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Of fish and mirrors: Fluoxetine disrupts aggression and learning for social rewards

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HIGHLIGHTS

- Learning and aggression for two different social reinforcers was assessed in *Betta splendens*, Siamese fighting fish.
- Exposure to a 10 μMol concentration of fluoxetine disrupted task performance and decreased aggression independent of social reinforcer type.
- Fluoxetine exposure produced a dramatic decrease in normal swimming behavior in experimental fish.
- We provide strong evidence for a motor inhibition as the main behavioral mechanism of action for fluoxetine's attenuation of aggression.

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ABSTRACT

Aggressive signaling is a key social behavior of male Siamese fighting fish (*Betta splendens*). Successfully establishing a territory and defending it from intruders has direct fitness effects, making *Betta splendens* a prime model for studies examining the biological underpinnings of aggressive behavior. Current research has outlined serotonin transporter pathways as one key component for the engagement and coordination of aggressive behavior in *Betta splendens*. Using the selective serotonin reuptake inhibitor fluoxetine, we examined the impact of 10 μmol exposures on associative learning and aggression between mirror and conspecific social reinforcers. Our results provide clear evidence that exposure to fluoxetine reduces aggression and impairs learning independent of social reinforce type. In addition, our results provide support for motor inhibition of aggressive behavior as the main behavioral mechanism of action for fluoxetine. Placed within the broader context of behavioral syndromes, our results, along with others, implicate serotonergic pathways as a key biological correlate of the bold-aggressive phenotype.

1. Introduction

The selective serotonin reuptake inhibitor fluoxetine has been well characterized in reducing the amount and patterning of aggressive behavior of male Siamese fighting fish [8,11,14,16,17]. Each of these reports has utilized a rather wide range of drug concentrations and exposure periods [8], lending credible support to the notion that serotoninergic alterations have a direct role in male Siamese fighting fish behavior. Despite the clear indication that fluoxetine exerts a physiological change in behavior, researchers have yet to specify the exact behavioral mechanism of action by which fluoxetine exerts its effects. Towards this end, Eisenreich and Szalda-Petree [8] proposed two possible explanations for fluoxetine's behavioral mechanism of action as being either motivational or motoric in nature. To date, evidence for either of these two accounts has been inconclusive; with some reports indicating both motor and motivational impairments in drug exposed fish.

One possible explanation for these mixed results is due to the natural confound of overt motor performance measures underlying the quantification of both motivational effects, as well as motor inhibition. For example, in a simple associative task involving swimming down an alley maze into a goal box, both the strength of learning as well as the quantification of motivation are dependent upon overt motor performance measures; in this case swimming speeds. The natural confound of performance and motivational quantification poses a potential problem for experimentally teasing apart the impact of motivational influences from overt motor performance effects within many experimental procedures in behavioral pharmacology [18]. However, within studies utilizing mirror social reinforcers the strong link between the eliciting properties of the mirror with the motivational and motor performance of the subject are exceptionally problematic, as the phase locked nature of mirror agonistic encounter does not allow

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for a differentiation between changes in motivation or motor inhibition (Fig. 1). Put simply, a mirror reinforced fish that fails to respond to a mirror stimulus is receiving a different stimulus than a fish that does respond aggressively. A point that has been well argued by studies examining the pitfalls of mirror elicited aggression [3,9]. In either case, if both a motivational shift and a motor inhibition predict the same response of less behavior; it is impossible to distinguish between the two explanations. With respect to the effects of fluoxetine on associative learning for aggressive rewards, as noted by Eisenreich & Szalda-Petree [8], it is possible that many of the reported motivational effects and deficits in acquisition could be due to an inhibitory effect on motor systems and not changes in motivational arousal to external stimuli.

