Science of the Total Environment 872 (2023) 162173

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Effects of the antidepressant fluoxetine on the swimming behaviour of the amphipod *Gammarus pulex*: Comparison of short-term and long-term toxicity in the laboratory and the semi-field

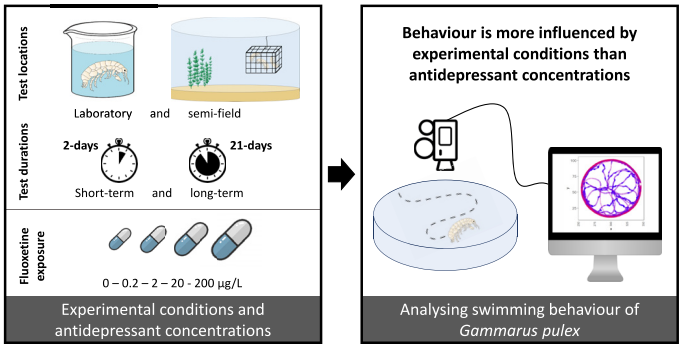
Lara M. Schuijt [a,*], Oluwafemi Olusoiji [b], Asmita Dubey [a,c], Pablo Rodríguez-Sánchez [d], Rima Osman [e], Paul J. Van den Brink [a,e], Sanne J.P. van den Berg [a,e]

[a] Aquatic Ecology and Water Quality Management group, Wageningen University and Research, P.O. box 47, 6700 AA Wageningen, the Netherlands
[b] Hasselt University, Martelarenlaan 42, 3500 Hasselt, Belgium
[c] Department of Aquatic Ecology, Netherlands Institute of Ecology (NIOO-KNAW), Droevendaalsesteeg 10, 6708 PB Wageningen, the Netherlands
[d] Netherlands eScience Center, Science Park 402, 1098 XH Amsterdam, the Netherlands
[e] Wageningen Environmental Research, Wageningen University and Research, P.O. Box 47, 6700 AA Wageningen, the Netherlands

HIGHLIGHTS

- Assessing effects of experimental conditions and fluoxetine on gammarid behaviour
- Experimental conditions have larger effects on behaviour of *Gammarus* than fluoxetine.
- Few significant impacts of fluoxetine on behavioural endpoints
- Behavioural results in line with unaffected *Gammarus* population in semi-field.
- Test duration and location affected behavioural outcomes after fluoxetine exposure.

GRAPHICAL ABSTRACT



ARTICLE INFO

Editor: Julian Blasco

Keywords:
Behavioural ecotoxicology
Antidepressants
Experimental conditions
Gammarus pulex
Baseline behaviour
Startle response

ABSTRACT

Fluoxetine is one of the worlds most prescribed antidepressant, and frequently detected in surface waters. Once present in the aquatic environment, fluoxetine has been shown to disrupt the swimming behaviour of fish and invertebrates. However, swimming behaviour is also known to be highly variable according to experimental conditions, potentially concealing relevant effects. Therefore, the aims of this study were two-fold: i) investigate the swimming and feeding behaviour of *Gammarus pulex* after exposure to the antidepressant fluoxetine (0.2, 2, 20, and 200 µg/L), and ii) assess to what degree the experimental test duration (short-term and long-term) and test location (laboratory and semi-field conditions) affect gammarid's swimming behaviour. We used automated video tracking and analysis to asses a range of swimming behaviours of *G. pulex*, including swimming speed, startle responses after light transition, acceleration, curvature and thigmotaxis. We found larger effects on the swimming behaviour of *G. pulex* due to experimental conditions than due to tested antidepressant concentrations. Gammarids swam faster, more straight and showed a stronger startle response during light transition when kept under semi-field conditions compared to the laboratory. Effects found for different test durations were opposite in the laboratory and semi-field. In the laboratory gammarids swam slower and spent more time at the inner zone of the arena after 2 days compared to 21 days while for the semi-field the reverse was observed. Fluoxetine had only minor impacts on the swimming behaviour of *G. pulex*, but experimental

* Corresponding author.
E-mail address: lara.schuijt@wur.nl (L.M. Schuijt).

http://dx.doi.org/10.1016/j.scitotenv.2023.162173
Received 29 August 2022; Received in revised form 18 January 2023; Accepted 7 February 2023
Available online 11 February 2023

conditions influenced behavioural outcomes in response to fluoxetine exposure. Overall, our results highlight the importance of standardizing and optimizing experimental protocols that assess behaviour to achieve reproducible results in ecotoxicology.

1. Introduction

Variation and changes in behaviour of individuals are important for fitness-related functions like growth (Stamps, 2007) and reproduction (Lind and Cresswell, 2005), as well as ecosystem functioning (Woodward, 2009). Behaviour of animals can change in response to human-induced alterations (Ford et al., 2021). These changes in behaviour might influence the survival and reproductive success of individuals, thereby affecting population dynamics (Tuomainen and Candolin, 2011). While some behavioural modifications might improve an organisms fitness (e.g., avoidance behaviour (Amiard-Triquet, 2009)), not all behavioural responses are beneficial (Badyaev, 2005). Environmental pollutants might interfere, for instance, with physiological systems, such as the sensory, endocrine or neurological system, subsequently leading to changes in behaviour like paralysis (Pisa et al., 2015) or altered feeding rates (Stanley et al., 2007). This is especially relevant for pharmaceuticals, as some are purposely designed to modify (human) behaviour (e.g., psychotropic drugs).

Worldwide pharmaceutical usage has increased substantially over the past years, and has resulted in a concomitant increase of pharmaceuticals in the environment (Patel et al., 2019). In freshwater systems, a wide range of pharmaceuticals has been detected (Fekadu et al., 2019; Hughes et al., 2013; Loos et al., 2009), including hormones, antibiotics, analgesics/anti-inflammatories and antidepressants (Wilkinson et al., 2022). Most pharmaceuticals enter freshwater as biologically active compounds and are specifically designed to interact with a specific process or pathway (Boxall et al., 2012). Even though environmental concentrations of pharmaceuticals in surface waters are detected at much lower than acute lethal concentrations (Brausch et al., 2012), many sublethal effects, including behavioural effects, have been reported at those low concentrations in aquatic organisms (e.g. Brodin et al. (2014); Kidd et al. (2007); Sehonova et al. (2018)).

Antidepressants make up one group of pharmaceuticals whose usage has exploded over the last decade, and can be found in aquatic systems (Mole and Brooks, 2019; Santos et al., 2010). Fluoxetine is one of the worlds most prescribed antidepressant and frequently detected in surface waters (Stewart et al., 2014). Fluoxetine is a Selective Serotonin Reuptake Inhibitor (SSRI) and inhibits the reuptake of the neurotransmitter serotonin. The neurotransmitter serotonin is present in vertebrates, as well as some invertebrates, and is involved in physiological processes, including behaviour, growth and reproduction (Corcoran et al., 2010; Fong and Ford, 2014). Concerns have therefore been raised about the potential effects of fluoxetine on aquatic organisms, especially regarding the behaviour of aquatic organisms.

Over the past years, research regarding the impacts of SSRIs on aquatic organisms has been increasing (Moreira et al., 2022; Sehonova et al., 2018) and impacts of SSRIs on normal functioning and behavioural changes of aquatic organisms have been reported (Silva et al., 2015). For fish it has been shown that fluoxetine can disrupt several ecologically important behaviours, including anxiety (Martin et al., 2019a), activity (de Farias et al., 2019) and foraging (Martin et al., 2019b). Fluoxetine has also been found to exhibit effects on the behaviour of multiple invertebrate species (Sehonova et al., 2018). In bivalves, effects of fluoxetine are observed in behaviours associated with feeding (Hazelton et al., 2013) and reproduction (Lazzara et al., 2012). In decapods, fluoxetine has been shown to affect swimming activity (Hamilton et al., 2016), aggression, anxiety (Hamilton et al., 2016; Peters et al., 2017), and anti-predator behaviours (Peters et al., 2017). In amphipods, fluoxetine is known to alter phototaxis, geotaxis and swimming behaviours *of Echinogammarus marinus* (Bossus et al., 2014; Guler and Ford, 2010), and levels of activity in *Gammarus pulex* (De Lange et al., 2006). This last species, *G. pulex*, is extensively used in aquatic ecotoxicology, primarily due to their important role in aquatic ecosystems and their widespread occurrence. It is for those reasons that we chose to use *G. pulex* as the model species in this study.

The use of behavioural endpoints to assess effects of pollutants on invertebrates is currently still limited by, among others, a lack of understanding of the studied species (Melvin and Wilson, 2013). This lack of understanding baseline behaviour can result in a large variability in the outcomes of behavioural assays, potentially concealing relevant effects (Van den Berg et al., 2023). It is, therefore, crucial to determine which experimental factors affect baseline behaviour. Consider, for example, the experimental testing period or the execution of laboratory versus field tests. In ecotoxicology, experimental testing duration may vary from short-term tests lasting 48 h, to long-term tests lasting up to several months. Similarly, large differences exist between tests performed under controlled laboratory conditions, versus tests performed under more realistic field conditions. The effects of such experimental conditions on behaviour is still largely unknown.

The main goals of this study were to i) investigate the swimming and feeding behaviour of *Gammarus pulex* after exposure to the antidepressant fluoxetine, and ii) assess to what degree experimental conditions (i.e., short-term (2 days) and long-term (21 days) exposure duration and laboratory and semi-field conditions) affects gammarid's swimming behaviour, as this comparison is rarely made in behavioural ecotoxicity studies (Ogungbemi et al., 2019). We predicted that fluoxetine would affect the feeding and swimming behaviour of *G. pulex*, as previous studies reported some behavioural changes of amphipods after fluoxetine exposure (Bossus et al., 2014; De Lange et al., 2006; Guler and Ford, 2010).

2. Material and methods

2.1. Species collection

G. pulex was collected by means of two sieves (mesh sizes 5 and 1 mm) in August and September 2019 from the Heelsumse Beek (51°58′40.8"N 5°45′31.6″E), a 2nd order, natural stream in the vicinity of Wageningen, The Netherlands. Organisms retaining on the 1 mm sieve were kept in buckets filled with water from the same stream and transported to the laboratory. The buckets were aerated and kept in a water bath to acclimatize at 19 ± 1 °C with a 18:6 light:dark cycle at a light level of 8 μmol s^{-1} m^{-2} for five days. Plants and organic material from the field site, in combination with dried leached *Populus* leaves, were provided as food ad libitum.

2.2. Experimental design

To assess the effects of fluoxetine and experimental conditions on the behaviour of *G. pulex* we performed four tests: an acute and chronic laboratory test, and an acute and chronic semi-field test. Acute experiments lasted 2 days (48 h) and chronic experiments lasted 21 days. In both the laboratory and the semi-field tests nominal fluoxetine exposure concentrations of 0.2, 2, 20, and 200 μg/L were used. This concentration range included environmental realistic exposures (Correia et al., 2022b) and higher concentrations with the highest being close to lethal concentrations [LC50] of 234 μg/L for *Ceriodaphnia dubia* (Brooks et al., 2003a).

In the laboratory experiment, both a negative control and a solvent control were tested, and each treatment was replicated four times. In the semi-field test, only a solvent control was included, and each fluoxetine treatment was replicated three times, whilst the solvent control was replicated six times (see Table S1).

