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Multi-generational impacts of exposure to antidepressant fluoxetine on behaviour, reproduction, and morphology of freshwater snail *Physa acuta*

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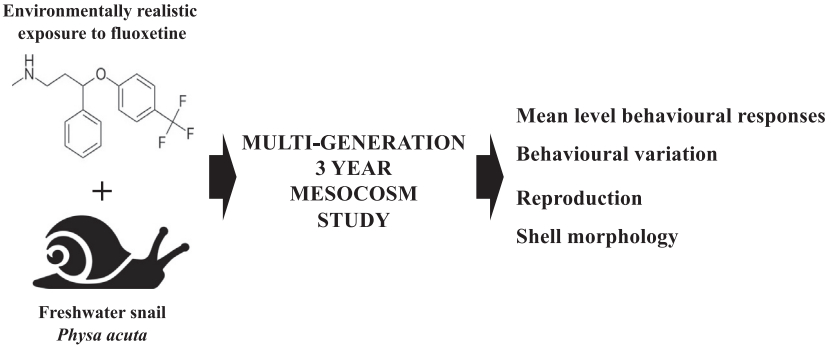
HIGHLIGHTS

- Pharmaceutical pollution represents a major global threat to the aquasphere.
- Long term exposure of aquatic snails, *Physa acuta*, to fluoxetine at environmentally relevant levels.
- Fluoxetine reduced within-individual variance and increased repeatability in behaviour.
- Fluoxetine decreased eggmass production.
- Fluoxetine had no clear impact on morphology.

GRAPHICAL ABSTRACT



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ABSTRACT

Contamination of the environment by pharmaceutical pollutants poses an increasingly critical threat to aquatic ecosystems around the world. This is particularly true of psychoactive compounds, such as antidepressant drugs, which have become ubiquitous contaminants and have been demonstrated to modify aquatic animal behaviours at very low concentrations (i.e. ng/L). Despite raising risks to the hydrosphere, there is a notable paucity of data on the long term, multigenerational effects of antidepressants at environmentally realistic concentrations. Moreover, current research has predominantly focused on mean-level effects, with little research on variation among and within individuals when considering key behavioural traits. In this work, we used a multigenerational exposure of a freshwater snail (*Physa acuta*) to an environmentally relevant concentration of the antidepressant fluoxetine (mean measured concentration: 32.7 ng/L, SE: 2.3). The snails were allowed to breed freely in large mesocosm populations over 3 years. Upon completion of the exposure, we repeatedly measured the locomotory activity (624 measures total), reproductive output (234 measures total) as well as morphometric endpoints (78 measures total). While we found no mean-level differences between treatments in locomotory activities, we did find that fluoxetine exposed snails ($n = 46$) had significantly reduced behavioural plasticity (i.e. V_W; within-individual variation) in activity levels compared to unexposed snails ($n = 32$). As a result, fluoxetine exposed snails demonstrated significant behavioural repeatability, which was not the case for unexposed snails. Further, we report a reduction in egg mass production in fluoxetine exposed snails, and a marginally non-significant difference in morphology between treatment groups. These results highlight the potential detrimental effects of long-term fluoxetine exposure on non-target organisms at environmentally realistic dosages. Additionally, our findings demonstrate the underappreciated potential for psychoactive contaminants to have impacts beyond mean-level effects, with consequences for population resilience to current and future environmental challenges.

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1. Introduction

Over the last decade, there are rapidly growing concerns over the quantity of psychoactive pharmaceuticals including drugs such as antipsychotics, anxiolytics, and antidepressants that are released to the aquatic environment (Bradley et al., 2020; Mole and Brooks, 2019; Ng et al., 2019; O'Flynn et al., 2021). Antidepressants and their metabolites are not able to be completely removed during the wastewater treatment process and are therefore released into the environment in wastewater effluents and remain bioactive (Klaminder et al., 2014; Kümmerer et al., 2018). This incomplete removal, combined with their extensive clinical use, has resulted in their pseudo-persistence in natural ecosystems especially those close to large urban agglomerations (Gunther et al., 2010). Further, the molecular targets of these drugs are evolutionarily conserved among diverse taxa and can thus modify their behaviours at very low concentrations (e.g. ng to μg/L) (Daughton and Ternes, 1999).

An example of a psychoactive pollutant of particular concern worldwide is fluoxetine, a selective serotonin reuptake inhibitor (SSRI). Fluoxetine is prescribed to treat mood disorders, such as anxiety and depression, in human and veterinary medicine (Karagiannis et al., 2015; Levy et al., 2019). Through inhibition of serotonin reuptake transport proteins, SSRIs increase serotonin neurotransmission. Serotonin is found in all animal phyla however the physiological processes regulated by this neurotransmitter can differ. For example, in fish species, fluoxetine has been shown to reduce anxiety behaviours and alter the release of sex hormones (Ansai et al., 2016; Foran et al., 2004; Martin et al., 2019b; Mennigen et al., 2010; Monson et al., 2019; Polverino et al., 2021; Wong et al., 2013). In arthropods and molluscs, the serotonergic system has been linked to ecologically important endpoints like reproduction (e.g. regulate egg laying and induction of penile erection) and behaviour (e.g. swimming speed and foraging) (Daughton and Ternes, 1999; De Castro-Català et al., 2017; Muschamp and Fong, 2001; Sánchez-Argüello et al., 2009).

Fluoxetine has been detected in aquatic environments around the world, typically ranging between 0.2 and 373.8 ng/L in freshwater systems (Mole and Brooks, 2019). It should be highlighted that the majority of studies reporting fluoxetine-induced changes in reproduction and behaviour employ acute exposure scenarios and are often performed at higher concentrations than are typically present in the environment (e.g. Ford et al., 2018; Méndez et al., 2013; Péry et al., 2008; Sánchez-Argüello et al., 2012). Given the potential pseudo-persistence of fluoxetine, there is a notable paucity of data on its long term, multigenerational effects at environmentally realistic concentrations. This is particularly important since it is likely that organisms living in contaminated environments are exposed to very low concentrations over multiple generations (Klaminder et al., 2015). Thus, there is a clear need for research addressing the impacts of long-term fluoxetine exposure at environmentally realistic concentrations on reproduction and behaviour.