One way to break free from the motivational and motor confound is to manipulate saliency and eliciting properties of the reinforcing stimuli independent of motor response processes. Within the experimental set up of Eisenreich & Szalda-Petree [8] this could be accomplished by comparing mirrors and conspecific presentations as social reinforcers. Eisenreich & Szalda-Petree [8] utilized a discrimination task that required fish to discriminate a predictive cue indicating the presence of a mirror from a non-predictive unreinforced cue. This allows for an assessment of motivational effects on the stimulus control of predictive cues signaling social reinforcement, in addition to measurement of aggressive display behavior. Building on this methodology a simple manipulation involving either a mirror presentation or access to viewing a conspecific fish can be examined within the same stimulus discrimination paradigm to better assess motivational effects from motor suppression.

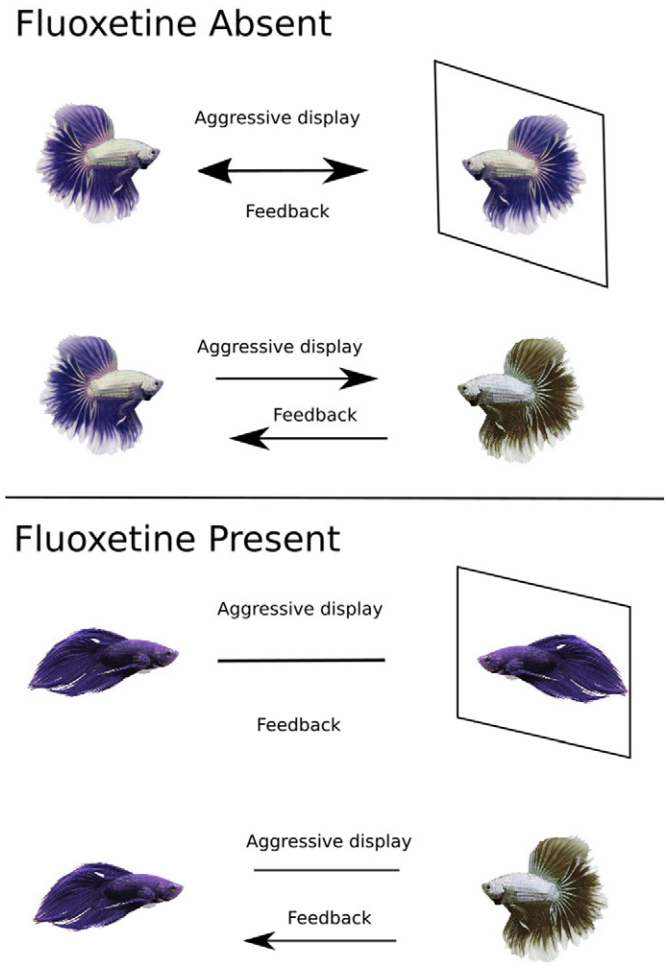

Fig. 1. Illustration of how fluoxetine impacts aggressive signaling to mirror and conspecific reinforcers. Top panel: feedback from mirror presentations are phased-locked with the aggressive display of the fish, while conspecific presentations allow for a natural exchange of sending and receiving of aggressive signals. Bottom panel: the addition of fluoxetine disrupts the sending of aggressive displays. Within a mirror reinforced preparations this leads to degradation in the feedback signal that is absent when conspecifics are utilized as the social reinforcer.

Past studies have demonstrated that Siamese fighting fish demonstrate greater acquisition in instrumental tasks reinforced with mirror over moving model presentations [19], and responded with greater amounts of gill flaring to mirrors then conspecifics (Dore, Lefebrve, & Ducharme [4]); providing evidence that the arousal of fish can be manipulated through social reinforcer selection. Likewise, Elwood et al. [9] and Arnott, Beatie, & Elwood [2] have provided evidence of different patterns of responding and lateralization of displays in agonistic encounters using conspecifics that are absent in mirror elicited displays. With respect to the work by Elwood and colleagues, the results indicate that the natural exchange of signaling behavior between conspecific males produces very different behavioral states than mirror-elicited behavior. Taken together, the noted differences between mirror elicited and conspecific elicited aggression provide a natural means by which social reinforcer saliency can be manipulated, and more importantly disentangled from possible motor inhibition effects due to the phase locked nature of mirror presentations.