2.2.1. Laboratory tests

Laboratory tests were performed from the end of August to the beginning of October 2019 in a water bath at 19 ± 1 °C with a 18:6 light:dark cycle at a light level of 8 μmol s^{-1} m^{-2}. We used 1.5 L glass jars filled with 1 L of groundwater water (pH of 8.0 and conductivity of 184 μS/cm) from the Sinderhoeve field station (www.sinderhoeve.org), Renkum, the Netherlands. Each jar was aerated and a metal mesh was added to act as substrate and offer protection. In the chronic laboratory test, 6 conditioned popular leaf discs acted as food source. For this, conditioned popular leaves were cut into circles of 20 mm diameter, weighted and kept in 200 mL pond water for a week to allow for colonization of microorganism. At the start of the experiment, 15 individuals were randomly selected from the buckets in which they were acclimatized and transferred into each jar, but individuals containing internal parasites were removed from the study. In addition, we took a subsample of 90 individuals for both the short-term and long-term test for which we measured the size. The average length of the gammarids at the start of the short-term test was 7.9 mm (±0.8), and 6.9 mm (±0.9) at the start of the long-term test. The total length of photographed *G. pulex* individuals was measures from the top of the cephalothorax to the base of the telson (Vellinger et al., 2013) by using the image analyser software ImageJ (http://rsbweb.nih.gov/ij/).

Application of fluoxetine was done just before the organisms were added to their jars. In the short-term laboratory test, the number of dead organisms were recorded after 48 h. For the long-term test, the number of dead organisms were recorded whilst refreshing the jars every week. During each refreshment, the gammarids were moved to cleaned jars containing newly applied fluoxetine, clean mesh and 6 new leaf discs. Abiotic water properties, including dissolved oxygen, electrical conductivity, pH and temperature, were measured directly after transferring gammarids into their jars and 1 day after application using a Multi 3630IDS (multi-parameter portable meter MultiLine®). In the long-term test, an additional measurement of abiotic conditions was done shortly before the weekly water renewal. The dissolved oxygen concentration should be above 5 mg/L and the pH above 6 as Sutcliffe and Carrick (1973) showed that *G. pulex* does not occur in streams with a pH < 5.7.

2.2.2. Semi-field tests

Semi-field tests consisted of exposing caged *G. pulex* individuals in aquatic mesocosms to which fluoxetine was applied. The mesocosm experiment was performed at the Sinderhoeve Experimental Station in Renkum, the Netherlands. We used 18 mesocosms (diameter 1.8 m, total depth 0.8 m, water depth 0.6 m, water volume ca. 1530 L) containing a 10 cm layer of fine sandy clay sediment and a water layer of 0.6 m originating from the experimental station's supply basin. In the mesocosms, fluoxetine was applied every week for 2 months. During these 2 months, we performed a short-term (2 days) and a long-term (21 days) test by inserting small cages containing 15 individuals *G. pulex* into the mesocosms. We refer to these tests as semi-field tests.

The short-term and long-term semi-field tests were performed between mid-July 2019 to mid-August 15, 2019. Gammarids were caged in stainless steel cylinders (length, 7.5 cm; diameter, 7.5 cm) with a 0.8 mm mesh size to guarantee free water flow, but to avoid the gammarids to escape. Each cage contained 15 individuals, plus 3 g of *Populus* leaves (conditioned leaves, dried at 60 °C). To minimize buoyancy and to stimulate biofilm growth, the cages were pre-soaked for 24 h in water from a control mesocosm before the start of the experiment. At the start of both the short-term and long-term semi-field tests, each mesocosm received 2 cages, one cage with gammarids and one cage containing leaves only. The latter served as a control for microbial and abiotic leaf mass loss over the exposure duration without the presence of organisms. Since the short- and long-term semi-field tests were started on the same day, only one sample was taken to determine the average length of the gammarids at the start of the experiment. The average length of a subsample of 45 gammarids was 8.5 mm (±1.2 mm) at the start of the short- and long-term semi-field tests. After an exposure period of 2- and 21-days, cages were collected, the number of surviving individuals were counted, and swimming behaviour was recorded. Remaining leaves in cages with *G. pulex* and cages with leaves only were dried at 60 °C for 72 h and weighted to determine feeding rates.

Water samples were collected 3 times a week from the mesocosms to measure fluoxetine concentrations. Additionally, once a week, general water quality parameters including pH, oxygen, temperature and conductivity were measured in the mesocosms to check whether dissolved oxygen concentrations are above 5 mg/L and the pH above 6.

2.3. Fluoxetine exposure, sampling and analysis

In both the laboratory and semi-field tests we aimed to achieve the following concentrations of fluoxetine after dosing: 0.2 μg/L, 2 μg/L, 20 μg/L and 200 μg/L. Stock solutions were made by using fluoxetine hydrochloride (Sigma-Aldrich, Product PHR1394, LOT#LRAA9180, GROS 484210, concentration 99.95 %) and acetone, to improve the solubility. Next, dosing solutions were prepared by using the stock solutions diluted with tap water and tap water with the same amount of acetone was applied to the solvent control.

Fluoxetine was applied once in the short-term laboratory test and water samples were taken with a glass pipet after one hour, at day 1 and day 2 to analytically measure the fluoxetine concentration. In the long-term laboratory test, fluoxetine was applied once a week for a 3-week period, and water samples were taken every week after one hour, at day 1, day 3 and day 7 after application. Tap water with the same amount of acetone was applied to the solvent control.

For the semi-field tests, fluoxetine was applied once a week to the mesocosms for a total of 8 weeks. During dosing, we poured the solution evenly over the water surface and mixed the compound through the water column by stirring with a steel rod. Sampling was done by collecting depth-integrated water samples by means of a Perspex® sampling tube. Water samples of all mesocosms were taken 1 h after application and 24 h before the next application (at day 6). Dosing was adjusted based on the concentration measured 24 h before next application, to achieve intended concentrations.

Immediately after water samples were collected in the laboratory- and semi-field tests, a sub-sample of 4.0 mL was transferred into 5-mL PP tube and spiked with 100 μL internal standard fluoxetine-d5 (Sigma Aldrich, article 613,347, Lot#LS-68-131) in methanol (200 ng/mL) and stored in the freezer (−20 °C) until further analysis. The highest concentration (200 μg/L) was 10× diluted whereafter all samples were directly injected onto an Agilent (Agilent Technologies, USA) liquid chromatograph coupled with an triple quad mass spectrometer 6410 or 6460 and equipped with Agilent electrospray ionization source ESI (6410) respectively jet stream electrospray ionization source AJS-ESI (6460). The limit of quantification was 0.07 μg/L.

2.4. Mortality and feeding rate

At the end of each experiment, the number of living organisms were counted. Amphipod feeding rates were calculated based on Maltby et al. (2002) and slightly modified by expressing the feeding rate per individual instead of per mg dry weight of *Gammarus*. Hence, feeding rates were expressed as mg dry leaf material per individual per day, and were corrected for microbial and abiotic leaf mass loss. Feeding rate (FR, mg DW food/ind/d) was calculated as:

$$FR = \frac{(L1 \times C) - L2}{I \times t}$$

where L1 is the dry weight of leaf material initially supplied (g), L2 is they dry weight of leaf material remaining after t days, I is the mean number of gammarids at the beginning and end of the test, t is the number of days, and C is the leaf weight change correction factor. This factor accounts for

microbial degradation and was calculated by dividing the final weight of the control leaves (without animals) by the initial weight.

2.5. Analysis of behaviour

Swimming behaviour was recorded using a Zebratower observation cabinet located in a room which could be completely darkened. The Zebratower observation cabinet consisted of an infrared panel with above an infrared sensitive camera connected to a video tracking software (Viewpoint). On the panel was room to record 20 observation arenas (glass Petri dishes with a diameter of 9 cm) simultaneously. The infrared panel and camera were situated in a cabinet with reflective walls so that the light intensity was homogenously distributed over the infrared panel and the 20 observation arenas.

We randomly selected 10 individuals from each jar or cage for the behavioural analyses and placed them individually in the observation arenas. Gammarids were transferred using a spoon to avoid inflicting damage, and the observation arenas contained filtered water with a depth of 1 cm (20 mL) from a control jar or control mesocosm. The water depth limited vertical swimming but allowed for free horizontal swimming. The gammarids were acclimatized in the Petri dish for 2 min before behavioural analysis started. During the behavioural analysis the organism's movement was recorded for 8 min during a 2-min dark/2-min light cycle. Hence, the total recording could be divided in four phases, of which phase 1 and phase 3 are the dark phases and phase 2 and 4 are the light phases. During the light phases, the light intensity was 50 μmol s^{-1} m^{-2}.

Swimming behaviour was analysed by means of six different endpoints: swimming speed, acceleration, curvature (the amount by which the swimming trajectory deviates from being a straight line), thigmotaxis (distance from the centre of the Petri dish), startle response magnitude (i.e., the drop in swimming speed directly after the light switches on), and startle response duration (i.e., time needed to recover to normal swimming speed). To obtain these endpoints, we extracted the x- and y-position over time of each individual by means of the video tracking software EthoVision R XT 11.5 (Noldus) (Van den Berg et al., 2023). The kinematics package (version 1.0, Rodriguez-Sanchez and Van den Berg, 2021) was subsequently used to derive the swimming speed (Feynman et al., 2011), acceleration (Feynman et al., 2011), curvature (Do Carmo, 2016), and thigmotaxis for each timepoint. Swimming speed, acceleration, curvature and thigmotaxis were analysed using 10-second time bins, whilst startle response magnitude and duration were analysed using 1-second time bins (Van den Berg et al., 2023). Extreme anomalous values generated by errors of the tracking software were excluded from the data analysis (as defined by values > mean ± 2*SD). An example of the output of the behavioural analysis of one individual can be found in Fig. 1, S1, S2 and S3.

Since gammarids were recorded for 8 min during a 2-min dark/2-min light cycle, this resulted in four light phases (first dark phase (0–120 s), first light phase (121–240 s), second dark phase (241–360), and second light phase (361–480), Fig. 1B) and three light-transitions with three startle responses (Fig. S4). Two startle responses were evoked by turning the light on (startle response 1 and 3) and one by turning the light off (startle response 2). To analyse these startle responses, we used the startle response magnitude and duration (Van den Berg et al., 2023). Startle response magnitude was calculated for each individual by dividing the swimming speed measured at the first second after the light-transition by the average swimming speed of the light or dark period before the light-transition. Startle response duration was calculated per individual by measuring the time between 1 s after light transition (at $t = 121$, $t = 241$ and $t = 361$) and the moment that the individual reached 90 % of its average swimming speed during the subsequent light or dark period (Pickell et al., 2016).

