Fish on Prozac: a simple, noninvasive physiology laboratory investigating the mechanisms of aggressive behavior in *Betta splendens*

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Lynn SE, Egar JM, Walker BG, Sperry TS, Ramenofsky M. Fish on Prozac: a simple, noninvasive physiology laboratory investigating the mechanisms of aggressive behavior in *Betta splendens*. Adv Physiol Educ 31: 358–363, 2007; doi:10.1152/advan.00024.2007.—The neuromodulator serotonin is an important regulator of aggressive behavior in vertebrates. Experimentally increasing synaptic levels of serotonin with fluoxetine, a selective serotonin reuptake inhibitor, has been shown to reliably decrease the expression of aggressive behavior. Here, we describe a method by which fluoxetine can be noninvasively administered to male *Betta splendens* (an attractive model for the study of aggressive behavior) and describe a simple laboratory exercise that allows students to experimentally investigate the physiological mechanisms of aggressive behavior. We demonstrate that relatively short-term exposure (3 h) of male bettas to as little as 3 μg/ml of fluoxetine-treated aquarium water is sufficient to reduce the expression of specific aggressive behaviors. We emphasize the physiological concepts that can be addressed with this exercise, including the role of the serotonergic system in regulating aggression, and the interplay of environmental contaminants and physiology in regulating the expression of behavior. We also highlight important aspects of experimental design. This exercise can be flexibly altered to accommodate one or several laboratory periods. It is also low cost, is low impact to the animals, and requires minimal preparation time for instructors.

fluoxetine

UNDERGRADUATE LABORATORY EXERCISES that illustrate basic principles in vertebrate physiology are often expensive, difficult to set up and replicate, time consuming, and involve invasive procedures. Here, we describe a laboratory exercise that allows students to effectively explore the mechanisms of aggressive behavior in vertebrates but that does not require expensive equipment, lengthy setup, or excessive handling or manipulation of animals.

The expression of agonistic behavior over territory boundaries allows individuals exclusive access to resources (e.g., food, mates, and nesting sites) that are important for both reproduction and survival. Territorial aggression may take on a variety of forms, including vigilance and patrolling behavior, exhibition of stereotyped displays, vocalizations, chasing intruders, and engaging in physical combat (2).

Useful model species for the study of aggression can be found throughout the vertebrate classes. However, in an undergraduate laboratory, low cost and ease of maintaining animals are important considerations. The Siamese fighting fish *Betta splendens* (hereafter also referred to by the common name, “betta”) has long been an attractive model for studying aggressive behavior in the laboratory (5). Bettas are found in freshwater ponds of Southeast Asia and are also cultivated as ornamentals and are readily available in pet stores around the world. Both wild and captive-bred males exhibit strong and stereotyped aggression in defending their territories against intruding male conspecifics (18, 28). Patterns of aggressive behavior include frontal displays (erecting the operculae, fins, and tail while facing an opponent), lateral or broadside displays (swimming with the side of the body facing opponent accompanied by erection of fins and tail), and physically attacking and biting the intruder. Males may also intensify scale coloration during an agonistic interaction (8, 28). Bettas are particularly useful for student laboratory investigations of agonistic behavior because aggressive behavior patterns in this species are easy to observe and quantify, and males will exhibit high levels of aggression when presented with a mirror or an appropriate releaser (1, 25, 31, 32).

The neuromodulator serotonin [5-hydroxytryptamine (5-HT)] is an important regulator of aggressive behavior in vertebrates. For example, across a variety of vertebrate classes, serotonergic activity is increased in less aggressive males compared with more aggressive males (4, 22, 33). Experimental increase of serotonin levels or serotonergic activity has also been shown to reduce aggression in fish, reptiles, birds, and mammals (9, 12, 13, 16, 23, 24, 29, 30). Fluoxetine (trade name: Prozac) is one of a class of drugs called “selective serotonin reuptake inhibitors” (SSRIs), which block the neuronal reuptake pump for serotonin, effectively increasing levels of serotonin at synapses and suppressing aggressive behavior (21, 26). Thus, fluoxetine can be a useful experimental tool for manipulating the action of serotonin on target cells and subsequently exploring the role this neurotransmitter has in regulating aggression in vertebrates.

