Long-term pharmaceutical contamination and temperature stress disrupt fish behaviour

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ABSTRACT

Natural environments are subject to a range of anthropogenic stressors, with pharmaceutical pollution being among the fastest-growing agents of global change. However, despite wild animals living in complex multi-stressor environments, interactions between pharmaceutical exposure and other stressors remain poorly understood. Accordingly, we investigated effects of long-term exposure to the pervasive pharmaceutical contaminant fluoxetine (Prozac®), and acute temperature stress, on reproductive behaviours and activity levels in the guppy (*Poecilia reticulata*). Fish were exposed to environmentally realistic fluoxetine concentrations (measured average: 38 or 312 ng/L) or a solvent control for 15 months using a mesocosm system. Additionally, fish were subjected to one of three acute (24 h) temperature treatments: cold stress (18 °C), heat stress (32 °C) or a control (24 °C). We found no evidence for interactive effects of fluoxetine exposure and temperature stress on guppy behaviour. However, both stressors had independent impacts. Fluoxetine exposure resulted in increased male coercive copulatory behaviour, while fish activity levels were unaffected. Under cold-temperature stress, both sexes were less active and males exhibited less frequent reproductive behaviours. Our results demonstrate that long-term exposure to a common pharmaceutical pollutant, and acute temperature stress, alter fundamental fitness-related behaviours in fish, potentially shifting population dynamics in contaminated ecosystems.

Introduction

Contamination of aquatic habitats by pharmaceuticals is a major environmental problem, evoking concern among scientists, health officials, and communities around the globe.[1–3] Most pharmaceuticals are incompletely metabolised when consumed and their metabolites can remain biologically active when excreted.[4,5] Wastewater treatment plants (WWTPs) are typically not designed to remove pharmaceutical compounds from sewage[6] and discharge of wastewater effluent into the environment is, therefore, a primary source of contamination.[7] Accordingly, pharmaceuticals such as antibiotics, painkillers, cardiovascular drugs, blood lipid regulators, and antidepressants, are frequently detected in surface and ground waters around the world.[3] The presence of these active pharmaceutical products in natural environments is problematic because of their capacity to induce a range of sub-lethal effects in exposed organisms.[1,8,9] Indeed, pharmaceuticals can disrupt fundamental behavioural processes, such as reproductive behaviour, aggression, boldness, activity levels, and feeding rates.[1,10] Changes to such behaviours can directly impact the strength and direction of selection, fitness, and even population viability, with potential for broader ecosystem and evolutionary consequences.[11–14]

Fluoxetine (Prozac®) is one of the most widely prescribed antidepressants globally,[15] being used to treat depression and anxiety-related disorders in humans and domesticated animals.[16,17] Fluoxetine is also a relatively stable compound (half-life of 68 days in water at pH 7 under light conditions)[18] that is commonly detected in freshwater environments worldwide.[19,20] In aquatic habitats, fluoxetine concentrations range between <1 ng/L to as high as 1400 ng/L,[3,20,21] although concentrations above 350 ng/L

tend to only occur in direct sewage effluent.[20] Fluoxetine inhibits the reuptake of neuronal serotonin (5-hydroxytryptamine), which acutely increases synaptic serotonin levels and, after 2–3 weeks, produces anxiolytic effects in humans.[16] The serotonergic system is conserved across all vertebrate classes[22] and, consequently, fluoxetine has the capacity to alter behaviour in a wide range of species.[23]

Continuous discharge of fluoxetine-contaminated effluent from WWTPs, coupled with the stability of dissolved fluoxetine, results in long-term ('pseudo-persistent') exposure of many aquatic environments.[8] Duration-dependent effects have been observed in mussels (*Mytilus californianus*), with some physiological changes manifesting only after 6 weeks of fluoxetine exposure.[24] Yet, most studies investigating impacts of fluoxetine are conducted using short-term exposure durations (i.e. <1 month) that represent a small fraction of the model species' lifespan.[25–30] This is problematic because effects that persist after long-term exposure may have important consequences on the lifetime fitness of individuals and population dynamics, making studies addressing effects of chronic exposure to pharmaceutical contaminants, such as fluoxetine, an urgent research priority.

Aquatic species are subjected to a range of environmental stressors contemporaneously.[31] It is, therefore, important to understand how species respond to pharmaceutical pollutants in the presence of other concurrent stressors, especially because joint effects of interacting stressors can be challenging to predict. In particular, effects of combined stressors can be less, or greater, than expected (i.e. antagonistic or synergistic, respectively), compared to stressors tested in isolation. For example, in the Mediterranean mussel (*Mytilus galloprovincialis*), concurrent exposure to fluoxetine and

the high blood pressure medication propranolol resulted in an antagonistic interaction with regard to cell signalling,[32] with the combined effect of both pharmaceuticals being less than what would be expected if the independent effects were simply summed together.

Temperature is an important stressor, especially in aquatic environments. Increased temperature variability represents a disproportionately greater threat to organisms than mean temperature increases,[33] and ambient environmental temperature is crucial to body temperature regulation in ectotherms.[34] In this respect, aquatic ectotherms are poorly adapted to cope with large temperature fluctuations,[35–37] especially in the context of reproduction.[38] Furthermore, temperature stress can compromise an individual's ability to respond effectively to other environmental stressors.[39] For instance, toxicity of pesticides to juvenile coho salmon (*Oncorhynchus kisutch*) was elevated at higher temperatures.[40] In zebrafish (*Danio rerio*), isolated exposures to high temperature or the endocrine disruptor progestin had a negative effect on female fecundity, whereas exposure to both of these stressors simultaneously resulted in complete reproductive failure.[41] Similarly, in the water flea *Daphnia magna*, fluoxetine and temperature variability had an adverse synergistic effect on reproductive success and population growth.[42] More generally, however, interactive effects between temperature and exposure to realistic levels of pharmaceutical pollution have received surprisingly little attention to date. Indeed, more work is clearly needed, given the prevalence of pharmaceutical contaminants in aquatic environments and the importance of temperature to ectothermic species.

