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Behavioral Effects of Fluoxetine on Aggression and Associative Learning in Betta splendens

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While the link between serotonin and the behaviors of aggression and learning has been elucidated, few studies have examined the impact serotonin has on learning for aggressive rewards. In particular, the SSRI fluoxetine has been demonstrated to reduce aggression as well the acquisition of instrumental responding for rewards indicating that this drugs behavioral effect may be related to motivational processes important for learning. To examine the relationship between fluoxetine and motivational process important for learning, two experiments were conducted using *Betta splendens*, a species well known for their robust aggressive response and drive to engage in aggressive behaviors, to examine the impact of fluoxetine on aggressive behavior and learning for aggressive rewards. Results from these two experiments provide evidence of a possible serotonergic input on dopamine circuits important for motivational processes related to learning.

The monoamine serotonin has been implicated as an important neurotransmitter in the mediation of a variety of behaviors. In particular, a growing body of research has indicated that serotonin plays an important role in associative learning processes related to appetitive and aversive stimuli, impulsivity, as well as the onset and maintenance of aggressive behaviors (Homberg, 2014; McDevitt and Neumaier, 2011; Olivier and Oorschot, 2005). In addition, serotonin's relationship with affective disorders, such as depression and anxiety, has been well characterized, indicating that serotonin may play a role in mediating affective states (Kranz et al., 2010). Due to such a wide impact on varying facets of behaviors, two distinct fields of research have developed examining the nature of serotonin in associative learning contexts and in the mediation of aggressive behaviors.

The Siamese fighting fish (*Betta splendens*) has long been used in animal models of aggression due to the robust behavioral display the eliciting stimuli of a conspecific male or mirror reflection will produce (Thompson,1963). Similar to rat models, aggression in male bettas has been studied using a variation on the resident intruder paradigm in which a male betta is placed in a home tank and allowed to establish a territory. After the male betta has established a territory, an intruder stimuli is introduced into the tank in the form of another conspecific male, a fake fish model, or the revealing of a mirror. Upon introduction of the intruder stimulus the resident male will approach the stimuli and engage in a stereotyped pattern of behavior consisting of increased coloration, erection of the dorsal fins, flaring of the operculae, tail flicks, broadside displays, and bites directed at the intruder (Clayton and Hinde, 1967). Thus, the typical chain of responses in betta fish aggression can be classified as consisting of stimulus recognition, consisting of approach towards the stimuli with increased body coloration and flaring of gills and

fins, and the execution of intruder directed signaling in the form of repeated gill flares, bites, tail flicks, and broadside displays.

Given the robust and typified pattern of behavior to aggression eliciting stimuli, a large literature has been devoted to examining the types of associations that can be made between seemingly neutral stimuli and aggression eliciting stimuli, between behavioral responses and aggression eliciting stimuli, and the stimulus characteristics that influence the eliciting effects of aggressive stimuli (Simpson, 1968). Due to this wealth of information on the behavioral variables important for aggression, *Betta splendens* represent an ideal model for exploring the role of serotonin in learning and aggression.

Serotonin, Learning, and Aggression

Serotonin and Learning

Research investigating the role of serotonin on associative learning has revealed a complex role of serotonin in the acquisition and extinction of responses as well as in decisional processing and the inhibition of learned responses (Homberg, 2014). Notably, research on serotonin and learning has revealed conflicting results for the a general role for serotonin in the mediation of reward processing and acquisition of responding that is dependent upon whether overall levels of central serotonin are increased or decreased. For example, past research examining increases in central serotonin levels through administration of the SSRI fluoxetine have been demonstrated to impair the acquisition of conditioned avoidance responses to electric shocks in rats (Nelson et al, 1997) and gold fish (Beulig and Fowler, 2008), as well as the acquisition of a lever pressing response for food rewards, while facilitating the extinction of the same response (Frick et al.,2014). Furthermore, in the same study by Frick et al. (2014) tianeptine, a selective serotonin reuptake enhancer, was found to facilitate the acquisition of the

lever pressing response while inhibiting extinction, indicating that opposing effects on acquisition depending upon the whether serotonin levels are increased, resulting in impairments, or decreased, resulting in facilitation.

However, not all experimental decreases of serotonin levels have shown a facilitative effect on learning. Bari et al. (2010) demonstrated that modulations of serotonin levels by use of the SSRI citalopram and 5,7 DHT did not impair the acquisition of a learned behavioral response but did alter the sensitivity of rats to omission of rewards in a probabilistic reversal learning task. In particular, Bari et al. found that with depletions of global serotonin levels following injections of 5,7 DHT subjects demonstrated a greater responsiveness to omission of rewards on the decision to switch, while increases of global serotonin levels with injections of citalopram resulted in a low sensitivity to reward omissions on the choice to switch. In conjunction with this finding, Rygula et al. (2014) demonstrated, using a variation of the probabilistic learning task of Bari et al (2010), that depletions of serotonin in the amygdala and orbital frontal cortex of new world monkeys resulted in a similar increased responsiveness to reward omissions and impairment to reversal learning. Furthermore, Izquierdo et al. (2012) demonstrated that serotonin depletions after injections of PCPA resulted in the failure of rats to successfully demonstrate reversal learning while leaving effortful responding for food rewards unchanged, thus indicating that the role of serotonin is more complex than a simple modulation of response acquisition.

Examined as a whole, the current body of research provides some evidence that serotonin's role in learning may be through the modulation of either motivational circuits controlling the arousal of the organism to unconditioned stimuli or motor circuits responsible for producing behavioral responses. Evidence for motoric effects comes from the variety of studies examining reversal learning, as the failed ability to cease responding on previously reinforced

options when they are no longer reinforced is interpreted as an inability to inhibit the previously learned response (Homberg, 2014; Bari et al., 2010). Furthermore, it appears that the failure to inhibit learned responses is seen with depletions of central serotonin levels, as increases in serotonin levels have been shown to increase reversal learning (Homberg, 2014). However, while increases in serotonin have been shown to increase reversal learning, they have also been shown to impair acquisition of conditioned associations (Nelson et al., 1997; Beulig and Fowler, 2008) and response reinforcer contingencies (Frick et al., 2014). In addition, in the study by Nelson et al., the impaired ability of rats to learn the escape responses was ruled out as being caused by altered sensory thresholds to the electric shock or motor impairments. As such, increases in central serotonin levels may impair acquisition through reducing the motivational component of learned responding through contingent feedback. As such the altered sensitivity of subjects to negative feedbacks in the study by Bari et al. (2010) as well as Frick et al. (2014) produced the impaired acquisition of responding since the contingent feedback loop necessary for learning was altered. Thus, serotonin may function to mediate both the sensitivity of subjects to behavioral contingencies as well as coordinate motoric aspects of executing learned behavioral responses.

Serotonin and Aggression

Serotonin's implication in aggression stemmed from early experimental observations of decreased levels of central serotonin and serotonin metabolites and a higher incidence of impulsive and aggressive behaviors in a variety of species (Soubrie, 1986; Miczek et al., 2002). This connection between lowered serotonin levels and aggression resulted in a theory termed the serotonin deficiency hypothesis (deBoer and Koolhas, 2005). Under the serotonin deficiency hypothesis, serotonin served in an inhibitory role in the facilitation of aggressive responses.

