Chemosphere xxx (2017) xxx-xxx

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com

An evaluation of behavioural endpoints: The pharmaceutical pollutant fluoxetine decreases aggression across multiple contexts in round goby (*Neogobius melanostomus*)

Erin S. McCallum , Aneesh P.H. Bose, Theresa R. Warriner, Sigal Balshine

Aquatic Behavioural Ecology Laboratory, Department of Psychology, Neuroscience and Behaviour, McMaster University, 1280 Main Street West, Hamilton, L8S4K1, ON, Canada

ARTICLE INFO

Article history:
Received 8 December 2016
Received in revised form 8 February 2017
Accepted 10 February 2017
Available online xxx

Handling Editor: Shane Snyder

Keywords:
PPCPs
SSRI
Ecotoxicology
Invasive species
Social affiliation
Activity

ABSTRACT

Fluoxetine (*Prozac*™) is designed to alter human behaviour; however, because many physiological pathways are conserved across vertebrates, this drug may affect the behaviour of fish living in fluoxetine-polluted environments. Although a number of studies have used behaviour to document the sub-lethal effects of fluoxetine, the repeatability of these effects across experiments, across behavioural contexts, and over different exposure durations are rarely considered. Here, we conducted two experiments and assessed how fluoxetine exposure affected a range of fitness-related behaviours in wild round goby (*Neogobius melanostomus*). We found that fluoxetine impacts round goby behaviour at high (40 μg/l) doses, but not at environmentally relevant low doses (1 μg/l). In both experiments, an acute 3-day exposure to fluoxetine reduced round goby aggression in multiple behavioural contexts, but had no detectable effect on overall activity or social affiliative behaviour. While a chronic 28-day exposure to fluoxetine exposure still reduced aggression, this reduction was only detectable in one behavioural context. Our findings demonstrate the importance of repeated behavioural testing (both between and within experiments) and contribute to a growing body of literature evaluating the effects of fluoxetine and other pharmaceuticals on animal behaviour.

1. Introduction

Human use of pharmaceuticals and personal care products (PPCPs) continues to escalate (OECD, 2013). Frequent use, ingestion and excretion, as well as improper disposal of these products burdens conventional wastewater treatment facilities that are rarely equipped to remove PPCPs from the water they treat (Jelic et al., 2012). Consequently, small but measureable amounts of active pharmaceuticals are now found in urban watersheds, with treated effluent acting as a major source of pharmaceuticals in the aquatic environment (Kolpin et al., 2002; Khetan and Collins, 2007; Metcalfe et al., 2010). Many pharmaceuticals are designed to modulate human physiology and behaviour (e.g. antidepressants, antibiotics, steroid hormones), and many of their biological targets (e.g., receptors, transporters, enzymes) are conserved across vertebrate taxa (Gunnarsson et al., 2008). Therefore, non-human vertebrates may also experience physiological and behavioural changes when exposed to PPCPs, raising concern over the impact of pharmaceuticals on aquatic species living near wastewater outfalls (Corcoran et al., 2010; Boxall et al., 2012; Arnold et al., 2013). While many pharmaceuticals are not lethal to organisms at concentrations typically found in environments receiving wastewater, chronic exposure may cause subtle or even large scale alterations to individual physiology and behaviour that could directly or indirectly impact fitness (Brodin et al., 2014).

Amid the various classes of pharmaceuticals detected in wastewater effluent, antidepressants have a particularly strong potential to alter wild fish behaviour, and these drugs are also increasingly being prescribed in developed countries (Hemels et al., 2005; Paulose-Ram et al., 2007; OECD, 2013). Fluoxetine (commercial name, *Prozac*™) is an antidepressant commonly used for the treatment of human depression and anxiety disorders (Milea et al., 2010). Fluoxetine and its main active metabolite, norfluoxetine, are measured in treated wastewater effluents and have been recorded downstream in surface waters at concentrations ranging from 0.001 μg/l up to 1.3 μg/l in Europe and North America (Kolpin et al., 2002; Christensen et al., 2009; Metcalfe et al., 2010). Fluoxetine has been found to bioconcentrate in the tissues of fish sampled downstream from wastewater outfalls (Brooks et al., 2005; Ramirez et al., 2009; Schultz et al., 2010). Fluoxetine can cause mortality in fish, but only at concentrations much higher than those reported in the environment (e.g., 48 h LC_{50} 705 μg/l, for fathead minnow, *Pimephales promelas,* Brooks et al., 2003; 96 h LC_{50} 2000 μg/l, for sheepshead minnow, *Cyprinodon variegatus,* Winder et al., 2009).

Fluoxetine modulates both physiology and behaviour by acting as a selective serotonin reuptake inhibitor (SSRI) that increases serotonin concentrations through blocking its reuptake in the synaptic cleft by the serotonin transporter (SERT; Stahl, 1998). The serotonin transporter is conserved across vertebrates, including fish (Mennigen et al., 2011). The serotonergic system is integral to many biological

Corresponding author. Aquatic Behavioural Ecology Laboratory, Department of Psychology, Neuroscience and Behaviour, Rm 102, Building PC-34, McMaster University, 1280 Main Street West, Hamilton, L8S4K1, ON, Canada.
Email address: mccalles@mcmaster.ca (E.S. McCallum)

http://dx.doi.org/10.1016/j.chemosphere.2017.02.059
0045-6535/

processes (e.g. appetite and metabolism, cardiovascular functioning, reproduction, social behaviours; Winberg and Nilsson, 1993; Berger et al., 2009; Lillesaar, 2011). Studies investigating the role of serotonin in fish physiology and behaviour have used fluoxetine treatments to manipulate serotonin, and this work has contributed significantly to our understanding of how fluoxetine exposure can impact fish in the wild. For example, fluoxetine suppresses fish appetite and reduces food intake, growth, and glucose metabolism (Gaworecki and Klaine, 2008; Mennigen et al., 2009, 2010a). Fluoxetine also alters reproductive physiology in male fish by reducing testosterone and milt production (Mennigen et al., 2010b), and increasing circulating estradiol and vitellogenin (Mennigen et al., 2010b; Schultz et al., 2011). Researchers have also demonstrated that fish treated with fluoxetine show decreased aggression, increased submission, increased sociability, and a muted physiological stress response (Perreault et al., 2003; Barbosa et al., 2012; Kohlert et al., 2012; de Abreu et al., 2014; Paula et al., 2015).

To understand how exposure to pharmaceuticals like fluoxetine impacts fish in the wild, researchers often use waterborne exposures to ecologically relevant drug concentrations. Fluoxetine has been shown to rapidly alter fish behaviour, even at concentrations of less than 1 μg/l. For example, after only 48 h of exposure to 0.5 μg/l, fluoxetine reduced aggressive displays in male Siamese fighting fish (*Betta splendens,* Dzieweczynski and Hebert, 2012). A 6–7 day exposure to a similar concentration decreased brood defense during parental care in Siamese fighting fish (Forsatkar et al., 2014; Greaney et al., 2015) and aggression towards a conspecifics in Arabian killifish (*Aphanius dispar,* Barry, 2013). Fish in the wild are likely exposed to pharmaceuticals over much longer durations. Research to date has shown that fish chronically exposed to fluoxetine for 21–28 days are less adept at avoiding predators (Weinberger and Klaper, 2014; Pelli and Connaughton, 2015), and a 21-day fluoxetine exposure resulted in reduced nest quality in three-spine stickleback (*Gasterosteus aculeatus,* Sebire et al., 2015).

Such exposure experiments are valuable, but these experiments are sometimes criticized for rarely replicating behavioural findings, testing behaviour at only one time point (e.g. acute exposure only), and only in a single behavioural context (Sumpter et al., 2014; Peakall, 1996). Such criticisms are important to address whether behavioural changes following pharmaceutical exposures are consistent and robust. In addition, there is an urgent need to develop reliable behavioural assays for more species, specifically for non-model fish species that inhabit affected waterways (Brooks, 2014).

To this end, we conducted two experiments aimed at identifying behavioural effects of exposure to fluoxetine in a wild fish, the round goby (*Neogobius melanostomus*). This benthic fish species is widespread throughout the Laurentian Great Lakes, Western Europe, and the Ponto-Caspian region of Eastern Europe (Corkum et al., 2004; Kornis et al., 2012). First, we conducted an acute, 3-day exposure to fluoxetine and assessed the impacts of this exposure on range of behaviours important for round goby fitness. Specifically, we tested the impacts of fluoxetine on social interactions with a conspecific, aggression in a contest over a valued shelter resource, and activity in an open-field. We predicted that fluoxetine exposure would increase the time spent interacting with conspecifics and reduce aggression in resource contests, as has been observed in other fish species (Dzieweczynski and Hebert, 2012; Barry, 2013; Forsatkar et al., 2014; Greaney et al., 2015). Second, we conducted another experiment assessing the effects of fluoxetine on round goby behaviour after three days (replicating our first experiment) and then again tested the same fish after 28 days of exposure. Thus, we repeated behavioural tests both within and between experiments to 1) assess the repeatability of certain assays, and 2) determine how fluoxetine exposure duration influenced behaviour. In the second experiment, we again assessed aggression in a resource contest but also assessed aggression using a mirror assay, a widely used method to gauge individual aggressiveness (Balzarini et al., 2014; Elwood et al., 2014). We predicted that we would observe reduced aggression after 3 days of exposure in both aggressive contexts (resource contest and mirror assay). Furthermore, we expected to observe less aggression after 28 days of exposure if fluoxetine's behavioural effects remain consistent across acute and chronic exposures (Dzieweczynski and Hebert, 2012; Forsatkar et al., 2014; Greaney et al., 2015). In both experiments, we also monitored fish activity to ensure that any decrease in aggression was not simply a result of a reduction in overall activity.