Here, we investigated how two important determinants of fitness, reproductive behaviour and activity, are influenced by two stressors, chronic fluoxetine exposure and acute temperature stress, in a freshwater fish. Specifically, guppies (*Poecilia reticulata*) sourced from mesocosm populations were exposed to environmentally realistic levels of fluoxetine (nominal concentrations: 30 ng/L or 300 ng/L) or left unexposed (i.e. solvent control only) for a period of 15 months and then underwent one of three temperature treatments. Fish were placed under cold stress (at 18 °C), heat stress (at 32 °C), or maintained at a control temperature (24 °C) for 24 h prior to experimental trials. We then investigated how reproductive behaviours and activity levels of guppies were impacted by the fluoxetine and temperature treatments. Because isolated exposure to fluoxetine and cold stress have been shown to generate opposite effects on reproductive behaviour in fish—i.e., increased male copulatory behaviour resulting from fluoxetine exposure[25,28,43] and decreased male sexual motivation due to cold stress[44]—we hypothesised that these two stressors would act antagonistically, with the effect of one countering the effect of the other when experienced in combination. We also predicted that heat stress and fluoxetine exposure would, in turn, interact synergistically to increase levels of courtship and copulation.[45] For activity levels, we tested the generality of previous findings suggesting that fluoxetine may not significantly affect fish activity.[30,46,47] We predicted, instead, that activity increases with temperature.[48]

Materials and methods

Study species

The guppy is a small poeciliid fish native to freshwaters of northern South America.[49] As a highly successful invader, the guppy is now found in tropical and subtropical regions around the world.[50] The preferred temperature range of guppies is 24–27 °C,[51–53] with females having fewer offspring per brood under heat stress (i.e. ≥32 °C)[54] and males subjected to cold stress (i.e. ≤20 °C) courting less.[44] Guppies undergo internal fertilisation, with males inseminating females using a modified anal fin, the gonopodium.[49] Male guppies exhibit two alternative mating strategies, either performing courtship displays to elicit consensual copulations with choosy females or carrying out coercive 'sneak' copulations that circumvent female mate choice.[55] Courtship displays involve the male positioning himself in the female's line of sight, bending his body into an s-shape and quivering (termed 'sigmoid display').[49] Sneak copulation attempts involve a male chasing a female from behind and attempting to insert his gonopodium into the female's genital pore without first performing courtship.[49] Because the latter strategy is associated with lower insemination efficiency and reduced offspring quality,[56] changes in the relative use of these two strategies can impact the quality and quantity of progeny[55], potentially altering population dynamics and size.

Mesocosm system and fluoxetine treatments

Guppies used in this experiment were sourced from mesocosm populations that had been maintained in a temperature-controlled greenhouse facility under natural (i.e. ambient) light conditions at Monash University, Melbourne, Australia. These mesocosm populations were founded using wild-caught guppies collected in November 2016 from Alligator Creek, a rainforest-fed stream located within Bowling Green Bay National Park,

Townsville, Australia (19°23'50.3" S, 146°56'56.5" E; collection permit: WITK17685216). Water samples taken from this site at the time of fish collection revealed no contamination with fluoxetine (Envirolab Services; all samples under the minimum detection limit of 2 ng/L, *n* = 5). After collection, fish were housed in 12 stainless steel mesocosm tanks (648 L; 180 cm × 60 cm × 60 cm), each of which was established with a founding population of 300 sexually mature guppies at an equal sex ratio, with these mesocosm populations having since been utilised for a series of experiments, including the present study. Mesocosm tanks 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 used to maintain water temperature. The temperature and pH of all tanks were tested weekly (temperature: mean = 23.4 °C, SD = 1.0 °C, *n* = 720; pH: mean = 7.36, range = 5.08–9.67, *n* = 720). Fish were fed *ad libitum* once every two days with commercial food pellets (Aquasonic Nutra Xtreme C1 pellets; 0.8 mm). Once per week, 20% water changes were conducted for each tank.

Mesocosm tanks were randomly allocated to one of three fluoxetine exposure regimes, a low-fluoxetine treatment (nominal concentration: 30 ng/L, *n* = 4 tanks), a high-fluoxetine treatment (nominal concentration: 300 ng/L, *n* = 4 tanks) or an unexposed treatment (i.e. solvent control, *n* = 4 tanks) from April 2017. The low-fluoxetine treatment is representative of concentrations commonly found in surface waters, whereas the high-fluoxetine treatment represents levels detected in effluent-dominated systems.[3,20,21] A population survey conducted in the month following behavioural experiments (August 2018) showed that adult densities within the twelve mesocosms

were similar across the treatments (mean ± SD: 78 ± 57, 66 ± 39, and 62 ± 26, for the control, low, and high treatments, respectively).

To maintain the desired fluoxetine water concentrations, dosing solutions were added to the low- and high-exposed tanks twice weekly. This involved fluoxetine hydrochloride (Sigma Aldrich; product number: F132, CAS: 56296-78-7) being dissolved in methanol to form two separate 100 mL stock solutions (20 and 200 mg/L for the low- and high-fluoxetine treatments, respectively), which were then used to create dosing solutions twice weekly. Dosing solutions were prepared by diluting 1 mL of either stock solution in 1 L of reverse-osmosis water. To eliminate any potential for solvent effects[57] 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 tanks twice weekly (equates to 0.0006% methanol by volume).