Support for the serotonin deficiency hypothesis was found with the advent of neurotoxic agents such as para-chlorophenylalanine which depletes serotonin from serotonergic cells resulting in increased aggression within subjects, as well as serenics, drugs found to have anti-aggressive effects by acting as an agonist at serotonin receptors (Olivier and Oorschot, 2005).

However, with the advent of better pharmacological manipulations there has been a mounting challenge to the serotonin deficiency hypothesis. Specifically, recent research into the serotonin system has revealed 14 different receptor subtypes widely dispersed throughout the brain (Olivier and Oorschot, 2005). Administration of specific drug agonists and antagonists targeted for individual serotonin receptor sub types has revealed receptor subtype specific roles in a multitude of behaviors. In particular, research has demonstrated that the serotonin 1a and 1b receptors appear to influence offensive aggressive behaviors through dual roles as autorecpetors on presynaptic serotonergic cells and heteroceptors on post synaptic nonserotonergic cells (Takahashi et al., 2011). Under this dual role, previous findings of lowered serotonin levels may actually have been due to increased serotonin activity at presynaptic autoreceptors on the serotonin system which would result in a net decrease of serotonin activity, akin to a negative feedback loop.

In a study by deBoer and Koolhas (2005), the dual effect of the serotonin 1a and 1b receptors was demonstrated in rats through the administration of multiple serotonin agonists and antagonists. The researchers grouped the subjects into low, average, and high aggression groups and then proceeded to measure aggressive responses as well as average motor behaviors during a resident intruder test. The research revealed two interesting trends. The first trend was a positive correlation between brain level concentrations of serotonin and the overall level of aggression, contrary to predictions from the serotonin deficiency hypothesis. The second trend was a

differing behavioral pattern of aggressive responses depending upon the receptor subtype targeted by the administered drug. With drug administrations of serotonin 1a agonists the researchers noted an overall decrease in motor behavior of subject animals, while administrations of serotonin 1b agonists resulted in an overall decrease in the duration of aggressive behaviors and an increase in the latency to attack.

In conjunction with the findings of deBoer and Koolhas (2005), Olivier and Oorschot (2005) provide further evidence of differing neural pathways in the serotonergic mediation of aggressive behaviors. In a review of the literature the authors highlight the findings of nonspecific reductions in aggression accompanied by general sedation in subjects given selective serotonin 1a receptor agonists. Furthermore, they note that in studies of solely offensive aggression triggered by electrical stimulation of the hypothalamus, administration of serotonin 1a receptor agonists result in little change of aggressive behavior while serenic drugs, which also target the serotonin 1b receptor, do result in reduced attack behavior without sedation effects. Likewise, studies of interspecies aggression, argued by some to be a heightened form of offensive aggression, are mediated only by administration of serotonin 1b agonists although at a higher dosage than normally used in resident-intruder tests. Thus the serotonergic mediation of aggression is likely caused by differing activity at the serotonin 1a and 1b receptor types, with serotonin 1a pathways regulating the maintenance of motor behaviors, as evidence by sedative effects serotonin 1a agonists exert, while the serotonin 1b pathway facilitates an arousal engagement pathway for aggressive behavior, as evidenced by the decreased duration of aggressive behaviors and increased latencies to attack observed with administration of serotonin 1b agonists.

Serotonin and Aggression in *Betta splendens*

Until recently the majority of experimental examinations of serotonin on aggression have been conducted using mammalian species, however with the expansion of genetic techniques and the growing concern over pharmaceutical waste in local water ways, exploration of the serotonergic pathways in a variety of teleost species, such as *Betta splendens*, has begun. Central to these investigations has been the question of whether teleost species share a conserved expression of serotonin pathways and whether pharmacological manipulations of those pathways will have a similar effect on fish behavior as in mammals.

Norton et al (2008) addressed the first concern by outlining the serotonin receptor 1a, 1b, and transporter pathways in the zebra fish brain utilizing a gene expression technique. The research indicated conserved expression of the serotonin 1b receptor along midline structures from the forebrain into the hind brain with a particularly dense expression in the habenula and down the descending dorsal conduction. Conversely the serotonin 1a receptor was found to have a conserved expression in medial brain structures surrounding the hypothalamus and the posterior tuberculum. Furthermore, the authors noted a dual expression of both receptor types in the inferior lobe of the hypothalamus.

Comparatively the posterior tuberculum has been shown to connect with the ascending dopaminergic pathways and is believed to be homologous to the substantia nigra and ventral tegmental area (Yamamoto, 2009). Likewise, the expression of serotonin 1b receptors in the forebrain appears to be analogous to limbic structures in mammals, while the habenula is believed to be associated with the control of reward and avoidance behavior. Thus, the serotonin system appears to be conserved between mammals and aquatic species (Norton et al., 2008).

Pharmacological investigations of the serotonin system in fish species have provided further evidence for the conserved nature of the serotonin system in the control of aggression. For example, Lyn et al (2007) demonstrated that chronic exposures to dissolved concentrations of fluoxetine, either 3 μg/mL for 3 hours or 6 μg/mL for 5 hours, resulted in reduced aggression, in the form of decreased number of broadside displays and turns in *Betta splendens.* Likewise, Kania et al. (2012) reported that daily administrations of 40 or 100 μg of fluoxetine for 28 days resulted in a significant increase to the latency to attack as well as reduced the frequency of displays towards a mirror, with the greatest effect occurring after the 14^{th} day of exposure for the 40 μg group, and Kholert et al. (2012) reported both a decrease in aggression as well as disruptions in normal motor behavior in fish exposed to either 350 or 705 μg/L concentrations of fluoxetine. Furthermore, Clotfelter et al. (2007) demonstrated a significant reduction in both the duration of displays as well as an increase in the latency to attack following administrations of the serotonin 1a agonist 8-OH-DPAT similar to findings in mammals. In addition, Clotfelter et al. (2007) also reported that injections of 4.3 mmol solutions of fluoxetine failed to impact the duration of aggressive displays but did result in significant reductions of forebrain levels of serotonin as well as overall reductions in levels of serotonin metabolites in both the forebrain and hindbrain of treated fish.

Applied to findings using mammalian species, it does appear that serotonin circuitry plays a role in the mediation of aggressive behaviors in *Betta splendens* with similar effects to those found in mammals of reduced aggression with increased levels of serotonin or administration of serotonin 1a receptor agonists. In addition the finding by Clotfelter et al. (2007) of lowered serotonin levels after treatment with fluoxetine corresponds with the proposed negative feedback role of serotonergic autoreceptors by De boer and Koolhaus (2005).

Furthermore, the work by Norton et al. (2008) indicates that the effects of serotonin manipulations may manifest in behaviorally different ways depending on the pathways activated. In particular it appears that general manipulations of serotonin, as is accomplished with fluoxetine treatments, may serve to mediate motoric initiation and continuation of aggressive behaviors or the appetitive properties of aggressive encounters. The findings of reductions in motoric components of aggression, e.g. reductions in turns and displays, as well as lessoned responsiveness to aggressive stimuli provide further support for this.

