Supplementary Material

Psychoactive pollution suppresses individual differences in fish behaviour

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Methods

Mesocosm populations

Population surveys conducted during the study—in 2018 and 2019—determined that fish densities were comparable across the exposure treatments (mean ± s.e.: 79 ± 14, 97 ± 17, and 74 ± 14, for unexposed, low fluoxetine, and high fluoxetine, respectively; $df_{2,23}$, ANOVA: $p = 0.562$).

Fluoxetine dosing and analytical verification of treatment levels

Nominal fluoxetine concentrations in mesocosm tanks were maintained via static renewal. For both fluoxetine treatments (low and high), mesocosm tanks were dosed twice weekly (as described in detail in [1,2]). Briefly, this involved preparing two separate stock solutions with either 2 mg or 20 mg of fluoxetine hydrochloride (Sigma Aldrich; product number: F132, CAS: 56296-78-7) dissolved in 100 mL of methanol, for the low and high treatments, respectively. Stock solutions were diluted to produce dosing solutions: 1 mL aliquot of stock solution (low or high) was diluted with 1000 mL of reverse osmosis water. Further, to control for any potential solvent effects, as well as to maintain consistent levels of handling across all mesocosm tanks, a solvent solution (1 mL of methanol in 1000 mL of reverse osmosis water) was also added to all unexposed tanks twice per week. Therefore, all 12 mesocosm tanks received 2 mL of methanol weekly (0.0006% methanol by mesocosm water volume).

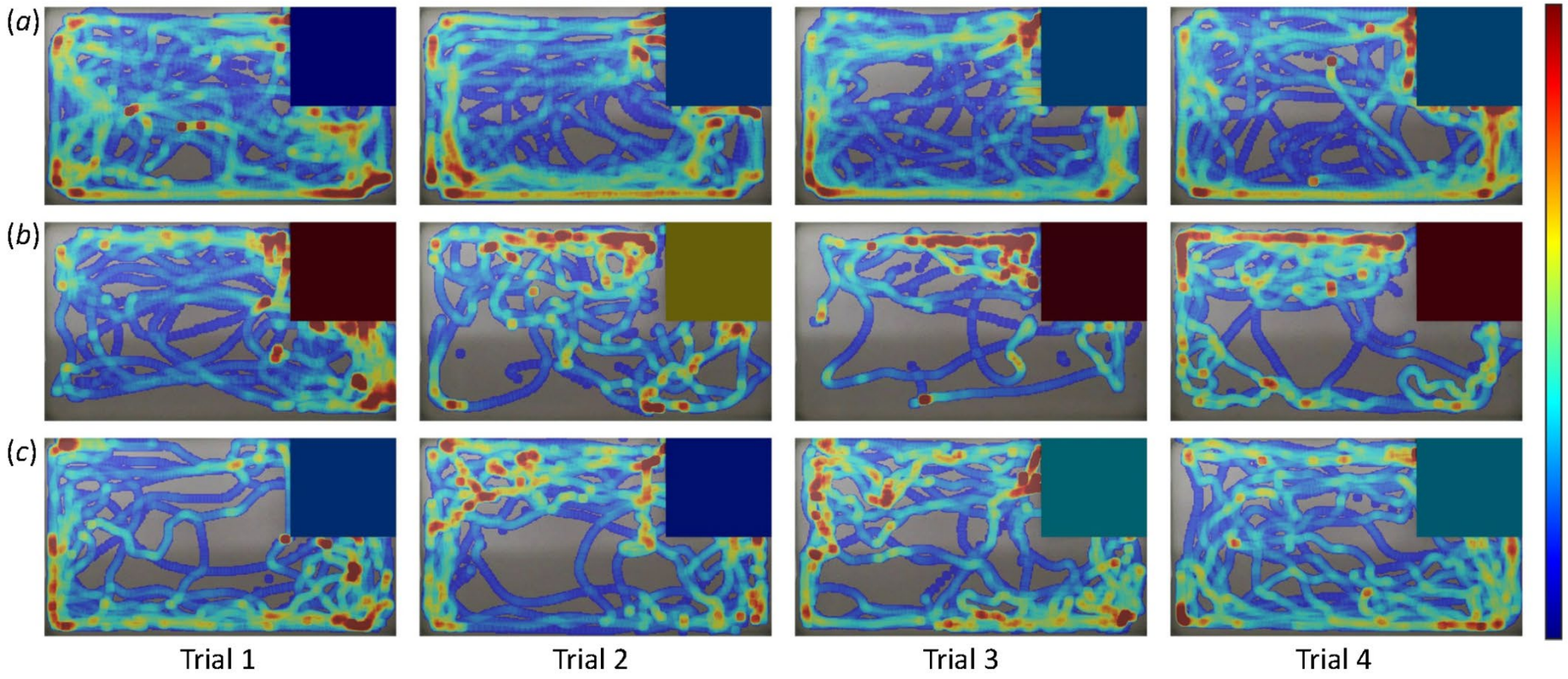
To verify concentrations of fluoxetine and norfluoxetine—fluoxetine's primary metabolite—over the two-year exposure period, water samples (40 mL) were drawn once per month from all mesocosm tanks in the low and high treatments. To ensure the absence of fluoxetine contamination in control tanks (unexposed treatment), water samples were also collected from these mesocosms every two months throughout the study. Water samples were stored at 4 °C in the dark and analysed with gas chromatography–tandem mass spectrometry (7000C Triple Quadrupole GC-MS/MS, Agilent Technologies, Delaware, USA; lower quantification limit: 2 ng l^{-1}). Water analysis was performed by Envirolab Services (MPL Laboratories; NATA accreditation: 2901; accredited for compliance with