Analytical verification of fluoxetine treatment levels

Throughout the experiment, water samples (40 mL) were drawn approximately once per month from each of the low- and high-fluoxetine treatment mesocosm tanks to determine the concentrations of fluoxetine and norfluoxetine (the major metabolite of fluoxetine)[19] using gas chromatography–tandem mass spectrometry (7000C Triple Quadrupole GC-MS/MS, Agilent Technologies, Delaware, USA; minimum detection limit: 2 ng/L). Control tanks were also sampled every second month using the same method, to ensure the absence of contamination. Water analyses were performed by Envirolab Services (MPL Laboratories; NATA accreditation: 2901; accredited for compliance with ISO/IEC: 17025) within 4 days of collection. A detailed description of the water analysis protocol is provided in Bertram et al.[25] Mean measured fluoxetine

197 concentrations in the low- and high-fluoxetine mesocosm tanks were 38 ng/L (SD = 24,
198 *n* = 60) and 312 ng/L (SD = 214, *n* = 60), respectively. Fluoxetine was not detected in
199 any of the control tanks (all samples under the minimum detection limit, *n* = 30).
200 Norfluoxetine was not observed in any of the tested samples. Fluoxetine readily sorbs to
201 sediment in water/sediment systems.[19,58] Hence, while the gravel substrate used in the
202 mesocosm system was important in simulating more natural environmental conditions, it
203 likely contributed to the variability in measured fluoxetine concentrations observed.

204 *Experimental procedure and temperature treatments*
205 The goal of the study was to investigate behavioural effects of long-term fluoxetine
206 exposure under ecologically realistic conditions comprising multiple overlapping and
207 interacting generations, which is reflective of guppy populations in nature.[49,59,60] Trials
208 were conducted in July 2018, resulting in a 15-month exposure protocol. Given an
209 approximate generation time of 4 months in guppies, up to 4 generations were present
210 within the mesocosm system at the time of trials.[59,60] One week prior to experimental
211 trials, sexually mature fish were caught and separated by sex into fine-mesh cylinders
212 (35 cm × 32 cm, diameter × height, water depth: 30 cm) within their respective
213 mesocosm tanks. To ensure that fish were sexually mature, we selected females that
214 were over 15 mm in standard length, and males displaying nuptial colouration and a
215 fully developed gonopodium.[49] Fish were then sourced from these cylinders for use in
216 trials, ensuring individuals were only tested once. Twenty-four hours prior to
217 experimentation, fish underwent temperature manipulations in 1 L cylindrical glass tanks
218 (10 cm × 30 cm, diameter × height, water depth: 20 cm, maximum 3 fish per tank), with
219 males and females housed in separate tanks. Three temperature treatments were

220 employed: heat stress (32 °C), cold stress (18 °C), and a control temperature treatment
221 (no change; 24 °C). The control treatment was chosen to represent the long-term
222 average temperature observed across mesocosm tanks (mean = 23.4 °C, SD = 1.0 °C,
223 *n* = 720). The heat stress treatment involved an 8 °C increase over the average
224 mesocosm tank temperature. This temperature increase was selected to simulate heat
225 stress but, importantly, was still below the critical thermal maximum of guppies (i.e. 38
226 °C).[61] The cold stress treatment involved a 6 °C reduction from the average mesocosm
227 tank temperature, which was chosen because guppies are more vulnerable to rapid
228 decreases in temperature than to rapid increases in temperature.[62] The temperature
229 changes of +8 °C and −6 °C used in these experiments are plausible in an
230 environmental context, with daily fluctuations of this scale having been observed within
231 the guppy's native range.[53] To avoid shock caused by instantaneous temperature
232 changes, temperature alterations occurred over a period of 6 h, which is common
233 practice in temperature manipulation experiments involving fish.[63,64] The tanks remained
234 at the new temperatures for the subsequent 18 h before fish were tested in behavioural
235 trials.

236 *Experimental trials*

237 Behavioural trials (*n* = 162) were conducted on the day after fish were exposed to
238 temperature manipulations, in glass tanks (60 cm × 30 cm × 30 cm, water depth: 15 cm)
239 filled with carbon-filtered water maintained at 18 °C (mean: 18.2 °C, range: 17.8–18.5
240 °C), 24 °C (mean: 24.3 °C, range: 24.0–24.5 °C) or 32 °C (mean: 32.3 °C, range: 31.8–
241 32.5 °C). The male and female used in each trial had been subjected to the same
242 fluoxetine and temperature treatment (solvent control treatment [i.e. 0 ng/L fluoxetine]:

control temperature: *n* = 20, low temperature: *n* = 17, high temperature: *n* = 17; low-fluoxetine treatment: control temperature: *n* = 16, low temperature: *n* = 20, high temperature: *n* = 19; high-fluoxetine treatment: control temperature: *n* = 19, low temperature: *n* = 18, high temperature: *n* = 16). Fish tested in each trial were sourced from different mesocosm tanks to ensure that male-female experimental pairs were novel to each other. This was done to control for familiarity, which is known to influence mate choice in guppies.[65] Each trial involved the male and female being placed into separate acclimation chambers (opaque cylinders; 7.5 cm × 20 cm, diameter × height, water depth: 15 cm) in an experimental tank matching the desired temperature used in the temperature manipulation. Fish were acclimated for 5 min, after which the acclimation chambers were removed so that the two fish were free to explore the trial tank and interact for 40 min. Tank water was replaced between each trial to prevent any potential for chemical cues to influence fish in subsequent trials.[66]

