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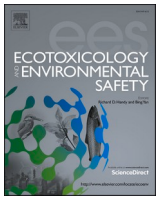

# Environmentally relevant concentrations of the tricyclic antidepressant, amitriptyline, affect feeding and reproduction in a freshwater mollusc

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ABSTRACT

Antidepressant drugs (ADDs) are one of the most extensively used pharmaceuticals globally. They act at particularly low therapeutic concentrations to modulate monoamine neurotransmission, which is one of the most evolutionary conserved pathways in both humans and animal species including invertebrates. As ADDs are widely detected in the aquatic environment at low concentrations (ng/L to low μg/L), their potential to exert drug-target mediated effects in aquatic species has raised serious concerns. Amitriptyline (AMI) is the most widely used tricyclic ADD, while monoamines, the target of ADDs, are major bioregulators of multiple key physiological processes including feeding, reproduction and behaviour in molluscs. However, the effects of AMI on feeding, reproduction and mating behaviour are unknown in molluscs despite their ecological importance, diversity and reported sensitivity to ADDs. To address this knowledge gap, we investigated the effects of environmentally relevant concentrations of AMI (0, 10, 100, 500 and 1000 ng/L) on feeding, reproduction and key locomotor behaviours, including mating, in the freshwater gastropod, *Biomphalaria glabrata* over a period of 28 days. To further provide insight into the sensitivity of molluscs to ADDs, AMI concentrations (exposure water and hemolymph) were determined using a novel extraction method. The Fish Plasma Model (FPM), a critical tool for prioritization assessment of pharmaceuticals with potential to cause drug target-mediated effects in fish, was then evaluated for its applicability to molluscs for the first time. Disruption of food intake (1000 ng/L) and reproductive output (500 and 1000 ng/L) were observed at particularly low hemolymph levels of AMI, whereas locomotor behaviours were unaffected. Importantly, the predicted hemolymph levels of AMI using the FPM agreed closely with the measured levels. The findings suggest that hemolymph levels of AMI may be a useful indicator of feeding and reproductive disruptions in wild population of freshwater gastropods, and confirm the applicability of the FPM to molluscs for comparative pharmaceutical hazard identification.

## 1. Introduction

Antidepressant drugs (ADDs) are a class of psychotropic drugs that are used majorly for the treatment of depressive disorders. They are one of the most widely prescribed and consumed pharmaceuticals worldwide (Lewer et al., 2015; Luo et al., 2020; Martin et al., 2019; Soleymani et al., 2018). They are particularly designed to modulate monoaminergic neurotransmission (Delgado, 2004), which is one of the most evolutionary conserved pathways across animal phyla (Caveney et al., 2006; D'Aniello et al., 2020; Tierney, 2018). The parent compounds and their pharmacologically active metabolites are further characterized by widespread occurrence in the aquatic environment (Deo, 2014; Ma et al., 2018), thereby increasing the potential for chronic exposure in aquatic species in which the drug targets are conserved. As evolutionary conservation of drug targets does not necessarily translate into conservation of biological responses in invertebrates (Baynes et al., 2019), a wide spectrum of different critical physiological processes are therefore potentially impacted in invertebrates by ADDs.

Amitriptyline (AMI) is the most widely prescribed tricyclic antidepressant (TCA), a major class of ADDs, used in the treatment of depression (Lalji et al., 2021; Lukmanji et al., 2020; Malhi et al., 2022). With strong medication efficacy, it is also used for the treatment of a wide range of high prevalence clinical conditions, sometimes comorbid with depressive disorders, including neuropathic pain, fibromyalgia, migraine and insomnia (Bogowicz et al., 2021; Goldenberg et al., 2004; Schneider et al., 2019), altogether resulting in high volume prescription

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and consumption. In humans, AMI acts by inhibiting pre-synaptic serotonin and norepinephrine reuptake transporters, thereby increasing synaptic serotonin and norepinephrine concentrations. It also exerts secondary inhibitory pharmacological actions on a range of receptors including post-synaptic alpha-adrenergic, histamine and muscarinic receptors, some of which are thought to be responsible for its adverse effects (Hillhouse and Porter, 2015). In addition to its various metabolites, it is excreted as nortriptyline (the major pharmacologically active metabolite), and unchanged, as the parent compound (Rudorfer and Potter, 1999). As excreted residues, following consumption by patients, are not completely removed by wastewater treatment processes, with levels of AMI in the region of 900 ng/L reported in treated effluent (Martínez-Bueno et al., 2012), they are ultimately released into the environment, and have been widely detected in the aquatic environment.

