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Uptake, depuration, and behavioural effects of oxazepam on activity and foraging in a tropical snail (*Melanoides tuberculata*)

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ABSTRACT

Pharmaceuticals are increasingly being detected in surface waters around the globe, giving rise to concerns that they may alter the physiology and behaviour of aquatic organisms exposed in the wild. Invertebrates represent important components of many ecosystems and bear a high potential for transmitting pharmaceutical contaminants to higher trophic levels. Here, we present a laboratory study in which we exposed a freshwater tropical snail, *Melanoides tuberculata*, to a serial dilution of the benzodiazepine oxazepam ranging from 50 ng/L to 5 mg/L. We tested for subsequent behavioural effects, including locomotor activity and foraging propensity, at two diurnal time points (day and night), and across three days. We found that the snails displayed a high level of behavioural tolerance to all treatments of oxazepam except at the highest exposure, where locomotor and foraging activity declined. We also detected a weak non-monotonic response curve suggestive of behavioural disinhibition at moderate exposure levels. Regardless of treatment, the snails were also less active after three days of exposure and more active during nighttime observations. We measured the uptake of oxazepam in tissues across treatments, showing that it bioconcentrated at up to 29 times the water exposure level (BCF range: 7 - 29). Finally, we characterized the uptake/depuration pharmacokinetics of oxazepam in snail tissues across time, which revealed that the snails reach a steady state equilibrium in < 8 hours of exposure and depurate at a similar rate. Overall, our study suggests that snails such as *M. tuberculata*, due to their behavioural resilience and high bioconcentration potential, could act as vectors for pharmaceutical transfer throughout the food web in pharmaceutical-polluted habitats (e.g., wastewater outfalls).

Introduction

The manufacturing and use of pharmaceuticals for human and veterinary treatment has increased drastically over the last 50 years (Bernhardt et al., 2017). Following consumption, pharmaceuticals enter the environment, especially aquatic habitats, through several now well-described pathways, which include municipal and hospital wastewater effluents, pharmaceutical manufacturing discharges, livestock and human biosolid run-offs, and landfill leachates (reviewed in Pal et al., 2010; Patel et al., 2019). Knowledge of these pathways and the detection of pharmaceuticals in various environmental matrices around the globe has raised concern among scientists and environmental managers over the impacts that these compounds can have on aquatic biota (aus der Beek et al., 2016; Boxall et al., 2012; Gunnarsson et al., 2019; Hughes et al., 2013; OECD, 2019). More recently, this concern has been especially focused on the sub-lethal effects of pharmaceuticals on the physiology and behaviour of animals exposed in the wild. This is because pharmaceuticals are designed to exert pharmacological effects at relatively low concentrations (Gunnarsson et al., 2019; Nilsen et al., 2018), and consequently, understanding the ecotoxicity of pharmaceuticals and their many effects on a variety of aquatic organisms, both vertebrates and invertebrates, is a timely and important issue (reviewed in Boxall et al., 2012; Patel et al., 2019).

A growing body of research has been assessing the bioconcentration and (eco)toxicity of pharmaceuticals in aquatic invertebrates (Bundschuh et al., 2016; Du et al., 2015; Dussault et al., 2008; Lagesson et al.,

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2016; Meredith-Williams et al., 2012; Miller et al., 2019; reviewed in Miller et al., 2018). Aquatic invertebrates bioconcentrate pharmaceuticals, and this bioconcentration can vary with a combination of factors, including internal physiological processes (e.g., pharmaceutical metabolism), habitat use, foraging strategy, and abiotic environmental conditions (Lagesson et al., 2016; Meredith-Williams et al., 2012). Many studies to date have shown that aquatic invertebrates uptake (and depurate) pharmaceuticals more quickly than vertebrates and they also tend to bioconcentrate to higher concentrations in their tissues (Heynen et al., 2016; McCallum et al., 2019; Miller et al., 2018). However, evidence for the opposite (i.e., lower bioconcentration compared to vertebrates) was shown in Cerveny et al. (2021), suggesting that pharmacokinetics in invertebrates might be species and/or compound dependent. Given that aquatic invertebrates often occupy low trophic levels, they could therefore act as vectors to transfer pollutants to higher trophic levels (Heynen et al., 2016). Moreover, this transfer could be facilitated if the pharmaceuticals being bioconcentrated alter behaviour, making the exposed animal less reactive or more noticeable to predators (see Amiard-Triquet, 2009; Saaristo et al., 2018).

Many studies have now investigated the impacts of commonly-prescribed psychiatric pharmaceuticals on aquatic invertebrate behaviour, especially antidepressants (Bossus et al., 2013; Buřič et al., 2018; Fong et al., 2017; Rivetti et al., 2016; reviewed in Fong and Ford, 2014). These studies have revealed that exposure to low, environmentally relevant concentrations (< 1 μg/L) of a variety of antidepressants (e.g., fluoxetine, sertraline, amitriptyline) can impair the reproductive, feeding, and locomotor behaviour of invertebrates from a range of taxonomic groupings, including bivalve molluscs, gastropods, and crustaceans. Relatively fewer studies have considered how benzodiazepines, another main class of psychiatric pharmaceutical, affect aquatic invertebrate behaviour (Brodin et al. 2014). Benzodiazepines are equally concerning, as they are commonly prescribed among the human population as sedatives to treat anxiety, insomnia, and muscle spasms (acting via agonism of the $GABA_A$ receptor to reduce anxiety and stress). As such, benzodiazepines are often measured in surface waters near pharmaceutical sources, such as wastewater effluents (61 ng/L, Fick et al., 2017) and can persist in aquatic sediments (1.2 ± 0.3 μg/kg, Klaminder et al., 2015). Moreover, a comparatively larger body of research has shown that benzodiazepines can affect fish behaviours in numerous species (Brandão et al., 2013; Brodin et al., 2013; Hellström et al., 2016; McCallum et al., 2019b; Sundin et al., 2019; Wu et al., 2020), often making fish more active, more bold, and less social. Two studies to date have investigated the behavioural effects of benzodiazepines on invertebrates, showing that 1) marbled crayfish (*Procambarus virginalis*) exposed to a benzodiazepine (~1 μg/L) were more active in an open arena (Kubec et al., 2019), and 2) wandering snails (*Radix balthica*) foraged less at high exposure concentrations (10 μg/L) but exhibited no change in their locomotion (Lebreton et al., 2021). To better establish whether exposure to benzodiazepines elicits a similar range of behavioural effects in aquatic invertebrates as in fish, additional research is needed.

