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RESEARCH ARTICLE

Dose-dependent fluoxetine effects on boldness in male Siamese fighting fish

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ABSTRACT

As the use of pharmaceuticals and personal care products (PPCPs) continues to rise, these compounds enter the environment in increasing frequency. One such PPCP, fluoxetine, has been found in detectable amounts in aquatic ecosystems worldwide, where it may interfere with the behavior of exposed organisms. Fluoxetine exposure has been found to influence boldness and exploration in a range of fish species; however, how it might alter behavior in multiple contexts or over time is rarely examined. To this end, the effects of fluoxetine on boldness over time were studied in male Siamese fighting fish. Three different groups of males (0, 0.5 and 5 µg l^{-1} fluoxetine) were tested in multiple boldness assays (empty tank, novel environment and shoal) once a week for 3 weeks to collect baseline measures and then at three different time points post-exposure. The effects of these varying exposure amounts on behavior were then examined for overall response, consistency and across-context correlations. Unexposed males were bolder in all contexts, were more consistent within a context, and had stronger between-context correlations than exposed males. Fluoxetine had dose-dependent effects on behavior, as males that received the higher dose exhibited greater behavioral effects. This study stresses the potential fitness consequences of fluoxetine exposure and suggests that examining behavioral effects of PPCPs under different dosing regimens and in multiple contexts is important to gain an increased understanding of how exposure affects behavior.

KEY WORDS: Inadvertent pharmaceutical exposure, Boldness, PPCPs, SSRI, Behavioral syndromes, Personality

INTRODUCTION

There has been growing concern over the past two decades regarding the prevalence of pharmaceuticals and personal care products (PPCPs) in waterways worldwide. Many PPCPs are still in their active form when they enter sewage treatment systems, where they have limited removal because of their water solubility and resistance to biodegradation (Heberer, 2002; Fent et al., 2006). PPCPs are designed to alter physiology and produce behavioral effects at low doses, and this, coupled with the high degree of evolutionary conservation across vertebrates, suggests that these compounds may influence non-target organisms (Ruhoy and Daughton, 2008; Boxall, 2009; Sumpter, 2009; Arnold et al., 2013). The chemical attributes of pharmaceuticals are very different from those of industrial chemicals, which leads to different influences in the environment and suggests that many environmental regulatory programs may not be effective or relative to these types of components (Brooks et al., 2009; Monteiro and Boxall, 2009). Much of the research on the effects of PPCPs on aquatic organisms has focused on the effects of estrogen mimics or the physiological rather than behavioral alterations caused by non-steroid PPCPs (Daughton and Ternes, 1999; Brooks et al., 2003). However, because many of the PPCPs found in aquatic environments have been specifically designed to alter behavior, behavioral endpoints are potentially more relevant indicators of species risk than physiological changes (Jones et al., 1991; Brooks et al., 2003; Lovern et al., 2007). Additionally, the use of standardized behavioral assays that incorporate behaviors of direct and indirect importance to ecosystem functioning could greatly improve our understanding of the effect of pharmaceutical exposure on wildlife (Brodin et al., 2014; Klaminder et al., 2014).

A class of PPCPs that is most concerning is selective serotonin reuptake inhibitors (SSRIs), such as fluoxetine; these compounds directly target the serotonergic system, which plays an important role in regulating many physiological and behavioral processes (Khan and Thomas, 1992; de Pedro et al., 1998; Kostich and Lazorchak, 2008; Fong and Ford, 2014). SSRIs inhibit the serotonin reuptake mechanism, resulting in a net increase of serotonin in the central nervous system (Kreke and Dietrich, 2008). SSRIs are among the most widely prescribed pharmaceuticals, with antidepressant use up over 60% the last decade (OECD, 2012). They have been found in wastewaters, drinking water and sediments worldwide, where they have dramatic effects on non-target organisms residing in these waters (Schultz and Furlong, 2008; Mennigen et al., 2011; Silva et al., 2012). The increased prescription rate is likely responsible for why SSRIs are one of the most commonly detected pharmaceuticals in the aquatic environment, with concentrations ranging between 0.15 and 32 ng l^{-1} in wastewater, 0.5 and 8000 ng l^{-1} in surface water, and 0.5 and 1400 ng l^{-1} in drinking water (Schultz et al., 2010; Silva et al., 2012). Common effects of exposure include delayed or abnormal reproductive or physiological development, reduced aggressiveness, and suppressed feeding rates (Demeestere et al., 2010; Fong and Ford, 2014; Silva et al., 2015). Indeed, the changes in behavior that typically accompany SSRI exposure, such as reduced foraging rates and decreased locomotion, could alter life history traits and may even have transgenerational effects (Schultz et al., 2011; Valenti et al., 2012; Lamichhane et al., 2014).

Fluoxetine, the active ingredient in Prozac, is one of the most widely prescribed SSRIs and is used to treat a range of anxiety-related disorders (Brooks, 2014). Fluoxetine exposure decreases feeding rates in multiple fish species, including goldfish and hybrid striped bass (Gaworecki and Klaine, 2008; Mennigen et al., 2010), which may reduce fitness if individuals are encountering prey less frequently and eating less. Short-term exposure to fluoxetine at low doses suppressed activity in Arabian killifish (Barry, 2013) and Siamese fighting fish (Kohlert et al., 2012). Zebrafish exposed to a higher dose (1.5 mg l^{-1}) of fluoxetine were less active and moved

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Journal of Experimental Biology (2016) 219, 797-804 doi:10.1242/jeb.132761

about the tank less than those exposed to a lower dose (0.5 mg l^{-1}) or no fluoxetine, with females being more affected than males (Dagh, 2013). Finally, fathead minnows exposed to fluoxetine for a month had reduced reproductive and predator avoidance behaviors, and these effects were dose dependent and more severe in males than females (Weinberger and Klaper, 2014), suggesting that it may be important to examine exposure effects at multiple doses and in both sexes. Additionally, examining the effects of fluoxetine on activity level and exploration is especially important as these behaviors have fitness implications and are thought to be associated with anxiety.