2. Methods

2.1. Fish collection and housing

We collected round goby between May 10, 2013, and June 10, 2013, (Experiment 1) and between July 10, 2014 and July 30, 2014 (Experiment 2) from LaSalle Park Marina, Hamilton, ON, Canada (43°18′1 N, 79°50′47 W) using baited minnow traps. For collection method details see McCallum et al. (2014) and Young et al. (2010). We transported the fish to McMaster University where we housed fish in same-sex groups of three (Experiment 1) or eight fish (Experiment 2) in 75 l aquaria (H30 cm x W62 cm x D46 cm). We equipped the housing aquaria with ~1 cm of natural gravel substrate, an airstone, plastic PVC tubes for shelter, and a static renewal filter (Aquaclear). We fed fish Nutrafin Basix Staple Food once daily and kept a 14L:10D light schedule. After 24 h acclimation to the laboratory, we weighed each fish to the nearest 0.01 g, measured their standard length to the nearest 0.01 cm, and uniquely tagged them using non-toxic acrylic paint (Wolfe and Marsden, 1998; Groen et al., 2012; Capelle et al., 2015) before returning them to their housing tanks. The visual tag was used to identify individuals throughout behavioural trials and later sampling. We monitored water quality, checking dissolved oxygen, temperature, and pH (LaMotte Pocket Tracer, Oakton PCTestr 35). Water quality measures were similar between experiments and across fluoxetine treatment groups (see Supplementary Table 1).

2.2. Fluoxetine exposures and experimental protocol

2.2.1. Experiment 1: acute exposure only

We exposed 88 round goby for 72 h to three fluoxetine treatments: a 0 μg/l control treatment, a 1 μg/l environmentally relevant low treatment, and a 40 μg/l high treatment. We used 44 males ($N_{control} = 15$, $N_{low} = 14$, $N_{high} = 15$) and 44 females ($N_{control} = 15$, $N_{low} = 14$, $N_{high} = 15$). We first prepared a fluoxetine 1 mg/ml stock solution by dissolving fluoxetine hydrochloride (99.9% purity, Sigma Aldrich) in anhydrous ethanol. Then, we prepared individual dosing aliquots for each treatment by dilution with ultrapure water (MilliQ). Control doses contained only ethanol and ultrapure water. We controlled for the amount of ethanol used across all doses which was 5×10^{-3}% (v/v) in the exposure tanks, an amount that was well below toxicity levels for fishes (Majewski et al., 1978). All doses were re-labeled to ensure we remained blind to treatment while conducting exposures and behavioural trials. We froze the individual dosing aliquots at −20 °C until their use at the beginning of an exposure period. We exposed fish in a static-renewal exposure in their 75 l housing tanks in the same-sex, groups of three. Treatments were randomly assigned to tanks, and we used 10 replicate exposure tanks per fluox-

etine treatment. We removed the activated carbon inserts from the filter of each tank during exposures. No fish died during the exposure period.

2.2.2. Experiment 2: acute and chronic exposure

We exposed 144 round goby for 28 days to the same three fluoxetine treatments used in Experiment 1. We used 69 females ($N_{control} = 24$, $N_{low} = 24$, $N_{high} = 21$), and 75 males ($N_{control} = 24$, $N_{low} = 24$, $N_{high} = 27$) in this experiment. We prepared fluoxetine doses as described above, and the experimenters were similarly blind to treatment. Fish were exposed in groups of eight in their 75 l housing tanks, and again the activated carbon was removed from the filters. Treatments were randomly assigned to tanks, and we used six replicate exposure tanks per fluoxetine treatment. Following the first exposure dosing, we re-dosed each tank every 72 h with half the original dose concentration (half-life of fluoxetine in a stocked tank, following Gaworecki and Klaine, 2008). In addition, we conducted two water changes throughout the 28-day exposure period: once every 12 days coinciding with a re-dosing day we replaced 30% of the tank water with de-chlorinated tap water.

We quantified fluoxetine in the tanks by taking grab water samples from three randomly selected tanks per treatment 1 h after dosing, and then again after 72 h but immediately before re-dosing. One low exposure, 1-h sample broke during transport before analysis was possible and was excluded from analysis. Fluoxetine samples were quantified following Metcalfe et al. (2010). Briefly, 20 ml samples were extracted using Oasis MCX SPE cation-exchange cartridges (Waters Scientific). The eluants were collected in a centrifuge tube, evaporated just to dryness, and then reconstituted in methanol. Samples were then transferred to an autosampler vial with an insert for analysis and analyzed by LC-MS/MS using a Q-Trap LC-MS/MS System. After 1 h of exposure, we found fluoxetine concentrations to be an average (±SE) of 0.00 (±0.00) µg/l for the 0 µg/l control treatment, 0.55 (±0.15) µg/l for the 1 µg/l low treatment, and 35.43 (±4.44) µg/l for the 40 µg/l high treatment. After 72 h of exposure, we found fluoxetine concentrations to be an average (±SE) of 0.00 (±0.00) µg/l for the 0 µg/l control treatment, 0.00 (±0.00) µg/l for the 1 µg/l low treatment, and 22.60 (±6.65) µg/l for the 40 µg/l high treatment. Five fish died during this exposure, but as these fish came from different tanks and treatment groups (1 from control, 3 from low, and 1 from high), it is highly unlikely that the mortality was related to the fluoxetine exposure.

2.3. Behavioural assays

Following each experiment, we immediately assayed a range of behavioural responses. In Experiment 1, we conducted a social interaction assay, a contest aggression assay, and an activity assay. In Experiment 2, we again conducted a contest aggression assay, as well as a mirror aggression assay (Fig. 1e). See the Supplementary Materials

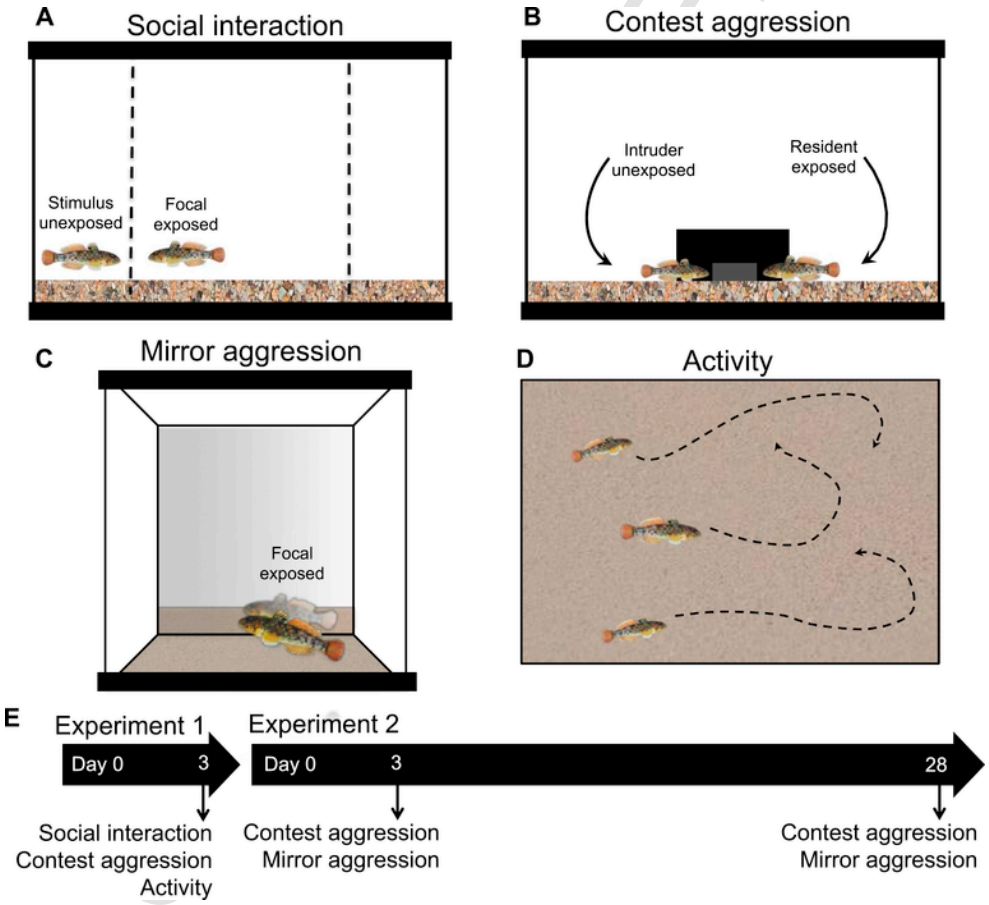

Fig. 1. Behavioural assays and an experimental timeline. **A)** Testing tank for the social interaction assay, dashed lines represent the two end compartments where the conspecific stimulus fish would have been placed. **B)** Testing tank for contest aggression assay, depicting the shelter resource and resident and intruder fish interacting. **C)** Testing tank for mirror aggression assay, showing a focal fish interacting with its mirror image (adapted and reprinted with permission from McCallum et al. (2017)). **D)** Testing tank for activity assay, an open-field without shelters as viewed from above. **E)** Experimental timeline for both Experiments 1 and 2 and the behavioural assays we conducted.