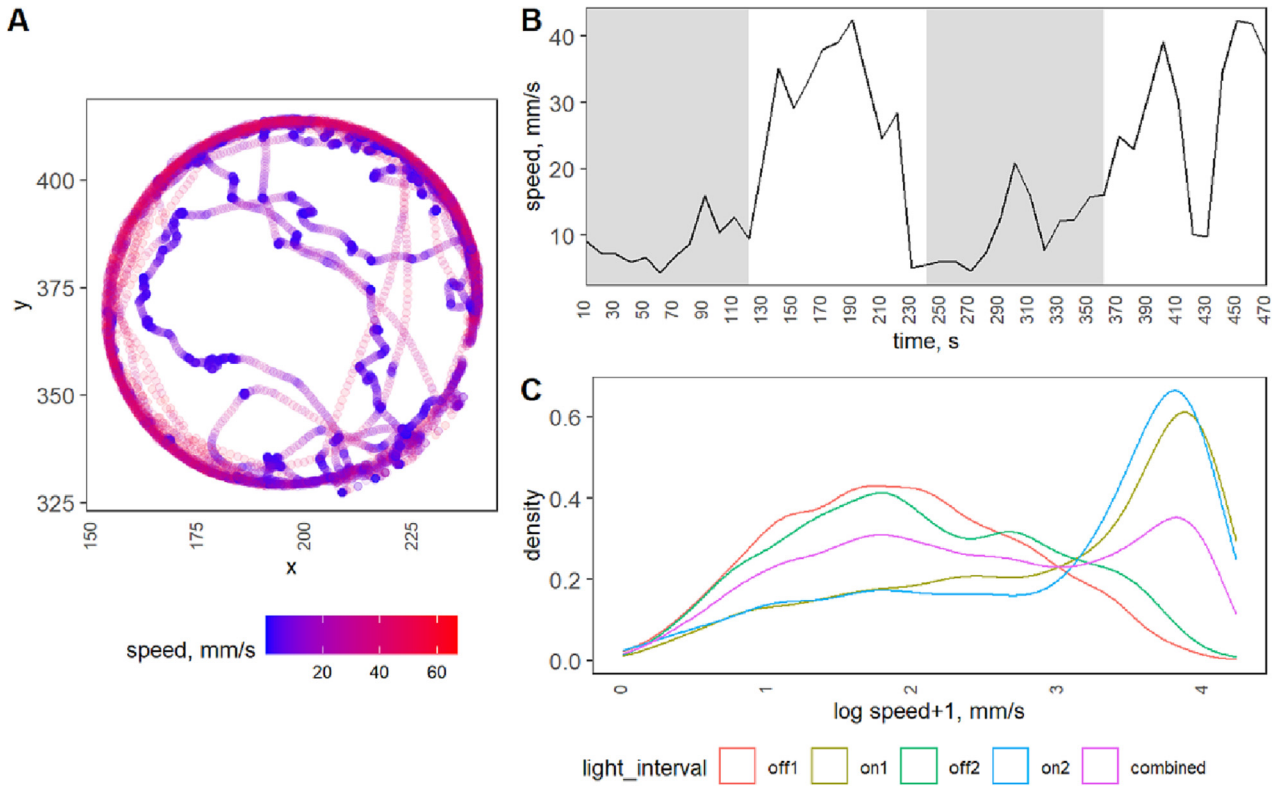


Fig. 1. An example of the behavioural analysis of one individual *Gammarus pulex*, showing the path the individual has travelled over the 8-minute measurement period, coloured according to absolute swimming speed (A), the speed over time (B), and the kernel density distribution of speed over time (C). The grey boxes in fig. B indicate the periods that the light was switched off. Fig. C shows a red line for the first dark period (phase 1), a yellow line for the first light period (phase 2), a green line for the second dark phase (phase 3), a blue line for the second light phase (phase 4) and a purple line for all time period combined.

2.6. Data wrangling and statistics

We calculated time-weighted average fluoxetine concentrations for the long-term (21 days) laboratory and semi-field test as in Roessink et al. (2013) using the following formula:

$$TWA_t = \frac{c_0}{k \times t_{TWA}} \left(1 - e^{(-k \times t_{TWA})}\right)$$

where TWA_t is the time-weighted average exposure concentration over a period of t days, c_0 is the concentration directly after dosing (μg/L), k is the dissipation rate coefficient (1/d) and t_{TWA} is the time over which the TWA was calculated (d). The dissipation rate coefficient, k, was calculated as:

$$k = \frac{-\ln\left(\frac{c_t}{c_0}\right)}{\Delta t}$$

where c_t is the concentration (μg/L) at the end of period Δt (d).

Feeding rate, size and survival were checked for normal distribution by using the Shapiro-Wilk test and homogeneity of variances by performing the Levene's test. In case that these assumptions were not met, we used a nonparametric Kruskal–Wallis H test and otherwise, when assumption were met, a one-way ANOVA. To test the effect of fluoxetine and exposure time on feeding rate, we performed a two-way ANOVA with a Tukey post hoc test, as the residuals were normally distributed and with an even variance between groups. Analyses were performed using R (version 4.0.2, R Core Team, 2020).

Before starting any statistical analysis on the different swimming behavioural endpoints, we performed two checks: i) whether there were any differences between the control and solvent control in the laboratory tests, and ii) whether there were any differences in the swimming behaviour of individuals due to sex. Considering the first check, we found no statistical differences between the control and the solvent control (Table S2). Therefore, these data were grouped together for further analyses, and we could conclude that the use of only a solvent control in the semi-field tests was appropriate. Considering the second check, sex distinction was based on 3 different morphological characteristics: (i) an individual was identified as male when a dense hairs on the middle back part of the second antennae was visible (Pinkster, 1970), (ii) and individual was identified as female with the existence female gonad tissue (Le Roux, 1933) or (iii) when eggs or a foetus was found. We used both sexes to increase the ecological relevance of the study and to investigate if any effects are sex dependent (Nielsen et al., 2018).

Individual variation of the behavioural swimming endpoints was characterized by the coefficient of variation (CV):

$$CV = \frac{\mathrm{SD}}{\mathrm{Y}}$$

where SD is the standard deviation and Y is the mean for each of the endpoints in the dark and light phases. Being standardized by its endpoint mean, the CV enables comparison of the degree of among-individual variation between the different swimming behavioural endpoints.

To meet assumptions of linear models, we had to transform the swimming behavioural endpoints. The endpoints acceleration and curvature were log transformed using the natural log, whilst speed and thigmotaxis were transformed using log(x + 1) and square root transformations respectively.

The swimming behavioural endpoints swimming speed, acceleration, thigmotaxis, curvature and startle responses were analysed using a linear mixed effect model in R using the lme function in the "nlme" package (version 3.1–148, Pinheiro et al. (2017)). To determine which covariance structure fitted best with our data, we visually inspected the variance pattern over time. Since the variances appeared to be homogenous, and the time intervals for the repeated measures were evenly spaced, we used a First Order Autoregressive AR(1) covariance structure. Next, we fitted the model with an AR-1 structure for the correlation of the measured endpoint over time under the following set of assumptions: (A) constant variance and random intercept over time, (B) constant variance with random intercept and slope in time, (C) different variance over 4 phases of light switching off and on, and (D) different variance over 2 phases of light switching off and on. Model C had the lowest AIC for all endpoints (Table S3 and S4), and was therefore used in the rest of the analyses. In the linear mixed effect model, light intervals (4 phases of light switching off and on), fluoxetine treatment, exposure duration (2 days versus 21 days) and/or test location (semi-field versus laboratory) were set as fixed effects. Individuals were nested within an experimental vessel (mesocosm or jar), which was included as a random factor in the model. For Post-Hoc analysis, we used the glht function in the "multcomp" package (version 1.4–14, Hothorn et al. (2016) to compare each fluoxetine treatment with the control treatment. *P*-values of ≤ 0.05 were considered significant.

All of our data handling, transformations, and statistics were performed in R (version 4.0.2, R Core Team, 2020), and all scripts are available on figshare (https://doi.org/10.6084/m9.figshare.20517939.v1).

3. Results

3.1. Experimental abiotic conditions, mortality and feeding rate

The minimal oxygen concentration was 6 mg/L in the semi-field tests and 8.8 mg/L in the laboratory, and therefore above the acceptable minimum concentration of 5 mg/L. The range (min-max) of abiotic conditions was larger for the semi-field tests compared to the laboratory tests (Table S5).

Fluoxetine concentrations in the laboratory were slightly below intended concentrations one hour after application (mean; 0.09, 0.95, 11.3 and 146 μg/L), whilst concentrations in the semi-field were relatively close to intended concentrations (0.23, 1.8, 19.4, 191 μg/L). In addition, dissipation of fluoxetine from the water phase was faster under laboratory conditions compared to semi-field conditions (Fig. 2). During the three-week exposure period of the long-term tests, time-weighted average fluoxetine concentrations were on average 60 % lower in the laboratory (0.34, 3.4 and 56 μg/L) compared to the semi-field (0.89, 8.0 and 121 μg/L). For the sake of clarity, results are referred to nominal values.

The semi-field tests showed a mean control mortality of <10 % and 15 % after 2- and 21-days, respectively. We did not find a significant effect from fluoxetine on survival in the semi-field tests (Fig. S5; Kruskal-Wallis H test for 2 days: $X^2(4) = 5.2, p = 0.27$ and 21 days: $X^2(4) = 5.5, p = 0.24$). In the laboratory tests, mortality was lower than 5 % and 15 % in all treatments after 2- and 21-days, respectively, and was non-significantly correlated with fluoxetine (Fig. S5; Kruskal-Wallis H test for 2 days: $X^2(4) = 2.2, p = 0.7$ and 21 days: $X^2(4) = 3.8, p = 0.43$).

We found no statistically significant difference in feeding rates between the different fluoxetine and control treatments after 2 days (Fig. S6A; Kruskal-Wallis H test: $X^2(4) = 7.2, p = 0.13$) and 21 days (Fig. S6B; One-way ANOVA: $F(4,13) = 1.4, p = 0.3$) exposure in the semi-field tests. Similar results were found in the laboratory tests, as we found no effects of fluoxetine on the feeding rate of *G. pulex* (Fig. S6C; Two-way ANOVA: $F(4,57) = 0.83, p = 0.5$), nor significant interaction effects between feeding rate and exposure time ($F(8,57) = 0.7$, p = 0.7). However, feeding rate significantly increased during the 21 days of exposure in the laboratory (Fig. S6C; $F(2,57) = 26.2, p < 0.001$) with significant differences between all sampling days (Tukey for 7–14 days: $p = 0.002$, 14–21 days: $p = 0.001$ and 7–21: $p < 0.001$).