The existence of consistent individual differences in behavior within a population, even in individuals of the same age or sex, has been widely studied by behavioral ecologists over the last decade (Gosling, 2001; Sih et al., 2004). Consistent behavioral variation within a population is beneficial, particularly if the environment is highly variable (Dingemanse and Réale, 2005). For example, in honeybees, some individuals locate flowers more quickly than others but are also less accurate at finding high-nectar flowers than those bees that take more time searching (Sih and Del Giudice, 2012). Additionally, risk-taking, activity level, and exploratory behavior are correlated in bluegill sunfish, with bolder individuals being more active and more willing to explore novel stimuli (Wilson and Godin, 2009). The term behavioral syndrome refers to a suite of correlated behaviors in individuals that is expressed consistently in a given context or across contexts (Sih et al., 2004). For example, a positive correlation between boldness and aggressiveness is found in numerous species; however, the strength of this correlation appears to vary as a function of predation intensity (Bell and Sih, 2007; Stamps and Groothuis, 2010). The bold–shy axis of behavior, referred to as introversion and extroversion in humans, is perhaps the most well-studied and most common source of distinct, stable, heritable variation in animals (Coleman and Wilson, 1998; Toms et al., 2010). Variation between individuals along the bold–shy axis may have important consequences in a range of contexts over an individual's lifespan because an individual's level of boldness may influence its success in competition, breeding, foraging, or response to environmental changes (Wilson et al., 1993). Thus, if exposure to PPCPs alters an individual's boldness or decreases the degree of behavioral variation present in the population, it could have dramatic, negative impacts on individual success and population survival. Indeed, examining how PPCPs and other contaminants might influence the degree of behavioral variation in a population has recently been identified as an important, yet underexplored, research question (Montiglio and Royauté, 2014).

Male Siamese fighting fish, *Betta splendens* Regan 1910, were used to investigate how fluoxetine dose (none, ecologically relevant, and pharmacological dose) influences behavior over time (i.e. consistency) and across contexts (i.e. behavioral syndrome). Siamese fighting fish of the veil-tail strain are an ideal subject for ecotoxicology studies using behavioral endpoints because they have well-defined behaviors (Simpson, 1968), consistent individual variation in multiple behaviors including boldness (e.g. Dzieweczynski and Hebert, 2012; Hebert et al., 2014), and few studies have examined the effects of PPCPs on fish from tropical regions (Brooks, 2014). Fluoxetine injected intramuscularly did not alter aggression levels in male Siamese fighting fish in one study (Clotfelter et al., 2007), but decreased aggression in another study (Kania et al., 2012), perhaps because of differences in the type of aggressive stimulus used and duration of exposure. Male Siamese fighting fish have been shown to be less aggressive (Dzieweczynski and Hebert, 2012), even after as little as 3 h of exposure to 3 μg ml^{-1} of fluoxetine-treated water (Lynn et al., 2007), and less active (Kohlert et al., 2012) after exposure to fluoxetine via water. Given that it has recently been found that female Siamese fighting fish are less active and explore less after fluoxetine exposure (Dzieweczynski et al., 2016), we hypothesized that similar reductions in behavior would be seen in males. Reductions in activity level after acute fluoxetine exposure appear to be common in fish (e.g. Arabian killifish, Brodin et al., 2014; zebrafish, Dagh, 2013). Activity may be an indirect measure of boldness in novel situations, as shy individuals typically freeze more whereas bold individuals are more active, and activity is positively correlated with boldness in numerous fish species (e.g. guppies, Budaev, 1997; threespine stickleback, Bell, 2005; zebrafish, Moretz et al., 2007). Additionally, we hypothesized that when fluoxetine was not present, males would behave consistently over time (i.e. repeatability) and that a given male would exhibit a similar level of boldness across assays (i.e. behavioral syndrome), as was previously found in this species (Hebert et al., 2014). Finally, as fluoxetine likely exerts greater effects at higher doses, it was expected that males in the 5 μg l^{-1} fluoxetine dose group would show more dramatic reductions in overall behavior and behavioral consistency than males in the 0.5 μg l^{-1} fluoxetine group.

MATERIALS AND METHODS

Subjects

Male Siamese fighting fish (n=60) from the veil-tail strain were obtained from a breeder (www.liveaquaria.com), housed individually in opaque cups (475 ml), fed Hikari Bio-Pure blood worms *ad libitum* daily, and given total water changes twice a week. Daily feedings did not occur the day before testing so that subjects would be more motivated. Males were kept in the laboratory (26.8°C; 14 h:10 h light:dark cycle) for 2 weeks to acclimate prior to testing. After this 2 week period, males (n=20 each group) were randomly assigned to the control group (no fluoxetine exposure), the low-dose group (0.5 μg l^{-1} fluoxetine), or the high-dose group (5 μg l^{-1} fluoxetine). The low dose is within the higher end of the range found in wastewater effluent (Fent et al., 2006; Schultz et al., 2010) whereas the high dose is a pharmacologically relevant dose. In a recent study, the sensitivity of fathead minnows to fluoxetine was similar to that of patients with anxiety disorders once clearance rates were accounted for, validating the read-across hypothesis and demonstrating the need to understand both pharmacologically and environmentally relevant doses (Margiotta-Casaluci et al., 2014). Testing occurred over a 6 week period, with 3 weeks of baseline data collection and 3 weeks of experimental trials. All procedures performed in this study were in accordance with the ethical standards of the University of New England, where this work was conducted, and covered under protocol UNE-20130910DZIET.