Aggression and Associative Learning

Aggression and Classical Conditioning

Studies of excitatory classical conditioning in *Betta splendens* follow a typical format in which a neutral stimulus, that is one which would not result in an aggressive response, is paired with an aggression provoking stimulus or unconditioned stimulus, typically a mirror or live conspecific. With repeated pairings the typified pattern of aggressive behavior comes to be elicited by the formerly neutral stimulus (Adler and Hogan, 1963). Likewise, studies of inhibitory classical conditioning use a similar format to excitatory preparations, where one neutral stimulus is always paired with the unconditioned stimulus and comes to elicit the aggressive response while the other neutral stimulus is never paired with the unconditioned stimulus and does not elicit the aggressive response (Adler and Hogan, 1963).

Research examining the excitatory conditioning of aggressive responses in *Betta splendens* has demonstrated a robust ability of subjects to learn predictive associations between neutral stimuli and aggression eliciting stimuli. Neutral stimuli such as electric shocks (Adler and Hogan, 1963) and different colored lights (Thompson and Sturm, 1965b) have all been shown to reliably produce conditioned aggressive responses when paired with either mirror access or a live

conspecific. Furthermore, Alder and Hogan (1963) demonstrated that subjects can learn an inhibitory relationship between electric shocks and mirror access, while Braud and Weibel (1969) demonstrated that previously established conditioned aggression to different colored lights could become associated with the aggression mediating effects of the drugs morphine and promethazine.

Aggression and Instrumental Learning

Examinations of instrumental learning in *Betta splendens* have typically been focused on examining whether the contingent relationship between performing a behavior, termed the instrumental response, and the opportunity to engage in aggression, in the form of a stimulus presentations, will lead to greater rates of performance of the instrumental response. In this paradigm the increased rate of instrumental performance is said to be reinforced by the opportunity to engage in aggression and as such the subject has learned the contingent relationship between the two behavioral occurrences.

Thompson (1963) and Thompson and Sturm (1965a) established the reinforcing effects of access to an intruder stimulus, in the form of a mirror image, moving model, or a stationary model, on the instrumental response of swimming through a ring. In all three conditions Thompson (1963) demonstrated that access to the intruder stimuli was reinforcing for the ring swimming response. However the most salient reinforcement conditions were the mirror presentation and moving model respectively, indicating that the reinforcing nature of the aggressive response was due to the type of eliciting stimuli.

Hogan (1967) further elucidated the reinforcing nature of aggression on operant responses in betta fish. Utilizing a similar response of swimming through a ring for access to a mirror, Hogan found that responding increased during periods of continuous reinforcement and

decreased significantly during the extinction period. Furthermore, in a replication of the study using yoked controls who received access to the mirror whenever the experimental yoke swam through the ring, Hogan (1967) demonstrated that responding increased significantly for experimental subjects but not for the yoked controls. From these findings Hogan concluded that the access to the mirror was serving as an appetitive stimulus for the behavior of swimming through a ring.

In a follow up study, Hogan et al. (1970) sought to examine the reinforcing effects of aggressive displays on an operant response as opposed to reinforcement in the form of food. The researchers found that on an increasing fixed ratio schedule the number of responses, in the form of swimming through a tunnel, remained relatively constant even as the amount of reinforcements tapered off in the aggressive display condition, while in the food reinforcement condition the number of responses increased as the fixed ratio schedule increased. Additionally in a follow up experiment on duration of reinforcement in the form of access to a mirror, the authors found that the amount of responding approaches an asymptote as duration of mirror presentations increase. Given these findings the authors concluded that the nature of aggressive displays as reinforcement is distinct from that of access to food. Specifically, the amount of responding as fixed ratios increase under the reinforcement condition of display deviates from that of food reinforcement indicating that the reinforcing nature of aggressive displays has a lower satiation point than food rewards. Applied to the reinforcing nature of aggressive displays, the satiation point may be contingent on the arousal of the organism in response to the eliciting stimuli, such that as durations of reinforcement increase the satiation point is reached creating an upper limit on responses.

Similar to the findings of Hogan et al. (1970) Clayton and Hinde (1967) found that the amount of aggressive responses to the mirror stimulus decreased over successive days of constant exposure. However, the overall duration of gill cover erection and the number of bites directed at the mirror increased with successive days. Based on these findings the increase in bites and duration of gill cover erections was likely indicative of an increased magnitude of aggression due to prolonged exposure to the eliciting stimuli in a frustration aggression paradigm similar to findings in rats (Miczek et al. 2002).

Recently Shapiro and Jensen (2009) conducted a series of experiments examining the reinforcing nature of aggressive displays on choice preference in a T-maze on betta fish. In the first set of experiments the researchers demonstrated a strong choice preference for the side of the T-maze that provided access to a mirror and a larger duration of access compared to no mirror access and a short duration access respectively. Building on this finding the researchers conducted a second set of experiments using a T-maze to examine choice preference for shorter or longer delays to a mirror presentation. The researchers found a general preference for sides with shorter delays to mirror access as opposed to longer delays. Paradoxically in a follow up study where delay was presented as a choice between a longer arm or shorter arm to traverse before mirror access was given, subjects exhibited a strong preference for sides that required traversing the longer tunnel, equivalent to a longer delay, than sides with a shorter tunnel. Drawing from these findings the researchers concluded that the nature of aggressive display opportunities may likely be shaped by ecologically relevant cues. As such, sides with access to a mirror and that require traversing a longer distance may be representative of defending a larger territory from an intruding conspecific and as such are experienced as being more reinforcing than the smaller territory displays.

Aggression and learning implications for Serotonin's role

Based on the literature it is clear that *Betta splendens* maintain an ability to make conditioned associations about stimuli within the environment that predict aggressive encounters, as well as behavioral contingencies that will result in aggressive encounters. Furthermore, aggression eliciting stimuli and opportunities to aggress due appear to function as a reinforcer for instrumental behaviors in *Betta splendens*, and of critical importance to the rewarding properties of the aggressive stimulus/encounter are: the duration of the encounter, the delay to the encounter, the stimulus properties of the aggressive stimuli, and the size of the arena in which the encounter occurs. Thus, it appears the behavioral factors that impact the rewarding value of the aggressive encounter can be broken into stimulus arousal factors, that is properties related to the aggression eliciting stimulus, as well as performance factors, that is components related to the actual performance of fighting behaviors.

Viewed from an ethological perspective, the role of learning in aggressive behavior is likely due to an increase arousal after exposure to an aggression predicting cue, such that the male fish is prepared for a more vigorous display. Indeed, Hollis et al. (1984) tested this hypothesis using blue gournami, a species related to *Betta splendens*, and demonstrated that male fish who received an excitatory stimulus presentation prior to fighting a pairmate, that had received an inhibitory stimulus presentation, showed a more robust aggressive display and had a greater percentage of fight outcomes than the inhibitory pairmate. Furthermore, Hogan (1986) demonstrated that *Betta splendens* exposed to a mirror before being given the option to choose either an aggressive reward or a food reward demonstrated a robust priming effect of greater choices of aggressive encounters after mirror exposure, indicating that like the Holis et al. the pre-exposure served to increase aggressive motivation and subsequent displays.

Applied to the role of serotonin in aggression and learning, any reduction in aggression by serotonergic agents within an associative learning task is likely to be accomplished through mediation of arousal to aggression eliciting cues or impairment to the actual performance of aggressive behaviors, such that either the formation of the predictive association for the aggressive encounter is impaired or the rewarding value of the aggressive act is shifted. As such, the mix of studies reporting a role for serotonin in the acquisition of associations, in mediating sensitivity to aversive experiences, or response inhibition may be representative of a mediational effect on arousal to environmental stimuli thus impacting the saliency of unconditioned stimuli. Within a learning context for aggressive rewards, this model predicts that variations in the level of arousal via serotonin manipulations will directly impair the encoding of predictive relationships as well as the overall vigor of aggressive displays.