Here, we aimed to fill several gaps in the literature by assessing the tissue uptake, depuration and behavioural effects of the benzodiazepine oxazepam in the tropical freshwater snail, *Melanoides tuberculata*. In general, tropical species have received only minimal research attention to date when investigating the behavioural implications of pharmaceutical exposure. Gastropods are also an excellent study system because, *i*) they are globally widespread, *ii*) they are relatively slow moving and therefore likely remain in exposed habitats for long durations of time, *iii*) they are often in direct contact with contaminated sediments, and *iv*) they are common prey items for predators and can therefore transfer pollutants throughout the food web. Indeed, Lagesson et al., (2016) and Du et al., (2015) have showed that snail spp. in the family Planorbidae bioconcentrated the highest amounts of pharmaceuticals when exposed to a pharmaceutical mixture in a natural pond setting or when collected near wastewater effluent discharges, respectively. *Melanoides tuberculata* is a tropical freshwater snail with a broad geographic distribution and established non-native populations in North America, as well as relatively high tolerances to environmental extremes (e.g., temperature, salinity, desiccation, Rader et al. 2003; U.S. Fish & Wildlife Service 2018). *M. tuberculata* is a prosobranch snail, with average body sizes typically ranging up to 40 mm across their populations, and can live for 2 – 3.5 years (Rader et al. 2003). *M. tuberculata* has a generalist diet and feeds on algae, detritus, and also decaying animal tissue (Rader et al. 2003). Most research on this species has focused on its biology and ecology in relation to its invasion success, and this wealth of information and its widespread, tropical distribution make *M. tuberculata* an excellent species for ecotoxicology. Here, we exposed *M. tuberculata* to the pharmaceutical oxazepam and treatments included controls, environmentally relevant concentrations, human therapeutic dosages, and supernormal exposures (treatments encompassed a serial dilution that ranged from 0ng/L to 5 mg/L). The snails were exposed for 72 hours, and we tested the effect of oxazepam exposure on snail activity soon after their initial exposure (~4 hrs post-exposure), and then again after 72 hours. Given that benzodiazepines are fast-acting pharmaceuticals in humans and the generally rapid uptake of pharmaceuticals in aquatic invertebrates (e.g., Heynen et al., 2016), we expected that behavioural effects would arise soon after initial exposure and be stable over the two testing time points. We also tested snails under both day and night conditions, as these snails are known to be more active during the crepuscular and nighttime periods (Rader et al., 2003). We predicted that oxazepam exposure would increase snail activity and foraging, and that exposed snails would be more active during the day when compared to control snails. However, benzodiazepines like oxazepam can also have sedative effects at higher concentrations (Calabrese, 2008); exposure may therefore also reduce activity and foraging at the highest concentration. Lastly, we conducted a complimentary oxazepam tissue uptake and depuration experiment to verify the pharmacokinetics of oxazepam in this species and under tropical (26°C) freshwater conditions.

2. METHODS

2.1. Experimental setup and exposure

Between August 21 and December 6, 2020, we exposed *M. tuberculata* to serial dilutions of oxazepam in the laboratory. The *M. tuberculata* used in this study originated from the aquarium trade and were part of a lab stock that has been continually maintained for over five years alongside a lab population of Tanganyikan cichlids. As such, the snails fed on the same food as the fish, daily *Artemia salina* nauplii and commercial cichlid flakes, as well as waste produced by the fish themselves. Fifty percent water changes were performed once a week. We collected 151 adult snails (mean ± std. dev.= 15.8 ± 2.3 mm shell length, range: 9.8 – 21.5 mm) from a large-scale, re-circulating, tap-water supplied, temperature-controlled, laboratory aquarium system that was also housing Tanganyikan cichlids (water chemistry was measured on 3 occasions throughout the experiment: mean ± std. dev., temperature (ºC): 26 ± 0; dissolved oxygen (mg/L): ≥ 10 ± 0; Cl_2 (mg/L): 0 ± 0; NO_3 (mg/L): 20 ± 8.7; NO_2 (mg/L): 0 ± 0; general hardness (°dH, where 1° dH = 17.8 ppm $CaCO_3$): > 14; carbonate hardness (°dH): 6.7 ± 1.2; pH: 8 ± 0). Each snail was exposed individually to one of seven exposure treatments (see following paragraph for details) of oxazepam in a plastic Petri dish (12 cm diameter) for 3 days, over which time we ran a battery of behavioural tests on each snail (one snail per Petri dish). The 3-day long exposure trials were run in batches where 12 to 43 snails were exposed to oxazepam in parallel on a given day. We ran 8 batches in total. Within each batch, the snails were also grouped into experimental blocks, and run side-by-side under one recording camera. Each experimental block contained a balance of the treatment groups, and each treatment's positioning within the block was randomized over each batch.

A stock solution of oxazepam (purchased from Sigma-Aldrich, CAS Number 604-75-1, purity > 98%, log K_{OW} = 2.24) was made fresh before each batch of snails was run by dissolving oxazepam powder in distilled water overnight using a magnetic stir plate. Exposures were created by pipetting a pre-calculated volume of the stock solution into each Petri dish containing aquarium water from their housing tank such that the final volume within each dish was 180 mL. Snails were exposed to 0 μg/L, 0.05 μg/L, 0.32 μg/L, 4.04 μg/L, 32.7 μg/L, 505 μg/L, or 5,814 μg/L, measured concentrations (average of 4 water samples, see section 2.5 for quantification methods). A larger volume of stock solution was required to make the highest concentration, and this necessarily meant that this treatment contained of a higher proportion of distilled water relative to aquarium water. To test for possible effects of distilled water versus aquarium water on our behavioural endpoints, we ran a control group with 100% aquarium water (N = 22 snails) and also a group with 100% distilled water (N = 9 snails). Once the exposure conditions were created in each Petri dish, the snails were placed in the centre of each dish to begin the exposure. Snails were held at 26°C (confirmed daily by checking the thermostat) in a climate-controlled room and given natural lighting from a north-facing window. While this necessarily meant that photoperiod varied across the experimental duration, we typically ran all exposure concentrations concurrently such that photoperiod variation was inherently accounted for in the study design. Sample sizes per treatment were 31 snails in the two control groups combined (see above), 22 snails in each of the 0.05 μg/L through to 505 μg/L groups, and 10 snails in the 5,814 μg/L group (only 10 snails were used due to the large quantity of oxazepam required to make this treatment). Snail size (shell length) did not differ significantly between any of the experimental groups (ANOVA, $F_{7,143} = 1.16$, $P = 0.33$).