Fluoxetine administration

A stock solution was created by adding 1.08 mg fluoxetine HCl (Sigma-Aldrich) to 8 ml of distilled water. The solution was stored at 4°C in a light-resistant bottle when it was not being used. After the 3 weeks of baseline trials, males from both experimental groups had fluoxetine (5 μg l^{-1} FLX: 73.7 μl stock solution; 0.5 μg l^{-1} FLX: 7.37 μl solution) added to their housing containers after daily total water changes had been completed over the total course of exposure. Males in the control group had 73.7 μl of distilled water added.

Experimental procedure

The effects of fluoxetine on behavior were examined using three different assays (empty tank, novel environment and shoal; Fig. 1).

Journal of Experimental Biology

Journal of Experimental Biology (2016) 219, 797-804 doi:10.1242/jeb.132761

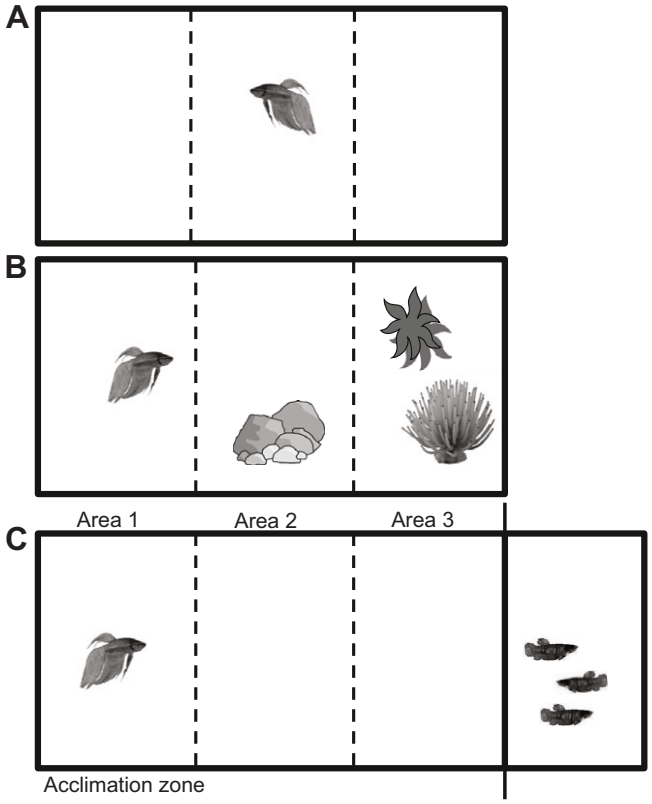

Fig. 1. Diagram of the different assays. (A) Empty tank, (B) novel environment and (C) shoal assay. Note that the subject was always released in Area 1 (acclimation zone). In the shoal assay, Area 1 was the area farthest from the shoal tank as the position of the shoal tank alternated across trials. Dashed lines represent the way each tank was visually divided into three areas (Areas 1–3). During the acclimation period, an opaque partition placed inside the tank in Area 1 restricted the subject to that area. Additionally, an opaque partition separated the shoal tank from the experimental tank in the shoal assay outside of the testing period.

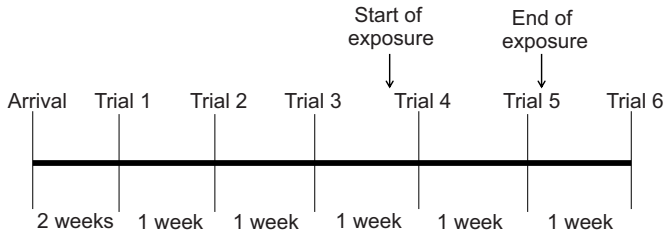

Fig. 2. Timeline of the experiment. Note that exposure in the two groups of exposed subjects occurred 24 h prior to the start of trial 4 and ended after trial 5.

These assays have been used before in this species (Dzieweczynski et al., 2016) and are standard assays for examining behavioral syndromes along with some measure of aggression (for a review, see Toms et al., 2010). Assays such as the empty tank test have been used to examine depression-like behaviors in zebrafish (Cachat et al., 2010), and these behaviors change with administration of fluoxetine (Egan et al., 2009). Each male was run in all three assays weekly for 6 weeks with the first 3 weeks serving as baseline trials (trials 1–3; pre-FLX or pre-control) and the experimental trials (trials 4–6; post-FLX or post-control) occurring over the final 3 weeks (Fig. 2). The order in which a given individual received the three assays was randomized each testing day. For the first experimental trial (trial 4), males in the two exposed groups had fluoxetine delivered into their housing containers 24 h prior to testing, whereas males in the control group received distilled water. Depending on the treatment group, either fluoxetine or distilled water was administered daily following complete water changes. After a week of exposure following trial 4, males were again tested in all three assays (trial 5; Fig. 2). Once trial 5 was completed, all subjects were returned to their containers where they were housed in water without FLX for 1 week and then tested in the three assays (trial 6).

