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Chronic exposure to a pervasive pharmaceutical pollutant erodes among-individual phenotypic variation in a fish☆

Hung Tan [a, *], Giovanni Polverino [b], Jake M. Martin [a], Michael G. Bertram [a, c], Sarah C. Wiles [a], Maria M. Palacios [d], Candice L. Bywater [a], Craig R. White [a, e], Bob B.M. Wong [a]

[a] School of Biological Sciences, Monash University, Melbourne, Australia
[b] Centre for Evolutionary Biology, School of Biological Sciences, University of Western Australia, Perth, Australia
[c] Department of Wildlife, Fish, and Environmental Studies, Swedish University of Agricultural Sciences, Umeå, Sweden
[d] School of Life and Environmental Sciences, Deakin University, Melbourne, Australia
[e] Centre for Geometric Biology, Monash University, Melbourne, Australia

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ABSTRACT

Pharmaceutical pollution is now recognised as a major emerging agent of global change. Increasingly, pharmaceutical pollutants are documented to disrupt ecologically important physiological and behavioural traits in exposed wildlife. However, little is known about potential impacts of pharmaceutical exposure on among-individual variation in these traits, despite phenotypic diversity being critical for population resilience to environmental change. Furthermore, although wildlife commonly experience multiple stressors contemporaneously, potential interactive effects between pharmaceuticals and biological stressors—such as predation threat—remain poorly understood. To redress this, we investigated the impacts of long-term exposure to the pervasive pharmaceutical pollutant fluoxetine (Prozac®) on among-individual variation in metabolic and behavioural traits, and the combined impacts of fluoxetine exposure and predation threat on mean metabolic and behavioural traits in a freshwater fish, the guppy (*Poecilia reticulata*). Using a mesocosm system, guppy populations were exposed for 15 months to one of two field-realistic levels of fluoxetine (nominal concentrations: 30 and 300 ng/L) or a solvent control. Fish from these populations were then tested for metabolic rate (oxygen uptake) and behaviour (activity), both before and after experiencing one of three levels of a predation treatment: an empty tank, a non-predatory fish (*Melanotaenia splendida*) or a predatory fish (*Leiopotherapon unicolor*). Guppies from both fluoxetine treatments had ~70% lower among-individual variation in their activity levels, compared to unexposed fish. Similarly, fluoxetine exposure at the higher dosage was associated with a significant (26%) reduction in individual-level variation in oxygen uptake relative to unexposed fish. In addition, mean baseline metabolic rate was disrupted in low-fluoxetine exposed fish, although mean metabolic and behavioural responses to predation threat were not affected. Overall, our study demonstrates that long-term exposure to a pervasive pharmaceutical pollutant alters ecologically relevant traits in fish and erodes among-individual variability, which may be detrimental to the stability of contaminated populations globally.

1. Introduction

Pharmaceutical pollution is now recognised as a major emerging threat to human and wildlife populations globally (Arnold et al., 2014; Boxall et al., 2012; Hughes et al., 2013; Küster and Adler, 2014). To date, over 600 pharmaceuticals have been detected in the environment, across all continents (aus der Beek et al., 2016; Bergmann et al., 2011; Boxall et al., 2012; Küster and Adler, 2014). Moreover, due to projected increases in pharmaceutical production and consumption, the quantity and diversity of pharmaceuticals in the environment are expected to increase

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* Corresponding author. School of Biological Sciences, Monash University, Melbourne, Victoria, 3800, Australia.
E-mail address: hung.tan@monash.edu (H. Tan).

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(Aitken and Kleinrock, 2015; Arnold et al., 2014). Antidepressants are among the most commonly prescribed pharmaceuticals and are frequently detected in natural environments around the world (González Alonso et al., 2010; Schlusener et al., 2015; Schultz et al., 2010). Environmental contamination by antidepressants is, therefore, cause for major concern.

One antidepressant pollutant that has been attracting increased attention from the scientific community is fluoxetine (Prozac®), a widely prescribed selective serotonin reuptake inhibitor (SSRI). As for many pharmaceuticals, waste water effluent is the primary pathway for fluoxetine to enter the environment (Schultz et al., 2010), and it has now been detected in aquatic environments worldwide, with concentrations in surface waters typically ranging from <1 to 350 ng/L (e.g. Blair et al., 2013; Fernández et al., 2010; Glassmeyer et al., 2005; Hughes et al., 2013; Kolpin et al., 2002; Metcalfe et al., 2010; Metcalfe et al., 2003; Mole and Brooks, 2019; Nödler et al., 2014; Yoon et al., 2010). Moreover, fluoxetine has been shown to bioaccumulate in tissues of exposed wildlife such as fish (e.g. Arnnok et al., 2017; Brooks et al., 2005; David et al., 2018; Martin et al., 2019b; Muir et al., 2017; Paterson and Metcalfe, 2008).

Given that some wild animals can accumulate and metabolise fluoxetine, they may be affected by its neuroendocrine-disrupting properties (Kreke and Dietrich, 2008; Mennigen et al., 2011). Indeed, fluoxetine has been found to induce both behavioural and physiological effects in many non-target species, as the serotonergic system, upon which fluoxetine acts, is highly conserved across vertebrate clades (Fong, 2001; Kreke and Dietrich, 2008; Lillesaar, 2011). For example, fluoxetine exposure can alter many behaviours, including activity (Barry, 2013; Chiffre et al., 2016; Clements and Schreck, 2007), aggression (Dzieweczynski and Hebert, 2012; Fairbanks et al., 2001; McCallum et al., 2017), foraging (Gaworecki and Klaine, 2008; Martin et al., 2019c), antipredator responses (Martin et al., 2017; Saaristo et al., 2017), and reproductive behaviour (Bertram et al., 2018a; Fursdon et al., 2019; Martin et al., 2019a). Fluoxetine exposure can also have adverse physiological effects, including altered neurodevelopmental (Bidel et al., 2016; Foster et al., 2010; Ko et al., 2014) and reproductive physiology (Campos et al., 2016; Lister et al., 2009; Mennigen et al., 2008). However, an important physiological trait that has received considerably less attention with regard to fluoxetine exposure is metabolic rate (but see Campos et al., 2013; Hird et al., 2016). This is surprising considering that the serotonergic system plays a key role in satiety signalling and energy expenditure (Lam and Heisler, 2007). Furthermore, energy metabolism is responsible for all biological activities and is closely associated with other behavioural and physiological traits of ecological importance (Auer et al., 2017; Mathot et al., 2019).