From different countries around the world, AMI levels ranging from 0.12 ng/L - 3.35 μg/L have been reported in surface water, while levels ranging from 0.149 - 0.504 ng/mL (biofluids) and 1.8–80 ng/g (tissues) have been reported in wildlife (Fig. 1). It is noteworthy that AMI, compared to most other pharmaceuticals, has a relatively long half-life (over 1 year) (Nałecz-Jawecki et al., 2008; Richardson and Bowron, 1985; Zuccato et al., 2001), contributing to its very high potential for chronic availability to non-target aquatic organisms. Furthermore, there is mounting evidence that environmental levels of human pharmaceuticals (ng/L to low μg/L) have the potential to elicit ecologically disruptive pharmacological, or drug target-mediated, effects (as opposed to toxicological effects) in aquatic organisms in which drug targets are evolutionarily conserved (Gunnarsson et al., 2008; Länge and Dietrich, 2002; McRobb et al., 2014; Verbruggen et al., 2018). This is because pharmaceuticals are usually designed to interact with specific targets at relatively low internal concentrations (Franzellitti et al., 2013; Regenthal et al., 1999). Consequently, it is now generally thought that pharmacological responses may occur in non-target species with conserved drug targets when plasma concentrations of pharmaceuticals reach levels that are similar to their human therapeutic plasma concentrations ($H_TPC$), depending, however, on inter-specific differences in sensitivity (Rand-Weaver et al., 2013; van den Berg et al., 2021). This 'Read-Across Hypothesis' has generated a lot of interest, and has been mostly explored in various species of fish using the predictive model known as the Fish Plasma Model (Coors et al., 2023; Henneberger et al., 2022; Huggett et al., 2003a,2003b; Malev et al., 2020; Margiotta-Casaluci et al., 2014; Nallani et al., 2016; Patel et al., 2016; Schreiber et al., 2011), while its applicability to molluscs remains unknown.

Interestingly, multiple key physiological processes are regulated by monoamines (the target of ADDs) in molluscs, which potentially makes them more vulnerable to ADD target-mediated effects compared to other animal phyla (Imiuwa et al., 2023). However, in molluscs, it remains to be understood what ADD target-mediated effects are for different classes of ADDs, whether such effects are only elicited at hemolymph levels of ADDs that are similar to their $H_TPC$, and whether the effects occur at environmentally relevant concentrations of ADDs. Furthermore, AMI has a particularly wide spectrum of monoamine-related drug targets including serotonin receptors (Honda et al., 2003), with a central role in multiple overarching physiological processes such as feeding, reproduction and locomotor-based behaviour in molluscs. Despite this, nothing is known about the effects of AMI on feeding, reproduction and locomotor-based reproductive behaviours in molluscs. Specifically, there are only a handful of reports on the effects of AMI in molluscs, with investigations currently limited to metamorphosis and a range of toxicological effects in mussels (Gilroy et al., 2017; Yamamoto et al., 1998; Yang et al., 2011, 2014); development in pacific oyster (C. Di Poi et al.,