Perreault et al. (24) demonstrated that both acute and chronic administration of fluoxetine via intraperitoneal injections significantly reduced the expression of aggressive behavior in a coral reef fish, the bluehead wrasse (*Thalassoma bifasciatum*). Clotfelter et al. (9), however, recently demonstrated that chronic administration of fluoxetine via intramuscular injections did not alter the expression of aggressive behavior in male *B. splendens*. This lack of effect may have been related to species-specific differences, issues of dose and/or duration of treatment, or route of treatment (9). Injections are often a useful and reliable method of treating animals. However,
injections for small fish present several problems in an undergraduate laboratory setting. For example, the handling associated with injections can impact subsequent behavior and can also affect the fishes’ mucous coat, potentially promoting infection and death following experimentation (S. E. Lynn and M. Ramenofsky, unpublished observations). To avoid these problems as well as to minimize stress associated with capture and handling, we have developed a technique that allows for epithelial uptake (presumably primarily via the gills) of fluoxetine in male bettas, followed by controlled testing for aggressive responses to a mirror challenge.

In addition to its utility as an experimental tool for investigating the role of serotonin in regulating aggression in fish, epithelial uptake of fluoxetine from the surrounding water may also have ecological relevance. SSRIs such as fluoxetine are the most widely prescribed antidepressants in the United States (17). Recent studies (6, 20) have indicated that antidepressants including fluoxetine are discharged in municipal wastewater treatment plant effluents and are widespread in surface waters of the United States. Thus, aquatic animals may be exposed to concentrations of SSRIs, at levels high enough to possibly alter both physiology and behavior. For example, populations of several species of fish living in a municipal effluent-dominated stream reportedly contained concentrations of fluoxetine (as well as other pharmaceuticals) at levels higher than 0.1 ng/g in liver, brain, and muscle tissues (7). Epithelial uptake represents a plausible mechanism for the uptake of environmental contaminants in body tissues of bettas (14, 15).

Thus, an undergraduate laboratory exercise that focuses on fluoxetine’s actions on the brain in fish can also prove useful to highlight the growing ecological relevance of this compound as an environmental contaminant. To this end, we describe a simple laboratory exercise that combines epithelial uptake of fluoxetine in male B. splendens with observations of staged aggression trials. This laboratory exercise allows students to experimentally investigate multiple physiological concepts. Depending on the focus and level of the course being taught experientially investigate multiple physiological concepts. Instructors may wish to highlight one or several of these concepts.

The aims of this laboratory exercise are to illustrate:
1. Interrelationships of physiology and behavior in B. splendens,
2. The role of the serotonergic system in the neural control of aggressive behavior in fish,
3. The mechanism of action of SSRIs and the importance of neurotransmitter reuptake pumps,
4. The interplay of environmental contaminants and animal physiology in regulating the expression of behavior in appropriate social contexts,
5. Aspects of experimental design and statistical analysis that are useful when sample sizes are low and individual variation is high.

These aims can be accomplished when students:
1. Use fluoxetine to manipulate brain physiology and subsequently record behavior as the experimental outcome (which specifically addresses aims 1–3),
2. Administer fluoxetine to bettas by treating the fishes’ water (which specifically addresses aim 4),
3. Actively participate in study design during a laboratory preparation exercise (which specifically addresses aim 5).

METHODS
Fish and Setup

We used a total of nine healthy male bettas obtained from two local pet stores in Wooster, OH. Males were housed singly in 10-gallon aquariums equipped with aquarium stones, dechlorinated water, and an ammonia/carbon filter (Ammocarb, Aquarium Pharmaceuticals). Tanks were covered on three sides with white paper so that fish could not see other males in neighboring tanks. One long side of the tank was left uncovered for behavioral observations. On this side of the tank, a scale was placed along the bottom of the tank to measure the position of the fish (to the nearest centimeter) relative to the mirror to determine the distance from the “intruder.” Males were held under these conditions for 14 days before the experiments were begun. They were fed fish food formulated for bettas (Betta bites, HBH pet products) daily for the duration of the study.

