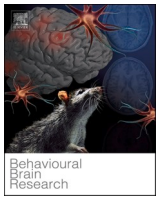
Behavioural Brain Research 442 (2023) 114317

Contents lists available at ScienceDirect

Behavioural Brain Research

journal homepage: www.elsevier.com/locate/bbr



Research report

Sub-chronic administration of fluoxetine does not alter prey-capture or predator avoidance behaviors in adult South African clawed frogs (*Xenopus laevis*).

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ARTICLE INFO

Keywords:
Xenopus laevis
Anxiety
Fluoxetine
Predator avoidance
Optic tectum
Behavioral test

ABSTRACT

Animals will halt foraging efforts and engage defensive behaviors in response to predator cues. Some researchers have proposed that the switch from appetitive to avoidance behavior resembles anxiety, but most work on this has been performed in a limited number of animal models, primarily zebrafish and rodents. We used adult South African clawed frogs (*Xenopus laevis*) to determine if the canonical anxiolytic fluoxetine alters predator-induced changes in appetitive and avoidance behavior in a laboratory-based trade-off task that mimics foraging/predator avoidance tradeoffs in the wild. We hypothesized that sub-chronic fluoxetine treatment (20 d) would not affect baseline behavior but would reverse predator-induced changes in food intake, appetitive and avoidance behavior, and the abundance of anxiety related gene transcripts in the optic tectum, a brain area central to ecological decision making in frogs. We found that fluoxetine significantly reduced baseline locomotion compared to vehicle-treated animals. Fluoxetine had no effect on appetitive and avoidance behaviors that were sensitive to predator cues in this assay and did not alter any of the anxiety-related transcripts in the tectum. We conclude that while peripheral sub-chronic administration of fluoxetine significantly reduces locomotion, it does not modify predator-induced changes in approach and avoidance behaviors in this assay. Our findings are not consistent with visual predator cues causing state anxiety in adult frogs.

1. Introduction

The American Psychiatric Association discriminates anxiety from other feelings of nervousness or anxiousness because this emotional state can be associated with excessive fear [1]. While it is difficult to envision a situation where excessive fear in the absence of a threat might be adaptive (in an evolutionary sense), there is considerable evidence that some anxiety-like behaviors heighten vigilance in response to predator cues and that such behaviors, and the neural circuits controlling them, are evolutionarily ancient. The lines of evidence suggesting an adaptive role for anxiety-like behavior are two-fold. First, despite anxiety being a more complex and less understood emotion than fear [2], we now know a considerable amount about the neuronal circuitry mediating anxiety-like behaviors in rodents. Many of these brain areas have homologs in teleost fishes, which diverged some 200 million years earlier than the first rodents, suggesting that anxiety circuits evolved millions of years ago. For example, nuclei involved in excitatory (bed nucleus of the stria terminalis, BnST; ventral tegmental area, VTA) and modulatory (locus coeruleus, LC) aspects of anxiety in rodents have identified homologs in teleosts (supracomissural portions of the ventral telencephalon are a homolog of the BnST, [3]; VTA in elasmobranchs, [4]; missing in teleosts, [5]; LC in teleosts, [6]).

The second line of evidence comes from behavioral studies in teleost fishes and suggest that aspects of anxiety-like behavior have been conserved for millions of years. Zebrafish (*Danio rerio*) have become a model system for studying the cellular and molecular basis for anxiety [7–9], but many studies also report the effects of anxiolytic drugs that enter watersheds as waste and modulate anxiety-like behavior in fishes [10,11] as do some studies on social behavior in fishes [12]. Several behavioral assays have been developed in zebrafish to study anxiety [8] and, perhaps more importantly, anxiety-like behaviors in zebrafish can be modulated by the same canonical anxiolytics used in humans to treat

Abbreviations: ANOVA, Analysis of variance; LGN, lateral geniculate nucleus; PVC, Polyvinyl chloride; RQI, RNA quality indicator; SSRI, Selective serotonin reuptake inhibitor; LGN, Lateral geniculate nucleus; SC, Superior colliculus.

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https://doi.org/10.1016/j.bbr.2023.114317
Received 28 July 2022; Received in revised form 23 December 2022; Accepted 24 January 2023
Available online 25 January 2023

anxiety disorders [13–15], suggesting conservation of the mechanisms involved in anxiety.

Identifying evolutionarily adaptive aspects of anxiety can benefit from behavioral models that use both appetitive (such as food) and aversive (such as a predator) stimuli in combination [16]. Such experimental designs model real-life decision making by animals in the wild, as many vertebrate species (including wild populations of the species most studied in the laboratory, i.e. (rats, mice, and zebrafish) must gather food under some degree of potential predation threat. We recently developed an ecologically-based tradeoff task to study visually-triggered anxiety-like behavior in adult African clawed frog *Xenopus laevis* [17]. This behavioral task employs some methodological details from previous work from Duggan et al. [18], such as a food stimulus to engage appetitive behavior, but uses a purely visual looming predator to engage avoidance behavior as well adult frogs to facilitate brain tissue collection for protein and transcript assessments. Using this task, we found that predator exposure failed to reliably alter food intake in adult *X. laevis*, even though juvenile frogs ate less during predator (using a live predator) exposure in several other studies by our research group [18–20]. Exposure to a visual predator stimulus did alter discrete aspects of behavior including decreased forelimb sweeping (a prey capture behavior in *X. laevis* [21]), decreased entrances into the predator zone are, decreased total time swimming, and increased time inactive [17].