3.2. Swimming behavioural endpoints

3.2.1. Effects of sex on swimming behaviour

We determined the sex of the 235 individuals used in the 21 days laboratory test. Of these, 108 were males and 127 were females. We did not find any sex-related differences in swimming speed, acceleration, thigmotaxis and curvature (Table S6). Also, the interaction between sex and fluoxetine

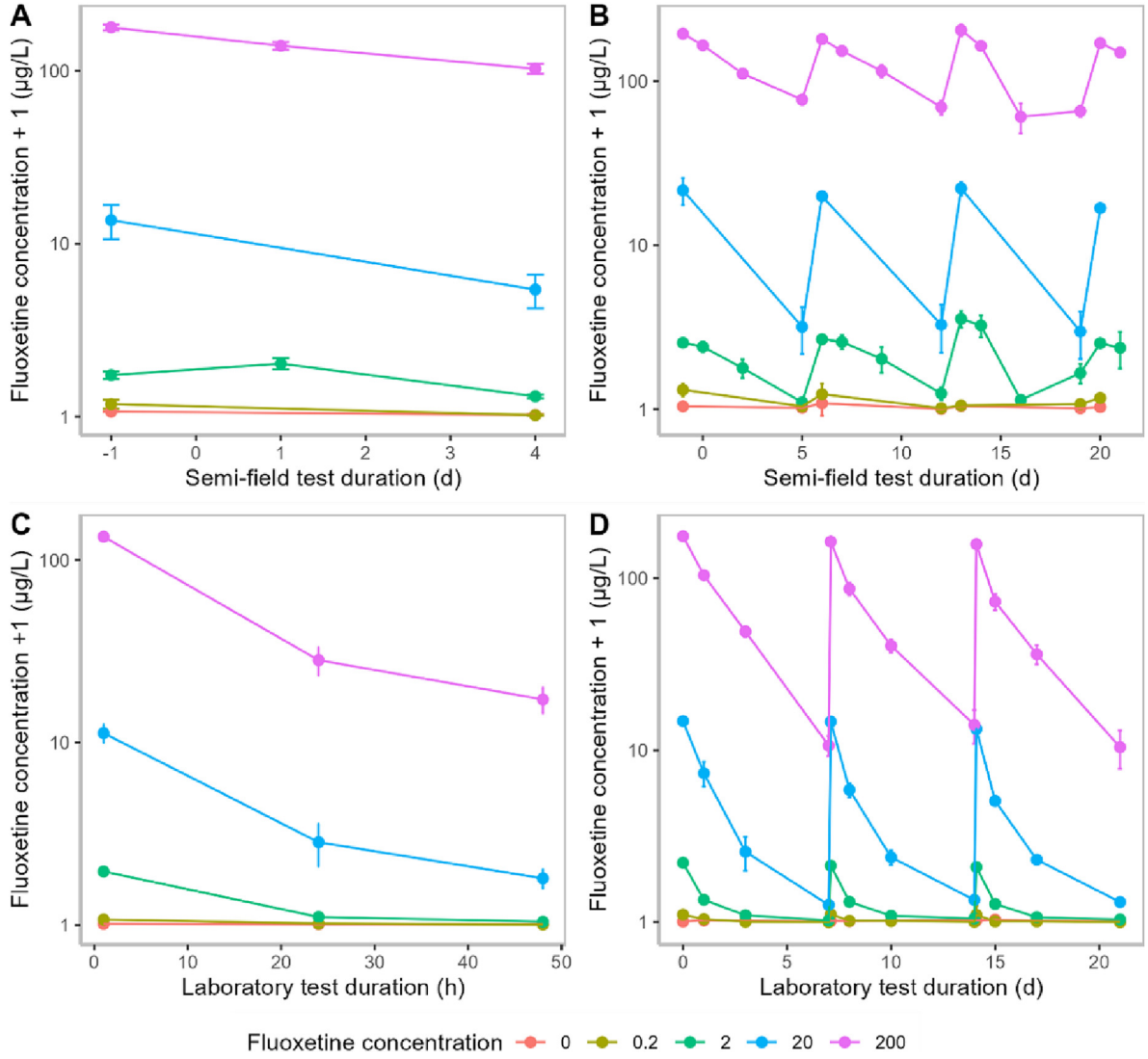


Fig. 2. Dynamics of fluoxetine in the water of the short-term (A) and long-term (B) semi-field tests and short-term (C) and long-term (D) laboratory tests. Mean values are shown and error bars indicate 2× the standard error (SE).

concentration was nonsignificant (Table S6), indicating no difference in sensitivity between males and females towards fluoxetine. Therefore, in the next analyses males and females were grouped together.

3.2.2. Effects of experimental conditions on baseline swimming behaviour

Of the control treatments, swimming behaviour was recorded for 80 gammarids for both the 2- and the 21-days tests in the laboratory, while 60 gammarids were recorded for the 2- and 21-days tests in the semi-field test. Generally, the gammarids of the control treatments showed a change in swimming behaviour in response to the light being switched on or off (Fig. 3). This effect was clearly visible for the average swimming speed and curvature, and to a lesser extent for acceleration and thigmotaxis (Fig. 3). The swimming speed of gammarids was higher under light compared to dark conditions (Fig. 3A). The opposite pattern was observed for curvature, with a higher curvature when the light was switched on compared to when the light was off (Fig. 3D).

3.2.2.1. Effect of test locations on swimming behaviour of control individuals. To determine to what degree the experimental set-up affected gammarid swimming behaviour, we explored the effects of test location (laboratory versus semi-field conditions) and test duration (2- versus 21-days) on swimming speed, acceleration, thigmotaxis, curvature, startle response duration, and startle response magnitude by analysing the data of the individuals in the control treatments.

Starting with test location, when comparing swimming behaviour between tests performed in the laboratory and under semi-field conditions, we found statistically significant differences for both short-term and long-term exposure (Table 1). A significant interaction between time bins and location was found for all swimming behavioural endpoints but for the startle response only for the short-term (2 days) test (Table 1).

For swimming speed, the interaction was caused by divergence in speed over the dark-light phases, with in the first dark phase the most pronounced differences between laboratory and semi-field conditions (Fig. 3A, S7A). In addition, mean speed was higher for individuals kept under semi-field conditions, compared to individuals kept under laboratory conditions, with largest differences found for the short-term (2 days) tests (Fig. 3A).

Individuals kept for 2 days under semi-field conditions accelerated significantly faster, especially during the first 140 s, compared to individuals kept in the laboratory (Table 1, Fig. 3B). In contrast, after 21 days the highest accelerations were found for individuals in the laboratory for a few time bins in the first light phase and the second dark phase (based on visual inspection of Fig. S7B).

The interaction effect between time bins and location for thigmotaxis (Table 1) caused a larger distance from the centre of the Petri dish for

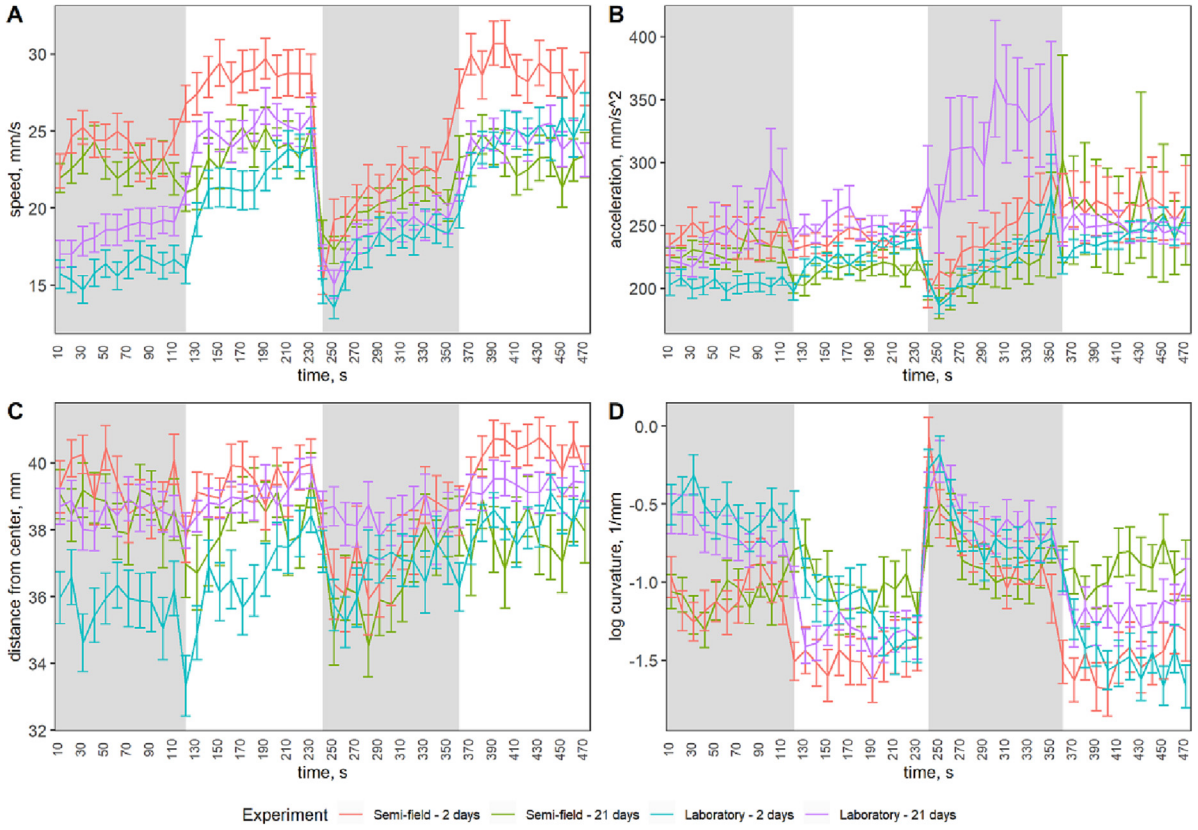


Fig. 3. Mean swimming behaviour of all control *G. pulex* individuals that were kept under laboratory or semi-field conditions over 10s time bins, with swimming behaviour described by swimming speed (A), acceleration (B), thigmotaxis (C), and log curvature (D). The grey boxes indicate the periods that the light was switched off, while the light was switched on in the white areas. Error bars indicate 2× the standard error (SE).

Table 1
Results of the mixed linear models that tested theeffects of test location (laboratory or semi-field), or test duration (2- or 21-days) on swimming behavioural endpoints of *G. pulex*. The values indicate the F-values, with the associated *p*-values in between brackets. The p-values are bold when indicating significance ($p < 0.05$).