Each assay comprised a 10 liter (50.5×30×25 cm) tank filled with treated tap water and divided into three areas of equal size (Areas 1–3) by drawing lines on the front of the tank (Fig. 1). All sides of the tank except the front were covered with opaque material to exclude external cues, and the observer remained out of sight of the test subjects. Testing in each assay started when a male was transferred from its home tank by a net and was placed in the leftmost area of the tank (Area 1). During a 5 min acclimation period, this area was separated from the rest of the tank by an opaque partition. Next, the partition was removed and the subject was able to swim throughout the assay tank for a 5 min trial. The subject was then removed from the tank and returned to its housing container until the next assay. Trials were recorded with a Sony Handycam (model DCR-SX60; 1080 pixel resolution, 60 pixel frame rate) digital camcorder on a tripod located in front of the test tank. Video trials were coded using the Event-PC event recorder program designed by Dr James Ha (University of Washington).

Empty tank assay

In the empty tank assay, the tank was kept devoid of stimuli (Fig. 1A). A trial started by placing a male in Area 1 behind an opaque partition. The subject remained in this area for 5 min, after which the partition was removed and it could move throughout the tank for the 5 min trial. The behaviors measured in this assay were the length of time that passed before a subject moved (latency), time spent in Area 1, time spent in Area 3, time spent in the top third of the water column, time spent in the bottom third of the tank, and time spent swimming (activity level).

Novel environment assay

In this assay, numerous stimuli were placed throughout a tank (Fig. 1B). These stimuli included rocks and artificial plants of varying sizes. A different environment was created each testing day to ensure that the tank set-up was always novel for each individual. The number of stimuli increased across the tank, with more items located in Area 3 than in Area 1. As in the empty tank assay, each trial comprised a 5 min acclimation period when the subject was restricted to Area 1 by an opaque partition, and a 5 min trial after the partition was removed. The same behaviors measured in the empty tank assay were measured in this assay.

Shoal assay

The shoal assay had a smaller tank (22×16×31 cm; 2.5 liters) located next to the test tank that was used to house a shoal of three female Siamese fighting fish (Fig. 1C). These shoal fish were familiar to one another but unfamiliar to all subjects. To control for side bias, the side of the test tank (e.g. Area 1 or Area 3) that the shoal tank was placed next to was randomized over testing days. Each male was acclimated for 5 min in the area furthest away from the shoal tank before the 5 min trial. Latency, time spent in the area closest to the shoal tank, time spent in the area furthest away from the shoal tank, time spent in the top third of the tank, time spent in

Journal of Experimental Biology (2016) 219, 797-804 doi:10.1242/jeb.132761

the bottom third of the tank, time spent within one body length of the shoal tank, and time spent swimming were all recorded.

Statistical analyses

Principal components analyses (PCAs) with varimax rotations were conducted to determine whether the behavioral measures within an assay were related to one another and thus could be reduced to composite variables. Each of the three PCAs run yielded two principal component (PC) scores with eigenvalues greater than one. The PC scores from these PCAs were normalized by log transformation and then used in additional statistical tests. To examine whether behavior changed over time and/or from fluoxetine exposure, two-way repeated-measures ANOVAs (RM-ANOVAs) with trial number (1–6) and exposure (control, 0.5 µg l^{-1} and 5 µg l^{-1}) as factors and a trial×treatment interaction effect were run on the PC scores in the three assays. *Post hoc* *t*-tests with Bonferroni corrections were conducted when significant RM-ANOVA results were found.

Spearman's rank correlations were run on the PC scores for each assay to examine whether pre- and post-measures within a context and measures across contexts were correlated. This provides information on whether the level of response is consistent over time and across contexts, as well as whether fluoxetine affected the relationship of behavior within and across contexts. To determine whether the resulting correlation coefficients were significantly different from one another, Fisher *r*-to-*z* transformations were performed for all pairs of correlation coefficients (Schuett and Dall, 2009; Nakagawa and Schielzeth, 2010).

Finally, repeatability values were calculated using the raw data rather than the PC scores in all assays. This provides a measure of consistent individual differences and the effects of fluoxetine on these differences. Repeatability partitions the total variation within a sample into that due to within-individual variation and that due to between-individual variation, providing a means of examining whether individuals in a population differ from one another (Boake, 1989). To calculate repeatability, one-way ANOVAs with individual as the main effect were run on the dependent measures in all assays. These variables were then used in the repeatability equation from Falconer and Mackay (1996). As with the correlation coefficients, repeatability values were compared using Fisher *r*-to-*z* transformations to determine whether the values differed significantly from one another (Schuett and Dall, 2009).

RESULTS

The three PCAs generated two PC scores (eigenvalue>1; Table 1). The first PC score (PC A1) found for the empty tank assay described 'boldness in empty tank' with high positive loadings for time in Area 3 and activity level, and high negative loadings for time in Area 1 and latency. The second PC score (PC A2) in this assay described 'swim depth in empty tank' with a high negative loading for time in the top third of the tank and a high positive loading for time in the bottom third of the tank. In the novel environment assay, the first PC score (B1) described 'boldness in novel environment' and the second PC score (B2) explained 'swim depth in novel environment' (Table 1). PC score C1 described 'boldness in shoal' with high positive loading for time spent in Area 3, time spent active, and time spent within one body length of the shoal, and high negative loading for time spent in Area 1 and latency. PC score C2 explained 'swim depth in shoal' with a high negative loading for time spent in the top third of the tank and a high positive loading for time in the bottom third of the tank.