Treatment Protocol and Behavioral Recording

A stock solution of 1 mg/ml fluoxetine HCl (Sigma F-132) was prepared in distilled water and kept in a foil-wrapped container at 4°C until use. We tested the efficacy of two different doses of fluoxetine, each with a different duration of exposure. Fish were exposed to fluoxetine-treated aquarium water or untreated aquarium water (control condition) for either 3 h (experiment A) or 5 h (experiment B). In both experiments, fish were removed from their home tanks with a dip net and quickly placed in a small, transparent container (200 ml total volume) with a tight-fitting lid that had a hole in the center (the containers in which bettas are often found at pet stores are ideal treatment containers). The closed container was then floated in the animal’s home tank for the duration of the treatment to ensure that water temperature and visual cues associated with the animal’s immediate surroundings were not drastically changed during the treatment period. In experiment A, males were placed in a solution of 3 μg/ml fluoxetine in aquarium water (experimental condition) or untreated aquarium water (control condition) for 3 h. In experiment B, males were placed in a solution of 6 μg/ml fluoxetine (experimental condition) or untreated aquarium water (control condition) for 5 h. At the conclusion of the treatment period, fish were removed from the treatment containers using a small dip net and were quickly placed in their home tanks. Fish were then allowed 15 min to acclimate to their home tanks.

Following the 15-min acclimation period, a mirror (24 × 27 cm) was placed in the water at one end of the tank with the reflective side facing the fish (so that the fish could immediately see its reflection), and the latency to the first aggressive display toward the mirror was recorded. Once the fish demonstrated a response toward the mirror, we recorded the following behaviors for 5 min: time (in s) with operculae flared and time (in s) spent in a broadside display within 10 cm of the mirror (Fig. 1). We also measured the number of 90° turns each male made during the threat display as an indicator of overall stimulation during the aggressive display period. We recorded distance from the mirror (in cm) every 5 s for the duration of the 5-min testing period to calculate the average distance each male kept from the mirror during the display period. At the conclusion of the behavioral trial, the mirror was removed from the tank.

The same males were tested under both control and fluoxetine conditions in both experiments (2 males were not tested in experiment A; thus, for experiment A, n = 7 males and for experiment B, n = 9 males). The order of treatment was randomized within each experiment, and fish were allowed at least 7 days between treatments prior to subsequent tests within an experiment. Experiment A was conducted in November 2005, and experiment B was conducted in April 2006. All procedures described here were approved by The College of Wooster’s Institutional Animal Care and Use Committee.
Pairs with operculae flared were normally distributed and thus tested using (see RESULTS). This will ensure that treatments are complete prior to setting fish up in treatment containers 3–5 h prior to behavioral testing. Instructors should home institution (e.g., approval through the school’s Institutional Animal Care and Use Committee or other such regulatory committee).

Fish should be set up in individual tanks as described above, preferably at least 7 days prior to the laboratory session. Instructors should set fish up in treatment containers 3–5 h prior to behavioral testing (see RESULTS). This will ensure that treatments are complete prior to the beginning of the laboratory period and will also enable students to be “blind” to the experimental treatment (fluoxetine or control) while performing observations. Gloves should be worn any time fluoxetine solutions are prepared or used by instructors and students.

Depending on the size of the laboratory, students may work in groups of 2–4. A group of four students is ideal if instructors wish for students to test all behavioral variables we described previously, although students and instructors may wish to focus on a smaller number of variables so that students can work in smaller groups. Because fluoxetine is a psychoactive compound, students should be given instructions for safe handling (e.g., gloves should be worn when transferring fish from treatment containers to tanks and, after use, nets should be placed on an absorbent surface rather than directly on the laboratory bench.) Each group will need the following: - One 10-gallon with a male betta, set up as described above. (Depending on time and resources available, student groups may use several tanks, each with a male betta.) If tanks are not marked with a scale prior to the beginning of the experiment, students should be provided with tape that can be marked every centimeter and placed along the base of the long side of the tank for determining distance from the mirror. (Students may also choose to draw a grid of 2.3 × 2.5-cm squares on the paper lining the back of the tank; the number of grids lines crossed during the trial can then be used as an index of activity.) - One mirror (24 × 24 cm) - One timer (for recording the latency to first response and for timing the subsequent 5-min display period) - Two stopwatches (1 stopwatch for timing opercular displays and 1 stopwatch for timing broadside displays)

Prior to the experiment begin run in the laboratory, it can be useful to allow students to observe and quantify aggressive behavior in one or two fish that will not be used as experimental subjects (using the equipment listed above). For laboratories in which resources are limited, instructors may wish to simply show students illustrations of aggressive postures (Fig. 1) or, prior to the laboratory period, make a video recording of aggressive displays in bettas that can be played during class time to demonstrate aggressive behavior to the students.