	Semi-field versus laboratory (location)		2 versus 21 days (duration)	
	2 days	21 days	Laboratory	Semi-field
Swimming speed				
Time bins	2.51(0.11)	7.14(**0.01**)	13.55(**<0.01**)	14.38(**<0.01**)
Location or duration[a]	32.66(**<0.01**)	10.07(**0.01**)	3.21(0.09)	0(1)
Time bins x location or duration[a]	29.26(**<0.01**)	19.41(**<0.01**)	11.74(**<0.01**)	12.96(**<0.01**)
Acceleration				
Time bins	0.16(0.69)	2.52(0.11)	2.99(0.08)	3.37(0.07)
Location or duration[a]	19.26(**<0.01**)	0(0.97)	6.06(**0.03**)	0.85(0.38)
Time bins x location or duration[a]	13.23(**<0.01**)	5.28(**0.02**)	8.37(**<0.01**)	1.8(0.18)
Thigmotaxis				
Time bins	2.1(0.15)	4.46(**0.03**)	3.22(0.07)	4.49(**0.03**)
Location or duration[a]	34.33(**<0.01**)	0.34(0.57)	19.13(**<0.01**)	0.27(0.61)
Time bins x location or duration[a]	11.47(**<0.01**)	7.49(**0.01**)	12.8(**<0.01**)	5.1(**0.02**)
Curvature				
Time bins	4.22(**0.04**)	10.87(**<0.01**)	11.54 < **0.01**)	11.99(**<0.01**)
Location or duration[a]	6.68(**0.02**)	8.86(**0.01**)	1.9(0.19)	0.06(0.81)
Time bins x location or duration[a]	29.76(**<0.01**)	28.61(**<0.01**)	16.32(**<0.01**)	14.87(**<0.01**)
Startle response magnitude				
Startle response number	17.91(**<0.01**)	1.02(0.36)	5.73(**<0.01**)	0.93(0.4)
Location or duration[a]	0.38(0.55)	4.52(0.05)	1.67(0.22)	0.07(0.8)
Startle response number x location or duration[a]	3.99(**0.02**)	1.9(0.15)	0.2(0.82)	7.67(**<0.01**)
Startle response duration				
Startle response number	18.43(**<0.01**)	1.26(0.29)	0.92(0.4)	1.52(0.22)
Location or duration[a]	25.32(**<0.01**)	3.08(0.1)	1.96(0.18)	3.02(0.11)
Startle response number x location or duration[a]	14.32(**<0.01**)	0.41(0.66)	0.08(0.92)	10.24(**<0.01**)

[a] For the semi-field versus laboratory analyses the factor location was used, whilst for the 2 versus 21 days the factor duration was used.

individual time bins within all phases in the short-term semi-field test, with the exception of the 2nd dark phase (Fig. 3C). This indicates that individuals swam more in the outer zone of the arena after being kept for 2 days under semi-field conditions compared to individuals that were kept for 2 days under laboratory conditions. However, after 21 days those differences were less clearly visible (Fig. S7C). Nevertheless, a significant interaction between time bins and location was found, and we measured a larger thigmotaxis in the 2nd dark phase for individuals that were kept in the laboratory, but this was only true for a few time bins (based on visual inspection of Fig. S7C).

The curvature was significantly lower for individuals in the semi-field tests compared to individuals kept under laboratory conditions after both 2- and 21-days (Fig. 3D), but this effect was caused mainly due to the differences in the first dark period (Fig. S7D). Hence, individuals kept under semi-field conditions swam more straight compared to individuals that were kept under laboratory conditions in the first dark period.

For the startle response magnitude, i.e., magnitude of change in swimming speed to the light switching on or off, post-hoc tests indicated that turning the light off (startle response 2) evoked a stronger startle response (more negative) for individuals kept under semi-field conditions compared to laboratory conditions (Fig. S8A). Interestingly, however, in all experiments, a larger drop in swimming speed was found when the light was switched off compared to the light being switched on, but this is only significant for individuals that were kept for 2 days under semi-field conditions (Fig. S8A). Lastly, when light was switched on (startle response 1 and 3) a larger startle response duration, i.e., the time an organism needs to recover from its startle response to its average swimming speed in the consecutive phase, was found for individuals kept under laboratory conditions in the short-term test (Fig. S8B).

3.2.2.2. Effect of test durations on swimming behaviour of control individuals. Test duration (2 and 21 days), also significantly affected the swimming behaviour of *G. pulex* (Table 1). A significant interaction between time bins and duration was found for swimming speed for individuals kept both under laboratory and semi-field conditions (Table 1). Indeed, swimming speed was higher in the light period for both test conditions (laboratory and semi-field; Fig. 3A) and differed significantly for a few time bins (based on visual inspection of Fig. S9A). In addition, individuals accelerated significantly faster after 21 days compared to 2 days in the laboratory, with largest differences found in the dark phases (Fig. S9B). For the semi-field, no significant differences were found for acceleration (Table 1).

Furthermore, for thigmotaxis an significant interaction effect between time bins and duration was found for both locations (Table 1). Thigmotaxis appeared to be larger in the laboratory after 21 test days than after 2 days, with animals swimming more on the outer zone of the arena (Fig. S9C). However, under semi-field conditions, only two of the time bins differed (based on visual inspection of Fig. S9C), both in the last light period, with a greater thigmotaxis for individuals in the short-term test.

For curvature, a significant interaction in the semi-field was caused by individuals swimming more straight in the light periods after 2 testing days compared to 21 days (Table 1, Fig. 3). Additionally, we found a significant interaction effect for individuals that were kept under laboratory conditions, with significant differences found at the first time bin of the first light period ($t = 130$) and for one time bin in the last light period (based on visual inspection of Fig. S9D).

Differences between startle responses of individuals tested for 2- or 21-days were only found for individuals kept under semi-field conditions (Table 1). For the startle response magnitude, as well as for the startle response duration, those differences were found when the light was turned off (startle response 2). After 2 test days, the magnitude was higher (Fig. S8A) and had a longer duration (Fig. S8B), indicating that individuals needed a longer time to recover compared to 21 days.

3.2.3. Effects of fluoxetine on swimming behaviour

In total, we recorded the swimming behaviour of 280 gammarids during two 2-min dark/2-min light cycles. In each of the short-term and long-term tests in the laboratory a total of 80 individuals were used, and 60 individuals in each of the short- and long-term semi-field tests. In general, the behavioural endpoints followed the same pattern during the dark/light cycles as described for the baseline behaviour (control data, paragraph 3.2.2), with light mostly affecting the endpoints swimming speed, thigmotaxis and curvature as indicated by the significant effect of time bins (Table 2).

Fluoxetine exposure did not affect swimming behavioural endpoints of *G. pulex* significantly over the total recorded time (Table 2). However, significant interactions between fluoxetine treatments and time bins were observed for the 21 days semi-field test and the 2 days laboratory test (Table 2). For one time bin in the first dark period, a smaller distance from the centre was found for individuals exposed to 200 μg/L compared to the control individuals kept under semi-field conditions for 21 days (Fig. S10, Table S7). For the short-term laboratory test, a significant interaction was found for swimming speed, acceleration and curvature (Table 2). For all three endpoints, at least one fluoxetine treatment differed from the control during one particular time bin (at 280 s) in the second dark period (Table S7). During this time bin, swimming speed and acceleration was lower for individuals exposed to 2 μg/L and 20 μg/L compared to control individuals (Fig. 4 and S11), while a higher mean curvature was found for the 20 μg/L treated individuals (Fig. S12). In addition, we found a lower acceleration for individuals exposed to 20 μg/L for another time bine (at 250 s) in the second dark period and in the first dark period (at 60s) for individuals exposed to 2 μg/L (Fig. S11, Table S7). Startle responses caused by light stimuli were not influenced by fluoxetine (Table 2), as we did not find an effect of fluoxetine on the startle response magnitude (Fig. S13), nor on the duration (Fig. S14).

Table 2
Linear mixed effect model output (F-values with *p*-values between brackets) for the different swimming behavioural endpoints of *G. pulex* for the semi-field and laboratory tests with a exposure duration of 2- and 21-days. The p-values are bold when indicating significance ($p < 0.05$).

	Semi-field tests		Laboratory tests	
	2 days	21 days	2 days	21 days
Swimming speed				
Time bins	3.07(0.08)	15.4(**<0.01**)	69.5(**<0.01**)	13.55(**<0.01**)
FLU concentration	0.18(0.95)	2.25(0.12)	2.23(0.1)	0.27(0.9)
Time bins x FLU concentration	1.02(0.39)	1.84(0.12)	2.85(**0.02**)	0.52(0.72)
Acceleration				
Time bins	0.11(0.75)	7.2(**0.01**)	44.11(**<0.01**)	2.04(0.15)
FLU concentration	0.43(0.79)	0.69(0.61)	1.97(0.14)	0.23(0.92)
Time bins x FLU concentration	1.32(0.26)	1.26(0.28)	3.7(**0.01**)	0.25(0.91)
Thigmotaxis				
Time bins	1.88(0.17)	4.71(**0.03**)	28.49(**<0.01**)	4.19(**0.04**)
FLU concentration	1.03(0.43)	1.63(0.23)	1.96(0.14)	0.85(0.51)
Time bins x FLU concentration	0.28(0.89)	2.75(**0.03**)	2.06(0.08)	1.71(0.15)
Curvature				
Time bins	5.94(**0.01**)	12.31(**<0.01**)	74.9(**<0.01**)	12.33(**<0.01**)
FLU concentration	0.17(0.95)	2.69(0.08)	2.3(0.1)	0.41(0.8)
Time bins x FLU concentration	0.83(0.51)	1.79(0.13)	3(**0.02**)	0.61(0.66)
Startle response magnitude				
Startle response number	18.91(**<0.01**)	0.98(0.38)	4.35(**0.01**)	7.77(**<0.01**)
FLU concentration	0.49(0.74)	0.3(0.87)	1.46(0.25)	0.49(0.74)
Startle response number x FLU concentration	0.61(0.77)	1.12(0.35)	0.65(0.74)	0.84(0.57)
Startle response duration				
Startle response number	19.98 (**<0.01**)	1.35(0.26)	1.23(0.29)	0.87(0.42)
FLU concentration	1.81(0.19)	0.66(0.63)	1.76(0.18)	0.59(0.67)
Startle response number x FLU concentration	0.8(0.6)	1.61(0.12)	1.27(0.26)	0.58(0.79)

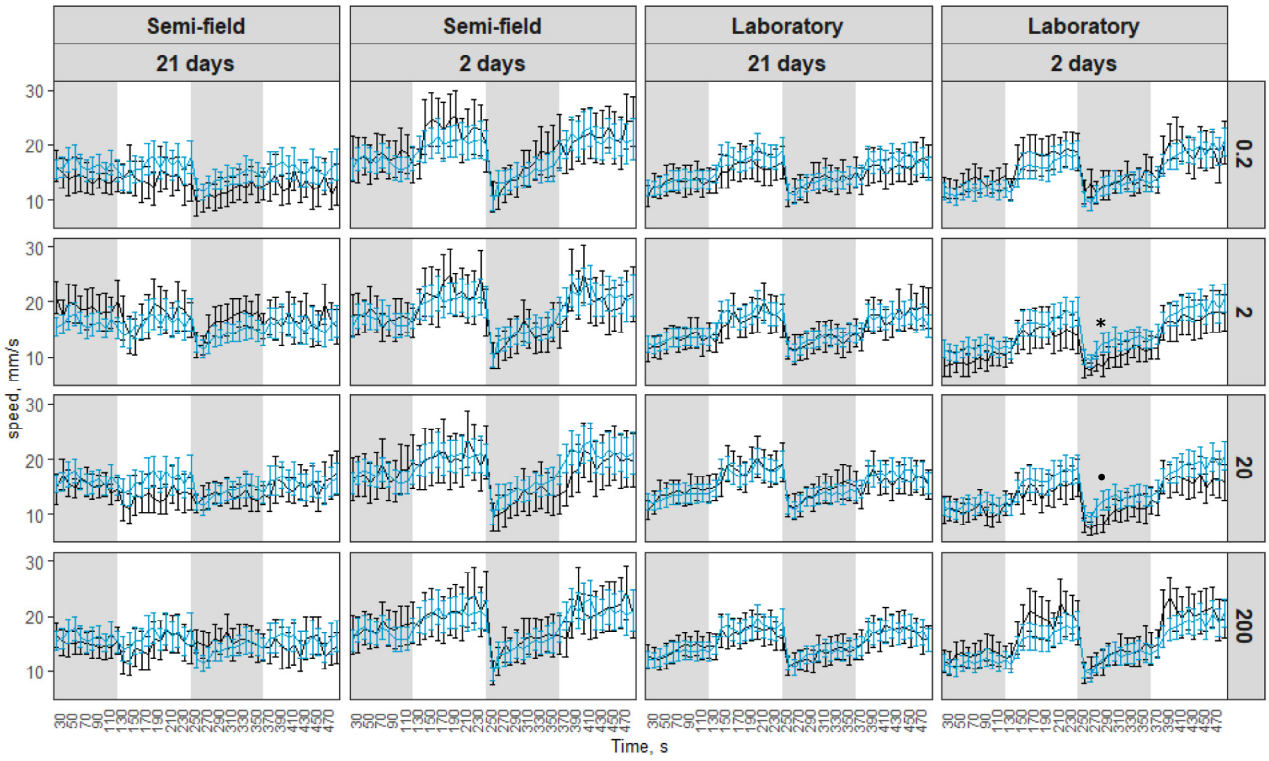


Fig. 4. Average swimming speed of *G. pulex* over time for chronic laboratory, acute laboratory, chronic semi-field, and acute semi-field. The panels show the average swimming speed of treated (black line) and untreated (blue line) individuals for each concentration. Error bars indicate the 95 % confidence intervals. * $p \leq 0.05$.