While efforts aimed at understanding the consequences of fluoxetine exposure on average metabolic and behavioural traits of animals are increasing, far less is known about its potential effects on phenotypic variation (both among and within individuals), as is also true for pharmaceutical pollutants more generally. This is surprising because phenotypic variability is essential for the stability and adaptive potential of animal populations in the face of environmental change (Allendorf et al., 2008; Anderson et al., 2008; Hilborn et al., 2003; Seebacher et al., 2014). For example, a decrease in phenotypic variability as a result of human-induced environmental change has the potential to undermine the stability of population dynamics, resulting in systematic population declines (Allendorf et al., 2008; Hilborn et al., 2003; Olsen et al., 2009). Antidepressants are designed to stabilise serotonin imbalances within the body (Kreke and Dietrich, 2008) and, as such, may be expected to reduce the natural variation in behavioural and physiological traits of animals that is typically observed in the wild. Impairments of the serotonergic system due to fluoxetine exposure may, therefore, force individuals from a given population to exhibit homogeneous physiological and behavioural responses. However, to date, no studies have explored possible effects of fluoxetine on individual-level variation in metabolic rate and behaviour.

In the wild, animals exposed to fluoxetine would also likely be exposed concurrently to other biological stressors, such as risk of predation. When animals are exposed to predators, their metabolic rate is often altered as certain physiological and behavioural processes are prioritised to guarantee survival (Alton et al., 2012; Killen et al., 2013; Thaler et al., 2012). For example, predation risk was found to select for low resting metabolic rates in mealworm beetles (*Tenebrio molitor*), as it was negatively correlated with total duration of immobility, thus reducing the likelihood of individuals being exposed to predation (Krams et al., 2013). In addition, acute increases in metabolic rate via elevated respiration and ventilation (i.e. fight or flight response) following exposure to predation risk have been widely documented in various species, such as within invertebrates, fish, reptiles, birds and mammals (see Hawlena and Schmitz, 2010). Given that fluoxetine has been shown to disrupt antipredator behaviours (Barry, 2014; Martin et al., 2017), and that there is an intimate link between metabolic rate and predation threat (Killen et al., 2013), understanding fluoxetine-induced shifts in metabolic rate may be valuable in revealing how physiological function is altered in fluoxetine-exposed animals under predation stress.

In the present study, we investigated whether long-term exposure to environmentally relevant concentrations of fluoxetine altered individual-level variation in metabolic and behavioural traits, and whether there were interactive effects of fluoxetine exposure and predation risk on mean metabolic and behavioural traits and responses to predation in guppies (*Poecilia reticulata*). First, we hypothesised that fluoxetine-exposed fish would exhibit lower among-individual variation in terms of both metabolic rate and behaviour. We also considered that chronic exposure to fluoxetine would result in a general decrease in the metabolic rate and activity levels of guppies. Moreover, for fluoxetine-exposed fish, we predicted that oxygen uptake and activity levels would remain stable regardless of the presence or absence of a predator (i.e. a reduced antipredator response).

2. Materials and methods

2.1. Study species

The guppy (*Poecilia reticulata*) was selected as the study species as they are widespread in tropical and subtropical regions of the world due to numerous deliberate and accidental human-mediated introductions (Deacon et al., 2011), and are known to inhabit systems impacted by chemical pollution (Araújo et al., 2009; Widianarko et al., 2000). Furthermore, guppies are a well-established model in behavioural and evolutionary ecology (Houde, 1997), including in terms of behaviours performed under predation risk (e.g. Botham et al., 2008; Godin, 1995; Magurran et al., 1992). Guppies used in this experiment (mean wet weight ± SD: 0.364 ± 0.188 g, $n = 189$) were sourced from a long-term mesocosm system founded by wild-caught individuals (see '*Mesocosm system*' below for details). Adult females were the focus of this experiment as they have been shown to represent more attractive prey than males due to their larger size (Pocklington and Dill, 1995) and are, therefore, more likely to be vulnerable to alterations in physiology and behaviour in the context of predation risk.

Eastern rainbowfish (*Melanotaenia splendida*) and spangled perch (*Leiopotherapon unicolor*) were used as non-predatory and

predatory stimulus fish, respectively, in experimental trials. The diet of the eastern rainbowfish is comprised entirely of small invertebrates and plant material (Davis et al., 2011), whereas the spangled perch is an aggressive omnivore that is known to prey on small fish, including guppies (Davis et al., 2011). Both species overlap in distribution with guppies from the source population used in this study (Allen et al., 2002). The rainbowfish was used to ensure that any shifts in metabolic rate and behaviour in guppies following exposure to the perch was due to perceived predation risk, and not simply due to the presence of a stimulus fish (Michelangeli and Wong, 2014). Stimulus fish were not exposed to fluoxetine to exclude the potential for contaminant-induced effects on their behaviour impacting the behaviour of the focal fish (*sensu* Bertram et al., 2018a; Bertram et al., 2018b; Fursdon et al., 2019; Tomkins et al., 2018; Tomkins et al., 2017). Both perch (mean standard length ± SE: 83.86 ± 3.12 mm, $n = 6$) and rainbowfish (79.30 ± 1.79 mm, $n = 6$) were wild-caught specimens purchased from a commercial supplier (AquaGreen, Darwin).

2.2. Mesocosm system

We conducted a laboratory mesocosm experiment in which populations of guppies were subjected to one of three fluoxetine exposure treatments over 15 months (representing approximately four overlapping generations; Reznick et al., 2001). The foundation population of this mesocosm system—3600 sexually mature wild guppies (50:50 sex ratio)—were collected from Alligator Creek (19°23′50″ S, 146°56′56″ E), Townsville, Australia (collection permit: WITK17685216). This initial foundation population was used to establish the mesocosm system, and this system has since been utilised for a series of experiments, including for this study. Water samples taken from the fish collection site revealed no contamination with fluoxetine (Envirolab Services, see '*Analytical verification of fluoxetine levels*' for details; all samples under the minimum detection limit of 2 ng/L, $n = 5$). The founder fish were transported in aerated containers to Monash University, Melbourne, Australia, and, upon arrival, were randomly distributed amongst 12 stainless steel mesocosm tanks (648 L; 180 × 60 × 60 cm; water depth: 30 cm), with 300 fish introduced into each tank at an equal sex ratio. The mesocosm system was kept under natural light:dark cycles in a temperature-controlled greenhouse facility. Tanks were monitored weekly for temperature (mean = 23.4 °C, SD = 1.0 °C, $n = 720$) and pH (mean = 7.36, range = 5.08–9.67, $n = 720$). Mesocosm tanks contained carbon-filtered fresh water aerated by commercial air pumps (Resun LP100), a 3 cm gravel substrate (7 mm grain size) and aquatic plants (*Taxiphyllum barbieri*) for refuge. Fish were fed *ad libitum* every second day (Aquasonic Nutra Xtreme C1 pellets; 0.8 mm).