If time is available (e.g., during a preparatory discussion prior to the day the laboratory exercise is to be held), students will benefit from being included in the design of the study. We find it useful to pose the following problems to students and allow them to work in small groups to come up with solutions. Because each problem builds off of the previous one, the students should be provided time to come up with ideas for problem 1, and then the class can discuss those ideas as a whole. Once the class has come to a consensus on how to address problem 1, the students can then brainstorm solutions to problems 2 and 3 in small groups. A final class discussion will allow the entire class to come to a consensus on how to design the experiment.
Experimental “Problems” Students Should Consider Before the Laboratory Exercise (and Discussion Points)

Problem 1. Serotonin seems to be linked to aggressive behavior in many species of vertebrates. List several different ways you might explore whether serotonin affects aggression in male *B. splendens* in a controlled laboratory experiment.

DISCUSSION POINT: The most common answer students will come up with is to inject a group of experimental fish with serotonin and a group of control fish with equal volumes of saline that is osmotically balanced for fish. Some students will also suggest injecting experimental fish with an SSRI and control fish with equal volumes of saline. Occasionally, students may suggest running dose-response curves of either serotonin or an SSRI to determine dose effects on aggressive behavior as well. During the class-wide discussion, pros and cons of each suggestion can be listed and evaluated as a group. This is a nice time to highlight that, when faced with a question that can be addressed experimentally, many different approaches may be appropriate. At this point, the instructor should guide the class toward using an SSRI.

Problem 2. Research indicates that injecting at least one species of fish with SSRIs can alter aggressive behavior (24). Your task is to determine if these drugs have a similar effect on aggression in male *B. splendens*. However, bettas are small and you are worried that the trauma of handling and administering injections may obscure your results or harm the fish. How might you design your study to explore the effects of SSRIs on aggression without injecting your fish?

DISCUSSION POINT: Particularly if students have already studied gill anatomy in relation to gas exchange, osmotic balance, or body temperature, the followup discussion to this problem may be rather short. This is a nice opportunity to remind students about the many repercussions for fish of having such a huge surface area for exchange exposed to the water around them.

Problem 3. Often, the expression of aggressive behavior varies widely across fish. For example, one male may be considerably more aggressive than another in the same situation, simply based on past experience or genetic makeup. How might you design your study to account for the fact that you are likely to see high levels of interindividual variability in your results?

DISCUSSION POINT: In our experience, when asked to design experiments, undergraduates often tend to specify that they should have an experimental group (e.g., fluoxetine) and a control group (e.g., no fluoxetine). If only one laboratory session can be devoted to this laboratory exercise, then this type of design is most appropriate. The discussion should also include issues of sample size and statistical analyses. (With this type of design, students and instructors may wish to increase the sample size if resources permit; increasing the number of fish used will make it less likely that high levels of interindividual variability in aggressive displays will obscure treatment effects. When this type of design is used, an unpaired *t*-test or a nonparametric Mann-Whitney *U*-test is appropriate for comparing the two treatment groups). However, if the laboratory exercise can extend over two laboratory sessions, this discussion represents an excellent time to talk about the merits of a paired design in which each fish is used as its own control. (When each fish is used as its own control, potential problems of interindividual variation in aggressive displays are minimized. For this type of design, a paired *t*-test or a nonparametric Wilcoxon test is appropriate.) This discussion should include issues of randomization, sample size, and statistical analyses as well. For classes in which students have a strong statistics background, instructors may wish to have students determine which statistical analysis is most appropriate (e.g., paired or unpaired test, parametric or nonparametric statistics). Alternatively, instructors can simply state which type of analysis students should conduct.
DISCUSSION