for specific protocol details for each behavioural assay. Briefly, we assessed the effect of fluoxetine on social interaction (Experiment 1) following an affiliation protocol commonly used to gauge social tendencies in fish (Svensson et al., 2000; Reddon et al., 2011; Capelle et al., 2015). We recorded the time round goby spent affiliating with a conspecific (an unexposed stimulus fish) to calculate a standard preference index ([time spent near the stimulus fish side/(time spent near the stimulus fish side + time spent near the empty side)]) and all aggressive interactions between the two fish (Fig. 1a). We tested for the effects of our fluoxetine exposure on round goby aggression using two methods: a contest aggression assay against a live opponent fish (Experiment 1 & 2) and a mirror aggression assay (Experiment 2). We used these two assays because they capture two aspects of aggressive behaviour. A contest interaction is most ecologically relevant and represents how fish would fight over territory and secure resources in the wild. However, contest aggression assays are more complex to conduct because they require careful opponent selection (i.e., body size differences between opponents must be controlled across treatments) and contests are inherently more variable because individuals may vary in their motivation to fight (Wilson et al., 2011). Mirror aggression assays have therefore been used as a simple and controlled method to assess aggressive behaviours in individual fish (Balzarini et al., 2014; Elwood et al., 2014). In the contest aggression assay, a fluoxetine-exposed fish was given an empty territory with a valued shelter/nest-box and was allowed to become resident over this area. We then introduced an unexposed intruder fish (Fig. 1b) and recorded the latency to begin the aggressive contest (a measure of motivation) and the number of aggressive acts performed throughout the trial. We specifically noted whether the aggression was being given from the exposed resident to the intruder or received by the exposed resident from the intruder, allowing us to better characterize dominance between the resident and intruder fish. We determined a contest winner, when one fish submitted and no longer retaliated. In the mirror aggression assay, we revealed a mirror to a fluoxetine-exposed fish and recorded all aggressive interactions with the mirror (Fig. 1c). Finally, we assessed the effect of our fluoxetine exposure on activity by recording how often the fish moved in an open-field tank (Experiment 1). Round goby travel along the benthic environment in a series or hops or short swims, thereby facilitating easy quantification of individual movements. Fish were tested for activity in groups of three, as round goby are known to be more naturally active when tested in a group than in isolation (Fig. 1d, Marentette et al., 2011). In all behavioural assays, we conducted trials from behind opaque barriers to limit external influence, we video recorded trials on a Canon HD recorder (Vixia HFS100 8.0 Megapixel), and all behaviours were scored by an observer blind to fluoxetine treatment and the hypotheses of the experiment (See Supplementary Materials for exact scoring protocol and Supplementary Table 2 for behavioural ethogram).

2.4. Post-behavioural processing

After each experiment, we euthanized all exposed fish with an overdose of benzocaine (0.025%, Sigma Aldrich) and dissected them to confirm sex and reproductive status. We measured body mass and gonad mass to the nearest 0.001 g. We calculated gonadosomatic index (GSI: gonad mass/body mass – gonad mass) and classified males as reproductive if their GSI was greater than 1%, and females as reproductive if their GSI was greater than 8% (Marentette and Corkum, 2008; Zeyl et al., 2014). In Experiment 1, the majority of the males (33 of 44) and the females (31 of 44) were non-reproductive, and the reproductive fish were evenly distributed across treatments (N_{High} = 8, N_{Low} = 8, $N_{Control}$ = 8). In Experiment 2, all 144 fish tested were non-reproductive. Non-reproductive fish have small urogenital papillae, and this led to three males being incorrectly sexed as females prior to exposure. They were later confirmed as males during dissection after trials (see sample size above in section 2.2.2).

2.5. Statistical analyses

All statistical analyses were conducted using R (version: 3.2.2, R Core Team, 2015). Some fish that underwent behavioural testing were excluded from the statistical analyses for not participating in the trials. We employed two inclusion criteria: 1) In the social interaction and mirror aggression assays, we analyzed data from fish that *moved at least once* during the trial to ensure that we were considering only the fish that were actively sampling their environment, 2) In the contest aggression assays, we analyzed data from resident fish that *interacted* with the intruder *at least once* during the trial to ensure that we were considering only fish that were aware of their opponents (see Table 1 for a description of sample sizes before and after inclusion criteria).

We first tested whether fluoxetine increased round goby social preference for conspecifics (Experiment 1). We fit a beta regression model (betareg package, Cribari-Neto and Zeileis, 2010) including the conspecific preference index as the dependent variable and fluoxetine treatment group and sex as predictors. We also tested whether fluoxetine reduced the amount of aggression performed towards the stimulus fish across the transparent barrier. For this we fit a generalized linear model assuming a negative binomial error distribution suitable for fitting over dispersed data. We included the number of aggressive acts performed as the dependent variable and fluoxetine treatment group and sex as predictors.

Second, we tested whether fluoxetine reduced fish aggression in contests over a resource (Experiment 1). We fit the following models for various dependent variables. The latency to begin an aggressive contest (seconds, ln-transformed) was fit with a linear regression. The likelihood of the exposed fish winning the contest was fit with a generalized linear model assuming a binomial error distribution (binary logistic regression). We fit the number of aggressive acts (either given from or received by the exposed resident fish) with a generalized linear model assuming a negative binomial error distribution. In all analyses, we included fluoxetine treatment group and sex as predictors, and we also included body size difference (absolute differ-

Table 1
Summary of fish used in all behavioural assays before and after the inclusion criteria were applied. -- indicates no inclusion criteria was applied.

	N exposed	*N* scored	Inclusion criteria	*N* analyzed			
				Control	Low	High	Total
Experiment 1 acute only							
Contest aggression	88	82	how many interacted?	18	17	21	56
Social interaction	88	88	how many moved once?	25	21	20	66
Activity	88	88	–	30	28	30	88
Experiment 2 acute & chronic							
Acute: Mirror aggression	144	139	how many moved once?	42	39	37	118
Acute: Contest aggression	144	137	how many fish interacted?	39	40	41	120
Chronic: Mirror aggression	144	137	how many moved once?	41	42	36	119
Chronic: Contest aggression	144	139	how many fish interacted?	41	42	37	120

ence in body mass, g, between opponents) as a covariate. When analyzing the number of aggressive acts, we also included a factor specifying whether the aggressive acts were given from or received by the exposed resident fish, and we specifically tested for an interaction between this factor and fluoxetine treatment group. This allowed us to test whether fluoxetine affects the dominance of the exposed fish over the intruder fish in the resource contest (e.g., reducing aggression given while increasing aggression received). We then replicated this contest aggression assay in Experiment 2, and in this experiment we repeated the assay at two time points (acute = 3 days, chronic = 28 days). In order to accommodate this design, we re-fit the above-described models using a mixed model framework; we included time point (acute vs. chronic) as a fixed effect and fish ID as a random intercept (package lme4, Bates et al., 2015; package glmmADMB, Fournier et al., 2012). When analyzing the observed number of aggressive acts for Experiment 2, we specifically tested for a three-way interaction among the variables time point, fluoxetine treatment group, and whether the aggression was given or received. This interaction term would indicate to us whether fluoxetine's effect of on fish dominance differed between the time points. We successfully controlled for differences in body mass between exposed residents and non-exposed intruder fish, and differences were consistent across treatment groups (ANOVA: Experiment 1: $F_{(2,53)} = 0.13$, $p = 0.88$; Experiment 2 Acute: $F_{(2, 113)} = 0.97$, $p = 0.38$; Experiment 2: Chronic: $F_{(2, 111)} = 1.80$, $p = 0.17$), and between sexes (Experiment 1: $F_{(2,53)} = 1.59$, $p = 0.21$; Experiment 2 Acute: $F_{(1, 113)} = 3.27$, $p = 0.073$; Experiment 2 Chronic: $F_{(1, 111)} = 3.11$, = 0.08).

Third, we tested whether fluoxetine reduced aggression in a mirror aggression assay (Experiment 2). Again, we fit various models for the different dependent variables. The latency to move towards the mirror (seconds, ln-transformed) was fit with a linear regression model. Both the number of aggressive acts performed towards the mirror and the number of non-aggressive movement behaviours performed away from the mirror (a proxy for overall activity level) were fit with generalized linear models assuming a negative binomial error distribution. As this mirror aggression assay was repeated at two time points, we used a mixed-model approach. We included fluoxetine treatment group and sex as predictors, as well as time point (acute 3 days vs. chronic 28 days) and its interaction with treatment group. We also included fish ID as a random intercept.

Lastly, we tested whether fluoxetine reduced overall activity and exploratory behaviour in an open-field style tank (Experiment 1). The number of movement actions performed by the exposed focal fish was fit with a generalized linear mixed model assuming a negative binomial error distribution. We included fluoxetine treatment group and sex as predictors. As fish were tested in groups of three, we included group ID as a random intercept. All non-significant interactions were removed from the models.

3. Results

3.1. Experiment 1: acute exposure only

In the social assay, fish spent on average 72% (±3.9% SE) of the total trial time associating with the stimulus fish. However, fluoxetine treatment did not affect the amount of time fish spent interacting (Beta regression: $N = 66$, High vs Control $Z = 1.01$, $p = 0.31$; High vs Low $Z = 0.42$, $p = 0.67$; Low vs Control, $Z = 0.57$, $p = 0.56$). Fish were mostly aggressive towards the stimulus fish across the barrier, and, interestingly, fish exposed to the high fluoxetine dose were less aggressive than the control fish (Negative binomial regression: $N = 66$, $Z = -2.12$, $p = 0.03$: Fig. 2). High treatment fish were not statistically different from low treatment fish ($Z = -0.72$, $p = 0.47$), and low treatment fish did not differ from controls ($Z = -1.39$, $p = 0.16$).