2.2. Behavioural trials: locomotor activity and thigmotaxis

We began each round of exposures between 10:00 and 11:00. We recorded locomotor activity of the snails in their Petri dishes at four times after beginning of the exposure: afternoon following exposure (~4 hours post-exposure, recordings started between 14:00 and 15:00), nighttime following exposure (~1 hour after sunset, recordings started between 19:00 and 20:00), afternoon post-72 hours following exposure (same recording start times), and nighttime post-72 hours following exposure (same recording start times, see Figure 1A). Top-mounted cameras (GoPro Hero 7) were used to record snail activity for ~90-minutes (ranged from 88 – 93 minutes), by taking one photo per minute of the Petrie dishes (using the 'linear' field of view setting on the cameras). From these time-series of photos, we measured the distances travelled by each snail every minute and then calculated the total distance travelled by each snail within their recordings. Note that the 12-cm diameter of the Petri dishes was much greater than the distances that the snails typically travelled in one minute (in our study ~2.2 cm per minute). Furthermore, the snails typically moved along the perimeter of the dish or linearly across the interior. Therefore, taking one photo per minute allowed us to track snail movement accurately. Such a camera setup to record locomotor activity was adapted from Lebreton et al. (2021). We also took note of whether the snails left contact with the walls of the Petri dish, and if so, how long they spent traversing the middle of the dish versus being in contact with the wall of the dish. This is a thigmotaxis or "wall hugging" assay, which is commonly used to measure anxiety in many species (Kohler et al., 2018a). We used a custom program written in Python to record the X-Y pixel coordinates of each snail in each photo frame (program by P. Nührenberg, Max Planck Institute for Animal Behavior, Konstanz, Germany). A dim red light was used to provide a minimal amount of illumination during the nighttime trials.

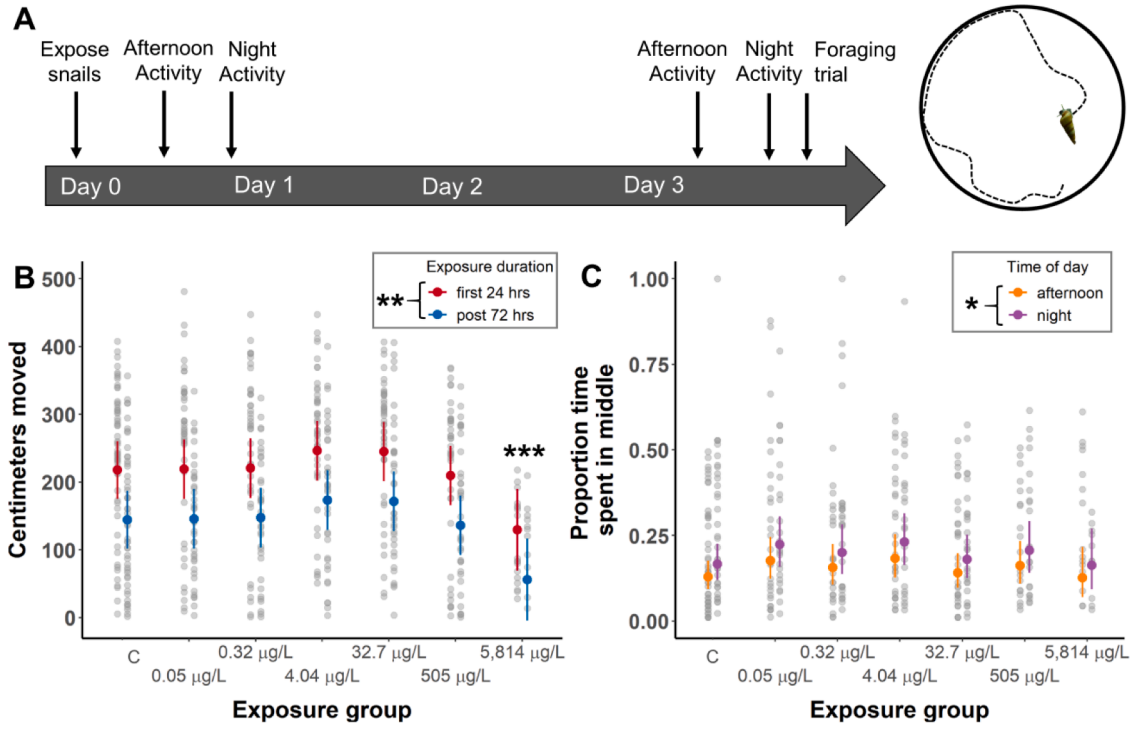

Figure 1. (A) Timeline of oxazepam exposure. Snails were tested within the first 24 hours of exposure, and then again after 72 hours. (B) Total locomotor activity, measured in centimeters moved, shown according to exposure group and split by exposure duration. (C) Proportion of time spent not in contact with the walls of the Petri dish (thigmotaxis), shown according to exposure group and split by time of day (i.e., afternoon or nighttime). Grey points show raw data values illustrating the considerable spread in the data, and the coloured points and whiskers show the model predicted means ± 95% confidence intervals. In panels (B) and (C), only the significant temporal predictor variable is visualized. "C" indicates the control group. *denotes a significant difference at $P < 0.05$, ** < 0.01, *** < 0.001. Sample sizes were N = 31 snails in the control group, N = 22 snails per intermediate exposure group, and N = 10 snails in the highest exposure group.

2.3. Behavioural trials: foraging task

The snails were not fed for the duration of the exposure. However, upon completion of the final activity recording (i.e., post-72 hours nighttime), we placed a small food item in the Petri dish, approximately two shell lengths away from the snail, and recorded for a further ~90-minutes (ranged from 89 – 93 minutes, with the exception of the first batch of snails, which were given 57 minutes). The food item consisted of a small piece (~2 mm^3) of blue mussel, *Mytilus edulis* (Frutti di Mare, Escal Seafood), pretreated with blue food colouring for contrast. The time (in minutes) between the start of the foraging trial and when the snail came into contact with the food item was recorded. After the foraging trial, each snail was removed and frozen at -20°C until further analysis.

2.4. Oxazepam uptake and depuration

To quantify oxazepam uptake and depuration in *M. tuberculata* tissue, we exposed 90 snails (mean ± std. dev.= 16.1 ± 1.9 mm shell length, range: 12 – 22 mm) to 0.40 μg/L oxazepam (measured concentration) for 7 days and then to unexposed aquarium water for another 7 days. The snails were housed in two separate tanks (15×20×25 cm, 45 snails per tank), which each contained 200 g of standard aquarium sand and 3 L of water. The tanks did not contain a filter but were equipped with air bubblers to gently aerate the water. Every other day, the snails were fed with a half pellet of sinking Pleco Tablets until satiation (Tetra®). Before exposures, we collected snails and water from our stock tank to serve as baseline values. During the first 7 days of uptake, we took a water sample from each tank immediately after dosing, and then again after 8 h, 24 h, 72 h, and 168 h. We also sampled three snails from each tank at 8 h, 12 h, 24 h, 48 h, 72 h, 120 h, and 168 h. The remaining snails were then transferred into two new identical tanks filled with sand and unexposed aquarium water. Again, we took a water sample from the stock tank from which we drew the unexposed aquarium water, and then sampled water from each depuration tank at 24 h, 72 h, and 168 h. We also sampled three snails from each tank at 4 h, 8 h, 12 h, 24 h, 48 h, 72 h, 120 h, and 168 h. We collected and analyzed snail tissue samples at more time points than water samples to better resolve the uptake and depuration curves, and we knew from previous work that benzodiazepines are stable in static aquaria over ~7 days (Brodin et al., 2013; McCallum et al., 2019b).