Throughout behavioural trials, tanks were video-recorded from above (Panasonic HC-V180), with male and female behaviour subsequently scored from recordings, blind to treatment, using behavioural observation key-logging software (BORIS v. 6.3).[67] Specifically, the time taken for the male to first attempt a sneak copulation and the number of attempted sneak copulations were scored for each trial. The time until the first courtship display and the number of courtship displays performed by each male were also recorded. Additionally, activity levels of the male and female were evaluated using 5 cm grid squares marked on the base of each experimental tank. We counted the number of 5 cm grid squares crossed by each fish for 1 min every 5 min over the 40-min trial, resulting in a total of 8 min of observations for each fish. We then estimated

activity as movement/time (cm/sec). After each trial, both of the fish were measured for body mass (±0.0001 g) and standard length (i.e. body length excluding tail; ±0.01 mm). The fish were then returned to their respective mesocosm source tanks, where they were isolated from untested fish.

Statistical analyses

Statistical analyses were performed in R 3.5.1.[68] As a proxy for body condition, we calculated a scaled mass index, which was done separately for males and females.[69] Specifically, we performed a standard major axis regression on the log of body mass and standard length of fish (*sma* function, *smatr* package), and calculated a sex-specific beta coefficient, which was then used (with mean standard length) to obtain the scaled mass for each fish. These scaled mass indices for males and females were initially included in all models but were later removed as they did not significantly improve their fit, as tested by Akaike information criterion comparisons.

Generalised linear mixed models (GLMMs) were used to test the effects of fluoxetine treatment, temperature treatment, and the interaction between them, for both sneak copulations and courtship displays, separately. For sneak copulations, a negative binomial distribution (NB GLMM; *nb.glmer* function, *MASS* package) was selected to account for overdispersion. For courtship displays, a binomial distribution was selected over a Poisson distribution because an insufficient number of fish conducted the behaviour for it to be analysed as a count variable (i.e. 19.8% across all groups). To account for possible mesocosm tank effects, the source tank IDs of male and female fish, as well as the combination of male and female source tank IDs, were included as

random effects in both GLMM models (see supplementary tables S1–S4 for random effects results).

To analyse potential effects of the fluoxetine and temperature treatments, and their interaction, on the time taken for males to perform their first sneak copulation and courtship display, we applied separate Cox's mixed effect (COXME) proportional hazard models (*coxme* function, *survival* package) for the two response variables (i.e. sneak attempts and courtship displays). Both models met the assumption of proportionality, as determined by examining the interaction between Schoenfeld residuals and log time (*cox.zph* function, *survival* package).

Data on fish activity levels were square-root transformed so that assumptions of normality and homogeneity of variance were satisfied (Shapiro-Wilk test, *shapiro.test* function and Bartlett test, *bartlett.test* function). We then analysed activity levels of fish using linear mixed effects models (LME; *lmer* function, *lme4* package). Fluoxetine and temperature treatments, the interaction between the two, and sex, were included as fixed effects, and mesocosm source tank ID was added as a random effect.

Where relevant, general linear hypothesis tests (GLHTs) with Tukey's post-hoc *p*-adjustments were used to generate pairwise comparisons (*glht* function, *multcomp* package).

Effects of fluoxetine exposure on morphology (weight, standard length, and scaled mass index) were investigated for each sex, using Kruskal-Wallis tests to account for non-normal distributions in data (KWT; *kruskal.test* function). Dunn's tests were used with Bonferroni corrections for pairwise comparisons (*dunnTest* function, *FSA* package).

Results

Reproductive behaviours

No significant interaction was detected between fluoxetine treatment and temperature treatment for the number of sneak copulations performed by males (NB GLMM: χ^2 = 3.885, *df* = 4, *p* = 0.43). We did, however, find a significant difference between the number of sneak copulations conducted by males in different fluoxetine treatments (NB GLMM: χ^2 = 7.843, *df* = 2, *p* = 0.019; Fig. 1a), with unexposed fish performing fewer sneaks than males in the low- and high-fluoxetine treatments (NB GLMM: *z* = −2.455, *p* = 0.037, and *z* = −3.129, *p* = 0.005, respectively). The low- and high-fluoxetine treatments did not differ significantly (NB GLMM; *z* = 0.3595, *p* = 0.63; Fig. 1a). Temperature treatment also significantly affected the number of sneak copulations performed by males (NB GLMM; χ^2 = 20.33, *df* = 2, *p* < 0.001; Fig. 1b). Males under low-temperature stress performed fewer sneak copulations than did those in the heat stress and control treatments (NB GLMM: *z* = 4.745, *p* < 0.001, and *z* = 3.370, *p* = 0.002, respectively; Fig. 1b). No significant difference was detected between the control and heat-stress treatments (NB GLMM; *z* = 1.728, *p* = 0.19; Fig. 1b).