Furthermore, studies investigating the impacts of fluoxetine on the behaviour of non-target organisms have focussed only on mean-level effects, ignoring much of the variation that exists both among and within individuals (Polverino et al., 2021). Until recently, individual variation in behaviour was largely considered as a statistical noise. However, this variation is now understood to be fundamentally important to species ecology and evolution (Kain and McCoy, 2016; Westneat et al., 2015). For example, previous research has demonstrated a prominent role for individual-level behavioural variation in population productivity and spread (Fogarty et al., 2011; Modlmeier et al., 2012). The disruption of behavioural variability within populations is particularly prevalent when considering psychoactive pollutants, like fluoxetine, which are specifically designed to reduce/moderate the presentation of extreme behavioural phenotypes (e.g. like depression) (Wong et al., 2005).

Here, we for the first time evaluated how long-term, mutigenerational exposure to fluoxetine alters the behaviour, reproduction and morphology in the freshwater snail (*Physa acuta*).

The concentration of fluoxetine in our mesocosm populations (Polverino et al., 2021; Tan et al., 2020; Wiles et al., 2020) represents those previously detected in aquatic ecosystems around the globe (Hughes et al., 2013; Mole and Brooks, 2019). Studies have shown fluoxetine is bioavailable and accumulates in the tissues of multiple non-target organisms in aquatic environments including fish, marine sponges and gastropods (Meredith-Williams et al., 2012; Rizzi et al., 2020; Yan et al., 2019). Findings suggest that acute exposure to both water-borne fluoxetine and fluoxetine at the sediment surface can also adversely affect reproduction in *Physa acuta* (Sánchez-Argüello et al., 2009, 2012). Thus, to test the impacts of long-term multigenerational exposure, we compared locomotor activity levels at both the mean and individual level, reproductive output, and morphology between fluoxetine exposed and unexposed snails. Our results demonstrate that fluoxetine exposed snails had significantly reduced behavioural plasticity and demonstrated significant behavioural repeatability. Moreover, the multi-generational exposure had also resulted in a reduction in egg mass production, and a marginally non-significant difference in morphometric indices. Our results highlight the potential detrimental effects of long-term fluoxetine exposure on non-target organisms at environmentally realistic dosages and demonstrate the underappreciated potential for psychoactive contaminants to have impacts beyond mean-level effects. The latter can have a potentially profound consequences for population resilience to current and future environmental challenges.

2. Materials and methods

2.1. Mesocosm system design

The mesocosm systems used in this experiment have been described in detail in Wiles et al. (2020) and Mason et al. (2021). In brief, each of the eight stainless steel mesocosm tanks (648 L; 180 cm × 60 cm × 60 cm) were filled with carbon-filtered fresh water to a depth of 30 cm and contained aquatic plants (Java moss, *Taxiphyllum barberi*) and a 3 cm layer of gravel substrate (~7 mm grain size). Commercial air pumps (Resun LP100) were used to aerate tanks, and aquarium heaters were used to maintain water temperature. Commercial food pellets (Aquasonic Nutra Xtreme C1 pellets; 0.8 mm) were introduced into the system every two days. There is some evidence to suggest that fluoxetine can bioconcentrate in plants. Thus, snails may have been exposed directly via water borne fluoxetine, and through dietary routes (Amy-Sagers et al., 2017). In order to maintain the desired fluoxetine water concentrations in the fluoxetine exposed mesocosms, dosing solutions were added to those tanks twice weekly. This involved fluoxetine hydrochloride (Sigma-Aldrich; product number: F132, CAS: 56296−78−7) being dissolved in methanol to form a 100 mL stock solution (20 mg/L), which was then used to create dosing solutions twice weekly. Dosing solutions were prepared by diluting 1 mL of the stock solution in 1 L of reverse-osmosis water. To eliminate any potential for solvent effects and to ensure consistency in the level of handling and disturbances across treatments, a solvent solution (1 mL of methanol in 1 L of reverse-osmosis water) was added to all control freshwater tanks twice weekly (equates to 0.0006% methanol by volume). Over the duration of the experiment, the mean measured concentration of fluoxetine was 32.7 ng/L (SE: 2.3 ng/L). Water analysis was performed using gas chromatography − tandem mass spectrometry (7000C Triple Quadrupole GC − MS/MS, Agilent Technologies, Delaware, USA; minimum detection limit: 2 ng/L) following protocols described in Bertram et al. (2018) and Martin et al. (2019a), and was conducted by Envirolab Services (MPL Laboratories; NATA accreditation: 2901; accredited for compliance with ISO/IEC: 17025).

2.2. Test organisms and exposure conditions

Native to the north-eastern United States, *Physa acuta* is an invasive aquatic snail species that is abundant in freshwater environments globally. The species is hermaphroditic and reproduces through both cross and self-fertilization (Escobar et al., 2009; Tsitrone et al., 2003). Over 3 years (corresponding to 3–54 generations), snail mesocosm populations (as described above) were continuously exposed to either fluoxetine (mean measured

concentration: 32.7 ng/L, SE: 2.29) or a freshwater control (i.e. 0 ng/L). Snails were sourced from one of eight mesocosm populations (four fluoxetine and four freshwater) established at Monash University, Melbourne, Australia.