Currently, no studies have been conducted to examine directly how serotonin impacts learning for aggressive rewards. However, there is some evidence from ablation studies that implicate serotonin pathways in these associative processes. For example, Van Cantfort and Bingham (2002) performed forebrain ablations on a group of betta fish and measured the overall latency to fin erection operculae spread, as well as the duration of fin erection and operculae spread in response to either a mirror presentation or the introduction of a conspecific. An analysis of the results revealed a significant increase in the latency of ablated fish for fin erection and operculae spread compared to sham operated and normal controls. Additionally the duration of fin erection and operculae spread also decreased, however the frequency of the aggressive display did not differ from that of sham operated and normal controls. Furthermore, the authors noted no difference in the motor behavior or normal activity of ablated fish to that of controls.

Similar to Van Cantfort and Bingham (2002), Hollis and Overmeir (1982) examined the effect of telencephalon ablation on the reinforcing effects of access to a mirror and overall aggressive displays. The researchers utilized a tunnel swimming task similar to Hogan (1967) in which responses were reinforced with access to a mirror for 15 seconds. Additionally master fish were paired with yoked controls that were reinforced whenever the master fish completed the operant response. The experiment revealed no difference in the responding of ablated masters to that of normal or sham operated fish, however the ablated yoked controls performed significantly less responses than the other yoked control groups; thus indicating a differential amount of operant response acquisition between the two ablated groups.

Following up this experiment, Hollis and Overmeir (1982) ran a second study examining the effect of telencephalon ablation on classical conditioning of aggressive displays. The researchers exposed the fish to a light followed by presentation of a mirror and measured the overall amount of aggressive responses to the unconditioned stimulus of the mirror and the conditioned stimulus of the light. The data revealed a distinct trend of lessoned displays of fin and gill cover erection for ablated fish to the unconditioned mirror stimuli as opposed to controls. Likewise, ablated fish failed to acquire a conditioned response of fin erection, however gill cover erection did appear to be classically conditioned to presentation of the light. Interestingly, the overall mean amount of displays for ablates in both the unconditioned stimulus and conditioned stimulus did not change indicating an overall lower level of arousal than compared to controls.

When compared with the findings by Norton et al. (2008), the findings of Van Camfort and Bingham (2002) and Hollis and Overmeir (1967) provide further evidence of a role for serotonin pathways in the acquisition, initiation, and maintenance of aggressive responding. In both studies the areas removed contain many of the serotonin pathways and structures outlined

by Norton et al (2008). Thus, the findings of increased latency to fin and gill erection by Van Cantfort and Bingham and the failure of ablates to develop a clear conditioned aggressive display are likely due to the destruction of serotonin pathways to limbic centers resulting in an overall decrease in arousal to the unconditioned stimuli.

Applied to pharmacological studies, the parallel results of decreased aggression with the destruction of serotonin circuits, ostensibly a reduction in serotonin signaling, and the upregulation of serotonin levels with SSRI administration indicates that the serotonin system may have multiple behavioral effects at different levels of signaling, such that at lower administrations of SSRI which lead to a decreased level of serotonin signaling (Clotfelter, 2007;.Homberg, 2014) the overall arousal of subjects to eliciting stimuli is lowered, resulting in the observed increases in latencies to attack and reductions in display behaviors while at higher dosages an overall impairment in motor behaviors is produced. Within an associative context, this dual role may manifest as altering the saliency of aggressive eliciting stimuli, thus resulting in impaired encoding of instrumental or conditioned response to stimuli, at low dosages to disrupted motor behavior, resulting in impairments to instrumental responding or conditioned responding, at higher dosages.

To examine this general model of the role serotonin plays in aggression and learning two studies were conducted. In the first study the concentration dependent relationship between fluoxetine administration and aggression was examined. Building off of the results from the first study the second study examined how the anti-aggressive effect of fluoxetine would impact associative learning for aggressive rewards with an emphasis on identifying the behavioral mechanism by which fluoxetine reduces aggression.

Experiment 1

Rational

Previous research on the effects of fluoxetine in *Betta splendens* has indicated a mediating role for serotonin in aggression. However, while this effect appears to be robust across multiple methodologies, no clear conclusion as to the concentration dependent nature of the effect on aggression can be reached. In particular, Dzieweczynski and Hebert (2012) reported that exposure to .54 μg/L of fluoxetine significantly reduced the duration of aggressive behaviors, while Kholert et al. (2012) reported effects on both aggression and motor behavior at concentrations of 350 and 705 μg/L. Yet, Clotfelter et al. (2007) reported only slight and non-significant reductions in aggression to fish injected with a solution of 4.3 mmol of fluoxetine. Based on these results, it appears that at higher doses, fluoxetine begins to disrupt motor behaviors while at lower doses the latency to engage in attack behavior is impaired, however these effects are once again based upon widely different exposure periods raising questions as to how much of the drug was bioavailable at the time of testing. Therefore, a study was conducted to examine the concentration dependent effects of fluoxetine on aggressive behavior in *Betta splendens* under constant exposure period.

Based on previous research it was hypothesized that fluoxetine exposure would lead to a reduction in the duration of aggressive displays and that these reductions would follow a concentration dependent function. Furthermore by exposing fish to set concentrations each day, it was theorized that a day by drug interaction, indicating a bio-accumulating effect of fluoxetine, would be detected.

Method

Subjects

Twenty five male *Betta splendens* were kept on a 12 hr light/dark cycle in individually labeled tanks maintained at approximately 26º C. The bettas had an average length of approximately 5cm and ranged in coloration from red to blue.

Apparatus and materials

Housing Each betta was housed singly in a tank containing a gravel floor, a T-maze, a water heater, a bubbler hooked up to an airstone, and a thermometer. The tanks had the approximate dimensions of 67.3 cm x 40.6 cm x 16.8 cm (LxWxH) and were filled with approximately 20 L of dechlorinated water. Inside each tank, subjects were housed within the T portion of a T-maze apparatus of approximate dimensions 53 cm x 20 cm x 10 cm (LxWxH). All subjects were fed a diet consisting of nine betta pellets given daily after the last experimental trial.

Drug preparation To examine the impact of fluoxetine exposure on aggression, the twenty five subjects were broken into a control and four experimental groups (n=5). fluoxetine HCL was obtained from TCI America and a stock solution with a concentration of .5 mMol was prepared. Drug exposures followed a protocol adopted from (Lyn et al., 2006) in which subjects were placed in separate dosing chambers containing 0, 5, 10, 15, or 20 μMol concentrations of the fluoxetine solution which were then floated inside of the T-maze. Exposures occurred for 30 minutes, after which subjects were released back into their tanks for two minutes before starting the first experimental trial. All drug exposures took place at approximately 8:30 am daily for fifteen days.

Procedure

Experimental trials consisted of lifting a guillotine door which revealed the straight alley portion of the T-maze at the end of which was placed a mirror (See figure 1). All experimental trials took place for three minutes and were recorded on a tablet computer. Experimental trials occurred three times daily at 9 am, 12 pm, and 3 pm. After the daily trials were completed all videos were uploaded on to a computer for video analysis. Video analysis consisted of measuring the duration of gill flares that were directed towards the mirror during the three minute period as previous research has indicated longer duration gill flares are a strong predictor of fight outcomes in *Betta splendens* (Abrahams et al., 2004).