The exposure protocol commenced 5 months after the founder population had been introduced into the mesocosm tanks. This involved each of the 12 independent mesocosm populations being randomly assigned to one of three treatments: solvent control (i.e. unexposed; $n = 4$ tanks), low-fluoxetine (nominal concentration: 30 ng/L; $n = 4$), or high-fluoxetine (nominal concentration: 300 ng/L; $n = 4$). These nominal fluoxetine levels were chosen to represent concentrations commonly detected in polluted surface waters (low fluoxetine), and heavily effluent-dominated systems that represent the higher range of environmental detections (high fluoxetine) (reviewed in Mole and Brooks, 2019).

The desired nominal fluoxetine concentrations in each mesocosm tank were maintained via static renewal. This involved stock solutions being created by dissolving 2 mg or 20 mg of fluoxetine hydrochloride (Sigma Aldrich; product number: F132, CAS: 56296-78-7) in 100 mL of methanol, for the low and high exposure treatments, respectively. Twice per week, for all fluoxetine exposure tanks, a 1 mL aliquot of stock solution (low or high) was diluted in 1000 mL of reverse osmosis water. To control for solvent effects and to maintain consistent levels of handling across treatments, twice weekly, a solvent solution (1 mL of methanol in 1000 mL of reverse-osmosis water) was added to all control tanks. Partial water changes (20% volume) were conducted for each tank once per week, prior to dosing.

Guppies used in this investigation were isolated within their respective mesocosm tanks for two weeks before experiments inside cylindrical fine-mesh (4 mm) stainless steel chambers (32 × 35 cm, height × diameter). Sampling fish from these chambers removed the possibility that individuals were unintentionally tested twice. Focal guppies were not fed for 24 h prior to experimentation to ensure a post-absorptive state, whereby energy is derived from breakdown of bodily reserves and not from digested food (Niimi and Beamish, 1974).

A population survey conducted one month after behavioural and physiological experiments (August 2018) showed that adult densities in the twelve mesocosm tanks were similar across exposure treatments (mean ± SD: 78 ± 57, 66 ± 39, and 62 ± 26, for control, low, and high treatments, respectively).

2.3. Analytical verification of fluoxetine levels

Water samples from each of the mesocosm tanks within the low and high exposure treatments, and from half of the tanks in the unexposed treatment (selected at random), were analysed once per month to assess fluoxetine concentrations, and to ensure the absence of contamination in the unexposed tanks. Briefly, water samples (40 mL) were drawn from each tank and analysed by Envirolab Services (MPL Laboratories; NATA accreditation: 2901; accredited for compliance with ISO/IEC: 17025) within 4 days of collection. Analysis was performed using gas chromatography–tandem mass spectrometry (7000C Triple Quadrupole GC-MS/MS, Agilent Technologies, Delaware, USA) with a minimum detection limit of 2 ng/L. A detailed description of this protocol is provided in Bertram et al. (2018a).

2.4. Experimental apparatus

Trials were conducted in 54 L glass aquaria (60 × 30 × 30 cm; water depth: 17 cm). Each experimental tank contained an air stone and a temperature probe (PT100 sensor; PreSens Precision Sensing GmbH), had 1 × 1 cm grid lines dividing the bottom of the tank, and opaque sides to prevent visual disturbances. Within the experimental tank, there was a submerged glass respirometry chamber (100 mL; 56 mm diameter Schott bottle, DWK Life Sciences), which housed the focal guppy during the trial (see Fig. S1 for a diagram of the experimental set-up). In addition, a separate smaller perforated glass tank (25.5 × 16 × 15 cm)—which was either empty, housed a non-predatory rainbowfish, or a predatory perch—was submerged within the experimental tank for the second stage of the experiment (see '*Experimental trials*', stage 2, below). Therefore, the focal guppy was exposed to both visual and chemical cues of the predator or non-predator during the trial. Reverse osmosis water (i.e. filtered water not containing fluoxetine) buffered with a water pH conditioner (goldfish gH conditioner; Aquapics) was used to fill experimental tanks, stimulus fish tanks, and respirometry chambers. Therefore, as with previously published ecotoxicological studies (e.g. Bertram et al., 2018a; Bertram et al., 2018b; Lagesson et al., 2019; Martin et al., 2017; Saaristo et al., 2019; Sundin et al., 2019), fish were not exposed to fluoxetine during experimental trials. The respirometry chambers had three holes in the lid to accommodate inflow and outflow tubing (3 mm diameter) and an

oxygen probe (Oxygen Dipping Probe DP-PSt7; PreSens Precision Sensing GmbH). Oxygen probes were connected to an oxygen meter (OXY-4 trace; PreSens Precision Sensing GmbH) and PreSens Measurement Studio 2 (v2.2.2.943; PreSens Precision Sensing GmbH) was used to record dissolved oxygen levels within the respirometry chamber. Water flow between the experimental tank and the respirometry chamber was driven by a manually controlled external pump (EHEIM universal pump). Following protocols adapted from Palacios et al. (2016) and Svendsen et al. (2016), dissolved oxygen levels within the respirometry chamber were continuously recorded every second while the pump was turned off, after which the chamber was flushed with aerated water from the experimental tank to prevent oxygen levels from reaching hypoxic conditions (i.e. falling below 80%; Svendsen et al., 2016). During the trial, respirometry chambers were video recorded (Panasonic HC-V180) to assess the behaviour of the focal guppy.

2.5. Experimental trials

We employed a 3 × 3 independent measures factorial design, which involved three chronic exposure treatments: a solvent control (i.e. unexposed), low-fluoxetine (nominal concentration: 30 ng/L) or high-fluoxetine (nominal concentration: 300 ng/L), and three acute predation treatments: an empty tank (i.e. no stimulus), a non-predatory stimulus or a predatory stimulus (i.e. each individual guppy from each exposure treatment was only tested under one predation treatment).