Twelve snails were collected from each mesocosm tank resulting in a total of 96 snails (48 per treatment). Once removed, these snails were allowed to acclimatise in individual 70 mL containers where they were kept for the duration of the measurements. Full water changes in these containers were carried out after every morning activity assay using fresh water obtained from the associated mesocosm. The 96 individual snails (48 per treatment group) were tested with respect to behaviour and reproduction (further detailed in the methods below) using a repeated measures experimental design.

2.3. Test chamber design and fabrication for assaying snail behaviour

All test chambers were designed using the CorelDraw X3 (Corel Corporation, Ottawa, Ontario, Canada) computer assisted design (CAD) package. Vessels were fabricated in a fully biocompatible poly(methyl methacrylate) (PMMA) transparent thermoplastic using a non-contact 30 W infrared laser machining system (Universal Laser Systems, Scottsdale, AZ, USA) as described before (Bai et al., 2020; Henry et al., 2019). Fabricated PMMA layers were thermally bonded at 100 °C in a fan assisted oven for 90 min to create watertight 12 well test chambers 28 mm in diameter and 18 mm in height (Fig. 1A). Multiple independent behavioural chambers were fabricated for use in the behavioural assays.

2.4. Behavioural assay

Snails were placed into 12 custom well plates (as described above) for behavioural testing. Each test chamber was filled with 5 mL (8 mm depth) with associated mesocosm water and covered with a clear plastic wrap to prevent the animals escaping from the chamber. Animals were allowed to acclimate for up to 2 min at 25.0 ± 0.5 °C in a temperature-stabilized room under even brightfield backlighting before first video recording. The behaviour of the snail was then recorded for 20 min. The locomotor activity of the snail was measured as the total distance moved (mm) during this period. Behavioural assays were repeated both in the morning (09:00–12:00) and afternoon (15:00–18:00) for every individual on each recording day (Fig. 1B). More specifically, behaviour of each snail was filmed over 4 experimental days with recordings taking place every third day (i.e. trials took place over a total of 12 days). Between each trial, animals were returned into their individual culture chambers.

2.5. High throughput video acquisition

Behavioural data were collected using a custom build high-throughput digital video imaging system, which consisted of a full spectrum LED (light-emitting diode) backlit illumination stage and a digital video camera mounted on a vibration-less photographic column (Polaroid M3, Polaroid Inc., USA) (Henry et al., 2019). The brightfield imaging was performed using a BlackMagic Micro Studio 4 K digital camera (BlackMagic Design, Australia). The camera was paired with a true 1:1 macro objective lens with focal length 30 mm (Olympus, Japan). The setup of the system enabled imaging of 12 chambers simultaneously, eliminating any need for a motorized stage and potentially disruptive manipulations of the test specimens. Native videos of 20 min in duration were recorded at standard resolution of 1920 × 1080 pixels (1080p) and a framerate of 25 fps. All video files were acquired using an external High-Definition Multimedia Interface (HDMI) recorder (Atomos Shogun, Melbourne, Australia) equiped with a programmable time-resolved video acquisition functionality. Native files were saved in .mov digital containers and encoded with a ProRes 422 HQ codec that provided no temporal compression artefacts (Inter frame-only encoding) and variable bitrate.

2.6. Behavioural data analysis

The standardisation of the high throughput behavioural recording set up (Fig. 1A) enabled raw videos to be analysed directly using a protocol described earlier (Henry and Wlodkowic, 2020). This was achieved using the animal tracking software Ethovision XT ver. 15 (Noldus Information Technology, The Netherlands) (Henry et al., 2019). Automatic frame-by-frame tracking produced time-stamped *x,y* coordinate pairs assigned to centroids of detected objects and provided a foundation for the reconstruction of graphical animal trajectories and behavioural parameters (i.e. average distance travelled) calculated for each test snail.

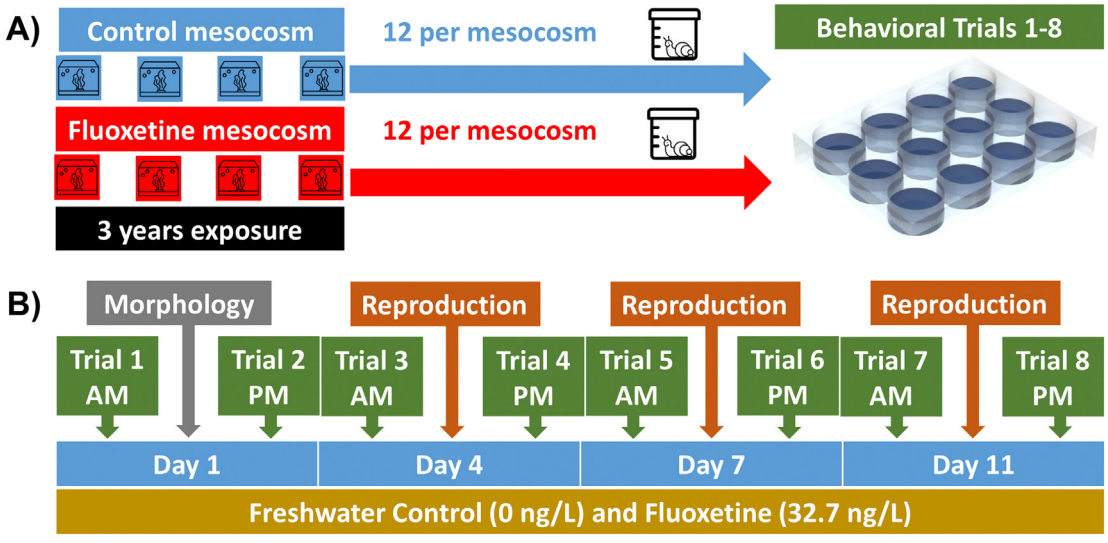

Fig. 1. Overview of experimental set up. (A) Snails were introduced and housed in control and fluoxetine exposed mesocosms over a period of three years. Twelve individuals were taken from each of the replicate mesocosms and housed in individual specimen containers for the duration of the experiment. (B) Experimental trials consisted of measuring snail morphology (on day 1), and repeated sampling of behaviour (twice daily on days 1, 4, 7 and 11) and reproductive output (on days 4, 7 and 11). Snails were maintained in the same water as the experimental mesocosms from which they originated (i.e. either fluoxetine exposed or unexposed) for the entire duration of the study.