Figure 1. Apparatus for Experiment 1

Analysis

All data were analyzed in SPSS using a mixed model ANOVA in which the change in the duration of gill flares was examined across the repeated measure of the three time points (9am, 12pm, and 3pm) within the second repeated measure of the fifteen trial days between the fixed factor of the five concentration levels (0, 5, 10, 15, 20 µMol). A Tukey's Post Hoc HSD test was preformed to examine the mean differences between the five concentration groups, and the effect size estimate Ω^2 was computed for the main effects. Xu (2003) demonstrated that Ω^2 serves as a

better estimate of effect size than traditional measures when data is highly correlated as is the case in this study due to nested repeated measures.

Results

The Concentration by Day by Time ANOVA revealed no significant interactions amongst the three factors or for the main effect of the three time points. The analysis did reveal a significant main effects for the factor of trial days, $F(14,122.946) = 2.734$, $p < .005$, $\Omega^2 = .0968$, and concentration levels, $F(4, 291.109) = 95.113$, $p < .001$, $\Omega^2 = .26$. The post hoc analysis revealed a grouping effect of the five concentration levels with mean differences between the 0 µMol and the 5, 10, 15, 20 µMol concentrations, no differences between the 5 and 10 µMol concentrations but significant differences between the these two concentrations and the 15 and 20 µMol concentrations respectively, and no differences in levels of aggression between the 15 and 20 µMol concentration groups (See figure 2).

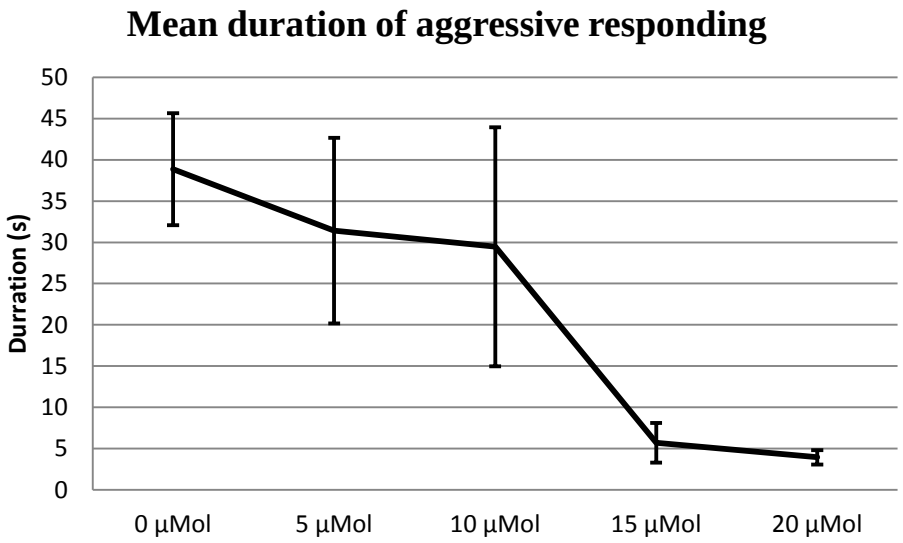

Figure 2. Concentration dependent decrease in the duration of aggressive responding towards a mirror after fluoxetine exposure. Error bars represent standard error of the means.

Discussion

The results from this study fit well with previous reports in which Fluoxetine was shown to reduce aggressive responding in *Betta splendens.* As predicted, increases in the concentration of fluoxetine did produce a concentration dependent reduction in aggression, with an almost complete reduction in aggression and a sedation effect on all motor behaviors at concentrations at or above 15 µMol. Additionally the finding of a grouping effect between the 0 µMol, the 5 and 10 µMol, and the 15 and 20 µMol concentrations further supports the concentration dependent relationship and sheds light on to the variety of behavioral effects that have been reported. In particular it appears that fluoxetine exerts its effect on different behavioral components of aggression, with lower concentrations producing slight decreases in durations and frequencies of displays and higher concentrations resulting in an almost complete inhibition of motoric behaviors. Given this, it is possible that fluoxetine at lower concentrations impacts an arousal/motivational circuit for initiating aggressive behavior in that there is a slight reduction in the duration of aggressive displays and frequencies without obvious motor disturbances.

Lastly, the finding of a significant reduction in aggression across the experimental days and no interaction between the experimental days and concentration levels or for the three time periods provides important insight into the bioavailability of fluoxetine under an acute exposure procedure. Mainly it appears that after a 30 minute exposure period fluoxetine is readily bioavailable, as indicated by no significant differences between the three time periods which covered immediately after exposure and 3 to 6 hours after initial exposure. Likewise, the failure to detect an interaction between the concentration levels and experimental days provides further evidence that the effect of the initial exposure remained relatively constant across days. Furthermore, the observed decrease in aggression across all subjects independent of drug

concentration did not appear to impact habituation to the mirror stimulus, as Hogan (1967) reported decreased aggression towards mirror presentations with repeated exposures in *Betta splendens.* Based on this it appears that using an acute daily exposure procedure, a concentration dependent reduction in aggression that is relatively constant from day to day and does not interact with the time dependent exposure period.

Experiment 2

Following the results from experiment 1, a follow up study was conducted to examine the impact of fluoxetine on aggression within an associative learning context. Of key interest was how the aggression reducing effects of fluoxetine would impact the function of an aggression eliciting stimulus as an unconditioned stimulus in a go-no go associative learning task. Previous research has implicated fluoxetine as playing a role in the acquisition of responding (Frick et al. 1997, Beulig and Fowler, 2009) as well as in reducing aggressive behavior through motor disturbances (Kohlert et al., 2010; experiment 1). Furthermore, findings from experiment 1 appear to indicate that at low concentrations fluoxetine may reduce the overall arousal of fish to aggressive stimuli. Given these findings, fluoxetine may exert its effects on learning and aggression through the alteration of stimulus saliency or through an inhibition of motor circuits depending on the concentration. To examine the relationship between fluoxetine and learning for aggressive rewards, a behavioral task was constructed using a straight alley maze and two discriminative stimuli (SD) paired with an excitatory unconditioned stimulus, a mirror, and an inhibitory unconditioned stimulus, a white wall, resulting in a excitatory discriminative stimulus (SD+) and an inhibitory discriminative stimulus (SD-).

Given that fluoxetine may impair the acquisition of associative relationships, a small N design in which subjects were trained on the behavioral task first and then exposed to a concentration of fluoxetine that was shown to reduce aggression but not produce overt motor deficits was implemented to isolate whether fluoxetine altered the saliency of the stimuli or inhibited motor circuitry. It was hypothesized that if fluoxetine impacted the saliency of the unconditioned stimulus mirror presentation, then changes in only responding to the SD+ would be produced. However, if changes in responding to both the SD+ and SD- occurred, then fluoxetine would be viewed as causing a general motor impairment.

Methods

Subjects

Eight male *Betta splendens* were obtained from a supplier and were kept on a 12 hr light/dark cycle in individually labeled tanks maintained at approximately 26º C. Fish had an average length of approximately 5cm and ranged in coloration from red to blue.