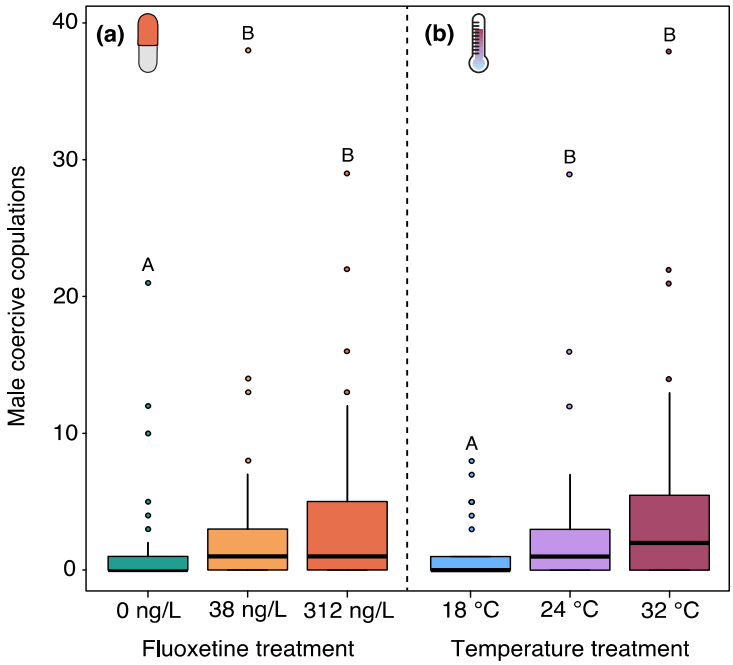

Figure 1. Number of male coercive 'sneak' copulations performed in the (a) unexposed (0 ng/L; *n* = 54), low-exposed (38 ng/L; *n* = 55) and high-exposed (312 ng/L; *n* = 53) fluoxetine treatments, and in the (b) low (18 °C; *n* = 54), control (24 °C; *n* = 56) and high (32 °C; *n* = 52) temperature treatments

We found no significant interactive effect between fluoxetine exposure and temperature treatment in terms of time taken to the first male sneak copulation (COXME: χ^2 = 1.491, *df* = 4, *p* = 0.83). Furthermore, fluoxetine treatment did not affect the time elapsed before males attempted a sneak copulation (COXME: χ^2 = 3.783, *df* = 2, *p* = 0.15; Fig. S1) but temperature treatment did (COXME: χ^2 = 13.17, *df* = 2, *p* = 0.001; Fig. 2). Specifically, males at 18 °C were significantly delayed in performing their first sneak relative to males at 32 °C (GLHT: *z* = 2.387, *p* = 0.043). Males in the 24 °C control

temperature treatment did not differ significantly from those at 18 °C (GLHT; z = 2.045, p = 0.098) or 32 °C (z = 0.5680, p = 0.83).

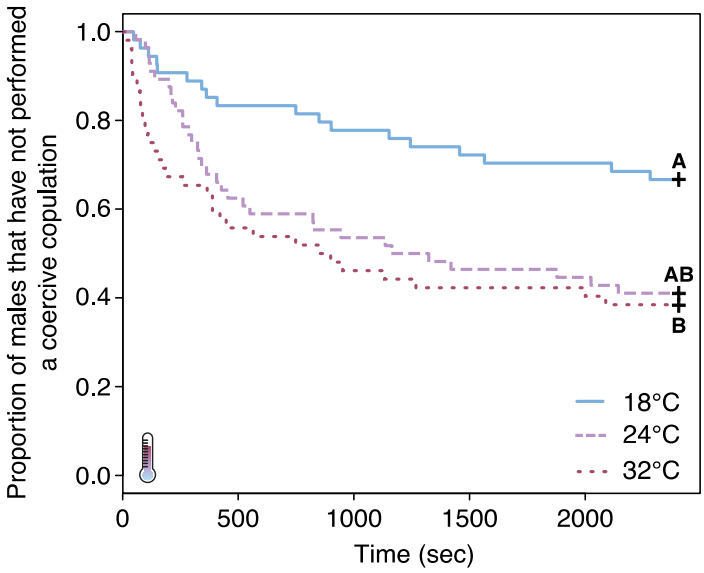

Figure 2. Time taken to first coercive 'sneak' copulation for males by temperature treatment, right-censored at 2400 sec. The solid line represents the low-temperature treatment (18 °C; n = 54), the dashed line represents the control temperature treatment (24 °C; n = 56) and the dotted line represents the high-temperature treatment (32 °C; n = 52)

There was no significant interaction between fluoxetine and temperature treatments regarding the proportion of males that performed courting behaviours (GLMM: χ^2 = 2.559, df = 4, p = 0.63), nor was there a significant effect of fluoxetine treatment (GLMM: χ^2 = 1.906, df = 2, p = 0.39), with 14.8% of males in the control treatment, 16.4% in the low-fluoxetine treatment, and 28.3% in the high-fluoxetine treatment

355 performing at least one courtship display. Temperature, however, did significantly
356 influence the proportion of males that performed courtship displays (GLMM: χ^2 = 7.284,
357 *df* = 2, *p* = 0.026). A significantly lower proportion of males in the low-temperature
358 treatment performed at least one courtship display (3.6%), relative to those in the
359 control treatment (30.9%; GLMM: *z* = 3.138, *p* = 0.002), and the high-temperature
360 treatment (25.0%; GLMM: *z* = 2.791, *p* = 0.005). No significant difference between the
361 control and high-temperature stress treatments was observed for courting behaviour
362 (GLMM: *z* = 0.6820, *p* = 0.52).

363 For the time elapsed until first courtship display, there was no significant interaction
364 between temperature and fluoxetine treatment (COXME: χ^2 = 3.877, *df* = 4, *p* = 0.42),
365 nor was there a significant main effect of fluoxetine exposure (COXME: χ^2 = 5.066, *df* =
366 2, *p* = 0.41; Fig. S2). A marginally non-significant main effect was, however, observed
367 for temperature treatment (COXME: χ^2 = 10.88, *df* = 2, *p* = 0.054, Fig. S3).