2.7. Reproduction

To test the effects of fluoxetine exposure on reproductive performance, we measured the number of eggmasses, the number of embryos, and the average quantity of embryos per eggmass produced through self-fertilization. From day 4 (Fig. 1B), eggmass deposits were collected from each snail's housing container. Specifically, housing water was carefully drained using a 74 μm mesh and the internal surface of the container examined for any remaining eggmasses, which were carefully extracted by scooping them from the edge of the container with a soft plastic spatula and rinsing with fresh water. Eggmasses were then placed into individual petri dishes and photographed using a Panasonic Lumix G7 digital camera (Panasonic Australia Pty Ltd) paired with a true 1:1 Macro objective lenses with focal length of 30 mm (Olympus Corp, USA). A custom Python script was used to batch-load embryo images, assist semi-automated counting and prevent false labelling of embryos.

2.8. Morphology

To test the effect of fluoxetine exposure on morphometric endpoints, we measured cone length, mass and calculated area and perimeter of the shell outline. To do this, on day 1 (Fig. 1B) the snails were placed in a petri dish and individually weighed on scales (Precision Weighing Balances, AND HM-300, ± 0.3 mg), measured (i.e. length) with digital calipers (Whitworth Digital Calipers, ± 0.03 mm), and photographed. The camera setup was standardized with brightfield backlighting to provide a high contrast imaging. Those images allowed for the outline of each shell to be clearly visible. The imaging was performed using a Canon EOS 7D Mk2 system, equipped with a CMOS APS-C type sensor (22.4 × 15.0 mm, 20.20 Megapixel resolution, individual pixel size of 4.1 μm) and paired with a true 1:1 Macro objective lenses with focal length of 90 mm (Tamron Corp, USA). ImageJ version IJ1.46r (NIH, USA) software was then used on the images to convert them to grayscale and analyze the resultant particle outline of the shell. This analysis resulted in obtaining the area, perimeter and associated circularity of the contrasted shell for every individual snail.

2.9. Statistical analysis

Data were analysed using R version 4.0.3 (R Core Team, 2018). In all models, continuous covariates were mean-centred (Mean = 0, SD = 1) prior to analysis to aid in the interpretation of model fixed-effects. Over the course of the experiment, some snails were lost due to early mortality. Although we had no a priori expectations for how fluoxetine would influence survival, we subsequently investigated treatment differences in mortality. We used a binomial generalized linear mixed-effects model with survival (survived = 1, died = 0) included as a response variable, while snail mass and mesocosm tank ID (1–8) included as fixed and random effects, respectively. There was a significant difference in survival between treatment groups over the duration of the experiment ($\chi^2 = 8.9$, $P = 0.003$) with 66.7% of snails in the control groups surviving compared to 95.8% of snails in the fluoxetine treatment group. Snails that died were excluded from further analysis resulting in a final sample size of 78 (control: $n = 32$, fluoxetine: $n = 46$).

Linear mixed-effects models were used to determine the effects of fluoxetine on snail locomotor activity levels. Activity data (i.e. distance travelled in mm) were square-root transformed to approximate a Gaussian error distribution, while trial number was left centred (i.e. trial 1 = 0) prior to analysis. The models included activity as a response variable, while mass, time of day (AM or PM), treatment, trial (1–8), and a treatment by trial interaction were included as fixed-effects. Mesocosm tank ID and individual snail ID were included as random intercepts, while trial was included as a random slope. A Bayesian generalized linear mixed-effects model was used to partition behavioural variation into its among- and within-individual components (*brms* package: (Bürkner, 2017)). This Bayesian model was structured as described directly above. However, we allowed variance among individual intercepts (V_A) and slopes (V_{Slope}), as well as residual within-individual (V_W) variance to differ among treatments. Further, we also allowed variance among mesocosm tanks (V_{Tank}) to differ between treatments. These variance estimates were used to calculate the repeatability of activity levels for each treatment. Repeatability (R) represents to proportion of behavioural variation due to among-individual differences. However, as individuals differed in how they changed their activity over repeated trials (i.e. random slopes, see results), the amount of among-individual differences changes as a function of trial number. We, therefore, used the equation described in Briffa et al. (2013) to calculate conditional repeatability at the intercept. The effect-size of the magnitude difference in variance and repeatability estimates (ΔV_A, ΔV_{Slope}, ΔV_W, ΔV_{Tank}, ΔR) was then calculated to statistically compare how behavioural variation changed between treatments. The Bayesian model was run for 5000 iterations (500 warmup) with a thinning interval of 2, on 4 chains using relatively uninformative, default priors. Model convergence was verified via trace plots, with R_{hat} values = 1. We report posterior means with 95% credibility intervals (95% CrI), with inference based on on-overlapping CrIs with zero.

The effects of fluoxetine on reproduction were investigated using generalized linear mixed-effects models. Reproductive endpoints (i.e. number of embryos, number of eggmasses, and the number of embryos per eggmass) were each included as response variables in three separate models. Models contained treatment, experimental day, and mass as fixed-effects, while mesocosm tank ID and individual ID were both included as random-effects. All models were initially fitted using a Poisson distribution. Where significant overdispersion was detected, models were re-fitted using a zero-inflated Poisson, negative binomial type I and II distributions. Final models for each response variable were selected based on QAICc and AICc scores.