Apparatus and Materials

Housing Each of the eight male *Betta splendens* were kept individually in tanks and maintained under the same housing conditions as experiment 1.

Drug preparation As with experiment one a stock solution of .5mMol fluoxetine HCL dissolved in dechlorinated water was prepared. The daily drug exposures followed an ABA format with the A portion consisting of a sham exposure of 200 mL of the subject's tank water and the B portion consisting of an exposure to a 10 μMol concentration of fluoxetine. All drug exposures were carried out in a similar matter to that described in experiment 1. Exposure to the fluoxetine concentration occurred after a baseline of five days of consistent responding to the

experimental task had been established for the subject and continued until a new period of five days of consistent responding to the experimental task was observed. After exposure to the fluoxetine concentration for the required criterion of five days of consistent responding, the subject was given sham exposures until the previously established baseline level of responding was recovered.

Procedure

To examine the impact of fluoxetine on associative learning processes a single subject ABA design was implemented, in which subjects were trained on the Go/No Go discrimination task to establish a baseline of responding, defined as five days of consistent performance. After the baseline was established subjects were then exposed to the 10 μMol concentration of fluoxetine until a new stable rate of responding was established. After establishing a stable rate of responding, subjects were then allowed to recover their baseline rate of responding in the non-drugged condition.

The Go/No-Go task required the bettas to swim down a straight alley maze through a portal door into a goal box (Figure 3). The goal box was equipped with either a mirror or a white wall as signaled by the color/pattern of the portal door (blue or black and white checkerboard pattern that covered both the front and back of the portal door). This allowed for the creation of two conditions, the Go condition in which one pattern of the portal door always predicted access to the mirror in the goal box, and a No Go condition in which the other patterned portal door always predicted access to the white wall. More succinctly the portal patterns were divided into an excitatory discriminative stimulus (SD+) that always predicted 30 s mirror access or an inhibitory discriminative stimulus (SD-) that always predicted no mirror access for 30 s. The two

SDs were counterbalanced across subjects such that half the subjects received blue checkerboard pattern as the SD+ and the remaining subjects received the black and white pattern as the SD+.

Experimental trials consisted of five exposures to the SD+/mirror pairing and five exposures to the SD-/no mirror pairing counterbalanced across days. The format for the trials consisted of guiding the fish into a start chamber and then lifting a guillotine door revealing the straight alley maze at the end of which the SD was placed creating a portal entrance into the goal box. Each of the trials was separated by a 30s time out period in which the fish was returned to the start chamber and had a maximum latency of 150 s for the subject to enter the goal box. The time taken for the subjects to swim down the maze into the goal box, as well as the duration of any aggressive responding to the SD+ stimulus before entering the goal box, and whether the subject responded aggressively to the mirror stimulus during the 30 s access in the goal box were recorded on a tablet computer.

Figure 3. Apparatus for Experiment 2.

Results

Of the eight fish that entered the experiment only four fish reliably learned the discrimination procedure and were used in the data analysis. The four fish that failed to show discrimination also exhibited a reluctance to aggress against the mirror stimuli placed within the goal box and would only aggress if forced. Thus, they may have been impacted by previous learning of a dominant-subordinate relationship or found the mirror stimulus to be aversive, as has been reported in the literature (Bornstein, 1981).

Examining the four fish that did demonstrate reliable discrimination, a clear pattern of responding can be seen as subjects were shifted across the ABA format for drug exposures. During the A portion of the experiment in which subjects were given sham exposures to the drug a clear pattern of discrimination between the two SD can be seen. With subjects showing a faster time to enter the goal box when the SD+ was present, a gradual increase in aggressive responding towards the SD+ before entering the goal box, and a consistent pattern of aggression towards the mirror once inside the goal box (See figure 3). As subjects were shifted into the B portion in which they were exposed to a 10 μMol concentration of fluoxetine a new pattern of responding emerged, in which the time taken to enter the goal box for both of the SD reached the maximum latency, the amount of aggressive responding to the SD+ decreased as well as the aggressive behavior towards the mirror (See figures 4-6). Furthermore, when reintroduced to the sham exposures the previously established pattern of behavior was recovered for all of the subjects with the exception of subject 1 who died before baseline could be reestablished (See figure 5).

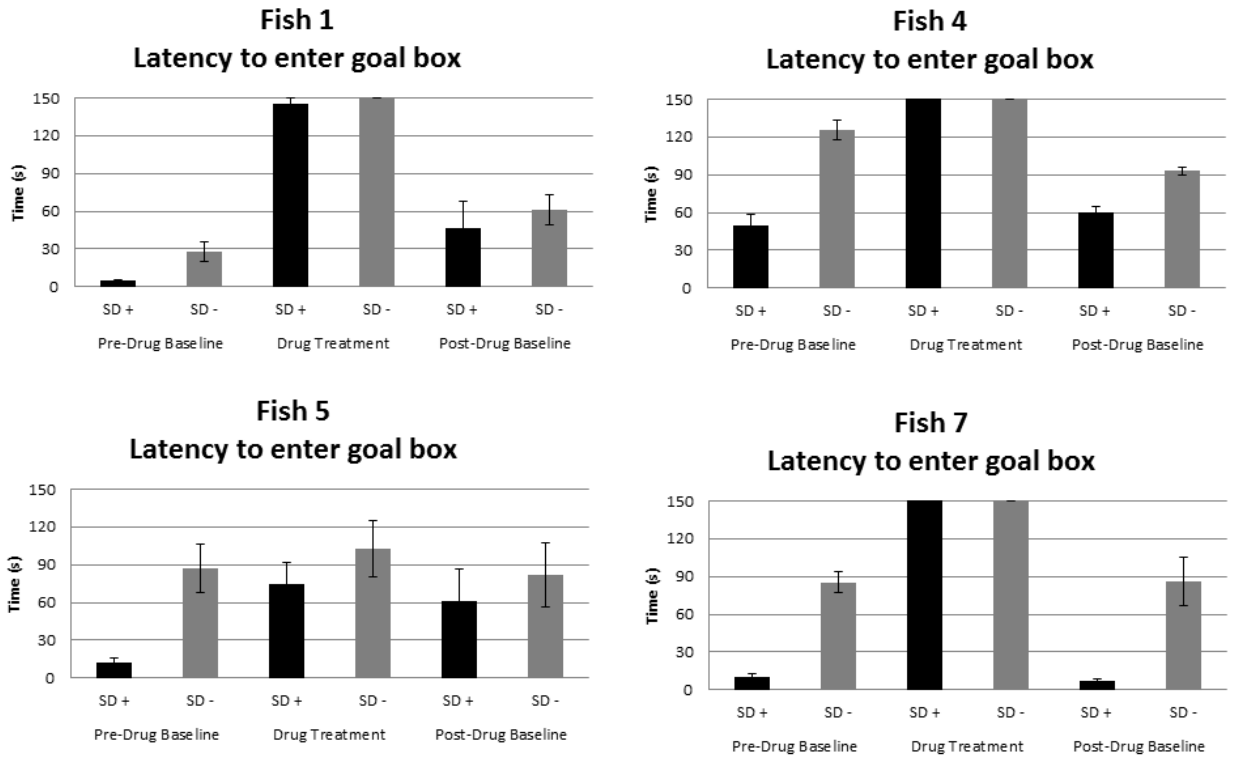

Figure 4. Five day average latency data for all four experimental subjects across the ABA experimental procedure. Lines indicate SEM.