Selective serotonin reuptake inhibitors (SSRIs) remain a most common treatment option for most anxiety disorders due to its lower risk of abuse and dependence [22,23]. SSRIs have a long history of use in animal research and reverse the effects of anxiogenic conditions in rodent preclinical models [22,24]. Serotonin also appears to play a role in anxiety-like behavior in fish based on extensive testing in *D. rerio*, although the specific effects of fluoxetine depend on which anxiety test is used and the fluoxetine dosage [9,25]. For example, fluoxetine treatment increases the amount of time *D. rerio* stays in the top area of the novel tank diving task and increases the amount of time spent in the lit portion of a light-dark test [9] whereas fluoxetine decreased time spent in the white compartment in a scototaxis test [25].

Adult *Rana pipiens* express serotonin binding sites in the optic tectum [26], the visual processing center of the amphibian brain and the major site for sensorimotor decision making involved in foraging/predator avoidance decisions. The fact that serotonergic drugs modulate synaptic activity in the *R. pipiens* tectum [27] suggests that these binding sites are biologically relevant. Sub-chronic (14 d) immersion exposure of Arabian toad (*Bufo arabicis*) tadpoles to fluoxetine reduced the hiding of tadpoles to visual and chemical cues of a natural predator (dragonfly larvae, *Anax imperator*) [28].

In this study we evaluated the effects of fluoxetine administered sub-chronically on food intake, baseline behavior, approach and avoidance behavior, and the abundance of anxiety-related mRNA transcripts in the optic tecta of adult *X. laevis*. We hypothesized that sub-chronic fluoxetine treatment would not affect baseline behavior but would reverse predator-induced changes in food intake, appetitive and avoidance behavior, and the abundance of anxiety related gene transcripts in the optic tectum.

2. Material and methods

2.1. Animals and care

Adult male *Xenopus laevis* frogs (n = 30) were obtained commercially (Xenopus Express, Inc., Brooksville, FL, USA). Frogs were maintained in flow through dechlorinated tap water in three separate fiberglass 300 L tanks (178 cm L x 46 cm W x 51 cm D) at a temperature of 23 ± 3 °C. Frogs were fed NASCO floating frog brittle three times a week during their acclimation period (7 d). After acclimation, frogs were maintained in the tanks for 20 d receiving injections and handled every other day. Frogs were maintained on a 12 hr light:12 hr dark cycle and all experiments were conducted in the dark phase under red light. The luminosity in the frog room during the light phase was approximately 350 lux and during testing was approximately 20 lux inside the tank during the dark phase. All procedures were approved by the Texas Tech Animal Care and Use Committee (protocol number 19069–07).

2.2. Freeze-brand labeling of animals

Copper branding irons, bent to form numerals 0–9, and liquid N2 were used for branding. On the fifth day after arriving, frogs were anesthetized in 0.05% MS-222 while branding surface of each branding iron was buried in the liquid nitrogen at least 1 min before use. Anesthetized frogs were branded on the ventral skin by touching the liquid N_2-cooled iron to the skin for 10 s. Frogs were returned to their home cage after recovery.

2.3. Administration of fluoxetine

Prior to starting the experiment all frogs were weighed and body mass values ranked from high to low. Frogs were systematically assigned to one of three groups; untreated, vehicle injected, and fluoxetine injected. There were no differences in body mass (n = 10 per group, 36.70 ± 1.210 g, 36.78 ± 1.528 g, 36.37 ± 1.331 g, respectively, mean + S.E.M.) prior to starting treatments.

Seven days after arrival, each frog in the fluoxetine treatment group (n = 10) was injected with 10 mg/kg of fluoxetine (fluoxetine HCl, Tocris; dissolved 0.6% NaCl) intraperitoneally (i.p., 250 μl injection volume) every other day for 20 d (Fig. 1). Thus, each frog received a total of 100 mg/kg fluoxetine over 20 d. The dosage was based upon [29]. Vehicle-control frogs (n = 10) received i.p. injections of 0.6% NaCl every other day for 20 d for a total of 10 saline injections. Untreated animals (n = 10) were removed out of the tank and handled similarly to the other groups before being placed back in the tank every other day at the same time of day as the drug and vehicle injected frogs. Normal swimming behavior was confirmed before and after each injection. 24 h after the last injection or handling session for the untreated control, frogs were individually isolated to prepare them for the tradeoff task. One animal died in each of the untreated and saline groups for a final sample size of n = 9 for untreated, n = 9 for vehicle-injected, and n = 10 for fluoxetine-injected.

2.4. Trade-off task

All lighting and temperature conditions were kept consistent with the acclimation phase. The trade-off task has been described in detail before [17] (Fig. 1). Briefly, 48 h prior to the trade-off task, frogs were individually isolated in glass aquaria (30.5 cm × 15.2 cm×20.3 cm, 38 L) containing 3 L deionized water with 0.33 g/L Instant Ocean. Twenty-four hours before the task, frogs were moved to the test arena, a glass aquarium (91.4 cm × 30.5 cm×40.6 cm, 114 L) containing 13 L deionized water with 0.33 g/L Instant Ocean. The sides of the test arena were covered with black plastic to avoid any possible light interference.