Each individual fish was tested over two stages. Stage 1 involved subjecting individual guppies from each of the three exposure treatments (n: unexposed = 63, low fluoxetine = 63, high fluoxetine = 63) to a metabolic test (rate of oxygen uptake) at rest prior to the introduction of a predation stimulus, to determine their resting metabolic rate. Rates of oxygen consumption were measured as a proxy for metabolic rate (Lighton, 2018). Additionally, the behaviour (activity) of each guppy was measured during the metabolic test (see below). Stage 1 of the experimental trial began with a 30 min acclimation of the focal guppy within the respirometry chamber. After acclimation, the respirometry chamber was flushed with water for 3 min. Focal guppies were then left undisturbed in the chambers for 1 h, during which time water oxygen content within the chamber was measured every second, and the guppy's behaviour was video-recorded. Metabolic rate was estimated as the oxygen uptake (mg O_2 kg^{-1} h^{-1}) of the focal guppy during the 1 h closed respirometry phase (adapted from Palacios et al., 2016; Svendsen et al., 2016). Linear least squares (LLS) regression was used to calculate the rate of oxygen uptake during the closed respirometry phase, excluding the first and last 6 min of the hour. Activity was recorded as the number of 1 cm grid squares crossed (Réale et al., 2007). The number of squares crossed between the 20th and 30th minute of the measurement hour was quantified using an event-logging software (BORIS v.7.4.7; Friard et al., 2016). Pilot studies determined that this 10-min period was a close approximation of the general activity level of fish over the full hour.

In stage 2, we tested whether the metabolic rate and activity levels of guppies from stage 1 changed in response to perceived predation risk. At the conclusion of stage 1, the respirometry chamber was flushed again for 3 min and the same focal guppy was presented with either an empty tank control (n: unexposed = 21, low = 21 and high = 21), a non-predatory rainbowfish (n: unexposed = 21, low = 21 and high = 21), or a predatory perch (n: unexposed = 21, low = 21 and high = 21). The procedure for recording the metabolic rate and behaviour of the focal guppy was identical to that of stage 1. Differences in metabolic rate and behaviour between the two stages of the experiment were then analysed to examine potential shifts in metabolism and/or behaviour as a response to perceived predation risk.

After completing the trials, standard length (±0.01 mm) and wet weight (±0.0001 g) were measured for each guppy. The body condition index of guppies was calculated by producing a least-squares regression of the wet weight (g) of all fish against their standard length (mm), with condition index representing the residuals of this regression line. The water in experimental tanks and respirometry chambers was replaced after each trial to remove any chemical cues left by the focal guppy and stimulus fish. To correct for background bacterial respiration, oxygen levels of chambers without guppies in each experimental tank were recorded for 1 h at the start and end of every third day of the experimental period (n = 64; adapted from Palacios et al., 2016).

2.6. Statistical analysis

Linear mixed-effect models (LMMs) were used to analyse individual-level variation and mean guppy oxygen uptake rates (MO_2; mg O_2 kg^{-1} h^{-1}) and activity levels across exposure and predation treatments. We ran separate LMMs with identical predictors (fixed and random structure) in which oxygen uptake (MO_2) and activity were included one-by-one as the dependent variable. In each model, the interaction between exposure treatment and trial stage, exposure treatment and predation treatment, and trial stage and predation treatment were included as fixed effects, while individual identities were included as a random effect (random intercepts). Trial stage accounted for the two separate stages performed in each experimental replicate (stage 1 and 2). The adjusted repeatability (Nakagawa and Schielzeth, 2010) was estimated for each trait by calculating the ratio of among-individual to total phenotypic variance, conditioned on the fixed effects. The importance of the contribution of consistent individual differences (random intercepts) to total phenotypic variance was assessed by determining the significance of the random effect of individual guppy ID using likelihood ratio tests (LRTs), and by comparing Akaike's Information Criterion (AICs) for models with and without the random effect. To disentangle the repeatability of metabolic rate from the repeatability of size, mass was first included as a fixed effect in the initial model with MO_2 as the dependent variable, but was not found to significantly affect MO_2 (t = 0.030, df = 182, p = 0.976), and was henceforth removed from all models. Additionally, the gravidity of female guppies used in experimental trials was visually assessed based on a swollen abdomen and presence of a gravid spot, and this was initially included as a variable during preliminary analyses. However, the gravidity status of guppies did not improve model fits and was subsequently removed from all models.

One of the main goals of our study was to test whether consistent among-individual differences in guppy oxygen uptake (MO_2) and activity, and their group-level plasticity, varied across fish from different exposure treatments. In other words, whether exposure to fluoxetine at low and high concentrations, in comparison to the unexposed treatment, altered the behavioural and metabolic responses of fish at both the individual and group levels. To accomplish this, we ran LMMs for oxygen uptake and activity for each exposure treatment separately. In each model, mesocosm, body condition index, and the interaction between trial stage and predation treatment were included as fixed effects, while the identities of focal individuals (guppy) and stimulus fish (eastern rainbowfish and spangled perch) were included as the two random effects (random intercepts). Adjusted repeatability and the significance of individual differences (i.e. random intercepts) were tested as above.

Data were analysed in R version 3.4.3 and LMMs were

performed using the *lme4* and *nlme* packages (Bates et al., 2015; Pinheiro et al., 2019, respectively). Where appropriate, data were checked for normality and homogeneity of variance (visual inspection of standard diagnostic plots). For all models, the significance of fixed effects was calculated from the *F*-statistic with the *lmerTest* package (Kuznetsova et al., 2017), using Satterthwaite's approximation for the denominator degrees of freedom. Where significant main effects were detected, pair-wise comparisons were performed using the *emmeans* package (Lenth, 2018).

3. Results

3.1. Chemical analyses

Mean measured exposure concentrations for the low- and high-fluoxetine treatments were 38.26 ng/L (SD = 23.87, $n = 60$) and 311.83 ng/L (SD = 214.14, $n = 60$), respectively. No fluoxetine contamination was detected in any control (i.e. unexposed) mesocosm tanks (all samples under quantification limit of 2 ng/L; $n = 30$). The variability in fluoxetine concentrations observed in this study is likely a result of exposure tanks containing gravel sediment—as fluoxetine is known to readily sorb to sediment (Kwon and Armbrust, 2006; Sánchez-Argüello et al., 2009)—and aquatic plants. While these factors were important in simulating ecologically relevant conditions, they likely increased variability in fluoxetine concentrations.