368 *Activity levels*

369 We found no significant interaction between fluoxetine treatment and temperature
370 treatment on fish activity levels (LME: χ^2 = 5.385, *df* = 4, *p* = 0.25). Fluoxetine exposure
371 did not influence activity (LME: χ^2 = 1.217, *df* = 2, *p* = 0.54; Fig. S4) but temperature did
372 (χ^2 = 118.6, *df* = 2, *p* < 0.001; Fig. 3). The cold-temperature treatment resulted in a
373 significant reduction in activity levels relative to the control and heat-stress treatments
374 (GLHT: *z* = 5.902, *p* < 0.001, and *z* = 4.904, *p* < 0.001, respectively) but there was no
375 significant difference between the control and heat-stress treatments with regard to
376 activity levels (GLHT: *z* = −0.8040, *p* = 0.70). Regardless of exposure treatment, female
377 fish were significantly less active than males (χ^2 = 7.577, *df* = 1, *p* = 0.006), and activity

level correlated with a variety of reproductive behaviours. Specifically, activity level was negatively correlated with time to first courtship display (Spearman's rank correlation: *rho* = –0.273, *df* = 160, $p < 0.001$) and time to first sneak copulation (Spearman's rank correlation: *rho* = –0.301, *df* = 160, $p < 0.001$). In addition, activity was positively correlated with the propensity to conduct at least one courtship display (Spearman's rank correlation: *rho* = 0.273, *df* = 160, $p < 0.001$), as well as the number of sneak copulations performed (Spearman's rank correlation: *rho* = 0.304, *df* = 160, $p < 0.001$).

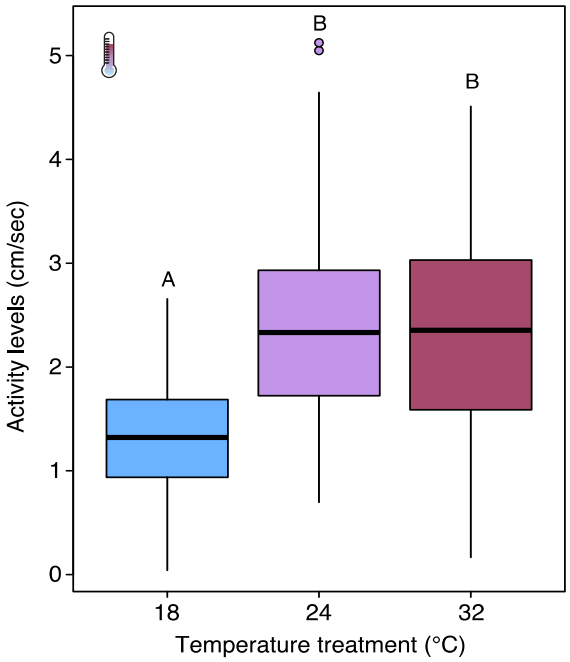

Figure 3. Activity levels of fish in the low-temperature treatment (18 °C; *n* = 108), the control temperature treatment (24 °C; *n* = 112), and the high-temperature treatment (32 °C; *n* = 104)

Morphology

391 Fluoxetine exposure did not impact weight (KWT: χ^2 = 2.527, *df* = 2, *p* = 0.28), standard
392 length (KWT: χ^2 = 1.925, *df* = 2, *p* = 0.38), or scaled mass (KWT: χ^2 = 0.9388, *df* = 2, *p*
393 = 0.63) in male guppies. Fluoxetine did, however, impact weight (KWT: χ^2 = 9.115, *df* =
394 2, *p* = 0.010) and standard length (KWT: χ^2 = 9.263, *df* = 2, *p* = 0.010) in females.
395 Specifically, females in the low-fluoxetine treatment were heavier (*z* = 2.995, *p* = 0.008)
396 and longer (*z* = 2.958, *p* = 0.009) than those in the control treatment. There was no
397 difference between unexposed and high-exposed fish (weight: *z* = 1.165, *p* = 0.73;
398 standard length: *z* = 0.8590, *p* = 0.39), or low- and high-exposed fish (weight: *z* = 1.810,
399 *p* = 0.21; standard length: *z* = 2.081, *p* = 0.11). Fluoxetine exposure did not have a
400 significant effect on female scaled mass (KWT: χ^2 = 5.539, *df* = 2, *p* = 0.063).

401

402 **Discussion**

403 Contrary to predictions, we did not find an interaction between fluoxetine exposure and
404 temperature stress on guppy reproductive behaviour. We did, however, find that both
405 stressors generated independent effects on reproductive behaviour. Specifically, for
406 fluoxetine, long-term (15-month) exposure resulted in an increase in male coercive
407 mating behaviour (i.e. sneak copulations) in both the low (38 ng/L) and high (312 ng/L)
408 treatments, but did not alter courtship behaviour. With regard to the effect of
409 temperature on reproductive behaviours, we found that acute (24 h) cold stress (i.e. 18
410 °C) resulted in reduced courtship by males, as well as a delay in the time taken to first
411 perform a coercive mating attempt. Activity levels, in turn, were not affected by the
412 interaction between fluoxetine exposure and temperature stress, or by fluoxetine

413 exposure independently. There was, however, an independent effect of temperature
414 stress on activity levels, with cold stress causing a reduction in activity in both males
415 and females.

416 To date, we know of only one other study addressing impacts of fluoxetine exposure
417 under varying temperature conditions. In contrast to the results of the present study,
418 Barbosa et al.[42] reported that chronic (fourth-generation) fluoxetine exposure interacted
419 synergistically with temperature variability to impair reproductive success in water fleas
420 (*Daphnia magna*). However, that study examined temperature variability and rate of
421 reproduction, rather than acute temperature stress and reproductive behaviour, which
422 may explain the disparity in results. We also cannot rule out differences in species-
423 specific sensitivities to fluoxetine or temperature stress.[30,52] In this regard, it is worth
424 noting that previous research in guppies has also failed to find interactions between
425 fluoxetine (61 ng/L and 350 ng/L for 28 days) and another common environmental
426 stressor, predation risk.[43] Yet, it is important to highlight that two stressors inducing
427 independent effects can nevertheless be detrimental, if an individual is exposed to both
428 stressors simultaneously.