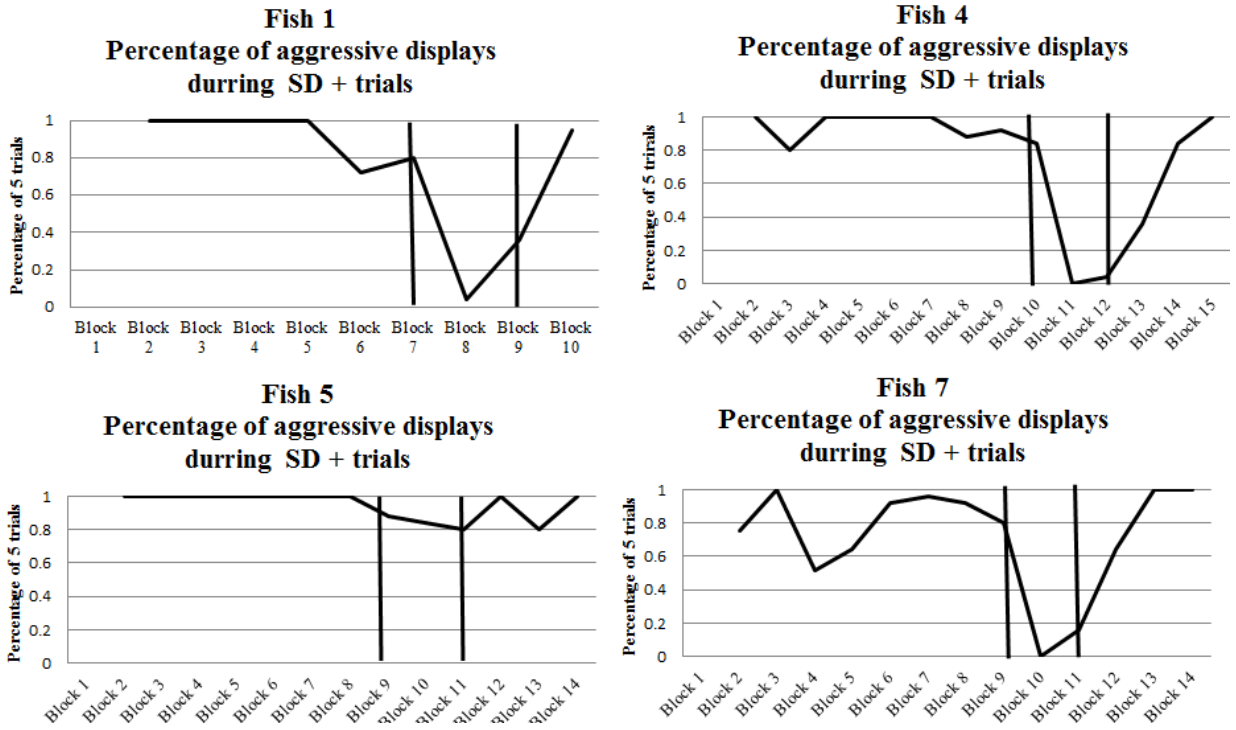

Figure 5. Percentage of aggressive displays during the SD+ trials over five day blocks. Black vertical lines indicate the onset and cessation of drug exposures.

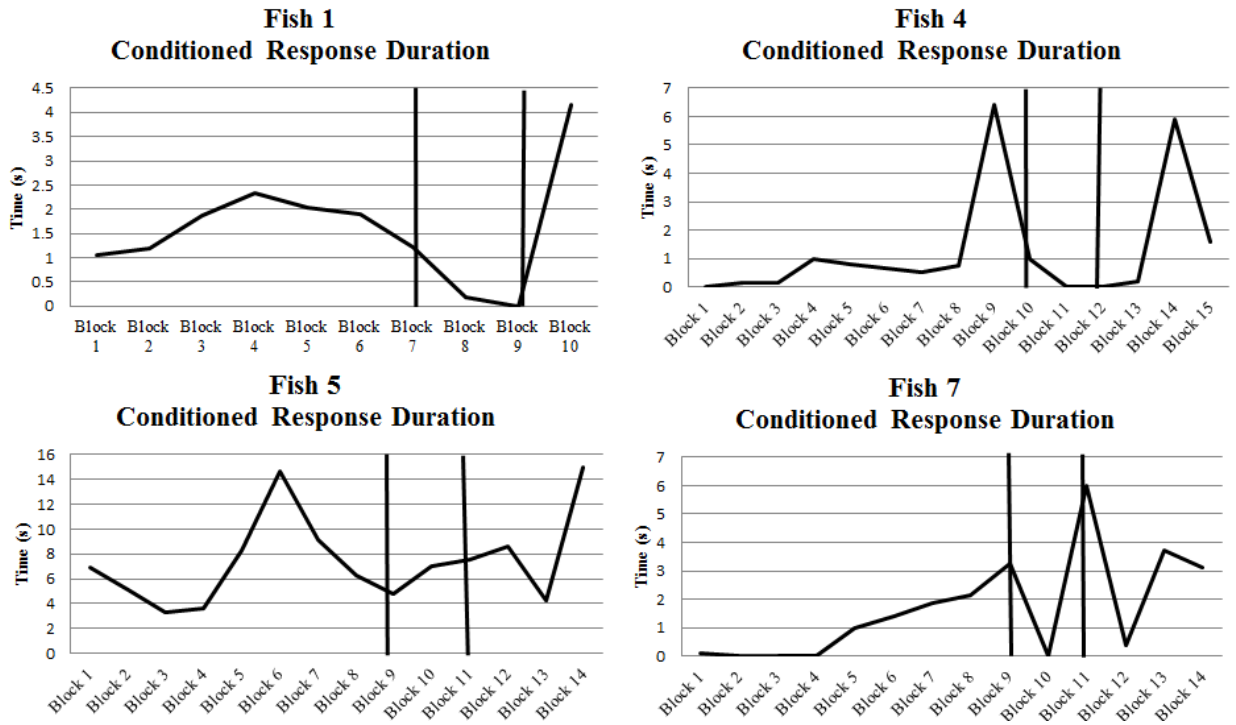

Figure 6. Duration of conditioned aggressive responses directed at the excitatory stimulus over five day blocks. Black vertical lines indicate the onset and cessation of drug exposures.

Discussion

Based upon the observed changes in responding to all aspects of the experimental task between the A and B portions, it is clear that Fluoxetine exposure does impact aggressive responding and associative learning. The present study provides strong evidence that fluoxetine reduced overall responding to both the discriminative stimuli as well as conditioned aggressive displays. In light of the results from experiment 1 and previous research on aggression and learning, fluoxetine may be producing its effects by either altering the experience of the unconditioned stimulus mirror presentations or by sedating the motoric behavior of the fish.

Under the first explanation, fluoxetine administrations are viewed to reduce the rewarding properties or saliency of the unconditioned stimulus. Functionally, this should produce a clear pattern of responding between the SD+ and SD- stimuli with faster responses

occurring for the SD+ at baseline and a pattern of similar responding to the SD+ and SD- after drug exposure with responding to the SD- remaining constant throughout. Thus, the change in responding to the SD+ only indicates that the relationship between the SD+ and the US of the mirror had been altered from a previously rewarding relationship to a non-rewarding one.