There were no significant differences in fluoxetine concentrations between replicate mesocosm tanks within each of the low- and high-fluoxetine treatment groups (Table S1).

3.2. Individual-level variation in oxygen uptake (MO_2) and activity

On average, we observed consistent among-individual differences in both MO_2 and activity levels, with repeatability ranging from 0.35 to 0.43, respectively (Table 1).

However, when testing whether consistent among-individual differences in behaviour and MO_2 varied across exposure treatments, we observed that consistent among-individual differences in activity levels were present in fish from the unexposed treatment, but not in fish exposed to either low or high fluoxetine concentrations (Table 2). In particular, fluoxetine exposure reduced among-individual variation in activity by approximately 70% in comparison to the unexposed treatment (Table 2). On the other hand, consistent among-individual variation in MO_2 was detected in fish from both the unexposed and low-fluoxetine treatments, but not in fish from the high-fluoxetine treatment (Table 2).

Notably, fixed effects did not explain a significant portion of the behavioural and metabolic variance observed in each of the three exposure treatments, except for group-level plasticity for activity levels observed in fish from the high-fluoxetine treatment, and increasing condition index resulting in a decrease in MO_2 in unexposed fish (Table 2).

3.3. Baseline oxygen uptake rate (MO_2) and changes in MO_2 in response to varying predation risk

Baseline MO_2 was significantly influenced by exposure treatment (Table 1; Fig. 1). On average, MO_2 of guppies from the low-fluoxetine treatment was lower in both the pre-predation stimulus stage ($t = -2.40$, $df = 328$, $p = 0.032$) and the post-predation stimulus stage ($t = -2.23$, $df = 328$, $p = 0.049$) compared to unexposed fish (Fig. 1). On the contrary, the baseline MO_2 of guppies from the high-fluoxetine treatment was not significantly different to those from the unexposed or low-fluoxetine treatments ($t = 0.95$, $df = 328$, $p = 0.537$; $t = -1.27$, $df = 328$, $p = 0.342$ respectively; Fig. 1).

The rate of oxygen uptake was significantly influenced by predation treatment, as indicated by the interaction between trial stage and predation treatment (Table 1). Specifically, guppies presented with a predator showed a significant increase in oxygen uptake from the pre-predation stimulus stage to the post-predation stimulus stage ($t = -4.02$, $df = 187$, $p < 0.001$; Fig. 2), regardless of the exposure treatment. By contrast, guppies presented with a non-predatory stimulus fish or an empty tank did not exhibit any significant change in oxygen uptake compared to the respective pre-predation stimulus baseline MO_2 ($t = -1.42$, $df = 187$, $p = 0.157$; $t = 0.65$, $df = 187$, $p = 0.520$, respectively; Fig. 2). However, we did not observe an overall significant interaction between exposure treatment and predation treatment in terms of the changes in MO_2 in response to varying predation threat (Table 1). Moreover, fluoxetine exposure did not significantly affect the differences in the MO_2 of guppies between the pre- and post-predation stimulus stages (Table 1; Fig. 1).

3.4. Changes in activity in response to varying predation threat

Activity was significantly influenced by predation treatment (Table 1). Interestingly, we found that the introduction of a predator or an empty tank did not induce any significant differences in guppy activity ($t = 1.74$, $df = 184$, $p = 0.084$; $t = 0.20$, $df = 184$, $p = 0.839$; Fig. S2). Instead, we found that guppy activity increased when presented with the non-predatory fish ($t = -3.46$, $df = 184$, $p < 0.001$; Fig. S2). Nevertheless, we did not observe an overall significant interaction between exposure treatment and predation treatment (Table 1). Fluoxetine exposure did not significantly influence mean changes in guppy activity when presented with a

Table 1
Results from LMMs for group- and individual-level variation with oxygen uptake (MO_2) and activity as dependent variables.

Model	Oxygen uptake (MO_2)				Activity			
Fixed effects	**Mean sq.**	***F***	***df*₁, *df*₂**	***p***	**Mean sq.**	***F***	***df*₁, *df*₂**	***p***
Exposure	23786	3.988	2, 183	**0.020**	19967	0.589	2, 180	0.556
Trial stage	44970	7.540	1, 187	**0.007**	25918	0.765	1, 184	0.383
Predation	8130	1.363	2, 183	0.258	282499	8.339	2, 180	**<0.001**
Exposure:trial stage	473	0.079	2, 187	0.924	26517	0.783	2, 184	0.459
Exposure:predation	412	0.069	4, 183	0.991	24176	0.714	4, 180	0.584
Predation:trial stage	32219	5.402	2, 187	**0.005**	241355	7.125	2, 184	**0.001**
Random effects	**Estimate ± SE**	**ΔAIC**	χ^2_1	***p***	**Estimate ± SE**	**ΔAIC**	χ^2_1	***p***
V_{among}	3164 ± 4.059	21.736	23.736	**<0.001**	25799 ± 11.682	35.774	37.774	**<0.001**
V_{within}	5964 ± 5.574				33876 ± 13.391			
Repeatability	**0.347**				**0.432**			

Table 2
Results from LMMs for group- and individual-level variation with oxygen uptake (MO_2) and activity as dependent variables, and fish from different fluoxetine treatments tested separately.