For snail morphology, a Principal Components Analysis (PCA) was performed to collapse morphological variables down to a composite score that collectively explained the largest proportion of variation in morphology. Prior to the PCA, a Kaiser-Meyer-Olkin (KMO) test was preformed to assess variables for factor adequacy. Variables with a KMO value <0.50 (i.e. shell circularity) were deemed inadequate for PCA and were not retained in further analyses. All other morphological variables (i.e. shell length, area, and perimeter) were included in the PCA followed by an oblique rotation. A single principal component (PC) was retained based on Kaiser-Guttman criterion (i.e. eigenvalues >1). The retained PC explained 89.35% of the morphological data with strong unidirectional loadings from all included variables (Table S1). This PC score represents overall size of each individual with positive values representing larger snails. Subsequently, the PC score was used in a linear mixed-effects model to investigate treatment differences in morphology. The model included the PC score as a response variable, while treatment (control or fluoxetine) and mesocosm tank ID were included as fixed and random effects, respectively. Similarly, a linear mixed-effects models was also performed on $\log_{10}$ transformed body mass to investigate any potential treatment effects on snail body mass.

3. Results

3.1. Mean level behavioural response

There was no significant interaction between treatment and trial number, nor a significant effect of treatment, on the average locomotor activity of the snails ($F_{1,76} = 1.35$, $p = 0.248$ and $F_{1,8} = 0.54$, $p = 0.481$, respectively). There was, however, a significant effect of trial number with a general increase in activity over successive trials ($F_{1,78} = 10.97$, $p = 0.001$; Fig. 4A). There was no effect of snail mass or time of day on average activity ($F_{1,71} = 0.40$, $p = 0.530$ and $F_{1,46} = 0.01$, $p = 0.950$, respectively).

3.2. Behavioural variation

Fluoxetine exposed snails demonstrated a decrease in within-individual variance compared to controls (ΔV_W [95% CrI] = 0.410 [0.222, 0.601];

Table 1
Variance and intercept repeatability estimates (± 95% CrIs) for each treatment. Repeatable estimates are indicated in bold.

Treatment	V_A (95% CrI)	V_W (95% CrI)	V_{Tank} (95% CrI)	V_{Slope} (95% CrI)	R (95% CrI)
Control	0.238 (0, 0.585)	0.848 (0.685, 1.026)	0.829 (0, 2.971)	0.014 (0, 0.032)	0.147 (0, 0.350)
Fluoxetine	0.329 (0.126, 0.566)	0.439 (0.367, 0.516)	0.549 (0, 2.033)	0.013 (0.004, 0.025)	**0.301 (0.068, 0.525)**

Table 1, Fig. 4C). In contrast, we found no substantial differences in variance among individual intercepts ($\Delta V_A = -0.091$ [−0.500, 0.369]; Table 1, Fig. 4B), slopes ($\Delta V_{Slope} = 0.001$ [−0.02, 0.024]; Table 1), nor among mesocosm tanks between treatments ($\Delta V_{Tank} = 0.281$ [−2.933, 4.090]; Table 1). Further, fluoxetine, but not control, snails were significantly repeatable in their activity levels at the intercept (Table 1; see Table S2 for trial specific repeatability estimates). The reduction in within-individual variance (i.e. V_W) in the fluoxetine treatment group largely contributed to fluoxetine exposed snails demonstrating a marginal increase in their repeatability at the intercept, relative to controls ($\Delta R = -0.154$ [−0.452, 0.172]; Table 1, Fig. 4D). However, there was substantial uncertainty around this estimate with CrI's including zero. Further, while both treatments groups became more repeatable over time, the marginally increased repeatability seen in fluoxetine exposed snails was maintained over the course of the experiment (see Table S2 for trial specific repeatability).

3.3. Reproduction

There was a significant effect of fluoxetine on the number of eggmasses produced, ($\chi^2 = 4.11$ $p = 0.043$; Fig. 3A), with an average of 0.65 ± 0.71 for control snails and 0.32 ± 0.58 for fluoxetine exposed snails. There was no significant difference between the number of embryos or average embryos per eggmass (all $p > 0.05$, Fig. 3B–C). There was, however, a significant overall negative effect of time, with the number of embryos produced decreasing over experimental days ($\chi^2 = 49.31$, $p < 0.001$; Fig. 3D).

3.4. Morphology

There was no significant effect of fluoxetine on snail mass ($F_{1,6} = 2.31$, $p = 0.179$), with a mean mass (±SD) of 0.11 ± 0.05 g and 0.07 ± 0.03 g for control and fluoxetine exposed snails, respectively (Fig. 2A). Further, there was a marginally non-significant difference between treatments in PC morphology scores, with unexposed snails being subtlety larger than fluoxetine exposed snails ($F_{1,6} = 5.48$, $p = 0.058$; Fig. 2B).

4. Discussion

The goal of this study was to assess the impact of long-term environmentally relevant exposure to the SSRI fluoxetine on behavioural activity, reproductive output, and shell morphology in aquatic snails. We detected no significant effect of fluoxetine exposure on mean locomotor activity levels of snails. We did, however, find a reduction in within-individual variance in fluoxetine exposed individuals relative to unexposed snails, whilst there was no difference in among-individual variance between treatment