Interestingly, the highest among individual variation, characterized by the mean coefficient of variation (CV), was generally found for the same treatments (2 and 20 μg/L) for all endpoints (Table S8). For example, speed, acceleration, thigmotaxis and curvature had the highest CV values for the test concentration of 2 and 20 μg/L for the 2 days laboratory test.

4. Discussion

As many contaminants, including pharmaceuticals, are typically found at levels below lethal concentration in the environment and cause subtle effects, non-lethal behavioural endpoints are becoming increasingly important in ecotoxicology (Ford et al., 2021; Schuijt et al., 2021). In this study, we show that effects found on swimming behaviour of *Gammarus pulex* were larger due to experimental conditions than due to tested antidepressant concentrations. Both test durations (2- and 21-days) and test locations (laboratory and semi-field) influence swimming behavioural endpoints, though larger effects were found for test locations than test durations. Contrary to our prediction, we observed only a few changes in swimming behaviour due to exposure to fluoxetine, while during most time bins the behavioural endpoints did not differ from the control (Table 3).

As expected, fluoxetine did not have an effect on the mortality of *G. pulex,* as acute toxicity tests reported lethal concentrations (LC50s) at a range of 234–43,000 μg/L for crustaceans (Brooks et al., 2003b). Our results are also in accordance with previous reports of low mortality (<15 %) after exposure to fluoxetine for the marine amphipod *Echinogammarus marinus* (from 0.01 to 10 μg/L) (Bossus et al., 2014; Guler and Ford, 2010) and *G. pulex* (0.1 μg/L)(De Castro-Català et al., 2017).

Neither did we find an effect of fluoxetine on the feeding rate of *G. pulex,* whilst De Castro-Català et al. (2017) found a reduction in feeding rate of *G. pulex* after fluoxetine exposure through both food and water (0.1 μg/L) simultaneously. However, when fluoxetine exposure was only through water, no effects on feeding rates were found either (De Castro-Català et al., 2017). Mixed results have also been found on the effect of fluoxetine on the feeding rates of *Daphnia magna*. For *Daphnia magna,* no effect of fluoxetine (10, 40, 80 μg/L) on feeding rates were found by Campos et al. (2012), whilst Stanley et al. (2007) reported an increase in grazing at mid-range concentrations of 200 μg/L, but not at high concentrations (450 μg/L).

We found among-individual behavioural variation to be generally highest in the fluoxetine treatment compared to the control. This increase in variability might be caused by behavioural changes of some individuals induced by fluoxetine. In a study with snails exposed to fluoxetine (0.003 μg/L) they found on average a higher among-individual variation in locomotor activity, but this was not significant.

Contrary to expectation, fluoxetine exposure (nominal concentrations: 0.2, 2, 20, and 200 μg/L), caused only a few significant impacts on the swimming behavioural endpoints that were tested. This result was surprising, as previous studies using similar fluoxetine concentrations reported effect on the swimming behaviour of amphipods (Bossus et al., 2014; De Lange et al., 2006; Guler and Ford, 2010). More specifically, Guler and Ford (2010) exposed *E. marinus* individuals to 0.01, 0.1, 1, 10 μg/L fluoxetine and found at 0.1 μg/L an increase in phototaxis behaviour as animals spent more time in the light after 7, 14 and an increased geotaxis at 0.1 μg/L after 7 days with animals higher up in a water column. Effects of fluoxetine on swimming velocity of *E. marinus* was found for all tested concentrations (0.001, 0.01, 0.1 and 1 μg/L) after 1 day exposure (Table 3), but no effects were found after 1 h or 8 days of exposure (Bossus et al., 2014). De Lange et al. (2006) tested a broad range of fluoxetine concentrations from 0.0001 to 1000 μg/L and observed for *G. pulex* a decrease in locomotion at 0.1 μg/L after 1.5 h exposure (Table 3). Contrastingly, similar to this study, a more recent study by Kohler (2019) observed few significant impacts of fluoxetine on behavioural endpoints of *E. marinus* and *G. pulex* (Table 3).

It is clear that some mixed results, with regard to the effects of fluoxetine on gammarids, can be found in the literature. A possible explanation for these discrepancies between studies could be due to differences in

Table 3
Summary of the results of fluoxetine on all evaluated endpoints for *G. pulex*. Arrows indicate a treatment-related increase (↑) or decrease (↓). We searched for literature on effects of fluoxetine on amphipods. When no literature on amphipods was available, we included other aquatic species.

Endpoint	Semi-field tests		Laboratory tests		Literature	
	2 d	21 d	2 d	21 d	Effects	Reference
Mortality	None	None	None	None	*G. pulex*: None at 0.1 μg/L	De Castro-Català et al. (2017)
					E. marinus: None (0.01–10 μg/L)	Bossus et al. (2014), Guler and Ford (2010)
Feeding rate	None	None	None	None	*G. pulex:* 0.1 μg/L ↓	De Castro-Català et al. (2017)
Swimming speed	None	None	2↓[a] and 20↓[a]	None	*E. marinus* exp. 1:	Kohler (2019)
					- 1 day: 1 μg/L ↑	
					- 14 days: 1 μg/L ↑(light) ↓(dark)	
					E. marinus exp. 2:	
					- 1 day: 1 μg/L ↑[a]	
					G. pulex:	
					- 1 day: 0.01 μg/L↓[a]	
					- 1 week: 1 μg/L↓[a]	
					G. pulex:	De Lange et al. (2006)
					- 1.5 h: 0.1 μg/L↓ (locomotion)	
					E. marinus:	Bossus et al. (2014)
					- 1 day: 0.001↑, 0.01↑, 0.1↑, 1↑ μg/L	
Acceleration	None	None	2↓[a] and 20↓[a]	None	*Nothobranchius furzeri*: 0.5 μg/L ↑ (maximum acceleration)	Thoré et al. (2021)
Thigmotaxis	None	200↓[a]	None	None	*E. marinus* exp. 1: 0.001 μg/L ↑ (less time in centre)	Kohler (2019)
					E. marinus exp. 2: 1 μg/L ↑	
					G. pulex: None (0.001–1 μg/L)	
Curvature	None	None	20↑[a]	None	*Danio rerio*: None on angles of the fish trajectory (0.1–10 μg/L)	Correia et al. (2022a)
					Aphanius dispar: None on angular acceleration (0.03 & 3 μg/L)	Barry (2013)
Startle responses	None	None	None	None	*Danio rerio* embryos: impaired the startle response (100 μg/L ↓)	Huang et al. (2019)
					Danio rerio embryos: reduced startle response (1–4000 μg/L ↓)	Zindler et al. (2020)

[a] Differences were found for only one or a few time bins.

experimental design and conditions. The different experimental conditions in our study, short- vs. long-term test (test durations) and semi-field vs. laboratory test (test locations), did influence the swimming behaviour of *G. pulex* (Table 4).

Individuals in the semi-field test swam faster and more straight compared to individuals in the laboratory and showed a stronger startle response evoked by turning off the light. One possibility is that the difference in light intensity between laboratory and semi-field conditions might explain the change in swimming speed and curvature. Individuals in the semi-field tests where kept in outdoor mesocosms with natural light conditions, hence, light intensity was about 100 times higher compared to laboratory conditions (can be >1000 μmol s^{-1} m^{-2} in absence of shade (Hill and Dimick, 2002) versus 8 μmol s^{-1} m^{-2} in the laboratory). Indeed, for larval zebrafish, it has been found that different light conditions during rearing can influence behavioural outcomes (Fraser et al., 2017). However, Fraser et al. (2017) assessed the effect the effect of day/night cycle vs. constant darkness, instead of different light intensities. With regard to *Gammarus*, it has been previously shown that the activity depends on light conditions, with organisms being more active during the night than in daylight (Holomuzki and Hoyle, 1990; Williams and Moore, 1985). Hence, the 50 μmol s^{-1} m^{-2} light intensity during our recoding of the behavioural endpoints is still substantially lower than daylight, which might have caused the observed higher swimming speed of individuals kept under semi-field conditions.

When comparing short- and long-term test durations, we found under laboratory conditions that gammarids swam and accelerated slower and spent more time at the inner zone of the arena after 2 days compared to 21 days. One explanation for this could be the difference in size of *Gammarus pulex* between the two experiments. However, in a previous study we found that size could not explain a large part of the among-individual variability in the swimming behaviour of *G. pulex* within a small size range of 1 mm (Van den Berg et al., 2023). Therefore, a likely cause of this lower activity in the 2 days test is a reduction in the amphipods' energy supply, as no food was provided in the 2 days test. Indeed, a reduction in swimming activity of *G. pulex* has been reported after 7 days without food (Peeters et al., 2009), and a reduction in ventilation activity after 2 days (Alonso et al., 2010). In the semi-field test, a less strong but reverse pattern was observed with higher swimming speed and individuals swimming more in the outer zone of the arena after 2 days compared to 21 days. Contrary to the 2 days laboratory test, food was provided during the 2 days semi-field test, potentially explaining this reversed pattern.

Experimental conditions also had an impact on whether we found an effect of fluoxetine on swimming behaviour. While no effects were found for the 2 days semi-field and the 21 days laboratory tests, we found for one time bin a decrease in thigmotaxis at 200 μg/L for the 21 days semi-field test and some significant effects during the 2 days laboratory test for three swimming behavioural endpoints (Table 3). Experimental conditions

Table 4
Summary of the effects on test location (semi-field versus laboratory) and test duration (2- versus 21-days) on swimming behavioural endpoints of the control gammarids. Arrows indicate whether behavioural endpoints are significantly higher (>) or lower (<) under semi-field conditions compared to laboratory conditions when assessing effect of test locations and are higher (>) or lower (<) after 2 days exposure compared 21 days exposure when assessing effect of test durations.