The present study was designed to disentangle motoric and motivational explanations for fluoxetine's behavioral role in male aggression by comparing between mirror and conspecific presentations as reinforcing stimuli within the Go No-Go discrimination task of Eisenreich and Szalda-Petree [8], in addition to measuring swimming behavior within a normal swim task. Under the proposed motivational salience account, fluoxetine functions to lower the arousal of fish to unconditioned stimuli in the external environment, and as such alters the eliciting power of social stimuli and the reinforcing nature of aggressive display behavior. Within our two tasks this should manifest in a drug by social reinforcer interaction in the discrimination task with no impairment to swimming behavior in the normal swim task when compared to controls. Stated plainly, subjects exposed to fluoxetine and reinforced with access to conspecifics should maintain the lowest amounts of aggressive behavior, express a reversed pattern of stimulus discrimination, and exhibit normal swim behavior. Alternatively, under the motor inhibition account there should be pronounced deficits in swimming behavior within the normal swim task, as well as clear reductions in both levels of aggression and discrimination independent of social reinforce type. More over, under a motor inhibition account we should see no effect of the stimulus type on experimental fish while exposed to fluoxetine.

2. Methods

2.1. Subjects

All subjects were cared and housed for in accordance with guidelines established by the University of Montana IACUC. 28 male *Betta splendens* were obtained from Live Aquaria, and housed individually on a 12 h light/dark cycle, with an average tank temperature 27 °C.

2.2. Apparatus and materials

2.2.1. Housing

Each fish was housed individually in their own tank of approximately 67.3 cm × 40.6 cm × 16.8 cm (L × W × H), containing 20 l of dechlorinated water. Each tank contained brown gravel, a T-Maze, heater, thermometer, and a bubbler hooked up to an airstone. The T-maze, in which the fish lived, measured approximately 53 cm × 20 cm × 10 cm (L × W × H). All fish were maintained on a diet of Tetra mini betta pellets, receiving 8 pellets a day.

2.2.2. Drug preparation

Fluoxetine HCL was obtained from TCI American, and a stock solution of 0.5 mmol was created. Drug exposures were accomplished by

mixing 4 ml of the stock solution with 196 ml of the tank water from individual fish in a separate dosing chamber to create a 10 μmol concentration of fluoxetine. Fish in the control group were given sham exposures consisting of placing the fish in a container filled with 200 ml of their own tank water. All exposures, sham or fluoxetine, occurred for 30 min approximately 3 h. before the start of daily trials.

2.3. Procedure

Prior to being entered into the experiments, all fish were screened for their aggressive behavior and overall health. Screening consisted of exposing the fish to a mirror for 1 min and recording the duration and latency of the display. Any fish that failed to respond consistently to the mirror presentation within a minimum of 20 s was eliminated from the study. All fish that successfully passed initial screening were then randomly assigned to one of four conditions, control mirror stimulus, control fish stimulus, experimental mirror stimulus, or experimental fish stimulus, n = 7 per group. All behavioral testing occurred within the home tank of subject fish.

To assess the impact of Fluoxetine on behavior across the two social reinforcers, an AB within between design was utilized, in which fish in the experimental group were first trained in the task while being exposed to a 10 μmol concentration of fluoxetine and then given sham exposures in part B. In a similar manner control fish were trained within the task and given sham exposures for the entire duration of the experiment. This allowed for a comparison of how fluoxetine impacts learning within the task between control and experimental groups, as well as an analysis of interaction effects between the drug treatment and social-reinforcer type. The shift from exposures of fluoxetine to sham exposures was determined by the establishment of consistent responding by the two control groups within the task for five days. Based on previous work in our lab, consistent performance typically occurs after 10 days of training. For the present study consistent behavior was established within 20 days and served as the transition point between the A and B portions.