Model	Oxygen uptake (MO_2)				Activity			
Unexposed								
Fixed effects	**Mean sq.**	***F***	***df*$_1$, *df*$_2$**	***p***	**Mean sq.**	***F***	***df*$_1$, *df*$_2$**	***p***
Trial stage	20440	2.805	1, 63	0.099	763	0.024	1, 60	0.879
Predation	2400	0.329	2, 59	0.721	75964	2.347	2, 8	0.158
Mesocosm	24139	3.313	3, 59	**0.026**	131645	4.067	3, 56	**0.011**
Condition index	50682	6.955	1, 59	**0.011**	72795	2.308	1, 56	0.139
Predation:trial stage	19748	2.710	2, 63	0.074	74714	2.308	2, 60	0.108
Random effects	**Estimate ± SE**	**ΔAIC**	χ^2_1	***p***	**Estimate ± SE**	**ΔAIC**	χ^2_1	***p***
V_{among}	3049 ± 6.797	0.100	4.797	**0.029**	35899 ± 23.875	15.800	17.749	**<0.001**
V_{within}	7287 ± 10.507				32366 ± 22.665			
Repeatability	**0.295**				**0.526**			
Low fluoxetine								
Fixed effects	**Mean sq.**	***F***	***df*$_1$, *df*$_2$**	***p***	**Mean sq.**	***F***	***df*$_1$, *df*$_2$**	***p***
Trial stage	15623	3.313	1, 60	0.074	77455	1.844	1, 60	0.180
Predation	4792	1.016	2, 56	0.369	146397	3.485	2, 11	0.066
Mesocosm	7361	1.561	3, 56	0.209	36185	0.861	3, 55	0.467
Condition index	7965	1.689	1, 56	0.199	33482	0.7971	1, 56	0.376
Predation:trial stage	6692	1.419	2, 60	0.250	100133	2.384	2, 60	0.101
Random effects	**Estimate ± SE**	**ΔAIC**	χ^2_1	***p***	**Estimate ± SE**	**ΔAIC**	χ^2_1	***p***
V_{among}	2857 ± 6.734	6.100	8.096	**0.004**	11035 ± 13.235	0.200	2.183	0.140
V_{within}	4715 ± 8.652				42005 ± 25.821			
Repeatability	**0.377**				0.208			
High fluoxetine								
Fixed effects	**Mean sq.**	***F***	***df*$_1$, *df*$_2$**	***p***	**Mean sq.**	***F***	***df*$_1$, *df*$_2$**	***p***
Trial stage	9691	1.602	1, 60	0.210	733	0.026	1, 60	0.872
Predation	2896	0.479	2, 56	0.622	47972	1.708	2, 11	0.226
Mesocosm	2031	0.336	3, 56	0.800	29535	1.052	3, 50	0.378
Condition index	7244	1.198	1, 56	0.276	16641	0.593	1, 51	0.445
Predation:trial stage	10936	1.808	2, 60	0.173	109498	3.899	2, 60	**0.026**
Random effects	**Estimate ± SE**	**ΔAIC**	χ^2_1	***p***	**Estimate ± SE**	**ΔAIC**	χ^2_1	***p***
V_{among}	2259 ± 5.988	1.700	3.726	0.054	9820 ± 12.485	1.500	3.465	0.063
V_{within}	6049 ± 9.799				28081 ± 21.116			
Repeatability	0.272				0.259			

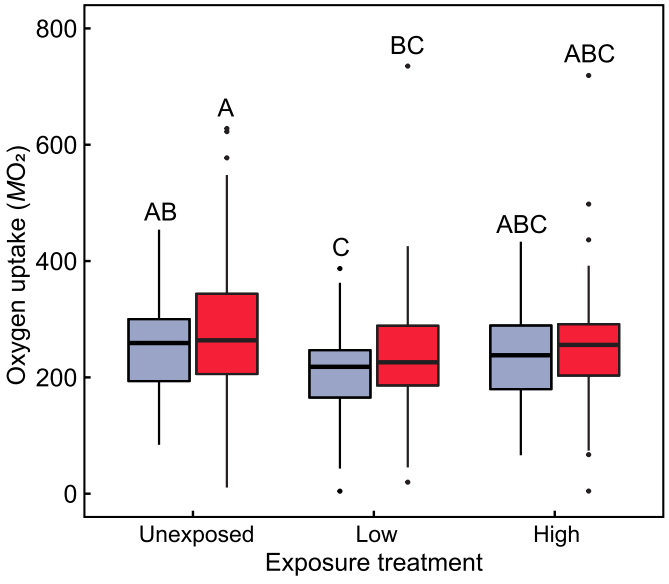

Fig. 1. Pre-predation stimulus (grey boxes) and post-predation stimulus (red boxes) rates of oxygen uptake (MO_2, mg O_2 kg^{-1} h^{-1}) of female guppies split by exposure treatment (unexposed = 0 ng/L, low fluoxetine = 38 ng/L, high fluoxetine = 312 ng/L; n = 63 per treatment). Treatment groups that do not share upper case letters are significantly different ($p < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

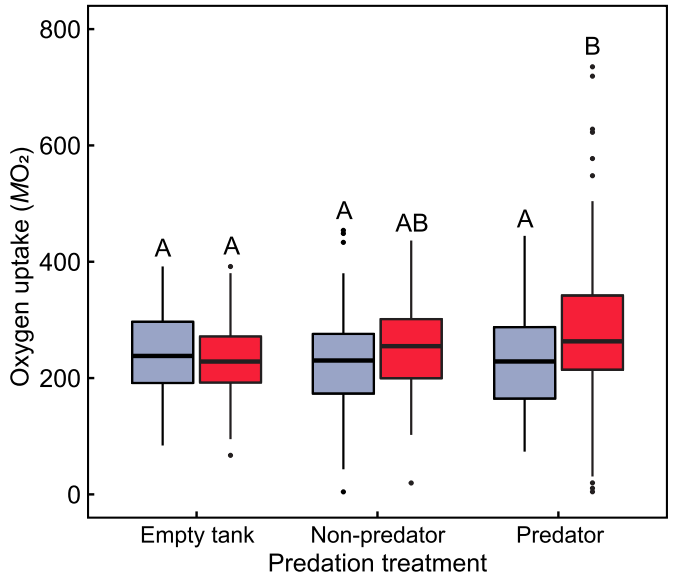

Fig. 2. Pre-predation stimulus (grey boxes) and post-predation stimulus (red boxes) rates of oxygen uptake (MO_2, mg O_2 kg^{-1} h^{-1}) of female guppies split by predation treatment (empty tank = control, non-predator = rainbowfish, predator = perch; n = 63 per treatment). Treatment groups that do not share upper case letters are significantly different ($p < 0.05$). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

perceived threat of predation (Table 1; Fig. S3).

4. Discussion

Here, we tested whether long-term (15-month) exposure to environmentally realistic concentrations of the globally pervasive pharmaceutical contaminant fluoxetine (Prozac®) altered the degree of among-individual variation in the metabolism and behaviour of guppies. We also tested whether fluoxetine exposure influenced both mean baseline levels of metabolic and behavioural traits of guppies, and mean trait responses as a function of perceived predation threat. In agreement with our predictions concerning the effects of fluoxetine on individual-level variation in metabolism and behaviour, we observed that consistent among-individual differences in activity levels (i.e. personality) were present in unexposed fish, but absent in fish from either of the fluoxetine treatments. Specifically, variation in activity rates among individuals exposed to fluoxetine was approximately 70% lower than in unexposed fish. Similarly, among-individual variation in MO_2 was detected in fish from the unexposed and the low-fluoxetine treatments, but was not detected in fish from the high-fluoxetine treatment. We also found evidence for a non-monotonic shift in mean baseline metabolic rate associated with fluoxetine exposure, whereby fish from the low-fluoxetine treatment, but not the high-fluoxetine treatment, showed lower oxygen uptake than unexposed fish. However, fluoxetine exposure did not influence mean changes in either oxygen uptake or activity levels of guppies presented with varying levels of predation threat.