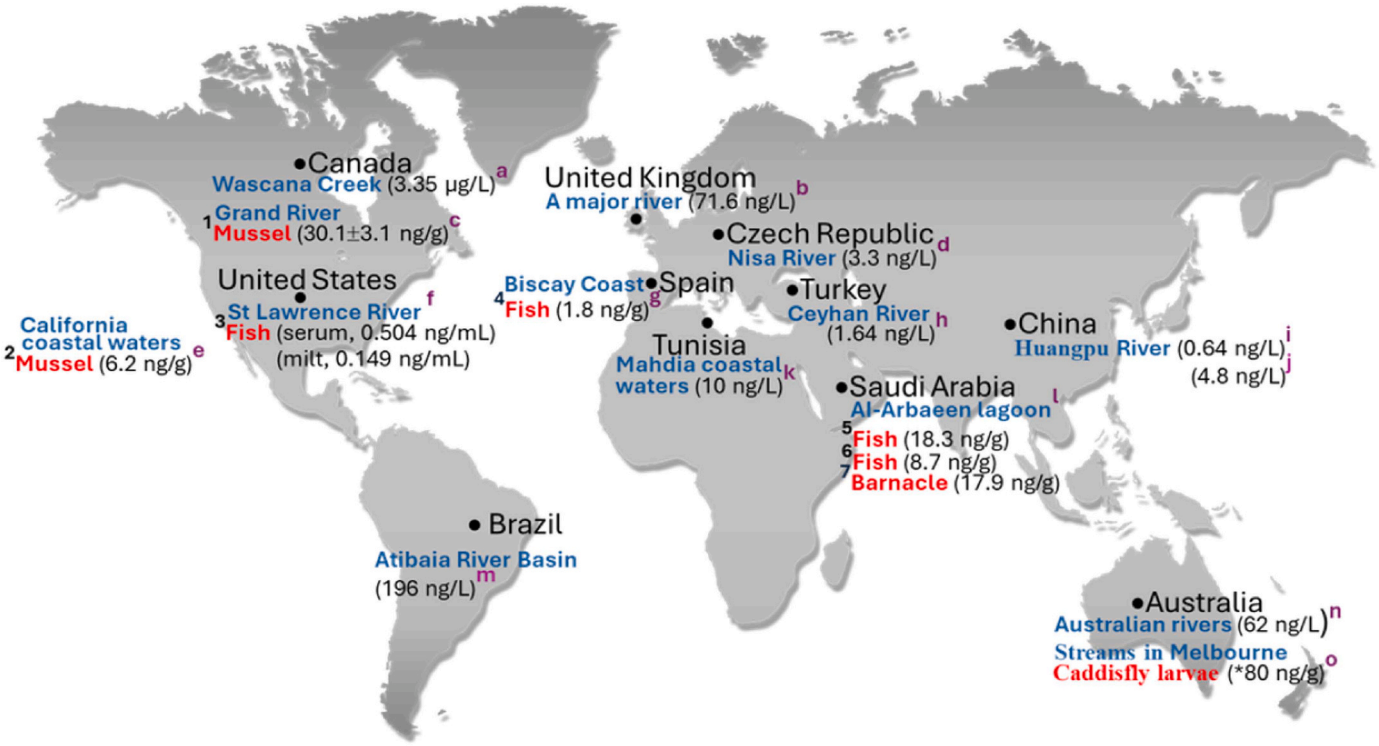


**Fig. 1.** Reported levels of AMI in surface water and wildlife. Values are presented as mean (±SD), or as maximum values where concentration range was reported. The lowest reported range is 0.12–0.64 ng/L (Huangpu River, China). *Value estimated from graphical data. [1]Freshwater mussel (*Lasmigona costata*), [2]marine mussels (*Mytilus* spp), [3]lake sturgeon (*Acipenser fulvescens*), [4]thicklip gray mullets (*Chelon labrosus*), [5]common silver biddy (*Gerres oyena*), [6]golden snapper (*Lutjanus johni*) and [7]*Balanus perforates*. [a](Perez et al., 2022), [b](Baker and Kasprzyk-Hordern, 2011), [c](de Solla et al., 2016), [d](Fedorova et al., 2014), [e](Maruya et al., 2014), [f](Banda et al., 2020), [g](Ziarrusta et al., 2016), [h](Guzel et al., 2019), [i](Ma et al., 2018), [j](Wu et al., 2017), [k](Afsa et al., 2020), [l](Ali et al., 2018), [m](Pivetta et al., 2020), [n](Scott et al., 2014) and [o](Richmond et al., 2018).

2014) and aspects of locomotion in snails (Fong et al., 2019). In all these reports, exposures were carried out at concentrations several orders of magnitude higher than measured environmental levels of AMI. There is also discourse in the literature to suggest that molluscs may be particularly sensitive to the effects of ADDs, and that levels in the environment that are deemed to be safe for fish may have adverse effects in molluscs (Carole Di Poi et al., 2013, 2014; Fong and Hoy, 2012; Franzellitti et al., 2014). However, internal concentrations of ADDs, including AMI, in hemolymph that are associated with apical effects have not been determined in molluscs.

Molluscs are an ecologically important group of animals with perhaps the greatest aggregate biological diversity which they amazingly display through their complex array of morphology, extensive micro-habitat heterogeneity, behaviour, reproduction and number of species (second only to arthropods) (Pandian, 2018). As they are largely lower trophic level organisms (Orlando-Bonaca et al., 2022; Rueda et al., 2009), they strategically provide materials and energy for higher trophic levels, with ecosystem and human health implications. Importantly, they represent the phylum with particularly high ecological predispositions to anthropogenic chemical assaults (Oehlmann and Schulte-Oehlmann, 2003), the effects of which are often less well-studied or understood compared to fish (OECD, 2010). The present study, therefore, seeks to (i) evaluate the effects of environmentally relevant concentrations of AMI on reproduction, feeding and key locomotor behaviours in the freshwater gastropod, *Biomphalaria glabrata*, (ii) determine whether hemolymph concentrations of AMI associated with apical effects are comparable to its $H_T$PC, and (iii) determine whether internal concentration of AMI in hemolymph can be predicted using the Fish Plasma Model. The study provides novel critical data needed to inform both ecological risk assessment and management of effects of environmental tricyclic ADDs on freshwater biodiversity.