2.5. Oxazepam quantification in snail tissue and water

We quantified oxazepam in snail tissues and exposure water from half of the experimental batches in the behavioural experiment (4 of 8 batches). We also quantified oxazepam in snail tissues and exposure water from the uptake/depuration experiment (details below).

We prepared water samples (from both the behavioural experiment and uptake/depuration experiment) for chemical analysis by thawing the sample and passing 5 mL of the sample through a 0.45 μm syringe filter (Filtropur S, Sarstedt, Nümbrecht, Germany). We then added 5 ng of oxazepam internal standard (50 μL of 0.1 μg/mL in methanol). Water samples from the two highest treatments were diluted 10x and 100x, respectively, with Ultrapure MilliQ water before filtering.

We prepared snail tissue samples (from both the behavioural experiment and the uptake/depuration experiment) following a previously established protocol for oxazepam (McCallum et al., 2019a). We thawed the snails, extracted the whole-body tissue from the shell, and removed the trapdoor. Snail tissues were too small for individual analysis; therefore, samples from the same treatments (within each experimental batch [behavioural experiment] or sampling time point [uptake/depuration experiment]) were pooled (N = 2-3 snails per sample). Tissues were combined, manually mixed and homogenized, and ~0.1 g of tissue was weighed and transferred to 2 mL polypropylene (PP) tubes. We added 5 ng of internal standard (50 μL of 0.1 μg/mL in methanol) to each sample. After, samples were extracted twice, sequentially using 1.5 mL of acetonitrile. Samples were homogenized for 4 min at 42 000 oscillations per minute with zirconium beads (Mini Beadbeater, Biospec, Bartlesville, OK) and then centrifuged at 17 500g for 10 min (Beckman Coulter Microfuge 22R Centrifuge). The supernatants from repeated extractions were combined for each individual sample, evaporated to dryness, and reconstituted in 150 μL of methanol. Final extracts were transferred to glass autosampler vials with a 200 μL insert and kept frozen at −18°C. The samples were centrifuged again before analysis to settle precipitated proteins and other solid particles in the sample. Blank samples were prepared alongside tissue samples using the extraction procedure but containing no tissue in the sample.

The method of liquid chromatography–tandem mass spectrometry (LC–MS/MS) that we used to analyse the snail tissue samples has been described previously (McCallum et al., 2019a). To analyze water samples, an online solid phase extraction system coupled with liquid chromatography-tandem mass spectrometry (SPE LC–MS/MS), also described in previous work (Khan et al., 2012) was used. The LC system used for analysis of tissue extracts consisted of an Ultimate 3000 pump (LPG-3400SD, Thermo Fisher Scientific, San Jose, CA) and a PAL HTC autosampler (CTC Analytics AG, Zwingen, Switzerland). Target analytes were separated using a Hypersil GOLD column (2.1 mm × 50 mm, 3 μm, Thermo Fisher Scientific) and analysed with a TSQ Quantiva triple quadrupole mass spectrometer equipped with heated-electrospray ionisation (Thermo Fisher Scientific). In addition to the above described, the SPE LC–MS/MS system also included 1a Surveyor LC-Pump (Thermo Fisher Scientific, San Jose, CA, USA) and an online SPE Hypersil GOLD C18 column (20 mm × 2.1 mm ID × 12 μm particles, Thermo Fisher Scientific, Waltham, MA, USA). Descriptions of the basic set-up of the electrospray ionisation interface and the gradient/flow of the mobile phase are presented in the Supplementary Materials (Tables S1-S3). We used liquid chromatography–mass spectrometry (LC–MS)-grade acetonitrile and methanol (LiChrosolv—hypergrade) purchased from Merck (Darmstadt, Germany). Formic acid (Sigma-Aldrich, Steinheim, Germany) was used to prepare the 0.1% mobile phases. Oxazepam (CAS 604-75-1) and the mass-labeled $^2H_5$-oxazepam internal standard (CAS 65854-78-6) were purchased from Sigma-Aldrich (Steinheim, Germany).

Quality assurance and quality control (QA/QC) of the analytical method in this work was evaluated based on its precision, linearity, limit of quantification (LOQ), and measurement of blank samples. Isotopic dilution (internal standard approach) was used to quantify oxazepam in water and tissue samples. Instrumental LOQ was derived from an eight-point standard curve. The peak area corresponding to the lowest point of the calibration curve that had a signal/noise ratio of at least 10 was then used for calculation of LOQs in the individual samples. Precision was expressed as a relative standard deviation of response factors calculated for each point of the calibration curve. Recovery of oxazepam for this extraction method and measurement method has been established previously for multiple tissue types (McCallum et al. 2019a).

2.6. Statistical analyses

The snail activity data was distinctly bimodal, as some snails moved around their Petri dishes during the behavioural recordings, while others were still. Because of this, we adopted a hurdle modelling approach where we first considered whether or not the snails moved as the response variable, and then followed this with an analysis of how far the snails travelled for only those snails that did move. We also applied a hurdle approach to the thigmotaxis data, wherein we first considered whether or not the snails left contact with the walls of the Petri dish and then, of those that did leave the wall, we tested the proportion of time they spent in the middle. Because snails were repeatedly observed over the three-day exposure, and run together in experimental blocks, we adopted a mixed-effects modelling approach in order to account for the non-independence among data points (Harrison et al. 2018). First, we fit

a binary generalized linear mixed effects model (GLMM) to the data on whether or not the snails moved during their recording trials (using the 'glmmTMB' R package, Brooks et al. 2017, assuming a 'cloglog' link function). To account for non-independence in our data, we included random intercepts of 'experimental block ID' and of 'snail ID' (note, we also included an observation level ID when overdispersion was detected). As predictor variables, we included 'treatment group' (categorical), 'snail size' (continuous: shell length in mm), 'exposure day' (categorical: 'first 24 hours' versus 'post-72 hours'), and 'trial time' (categorical: afternoon versus nighttime). We also tested for the interaction between 'treatment group' and 'snail size' based on whether its inclusion significantly improved model fit (tested with a likelihood ratio test). If the interaction did not improve model fit, it was dropped from the final model. We did not test for additional interactions among predictors in our models due to the large number of model contrasts that this would have required. Model diagnostics were checked using the 'DHARMa' and 'performance' R packages (Hartig 2020; Lüdecke et al. 2020). Next, we examined only the snails that did move, and fit their total distances moved (in cm) with a linear mixed effects model (LMM), assuming a 'gaussian' error distribution, but otherwise using the same model structure as above.