ISO/IEC: 17025), within four days of collection. A detailed description of the water analysis protocol is provided in [3].

Chemical analyses

Mesocosm tanks from the same exposure treatment did not differ significantly in fluoxetine concentration throughout the study (mean ± s.e.: 40 ± 3 ng l^{-1} and 366 ± 28 ng l^{-1}; ANOVA: $p = 0.415$, $df_{3,89}$ and $p = 0.817$, $df_{3,89}$ for low and high fluoxetine, respectively). Nevertheless, we have accounted for the small variation in fluoxetine levels in our analyses, confirming that differences across mesocosm tanks had no effect on fish behavioural and size variation (Tables S3 and S4). Small variation in fluoxetine concentrations are likely the result of the scale and ecological realism of the mesocosm system, with numerous adult fish exposed in large tanks with gravel substrate and natural vegetation, to which fluoxetine can readily sorb [4,5]. Water analysis of control (unexposed) mesocosm tanks indicated no fluoxetine contamination.

Figure S1: Snapshots of the open-field arenas with examples of activity level and refuge use in one individual from each of the (a) unexposed, (b) low-fluoxetine, and (c) high-fluoxetine treatments, respectively, over the four trials. In a trial, a fish was allowed to freely explore the arena for 20 minutes, where we measured its activity level (distance moved in cm) and risk-taking (use of the refuge in seconds, and consequently not exploring open spaces that are unfamiliar and potentially dangerous): the arena was white, while the refuge—top-right corner—was black. Shown are the tracking outputs produced by EthoVision for each of the four trials, and heat maps generated by MATLAB: red indicates high and blue indicates low traffic.



Results

Table S1: Comparison of LMMs with different random (co)variance structures for activity (distance moved) and risk-taking (refuge use). We compared two models with both random intercepts (ID) and slopes (treatment, class, or trial) across individuals against a reduced model with only random intercepts. We also compared the model with random intercepts with a model in which random intercepts were excluded. Significant improvements in the model fit are tested with both Akaike information criteria (ΔAIC) and likelihood-ratio tests (*p*). Treatment (unexposed, low fluoxetine, high fluoxetine), class (juveniles, males, females), mesocosm population (four per treatment), trial (four repeated measures per individual), and time of day are included as fixed effects in all models. Significance was set at $\alpha < 0.05$ and significant results are in bold; the model with random intercepts and treatment as the random slope was best supported as the most parsimonious model for activity, while only random intercepts but not random slopes explained a significant portion of the variance in risk-taking. The random structure is written in the syntax of the *lme4 R* package.

Model	χ_1^2	ΔAIC	*p*
Activity: distance moved			
(1|ID)	118.040	116.04	**< 0.001**
(treatment|ID)	5.998	2.000	**0.049**
(class|ID)	4.814	< 0.001	0.090
(trial|ID)	1.424	2.600	0.491
Risk-taking: refuge use			
(1|ID)	45.081	43.081	**< 0.001**
(treatment|ID)	1.080	2.900	0.583
(class|ID)	0.323	3.700	0.851
(trial|ID)	3.353	0.700	0.187

Table S2: Changes in variance components between and within individuals, and in the overall measure of repeatability for activity (distance moved) and risk-taking (refuge use) across treatments (unexposed, low fluoxetine, high fluoxetine) with a categorical variable as a predictor. Medians (± s.e.) are indicated for the intercepts and slopes of the regression lines. Significance was set at $\alpha < 0.05$ and significant results are in bold. Because of the nature of the risk-taking variable, high values represent low risk-taking.