## 2. Material and methods

### 2.1. Chemicals and reagents

Amitriptyline HCl (AMI) (purity > 99.9 %) was purchased from Tocris Bioscience (Abingdon, UK) while the isotopic internal standards (IS), amitriptyline-$d_6$ HCl (AMI-$d_6$), was purchased from QMX Laboratories Ltd (Thaxted, UK). Acetonitrile, methanol and HPLC grade water were purchased from Fisher Scientific (Loughborough, UK). Deionized water was obtained from a Milli-Q reference ultrapure water purification system (Millipore, Billerica, MA, USA), while Strata-X Polymeric Reverse Phase extraction cartridge (60 mg/3 mL) was purchased from Phenomenex (Macclesfield, UK).

### 2.2. Biomphalaria glabrata (Say, 1818) husbandry

*B. glabrata*, a freshwater pulmonate gastropod, is a molluscan model. Its biology, including sensitivity to environmental contaminants, is well-documented (Adema et al., 2017; Aisemberg et al., 2005; de de de Freitas Tallarico et al., 2014; de Siqueira et al., 2021). It is particularly suited for laboratory studies on account of its ease of husbandry, high reproductive capacity, translucent egg masses which can be visualized during embryonic development, and short regeneration time. The BBO2 strain used in the present study was originally obtained from the Natural History Museum, London, and has been housed for several years in the Aquatic Research Unit at Brunel University London (UK). The snails are maintained in a flow through aquaria system supplied with reverse osmosis-filtered dechlorinated tap water at 27°C, with a constant photoperiod of 16:8 L:D. They are fed three times (*ad libitum*) a week with fish flakes (TetraMin) after cleaning the glass tanks.

#### 2.2.1. Rationale for exposure concentrations and selected apical endpoints

The exposure concentrations were chosen to reflect environmentally relevant levels of AMI. As 3.35 μg/L of AMI was reported in surface water after the commencement of the present study (Perez et al., 2022), 10 and 100 ng/L test concentrations were selected based on previously reported levels of AMI in surface water (0.12–196 ng/L), while 500 and 1000 ng/L were selected based on reported levels (in the region of 900 ng/L) in treated effluents (Ma et al., 2018; Martínez-Bueno et al., 2012; Pivetta et al., 2020). Feeding, complex locomotor behaviour and reproduction were selected as apical responses being key physiological processes in molluscs that are regulated by the monoaminergic system, the principal target of ADDs in humans (Imiuwa et al., 2023).

#### 2.2.2. AMI exposure experiment

A total of ninety 4-month old adult snails (Mean±SD; shell diameter: 17.79±0.55 mm, body weight: 0.78±0.07 g) were selected from the same cohort. Six snails were randomly allocated to a 1 L beaker filled with 1000 mL reverse osmosis filtered dechlorinated tap water. The resulting fifteen 1 L glass beakers were immersed in two open-top flow-through metallic tanks supplied with water from a temperature-regulated overhead tank (26±0.7°C). The beakers were covered with metal gauze and gently aerated. Upon transfer into the beakers, the snails were fasted and acclimated. Acclimation continued until oviposition was observed in all beakers. Thereafter, baseline reproductive data was collected for one week which was used to normalize reproductive output across treatment groups (0, 10, 100, 500 and 1000 ng/L) in triplicate before the exposure period of 28 days commenced. The test medium was fully renewed every 48 hours (3–4 times per week) during which snails in a beaker were transferred into another freshly prepared 1 L beaker containing the appropriate exposure medium (reverse osmosis filtered de-chlorinated tap water with and without freshly prepared AMI, at 26±0.7°C). Each freshly prepared 1 L beaker was immediately placed in the metallic tanks supplied with temperature-regulated water, with the old beaker removed. Test medium water quality parameters, including dissolved oxygen (DO) and temperature, were monitored with a HACH (HQ40d) meter and Thermocouple (TPI 343) thermometer respectively; while pH, ammonia, nitrite and nitrate were monitored with Aquarium water test kits (OECD, 2010). The water quality measurements were taken before each medium renewal in the control and treatment (highest test concentration) groups. As *B. glabrata* is not protected by the UK Animal (Scientific Procedures) Act 1986, it was not necessary to obtain ethical approval for this study. All experimental procedures were, however, conducted in line with OECD general guidelines on molluscs toxicity testing (OECD, 2010).