429 For fluoxetine, our study shows that, irrespective of temperature, environmentally
430 realistic exposure levels (i.e. 38 ng/L and 312 ng/L) can disrupt reproductive behaviours
431 in fish, with exposed males increasing their use of a coercive mating strategy. In this
432 regard, it is important to note that effects of fluoxetine on reproductive traits can vary
433 between species. For instance, fluoxetine has been shown to increase nest defence
434 behaviours in fathead minnows (*Pimephales promelas*; 1000 ng/L for 28 days),[70] reduce
435 courtship displays in starlings (*Sturnus vulgaris*; 2700 ng/day for 28 weeks),[71] and

436 increase copulatory behaviours in livebearing fish (479 ng/L for 30 days;[25] 350 ng/L for
437 28 days;[43] 31 ng/L for 35 days[28]), while shorter-term exposure has been shown to have
438 no effect on reproductive behaviours in Siamese fighting fish (*Betta splendens;* 540 ng/L
439 for 5 h).[26] The mechanisms by which selective serotonin reuptake inhibitors alter
440 reproductive traits are not fully understood.[23,72,] One possible explanation is that
441 fluoxetine can influence circulating levels of hormones via the hypothalamic–pituitary–
442 gonadal axis by affecting the retention of serotonin and, more generally, the
443 serotonergic system.[23,73] For example, in fish, increases in extracellular serotonin can
444 stimulate the release of gonadotropin-releasing hormones, gonadotropic hormones, and
445 androgens. Such hormonal changes can, in turn, alter levels of sexual motivation[74] and
446 potentially modify how attractive individuals are to the opposite sex by, for example,
447 altering chemical and visual cues of sexual fitness in males and females.[75] However,
448 pinpointing precise physiological factors and hormones affected by fluoxetine is
449 challenging. For example, female starlings exposed to fluoxetine were courted less by
450 males than were unexposed females, but no differences in body condition or levels of
451 circulating testosterone or oestradiol (sex hormones) were observed between females
452 from different exposure treatments (2700 ng/day for 28 weeks).[71] Further research
453 targeted at identifying what physiological changes underpin observed effects of
454 fluoxetine on reproductive behaviours would be valuable in understanding differences
455 between species, and in determining which species may be particularly susceptible to
456 fluoxetine-mediated alterations to reproductive processes.

457 While the physiological processes underpinning fluoxetine's effects on reproductive
458 traits remain unclear, this study has nonetheless shown that long-term exposure to

fluoxetine generates an increase in coercive reproductive behaviour in male guppies. The relative shift towards this unsolicited sneaking strategy over cooperative mating (i.e. male courtship) could impair fitness by, for example, reducing the ability of females to exercise mate choice. Female mate choice plays an important role in reproduction and, when females are unable to select males displaying indicators of high fitness to mate with, the quality and quantity of offspring may be impacted.[13] Additionally, increases in male sneaking behaviour often result in females spending more time actively avoiding males, with consequences for female fitness even in a non-reproductive context. For example, female guppies will alter their habitat use to areas where predation risk is high to avoid sexual harassment by males,[76] and suffer reduced foraging opportunities when targeted by male sneaking behaviours.[77] Female avoidance tactics further impair male fitness by reducing interaction between the sexes and mating opportunities for males. Moreover, sneak copulations confer a lower insemination efficiency compared to post-courtship copulations.[56] Hence, although fluoxetine exposure increased the number of male copulation attempts performed, it may actually reduce overall male fitness.

Because effects of fluoxetine may be dependent on exposure duration, we employed a long-term 15-month experiment to identify effects of chronic exposure on reproductive traits. Recently, a shorter-term experiment on guppies found that 28 days of fluoxetine exposure at 350 ng/L caused males to perform more frequent sneaking behaviour than unexposed fish, but this effect was not seen in males exposed at the lower concentration of 61 ng/L.[43] The latter finding contrasts with our results in that 15-month exposure to 38 ng/L of fluoxetine did increase sneaking behaviour in the present study. Given that both studies were conducted in a similar fashion and on the same species,

482 we contend that exposure duration is the most likely explanation for the different results
483 observed, with male guppy reproductive behaviour being relatively more vulnerable to
484 disruption by longer-term fluoxetine exposure. Our study, therefore, provides new
485 evidence for time-dependent effects of fluoxetine exposure on behavioural traits, and
486 underscores the importance of longer-term studies for understanding impacts of
487 environmentally realistic pharmaceutical contamination. In this regard, it is important to
488 note that this study was specifically designed to simulate a realistic exposure scenario,
489 with up to four overlapping generations exposed and allowed to interact, as is reflective
490 of natural populations. However, future studies investigating long-term effects of
491 pharmaceutical exposure and disentangling plastic versus genetic responses to
492 contamination will also be valuable.

