Research International, 22*(21), 17192-17201.

De Solla, S. R., Gilroy, È. A. M., Klinck, J. S., King, L. E., Mcinnis, R., Struger, J., Backus, S. M., & Gillis, P. L. (2016). Bioaccumulation of pharmaceuticals and personal care products in the unionid mussel *Lasmigona costata* in a river receiving wastewater effluent. *Chemosphere, 146*, 486-496.

Dzieweczynski, T. L., Earley, R. L., Green, T. M., & Rowland, W. J. (2005). Audience effect is context dependent in Siamese fighting fish, *Betta splendens*. *Behavioral Ecology, 16*(6), 1025-1030.

Dzieweczynski, T. L., Gill, C. E., and Perazio, C. E. (2012). Opponent familiarity influences the

audience effect in male–male interactions in Siamese fighting fish. *Animal Behaviour, 83*(5), 1219-1224. doi:10.1016/j.anbehav.2012.02.013.

Dzieweczynski, T. L., & Hebert, O. L. (2012). Fluoxetine alters behavioral consistency of aggression and courtship in male Siamese fighting fish, *Betta splendens*. *Physiology & Behavior, 107*(1), 92-97. doi:10.1016/j.physbeh.2012.06.007.

Dzieweczynski, T., and Perazio, C. (2012). I know you: familiarity with an audience influences male-male interactions in Siamese fighting fish, *Betta splendens*. *Behavioral Ecology & Sociobiology, 66*(9), 1277-1284. doi:10.1007/s00265-012-1381-9

Eisenreich, B. R, Greene, S., & Szalda-Petree, A. (2017). Of fish and mirrors: Fluoxetine disrupts aggression and learning for social rewards. *Physiology & Behavior, 173*, 258-262.

Eisenreich, B. R., & Szalda-Petree, A. (2015). Behavioral effects of Fluoxetine on aggression and associative learning in Siamese fighting fish (*Betta splendens*). *Behavioural Processes, 121*, 37-42. doi:10.1016/j.beproc.2015.10.008.

Ferrari, B., Paxe´us, N., Lo Guidice, R., Pollio, A., & Garric, J. (2003). Ecotoxicological impact of pharmaceuticals found in treated wastewaters: study of Carbamazepine, Clofibric acid, and Diclofenac. *Ecotoxicology and Environmental Safety, 55*(3), 359-370.

Fick, J., Lindberg, R. H., Parkkonen, J., Arvidsson, B., Tysklind, M., & Larsson, D. G. J. (2010). Therapeutic levels of Levonorgestrel detected in blood plasma of fish: Results from screening rainbow trout exposed to treated sewage effluents. *Environmental Science & Technology*, *44*(7), 2661− 2666.

Fong, P., & Hoy, C. (2012). Antidepressants (Venlafaxine and Citalopram) cause foot

detachment from the substrate in freshwater snails at environmentally relevant concentrations. *Marine and Freshwater Behaviour and Physiology, 45*(2), 145-153.

Fong, P., Huminski, P., & D'Urso, L. (1998). Induction and potentiation of parturition in fingernail clams (*Sphaerium striatinum*) by selective serotonin re□uptake inhibitors (SSRIs). *Journal of Experimental Zoology, 280*(3), 260-264.

Forsatkar, M., Nematollahi, M., Amiri, B., & Huang, W. (2014). Fluoxetine inhibits aggressive behaviour during parental care in male fighting fish (*Betta splendens*, Regan). *Ecotoxicology*, *23*(9), 1794-1802. doi:10.1007/s10646-014-1345-0.

Gagne´, F., Blaise, C., Fournier, M., & Hansen, P. D. (2006). Effects of selected pharmaceutical products on phagocytic activity in *Elliptio complanata* mussels. *Comparative Biochemistry and Physiology, Part C, 143*(2), 179-186.

Garcia, S. N., Foster, M., Constantine, L. A., & Huggett, D. B. (2012). Field and laboratory fish tissue accumulation of the anti-convulsant drug Carbamazepine. *Ecotoxicology and Environmental Safety, 84*, 207-211.