We note that repeatability estimates of MO_2 and activity were obtained from two consecutive measurements. Therefore, the short time interval between measurements may have contributed to the relatively high repeatability scores observed for both MO_2 (0.35; see White et al., 2013) and activity (0.43). In this regard, the finding that consistent among-individual differences in activity were present in unexposed fish, but absent in fish from both fluoxetine exposure treatments suggests that exposure to fluoxetine had a dramatic impact on behavioural variation at the individual level. Hence, long-term exposure to fluoxetine (i.e. 15 months, approximately four overlapping generations) appears to have reduced behavioural variability among individuals and homogenised their behavioural responses. A similar effect of fluoxetine exposure was also detected on among-individual variation in metabolic rate, although disruption of individual-level variation in MO_2 was only observed in the high-fluoxetine treatment. Behavioural responses are typically more plastic than physiological responses (Sih et al., 2015; Stoks et al., 2005), as behavioural adjustments are more readily made in the face of environmental change to buffer individuals from adverse effects, thereby reducing selection pressure on potential plasticity in physiological traits such as metabolic rate (Bogert, 1949; Gunderson and Stillman, 2015). Thus, only the higher concentration of fluoxetine impaired individual variation in metabolic rates, although such concentrations are still environmentally relevant. Although not significant, it is important to note that repeatability of MO_2 in the high-fluoxetine treatment was, however, high, so that disruptive effects of fluoxetine on individual-level variation in metabolic rate should be taken with caution at this stage until future studies are able to confirm this finding.

Since phenotypic variability plays a critical role in the persistence of animal populations under environmental change (Mimura et al., 2017), the loss of such variability can contribute substantially to the decline of animal populations in the wild, including in fish (Anderson et al., 2008; Carlson et al., 2011; Olsen et al., 2009). A reduction in phenotypic (and genetic) diversity of Chinook salmon (*Oncorhynchus tshawytscha*) associated to dams, habitat loss, and hatchery production has been suggested to be the primary cause for the collapse of wild populations whose adaptive capacity and life history portfolios were compromised (see Carlson and Satterthwaite, 2011 and references therein). Similarly, among-individual variation in migration strategies and life-history diversity within populations has been documented to stabilise population dynamics in anadromous fish, such as abundance and biomass (Moore et al., 2014). Variation in metabolic rate was also shown to be an important mechanism in producing among-individual variation in coping strategies for overwintering brown trout (*Salmo trutta*) when facing scarcity of food resources (Auer et al., 2016). As such, our results point out a hidden but serious threat posed by fluoxetine pollution on the persistence of wild animals in polluted habitats, and suggests that exploring individual-level variation in phenotypic variability of animals is urgently needed to fully understand the impacts of pharmaceutical pollution (Olsen et al., 2009).

Guppies from the low-fluoxetine treatment exhibited a significantly lower MO_2, indicating a lower baseline metabolic rate. Previous studies have, however, reported contrasting results. For example, marine worms (*Hediste diversicolor*) were shown to increase their oxygen consumption following exposure to fluoxetine at 10 000 ng/L (Hird et al., 2016). Similarly, water fleas (*Daphnia magna*) exposed to fluoxetine at 80 000 ng/L increased oxygen consumption rates (Campos et al., 2012), with deregulation of genes involved in the Krebs cycle as a possible mechanism for the metabolic change (Campos et al., 2013). The difference between these studies and the present may be a result of the concentrations employed. In the present study, the concentration of fluoxetine found to produce a decreased metabolic rate was 38 ng/L, which was approximately 260 times lower than the minimum concentration of fluoxetine found to increase metabolic rate in previous studies. Further, we saw evidence for a non-monotonic response to fluoxetine (i.e. a nonlinear relationship between fluoxetine dosage and the response; Rivetti et al., 2016; Vandenberg et al., 2012), which suggests that the effects seen at higher dosages may not reflect those seen at lower dosages.

A non-monotonic dose response has been reported as characteristic of fluoxetine's mode of action on several other metabolic functions and behaviours of exposed animals (Bertram et al., 2018a; Bossus et al., 2014; Guler and Ford, 2010; Martin et al., 2019b; Martin et al., 2017; Mennigen et al., 2010; Painter et al., 2009; Rivetti et al., 2016; Saaristo et al., 2017). For example, goldfish (*Carassius auratus*) exposed to fluoxetine at a concentration of 540 ng/L exhibited a decrease in plasma glucose levels possibly linked to a reduction in gluconeogenesis, whilst this effect was not seen in goldfish exposed to 54 000 ng/L of fluoxetine (Mennigen et al., 2010). Similarly, fluoxetine-exposed eastern mosquitofish (*Gambusia holbrooki*) exhibited a reduction in antipredator behaviour at a lower dosage (25 ng/L), which was not seen in fish exposed to a higher dosage (226 ng/L; Martin et al., 2017). Thus, with increasing evidence that fluoxetine exposure can result in non-monotonic responses, it is important that future studies employ a dosage range reflecting levels found in the environment when seeking to identify ecological impacts of this contaminant, as is also true for other pharmaceutical pollutants with similar modes of action.

In the present study, shifts in metabolic rate in response to perceived predation risk were not significantly influenced by exposure to fluoxetine, as indicated by the lack of interaction between the exposure and predation treatments. Specifically, guppies presented with the predatory perch exhibited a predator-stress response regardless of fluoxetine exposure, as evidenced by an increase in their metabolic rate, which is indicative of the fight-or-flight response (Hawlena and Schmitz, 2010; Slos and Stoks, 2008; Van Dievel et al., 2016). Previous studies have found that

differences in lipid and amino acid metabolic synthesis between exposed and unexposed zebrafish (*Danio rerio*) were associated with differences in swimming behaviour and activity as an indicator of anxiety within the same study (Wong et al., 2013). However, to date, there have been no studies investigating these alterations in metabolism in relation to actual predation stress. Thus, it is possible that although there may be effects of fluoxetine on metabolism when animals are not exposed to an additional stressor, the underlying differences may not be sufficient to disrupt metabolic changes in response to predation threat.