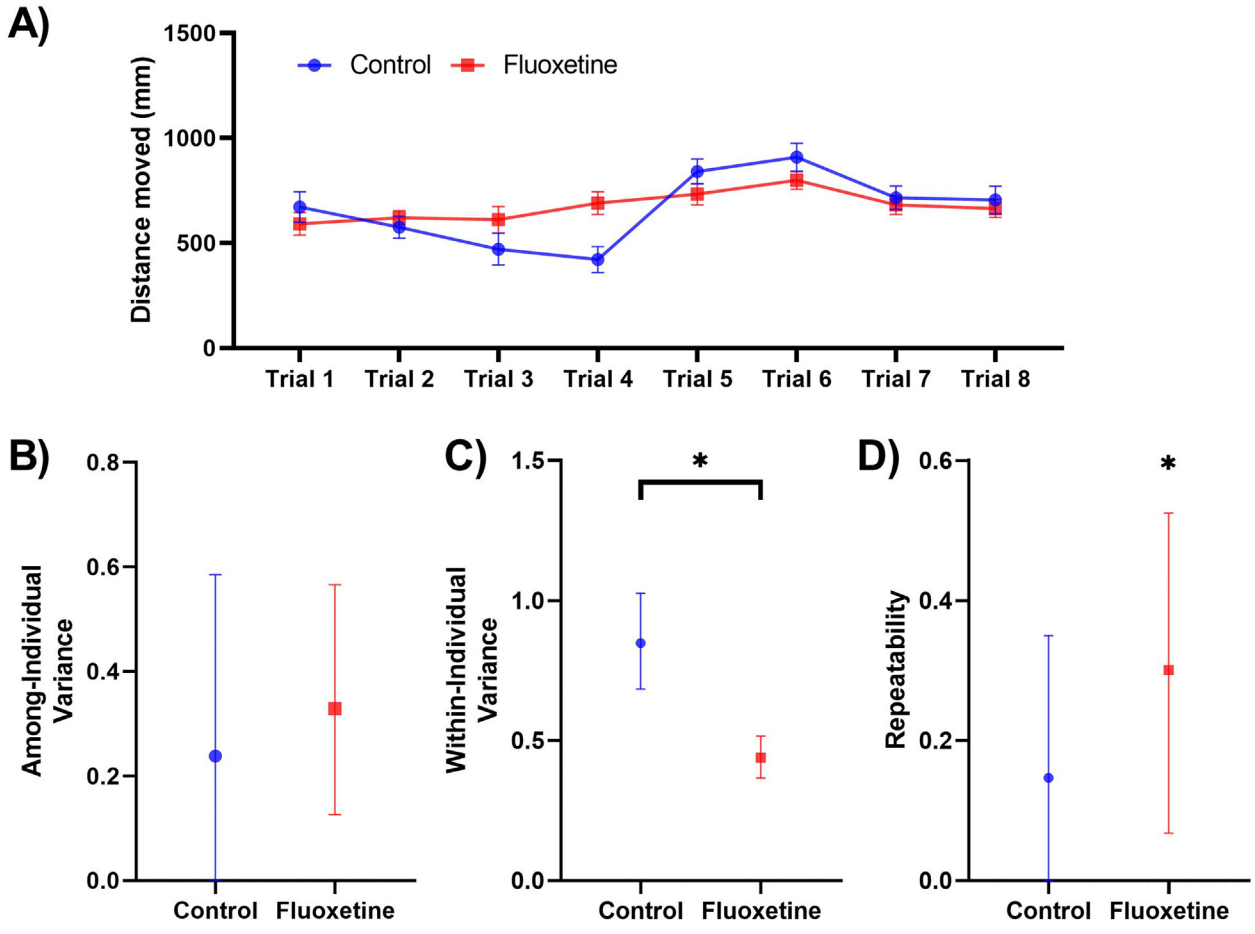

Fig. 2. Behaviour. (A) Total locomotor activity over time, and (B) among-individual variance, (C) within-individual variance, and (D) and repeatability (at the intercept) of activity of fluoxetine exposed and unexposed snails. Asterisks denotes non-overlapping confidence intervals with zero.