Model	Intercept ± s.e.	Slope ± s.e.	*p*
Activity: distance moved			
$V_{between}$	343 403.9 ± 880.327	–198 779 ± 1 050.181	**0.033**
V_{within}	287 853.6 ± 316.534	64 955.07 ± 524.901	0.204
Repeatability	0.502 ± 0.001	–0.242 ± 0.001	**0.021**
Risk-taking: refuge use			
$V_{between}$	3.937 ± 0.023	3.096 ± 0.038	0.381
V_{within}	22.405 ± 0.026	1.714 ± 0.038	0.643
Repeatability	0.139 ± 0.001	0.068 ± 0.001	0.503

Table S3: Pairwise comparisons between variance components (within and between individuals) and repeatability estimates for activity (distance moved) and risk-taking (refuge use) across treatments (unexposed, low fluoxetine, high fluoxetine). Mean estimates and their 95% credible intervals are represented, and significant results (bold) correspond to estimated differences whose credible intervals do not overlap with zero.

Models	Pairwise comparisons	Estimate	95% CIs
Activity: distance moved			
$V_{between}$	Unexposed-Low	**172 168.100**	**12 196.090, 429 603.500**
	Unexposed-High	**233 493.500**	**38 073.480, 432 233.400**
	Low-High	–537.279	–130 613.300, 164 493
V_{within}	Unexposed-Low	–61 931.780	–166 754.400, 39 138.950
	Unexposed-High	–59 393.510	–167 617.200, 32 214.990
	Low-High	10 665.240	–109 928.300, 112 270
Repeatability	Unexposed-Low	**0.256**	**0.038, 0.442**
	Unexposed-High	**0.279**	**0.068, 0.447**
	Low-High	0.009	–0.170, 0.232
Risk-taking: refuge use			
$V_{between}$	Unexposed-Low	–2.681	–10.650, 4.539
	Unexposed-High	–5.280	–12.814, 2.638
	Low-High	–0.181	–10.425, 6.890
V_{within}	Unexposed-Low	–1.756	–9.229, 5.535
	Unexposed-High	2.278	–4.803, 8.573
	Low-High	3.554	–3.109, 10.870
Repeatability	Unexposed-Low	–0.059	–0.266, 0.132
	Unexposed-High	–0.188	–0.339, 0.064
	Low-High	–0.070	–0.285, 0.124

Table S4: Correlation estimates (phenotypic, and between and within individuals) for activity (distance moved) and risk-taking (refuge use) across treatments (unexposed, low fluoxetine, high fluoxetine). The best estimate of correlation coefficients and their 95% credible intervals are represented for each treatment. Significant results (bold) correspond to correlation coefficients whose confidence intervals do not overlap with zero. Because of the nature of the risk-taking variable, high values represent low risk-taking.

Correlations	Unexposed	Low fluoxetine	High fluoxetine
Phenotypic	–0.027 (–0.163, 0.117)	–0.106 (–0.240, 0.039)	–0.0168 (–0.163, 0.131)
Between individual	–0.029 (–0.999, 0.891)	**–0.482** (**–0.999, –0.076**)	–0.171 (–0.695, 0.335)
Within individual	–0.032 (–0.187, 0.116)	0.024 (–0.139, 0.172)	0.038 (–0.116, 0.186)

Figure S2: Pairwise differences in activity (a; distance moved), risk-taking (b; refuge use), and (c) body size of fish across treatments (unexposed, low fluoxetine, high fluoxetine). Estimated marginal means (EMMs + SE) account for the contribution of the other fixed and random effects included in each model: class (juveniles, males, females), mesocosm population (four per treatment), trial (four repeated measures per individual), time of day, and random intercepts (individual ID) for activity and risk-taking; and class and random intercepts (mesocosm population ID) for body size. We ran pairwise comparisons with the conservative Bonferroni method. Significance was set at $\alpha < 0.05$, and means not sharing a common superscript are significantly different. Because of the nature of the risk-taking variable, high values represent low risk-taking.