Similarly, we saw no impact of fluoxetine exposure on the behavioural responses of guppies to the predation threat. Specifically, there were no significant differences in the change in activity levels across the exposure treatments, regardless of predation treatment. This result was again contrary to the findings of previous studies, even those that used fluoxetine exposure concentrations similar to this study (e.g. Barry, 2014; Martin et al., 2017; Peters et al., 2017). For example, estuarine crabs (*Hemigrapsus oregonensis*) exposed to 30 ng/L of fluoxetine over 9 weeks were more active irrespective of predator presence or time of day, whilst unexposed crabs were more risk averse and active only at night when the predator was inactive (Peters et al., 2017). Similarly, eastern mosquitofish exposed to 25 ng/L of fluoxetine over a 28-day period showed an increase in activity compared to unexposed fish (Martin et al., 2017). However, a key difference between the present study and those listed above is the duration of exposure. In the present study, mesocosm populations were exposed over a 15-month period, with focal guppies being sourced from a generation of fish born within the mesocosm system, not from the wild founder population. As such, there are likely transgenerational effects of fluoxetine within this study, although such effects were not directly explored. Past studies on other SSRIs have shown that effects can differ over multiple generations, with possible adaptation or increased plasticity depending on the exposure concentration. For example, when offspring daphnids (*Daphnia magna*) from mothers exposed to 300 ng/L of the SSRI sertraline were removed from exposure conditions, there was a decrease in fecundity when compared to both the offspring daphnids still being exposed to sertraline and the control offspring from unexposed generational lines (Minguez et al., 2015). This suggests that there was possibly adaptation to the pharmaceutical or an increase in plasticity within the offspring of the sertraline-exposed mothers. Similarly, it is possible that fluoxetine-exposed guppies in this study had adapted to fluoxetine, as they had developed entirely within the fluoxetine exposure conditions (Minguez et al., 2015). Thus, possible adaptation to the environmentally realistic concentrations of fluoxetine over multiple generations may explain why fluoxetine did not affect metabolic and behavioural responses to predation, a result contrary to previous studies that found a reduction in antipredator behaviours (e.g. Painter et al., 2009; Pelli and Connaughton, 2015; Peters et al., 2017). However, to date, there has been a lack of studies directly exploring transgenerational effects of fluoxetine, and pharmaceutical contaminants in general, on behavioural and physiological variation under chronic environmentally relevant exposure conditions (discussed in Saaristo et al., 2018). As this study did not directly compare effects of fluoxetine exposure between generations, it is difficult to ascertain whether there has indeed been adaptation to fluoxetine, or if an increase in plasticity within the offspring of exposed parents resulted in fluoxetine's lack of influence on the antipredator responses of the tested guppies. More broadly, effects of fluoxetine have previously been reported to be duration-dependent, as long-term effects are often only seen after several weeks of exposure, resulting from alterations in the expression of serotonin receptors (Stewart et al., 2014). As there are distinctly different mechanisms of action between acute and chronic fluoxetine exposure, this may, in part, also explain differences in the impacts of fluoxetine seen across studies (Stewart et al., 2014).

We also report that guppies were more active in the non-predator treatment (i.e. rainbowfish) compared to guppies in the predator treatment (i.e. spangled perch) and in the empty tank control. Increased activity level in the presence of the non-predator is unlikely to be a stress response, as previous work with guppies (from this source population) has shown that rainbowfish are not perceived as a predatory threat (Fursdon et al., 2019). Instead, increased activity in the presence of the non-predator is more likely to be a result of heterospecific signalling/information (Huang et al., 2012), with the guppies' perception of predation risk being reduced by the presence of a non-predatory heterospecific (Huang et al., 2012; Michelangeli and Wong, 2014).

5. Conclusions and future directions

In summary, we found a decrease in among-individual variation in metabolic rate and activity levels of fish from the fluoxetine treatments compared to controls. These results suggest that fluoxetine reduces variation in metabolic rate and behaviour at the individual level by homogenising animal responses. The homogenisation of ecologically important physiological and behavioural responses reported here could have adverse consequences on population dynamics and resilience of animal populations against environmental changes in the long-term. We also report a non-monotonic effect of fluoxetine on baseline metabolic rate, with a decrease in metabolic rate detected at the low-fluoxetine dosage, but not the high-fluoxetine dosage. Further, the direction of the shift in metabolic rate observed here is contrary to shifts reported in previous studies which employed exposure dosages magnitudes higher than the present study, highlighting the importance of testing effects of fluoxetine exposure at environmentally realistic concentrations. More broadly, the subtle non-monotonic impact on metabolic rate, and substantial impacts on among-individual variation, documented suggest that adverse effects of chronic fluoxetine exposure may be more likely to result from disruptions to among-individual variation than drastic changes in mean levels of physiological and/or behavioural traits. It is also important to note that, while this study involved four overlapping generations exposed simultaneously and allowed to interact, thereby simulating an environmentally realistic exposure scenario, future research specifically designed to disentangle plastic versus genetic responses to long-term pharmaceutical contamination will be valuable. Furthermore, given that previous studies have shown sex-specific differences in how fluoxetine affects certain behavioural traits, albeit over short-term exposure durations (see Martin et al., 2019b; Martin et al., 2017), the potential for long-term fluoxetine exposure to induce sex-specific effects also warrants further investigation. In summary, the results of the present study emphasise the need to further explore impacts of pharmaceutical pollution within ecologically relevant systems, taking into consideration not only effects on mean phenotypic values, but also changes in phenotypic variation within populations.

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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.

CRediT authorship contribution statement

Hung Tan: Conceptualization, Methodology, Software, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing, Visualization, Project administration. **Giovanni Polverino:** Methodology, Validation, Formal analysis, Data curation, Writing - original draft, Writing - review & editing, Visualization. **Jake M. Martin:** Conceptualization, Methodology, Writing - review & editing, Visualization. **Michael G. Bertram:** Writing - original draft, Writing - review & editing, Visualization. **Sarah C. Wiles:** Investigation, Writing - review & editing. **Maria M. Palacios:** Conceptualization, Methodology, Writing - review & editing. **Candice L. Bywater:** Resources, Writing - review & editing. **Craig R. White:** Resources, Writing - review & editing, Methodology. **Bob B.M. Wong:** Conceptualization, Methodology, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2020.114450.

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