Endpoint	Test location		Test duration	
	2 days	21 days	Semi-field	Laboratory
Swimming speed	Semi-field > Lab	Semi-field > Lab	2 > 21 days[a]	2 < 21 days[a]
Acceleration	Semi-field > Lab	Semi-field < Lab[a]	NS	2 < 21 days
Thigmotaxis	Semi-field > Lab	Semi-field < Lab[a]	2 > 21 days[a]	2 < 21 days
Curvature	Semi-field < Lab	Semi-field < Lab	2 < 21 days[a]	None
Startle response magnitude	Semi-field > Lab	None	2 > 21 days	None
Startle response duration	Semi-field < Lab	Semi-field < Lab	2 > 21 days	None

[a] Differences were found only for a few time bins.

clearly had an effect on the fate of fluoxetine in the water. Time-weighted average fluoxetine concentrations were 60 % lower in the laboratory compared to the semi-field. Even though the effects of fluoxetine were observed for only a few time bins, this illustrates the importance of experimental conditions when measuring behavioural endpoints. Also, Fraser et al. (2017) stressed the importance of methodological practices, as they found a considerable effect of experimental conditions on behaviour outcomes after exposure of larval zebrafish to toxic compounds, but also on baseline behaviour.

The impact of experimental design on behavioural outcomes can also be observed after comparing two studies, performed in the same laboratory, investigating the effect of fluoxetine on *E. marinus* (Bossus et al., 2014; Kohler, 2019). Whereas Bossus et al. (2014) found an increase in swimming activity, this effect was not observed in the study of Kohler (2019) as shown in Table 3. Although in both studies animal exposure and compound preparation were the same across the experiments, species collection, length of acclimation and shape and size of arenas differed. Next to that, a review on zebrafish embryo's, one of the most used species in toxicant induced behavioural changes testing, showed as well that experimental conditions have a high impact on the outcome and thus comparability between studies (Ogungbemi et al., 2019). Ultimately, understanding and controlling experimental conditions, consisting of biological factors (e.g. rearing, time of day for behaviour analysis, tested endpoints) and technical factors (e.g. test duration, light conditions, test location, size and shape of arena) (Ogungbemi et al., 2019), in combination with harmonizing and standardizing protocols would increase the comparability between behavioural ecotoxicology studies (Ford et al., 2021; Moermond et al., 2016).

In the end, the main goal of most behavioural studies is to translate effects found after exposure into long-term effects on the individual fitness, population viability and/or ecological impacts. In this study, *G. pulex* individuals were caged and exposed in aquatic mesocosms to which fluoxetine was applied, whereafter the swimming behaviour was recorded. Next to the caged *G. pulex* individuals, the mesocosms contained also an uncaged *G. pulex* population which was monitored over the course of the experiment as part of another study (Schuijt, personal communication). No fluoxetine related population changes of *G. pulex* were found in the mesocosms (Schuijt, personal communication). Hence, the very few significant impacts of fluoxetine on the behaviour of *G. pulex* observed are corresponding with the absence of population effects in the mesocosms.

5. Conclusions

The present study highlights the importance of experimental conditions when measuring behavioural endpoints. While effects of fluoxetine on swimming behaviour of *Gammarus pulex* were only observed for some sporadic time bins, effects of test durations (2 versus 21 days) and test locations (laboratory versus semi-field) on swimming behaviour was evident. With an increasing number of studies reporting behavioural data as an endpoint for assessing effects of chemical stressors, the results demonstrate how differences in experimental conditions might lead to different conclusions. Hence, we emphasise the importance to include and have knowledge of temporal, biological, experimental, multi-laboratory and among-individual variation in behavioural studies. In addition, as many factors can influence the behaviour of organisms, standardization is essential to improve reproducibility and reliability of behavioural endpoints.

CRediT authorship contribution statement

Lara Schuijt: Conceptualization, Investigation, Methodology, Statistical analysis, Writing - original draft. Oluwafemi Olusoiji: Statistical analysis. Asmita Dubey: Methodology, Writing - review & editing. Pablo Rodríguez-Sánchez: Software, Writing - review & editing. Rima Osman: Methodology. Paul Van den Brink: Writing - review & editing. Sanne van den Berg: Conceptualization, Investigation, Methodology, Software, Statistical analysis, Writing - original draft.

Data availability

We made all raw data and R scripts used to process this data publicly available on figshare (https://doi.org/10.6084/m9.figshare.20358942.v1).

Declaration of competing interest

There is no conflict of interest to declare.

Acknowledgements

This project was funded by TKI (Topconsortium voor Kennis en Innovatie) Deltatechnology, jointly financed by the Dutch government and commercial parties through the Precaution project: PREdicting the Chemical sensitivity of AqUaTIc OrgaNisms. This project was also funded by the Netherlands Organization for Scientific Research (NWO) domain TTW through the EMERCHE project: Effect-directed Monitoring tools to assess Ecological and human health Risks of CHemicals of Emerging concern in the water cycle (File number 15760).

Appendix A. Supplementary data

All of our data handling, transformations, and statistics were performed in R (version 4.0.2, R Core Team, 2020), and all scripts and raw data are available on figshare (https://doi.org/10.6084/m9.figshare.20517939.v1). Supplementary data to this article can be found online at doi:https://doi.org/10.1016/j.scitotenv.2023.162173.

References

Alonso, A., Garcia-Johansson, V., De Lange, H.J., Peeters, E.T., 2010. Effects of animal starvation on the sensitivity of the freshwater amphipod Gammarus pulex to cadmium. Chem. Ecol. 26 (3), 233–242.
Amiard-Triquet, C., 2009. Behavioral disturbances: the missing link between sub-organismal and supra-organismal responses to stress? Prospects based on aquatic research. Hum. Ecol. Risk. Assess. 15 (1), 87–110.
Badyaev, A.V., 2005. Stress-induced variation in evolution: from behavioural plasticity to genetic assimilation. Proc. R. Soc. B Biol. Sci. 272 (1566), 877–886.
Barry, M.J., 2013. Effects of fluoxetine on the swimming and behavioural responses of the Arabian killifish. Ecotoxicology 22 (2), 425–432.
Bossus, M.C., Guler, Y.Z., Short, S.J., Morrison, E.R., Ford, A.T., 2014. Behavioural and transcriptional changes in the amphipod Echinogammarus marinus exposed to two antidepressants, fluoxetine and sertraline. Aquat. Toxicol. 151, 46–56.
Boxall, A.B., Rudd, M.A., Brooks, B.W., Caldwell, D.J., Choi, K., Hickmann, S., Innes, E., Ostapyk, K., Staveley, J.P., Verslycke, T., 2012. Pharmaceuticals and personal care products in the environment: what are the big questions? Environ. Health Perspect. 120 (9), 1221–1229.
Brausch, J.M., Connors, K.A., Brooks, B.W., Rand, G.M., 2012. Human pharmaceuticals in the aquatic environment: a review of recent toxicological studies and considerations for toxicity testing. Rev. Environ. Contam. Toxicol. 218, 1–99.
Brodin, T., Piovano, S., Fick, J., Klaminder, J., Heynen, M., Jonsson, M., 2014. Ecological effects of pharmaceuticals in aquatic systems—impacts through behavioural alterations. Philos. Trans. R. Soc., B Biol. Sci. 369 (1656), 20130580.
Brooks, B.W., Foran, C.M., Richards, S.M., Weston, J., Turner, P.K., Stanley, J.K., Solomon, K.R., Slattery, M., La Point, T.W., 2003a. Aquatic ecotoxicology of fluoxetine. Toxicol. Lett. 142 (3), 169–183.
Brooks, B.W., Turner, P.K., Stanley, J.K., Weston, J.J., Glidewell, E.A., Foran, C.M., Slattery, M., La Point, T.W., Huggett, D.B., 2003b. Waterborne and sediment toxicity of fluoxetine to select organisms. Chemosphere 52 (1), 135–142.
Campos, B., Pina, B., Barata, C.C., 2012. Mechanisms of action of selective serotonin reuptake inhibitors in Daphnia magna. Environ. Sci. Technol. 46 (5), 2943–2950.
Corcoran, J., Winter, M.J., Tyler, C.R., 2010. Pharmaceuticals in the aquatic environment: a critical review of the evidence for health effects in fish. Crit. Rev. Toxicol. 40 (4), 287–304.
Correia, D., Domingues, I., Faria, M., Oliveira, M., 2022a. Chronic effects of fluoxetine on Danio rerio: a biochemical and behavioral perspective. Appl. Sci. 12 (4), 2256.
Correia, D., Domingues, I., Faria, M., Oliveira, M., 2022b. Effects of fluoxetine on fish: what do we know and where should we focus our efforts in the future? Sci. Total Environ. 159486.
De Castro-Català, N., Muñoz, I., Riera, J., Ford, A., 2017. Evidence of low dose effects of the antidepressant fluoxetine and the fungicide prochloraz on the behavior of the keystone freshwater invertebrate Gammarus pulex. Environ. Pollut. 231, 406–414.
De Lange, H., Noordoven, W., Murk, A., Lürling, M., Peeters, E., 2006. Behavioural responses of Gammarus pulex (Crustacea, Amphipoda) to low concentrations of pharmaceuticals. Aquat. Toxicol. 78 (3), 209–216.
Do Carmo, M.P., 2016. Differential Geometry of Curves and Surfaces: Revised and Updated Second Edition. Courier Dover Publications.