For thigmotaxis, we tested whether or not the snails left contact with the walls of the Petri dish by fitting a binary GLMM (snail size was scaled, such as mean = 0 and standard deviation = 1). We then asked how long the snails remained in the middle of the dish (for those that did leave the wall), by fitting a binomial GLMM assuming a 'logit' link function to the minutes that they spent in the middle of the dish relative to the minutes they spent in contact with the walls. In all these models, we used the same general model structure as described above.

Since we ran two control groups, one with 100% aquarium water and the other with 100% distilled water, we also tested whether the behaviour of the snails in each of these two groups differed significantly from each other. We did this by fitting the same four models described above to the behavioural data of just the control snails and made comparisons between the two groups. In all cases, the two groups of control snails did not differ significantly from one another (all $P > 0.22$, see Supplementary Materials for further details on similarities between the two control groups). While a 100% distilled water exposure is not ecologically typical for these snails, this was a necessary procedural control. We therefore pooled the control snails together into one group for the above analyses to make comparisons with the exposed groups.

Survival analyses are appropriate for analyzing time-until-an-event data, and so we analyzed foraging (i.e., the time that it took the snails to reach the food item, in minutes) using a univariate cox proportional hazards model. We assumed right-censored data for all snails that did not reach the food item within their recording period (using the 'survival' R package, Therneau & Grambsch 2000).

Data from the tissue uptake and depuration experiment was descriptively summarized. Results from both exposure aquaria replicates were pooled when summarizing and visualizing the results. Wet-weight bioconcentration factor (BCF) was calculated by dividing the average measured concentration in snail tissue by the average water exposure concentration for both the behavioural and tissue uptake/depuration experiments. We opted against lipid-normalization of BCF because of the challenges associated with accurately quantifying lipid contents from such small animals (~50 mg per snail) along with the typically low lipid contents of snail tissues (Paszkiewicz et al. 2014).

RESULTS

Instrumental analysis

The analytical method achieved excellent performance parameters. For the water analysis, the method was linear ($R^2 > 0.999$; RSD = 4%) between 0.005 and 50 μg/L of the standard curve, with the LOQ in individual samples ranging from 0.008 to 0.02 μg/L. For the tissue analysis, an LOQ from 0.2 to 1.2 ng/g was achieved in individual samples and the method was linear ($R^2 > 0.999$) between 0.5 and 50 ng/g of the standard curve, with a relative standard deviation (RSD) of 3%. No quantifiable concentrations of oxazepam were found in any of the measured blank samples. We detected oxazepam above the limit of quantification in two of the four control water samples (0.007 and 0.031 μg/L) and in one of the corresponding snail tissue samples (1.3 μg/kg). This contamination was likely caused by microscopic droplet transfer between dishes that were placed close together to enable filming of behaviour.

Locomotor activity and thigmotaxis

No snails died in any of the exposure groups prior to the end of the trials. Each of the 151 snails used in this study were recorded on four occasions (Figure 1A), resulting in a total of 604 behavioural observations. Out of these 604 observations, the snails remained immobile in 68 (11.3%) of them. However, of the snails that did move during their activity observations, they moved an average (± std. dev.) of ~202 ± 113 cm (range: 0.9 – 481 cm). This corresponds to an average movement velocity of ~2.2 ± 1.2 cm per minute (range: 0.01 – 5.41 cm per minute). The probability that a snail would move did not differ significantly between the control group and the exposure groups (Table 1A). Snails were less likely to move on the third day of exposure relative to the first day, but no difference could be detected between the afternoon trials and the nighttime trials (Table 1A). Of the snails that did move, those exposed to the highest concentration travelled a significantly shorter distance than control snails (Table 1B, Figure 1B). Similarly, snails moved a shorter distance on day three of exposure compared to the first 24 hours (Table 1B).

When examining thigmotaxis, we detected a significant interaction effect between oxazepam exposure and snail size (Table 2A). This interaction suggested that increasing body size was associated with a lower probability that snails would leave contact with the walls of the Petri dishes, though this was only observed in exposure concentrations of 32.7 μg/L and 505 μg/L compared to the control group. Of the snails

Table 1
Statistical output of mixed-effects models for activity data, (A) examining whether or not snails moved during a trial, and (B) of the snails that did move, how far they travelled. Model estimates and standard errors (Est. ± SE), z-statistics (z), and *P*-values are provided. Note that the estimates reported are relative to the control group, the first exposure day, and the daytime trial times (i.e., they are the reference groups in these analyses). Significant *P*-values at α = 0.05 as shown in bold, *P*-values between 0.05-0.08 are italicized.

Model contrasts	Est. ± SE	z	*P*
(A) *Proportion of snails that moved*			
Intercept	2.86 ± 0.87	3.27	**0.001**
Exposure 0.05 μg/L	0.004 ± 0.33	0.011	0.99
Exposure 0.32 μg/L	-0.017 ± 0.33	-0.051	0.96
Exposure 4.04 μg/L	0.14 ± 0.35	0.41	0.68
Exposure 32.7 μg/L	-0.084 ± 0.32	-0.26	0.79
Exposure 505 μg/L	-0.069 ± 0.33	-0.21	0.83
Exposure 5,814 μg/L	-0.80 ± 0.43	-1.83	0.067
Shell size (mm)	-0.058 ± 0.049	-1.17	0.24
Exposure day (3)	-1.03 ± 0.29	-3.57	**0.0004**
Trial time (night)	-0.064 ± 0.15	-0.42	0.68
(B) *Centimeters moved*			
Intercept	181 ± 47.3	3.82	**0.0001**
Exposure 0.05 μg/L	1.33 ± 15.4	0.093	0.93
Exposure 0.32 μg/L	3.02 ± 15.4	0.20	0.84
Exposure 4.04 μg/L	28.6 ± 15.4	1.86	*0.063*
Exposure 32.7 μg/L	27.2 ± 15.3	1.78	*0.075*
Exposure 505 μg/L	-8.27 ± 15.4	-0.53	0.60
Exposure 5,814 μg/L	-88.3 ± 23.8	-3.71	**0.0002**
Shell size (mm)	2.36 ± 2.61	0.90	0.37
Exposure day (3)	-73.3 ± 27.3	-2.68	**0.0074**
Trial time (night)	-3.13 ± 4.69	-0.66	0.51

Table 2
Statistical output of mixed-effects models for thigmotaxis data, (A) examining whether or not snails left contact with the wall of their Petri dishes, and (B) examining, of the snails that did leave the wall, the proportion of time that they spent in the centre of the dish. Model estimates and standard errors (Est. ± SE), z-statistics (z), and *P*-values are provided. Note that the control group, the first exposure day, and the afternoon trial times are treated as the reference groups in these analyses. Significant *P*-values at α = 0.05 as shown in bold.