The test arena was divided into two areas of equal size, a predator zone that consisted of the area directly below the predator stimulus (described below) and the safe zone consisted of the area around the hide, with no physical barrier between the two divisions [17]. A hide (polyvinyl chloride (PVC) pipe, 9 cm × 8.3 cm diameter) was placed in the middle of the safe zone also equidistant from the sides of the tank [17].

The trade-off task was divided into three timed periods: a 10 min period with no food or predator stimulus (time period A), a 30 min period with food in the presence of a visual predator stimulus (time period B) and a 30 min period with fresh food but no visual predator stimulus (time period C). Frogs received approximately 3.5 g fresh chicken liver (Pilgrim's Pride Corporation, Greenly, CO) as the food stimulus attached to a metal washer (1.9 cm × 5.08 cm) during the time

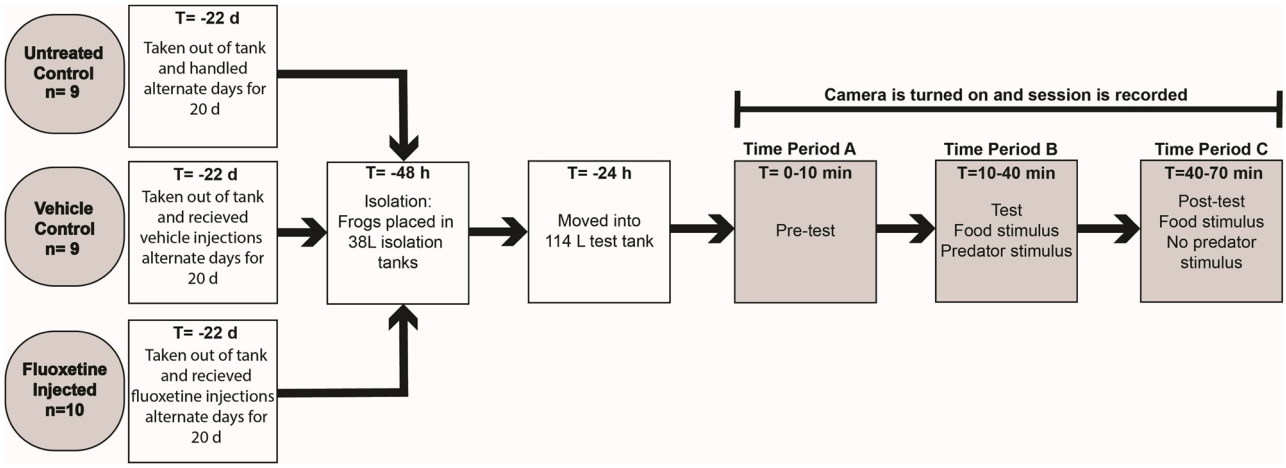

Fig. 1. Timeline for the tradeoff task. Animals were first isolated from the group housing 48 h before the testing began. The animals were moved to the test arena for 24 h prior to the test for acclimation. We recorded three simultaneous time points on the test day: time period A (no stimulus, 10 min), time period B (presence of predator and food, 30 min) and time period C (new food, no predator, 10 min). After time period C frogs were anesthetized, euthanized, and tissue was collected for RNA extraction.

periods B and C. The metal washer prevented the food from floating away from the predator zone during the tradeoff task. Each liver piece was weighed individually before and after the test.

The looming predator stimulus (preserved common snipe, *Gallinago gallinago*) was mounted on a 32 cm long wooden dowel connected to a servomotor (Hitec, SubMicroServoU, USA) allowing the head to pivot 90° back and forth every 30 s over the predator zone of the test arena. The rationale for choosing this stimulus was described previously [17]. The pivoting motion of the predator stimulus was controlled using a microcontroller (Arduino, Duemilanove, Italy). Two Energizer Vision HD+ © red light night vision headlamps were placed diagonally above the test tank between 15:00 h and 19:00 h and behavior recorded in the dark using a 12-megapixel Samsung Galaxy S9 phone camera recording at 720 P that was located above the tank.

2.5. Tissue collection

Animals were weighed immediately after the behavioral test and anesthetized in 0.5% MS-222 (with equal parts $NaHCO_3$) and euthanized by decapitation. Brains were excised and placed in RNAlater© in a Petri dish and the optic tecta removed by dissection. Both optic tecta were frozen in 10 volumes of RNAlater© in a RNAse free 1.5 mL tubes at − 20 °C overnight and stored at − 80 °C long term.

2.6. RNA extraction

Tissue stored frozen in RNAlater was thawed on ice and RNA was extracted using RNAqueous-4PCR DNA-free RNA Isolation for RT-PCR kits (ThermoFisher) as described previously [29]. This included a DNAase step to eliminate genomic DNA. Murine RNase inhibitor (New England Biolabs, 1 unit/1 mL) was added to the freshly extracted RNA and the RNA stored at − 80 °C. RNA concentration was determined using a NanoDrop ND-1000 spectrophotometer. RNA samples were reverse transcribed to cDNA using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. A control RNA group treated with the High Capacity cDNA Reverse Transcription Kit but not reverse transcriptase was checked by qRT-PCR. cDNA was stored at − 20° C.