### 2.3. Feeding

The snails were fed blanched organic Cos lettuce (Sainsbury, London) after the first 48 hours during acclimation, and subsequently, after each test medium renewal during exposure. Briefly, the midribs of the lettuce leaves were removed as hard parts, the leaves were cut into smaller sizes that could readily fit into the test vessels, and were then blanched with boiling water (to make them sink to the bottom of the beakers). The mass of blanched lettuce leaves was then drained to remove excess water and weighed. Snails were fed 2.0–2.6 g of the lettuce per beaker. At each exposure medium renewal, uneaten food and feacal matter were collected and then filtered to separate the food from the feacal matter. Uneaten food was drained to remove excess water and weighed. Food intake per beaker was calculated as the difference between the weight of the original food provided and the uneaten food. As the average body weight (Mean±SD) of snail per replicate was 4.66 ±0.26 g at the start of exposure, it was not necessary to further standardize food intake per gram of body weight.

#### 2.3.1. Locomotor behaviours

To measure the effects of exposure of *B. glabrata* to AMI on key ecologically relevant locomotor behavioural patterns, we developed a novel *in situ* approach to monitor the behaviour of snails in the beakers without the need for manipulation or interference during chronic

exposure. Snails were observed every other day from the start of the exposure, and the number of snails displaying the following behaviours were counted: (i) snails attached to, or crawling on, the wall of the glass beaker, to evaluate effects on substrate-dependent locomotion,(ii) the position of snails in the water column (bottom, middle and upper layers), with the beakers divided vertically into three equal layers externally using rubber bands, to evaluate effects on substrate-independent direct ascent and descent locomotion patterns through the water column, and (iii) snails displaying shell mounting or intromission, to evaluate effects on social and complex mating behaviours (Soldatenko and Petrov, 2012). Snails were observed three times a day throughout the period of exposure to increase the frequency of observation, and the total count per day was summed for each beaker. This approach was developed in order to provide a comprehensive and ecologically relevant movement evaluation as opposed to the widely reported solitary movement trial in which snails are repeatedly transferred between culture and trial vessels, and observed over a short period of time (including acclimation period) (Alberto-silva et al., 2015; Fong et al., 2017; Henry et al., 2022; Lebreton et al., 2021).

#### 2.3.2. Reproduction

During each test medium renewal (3–4 times per week), egg masses were collected from the wall of each beaker with a surgical blade, while those attached to the shell of the snails, were carefully hand-picked. The egg masses were counted and immediately fixed in 35 % ethanol. At the end of the exposure study, the fixed egg masses were examined under the microscope (Olympus SZX12) and photographed with a microscope-mounted camera (Euromex). The photographed images were thereafter processed with imageFocus (version 4.0) to count the number of eggs in the egg masses, and derive the cumulative number of eggs produced in each beaker during the course of the exposure.

#### 2.3.3. Water and hemolymph samples

Water samples (800–1000 mL) of all replicates of the control, lowest and highest test concentrations were collected for both freshly prepared exposure medium and the old exposure medium during medium renewal weekly throughout the period of exposure. The samples were collected in low density polyethylene (LDPE) bottles and stored at −20°C for subsequent analysis. At the end of exposure, the snails were bled for hemolymph collection after morphometric measurements were taken. Hemolymph samples (at least 100 μL per snail) were collected in microtubes and stored at −20°C for subsequent analysis. Some water samples after SPE extraction could not be analyzed for technical issues. Hemolymph samples from the six individual snails per replicate were pooled for both control and all test concentrations for analysis.