Likewise under the second explanation, fluoxetine administrations are viewed as producing a general sedative effect on all motor behavior. As such, the pattern of responses seen under this explanation would be one of differential responding between SD+ and SD- at baseline, and then a general reduction of responding to both SD+ and SD- with drug administrations.

The evidence from experiment 2 can be viewed as fitting with either of these two explanations. In examining the overall patterns of responding it is clear that exposures to the 10µMol concentrations of fluoxetine did produce a change in responding to both the SD+ and SD-. Thus, the observed changes could have occurred due to a general sedative effect on motor behavior as reported by Kohlert et al. (2010). However, care should be taken before invoking an explanation of motor sedation, as in the first experiment administrations of 10µMol of fluoxetine were found to produce only a slight reduction in aggression, approximately an 8 second average reduction in display times, and the researchers did not notice any obvious impairment in motor movement as was observed in the 15 and 20 µMol groups. Furthermore, some of the change in the responding to the SD- after drug administrations could have been produced by a reduction of anticipatory responding to the general experimental procedure. Hogan et al. (1986) demonstrated that mirror exposures could serve to prime the choice preference of *Betta splendens* for aggressive encounters and Hollis et al. (1984) demonstrated that exposure to excitatory conditioned stimuli would lead to greater aggressive displays. Applied within this research study, it is possible that the daily alterations between first presentation of SD+ and SD- produced a

general association between running the experimental trials and the opportunity to aggress. The researchers did note some evidence for this hypothesis in the form of patrolling behavior during SD- trials. As such it is possible that the drug administration may have altered the sensitivity of subjects to the mirror presentations and resulted in an overall decrease in the level of arousal. This explanation is similar to the Beulig and Fowler (2010) report of impaired learning of avoidance responses to electric shocks with treatment of fluoxetine which was believed to be caused by lowered sensitivity and arousal to the unconditioned stimulus. Thus, if fluoxetine reduced the sensitivity of individuals to mirror presentations, then the reward motivating properties of the mirror stimulus were weakened producing a decrease in responding to the SD+ while also resulting in an overall decrease in the anticipation of the aggressive encounter and subsequent decrease in patrolling behavior. Of the four subjects, one of the subject's patterns of responses matches this prediction perfectly lending some plausibility to the argument. However, care should still be taken as future research is needed to rule out the motor sedation hypothesis.

General discussion

Currently the role of serotonin in learning and aggression has not been fully elucidated. Past investigations have established that serotonin does impact the acquisition of associative relationships, the initiation of behavioral responses, and appears to alter the effectiveness of punishments or negative feedbacks. Likewise, serotonin has been demonstrated to play a key role in the onset and maintenance of aggressive behaviors, with apparent differentiation between behavioral effects depending on the sub receptor that is targeted. In light of the findings from the two experiments in this study, it appears that manipulations of serotonin levels through the drug fluoxetine: 1) produce a graded deficit on motor behavior and a reduction in aggressive responding, and 2) potentially impact the saliency or reward value of unconditioned stimuli.

Given these results it is possible that the impairments in learning acquisition, responsiveness to negative feedback, and inhibition of behavioral responses as well as the reduction in aggression are all elements of a serotonergic input on dopaminergic pathways important for the motivational aspects of behavior.

Dopamine has long been implicated an important component for reward based learning and motor movement (Vaccarino et al., 1989). Stemming from early research on brain stimulation reward learning, current research has outlined two important ascending dopamine pathways critical for reward learning, the mesolimbic and nigrostriatal pathways. Examinations of the ascending mesolimbic dopamine pathways have indicated that it plays an important role in facilitating the motivational aspects of reward learning. In particular, it appears that this pathway mediates the arousal of the organism to unconditioned stimuli and conditioned stimulus presentations such that conditioned stimulus presentations produce a "wanting" state in anticipation of the unconditioned stimulus (Anselm, 2013; Berridge, 2004). Contrarily the ascending nigrostriatal dopamine pathways appears to facilitate memory consolidation for response reinforcer associations and the coordination of behavioral responses (Vaccarino et al., 1989; Salmone, 1992). Taken together, these two ascending pathways appear to both motivate responding to stimuli that have been associated with an unconditioned stimulus as well as facilitate learned behavioral response.

While dopamine has long been studied in the context of reward learning, serotonin may also play an important functional role in modulating dopamine activity within the learning context. Serotonin manipulations in the form of fluoxetine and the serotonin 1a agonist WAY 100635 have been shown to reduce cocaine induced locomotor activity in rats (Herges and Taylor, 1997), and administrations of fluoxetine have been demonstrated to alter the activity of

dopamine neurons in the substantia nigra and ventral tegmental area of rats. Likewise, fluoxetine administrations after injections of cocaine was shown to reduce the subject pleasure of the cocaine in humans (Walsh et al, 1994), while the serotonin 1b agonist CP94253 has been demonstrated to attenuate bar pressing for cocaine administrations in rats (Parsons et al., 1998). Thus, it appears that serotonin activity does modulate the two ascending dopamine pathways described above.

Applied within the context of the present investigation, it is probable that the behavioral effects of serotonergic drugs on aggression and learning are due to the modulations of dopamine pathways. Ferrari et al. (2003) demonstrated that exposures to regularly scheduled aggressive encounters resulted in increased dopamine release in the nucleus accumbens one hour before the encounter would normally occur. Due to its location within the mesolimbic ascending pathways Ferrari et al. hypothesized that the observed change in dopamine was functioning as an anticipatory response to prepare the animal for the aggressive encounter and carried with it an appetitive signal. Thus, the present findings of reduced aggression with administrations of fluoxetine and altered responding within an associative learning task may be due to the effects of the serotonin signal pathways on dopamine circuitry. In particular, the findings of slightly reduced aggression after exposure to 5 and 10 μMol concentrations and complete disruption of motoric behavior after exposure to 15 and 20 μMol concentrations of fluoxetine is suggestive of a differential disruption within the mesolimbic dopamine pathway and the nigrostriatal pathway respectively. Furthermore, the findings from the second experiment provide further evidence for a modulating role on mesolimbic dopamine pathways as subjects did not demonstrate any overt motor impairment to normal behavior but showed a clear cessation of responding after drug treatment. As such, exposures to low concentrations of fluoxetine may result in a modulation of

the mesolimbic dopamine circuit thus producing a disruption of motivational factors key for learning about and preforming aggressive behaviors, while exposures to higher concentrations modulate both the mesolimbic and nigrostriatal pathways thus producing impairments in motoric performance.

Future studies should be conducted to test this model as it predicts that manipulations of mesolimbic dopamine signaling, either directly or through serotonin agonists, should produce a similar impairment in both learning and motivational aspects related to reward saliency while leaving motoric responding unimpeded. Furthermore, these effects should cross over from aggressive rewards to other reward systems. Applied within the framework of aggressive behavior, it is probable that the anti-aggressive effects of many serotonergic agents are due to alterations in dopamine pathways in the form of modulating the overall arousal the aggressive stimulus provides. As such, the serotonin system may be functioning as an affective input on dopamine pathways that facilitate goal seeking behavior and the saliency of unconditioned stimuli that supports the initiation and consummation of aggressive behavior. Future research aimed at examining this model may provide useful insight into the affective factors that influence learning and motivated behavior in a variety of species.

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