We have demonstrated that a 3-h exposure to 3 μg/ml fluoxetine administered via aquarium water significantly altered the expression of aggressive behavior in male bettas in a laboratory setting. Specifically, when fish were treated with fluoxetine, broadside displays made within 10 cm of the mirror and 90° turns were diminished relative to control conditions. Increasing the dosage and duration of fluoxetine administration resulted in similar effects on broadside displays made within 10 cm of the mirror and 90° turns. We also found a marginally nonsignificant effect of the lower dose of fluoxetine on increasing the distance from the mirror during the agonistic display but no such effect with the higher dose. Bettas generally show very low levels of swimming activity when unstimulated (e.g., when observed without the mirror, few males swim or made any turns, regardless of treatment or dose; unpublished observations). Generally, low activity levels in untreated males in combination with the fact that all males displayed aggressive behavior for the duration of the 5-min experimental trials and had a similar latency to respond, regardless of treatment, suggests that the reduction in turns represents a reduction in aggressive display activity, not a reduction in overall activity.

Our data suggest that the serotonergic system is important in modulation of male-male aggression in bettas. Thus, our data support the one previously published study (24) that indicated that acute fluoxetine treatment can significantly alter aggressive behavior in fish. Interestingly, a recent study (9) investigating the role of the serotonergic system in regulating aggression in male bettas demonstrated that, whereas acute treatment with serotonin and a 5-HT1A receptor agonist [8-hydroxy-2-(di-n-propylamino)-tetraline] both decreased aggressive behavior, chronic administration of fluoxetine did not. Further investigations utilizing different routes of administration, doses, and lengths of exposure of fluoxetine in male bettas may provide interesting insights into the interrelationships of serotonergic modulation of aggression in vertebrates.

In our study, fluoxetine affected some measures of stereotyped aggression but not all measures. The latency to the first response, average distance from the mirror, and time with operculae flared were unaffected by either dose of fluoxetine used. Our data suggest that different agonistic displays in male bettas (e.g., opercular displays vs. broadside displays) may be regulated through different mechanisms, and this represents an interesting area for future study. Research in mammalian models has suggested that the effects of serotonin in modulating aggression, while complex, probably occur through the 5-HT1A and 5HT1B receptors (e.g., Refs. 19, 26, and 27) with a number of different hormones such as testosterone and estradiol (e.g., Refs. 3 and 10) and neurotransmitters such as serotonin and dopamine (e.g., Ref. 11) interacting to regulate the suite of behaviors associated with an aggressive response.

We have also demonstrated that epithelial uptake of fluoxetine (presumably via the gills) is a physiologically relevant route for fish. This finding has implications both for fish found in bodies of water with high levels of SSRI contamination as well as for the efficacy of administering fluoxetine to small fish for undergraduate physiology laboratories. Because we generally found similar effects of fluoxetine on behavior at both low (3 μg/ml for 3 h) and high doses (6 μg/ml for 5 h), to keep costs down and to reduce laboratory preparation time, we recommend using the lower dose for student laboratories.

We have conducted a similar exercise in laboratory classes in which we utilized injections of fluoxetine to small fish followed by observations of agonistic behavior. We feel that the design we present here is more attractive for undergraduate teaching for a variety of reasons. For example, the procedures we describe are noninvasive and low impact, effectively eliminating many problems in behavioral analysis or fish health that can occur as a result of handling and injecting fish. In addition, allowing fish to take up fluoxetine from the water allows for analysis and discussion of results to also incorporate issues of environmental contaminants and possible effects on physiology.

In summary, this laboratory exercise has the advantage of being noninvasive, low cost, and relatively simple to set up. In addition, we feel that this exercise provides an excellent opportunity for effectively incorporating student input into the design of laboratory experiments. Instructors may wish to modify behavioral observations or allow students to develop their own measures of aggression after exploring the literature on agonistic displays in bettas and/or observing agonistic displays at the beginning of the laboratory period. Assessment of student performance and comprehension of the principles illustrated by this laboratory exercise can be achieved through the evaluation of written laboratory reports that include appropriate literature sources, statistical analysis, and discussion of the results in the context of the larger body of knowledge on serotonergic modulation of aggression in vertebrates.

Fig. 3. Average distance from the mirror of male B. splendens exposed to 3 μg/ml fluoxetine in aquarium water or untreated aquarium water for 3 h (n = 7; top) and exposed to 6 μg/ml fluoxetine in aquarium water or untreated aquarium water for 5 h (n = 9; bottom) during 5-min aggressive display to a mirror placed in the home tank. Bars show means ± SE.
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REFERENCES