2.7. Primer design and quantitative real time PCR (qRT-PCR) assay

Primers were constructed for serotonin-1A receptor (5-HT1A) (*htr1a*), dopamine D1 (*drd1*) and D2 receptor transcripts (*drd2*) using PrimerBlast (Table 2). Primers for ribosomal protein L8 (*rpl8)* and corticotropin releasing factor (*crf*) were acquired from previous literature [30–32]. *Rpl8* was used as a reference gene since it has been shown it has a constant and ubiquitous expression in *X. laevis* [33]. GenBank Accession Numbers for the *rpl8*, *crf*, *htr1a*, *drd1* and *drd2* are as follows U00920.1, S50096.1, NM_001085830.1, XM_018251699, NM_001101742. These genes were selected due to their established role

Table 1
Ethogram for the quantification of predator avoidance and prey capture in adult *X. laevis* frogs.

ME	Behavior	Code	Time of measure	Descriptive/Definition
	Latency to move*	a	duration	Start as soon as liver is dropped in the tank, stopped as soon as frog exhibits directional movement
	Latency to contact*	q	duration	Start as soon as liver is dropped in the tank, stopped as soon as 1/3 of frogs' body (head/arms) are in contact with liver
	Sweeping*	k	count	Random appetitive searching component that involves forelimb extension in quick succession
	Contact with food*	u	duration	Frog is touching or holding food, first 1/3 of frog body is in continuous contact with the food
@	Swimming towards safe zone	d	duration	Frog is actively swimming towards the safe side of the tank
@	Swimming towards the predator zone	f	duration	Frog is actively swimming towards the predator side of the tank
@	Inactive*	c	duration	Frog is resting on substrate or surface, and frog is still/not moving
	Time spent in safe zone		duration	Total time frog spends in the safe side of area, calculated manually
	Number of entrances into predator side		count	Amount of times frog enters the predator area (must be 3/4th of the frogs' body and continuous movement)

ME denotes behaviors that are mutually exclusive, if behaviors share a symbol (@), they cannot occur at the same time. Code refers to the keystroke entered by the researcher during the behavioral analysis step. Behaviors denoted with an asterisk (*) are prey capture behaviors identified in previous studies [21].

Table 2
Oligonucleotide primers used to amplify *X. laevis* genes.

Gene	GenBank Accession Number	FWD primer (5′−3′)	REV primer (5′−3′)	Product Length	Reference	Intron spanning
rpl8	U00920.1	GACATTATCCATGATCCAGG	GGACACGTGGCCAGCAGTTT	480	31	Y
crf	S50096.1	TCTCCTGCCTGCTCTGTCCAA	CTTGCCATTTCTAAGACTTCACGG	321	30	N
htr1a	NM_001085830.1	TCTGACGCACTCGCTCGCTTC	TCCTCGGGCGACACTCCT	157	This paper	Y
drd1	XM_018251699	GAACGTTAAGCAACGCCCTC	CACTTCTACTATAAGGCTCAGTTGC	176	This paper	N
drd2	NM_001101742	GACAAGTGCACTCACCCTGA	CGGTGGAGATGTGCTTGACA	159	This paper	Y

in preclinical models of anxiety and depression (*htr1a*, [34,35]); *drd1* and *drd2*, especially D1-D2 heterodimers, [36]; *crf*, [37]).

Primer and template concentrations were measured using a Nanodrop ND-1000 spectrophotometer. Polymerase chain reaction (PCR) reactions were carried out on 96 well optical plates (Applied Biosystems, Grand Island, NY), consisting of 1ul diluted cDNA template (200 ng cDNA), 1ul of forward primer (200 nM), 1ul of reverse primer (200 nM), 12.5 μl of SYBR green PCR master mix, and nuclease free water for a total volume of 25 μl. Non-template controls included all other reagents except cDNA templates, which were replaced with nuclease-free water instead. Plates were then centrifuged and loaded onto an CFX96 RT-PCR detection system (Biorad). Amplification efficiency for the primer sets were determined using a 10-fold serial dilution (300, 30, 3, 0.3, and 0.03 ng) of the template and calculating the slope of the regression plotting Ct values against the log of the template amount [17]. Cycle threshold values were normalized using the *rpl8* reference gene and expressed as a percentage of control values using the ΔΔCt method [38] and by using a linear ratio method [39]. Ct values above 31 were not used in the analyses.

2.8. Behavioral analysis

We scored behaviors using JWatcher (Macquarie University, http://galliform.bhs.mq.edu.au/jwatcher/) software was as per the handbook's instructions [40]. All videos were assigned a randomized code and behavioral scoring was conducted after the live portion of the experiment was over. Behavior was analyzed using an ethogram based on previous literature with modifications [18,20] (Table 1). Behavioral data were double-blinded and analyzed by three separate individuals without observation from others, and the scoring methods between the observers were correlated using an inter-rater agreement calculated on JWatcher (inter-rater agreement % $\geq$0.84). The duration measurements were measured manually with a stopwatch. All durational behavioral measurements were corrected for time spent in the tank or total time spent moving as appropriate. The number of forelimb sweeps and entrance into the predator section were recorded as counts.

2.9. Statistical analysis

Statistical analyses were performed using SPSS (v. 29). Before modeling we first attempted factor reduction using principal component analysis (PCA) on the dependent variable dataset using SPSS, however the Kaiser-Meyer-Olkin measure of sampling adequacy (KMO = 0.466) indicated inadequate sampling for PCA.