de Farias, N.O., Oliveira, R., Sousa-Moura, D., de Oliveira, R.C.S., Rodrigues, M.A.C., Andrade, T.S., Domingues, I., Camargo, N.S., Muehlmann, L.A., Grisolia, C.K., 2019. Exposure to low concentration of fluoxetine affects development, behaviour and acetylcholinesterase activity of zebrafish embryos. Comp. Biochem. Physiol. C: Toxicol. Pharmacol. 215, 1–8.
Fekadu, S., Alemayehu, E., Dewil, R., Van der Bruggen, B., 2019. Pharmaceuticals in freshwater aquatic environments: a comparison of the African and European challenge. Sci. Total Environ. 654, 324–337.
Feynman, R.P., Leighton, R.B., Sands, M., 2011. The Feynman Lectures on Physics. The New Millennium Edition: Mainly Mechanics, Radiation, and Heatvol. I. Basic Books.
Fong, P.P., Ford, A.T., 2014. The biological effects of antidepressants on the molluscs and crustaceans: a review. Aquat. Toxicol. 151, 4–13.
Ford, A.T., Ågerstrand, M., Brooks, B.W., Allen, J., Bertram, M.G., Brodin, T., Dang, Z., Duquesne, S., Sahm, R., Hoffmann, F., 2021. The role of behavioral ecotoxicology in environmental protection. Environ.Sci.Technol. 55 (9), 5620–5628.
Fraser, T.W., Khezri, A., Jusdado, J.G., Lewandowska-Sabat, A.M., Henry, T., Ropstad, E., 2017. Toxicant induced behavioural aberrations in larval zebrafish are dependent on minor methodological alterations. Toxicol. Lett. 276, 62–68.
Guler, Y., Ford, A.T., 2010. Anti-depressants make amphipods see the light. Aquat. Toxicol. 99 (3), 397–404.
Hamilton, T.J., Kwan, G.T., Gallup, J., Tresguerres, M., 2016. Acute fluoxetine exposure alters crab anxiety-like behaviour, but not aggressiveness. Sci. Rep. 6 (1), 1–6.
Hazelton, P.D., Cope, W.G., Mosher, S., Pandolfo, T.J., Belden, J.B., Barnhart, M.C., Bringolf, R.B., 2013. Fluoxetine alters adult freshwater mussel behavior and larval metamorphosis. Sci. Total Environ. 445, 94–100.
Hill, W.R., Dimick, S.M., 2002. Effects of riparian leaf dynamics on periphyton photosynthesis and light utilisation efficiency. Freshw. Biol. 47 (7), 1245–1256.
Holomuzki, J.R., Hoyle, J.D., 1990. Effect of predatory fish presence on habitat use and diel movement of the stream amphipod,Gammarus minus. Freshw. Biol. 24 (3), 509–517.
Hothorn, T., Bretz, F., Westfall, P., Heiberger, R.M., Schuetzenmeister, A., Scheibe, S., Hothorn, M.T., 2016. Package 'multcomp'.
Huang, I.J., Sirotkin, H.I., McElroy, A.E., 2019. Varying the exposure period and duration of neuroactive pharmaceuticals and their metabolites modulates effects on the visual motor response in zebrafish (Danio rerio) larvae. Neurotoxicol. Teratol. 72, 39–48.
Hughes, S.R., Kay, P., Brown, L.E., 2013. Global synthesis and critical evaluation of pharmaceutical data sets collected from river systems. Environ.Sci.Technol. 47 (2), 661–677.
Kidd, K.A., Blanchfield, P.J., Mills, K.H., Palace, V.P., Evans, R.E., Lazorchak, J.M., Flick, R.W., 2007. Collapse of a fish population after exposure to a synthetic estrogen. Proc. Natl. Acad. Sci. 104 (21), 8897–8901.
Kohler, S.A., 2019. Using Advanced Techniques for the Development of Behavioural Assays in Ecotoxicology: Assessing the Impacts of Psychotropic Compounds on Crustacean Behaviour. University of Portsmouth.
Lazzara, R., Blázquez, M., Porte, C., Barata, C., 2012. Low environmental levels of fluoxetine induce spawning and changes in endogenous estradiol levels in the zebra mussel Dreissena polymorpha. Aquat. Toxicol. 106, 123–130.
Le Roux, M., 1933. Recherches sur la sexualité des gammariens: croissance, reproduction, déterminisme des caractères sexuels secondaires, Labor. d'évolution des êtres organisés.
Lind, J., Cresswell, W., 2005. Determining the fitness consequences of antipredation behavior. Behav. Ecol. 16 (5), 945–956.
Loos, R., Gawlik, B.M., Locoro, G., Rimaviciute, E., Contini, S., Bidoglio, G., 2009. EU-wide survey of polar organic persistent pollutants in European river waters. Environ. Pollut. 157 (2), 561–568.
Maltby, L., Clayton, S.A., Wood, R.M., McLoughlin, N., 2002. Evaluation of the Gammarus pulex in situ feeding assay as a biomonitor of water quality: robustness, responsiveness, and relevance. Environ. Toxicol. Chem. Int. J. 21 (2), 361–368.
Martin, J.M., Bertram, M.G., Saaristo, M., Fursdon, J.B., Hannington, S.L., Brooks, B.W., Burket, S.R., Mole, R.A., Deal, N.D., Wong, B.B., 2019a. Antidepressants in surface waters: fluoxetine influences mosquitofish anxiety-related behavior at environmentally relevant levels. Environ.Sci.Technol. 53 (10), 6035–6043.
Martin, J.M., Saaristo, M., Tan, H., Bertram, M.G., Nagarajan-Radha, V., Dowling, D.K., Wong, B.B., 2019b. Field-realistic antidepressant exposure disrupts group foraging dynamics in mosquitofish. Biol. Lett. 15 (11), 20190615.
Melvin, S.D., Wilson, S.P., 2013. The utility of behavioral studies for aquatic toxicology testing: a meta-analysis. Chemosphere 93 (10), 2217–2223.
Moermond, C.T., Kase, R., Korkaric, M., Ågerstrand, M., 2016. CRED: criteria for reporting and evaluating ecotoxicity data. Environ. Toxicol. Chem. 35 (5), 1297–1309.
Mole, R.A., Brooks, B.W., 2019. Global scanning of selective serotonin reuptake inhibitors: occurrence, wastewater treatment and hazards in aquatic systems. Environ. Pollut. 250, 1019–1031.
Moreira, D.G., Aires, A., de Lourdes Pereira, M., Oliveira, M., 2022. Levels and effects of antidepressant drugs to aquatic organisms. Comp. Biochem. Physiol. C: Toxicol. Pharmacol. 109322.
Nielsen, S.V., Kellner, M., Henriksen, P.G., Olsén, H., Hansen, S.H., Baatrup, E., 2018. The psychoactive drug escitalopram affects swimming behaviour and increases boldness in zebrafish (Danio rerio). Ecotoxicology 27 (4), 485–497.
Ogungbemi, A., Leuthold, D., Scholz, S., Küster, E., 2019. Hypo-or hyperactivity of zebrafish embryos provoked by neuroactive substances: a review on how experimental parameters impact the predictability of behavior changes. Environ. Sci. Eur. 31 (1), 1–26.
Patel, M., Kumar, R., Kishor, K., Mlsna, T., Pittman Jr., C.U., Mohan, D., 2019. Pharmaceuticals of emerging concern in aquatic systems: chemistry, occurrence, effects, and removal methods. Chem. Rev. 119 (6), 3510–3673.
Peeters, E.T., De Lange, H., Lürling, M., 2009. Variation in the behavior of the amphipod Gammarus pulex. Hum. Ecol. Risk. Assess. 15 (1), 41–52.
Peters, J.R., Granek, E.F., de Rivera, C.E., Rollins, M., 2017. Prozac in the water: chronic fluoxetine exposure and predation risk interact to shape behaviors in an estuarine crab. Ecol. Evol. 7 (21), 9151–9161.
Pickell, P.D., Hermosilla, T., Frazier, R.J., Coops, N.C., Wulder, M.A., 2016. Forest recovery trends derived from landsat time series for North American boreal forests. Int. J. Remote Sens. 37 (1), 138–149.
Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D., Heisterkamp, S., Van Willigen, B., Maintainer, R., 2017. Package 'nlme'. Linear and Nonlinear Mixed Effects Models, Version 3(1).
Pinkster, S., 1970. Redescription of Gammarus pulex (Linnaeus, 1758) based on neotype material (Amphipoda). Crustaceana 177–186.
Pisa, L.W., Amaral-Rogers, V., Belzunces, L.P., Bonmatin, J.-M., Downs, C.A., Goulson, D., Kreutzweiser, D.P., Krupke, C., Liess, M., McField, M., 2015. Effects of neonicotinoids and fipronil on non-target invertebrates. Environ. Sci. Pollut. Res. 22 (1), 68–102.
R Core Team, 2020. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.
Rodriguez-Sanchez, P., Van den Berg, S.J.P., 2021. Kinematics: Studying Sampled Trajectories., R. https://CRAN.R-project.org/package=kinematics.
Roessink, I., Merga, L.B., Zweers, H.J., Van den Brink, P.J., 2013. The neonicotinoid imidacloprid shows high chronic toxicity to mayfly nymphs. Environ. Toxicol. Chem. 32 (5), 1096–1100.
Santos, L.H., Araújo, A.N., Fachini, A., Pena, A., Delerue-Matos, C., Montenegro, M., 2010. Ecotoxicological aspects related to the presence of pharmaceuticals in the aquatic environment. J. Hazard. Mater. 175 (1–3), 45–95.
Schuijt, L.M., Peng, F.-J., van den Berg, S.J., Dingemans, M.M., Van den Brink, P.J., 2021. (Eco) toxicological tests for assessing impacts of chemical stress to aquatic ecosystems: facts, challenges, and future. Sci. Total Environ. 148776.
Sehonova, P., Svobodova, Z., Dolezelova, P., Vosmerova, P., Faggio, C., 2018. Effects of waterborne antidepressants on non-target animals living in the aquatic environment: a review. Sci. Total Environ. 631, 789–794.
Silva, L.J., Pereira, A.M., Meisel, L.M., Lino, C.M., Pena, A., 2015. Reviewing the serotonin reuptake inhibitors (SSRIs) footprint in the aquatic biota: uptake, bioaccumulation and ecotoxicology. Environ. Pollut. 197, 127–143.
Stamps, J.A., 2007. Growth-mortality tradeoffs and 'personality traits' in animals. Ecol. Lett. 10 (5), 355–363.
Stanley, J.K., Ramirez, A.J., Chambliss, C.K., Brooks, B.W., 2007. Enantiospecific sublethal effects of the antidepressant fluoxetine to a model aquatic vertebrate and invertebrate. Chemosphere 69 (1), 9–16.
Stewart, A.M., Grossman, L., Nguyen, M., Maximino, C., Rosemberg, D.B., Echevarria, D.J., Kalueff, A.V., 2014. Aquatic toxicology of fluoxetine: understanding the knowns and the unknowns. Aquat. Toxicol. 156, 269–273.
Sutcliffe, D., Carrick, T., 1973. Studies on mountain streams in the English Lake District I. pH, calcium and the distribution of invertebrates in the River Duddon. Freshw. Biol. 3.
Thoré, E.S., Van Hooreweghe, F., Philippe, C., Brendonck, L., Pinceel, T., 2021. Generation-specific and interactive effects of pesticide and antidepressant exposure in a fish model call for multi-stressor and multigenerational testing. Aquat. Toxicol. 232, 105743.
Tuomainen, U., Candolin, U., 2011. Behavioural responses to human-induced environmental change. Biol. Rev. 86 (3), 640–657.
Van den Berg, S.J., Rodriguez-Sanchez, P., Zhao, J., Olusoiji, O., Peeters, E.T., Schuijt, L.M., 2023. Among-individual variation in the swimming behaviour of the amphipod Gammarus pulex under dark and light conditions: implications for ecotoxicology accepted at. Sci. Total Environ.
Vellinger, C., Gismondi, E., Felten, V., Rousselle, P., Mehennaoui, K., Parant, M., Usseglio-Polatera, P., 2013. Single and combined effects of cadmium and arsenate in Gammarus pulex (Crustacea, Amphipoda): understanding the links between physiological and behavioural responses. Aquat. Toxicol. 140, 106–116.
Wilkinson, J.L., Boxall, A.B., Kolpin, D.W., Leung, K.M., Lai, R.W., Galbán-Malagón, C., Adell, A.D., Mondon, J., Metian, M., Marchant, R.A., 2022. Pharmaceutical pollution of the world's rivers. Proc. Natl. Acad. Sci. 119 (8), e2113947119.
Williams, D.D., Moore, K.A., 1985. The role of semiochemicals in benthic community relationships of the lotic amphipod Gammarus pseudolimnaeus: a laboratory analysis. Oikos 280–286.
Woodward, G., 2009. Biodiversity, ecosystem functioning and food webs in fresh waters: assembling the jigsaw puzzle. Freshw. Biol. 54 (10), 2171–2187.
Zindler, F., Stoll, S., Baumann, L., Knoll, S., Huhn, C., Braunbeck, T., 2020. Do environmentally relevant concentrations of fluoxetine and citalopram impair stress-related behavior in zebrafish (Danio rerio) embryos? Chemosphere 261, 127753.