493 In contrast to reproduction, fluoxetine exposure had no effect on activity levels in male
494 or female guppies. This is consistent with studies in zebrafish (100 000 ng/L for 2
495 weeks),[78] killifish (*Aphanius dispar*; 300 ng/L for 7 days),[46] and mosquitofish (*Gambusia*
496 *holbrooki*; 31 and 374 ng/L for 35 days),[28] in which no impacts of fluoxetine on activity
497 were observed. In contrast, activity levels increased in mosquitofish after 28 days of
498 exposure to a low level of fluoxetine (25 ng/L), although no change in activity was seen
499 at a higher dosage (226 ng/L).[79] These studies highlight the potential for fluoxetine to
500 induce non-monotonic effects (i.e. where the slope of a dose–response curve changes
501 direction within the range of tested doses) and generate contrasting results depending
502 on exposure concentration and duration, and the species tested. While fluoxetine was
503 not found to affect activity in the present study, it is important to emphasise that activity
504 is just one aspect of spatial use. In particular, Egan et al.[78] found that fluoxetine-

505 exposed zebrafish were quicker to enter the top half of a trial tank and spent more time
506 in the upper portion of the water column. Fish that spend more time near the water's
507 surface are more vulnerable to aerial predators[80] and, therefore, exposure to fluoxetine
508 may increase vulnerability to predation. These potentially costly alterations to behaviour
509 would not have been identified if only activity levels had been measured, suggesting
510 that future research may benefit from investigating how other aspects of swimming
511 performance, movement, and spatial use respond to fluoxetine exposure.

512 Regarding independent effects of temperature, we found that cold stress leads to a
513 reduction in reproductive-related traits in fish, whereas elevated temperatures did not
514 affect these traits. Reproductive processes are sensitive to temperature and are often
515 impaired when temperature falls outside of an organism's optimal range.[81,82] For
516 example, courting behaviours are lower in guppies exposed to temperature
517 decreases,[44] and reproductive performance is hindered in female pejerrey (*Odontesthes*
518 *bonariensis*) under heat stress.[83] It may, therefore, seem counterintuitive that the heat-
519 stress treatment employed in our experiment did not alter reproductive behaviour.
520 However, this may be because the temperature used was not sufficiently high to induce
521 a notable stress response in guppies.

522 Guppies under low-temperature stress showed reduced reproductive behaviours
523 relative to other treatments. Such temperature stress in aquatic organisms can generate
524 responses including changes to behaviour, metabolic rate, and the expression of heat
525 shock proteins.[44,84] Under acute stress, these changes are usually temporary and are
526 reversed when ambient temperature returns to normal.[84] The temperature
527 manipulations used in this study were acute, indicating that the reproduction-related

528 behavioural changes observed may not persist once temperatures return to the long-
529 term average of 24 °C. Future research may, therefore, benefit from investigating
530 whether such temperature-mediated short-term adjustments in mating behaviours have
531 long-term impacts on the reproductive success of individuals and populations.

532 In line with our predictions, fish exposed to cold-temperature stress were significantly
533 less active than those undergoing heat stress or maintained at an intermediate control
534 temperature, irrespective of fluoxetine treatment. Temperature variation results in shifts
535 to metabolic rate in aquatic ectotherms, and behavioural changes, such as adjusted
536 activity, are a key mechanism used by animals to restore metabolic homeostasis.[85]
537 Swimming speed increases with temperature, meaning that fish at low temperatures
538 tend to have lower cruising speeds,[86] which is consistent with the results of the current
539 experiment. This reduction in swimming speed may have fitness consequences
540 because fish that are slower when encountering predators are less likely to escape
541 and/or survive.[87]

542 The 15-month fluoxetine exposure resulted in sex-specific, non-monotonic changes to
543 fish morphology, with females in the low-fluoxetine treatment being heavier and longer
544 than unexposed fish. This contrasts with research on goldfish (*Carassius auratus*), in
545 which exposure resulted in decreased weight gain (540 000 ng/L for 28 days),[88] and
546 juvenile guppies, which had reduced weight and standard length under fluoxetine
547 exposure (30 and 500 ng/L for 35 days).[89] It is worth noting that fluoxetine has
548 previously been reported to alter foraging dynamics in mosquitofish (215 ng/L) following
549 a 28-day exposure, while no associated changes in morphological traits (i.e. weight,
550 body length and body condition) were detected.[90] It is important to point out, however,

551 that the earlier studies on goldfish, guppies and mosquitofish employed relatively short-
552 term exposures. Further research into the mechanisms causing morphological changes
553 in long-term fluoxetine exposure would be valuable in identifying why the observed non-
554 monotonic, sex-specific differences arise. It is also important to highlight that fluoxetine-
555 induced non-monotonic effects have previously been reported in a wide range of
556 species, especially in the context of behavioural traits.[79,91–97] The mechanism(s) driving
557 these types of fluoxetine-induced non-monotonic effects is/are not yet fully understood.
558 However, a number of mechanisms that are known to drive other non-monotonic
559 effects[98] have the potential to apply to fluoxetine.

560 In summary, we found no interaction between chronic exposure to the pervasive
561 pharmaceutical contaminant fluoxetine and acute temperature stress on reproductive
562 behaviour or activity levels in guppies. However, long-term (15-month) exposure to
563 fluoxetine led to an increase in the frequency of coercive sneak copulations carried out
564 by male guppies at both of the environmentally realistic dosages tested, while male
565 courtship behaviour, and activity levels in both sexes, were not affected. Regarding
566 effects of temperature, males exposed to acute (24 h) cold stress were slower to first
567 perform a coercive copulation (relative to males in the heat-stress treatment), performed
568 fewer such copulations, and were less likely to perform courtship behaviour. In addition,
569 cold-temperature stress was associated with reduced activity levels in both males and
570 females. In combination, our findings demonstrate complex independent effects of
571 multiple stressors on ecologically important behavioural processes in fish. Despite a
572 growing appreciation of the importance of a multi-stressor approach, there remains a
573 dearth of knowledge on this topic, particularly for novel stressors like pharmaceutical

574 pollutants, as well as the direct and indirect effects they can generate. Such studies are
575 clearly necessary, however, if we are to gain a more holistic understanding of the
576 potential impacts of pharmaceutical contaminants on wildlife populations around the
577 globe.

578

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589

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