Goldstein, S. R. (1975). Observations on the establishment of a stable community of adult male and female siamese fighting fish (*Betta splendens*). *Animal Behaviour, 23*(1), 179-185. doi:10.1016/0003-3472(75)90063-9.

González-Alonso, S., Merino, L. M., Esteban, S., López, A. M., Barceló, D., Durán, J. J., López-Martínez, J., Aceña, J., Pérez, S., Mastroianni, N., Silva, A., Catalá, M., & Valcárcel, Y. l. (2017). Occurrence of pharmaceutical, recreational and psychotropic drug residues in surface water on the northern Antarctic Peninsula region. *Environmental Pollution, 229*, 241-254.

Hogan, J. (1967). Fighting and reinforcement in the siamese fighting fish (*Betta splendens*).

Journal of Comparative and Physiological Psychology, 64(2), 356-359.

Hollis, K., Martin, K., Cadieux, E., & Colbert, M. (1984). The biological function of Pavlovian conditioning: Learned inhibition of aggressive behavior in territorial fish. *Learning and Motivation, 15*(4), 459-478.

Huerta-Fontela, M., Galceran, M., Martin-Alonso, J., & Ventura, F. (2008). Occurrence of psychoactive stimulatory drugs in wastewaters in north-eastern Spain. *The Science of the Total Environment, 397*(1-3), 31-40.

Huggett, D., Brooks, B., Peterson, B., Foran, C., & Schlenk, D. (2002). Toxicity of select beta adrenergic receptor-blocking pharmaceuticals (B-Blockers) on aquatic organisms. *Archives of Environmental Contamination and Toxicology, 43*(2), 229-235.

Karagiannis, C., Burman, O., & Mills, D. (2015). Dogs with separation-related problems show a "less pessimistic" cognitive bias during treatment with fluoxetine (Reconcile (TM)) and a behaviour modification plan. *Bmc Veterinary Research, 11*, 80.

Koçolu, E., Bakirdere, S., & Keyf, S. (2017). A novel liquid-liquid extraction for the determination of Sertraline in tap water and waste water at trace levels by GC-MS. *Bulletin of Environmental Contamination and Toxicology, 99*(3), 354-359.

Lahti, M., Brozinski, J., Jylhae, A., Kronberg, L., & Oikari, A. (2011). Uptake from water, biotransformation, and biliary excretion of pharmaceuticals by rainbow trout. *Environmental Toxicology and Chemistry, 30*(6), 1403-1411.

Logarinho, F., Rosado, T., Lourenço, C., Barroso, M., Araujo, A. R. T. S., & Gallardo, E. (2016). Determination of antipsychotic drugs in hospital and wastewater treatment plant samples by gas chromatography/tandem mass spectrometry. *Journal of Chromatography B, 1038*(C), 127-135.

Mackul'Ak, T., Bodik, I., Hasan, J., Grabic, R., Golovko, O., Vojs-Stanova, A., Gál, M., Naumowicz, M., Tichý, J., Brandeburová, P., & Hives, J. (2016). Dominant psychoactive drugs in the Central European region: A wastewater study. *Forensic Science International, 267*, 42-51.

Margiotta-Casaluci, L., Owen, S. F., Cumming, R. I., De Polo, A., Winter, M. J., Panter, G. H., Rand-Weaver, M., & Sumpter, J. (2014). Quantitative cross-species extrapolation between humans and fish: The case of the anti-depressant Fluoxetine. *PLoS One, 9*(10), E110467.

Matos, R. J., Peake, T. M., & McGregor, P. K. (2003). Timing of presentation of an audience: Aggressive priming and audience effects in male displays of Siamese fighting fish (*Betta splendens*). *Behavioural Processes, 63*(1), 53-61. doi:10.1016/S0376-6357(03)00029-9.

Mazzitelli, J., Bonnafe, E., Klopp, C., Escudier, F., & Geret, F. (2017). De novo transcriptome sequencing and analysis of freshwater snail (*Radix balthica*) to discover genes and pathways affected by exposure to Oxazepam. *Ecotoxicology, 26*(1), 127-140.