Following the procedure of Eisenreich and Szalda-Petree [8], a go no-go task was utilized. In short, the task consisted of training fish to swim down a straight alley-way maze, with removable portal doors into a goal box within which either a social reinforcer (mirror or conspecific) or a blank white wall was placed. This allowed for the examination of SD+ "go" trials in which the pattern on the portal door signaled the presence of the social reinforcer, and SD− "no-go" in which the portal door pattern signaled the absence of the social reinforcer. All discriminative stimuli were counterbalanced across the control and experimental groupings and trials were run in two blocks of 5. That is fish were exposed to 5 trials of the SD+ stimulus and then 5 trials of the SD− stimulus with an ITI of 60 s. Social reinforcement in the form of a mirror or conspecific was presented after entry into the goal box in SD+ for 30 s, while SD− trials consisted of a 30 s presentation of a white wall. During task performance the time taken to swim down the maze into the goal box, as well as the aggressive behavior towards the social stimulus was measured. Aggressive behavior was quantified as the presence of gill flaring during the 30 s social reinforcement period.

To create the conspecific social reinforcer, fish were screened in the same manner as described above and housed in separate tanks from subject fish. At the start of daily experimental trials, these stimulus fish were placed into clear containers filled with their own tank water and placed at the end of the alley maze to form the back wall of the goal box during SD+ trials. Stimulus fish were alternated between each subject fish in the conspecific social reinforcer condition following a block design. Conspecific socially reinforced subject fish were broken into groups of four, two experimental fish and two controls, and were randomly assigned to a group of five stimulus fish. This allowed for a daily rotation through a consistent set of five conspecifics throughout the entire experiment.

To assess the impact of Fluoxetine on normal locomotive behavior, fish were placed for 2 min periods into the rectangular T-portion of their home tank and video recorded. Video recording occurred after the establishment of stable behavior in the discrimination task and occurred on the last three days of part A and part B. Movement was assessed by overlaying a 1 cm × 1 cm grid over the recorded video and counting the number of grid crossings within the recorded 2 min period. In order to minimize experimenter bias, all behavior was recorded by assistants who were blind to the experimental condition of the fish.

3. Analysis

Go No-Go task data was averaged over the last five days of experimental sessions for both part A and part B, while movement data for the normal swim task was averaged over the last three test days in both part A and part B. Due to equipment failure, one day of movement data was unavailable for six control fish in part B (four from the mirror group and two from the conspecific group). Data for these six subjects was excluded from analysis in part B. Averages were computed for all fish on the experimental measures of latency to enter the goal box for SD+ and SD− trials, the percentage of SD+ trials in which the fish responded aggressively to the social stimulus, and the number of grid crossings during the normal swim task. A 2(group) × 2(reinforcer type) repeated measures ANOVA was used to examine differences between the control and experimental groups and stimulus types across the two time points. Additionally to further assess any impacts on learning, a ratio of latencies in SD− trials to the total latency of both SD+ and SD− trials for the first 20 and last 20 days was computed across two-day blocks. This created a preference ratio that is bounded between 1 and 0 such that as the latency to SD+ trials decreased the ratio approaches 1, thus indicating a motivating preference for the signaled reinforcer, similar to ratios utilized in assessing pavlovian instrumental transfer effects [12]. Preference ratios were then utilized to predict the percentage of fighting during SD+ trials using a linear regression model that included the interaction term between experimental grouping and social stimulus type. This allowed for an analysis of changes across the four conditions over the daily sessions.

4. Results

The analysis of latency data revealed a significant interaction effect across the two time points between the control and experimental drug groups and the two stimulus reinforce types, $F(1,24) = 5.492$,