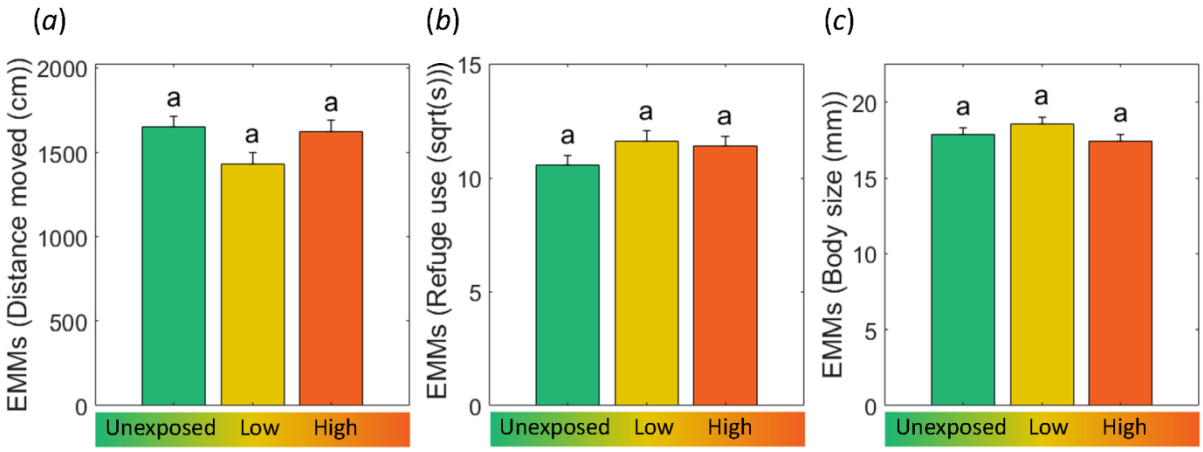


Table S5: Results from the fixed factor structure of LMMs with activity (distance moved) and risk-taking (refuge use) for each treatment as dependent variables. Class (juveniles, males, females), mesocosm population (four per treatment), trial (four repeated measures per individual), and time of day are included in all models as fixed effects. Random intercepts are included for each individual, which allowed variance partitioning. Analysis of variance was performed with Satterthwaite's method. Significance was set at $\alpha < 0.05$ and significant results are in bold. Because of the nature of the risk-taking variable, high values represent low risk-taking.

Model	Fixed effects	Mean sq.	Num_{df}, Den_{df}	*F*	*p*
Activity: distance moved					
Unexposed	Class	50 218	2, 60	0.178	0.837
	Mesocosm	501 628	2, 60	1.781	0.177
	Trial	1 615 281	3, 178	5.734	**< 0.001**
	Time of day	107 661	4, 60	0.382	0.820
Low fluoxetine	Class	1 273 415	2, 53	3.701	**0.031**
	Mesocosm	452 969	2, 53	1.316	0.277
	Trial	1 205 325	3, 166	3.503	**0.017**
	Time of day	183 465	4, 53	0.533	0.712
High fluoxetine	Class	1 194 583	2, 58	3.456	**0.038**
	Mesocosm	919 365	2, 58	2.659	0.078
	Trial	1 448 457	3, 174	4.190	**0.007**
	Time of day	633 969	4, 58	1.834	0.135
Risk-taking: refuge use					
Unexposed	Class	23.768	2, 58	1.102	0.339
	Mesocosm	60.610	2, 58	2.811	0.068
	Trial	80.213	3, 176	3.720	**0.013**
	Time of day	5.072	4, 58	0.235	0.917
Low fluoxetine	Class	45.796	2, 59	1.954	0.151
	Mesocosm	25.005	2, 58	1.067	0.351
	Trial	15.686	3, 173	0.670	0.572
	Time of day	49.527	4, 59	2.114	0.090
High fluoxetine	Class	14.180	2, 57	0.715	0.494
	Mesocosm	8.048	2, 58	0.406	0.668
	Trial	46.706	3, 174	2.354	0.074
	Time of day	7.063	4, 58	0.356	0.839

Table S6: Results from the LMM with body size as the dependent variable. Treatment (unexposed, low fluoxetine, high fluoxetine), class (juveniles, males, females), and their interaction are included as fixed effects. Random intercepts are also included for each mesocosm, which allowed accounting for repeated measures and variance partitioning: intercepts ($V_{between}$), residuals (V_{within}), and repeatability. Test statistics (χ_1^2) and significance levels of the random effects (intercepts) were estimated using LRTs (*p*) and Akaike information criteria (ΔAIC) between the LM and LMM. Analysis of variance was performed with Satterthwaite's method. Significance was set at $\alpha < 0.05$ and significant results are in bold; body size increased on average from juveniles to adult females as is typical in this species (estimate ± s.e.: 9.619 ± 0.499, 5.110 ± 0.496, and 4.510 ± 0.499; df_{171}; $p < 0.001$, $p < 0.001$, and $p < 0.001$; for females–juveniles, females–males, and males–juveniles, respectively), irrespective of treatment and mesocosm population.

Body size

Fixed effects	Mean sq.	*df*	*F*	*p*
Treatment	12.150	2, 9	1.646	0.246
Class	1376.310	2, 171	186.416	**< 0.001**
Treatment × class	3.570	4, 171	0.483	0.748
Random effects	**Estimate**	χ_1^2	**ΔAIC**	***p***
$V_{between}$	0.236	2.856	0.856	0.091
V_{within}	7.383	-	-	-
Repeatability	0.031	-	-	-

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