Mennigen, J., Stroud, P., Zamora, J., Moon, T., & Trudeau, V. (2011). Pharmaceuticals as neuroendocrine disruptors: Lessons learned from fish on Prozac. *Journal of Toxicology and Environmental Health, Part B, 14*(5-7), 387-412.

Miguel-Queralt, S., & Hammond, G. (2008). Sex hormone-binding Globulin in fish gills is a portal for sex steroids breached by xenobiotics. *Endocrinology, 149*(9), 4269-4275.

Minguez, L., Poi, C., Farcy, E., Ballandonne, C., Benchouala, A., Bojic, C., Cossu-Leguille, C., Costil, K., Serpentini, A., Lebel, J. M., & Halm-Lemeille, M. P. (2014). Comparison of the sensitivity of seven marine and freshwater bioassays as regards antidepressant toxicity assessment. *Ecotoxicology, 23*(9), 1744-1754.

Nakamura, Y., Yamamoto, H., Sekizawa, J., Kondo, T., Hirai, N., & Tatarazako, N. (2008). The effects of pH on Fluoxetine in Japanese medaka (*Oryzias latipes*): Acute toxicity in fish larvae and bioaccumulation in juvenile fish. *Chemosphere, 70*(5), 865-873.

Oetken, M., Nentwig, G., Löffler, D., Ternes, T., & Oehlmann, J. (2005). Effects of pharmaceuticals on aquatic invertebrates. Part I. The antiepileptic drug Carbamazepine. *Archives of Environmental Contamination and Toxicology, 49*(3), 353-361.

Schebb, N. H., Flores, I., Kurobe, T., Franze, B., Ranganathan, A., Hammock, B. D., & Teh, S. J. (2011). Bioconcentration, metabolism and excretion of Triclocarban in larval Qurt medaka (*Oryzias latipes*). *Aquatic Toxicology, 105*(3), 448-454.

Snekser, J. L., McRobert, S. P., & Clotfelter E. D. (2006). Social partner preferences of male and female fighting fish (*Betta splendens*). *Behavioural Processes, 72*(1), 38-41. doi:10.1016/j.beproc.2005.11.014.

Subedi, B., & Kannan, K. (2015). Occurrence and fate of select psychoactive pharmaceuticals and antihypertensives in two wastewater treatment plants in New York State, USA. *The Science of the Total Environment, 514*(23), 273-80.

Subedi, B., Lee, S., Moon, H., & Kannan, K. (2013). Psychoactive pharmaceuticals in sludge and their emission from wastewater treatment facilities in Korea. *Environmental Science & Technology, 47*(23), 13321-9.

Tanoue, R., Nomiyama, K., Nakamura, H., Kim, J., Isobe, T., Shinohara, R., Kunisue, T., & Tanabe, S. (2015). Uptake and tissue distribution of pharmaceuticals and personal care products in wild fish from treated-wastewater-impacted streams. *Environmental Science & Technology, 49*(19), 11649.

Vymazal, J., Březinová, T., Koželuh, M., & Kule, L. (2017). Occurrence and removal of pharmaceuticals in four full-scale constructed wetlands in the Czech Republic – the first year of monitoring. *Ecological Engineering, 98*, 354-364.

Wang, J., & Gardinali, P. (2013). Uptake and depuration of pharmaceuticals in reclaimed water by mosquito fish (*Gambusia holbrooki*): A worst-case, multiple-exposure scenario. *Environmental Toxicology and Chemistry, 32*(8), 1752-8.

Wick, A., Fink, G., Joss, A., Siegrist, H., & Ternes, T. (2009). Fate of beta blockers and psycho-active drugs in conventional wastewater treatment. *Water Research, 43*(4), 1060-74.

Wu, M., Xiang, J., Que, C., Chen, F., & Xu, G. (2015). Occurrence and fate of psychiatric pharmaceuticals in the urban water system of Shanghai, China. *Chemosphere, 138*, 486-93.