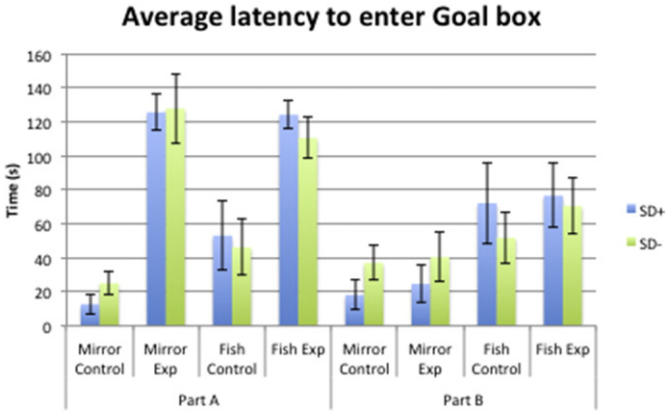

Fig. 2. Average latency to enter the goal box for the excitatory "go" stimulus signaling the presence of the social reinforcer (blue bars) and the inhibitory "no-go" stimulus signaling the absence of the social reinforcer (green bars) across the two experimental groups and social reinforcers. Fluoxetine exposures occurred for the experimental group only in Part A. Error bars are the SEM.

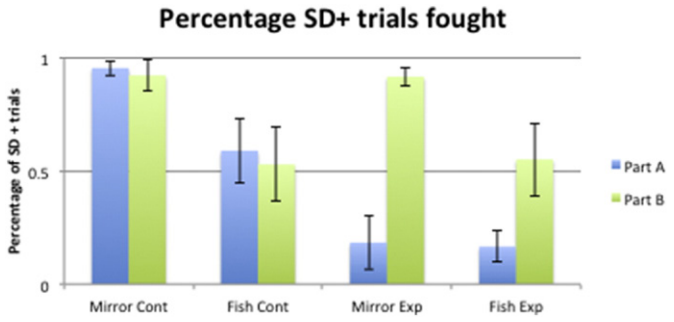

Fig. 3. Fight percentages across the four experimental conditions between fluoxetine exposures in part A (Blue bars) for the experimental group and sham exposures in part B (Green Bars). Error bars are SEM.

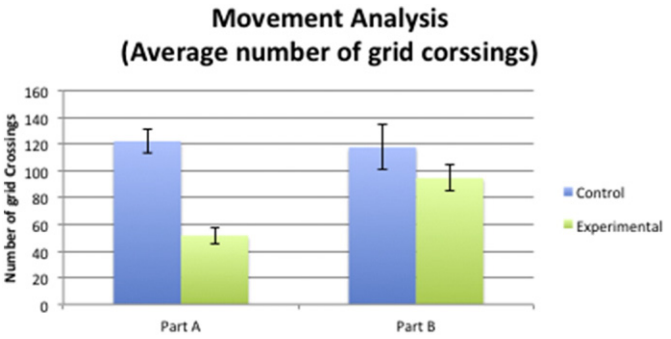

Fig. 5. Average number of grid crossings between experimental (green bars) and control (blue bars) within the rectangular portion of their home tank across fluoxetine exposures for the experimental group in part A and sham exposures in part B.

$p = 0.028$, $\omega^2 = 0.0547$. This 2(group) × 2(reinforce type) within subjects interaction effect broke down into a between subjects effect of significantly longer latencies for the experimental group when exposed to fluoxetine in part A than in part B ($F(1,24) = 47.020$, $p < 0.001$, $\omega^2 = 0.4105$), with a significantly greater overall decrease in latency occurring for subjects reinforced with the mirror social stimulus in part B, $t(12) = 2.5603$, $p = 0.025$, $d = 2.569$, (Fig. 2). Concurrently the analysis of fight percentages using the same 2(group) × 2(reinforce type) repeated measures ANOVA revealed a significant interaction between the control and experimental groups across the two time points, $F(1,24) = 37.998$, $p < 0.001$, $\omega^2 = 0.4061$, with the experimental group demonstrating suppressed fighting compared to control subjects when exposed to fluoxetine in part A that was abolished in part B, further supporting previous results demonstrating fluoxetine's effect in suppressing aggression. Contrary to expectations, there was only a marginally significant effect of the reinforcer type between the two groups across the two time points on fighting behavior, $F(1,24) = 3.366$, $p = 0.079$, $\omega^2 = 0.0454$, indicating a conserved effect of fluoxetine on aggression independent of the social reinforcer (Fig. 3).