Model contrasts	Est. ± SE	z	*P*
(A) *Proportion of snails that left the wall*			
Intercept	0.34 ± 0.20	1.67	0.096
Exposure 0.05 μg/L	-0.32 ± 0.24	-1.31	0.19
Exposure 0.32 μg/L	-0.45 ± 0.26	-1.75	0.080
Exposure 4.04 μg/L	-0.13 ± 0.25	-0.54	0.59
Exposure 32.7 μg/L	0.11 ± 0.26	0.43	0.66
Exposure 505 μg/L	-0.69 ± 0.26	-2.71	**0.0068**
Exposure 5,814 μg/L	0.29 ± 0.45	0.64	0.52
Shell size (scaled)	0.11 ± 0.15	0.73	0.47
Exposure day (3)	0.14 ± 0.18	0.78	0.44
Trial time (night)	-0.18 ± 0.13	-1.45	0.15
Exposure 0.05 μg/L * Shell size (scaled)	-0.06 ± 0.23	-0.24	0.81
Exposure 0.32 μg/L * Shell size (scaled)	0.23 ± 0.27	0.86	0.39
Exposure 4.04 μg/L * Shell size (scaled)	-0.50 ± 0.27	-1.84	0.067
Exposure 32.7 μg/L * Shell size (scaled)	-0.60 ± 0.24	-2.55	**0.011**
Exposure 505 μg/L * Shell size (scaled)	-0.50 ± 0.25	-2.02	**0.043**
Exposure 5,814 μg/L * Shell size (scaled)	-0.43 ± 0.63	-0.68	0.50
(B) *Proportion of time in the middle of the arena*			
Intercept	-1.88± 0.56	-3.27	**0.001**
Exposure 0.05 μg/L	0.37 ± 0.23	1.59	0.11
Exposure 0.32 μg/L	0.22 ± 0.24	0.95	0.34
Exposure 4.04 μg/L	0.41 ± 0.23	1.78	0.075
Exposure 32.7 μg/L	0.10 ± 0.23	0.42	0.67
Exposure 505 μg/L	0.26 ± 0.24	1.08	0.28
Exposure 5,814 μg/L	-0.029 ± 0.31	-0.09	0.93
Shell size (mm)	-0.0015 ± 0.035	-0.044	0.96
Exposure day (3)	0.35 ± 0.18	1.89	0.059
Trial time (night)	0.30 ± 0.13	2.36	**0.018**

that did move and leave contact with the walls, they spent significantly more time in the middle of the arena during the nighttime trials when compared to the afternoon trials (Table 2B, Figure 1C).

Foraging

Snails exposed to the highest concentration took significantly longer to reach the food item than the control snails in the foraging task (Table 3, Figure 2).

Tissue uptake and depuration

Oxazepam concentrations were stable in the water column over the 7-day uptake period (Figure 3A). Snails rapidly bioconcentrated and depurated oxazepam and reached a steady-state equilibrium with the water concentration in less than 8 hours (Figure 3A). We considered the snails to have reached steady-state equilibrium with their exposure environment when their tissue concentrations reached a visual plateau. There was notably more oxazepam in one snail sample during the first sampling timepoint (18 μg/kg). As with uptake, we observed rapid depuration in the clean water phase (Figure 3B). The BCF for snails during the uptake phase is summarized in Table 4. We measured a small amount of oxazepam in the baseline (pre-exposure) water sample from the uptake experiment (0.014 μg/L). However, there was no oxazepam measured in baseline snail tissues from this water source, likely indicating contamination during sample preparation.

Across treatments in the behavioural trials, the BCF for snails ranged from 7-29 (Table 4). BCF tended to decline with increasing exposure concentration (Table 4).

Table 3
Statistical output of Cox proportional hazard model examining effects of oxazepam exposure on snail performance in foraging task. Note that all contrasts are being made in comparison to the control group. Hazard ratio indicates the relative probability that, at any given time point, a snail in an exposure group will reach the food item compared to a snail in the control group. Model estimates and standard errors (Est. ± SE), z-statistics (z), and *P*-values are provided. Significant *P*-values at α = 0.05 as shown in bold.

Model contrasts	Est. ± SE	z	P	Hazard ratio relative to control group
Foraging task				
Exposure 0.05 μg/L	0.018 ± 0.36	0.051	0.96	1.02
Exposure 0.32 μg/L	0.13 ± 0.35	0.37	0.71	1.14
Exposure 4.04 μg/L	0.15 ± 0.35	0.43	0.66	1.16
Exposure 32.7 μg/L	0.06 ± 0.35	0.18	0.85	1.07
Exposure 505 μg/L	-0.42 ± 0.39	-1.08	0.28	0.65
Exposure 5,814 μg/L	-1.50 ± 0.74	-2.02	**0.043**	**0.22**

DISCUSSION

We investigated the behavioural effects of exposure to the benzodiazepine oxazepam, as well as its uptake and depuration pharmacokinetics, in *Melanoides tuberculata*, a tropical freshwater snail. Focusing first on the behavioural results, we found that snail behaviour was variable across the trials, and this variation was only slightly correlated to the oxazepam exposure regime. Snail locomotor activity did not clearly become affected by oxazepam until the very highest exposure, ~5.8 mg/L. Likewise the snails took significantly longer to reach the food item in the foraging task at this highest exposure. Our highest concentration is three orders of magnitude higher than what has been reported in surface waters. The highest concentration reported to date for oxazepam is 7.4 μg/L in wastewater effluent entering a river in France (Mazzitelli et al., 2018), and 1.76 μg/L was the highest concentration of oxazepam measured in an earlier Europe-wide survey of wastewater effluents (Loos et al., 2013). Additionally, the maximum concentration of diazepam (another benzodiazepine, of which oxazepam is a main metabolite) exceeded 39 μg/L in samples collected from effluents entering freshwater rivers in Nigeria (Ogunbanwo et al., 2020). Interestingly, we found a weak signature of a non-monotonic response. When examining locomotor activity (distance moved), snails at 4.04 μg/L and 32.7 μg/L tended to move further than controls, but this did not reach statistical significance ($P = 0.063$, $P = 0.075$, respectively), and so these results should be interpreted with care. However, non-monotonic response curves, where low concentrations stimulate behaviour and higher concentrations sedate behaviour, are a common phenomenon with benzodiazepines and psychiatric pharmaceuticals (as seen in humans, mammalian model organisms, and fish, Calabrese, 2008; Fong and Ford, 2014).

Snails exposed to 32.7 μg/L and above in our study likely experienced a human therapeutic concentration of oxazepam or higher (which ranges between 200 – 1500 μg/L in human plasma, Regenthal et al., 1999; Smink et al., 2008), while those exposed to 505 or 5814 μg/L bioconcentrated levels in tissue that would be toxic in humans (< 2000 μg/L in human plasma, Regenthal et al., 1999). Our results suggest that *M. tuberculata* show a behavioural tolerance of benzodiazepine exposure, at least for activity and foraging in our laboratory setup. Recently, Lebreton et al., (2021) found that snail locomotion, as measured via a similar behavioural assay as the one we used here, was not impacted when freshwater wandering snails (*Radix balthica*) were exposed to 0.8 μg/L or 10 μg/L of oxazepam. These results are consistent with ours because we only observed effects at a much higher concentration. The authors also found that feeding was stimulated in snails at their low concentration, but inhibited at their high concentration. Although, these foraging results are at odds with ours, Lebreton et al.'s feeding assay examined the amount of food consumed, while ours focused on the speed at which the food item could be located (which is more dependent on locomotion).