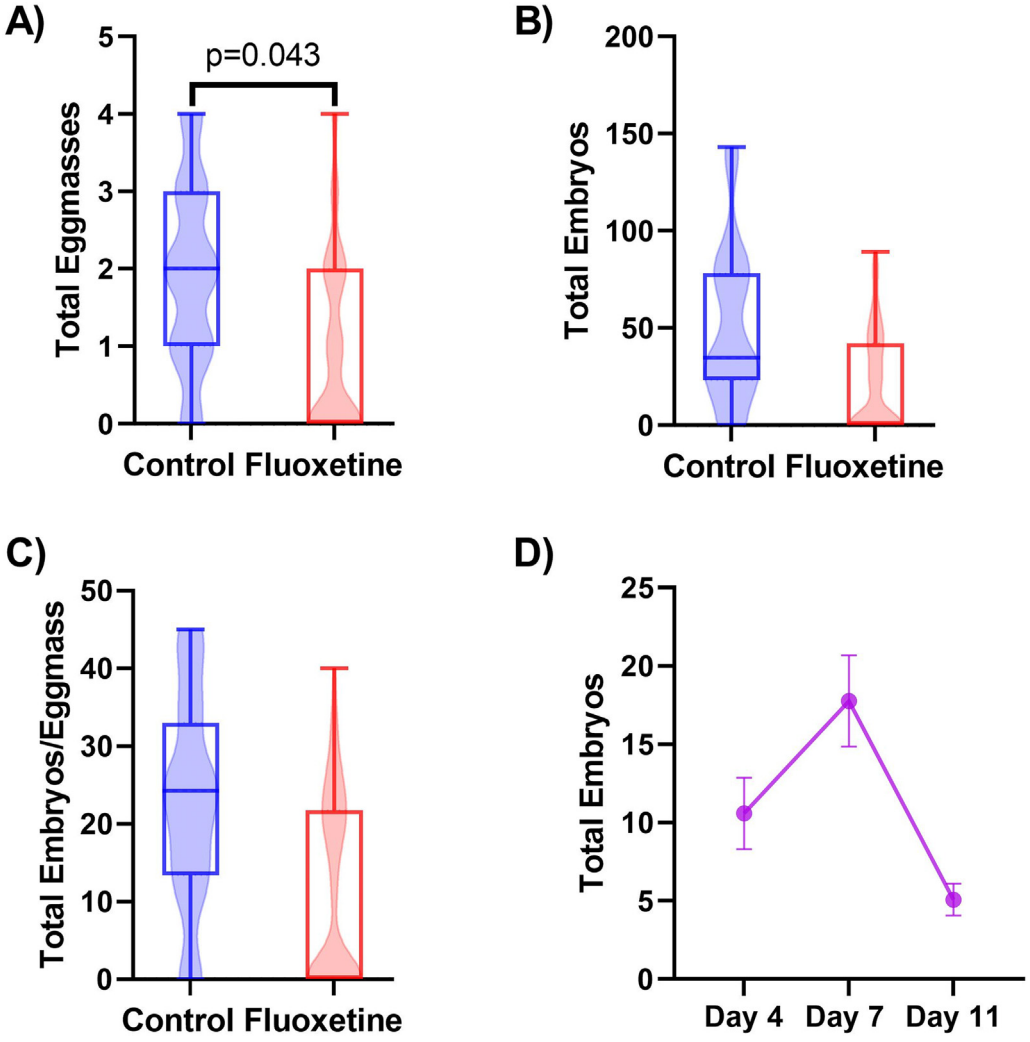

Fig. 3. Reproductive output. (A) Total number of eggmasses, (B) total number of embryos and (C) the number of embryos per eggmass in fluoxetine exposed and unexposed snails. (D) Total number of embryos over time with treatments combined.

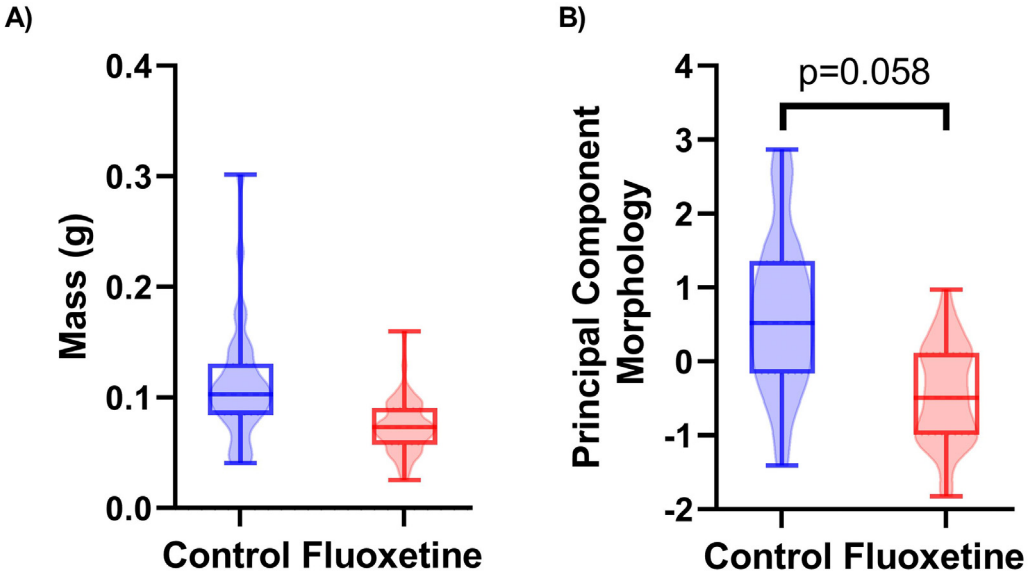

Fig. 4. Morphology. (A) Mass and (B) principal component analysis of morphological endpoints (shell length, area and perimeter) in fluoxetine exposed and unexposed snails.

groups. We also found that activity was repeatable in fluoxetine exposed, but not unexposed, snails. For reproduction, we found there were fewer eggmasses produced by fluoxetine exposed individuals, however there was no difference found in total embryos or the ratio of embryos per eggmass produced. Lastly, for morphology, we found no significant difference between treatment groups in snail mass and a marginally non-significant change in shell size.

4.1. Mean level behavioural response

There was no significant effect of fluoxetine treatment on mean-level locomotor activity of snails. This result is in-line with work on water fleas (*Daphnia magna*) which, following a 5 month multigenerational fluoxetine exposure (54 ng/L), found no change in mean swimming speed of the F3 generation (Heyland et al., 2020). This contrasts with previous studies on amphipods (*Gammarus pulex*), which found an increase in swimming speed following a 14 day fluoxetine exposure (100 ng/L) (De Castro-Català et al., 2017). Furthermore recent studies on marine snails (*Urosalpinx cinerea* and *Lithopoma americanum)*, showed that upon acute 4 h exposure to varied concentrations of fluoxetine (>3.45 μg/L) both species demonstrated a significant reduction in locomotory speed (Fong et al., 2015). An acute exposure study on zebrafish (*Danio rerio*) larvae demonstrated a reduction in mean locomotion by fluoxetine exposed (>0.88 μg/L, 120 h) individuals (de Farias et al., 2019). It should be noted that the differences found in standard locomotion of the latter two studies were based on treatments that are an entire order of magnitude higher in concentration level compared to our's. The above examples highlight that, unlike many acute exposure experiments, the effects of chronic exposure to environmentally relevant levels of fluoxetine are often too subtle to observe at a mean level and thus require further analysis. Moreover, it is also important to note that, clinically, fluoxetine manifests its initial antidepressant effect within 2 to 4 weeks of administration (Gardier et al., 1996). As a result, short term exposure scenarios may fail to recapitulate the time that is required to manifest the biological activity of SSRI's drugs (Jangid et al., 2013; Machado-Vieira et al., 2010).