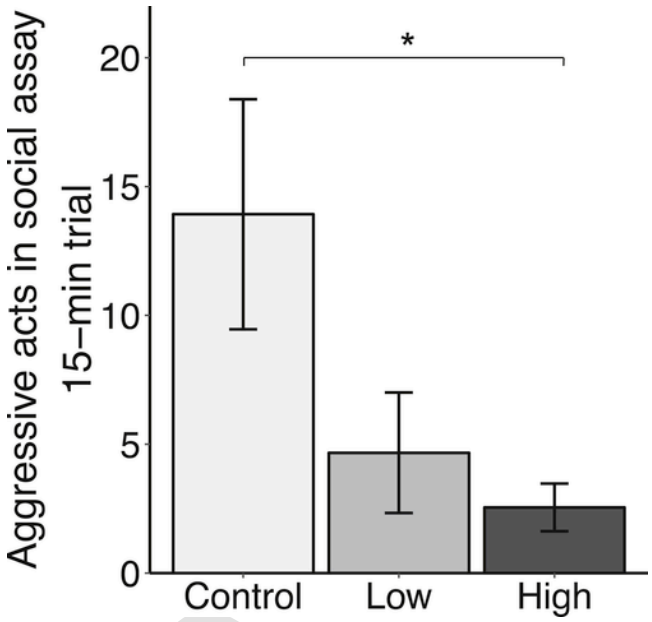

Fig. 2. Number of aggressive acts towards the conspecific stimulus fish in the social interaction assay, plotted by treatment. Brackets show a reduction in aggression towards the stimulus fish following an acute 3-day fluoxetine exposure. *$p < 0.05$. Error bars represent ± SE.

In the contest aggression assay, we found an interaction among the variables fluoxetine treatment and the number of aggressive acts between resident and intruder fish. Contests where a resident had been exposed to the high dose of fluoxetine involved fewer aggressive acts from the resident and more aggressive acts from the non-exposed intruder, compared to control treatment contests. (Negative binomial GLMM: Treatment-by-contest aggression interaction, $N = 56$, High vs Control $Z = 2.07$, $p = 0.039$; High vs Low $Z = 0.53$, $p = 0.60$; Low vs Control, $Z = 1.53$, $p = 0.13$, Fig. 3a). These results suggest that fluoxetine made resident fish less aggressive towards intruders, and intruders responded with increased aggression of their own. However, fluoxetine treatment did not impact how quickly aggression was initiated (Linear model: $N = 56$, High vs Control, $t = 0.57$, $p = 0.57$; High vs Low: $t = 0.54$, $p = 0.59$; Low vs Control: $t = 0.050$, $p = 0.96$). Also, although fish exposed to the high dose of fluoxetine were less aggressive, exposure did not significantly impact the likelihood of the exposed resident winning the contest (Binary logistic regression: $N = 56$, High vs. Control $Z = -1.68$, $p = 0.09$; Low vs. Control $Z = -0.55$, $p = 0.58$; High vs. Low $Z = -1.16$, $p = 0.25$). Resident fish won the majority of contests (taking 88% of control, 81% of low dose, and 65% of high dose contests).

In the open tank activity assay, the fish moved an average of 17 times (±2 SE) per 5-min period. Activity levels were not influenced by acute exposure to fluoxetine at any dose (Negative binomial GLMM: $N = 88$: High vs Control $Z = 0.81$, $p = 0.42$; High vs Low $Z = -0.29$, $p = 0.77$; Low vs Control $Z = 1.09$, $p = 0.28$). There was no effect of sex in any of the above analyses for Experiment 1 ($p > 0.05$), except that males were more aggressive towards their intruder fish than females were in the contest aggression assay (Effect of sex: $Z = 2.25$, $p = 0.024$).

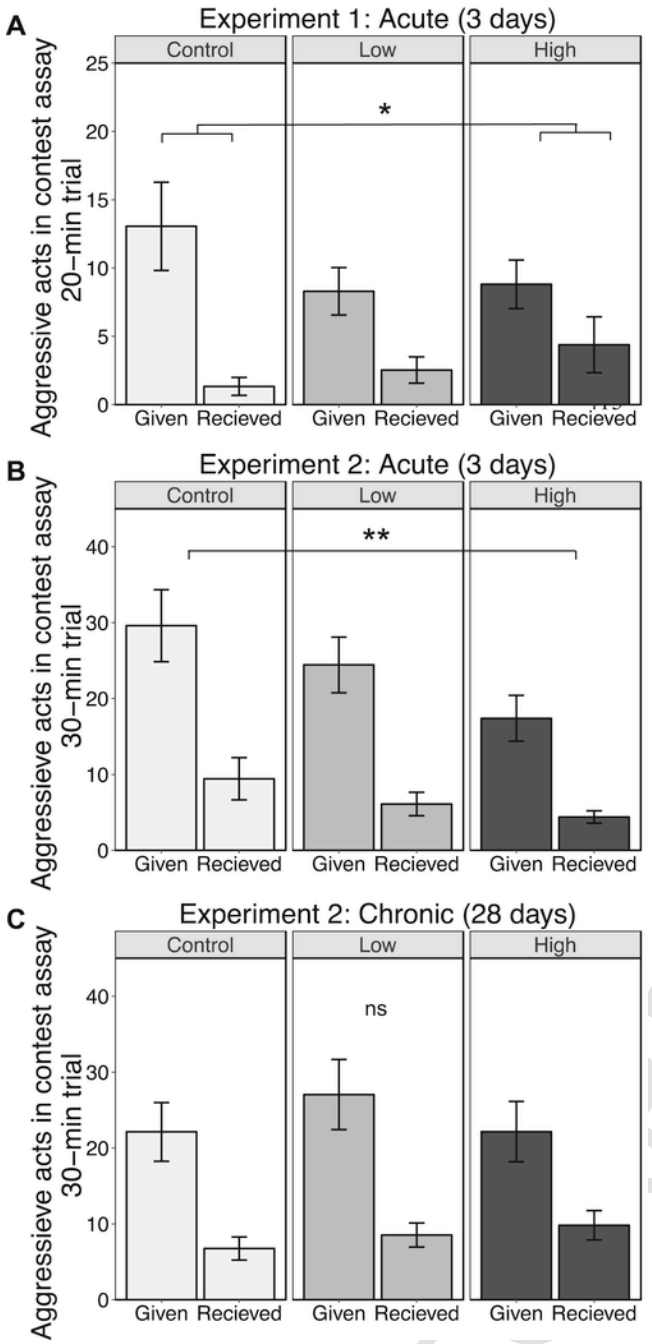

Fig. 3. Contest aggression in Experiments 1 and 2. *p < 0.05, **p < 0.01, ns = no significant difference in contest aggression in relation to treatment. Error bars represent ± SE. **A)** Aggressive acts plotted by aggressive acts given and aggressive acts received, and faceted by fluoxetine treatment for the acute exposure in Experiment 1. Brackets denote an interaction between treatment and the aggression performed by residents and intruders during the contests. **B)** Aggressive acts plotted by aggressive acts given and aggressive acts received, and faceted by fluoxetine treatment for the acute time-point in Experiment 2. Brackets show a reduction in contest aggression by both residents and intruders. **C)** Aggressive acts plotted by aggressive acts given and aggressive acts received, and faceted by fluoxetine treatment for the chronic time-point in Experiment 2.

3.2. Experiment 2: acute and chronic exposure

We found no evidence for a three-way interaction between fluoxetine treatment, the aggressive acts between the resident and intruder, and exposure duration (Negative binomial GLMM: $N = 140$, High vs. Control: $Z = 1.03$, $p = 0.30$; Low vs. Control: $Z = 0.77$, $p = 0.44$; High vs. Low, $Z = 0.27$, $p = 0.79$). Therefore, we analyzed the acute and chronic time points separately to simplify the interpretation of the analysis. Unlike Experiment 1, we found no indication that fluoxetine influenced fish dominance in a contest, i.e., there was no interaction between fluoxetine treatment and the aggressive acts performed between resident and intruder fish (Negative Binomial GLMM: Treatment-by-contest aggression interaction: $N = 120$, High vs Control, $Z = 0.23$, $p = 0.82$; Low vs Control, $Z = -0.60$, $p = 0.55$; High vs Low: $Z = 0.84$, $p = 0.40$). Instead, the contests after an acute (3 days) exposure to the high dose of fluoxetine were less intense, involving fewer aggressive acts from *both* the resident and the intruder fish, when compared to contests from the control treatment (Control vs High: $Z = -2.31$ $p = 0.021$; Control vs Low: $Z = -0.71$, $p = 0.48$; High versus Low: -1.61, $p = 0.11$, Fig. 3b). This indicates that fluoxetine again reduced resident aggression towards intruders after a 3-day exposure, but intruders did not respond by increasing their aggression. After the chronic (28 day) exposure, we found no interaction between fluoxetine treatment and aggressive acts between resident and intruder fish (Negative Binomial GLMM: Treatment-by-contest aggression interaction, $N = 120$, High vs. Control: $Z = 0.97$, $p = 0.33$; Low vs. Control: $Z = 0.21$, $p = 0.84$, High versus low: $Z = 0.78$, $p = 0.43$). Furthermore, after 28 days, aggressive contests under high fluoxetine treatment were just as intense, i.e., involved similar numbers of aggressive acts by *both* resident and intruder fish, when compared to other treatment conditions (High vs. Control $Z = 0.36$, $p = 0.72$; Low vs. Control $Z = 0.95$, $p = 0.34$, High vs Low $Z = -0.56$, $p = 0.58$, Fig. 3c).