We observed several notable temporal effects in the behaviour of the snails. First, snail locomotor activity generally decreased with duration of

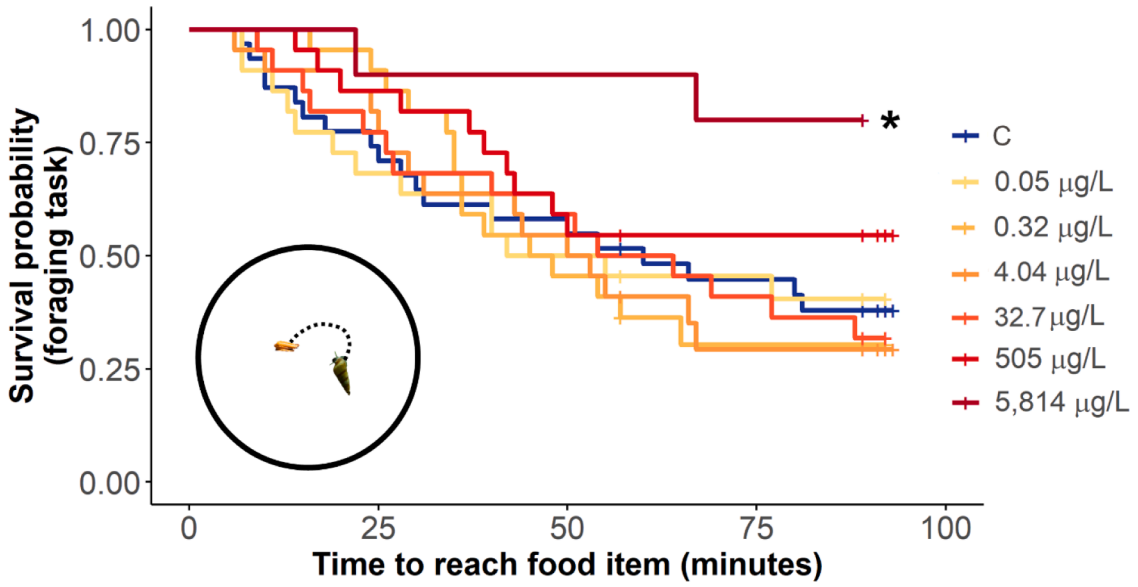

Figure 2. Time until food item was reached in foraging task shown according to exposure group (plotted as Kaplan-Meier survival probabilities, which indicate the probability that snails in each group will reach their food item at some time *beyond* each time step (in minutes)). + denotes a right-censored datapoint. *denotes a significant difference at $P < 0.05$. Inlay in bottom left shows a depiction of the foraging task.

exposure: all snails moved less after three days of exposure relative to the first day. Second, we observed that snails spent more time in the centre of the Petrie dish during the nighttime trials relative to the afternoon trials. Although this is a small effect (Figure 1C), it is consistent with the behavioural patterns of this species being most active and foraging during the crepuscular period. These two findings underscore the importance of carefully designing behavioural assays to consider time-related effects, such as habituation or diurnal patterns of an organism's natural behaviour. Such issues of study design have recently been in focus for both invertebrate and vertebrate behavioural ecotoxicology, as the field strives to standardize approaches to behavioural testing (Kohler et al., 2018a, 2018b; Melvin, 2017; Thoré et al., 2021). While we controlled for snail body size in all our analyses, we detected no effect of body size on the behavioural endpoints studied, except for an interaction effect: smaller snails were more likely to leave contact with the walls of their Petri dishes in the 33 µg/L and 505 µg/L exposure groups. The danger of leaving the wall of the Petri dish may be perceived differently for larger versus smaller snails (e.g., larger snails may be more noticeable to predators in the open), and exposure to oxazepam may have reduced the threshold at which snails choose to leave the wall, which could differ by body size. This intriguing idea requires further investigation of the behavioural responses of *M. tuberculata* under differing levels of predation threat before clear conclusions can be drawn.

Our pharmacokinetic data show that oxazepam was bioconcentrated at high levels (for a pharmaceutical) in *M. tuberculata* tissue, and it is both taken up and depurated very rapidly from tissues. Previous research by Lagesson et al., (2016) found that *Planorbidae* snails bioconcentrated high amounts of oxazepam in a natural pond exposure, with a bioaccumulation factor (also including food sources) of ~38 for sampled snails. However, BCFs for oxazepam appear to be variable depending on the aquatic invertebrate being studied, previously reported to be ~22 in the amphipod *Gammarus fossum* (Sordet et al., 2016) and only ~0.5 in dragonfly larvae (*Sympetrum* spp., Cerveny et al. 2021; *Aeshna grandis,* Heynen et al., 2016). We noted some variability in BCF (range 7-29) depending on the exposure concentration within the behavioural experiment and between the behaviour and uptake/depuration experiments (see Table 4). Oxazepam is known to sorb onto and be persistent in sediments that snails would come in direct contact with, often grazing on items directly on top of the sediment. This may perhaps modulate each snail's individual exposure depending on the extent of dermal contact (e.g., burrowing vs surface movement) and food intake in addition to the waterborne exposure. Unlike prior work on fish exposed to oxazepam, the snails in our study rapidly reached a steady-state equilibrium with the exposure environment (i.e., < 8 hours versus ~ 5 days for fish; Brodin et al., 2013). Snails, and other aquatic invertebrates, may bioconcentrate these drugs rapidly and to a greater extent if they do not possess metabolic pathways with the same capacity for biotransformation of the parent drugs or efficiency of their excretion (Hutchinson et al., 2014). Oxazepam is transformed in mammals and humans via phase 2 metabolism by glucuronidation; however, very little is known about phase 2 metabolism of pharmaceuticals in snails and aquatic organisms more generally (Arnold et al., 2014).