Table 1. Results of principal components analyses for the different assays

Behavior	PC1	PC2
Empty tank	A1	A2
Time in Area 1	**−0.925**	−0.031
Time in Area 3	**0.921**	0.010
Time in top	0.024	**−0.922**
Time in bottom	−0.129	**0.918**
Latency	−0.353	0.020
Time active	**0.594**	−0.166
Eigenvalue	2.00	1.72
Variance explained (%)	33.34	28.68
Novel environment	B1	B2
Time in Area 1	**−0.917**	−0.050
Time in Area 3	**0.846**	−0.280
Time in top	0.108	**−0.841**
Time in bottom	0.105	**0.873**
Latency	−0.454	0.328
Time active	**0.581**	−0.042
Eigenvalue	2.02	1.66
Variance explained (%)	33.61	27.65
Shoal	C1	C2
Time in Area 1	**−0.926**	0.069
Time in Area 3	**0.955**	−0.074
Time in top	−0.205	**−0.784**
Time in bottom	−0.248	**0.806**
Latency	−0.251	0.357
Time active	**0.675**	−0.278
Time by shoal	**0.921**	−0.053
Eigenvalue	3.11	1.56
Variance explained (%)	44.50	22.33

PC scores with eigenvalues >1.00 for each treatment were used in further analyses. Measures included in these PC scores with values higher than ±0.500 were considered to have high loadings, and these values are in bold.

Overall response

Boldness in the empty tank assay (PC A1) was influenced by trial number ($F_{5,59}$=37.56, P<0.0001), exposure ($F_{2,59}$=58.10, P<0.0001), and their interaction ($F_{10,59}$=12.89, P<0.0001). Males in the control group exhibited greater boldness levels in all trials than males in the 0.5 µg l^{-1} FLX group (Bonferroni: t≥2.91, n=20, P≤0.002; Fig. 2) and males in the 5 µg l^{-1} FLX group (t≥7.51, n=20, P≤0.0001) after exposure (i.e. trials 4–6). Additionally, pre-exposure boldness was higher than post-exposure boldness in both exposure groups (0.5 µg l^{-1}: t≥3.77, n=20, P≤0.0001; 5 µg l^{-1}: t≥7.55, n=20, P≤0.0001). Boldness was higher in trials 4 and 6 than in trial 5 in the 0.5 µg l^{-1} FLX group (t≥7.55, n=20, P≤0.0001; Fig. 3), suggesting that exposure period is important at this dose. Boldness levels for males in the 0.5 µg l^{-1} FLX group in trials 4 and 6 were also higher than post-exposure boldness in the 5 µg l^{-1} FLX group (t≥4.60, n=20, P≤0.0001). Swim depth in the empty tank (PC A2) was not affected by trial number ($F_{5,59}$=0.06, P=0.99), exposure ($F_{2,59}$=0.35, P=0.71) or a trial×exposure interaction effect ($F_{10,59}$=0.55, P=0.85).

In the novel environment assay, boldness was affected by trial number ($F_{5,59}$=37.28, P<0.0001), exposure ($F_{2,59}$=67.09, P<0.0001) and their interaction ($F_{10,59}$=13.74, P<0.0001). Boldness was higher in all trials within the control group than in the post-exposure trials for both the 0.5 µg l^{-1} FLX (t≥5.01, n=20, P≤0.0001) and the 5 µg l^{-1} FLX groups (t≥6.00, n=20, P≤0.0001; Fig. 4). No difference was found between the two exposure groups in the post-exposure trials (t≤0.77, n=20, P≥0.22). No trial ($F_{5,59}$=1.58, P=0.16), exposure ($F_{2,59}$=0.85, P=0.43) or trial×exposure interaction ($F_{10,59}$=0.97, P=0.47) effects were found for swim depth in the novel environment assay.

Journal of Experimental Biology

Journal of Experimental Biology (2016) 219, 797-804 doi:10.1242/jeb.132761

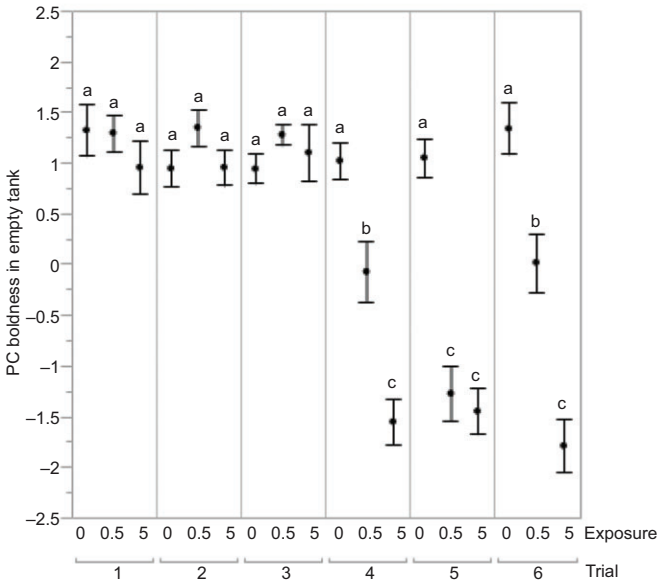

Fig. 3. Mean boldness scores (PC A1) in all trials (1–6) for the three exposure groups (0, 0.5 and 5 µg l^{-1}; *n*=20 each group) in the empty tank assay. Error bars are ±1 s.e.m. Significant relationships are indicated by differing letters.