Fluoxetine treatment did not impact how quickly aggression was initiated in the contest assay (Linear mixed effects model: $N = 140$; High vs Control $t = 1.76$, $p = 0.082$; Low vs Control $t = 1.01$, $p = 0.31$; High vs Low: $Z = 0.76$, $p = 0.45$). However, the fish took longer to begin contests after 28 days of exposure than after 3 days of exposure (effect of exposure time: $t = 2.53$, $p = 0.013$); contests began on average after 498 s (±40 SE) seconds to begin after 3 days and after 585 s (±40 SE) to start after 28 days. Residents won more contests than intruders (taking 82% of control, 80% of low dose, and 75% of high dose contests), and fluoxetine treatment did not significantly affect the likelihood of the resident winning the contest (Binary logistic GLMM: $N = 140$, High vs Control, $Z = -1.21$, $p = 0.23$; Low vs Control $Z = -0.48$, $p = 0.63$; High vs Low: $Z = -0.77$, $p = 0.44$). Residents were equally likely to win contests at the acute and chronic time points (effect of exposure time: $Z = -0.60$, $p = 0.55$).

In contrast to aggression in contests over a resource, the fish treated with the high dose of fluoxetine were less aggressive towards a mirror image (second aggression assay) after exposure for 3 days and were also still less aggressive after 28 days (Negative binomial GLMM: $N = 135$, High vs Control $Z = -3.40$, $p = 0.00067$; High vs Low $Z = -3.16$, $p = 0.0016$; Low vs Control $Z = -0.24$, $p = 0.81$; Fig. 4; effect of exposure time: $Z = -0.21$, $p = 0.84$). In this experiment, fish exposed to the high dose and to the low dose of fluoxetine took longer to begin moving towards their mirror image and initiating aggression compared to controls, but this effect was only apparent after 28 days of exposure (Linear mixed effects model: $N = 135$: Time-by-treatment interaction for High vs Control: $Z = 2.31$, $p = 0.045$; Low

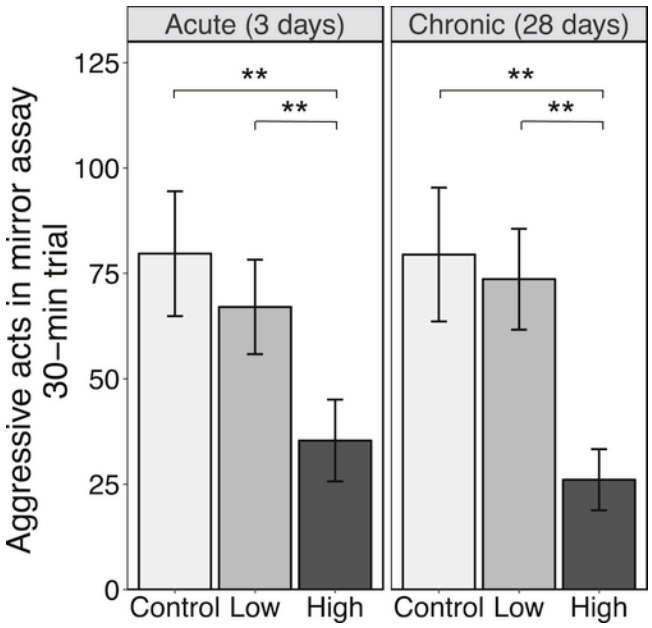

Fig. 4. Mirror aggression results from Experiment 2. Aggressive acts towards the mirror plotted against treatment, faceted by exposure duration. Brackets show reduction in aggression towards a mirror at both acute and chronic exposure durations. **p < 0.01, error bars represent ± SE.

vs Control: $Z = 1.96$, $p = 0.050$; High vs Low: $Z = 0.07$, $p = 0.95$). Consistent with our activity assay in Experiment 1, we found no effect of fluoxetine on non-aggressive activity at either time point (Negative binomial GLMM: $N = 135$, High vs Control $Z = -1.34$, $p = 0.18$: Low vs. Control: $Z = 0.46$, $p = 0.65$; High vs Low $Z = -1.79$, $p = 0.073$; effect of exposure time $Z = -0.93$, $p = 0.53$). There was no effect of sex in the above analyses for Experiment 2 ($p > 0.05$).

4. Discussion

4.1. Responses to fluoxetine exposure concentrations

Through its role in modulating the serotonergic system, fluoxetine exposure alters multiple physiological and behavioural processes important for fitness in fish (Gaworecki and Klaine, 2008; Mennigen et al., 2009, 2010a; 2010b, 2011; Schultz et al., 2011; de Abreu et al., 2014; Paula et al., 2015). In our study, we found that fluoxetine reduced round goby aggression after exposure to the high dose (40 μg/l), but found little evidence that exposure impacted aggression after exposure to an environmentally relevant low dose (1 μg/l). However, low dose fish were often behaviorally intermediate between control and high dose fish, indicating that we may have lacked power to identify differences. This pattern of results (impact observed at the high dose) was generally consistent between our two exposure experiments (Experiment 1 and Experiment 2) and between exposure durations (3 versus 28 days) within Experiment 2.

In humans, behavioural changes during fluoxetine treatment occur when plasma concentrations of fluoxetine reach between 0.09 μg/l - 0.30 μg/l (Amsterdam et al., 1997; de Freitas et al., 2010). Recently, Margiotta-Casaluci et al. (2014) showed that fathead minnow were more exploratory in a novel tank (a model for studying anxiety responses in fish) when their plasma fluoxetine concentrations reached levels similar to those needed to elicit therapeutic responses in humans. This 'therapeutic' effect in the fish only occurred at water concentrations greater than 30 μg/l in their study. Using calculations from the Fish Plasma Model (Huggett et al., 2003; see Supplementary Materials), we estimated that the steady state concentration of fluoxetine in round goby plasma was 0.011 μg/l and 0.42 μg/l for those fish exposed to our low 1 μg/l dose and high 40 μg/l dose, respectively. Therefore, round goby exposed to 40 μg/l may have experienced a plasma concentration of fluoxetine similar to a human therapeutic dose, as noted for fathead minnow in Margiotta-Casaluci et al. (2014). Select other studies have reported changes in fish behaviour at fluoxetine exposure concentrations of 1 μg/l or even lower (e.g guppy Pelli and Connaughton, 2015; Siamese fighting fish, Dzieweczynski and Hebert, 2012; Greaney et al., 2015; fathead minnow Weinberger and Klaper, 2014). However, in a current review of fluoxetine and its effects in fish, Sumpter et al. (2014) noted that most of the documented behavioural effects occur at water concentrations of 30 μg/l to 100 μg/l.

4.2. Responses to fluoxetine across experiments and testing contexts

We used a range of behavioural assays in our study to characterize round goby responses to fluoxetine, and we replicated two separate exposure experiments. We found that fluoxetine exposure primarily affected aggressive interactions, while having little effect on fish activity in an open-field or on the time fish spent interacting with a conspecific across a barrier. Therefore, the dampened aggression we observed was not a consequence of a more general reduction to fish activity. Altered aggression in round goby may be particularly important for survival and reproductive success, as these fish need to acquire and then vigorously defend shelters, which are used for protection from aquatic and avian predators as well as for mating and parental care (MacInnis and Corkum, 2000; Somers et al., 2003; Reyjol et al., 2010; Kornis et al., 2012). Across vertebrates, aggressiveness is a trait commonly associated with dominance and reproductive success, and aggression often correlates with an individual's ability to secure resources for breeding or for protection from predation (Clutton-Brock, 1988; Arnott and Elwood, 2008).

We found that fluoxetine treatment quickly reduced round goby aggression after exposure to the high dose (40 μg/l) for only 3 days. Aggression was reduced in multiple behavioural contexts: aggression towards a conspecific in a social interaction assay, aggression towards an intruder in a resource contest, and aggression towards a mirror reflection. Dzieweczynski and Hebert (2012) and Greaney et al. (2015) have documented a similar quick, acute reduction in aggression with male Siamese fighting fish; the fish exposed to 0.5 μg/l displayed reduced territorial aggression after only 48 h and also after 6 days of exposure. Likewise, Barry (2013) found reduced chasing behaviours in Arabian killifish after an exposure to 3 μg/l for seven days. At a much higher exposure, Kohlert et al. (2012) noted that Siamese fighting fish exposed to 350 μg/l and 705 μg/l decreased aggression towards a mirror after 11 days of exposure. The reduction in aggression we observed may be attributed to the actions of fluoxetine on the serotonergic system. By blocking the reuptake of serotonin via the serotonin transporter, fluoxetine acutely increases serotonergic signaling, which appears to have a highly conserved effect of reducing aggression in the short-term (Gaworecki and Klaine, 2008; Winder et al., 2009; Mennigen et al., 2011; Mitchell and Redfern, 2005).