The dependent variables evaluated were food intake (periods B and C only, mass consumed/body mass), latency to contact food, time in contact with food (B and C only), number of forelimb sweeps, number of entrances into predator section, latency to move, percent of total time swimming, percent total time inactive, percent time swimming toward the safe zone, percent time swimming toward predator zone, percent time spent in safe zone, percent time in predator zone, and the relative ct values for the transcript analysis. The number of sweeps and number of entrances into the predator section were analyzed as raw counts. Latency to move, total time swimming, time inactive, and total time in safe section were analyzed as a percentage of the total time in the tank. The percent time swimming toward the safe zone or predator zone were analyzed as a percentage of the total time spent swimming. The independent variables were fluoxetine treatment (between subjects) and test period (within subjects).

We explored behavioral data for normality and homogeneity of variance and found that the assumptions of normality required for general linear mixed effect modeling were not met after data transformation. Thus, we used generalized estimating equations (GEE) and generalized linear mixed effect modeling (GLZX) to evaluate the behavioral data with fluoxetine treatment as a fixed variable and time period as a repeated measures variable, and animal id as a random factor in mixed effect models. Estimated marginal means were contrasted pairwise with Sidak's correction for multiple comparisons. Count data (sweeps, entrances into predator zone) were analyzed using a negative binomial probability with a log link function. We analyzed proportion using a gamma distribution with log link. For gamma distributions, any zero values were replaced by $n/4$ where n is the lowest measured value in the dataset. Quasi likelihood under independence model criterion and Akaike information criterion values were used to assess goodness of fit in GEE and GLZX, respectively. qRT-PCR data were analyzed using Kruskal-Wallis tests as the data failed normality assumptions after transformation.

3. Results

3.1. Effects of fluoxetine on sweeps and number of entrances into the predator zone

All statistical results are summarized in Table S1 (Supplemental Materials). Only one of the 28 frogs ate food during period B, none ate during period C. Thus, food intake and latency to contact food were not analyzed, although forelimb sweeps, a food gathering behavior, was analyzed. While there was no main effect of fluoxetine treatment on the number of sweeps ($p = 0.353$), there was a significant main effect of time period ($\chi^2 = 70.39$, df = 2,79, $p < 0.001$). Pairwise comparisons with Sidak correction revealed that frogs carried out more sweeps in period C than in period A (no food, no predator, $p < 0.001$) or period B (food+ predator, $p = 0.009$) (Fig. 2A). As with sweeps, there was no main effect of fluoxetine treatment on the number of entrances into the predator section ($p = 0.838$) although there was a significant main effect of time period ($\chi^2 = 15.35$, df = 2,79, $p < 0.001$). Based upon pairwise comparisons with Sidak correction, frogs moved more often into the predator section in the absence of a predator and the presence of food (period C) than in period A ($p = 0.008$) or period B ($p = 0.002$) (Fig. 2B).

3.2. Effects of fluoxetine on other behaviors

Both the main effects of fluoxetine treatment ($F_{2,79} = 7.258$, $p < 0.001$) and time period ($F_{2,79} = 18.64$, $p < 0.001$) on total time swimming were statistically significant (Fig. 3, Table S1). Fluoxetine treatment significantly reduced total time swimming relative to untreated ($p = 0.007$) and saline-treated ($p = 0.029$) frogs. Total time swimming was significantly reduced during period B relative to period C ($p < 0.001$) and period A ($p < 0.001$).

There was an overall main effect of drug treatment on latency to move ($\chi^2 = 8.614$, df= 2,79, $p = 0.013$) but pairwise comparisons using

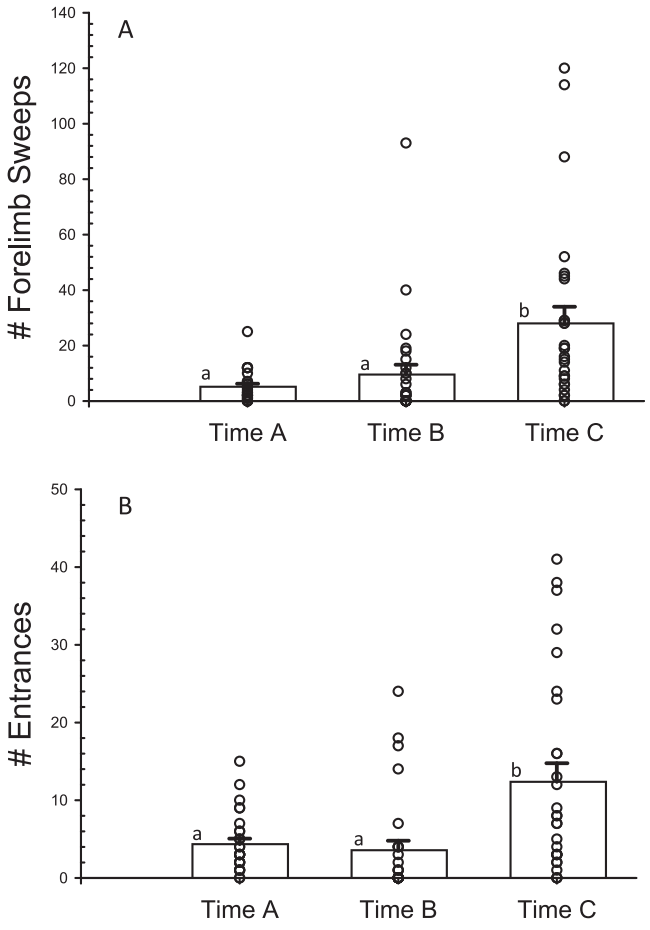

Fig. 2. The main effects of time period on the number of forelimb sweeps (Fig. 2A) and the number of entrances into the predator zone (Fig. 2B) . Bars with different superscripts are statistically different following pairwise comparison with Sidak correction. Bars represent the mean ± S.E.M. of 28 animals per time period.