Boldness in the shoal assay was influenced by trial number ($F_{5,59}$=61.55, P<0.0001), exposure ($F_{2,59}$=65.13, P<0.0001), and their interaction ($F_{10,59}$=15.67, P<0.0001). Males in the control group were bolder in all trials than males in the 0.5 µg l^{-1} FLX group (t≥7.33, n=20, P≤0.0001; Fig. 5) or the 5 µg l^{-1} FLX group (t≥7.76, n=20, P≤0.0001) were in the post-exposure trials. Post-exposure trials for the 0.5 µg l^{-1} FLX group and the 5 µg l^{-1} FLX group did not differ in terms of boldness (t≤0.17, n=20, P≥0.57; Fig. 4). As in the other two assays, no trial ($F_{5,59}$=1.21, P=0.31), exposure ($F_{2,59}$=0.97, P=0.38) or interaction effect ($F_{10,59}$=1.07, P=0.39) was found for swim depth in the shoal assay.

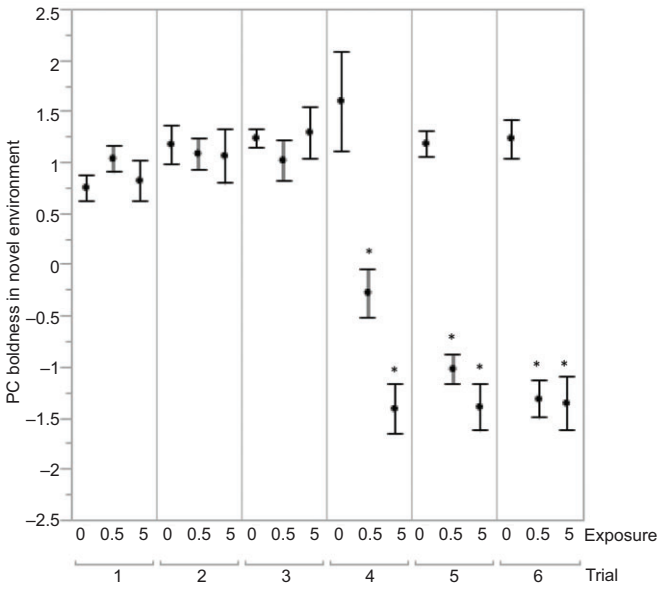

Fig. 4. Mean boldness scores (PC B1) in all trials (1–6) for the three exposure groups (0, 0.5 and 5 µg l^{-1}; *n*=20 each group) in the novel environment assay. Error bars are ±1 s.e.m. Significant relationships are indicated by asterisks.

Correlations

Spearman's rank correlations were conducted on the pre- and post-measures of boldness (i.e. PC1) in each assay. To examine whether behavior was consistent over time, the pre- and post-measures within a given treatment were compared and the significance of the resulting correlation coefficients was determined through Fisher r-to-z transformations. Pre- and post-levels of boldness were positively correlated in all three assays within the control group (Spearman: empty tank: r_S=0.73, P<0.0001; novel environment: r_S=0.34, P=0.01; shoal: r_S=0.72, P<0.0001) but not in the 0.5 µg l^{-1} FLX (empty tank: r_S=0.14, P=0.28; novel environment: r_S=−0.23, P=0.07; shoal: r_S=0.16, P=0.21) or 5 µg l^{-1} FLX groups (empty tank: r_S=0.08, P=0.54; novel environment: r_S=0.07, P=0.61; shoal: r_S=−0.14, P=0.30). The correlation coefficient for boldness in the control group was significantly greater than that in the two exposure groups (empty tank: z≥4.21, P≤0.0001; novel environment: z≥1.62, P≤0.05; shoal: z≥3.98, P≤0.0001).

Spearman's correlations were also conducted to determine whether response in one context was associated with response in another context, and whether this relationship was influenced by FLX exposure. All pre-measures had significantly high positive correlations when boldness levels between the empty tank and novel environment, empty tank and shoal, and novel environment and shoal were compared (r_S≥0.26, P≤0.04). Levels of boldness were also positively correlated across all contexts in the control group (r_S≥0.36, P≤0.004) but not in the two exposure groups (0.5 µg l^{-1} FLX: r_S≤0.24, P≥0.07; 5 µg l^{-1} FLX: r_S≤0.10, P≥0.47) in the post trials. The correlation coefficients for the control group were significantly higher than those post-exposure in the 5 µg l^{-1} FLX group (z≥1.58, P≤0.05) but not in the 0.5 µg l^{-1} FLX group (z≤0.81, P≥0.21), with the exception of boldness in the empty tank versus the shoal assay, for which the correlation coefficient for the 0.5 µg l^{-1} FLX group differed from that of the control group (z≥2.27, P≤0.01).