In addition to the above results, the regression analysis of the preference ratio, drug grouping, social reinforcer, and drug by social reinforcer interaction in predicting fighting behavior provided further evidence of fluoxetine's effect on motor performance. Within the first 20 days the regression equation provided a significant estimate of fighting behavior $F(4,35) = 17.68$, $p < 0.001$, $R^2 = 0.668$, with significant slopes for both the preference ratio, $\beta = 1.163$, $t = 2.624$, $p = 0.012$, and drug grouping, $\beta = 0.142$, $p < 0.001$. While the slopes for both the social reinforcer type and the interaction of drug grouping and stimulus type were not significant (Fig. 4). During the last 20 days, the regression equation retained a significant predictive estimate of fighting behavior, $F(4,35) = 12.226$, $p < 0.001$, $R^2 = 0.5828$, with significant slopes for the preference ratio $\beta = 1.067$, $t = 2.963$, $p = 0.005$, as well as the social reinforcer type, $\beta = -0.0614$, $t = -2.25028$, $p = 0.03$ (Fig. 4).

Lastly, the movement analysis revealed a significant reduction in the overall number of grid crossings between the experimental subjects exposed to fluoxetine and sham exposed controls, $t(26) = 6.558$, $p < 0.001$, $d = 2.482$ in part A. When transitioned to sham exposures in part B, the experimental subjects exhibited no significant difference in comparison to control subjects $t(18) = 1.247$, $p = 0.228$, $d = 0.588$ (Fig. 5).

5. Discussion

A variety of reports have demonstrated the SSRI fluoxetine impacts aggression in Siamese fighting fish. Eisenreich and Szalda-Petree [8] put forward two possible behavioral mechanisms by which fluoxetine may be altering behavior within associative contexts, namely through motivational or motoric pathways. In pursuit of distinguishing between these two explanations, the present study chose to capitalize on known behavioral differences in fighting fish reinforced with mirror or conspecific social stimuli. Principally, mirror social reinforcement is limited in the amount of natural signaling feedback between the sender and the mirror reflection due to the phase locked nature of the mirror. By utilizing conspecific social reinforcement, the phased lock coupling of sender and the feedback signal can be compared to a natural flow of signaling between a subject sender and stimulus receiver. Within the context of motivational and motoric mechanisms, two fundamental patterns of behavior arise from this experimental set up. First under the motivational account, fluoxetine functions to reduce the rewarding value of engaging in aggressive display behavior by altering the saliency of social stimuli. Within the experimental setup, motivational effects should manifest as a drug by social reinforcer type interaction, with drugged conspecific reinforced fish exhibiting reverse patterns of responding when compared to drugged mirror reinforced fish within the Go No-Go task. Furthermore, within the normal swimming task, there should

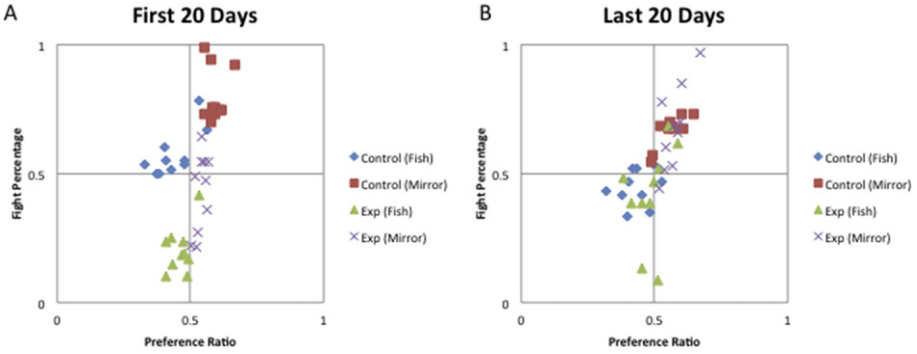

Fig. 4. Average daily preference ratios plotted against fight percentages for each of the four stimulus × drug groupings from the first 20 days of the experiment (plot A) and the last 20 days (plot B). The preference ratio is bounded between 1 indicating shorter latencies during social reinforced trials than non-reinforced trials and 0 indicating the opposite.

be no differences in the amount of swimming behavior between control and experimental fish under a motivational mechanism. By contrast, the motor account of fluoxetine's action predicts pronounced deficits in motor behavior between the control and experimental groups within both the normal swim task and response latencies within the Go No-Go task independent of reinforcer type. With respect to these two patterns, our results appear to favor a motor impairment account for fluoxetine's behavioral mechanism of action.