4.2. Behavioural variation

Interestingly, whilst there were no differences found in average activity levels between groups, fluoxetine exposure affected behavioural variation. More specifically, there were reduced levels of within-individual variation in fluoxetine exposed snails identified when compared to controls. In contrast, there was no difference in the amount of variation among individuals in activity between the treatment groups. Notably, we also found that only fluoxetine exposed snails were significantly repeatable in their locomotor activity at the intercept. Similar reductions in within-individual variance have also been found in other invertebrate responses upon exposure to environmental pollutants. For example, following exposure to microplastics, the startle response of hermit crabs (*Pagurus bernhardus)* was shown to decrease in variation within individuals while exhibiting no differences between individuals (Nanninga et al., 2020). These results are in contrast with previous work on fluoxetine exposed fish species such as guppies (*Poecilia reticulata*), which, although finding no within-individual differences in variation, did exhibit a decrease in repeatability (Polverino et al., 2021). This behavioural phenotypic comparison lends further support to mechanistic studies (e.g. Sánchez-Argüello et al., 2009) reasoning that invertebrates are neuro-physiologically affected by fluoxetine differently than vertebrates. It has been shown that increased behavioural variation in a species is important for the ability of an organism to adapt to changes in their environment (Dingemanse and Wolf, 2013). Within individual variation in behaviour has been noted to assist an organism's ability to persist through adverse conditions prior to an evolutionary genetic change. Subsequently, the lack of within individual variance suggests these populations may be less able to cope with environmental challenges (Snell-Rood, 2013; Westneat et al., 2015; Wolf and Weissing, 2012).

4.3. Reproduction

There were significantly less eggmasses produced by snails exposed to the fluoxetine treatment. However, there was no differences found in the total embryos produced within these eggmasses, or in the number of embryos per eggmass. Therefore, although embryo quantity per eggmass is conserved across groups, the fluoxetine group had a decreased overall reproductive output in number of eggmasses. Here, our results are consistent with prior research on another freshwater snail (*Potamopyrgus antipodarum*), which similarly found a reduction in the number of offspring produced following a fluoxetine (68 μg/L) exposure period of 6 weeks (Péry et al., 2008). Our results are also consistent with an asexual reproduction study on planarians (*Schmidtea mediterranea*) which showed that individuals exposed over a 9 day period to fluoxetine (10 μg/L) had a reduced rate of fission occurrence (Ofoegbu et al., 2019). Again, it is important to highlight the studies above have a shorter exposure period and use concentrations of fluoxetine an order of magnitude higher than our study. A 5 month multigenerational fluoxetine exposure test on the common water flea (*D. magna*), at similar concentrations (54 ng/L) to our work, found fluoxetine had significant impacts on reproductive interactions in subsequent generations (Heyland et al., 2020). Generally, these results suggest that a reduction in reproduction reported previously in acute experiments with high dose rates can also occur when animals are chronically exposed across multiple generations to lower, more environmentally relevant exposure concentrations.

4.4. Morphology

There was no effect of chronic fluoxetine exposure on snail mass. However, there was a marginally non-significant difference in shell size (i.e. PC of cone length, area and perimeter) between fluoxetine exposed and unexposed snails. As far as we are aware, only a few published studies have previously examined morphological changes following exposure durations that are directly comparable to those used in our study. One such example, looking at chronic exposure to fluoxetine in a mesocosm over 123 days, found no malformations in development of the tadpole (*Rana pipiens*), however the tadpoles, exposed to 80 ng/L fluoxetine, gained weight significantly slower than the control group (Foster et al., 2010).

Aside from mass, no differences in morphology (morphology scored on length and malformations) were reported in fluoxetine exposed brown hydra (*Hydra oligactis*) or water fleas (*D. magna*) exposed to fluoxetine for 14 day at 10 μg/L and 36 days at 36 μg/L, respectively (Flaherty and Dodson, 2005; Lee et al., 2020).

Interestingly, previous work in zebrafish showed that 30 day exposure to fluoxetine (100 μg/L) caused a significant increase in mass (de Farias et al., 2020), whereas no difference in mass was reported in polychaetes (*Capitella teleta*) exposed to fluoxetine in feeding trials for 18 days (mixed feed with fluoxetine dry weight of up to 3.3 μg/g) (Méndez et al., 2013). Clearly, if we are to gain a better understanding of the effects of fluoxetine exposure on morphology, further research is needed to bridge the gap between those studies that have been conducted over shorter exposure durations at relatively high concentrations and studies such as ours, that have employed longer term exposure durations over multiple generations at more field-relevant concentrations.

5. Conclusions

This study aimed to determine what effects long-term exposure to fluoxetine has on the behaviour, reproductive output and morphology of an aquatic snail. Overall, our findings indicate chronic 3-year fluoxetine exposure (Mean: 32.7 ng/L, SE: 2.3) did not affect mean level locomotor activity changes or among-individual variance in the behaviour of individual snails. There was, however, reduced within-individual variance in behaviour, and hence, increased repeatability of activity levels in fluoxetine exposed individuals. From a reproductive context, there were fewer eggmasses produced by fluoxetine exposed individuals, but no change in the total

embryos or the number of embryos per eggmass produced. There was also a marginally non-significant difference in shell size. This study highlights that multigenerational exposure to environmentally realistic concentrations of fluoxetine can impact key reproductive endpoints in aquatic invertebrates. Further, we demonstrate the potential subtle impacts that these pollutants may have on individual level variation, even when no mean-level effects are apparent. We postulate that more multigenerational studies on a wider variety of species are required to elucidate and model aquatic environmental risks associated with the long-term impacts of widespread and growing pollution by psychoactive chemicals.

CRediT authorship contribution statement

JH conduced the experiments, data analysis and wrote the manuscript; JAB conducted the statistical analysis and reviewed the manuscript; YB conduced the experiments and data analysis; JMM conducted the statistical analysis and reviewed the manuscript; BBMW designed the study, drafted and reviewed the manuscript; DW designed and managed the study, drafted and reviewed the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2021.152731.

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