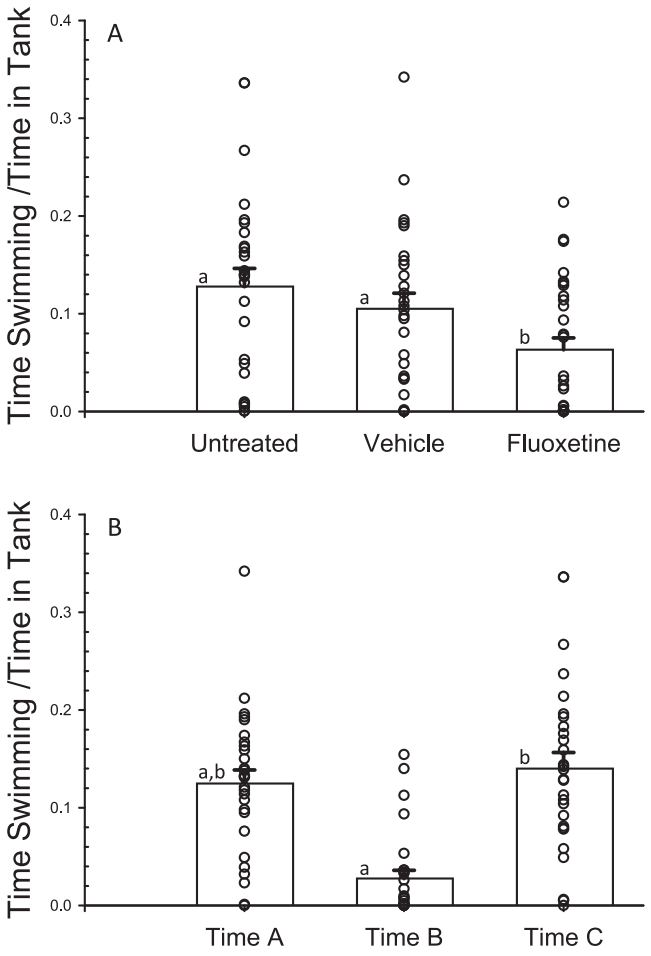

Fig. 3. Main effects of fluoxetine treatment (3 A) and time period (3B) on total time swimming. Bars with different superscripts are statistically different following pairwise comparison with Sidak correction. Bars represent 27–30 animals per group for the main effect of drug treatment or 28 animals per group for time period + S.E.M.

the Sidak correction revealed no significant differences between the untreated, saline-injected, and fluoxetine-injected treatments ($p = 0.060$ A vs B and C, $p = 0.254$ B vs C). There was no main effect of time period on latency to move ($p = 0.376$, Table S1).

The main effect of drug treatment on time spent inactive was not statistically significant ($p = 0.873$, Table S1) although there was a main effect of time period on time spent inactive ($\chi^2 = 18.64$, df = 2,79, $p < 0.001$, Fig. 4). Frogs spent more time inactive in the presence of a predator during period B relative to period A ($p = 0.013$) and period C ($p < 0.001$) (Fig. 4).

There were no significant main effects of fluoxetine treatment or time period on time spent moving to the predator section, time spent moving to the safe section, or time spent in safe section (Table S1).

3.3. The effect of fluoxetine administration on relative transcript abundance in the optic tectum

Analysis of delta delta Ct values using the KW test revealed no treatment effects on any of the transcripts (*crf*, $p = 0.8986$; *htr1a*, $p = 0.3448$; *drd1*, $p = 0.5703$; *drd2*, $p = 0.9921$) (Fig. 5).

4. Discussion

This study sought to identify if repeated administration of the SSRI fluoxetine affects: (1) food intake, (2) baseline locomotion, (3) prey-capture and avoidance behaviors, and (4) transcriptional changes of

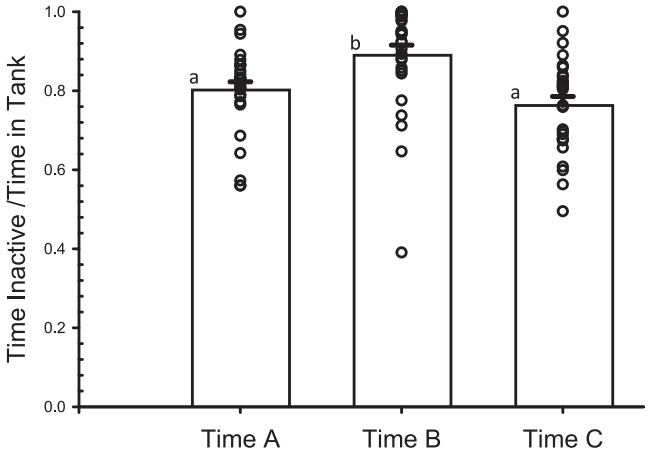

Fig. 4. The main effects of time period on total time inactive (Fig. 2A). Bars with different superscripts are statistically different following pairwise comparison with Sidak correction. Bars represent the mean + S.E.M. of 28 animals per time period.