Repeatability

Repeatability values were used to examine consistent individual differences in behavior within an assay for the different exposure

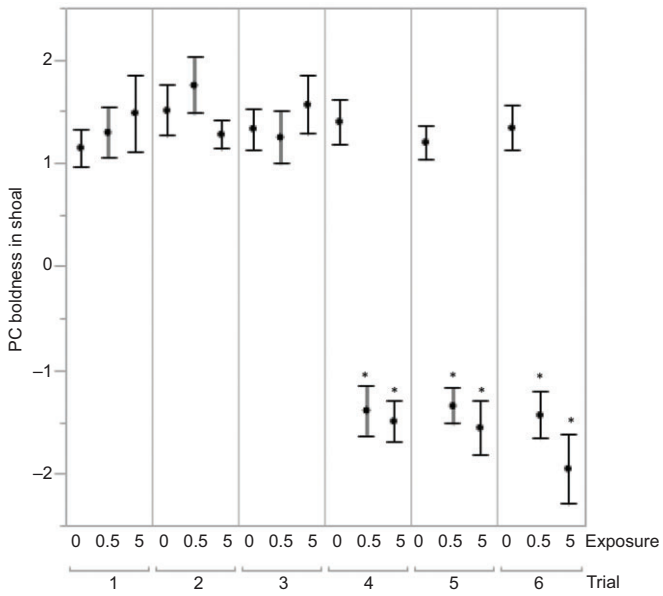

Fig. 5. Mean boldness scores (PC C1) in all trials (1–6) for the three exposure groups (0, 0.5 and 5 µg l^{-1}; *n*=20 each group) in the shoal assay. Error bars are ±1 s.e.m. Significant relationships are indicated by asterisks.

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regimes. Because the PCAs indicated an inverse relationship between sets of variables (e.g. time in top of tank versus time in bottom of tank), repeatability values were only calculated for time in Area 3, time spent in the bottom third of the tank, and time spent active. Repeatability values before exposure were quite high for both time spent in Area 3 and time spent active (Table 2). High repeatability values mean that more of the variation in a sample is due to between-individual variation rather than within-individual variation. Fisher *r*-to-*z* transformations were used to compare repeatability values within an assay for the three behaviors analyzed. Repeatability values did not differ for time spent in the bottom third of the tank in any of the three assays (empty tank: $z \leq 1.13$, $P \geq 0.13$; novel environment: $z \leq 1.29$, $P \geq 0.10$; shoal: $z \leq 0.61$, $P \geq 0.28$). In all three assays, the repeatability value for activity level found after exposure in the 5 µg l^{-1} FLX group was significantly lower than before exposure or the before or after repeatability values for the control group (empty tank: $z \geq 1.63$, $P \leq 0.05$; novel environment: $z \geq 1.67$, $P \leq 0.05$; shoal: $z \geq 1.79$, $P \leq 0.04$). No difference was found for the after exposure repeatability value in the 0.5 µg l^{-1} FLX group and any of the other repeatability values for activity level in any of the three assays ($z \leq 1.30$, $P \geq 0.10$). Repeatability values were significantly lower after exposure in both treatment groups than before exposure or the before or after values for the control group for the amount of time spent in Area 3 in all three assays (empty tank: $z \geq 2.31$, $P \leq 0.01$; novel environment: $z \geq 2.17$, $P \leq 0.02$; shoal: $z \geq 2.06$, $P \leq 0.02$).

DISCUSSION

Fluoxetine exposure has been found to impact multiple fitness-related behaviors, such as foraging and activity, in aquatic organisms (Brooks, 2014). However, there is still much that remains unknown in regards to what specific behaviors are affected and how context and dosing regimen might influence these effects. In the present study, male Siamese fighting fish were exposed to a low or high dose of fluoxetine, and their behavior in a series of assays was compared with that of unexposed males to determine how exposure influenced the overall amount, consistency, and relationships between behaviors. In general, males exposed to fluoxetine were less bold and less consistent in their behavioral responses, and the correlations between boldness over time and across assays were weaker than those for unexposed males. Both concentration of fluoxetine and duration of exposure appeared to be important in generating behavioral changes, although the degree of influence varied based on the behavior measured and the assay used. This stresses the importance of examining the consequences of PPCP exposure on various dosing and exposure scales as well as measuring multiple behaviors in multiple contexts to understand the fitness-related impacts. Changes in overall level of response, consistency, and relationships of behavior across contexts may produce individual- and population-level consequences if these behaviors are crucial to survival in complex physical and/or social environments. If exposure affects boldness, fitness may be decreased as individuals that fail to forage, avoid predators or attract mates will obviously have decreased fitness (Arnold et al., 2014).

Males in both fluoxetine exposure groups were less bold than unexposed males, meaning that fluoxetine decreased activity and movement around the tank. Boldness was lower after exposure in all three assays in both the low- and high-dose groups. This is especially alarming as fluoxetine has been found at levels between 0.15 and 32 ng l^{-1} in North American waterways (Silva et al., 2012). In two of the three assays, the effect of fluoxetine on overall level of response did not differ across the two doses. A dose-dependent effect was only found in the empty tank assay in the low-dose group after a week of exposure, whereas boldness was significantly reduced in all three post-exposure trials in the higher-

Table 2. Repeatability values for behavior in the assays for the three exposure groups before and after exposure (pre-control, control, pre-0.5 µg l^{-1}, 0.5 µg l^{-1}, pre-5 µg l^{-1} and 5 µg l^{-1})