In contrast to many acute exposure studies, few studies have addressed whether fluoxetine similarly affects aggression in fish after a chronic exposure (>21 days). After 28 days of exposure in Experiment 2, we found that fish showed reduced aggression *only* in the mirror assay and not in the contest aggression assay. There is grow-

ing evidence in mammals that individuals chronically exposed (1 month or more) to selective serotonin reuptake inhibitors like fluoxetine can exhibit a behavioural recovery or even display behavioural effects in the opposite direction from those following an acute exposure (reviewed in: Mitchell and Redfern, 2005). Such a "recovery process" could be mediated by negative feedback, where serotonin autoreceptors decrease serotonin production in order to return serotonin to pre-treatment or lower levels (Hjorth et al., 2000; Mitchell and Redfern, 2005). However, we find it unlikely that a negative-feedback process is driving the lack of treatment effects in the chronic contest aggression assay. Instead, we suggest that this inconsistency was more likely driven by behavioural variability introduced by intruder fish. In support of this, we found that the amount of aggression performed by the exposed resident fish towards intruder fish was not correlated between the two testing time points in Experiment 2 (Pearson's R = 0.18, $p = 0.084$). Whereas, aggression performed by the same resident fish towards the mirror in the mirror assay was highly correlated between the two time points (Pearson's R = 0.40, $p < 0.001$). Intruder behaviour also varied between the acute exposure in Experiment 1 and the acute exposure in Experiment 2: intruders *increased* their aggression towards the resident in Experiment 1, while intruders *decreased* their aggression towards the resident in Experiment 2. We selected intruder fish for our resource contests in a consistent manner across experiments and exposure regimes and were always careful to control for resident-intruder size differences. However, considerable variability in aggression between contestants has been observed in many behavioural ecological studies (Wilson et al., 2011; Balzarini et al., 2014), and such variation presents a challenge when measuring how environmental pollutants like pharmaceuticals affect complex aggressive interactions between fish. Mirror assays provide a fast and standardized way of measuring aggressive behaviours in fish; however, mirror images do not accurately capture the complexity of dyadic resource contests.

4.3. Conclusions

We studied the impact of fluoxetine exposure at environmentally and therapeutically relevant concentrations on a wild fish species, the round goby. We tested multiple behavioural contexts, durations and replicated exposures in two experiments. We conclude that mirror assays provide a more consistent indicator of aggressive motivation. However, aggressive contests between two fish still better capture the effects of exposures on ecologically relevant outcomes of aggressive interactions (i.e., acquiring resources). While the adult round goby in our study appeared unaffected by a low environmentally relevant dose of fluoxetine, future research testing a wider range of fluoxetine concentrations and age classes of round goby would help elucidate at what exposure concentrations and life stages behavioural effects become apparent (for example, determining effective concentrations for behavioural effects, EC_{50}). Our research can be added to a growing body of literature indicating that fluoxetine has little notable impact on fish aggression at doses lower than 30 μg/l (Sumpter et al., 2014), with a few specific species exceptions (Siamese fighting fish; Dzieweczynski and Hebert, 2012; Greaney et al., 2015; and Arabian killifish, Barry, 2013). Partnering future behavioural work with measurements of serotonin will be especially informative for ascertaining a mechanism of action for altered aggressive behaviours. Moreover, future work exploring how exposure of intruder fish to fluoxetine, alongside resident fish, affects aggressive resource contests will be important for further extending these behavioural findings to fish in the wild. As behavioural assays are increasingly incorporated into studies of aquatic toxicology, we emphasize the need for reliable and repeatable assays. Ideally, these assays will be suited to test behavioural effects in a standardized manner across a wide variety of organisms.

Acknowledgements

The research described here followed procedures approved by the Animal Research Ethics Board (AREB) at McMaster University (AUP: 13-12-51) and are in accordance with the Canadian Council on Animal Care. The authors would like to thank J. Wilson for comments on experimental design; C. Metcalfe, B. Seaborn, & T. Sultana for analyzing fluoxetine in the water samples; J. Wilson, J. Marentette, P. Andrews and B. Galef for comments on this manuscript; B. Bolker & J. Dushoff for assistance with statistical analyses; P. Capelle, K. Goodick, N. Lobban, and M. Lu for assistance with field collections and behavioural scoring; and two anonymous reviewers for reviewing our manuscript. This work was funded by an NSERC Discovery Grant and the Canada Research Chair Program to S. Balshine, and an NSERC PGS-D to E. McCallum.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.chemosphere.2017.02.059.

References

Amsterdam, J.D., Fawcett, J., Quitkin, F.M., Reimherr, F.W., Rosenbaum, J.F., Michelson, D., Hornig-Rohan, M., Beasley, C.M., 1997. Fluoxetine and norfluoxetine plasma concentrations in major depression: a multicenter study. Am. J. Psychiatr. 154 (7), 963–969. http://dx.doi.org/10.1176/ajp.154.7.963.

Arnold, K.E., Boxall, A.B.A., Brown, A.R., Cuthbert, R.J., Gaw, S., Hutchinson, T.H., Jobling, S., Madden, J.C., Metcalfe, C.D., Naidoo, V., Shore, R.F., Smits, J.E., Taggart, M.A., Thompson, H.M., 2013. Assessing the exposure risk and impacts of pharmaceuticals in the environment on individuals and ecosystems. Biol. Lett. 9, 20130492. http://dx.doi.org/10.1098/rsbl.2013.0492.

Arnott, G., Elwood, R.W., 2008. Information gathering and decision making about resource value in animal contests. Anim. Behav. 76 (3), 529–542. http://dx.doi.org/10.1016/j.anbehav.2008.04.019.

Balzarini, V., Taborsky, M., Wanner, S., Koch, F., Frommen, J.G., 2014. Mirror, mirror on the wall: the predictive value of mirror tests for measuring aggression in fish. Behav. Ecol. Sociobiol. 68 (5), 871–878. http://dx.doi.org/10.1007/s00265-014-1698-7.

Barbosa, A., Alves, F.L., Pereira, A.D.S.F., Ide, L.M., Hoffmann, A., 2012. Behavioral characterization of the alarm reaction and anxiolytic-like effect of acute treatment with fluoxetine in piauçu fish. Physiol. Behav. 105 (3), 784–790. http://dx.doi.org/10.1016/j.physbeh.2011.10.007.

Barry, M.J., 2013. Effects of fluoxetine on the swimming and behavioural responses of the Arabian killifish. Ecotoxicology 22, 425–432. http://dx.doi.org/10.1007/s10646-012-1036-7.

Bates, D., Maechler, M., Bolker, B., Walker, S., 2015. Fitting linear mixed-effects models using lme4. J. Stat. Softw. 67 (1), 1–48. Stable URL: https://arxiv.org/pdf/1406.5823.

Berger, M., Gray, J.A., Roth, B.L., 2009. The expanded biology of serotonin. Annu. Rev. Med. 60, 355–366. http://dx.doi.org/10.1146/annurev.med.60.042307.110802.

Boxall, A.B.A., Rudd, M.A., Brooks, B.W., Caldwell, D.J., Choi, K., Hickmann, S., Innes, E., Ostapyk, K., Staveley, J.P., Verslycke, T., Ankley, G.T., Beazley, K.F., Belanger, S.E., Berninger, J.P., Carriquiriborde, P., Coors, A., DeLeo, P.C., Dyer, S.D., Ericson, J.F., Gagné, F., Giesy, J.P., Gouin, T., Hallstrom, L., Karlsson, M.V., Larsson, D.G.J., Lazorchak, J.M., Mastrocco, F., McLaughlin, A., McMaster, M.E., Meyerhoff, R.D., Moore, R., Parrott, J.L., Snape, J.R., Murray-Smith, R., Servos, M.R., Sibley, P.K., Straub, J.O., Szabo, N.D., Topp, E., Tetreault, G.R., Trudeau, V.L., Van Der Kraak, G., 2012. Pharmaceuticals and personal care products in the environment: what are the big questions?. Environ. Health Perspect. 120 (9), 1221–1229. http://dx.doi.org/10.1289/ehp.1104477.

Brodin, T., Piovano, S., Fick, J., Klaminder, J., Heynen, M., Heynen, M., Jonsson, M., 2014. Ecological effects of pharmaceuticals in aquatic systems − impacts through behavioural alterations. Philos. Trans. R. Soc. B Biol. Sci. 369, 20130580. http://dx.doi.org/10.1098/rstb.2013.0580.

Brooks, B.W., 2014. Fish on prozac (and zoloft): ten years later. Aquat. Toxicol. 151, 61–67. http://dx.doi.org/10.1016/j.aquatox.2014.01.007.

Brooks, B.W., Chambliss, C.K., Stanley, J.K., Ramirez, A., Banks, K.E., Johnson, R.D., Lewis, R.J., 2005. Determination of select antidepressants in fish from an effluent-dominated stream. Environ. Toxicol. Chem. 24 (2), 464–469. http://dx.doi.org/10.1897/04-081R.1.

Brooks, B.W., Turner, P.K., Stanley, J.K., Weston, J.J., Glidewell, E.A., Foran, C.M., Slattery, M., La Pointa, T.W., Huggett, D.B., 2003. Waterborne and sediment toxicity of fluoxetine to select organisms. Chemosphere 52, 135–142. http://dx.doi.org/10.1016/S0045-6535(03)00103-6.

Capelle, P.M., McCallum, E.S., Balshine, S., 2015. Aggression and sociality: conflicting or complementary traits of a successful invader?. Behavior 152 (2), 127–146. http://dx.doi.org/10.1163/1568539X-00003235.