anxiety-related peptides in the optic tectum in *Xenopus laevis* frogs. Not enough frogs ate to determine any effect on food intake, and there were no effects of fluoxetine treatment on forelimb sweeping, a prey-capture

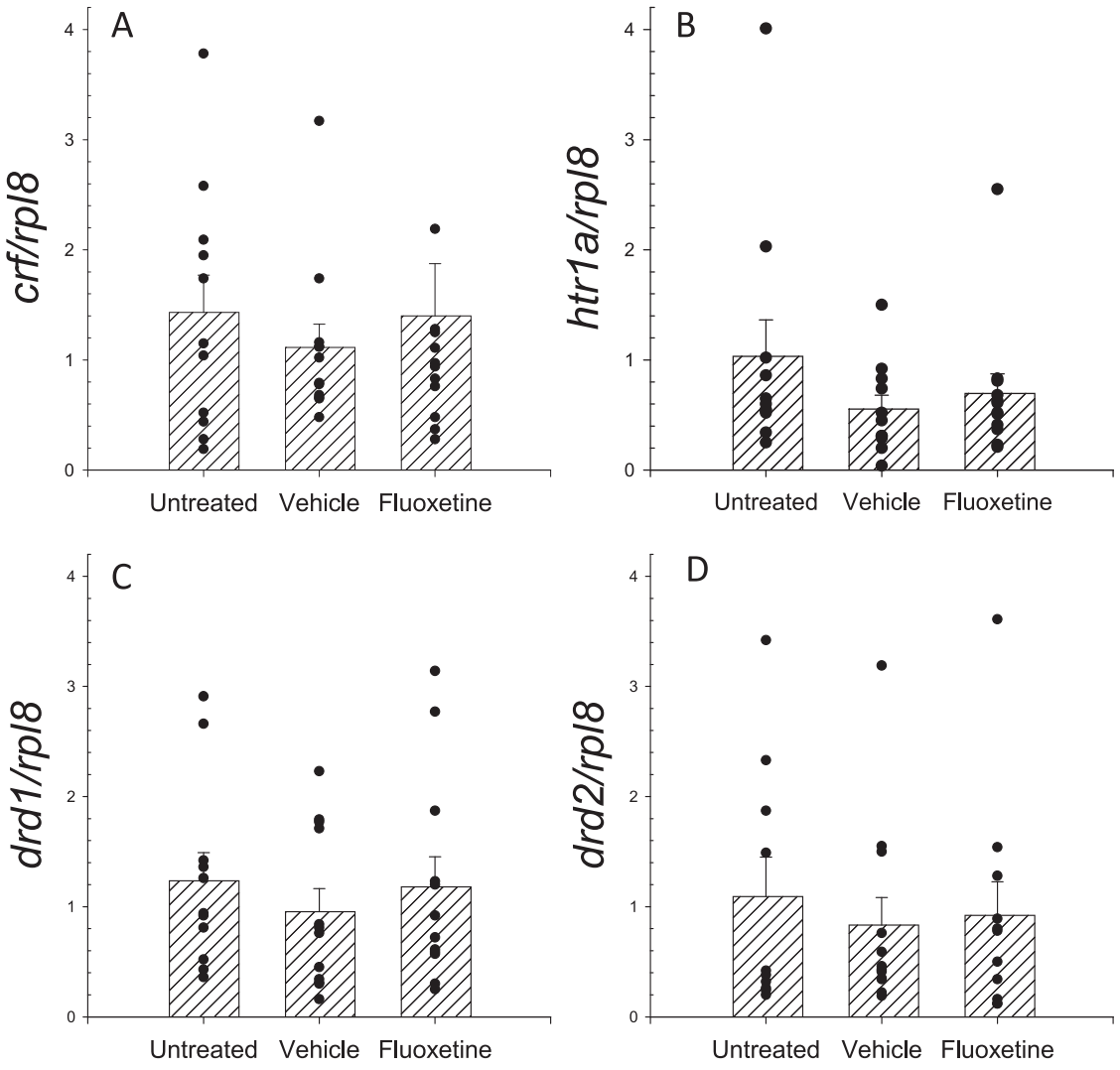

Fig. 5. Differences in transcript abundance of A) corticotropin releasing factor (*crf*), B) serotonin 1a receptor (*htr1a*), C) dopamine D1 receptor (*drd*1), and D) dopamine D2 (*drd2*) relative to the reference gene, ribosomal protein L8 (*rpl8*) in the optic tecta of control and predator-exposed *X. laevis*. Data are represented as mean ± S.E.M. of n = 6–7 animals.

behavior in this species and a behavior sensitive to predator exposure in this assay. These results, along with our previous work, indicate that food intake is not a reliable measure of appetitive behavior in this assay, despite food intake being sensitive to predator presence and highly reproducible in juvenile *X. laevis* frogs [17–20]. Moreover, forelimb sweeping is one of the behavioral endpoints that we find sensitive to predator exposure in adults in our previous [17] and current work.

The only behavioral effect of fluoxetine relative to vehicle injected frogs in period A was a reduction in swimming time. Fluoxetine administered by immersion for 12 d increased swimming during the dark period, but not the light period, in juvenile *D. rerio* [9] but significantly decreased the total distance swam in zebrafish larvae after a 96 h treatment [41]. Similarly, immersion exposure of Siamese fighting fish (*Betta splendons*) [42], sheepshead minnows (*Cyprinodon variegatus*) [43], and guppies (*Poecilia reticulata*) [44] to fluoxetine decreased locomotion. Similar effects were observed after i.p. administration in fish [45]. Moreover, this effect of fluoxetine is not restricted to aquatic vertebrates, as it also has been seen in some, but not other, mouse strains after chronic administration [46].