	Empty tank			Novel environment			Shoal		
Behavior	*R*	*F*	*P*	*R*	*F*	*P*	*R*	*F*	*P*
Pre-control									
Time in Area 3	**0.783**	11.82	<0.0001	**0.712**	8.42	<0.0001	**0.764**	10.71	<0.0001
Time in bottom	0.227	1.88	0.05	0.433	3.29	0.001	0.489	5.68	<0.0001
Time active	**0.582**	5.17	<0.0001	**0.554**	4.72	<0.0001	**0.621**	5.91	<0.0001
Control									
Time in Area 3	**0.784**	11.86	<0.0001	**0.676**	7.26	<0.0001	**0.739**	9.5	<0.0001
Time in bottom	0.236	1.93	0.04	0.142	1.5	0.14	0.33	2.48	0.01
Time active	**0.646**	6.48	<0.0001	**0.539**	4.5	<0.0001	**0.557**	4.77	<0.0001
Pre-0.5 µg l^{-1}									
Time in Area 3	**0.752**	10.1	<0.0001	**0.682**	7.43	<0.0001	**0.765**	10.75	<0.0001
Time in bottom	0.141	1.67	0.1	0.264	2.07	0.03	0.45	4.57	<0.0001
Time active	**0.519**	4.24	<0.0001	**0.548**	4.63	<0.0001	**0.598**	5.47	<0.0001
0.5 µg l^{-1}									
Time in Area 3	0.025	1.08	0.41	0.078	1.25	0.27	0.158	1.56	0.12
Time in bottom	0.484	3.81	0.0002	0.249	1.99	0.03	0.314	2.38	0.01
Time active	0.357	2.66	0.005	0.155	1.55	0.12	0.111	1.38	0.19
Pre-5 µg l^{-1}									
Time in Area 3	**0.76**	10.52	<0.0001	**0.723**	8.82	<0.0001	**0.728**	9.01	<0.0001
Time in bottom	0.322	2.43	0.01	0.213	1.81	0.06	0.484	5.39	<0.0001
Time active	**0.564**	4.88	<0.0001	**0.563**	4.89	<0.0001	**0.566**	4.92	<0.0001
5 µg l^{-1}									
Time in Area 3	–0.067	0.81	0.68	0.146	1.51	0.14	0.159	1.57	0.12
Time in bottom	0.119	1.41	0.18	0.06	1.19	0.31	–0.176	0.55	0.91
Time active	0.081	1.26	0.26	0.029	1.09	0.39	0.184	1.68	0.08

Repeatability (*R*) as well as the ANOVA outputs (*F*) and associated *P*-values are reported. Repeatability values greater than 0.500 are in bold; these values indicate that over 50% of the variance in the sample is explained by between-individual variation.

Journal of Experimental Biology

dose group. What might make this assay different from the others should be examined further. Perhaps fluoxetine exerts a stronger effect when stimuli are present, as in the other two assays, compared with the empty tank assay, which is devoid of stimuli and conspecifics. Endler guppies exposed to another SSRI, citalopram, exhibited behavioral changes in a novel tank but not in a social preference assay, supporting the hypothesis that SSRIs may differentially affect behavior in different contexts (Olsén et al., 2014). Decreased movement after fluoxetine exposure has been found in other fish species (e.g. Clements and Schreck, 2007; Kohlert et al., 2012) and may be disadvantageous because decreases in activity level and exploration are frequently associated with reductions in the ability to acquire food and mates (Sih et al., 2004).

The amount of fluoxetine males were exposed to was important when behavioral consistency and relationships of behavior between assays were examined. Fluoxetine exposure affected the relationship of boldness over time in both experimental groups, but disrupted the behavioral syndrome found for boldness across contexts in the control group and before exposure in the high-dose group only. Although correlations were lower in the low-dose fluoxetine group than in the control group, when the relationship between behavior across contexts was examined, these correlations were only significantly lower in the high-dose group. The post-exposure repeatability values for activity level in all three assays were lower in the experimental groups than in the control group, although this was only significant for the high dose. In contrast, repeatability vales for the amount of time spent in Area 3, a measure of exploration, were significantly lower in both experimental groups, perhaps because of decreased locomotion (Barry, 2013), again demonstrating why it is important to examine multiple behaviors and multiple measures. This also suggests that exploration may be affected more dramatically than activity level by fluoxetine exposure and that it is boldness rather than decreased locomotion that is affected in this species.

In conclusion, fluoxetine exposure was found to generate effects on multiple levels, from overall boldness to consistency, when the behavior of male Siamese fighting fish was assessed in three different assays. Although for some measures the higher dose of fluoxetine appeared to have a greater effect than the lower dose, this was not always the case, stressing the importance of examining the consequences of PPCP exposure on multiple measures and in multiple contexts. For example, a dose effect on overall level of boldness was only found in the empty tank assay, whereas both doses generated similar decreases in behavior in the novel environment and shoal assays. Perhaps most importantly and alarmingly, the effects of exposure lasted even after fluoxetine was removed, although it should be noted that males were only returned to clean water for 1 week. This demonstrates that even short-term exposure to small doses of fluoxetine may have severe consequences as changes in activity levels and exploratory behavior may impact survival. As such, even brief periods of exposure could potentially produce chronic effects, especially as boldness is important in migration, aggression and predator evasion. For example, population-wide reductions in boldness may lead to fewer individuals searching for food sources or less aggressive males that no longer form and defend territories. Finally, this study suggests that serotonin may play an important role in generating or maintaining behavioral syndromes because fluoxetine exerts its effects via the serotonergic pathways. The mechanisms behind how fluoxetine alters boldness as well as studies on the more chronic effects of this SSRI and other PPCPs should be examined in the future.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
T.L.D. designed the experiment, analyzed the data, and wrote the majority of the manuscript. B.A.C. and J.L.K. collected data, coded behavioral trials, and assisted with experimental design and manuscript preparation.

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