Christensen, A.M., Markussen, B., Baun, A., Halling-Sørensen, B., 2009. Probabilistic environmental risk characterization of pharmaceuticals in sewage treatment plant discharges. Chemosphere 77 (3), 351–358. http://dx.doi.org/10.1016/j.chemosphere.2009.07.018.

Clutton-Brock, T.H., 1988. Reproductive Success: Studies of Individual Variation in Contrasting Breeding Systems. University of Chicago Press.

Corcoran, J., Winter, M.J., Tyler, C.R., 2010. Pharmaceuticals in the aquatic environment: a critical review of the evidence for health effects in fish. Crit. Rev. Toxicol. 40 (4), 287–304. http://dx.doi.org/10.3109/10408440903373590.

Corkum, L.D., Sapota, M.R., Skora, K.E., 2004. The round goby, Neogobius melanostomus, a fish invader on both sides of the Atlantic Ocean. Biol. Invas. 6 (2), 173–181. http://dx.doi.org/10.1023/B:BINV.0000022136.43502.db.

Cribari-Neto, F., Zeileis, A. 2010. Beta Regression in R. J. Statistical Softw., 34(2), 1–24.

de Abreu, M.S., Koakoski, G., Ferreira, D., Acosta Oliveira, T., Santos Da Rosa, J.G., Gusso, D., Giacomini, A.C.V., Piato, A.L., Barcellos, L.J.G., 2014. Diazepam and fluoxetine decrease the stress response in zebrafish. PLoS One 9 (7), 1–5. http://dx.doi.org/10.1371/journal.pone.0103232.

de Freitas, D.F., Porto, C.E.D., Vieira, E.P., de Siqueira, M.E.P.B., 2010. Three-phase, liquid-phase microextraction combined with high performance liquid chromatography-fluorescence detection for the simultaneous determination of fluoxetine and norfluoxetine in human plasma. J. Pharm. Biomed. Anal. 51 (1), 170–177. http://dx.doi.org/10.1016/j.jpba.2009.07.017.

Dzieweczynski, T.L., Hebert, O.L., 2012. Fluoxetine alters behavioral consistency of aggression and courtship in male Siamese fighting fish, Betta splendens. Physiol. Behav. 107 (1), 92–97. http://dx.doi.org/10.1016/j.physbeh.2012.06.007.

Elwood, R.W., Stoilova, V., Mcdonnell, A., Earley, R.L., Arnott, G., 2014. Do mirrors reflect reality in agonistic encounters? A test of mutual cooperation in displays. Anim. Behav. 97, 63–67. http://dx.doi.org/10.1016/j.anbehav.2014.07.028.

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

Fournier, D.A., Skaug, H.J., Ancheta, J., Ianelli, J., Magnusson, A., Maunder, M., Nielsen, A., Sibert, J., 2012. AD Model Builder: using automatic differentiation for statistical inference of highly parameterized complex nonlinear models. Optim. Methods Softw. 27, 233–249. http://dx.doi.org/10.1080/10556788.2011.597854.

Gaworecki, K.M., Klaine, S.J., 2008. Behavioral and biochemical responses of hybrid striped bass during and after fluoxetine exposure. Aquat. Toxicol. 88 (4), 207–213. http://dx.doi.org/10.1016/j.aquatox.2008.04.011.

Greaney, N.E., Mannion, K.L., Dzieweczynski, T.L., 2015. Signaling on Prozac: altered audience effects on male-male interactions after fluoxetine exposure in Siamese fighting fish. Behav. Ecol. Sociobiol. 69 (12), 1925–1932. http://dx.doi.org/10.1007/s00265-015-2005-y.

Groen, M., Sopinka, N.M., Marentette, J.R., Reddon, A., Brownscombe, J., Fox, M., Marsh-Rollo, S.E., Balshine, S., 2012. Is there a role for aggression in round goby invasion fronts?. Behavior 149, 685–703. http://dx.doi.org/10.1163/1568539X-00002998.

Gunnarsson, L., Jauhiainen, A., Kristiansson, E., Nerman, O., Larsson, D.G.J., 2008. Evolutionary conservation of human drug targets in organisms used for environmental risk assessments. Environ. Sci. Technol. 42 (15), 5807–5813. http://dx.doi.org/10.1021/es8005173.

Hemels, M.E.H., Koren, G., Einarson, T.R., 2005. Increased use of antidepressants in Canada: 1981 – 2000. Ann. Pharmacother. 36, 1375–1379. http://dx.doi.org/10.1345/aph.1A331.

Hjorth, S., Bengtsson, H.J., Kullberg, A., Carlzon, D., Peilot, H., Auerbach, S.B., 2000. Serotonin autoreceptor function and antidepressant drug action. J. Psychopharmacol. 14 (2), 177–185. http://dx.doi.org/10.1177/026988110001400208.

Huggett, D.B., Cook, J.C., Ericson, J.F., Williams, R.T., 2003. Theoretical model for prioritizing potential impacts of human pharmaceuticals to fish. Hum. Ecol. Risk Assess. 9 (7), 1789–1799. http://dx.doi.org/10.1080/714044797.

Jelic, A., Gros, M., Petrovic, M., Ginebreda, A., Barcelo, D., 2012. Occurrence and elimination of pharmaceuticals during conventional wastewater treatment. In: In: Guasch, H., Ginebreda, A., Geiszinger, A. (Eds.), Emerging and Priorty Pollutatns in Rivers. vol. 19. Springer Berlin Heidelberg, Berlin, Heidelberg, pp. 1–23.

Khetan, S.K., Collins, T.J., 2007. Human pharmaceuticals in the aquatic environment: a challenge to green chemistry. Chem. Rev. 107 (6), 2319–2364. http://dx.doi.org/10.1021/cr020441w.

Kohlert, J.G., Mangan, B.P., Kodra, C., Drako, L., Long, E., Simpson, H., 2012. Decreased aggressive and locomotor behaviors in Betta Splendens after exposure to fluoxetine. Psychol. Rep. 110 (1), 51–62. http://dx.doi.org/10.2466/02.13.PR0.110.1.51-62.

Kolpin, D.W., Furlong, E.T., Meyer, M.T., Thurman, E.M., Zaugg, S.D., Barber, L.B., Buxton, H.T., 2002. Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999-2000: a national reconnaissance. Environ. Sci. Technol. 36 (6), 1202–1211. http://dx.doi.org/10.1021/es011055j.

Kornis, M.S., Mercado-Silva, N., Vander Zanden, M.J., 2012. Twenty years of invasion: a review of round goby Neogobius melanostomus biology, spread and ecological implications. J. Fish. Biol. 80 (2), 235–285. http://dx.doi.org/10.1111/j.1095-8649.2011.03157.x.

Lillesaar, C., 2011. The serotonergic system in fish. J. Chem. Neuroanat. 41 (4), 294–308. http://dx.doi.org/10.1016/j.jchemneu.2011.05.009.

MacInnis, A., Corkum, L., 2000. Fecundity and reproductive season of the round goby Neogobius melanostomus in the upper Detroit River. Trans. Am. Fish. 129, 136–144. http://dx.doi.org/10.1577/1548-8659(2000)129<0136:FARSOT>2.0.CO;2.

Majewski, H.S., Klaverkamp, J.F., Scott, D.P., 1978. Acute lethality, and sub-lethal effects of acetone, ethanol, and propylene glycol on the cardiovascular and respiratory systems of rainbow trout (Salmo gairdneri). Water Res. 13, 217–221. http://dx.doi.org/10.1016/0043-1354(78)90089-1.

Marentette, J.R., Corkum, L.D., 2008. Does the reproductive status of male round gobies (Neogobius melanostomus) influence their response to conspecific odours?. Environ. Biol. Fish. 81 (4), 447–455. http://dx.doi.org/10.1007/s10641-007-9240-7.

Marentette, J.R., Wang, G., Tong, S., Sopinka, N.M., Taves, M.D., Koops, M.A., Balshine, S., 2011. Laboratory and field evidence of sex-biased movement in the invasive round goby. Behav. Ecol. Sociobiol. 65 (12), 2239–2249. http://dx.doi.org/10.1007/s00265-011-1233-z.

Margiotta-Casaluci, L., Owen, S.F., Cumming, R.I., De Polo, A., Winter, M.J., Panter, G.H., Rand-Weaver, M., Sumpter, J.P., 2014. Quantitative cross-species extrapolation between humans and fish: the case of the anti-depressant fluoxetine. PLoS One 9 (10), e110467. http://dx.doi.org/10.1371/journal.pone.0110467.

McCallum, E.S., Charney, R.E., Marenette, J.R., Young, J.A.M., Koops, M.A., Earn, D.J.D., Bolker, B.M., Balshine, S., 2014. Persistence of an invasive fish (Neogobius melanostomus) in a contaminated ecosystem. Biol. Ivas. 16 (11), 2446–2449. http://dx.doi.org/10.1007/s10530-014-0677-2.

McCallum, E.S., Du, S.N.N., Vaseghi-Shanjani, M., Choi, J.A., Warriner, T.R., Sultana, T., Scott, G.R., Balshine, S., 2017. In situ exposure to wastewater effluent reduces survival but has little effect on the behaviour or physiology of an invasive Great Lakes fish. Aquat. Toxicol. 184, 37–48.

Mennigen, J.A., Sassine, J., Trudeau, V.L., Moon, T.W., 2010a. Waterborne fluoxetine disrupts feeding and energy metabolism in the goldfish Carassius auratus. Aquat. Toxicol. 100 (1), 128–137. http://dx.doi.org/10.1016/j.aquatox.2010.07.022.