To our knowledge this is the first report demonstrating that fluoxetine decreases locomotion in adult anurans. Aliko et al. [47] reported that a 7-d immersion exposure to fluoxetine reduced locomotion in larval *Bufo bufo*. Interestingly, fluoxetine on its own has no effect on locomotion when administered i.c.v. in rough-skinned newts (*Taricha granulosa*) [48], so there is much to learn regarding the site(s) of fluoxetine's effect on locomotion, especially since fluoxetine crosses the blood brain barrier [49] after peripheral administration. One of the earliest reported side effects of fluoxetine in preclinical studies was reduced meal size [50]. To the extent that locomotion and foraging are linked in this species, it may be that fluoxetine reduces the motivation to forage in *X. laevis*, although any effect would likely be subtle as we found no effect on forelimb sweeps, a prey capture behavior in this species. Immersion-exposure to fluoxetine reduces body mass, presumably due to reduced foraging, in larval *X. laevis* [51], which is consistent with the notion that fluoxetine effects on locomotion and foraging are related in amphibians.

In our previous work using this tradeoff task [17], we identified four appetitive and avoidance behaviors impacted by exposure to the predator stimulus: a reduction in forelimb sweeping, a foraging behavior, fewer entrances into the predator zone, a reduction in total time swimming, and an increase in time spent inactive. Fluoxetine failed to alter a statistically significant change any of these endpoints during or after predator exposure relative to vehicle controls. Exposure to a predator stimulus has been used previously to elicit so-called 'state' anxiety in many vertebrate models. The effects of fluoxetine in these models is variable, and sometimes complex in that it depends not only

upon dose but dosage [52]. Our data are not consistent with a role for serotonin in predator-induced changes in behaviors in adult *X. laevis.* although more work using serotonin receptor antagonists is needed to confirm this. Our findings are consistent with the results of previous work failing to alter the performance of *X. laevis* in an a plus maze and scototaxis test using fluoxetine administered 24–1 h prior to testing [53]. Thus, we cannot conclude that any of these tests elicit anxiety-like behavior in *X. laevis*.

Our choice of gene transcripts was based upon the role of these genes in preclinical models of anxiety and depression [34–37]. None of these markers changed within the OT after sub-chronic fluoxetine treatment. In particular, expression of the 5-HT autoreceptor 5-HTR1A is a well-known marker of changes in extracellular 5-HT, since it is present presynaptically on nerve terminals that release 5-HT and can be considered a 'first responder' to changes in synaptic 5-HT. Fluoxetine treatment reduces 5-HTR1A expression in mice [54]. The fact that we observed no effect of fluoxetine on *htr1A* expression in the OT is consistent with the idea that this SSRI did not significantly alter synaptic 5-HT levels at the end of the sub-chronic treatment.

5. Conclusions

In this study we evaluated the effects if sub-chronic fluoxetine administration affects a) baseline appetitive and avoidance behavior, b) reverses predator-induced changes in these behaviors, c) or affects transcription of anxiety-related transcripts in adult *Xenopus laevis* frogs. We found sub-chronic administration of fluoxetine reduced baseline swimming activity relative to vehicle control but failed to alter any of the predator-induced behaviors observed. There were no differences in transcript abundance between any of the treatment groups. We conclude that fluoxetine administration reduces locomotion in adult *X. laevis* but has no effect on the response to a visual predator stimulus in this species.

Ethics approval and consent to participate

Not applicable.

Funding

This work was supported by Texas Tech Department of Biological Sciences, Texas Tech University Association of Biologists, and the National Science Foundation [IOS #1656734].

CRediT authorship contribution statement

Nikhil Menon funded the study, designed the study, conducted the in life portion of the study, conducted the measurements, performed the initial analyses, assisted with the GLZX and GEE, analyses, wrote the original manuscript, assisted with revision. Caoyuanhui Wang assisted with statistical analysis, specifically GLZX and PCA in R. Dr. Carr assisted with experimental design, supplied some funding, edited and co-wrote the original manuscript, helped with initial analysis, and conducted revised GLZX and GEE in modeling in SPSS. NM conceptualized, investigated, ran the formal data analysis and drafted the manuscript. JAC provided supervision, some resources, assisted with formal statistical analyses, edited the manuscript. The authors read and approved the final manuscript.

Data Availability

Data are included as a supplemental file.

Acknowledgements

We thank the Texas Tech University Department of Biological Sciences and the Texas Tech University Association of Biologists for funds to NM. JAC was supported by National Science Foundation IOS #1656734. We also acknowledge Drs. Karina Alvina, Lauren Gollahon, Naima Moustaid-Moussa, and Ernest Smith for assistance with experimental design and Marcos Arciniega, Briana Lacy, and Hailey Luckie for help with behavior analysis. Finally we thank the anonymous reviewers who sacrificed their time to produce extraordinarily careful reviews of our paper. This work was completed in partial fulfilment of the requirements for a doctoral degree in the Department of Biological Sciences at Texas Tech University (NM).

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bbr.2023.114317.

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