In conclusion, we have provided a comprehensive overview of the behavioural effects and pharmacokinetics of oxazepam in a tropical aquatic invertebrate. We found that *M. tuberculata* quickly bioconcentrated (and depurated) oxazepam, and this combined with their low trophic position suggests that they could be a potent vector for pharmaceutical transmission through the food chain in environments continuously polluted by pharmaceuticals. However, the lack of behavioural effects in the snails at environmentally relevant exposure concentrations (< 1 µg/L) indicates some level of behavioural tolerance. Therefore, it may be unlikely for aberrant behaviours to enhance the trophic transfer of this pharmaceutical pollutant (i.e., by being less reactive or more noticeable to predators). Further predator-prey interaction studies are needed to verify any behavioural effects and the extent of oxazepam's trophic transfer to tissues in other predatory organisms. Moreover, research on the metabolism of oxazepam (e.g., investigating possible metabolic pathways and if/how they differ from mammals) in invertebrates would be useful for further understanding the pharmacokinetics of this compound in non-target organisms. Even though our study was conducted at tropical water temperatures (26°C), our behavioural findings aligned with a previous study on a temperate snail species (Lebreton et al., 2021). We found no literature on the acute pharmacokinetics of oxazepam in temperate snails for which we could compare the uptake and depuration patterns we observed here; however, the timing (but not magnitude) of uptake did mirror previous research on temperate dragonfly larvae (Cerveny et al. 2021; Heynen et al., 2016). Altogether, we underscore the need for further research on the uptake, depuration, and behavioural effects of benzodiazepine pharmaceutical pollutants in aquatic invertebrates.

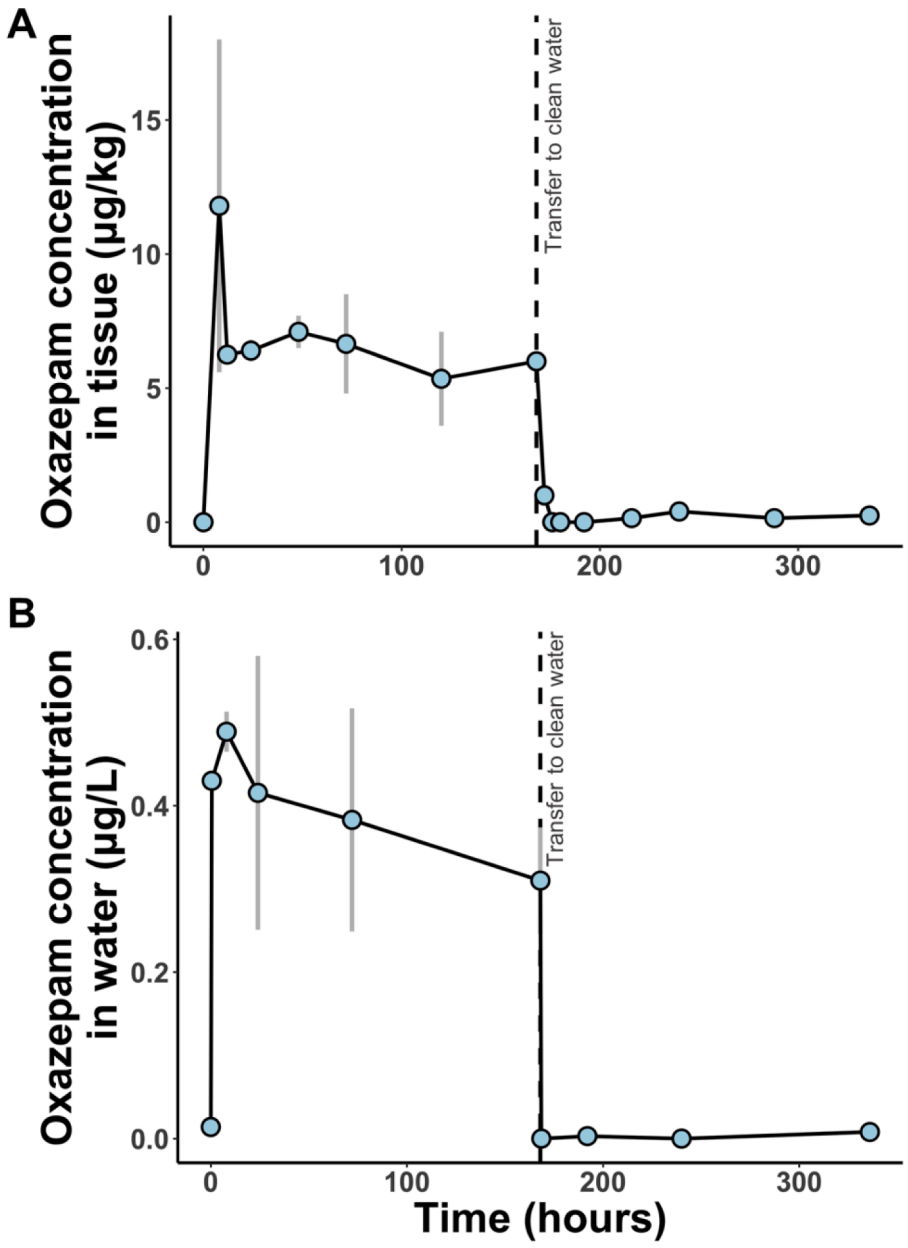

Figure 3. Concentrations of oxazepam in (A) snail tissue and (B) water plotted against sampling time during the uptake and depuration experiment. Note that each dot represents the average of two samples, each comprised of 2-3 snails, pooled. Grey bars indicate the range in measured concentrations between the two samples. Vertical dashed line indicates when snails were transferred to aquaria with no oxazepam for the depuration phase.

Table 4
Concentrations (mean ± std. dev.) of oxazepam measured in exposure water and snail tissues with sample sizes, and bioconcentration factor (BCF) from the behavioural experiment and uptake/depuration experiment (note: only data from the uptake phase is shown).

	Behavioral Experiment						Uptake Experiment
Water concentration (µg/L)	0.05 ± 0.02 *n = 4*	0.32 ± 0.07 *n = 4*	4.04 ± 1.96 *n = 4*	32.7 ± 11.5 *n = 4*	505 ± 87.5 *n = 4*	5 814 ± 464 *n = 4*	0.40 ± 0.13 *n = 8*
Tissue concentration (µg/kg)	1.23 ± 0.42 *n = 4*	9.28 ± 5.78 *n = 4*	66.5 ± 14.1	662 ± 172 *n = 4*	5 073 ± 869 *n = 4*	41 764 ± 5 954 *n = 4*	7.08 ± 3.36 *n = 14*
BCF	24.5	29.0	16.6	20.1	10.0	7.18	17.7

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Data Availability

All datasets presented here are freely available in the supplementary materials.

Ethics

This study was performed under permit no. G 19/79 issued by the Tierschutzgesetzes (TierSchG) Baden-Württemberg to A. Bose and E. McCallum.

CRediT authorship contribution statement

Aneesh P.H. Bose: Conceptualization, Methodology, Investigation, Formal analysis, Visualization, Writing – original draft, Writing – review & editing, Funding acquisition, Project administration. **Tomas Brodin:** Methodology, Writing – review & editing, Resources, Funding acquisition. **Daniel Cerveny:** Investigation, Writing – review & editing. **Erin S. McCallum:** Conceptualization, Methodology, Investigation, Visualization, Writing – original draft, Writing – review & editing, Project administration.

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.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.envadv.2022.100187.

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