Mennigen, J.A., Lado, W.E., Zamora, J.M., Duarte-Guterman, P., Langlois, V.S., Metcalfe, C.D., Chang, J.P., Moon, T.W., Trudeau, V.L., 2010b. Waterborne fluoxetine disrupts the reproductive axis in sexually mature male goldfish, Carassius auratus. Aquat. Toxicol. 100 (4), 354–364. http://dx.doi.org/10.1016/j.aquatox.2010.08.016.

Mennigen, J.A., Harris, E.A., Chang, J.P., Moon, T.W., Trudeau, V.L., 2009. Fluoxetine affects weight gain and expression of feeding peptides in the female goldfish brain. Reg. Pept. 155 (1), 99–104. http://dx.doi.org/10.1016/j.regpep.2009.01.001.

Mennigen, J.A., Stroud, P., Zamora, J.M., Moon, T.W., Trudeau, V.L., 2011. Pharmaceuticals as neuroendocrine disruptors: lessons learned from fish on Prozac. J. Toxicol. Environ. Health Part B. Crit. Rev. 14 (5–7), 387–412. http://dx.doi.org/10.1080/10937404.2011.578559.

Metcalfe, C.D., Chu, S., Judt, C., Li, H., Oakes, K.D., Servos, M.R., Andrews, D.M., 2010. Antidepressants and their metabolites in municipal wastewater, and downstream exposure in an urban watershed. Environ. Toxicol. Chem. 29 (1), 79–89. http://dx.doi.org/10.1002/etc.27.

Milea, D., Verpillat, P., Guelfucci, F., Toumi, M., Lamure, M., 2010. Prescription patterns of antidepressants: findings from a US claims database. Curr. Medic. Res. Opin. 26 (6), 1343–1353. http://dx.doi.org/10.1185/03007991003772096.

Mitchell, P.J., Redfern, P.H., 2005. Animal models of depressive illness: the importance of chronic drug treatment. Curr. Pharm. Des. 11 (2), 171–203. http://dx.doi.org/10.2174/1381612053382250.

OECD, 2013. Health at a Glance 2013: OECD Indicators. OECD Publishinghttp://dx.doi.org/10.1787/health_glance-2013-en.

Paula, J.R., Messias, J.P., Grutter, A.S., Bshary, R., Soares, M.C., 2015. The role of serotonin in the modulation of cooperative behavior. Behav. Ecol. 26 (4), 1005–1012. http://dx.doi.org/10.1093/beheco/arv039.

Paulose-Ram, R., Safran, M.A., Jonas, B.S., Gu, Q., Orwig, D., 2007. Trends in psychotropic medication use among U.S. adults. Pharmacoepidemiol. Drug Saf. 16, 560–570. http://dx.doi.org/10.1002/pds.1367.

Peakall, D.B., 1996. Disrupted patterns of behavior in natural populations as an index of ecotoxicity. Environ. Health Perspect. 104 (Suppl. 2), 331. http://dx.doi.org/10.2307/3432653.

Pelli, M., Connaughton, V.P., 2015. Chronic exposure to environmentally-relevant concentrations of fluoxetine (Prozac) decreases survival, increases abnormal be-

haviors, and delays predator escape responses in guppies. Chemosphere 139, 202–209. http://dx.doi.org/10.1016/j.chemosphere.2015.06.033.
Perreault, H., Semsar, K., Godwin, J., 2003. Fluoxetine treatment decreases territorial aggression in a coral reef fish. Physiol. Behav. 79 (4–5), 719–724. http://dx.doi.org/10.1016/S0031-9384(03)00211-7.
R Core Team, 2015. R: a Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. URL: https://www.R-project.org/.
Ramirez, A., Brain, R., Usenka, S., Mottaleb, M., O'Donnell, J., Stahl, L., Wathen, J.B., Snyder, B.D., Pitt, J.L., Perez-Hurtado, P., Dobbins, L.L., Brooks, B.W., Chambliss, C.K., 2009. Occurrence of pharmaceuticals and personal care products in fish: results of a national pilot study in the United States. Environ. Toxicol. Chem. 28 (12), 2587–2597. http://dx.doi.org/10.1897/08-561.1.
Reddon, A.R., Balk, D., Balshine, S., 2011. Sex differences in group-joining decisions in social fish. Anim. Behav. 82 (2), 229–234. http://dx.doi.org/10.1016/j.anbehav.2011.04.017.
Reyjol, Y., Brodeur, P., Mailhot, Y., Mingelbier, M., Dumont, P., 2010. Do native predators feed on non-native prey? The case of round goby in a fluvial piscivorous fish assemblage. J. Gt. Lakes Res. 36 (4), 618–624. http://dx.doi.org/10.1016/j.jglr.2010.09.006.
Schultz, M.M., Furlong, E.T., Kolpin, D.W., Werner, S.L., Schoenfuss, H.L., Barber, L.B., Blazer, V.S., Norris, D.O., Vajda, A.M., 2010. Antidepressant pharmaceuticals in two US effluent-impacted streams: occurrence and fate in water and sediment, and selective uptake in fish neural tissue. Environ. Sci. Technol. 44 (6), 1918–1925. http://dx.doi.org/10.1021/es9022706.
Schultz, M.M., Painter, M.M., Bartell, S.E., Logue, A., Furlong, E.T., Werner, S.L., Schoenfuss, H.L., 2011. Selective uptake and biological consequences of environmentally relevant antidepressant pharmaceutical exposures on male fathead minnows. Aquat. Toxicol. 104 (1), 38–47. http://dx.doi.org/10.1016/j.aquatox.2011.03.011.
Sebire, M., Elphinstone Davis, J., Hatfield, R., Winberg, S., Katsiadaki, I., 2015. Prozac affects stickleback nest quality without altering androgen, spiggin or aggression levels during a 21-day breeding test. Aquat. Toxicol. 168, 78–89. http://dx.doi.org/10.1016/j.aquatox.2015.09.009.
Somers, C.M., Lozer, M.N., Kjoss, V.A., Quinn, J.S., 2003. The invasive round goby (Neogobius melanostomus) in the diet of nestling double-crested cormorants (Phalacrocorax auritus) in Hamilton Harbour, lake Ontario. J. Gt. Lakes Res. 29 (3), 392–399. http://dx.doi.org/10.1016/S0380-1330(03)70446-8.
Stahl, S.M., 1998. Mechanism of action of serotonin selective reuptake inhibitors: serotonin receptors and pathways mediate therapeutic effects and side effects. J. Affect. Disord. 51, 215–235. http://dx.doi.org/10.1016/S0165-0327(98)00221-3.
Sumpter, J.P., Donnachie, R.L., Johnson, A.C., 2014. The apparently very variable potency of the anti-depressant fluoxetine. Aquat. Toxicol. 151, 57–60. http://dx.doi.org/10.1016/j.aquatox.2013.12.010.
Svensson, P.A., Barber, I., Forsgren, E., 2000. Shoaling behaviour of the two-spotted goby. J. Fish. Biol. 56 (6), 1477–1487. http://dx.doi.org/10.1006/jfbi.2000.1274.
Weinberger, J., Klaper, R., 2014. Environmental concentrations of the selective serotonin reuptake inhibitor fluoxetine impact specific behaviors involved in reproduction, feeding and predator avoidance in the fish Pimephales promelas (fathead minnow). Aquat. Toxicol. 151, 77–83. http://dx.doi.org/10.1016/j.aquatox.2013.10.012.
Wilson, A.J., de Boer, M., Arnott, G., Grimmer, A., 2011. Integrating personality research and animal contest theory: aggressiveness in the green swordtail Xiphophorus helleri. PLoS One 6 (11), e28024. http://dx.doi.org/10.1371/journal.pone.0028024.
Winberg, S., Nilsson, G.E., 1993. Roles of brain monoamine neurotransmitters in agonistic behaviour and stress reactions, with particular reference to fish. Comp. Biochem. Physiol. C. – Pharmacol. Toxicol. Endocrinol. 106 (3), 597–614. http://dx.doi.org/10.1016/0742-8413(93)90216-8.
Winder, V.L., Sapozhnikova, Y., Pennington, P.L., Wirth, E.F., 2009. Effects of fluoxetine exposure on serotonin-related activity in the sheepshead minnow (Cyprinodon variegatus) using LC/MS/MS detection and quantitation. Comp. Biochem. Physiol. C. – Pharmacol. Toxicol. Endocrinol. 149 (4), 559–565. http://dx.doi.org/10.1016/j.cbpc.2008.12.008.
Wolfe, K.R., Marsden, E.J., 1998. Tagging methods for the round goby (Neogobius melanostomus). J. Gt. Lakes Res. 24 (3), 731–735. http://dx.doi.org/10.1016/S0380-1330(98)70857-3.
Young, J.A.M., Marentette, J.R., Gross, C., McDonald, J.I., Verma, A., Marsh-Rollo, S.E., Macdonald, P.D.M., Earn, D.J.D., Balshine, S., 2010. Demography and substrate affinity of the round goby (Neogobius melanostomus) in Hamilton Harbour. J. Gt. Lakes Res. 36 (1), 115–122. http://dx.doi.org/10.1016/j.jglr.2009.11.001.
Zeyl, J.N., Love, O.P., Higgs, D.M., 2014. Evaluating gonadosomatic index as an estimator of reproductive condition in the invasive round goby, Neogobius melanostomus. J. Gt. Lakes Res. 40 (1), 164–171. http://dx.doi.org/10.1016/j.jglr.2013.12.004.