Principally, within the normal swim task we found clear reductions in the overall amount of swimming behavior between control and experimental fish that were abolished with the removal of fluoxetine exposures, an effect that is hard to explain through a motivational mechanism. In a similar manner there was a marked reduction of aggressive responding to both social reinforcers, and an increase in the latency to enter the goal box between experimental subjects and controls. Furthermore, the obtained latencies for either discriminative stimulus in both this experiment and in Eisenreich and Szalda-Petree [8] were close to or at the maximum allowed latency for the trial for fish exposed to fluoxetine. In examining differences between the two social reinforcers, we saw a similar pattern of responding in both experimental groups between fluoxetine exposures and sham exposures. While exposed to fluoxetine, subjects exhibited decreased aggression and longer latencies to enter the goal box regardless of social reinforcer types. However, when shifted to sham exposures the two experimental groups exhibited marked increases in aggression that matched their social reinforcer controls, as predicted from the motor sedation account.

Taken together, the above pattern of results fits nicely with a motor inhibition hypothesis for serotonergic function. Under a motor inhibition account, serotonergic projections serve to modulate motor response circuits for controlling the coordination and vigor of behavior. Applied within the associative structure of our task, the exposure to fluoxetine serves as a brake on the engagement and performance of aggressive behavior, and as such breaks up the typical stimulus control of the discriminative stimuli. In the case of mirror reinforced subjects, the suppression of motor performance results in both the mirror losing its salience as a social reinforcer and produces the robust rebound in aggressive responding towards the mirror with the removal of fluoxetine, due to the phased locked nature of the mirror's salience with motor performance. Furthermore, while motor performance is suppressed normal associative processes related to the establishment of dominance hierarchies appear to be unimpaired, as subjects reinforced with conspecific presentations demonstrated a rebound effect in aggressive behavior similar to the level of conspecific reinforced controls after fluoxetine removal. This latter observation implies that within our chosen model system fluoxetine's suppression of motor behavior modulates associative learning through signaling feedback from the social reinforcer, and may explain some of the social context dependent effects of fluoxetine on aggression and courtship behavior noted in the literature [11].

Applied to the wider role of how the serotonergic system functions within complex behavior, the role of fluoxetine in suppressing motor behavior fits well with the wide variety of reports across aquatic species and opens an interesting avenue for future studies. In particular, recent reports have indicated that fluoxetine exposure reduces boldness via decreased exploration and swimming behavior within novel environments and social contact tasks in both male and female Siamese fighting fish [6,7], as well as in the Japanese rice fish medakka [1]. Furthermore, Dugatkin and Alfieri [5] demonstrated a positive link between boldness in predator inspection and associative learning in guppies (*Poecilia reticulate*), while Frost et al. [10] have demonstrated links between boldness and aggression in trout (*Onchorhyncus mykiss*). Our own work [8] has demonstrated clear impairments to associative process for social rewards after fluoxetine exposure, providing support to the notion of a combined boldness-aggression behavioral axis that is modulated by serotonergic circuitry. It may be that serotonergic pathways serve to modulate the activation of motor patterns in response to environmental stimuli similar to the proposal by Homberg [13], in which serotonin is argued to govern a vigilance system that controls behavior in response to internal and external stimuli. Indeed, the work by Kravitz and colleagues (see Kravitz et al., [15] for review) has isolated such a role for serotonergic neurons in the mediation of social aggression and dominance in lobsters. With respect to our own work, it is likely that serotonergic pathways and corresponding activity underlie a common biological marker of a bold-aggressive behavioral syndrome. Future studies should focus on elucidating how modulations of motor pathways contribute to the complex array of behaviors serotonin has been implicated in.

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