#### 2.3.4. The fish plasma model (FPM)

The potential of the test concentrations (10–1000 ng/L) of AMI to reach its $H_TPC$ (50–300 ng/mL) (David et al., 2018; Regenthal et al., 1999) in *B. glabrata* hemolymph was evaluated using the FPM. Briefly, the FPM, which is used to evaluate the potential of a pharmaceutical to reach the $H_TPC$ in fish plasma and elicit a pharmacological response assuming the drug targets are conserved, is essentially an effect ratio (ER) (Huggett et al., 2003a,2003b) defined as:

$$ER = H_TPC/F_{SS}PC \quad (1)$$

Where $H_TPC$ is the Human Therapeutic Plasma Concentration (which is generally available for pharmaceuticals), and $F_{SS}PC$ is the predicted Fish Steady State Plasma Concentration of a pharmaceutical, and it is derived as follows:

$$F_{SS}PC = EC \times (P_{Blood:Water}) \quad (2)$$

Where EC is a given Environmental Concentration of the pharmaceutical to which the fish is exposed, and ($P_{Blood:Water}$), the partition of the pharmaceutical between aqueous phase (water) and blood in fish, is estimated as follows:

$$\log P_{Blood:Water} = 0.73 \times \log K_{OW} - 0.88 \quad (3)$$

Note that Eq. (3), originally described to model the partition of an organic compound between water and branchial blood in fish (rainbow trout) (Fitzsimmons et al., 2001), is used to predict $F_{SS}PC$. Interestingly, then, the effect ratio (ER) of the FPM would be a general plasma concentration ratio if the $F_{SS}PC$ component of the model was available for organisms, in which drug targets are evolutionary conserved, other than fish. This is, however, not the case as steady state plasma concentration of an organic compound has only been comprehensively described in fish. Intuitively, nonetheless, the $F_{SS}PC$ component of the plasma model may be used in organisms other than fish to theoretically determine the applicability of the FPM to the organisms, and as result, evaluate their sensitivity to pharmaceuticals relative to fish and humans. The $F_{SS}PC$ component, herein described as $M_{SS}HC$ (mollusc steady state hemolymph concentration) was, however, used in the present study to assess the applicability of the FPM to molluscs, and to further evaluate molluscs' sensitivity to AMI relative to fish and humans on account of the following considerations: (i) the partition of organic compounds between two solvent phases such as water (aqueous) and organic solvents takes the same general form (Collander, 1950; Fitzsimmons et al., 2001); (ii) the relationship, $\log P_{Blood:Water} = 0.73 \times \log K_{OW} - 0.88$, used in the fish plasma model to estimate partition between aqueous phase and blood in fish across the gills, is a two-solvent partition system in which the organic phase is the protein and lipid material in the blood of the fish (Briggs, 1981; Fitzsimmons et al., 2001); (iii) fish plasma is similar, in many key characteristics, to molluscan heamolymph (Machałowski and Jesionowski, 2021; Sheikh et al., 2022); and finally (iv), the fish gills, which are an important component of this two-solvent partition system, are also similar to molluscan gills (ctenidia), both in terms of diagnostic characteristics and their morphological variations (Ponder et al., 2019). Furthermore, as the $\log K_{OW}$ of ionizable pharmaceuticals have been shown to be affected by pH (Chen and Lin, 2016), ionization-corrected $\log K_{OW}$ ($\log D_{7.4}$) of AMI (2.50) was used (Escher et al., 2020; Tsopelas et al., 2015).

### 2.4. Liquid chromatography-tandem mass spectrometry (LC-MS/MS)

For liquid chromatography-tandem mass spectrometric analysis, an Agilent 1100 Series HPLC System with a Kinetex 2.6 μm $C_{18}$ 100 Å (50 ×2.1 mm) column coupled to an AB SCIEX API 5000 mass spectrometer (Sciex, Framingham, USA), was used. The column temperature was set at 40°C while autosampler was set at 4°C with an injection volume of 20 μl. Chromatographic separation was performed through a gradient elution in filtered milli-Q water (mobile phase A) and acetonitrile (mobile phase B) at a constant flow rate of 0.8 mL/min. The following gradient was applied: 90 % filtered milli-Q water from 0.00 to 3.49 min; then, 100 % acetonitrile from 3.50 to 6.51 min; while filtered milli-Q water increased again to 100 % from 6.51 to 8.60 min, and the total run time was nine minutes. MS was performed using positive ion electrospray ionization and multiple reaction monitoring (MRM). The acquisition parameters were as follows: dwell time (50.0 msec), delustering potential (131.0 V), collision energy (31.0 V) and collision exit potential (22.0 V) for AMI; delustering potential (26.0 V), collision energy (15.0 V) and collision exit potential (22.0 V) for AMI-$d_6$. One major transition (*m/z*) 278.2–105.1 and 284.4–91.2 for the analyte and the internal standard respectively, was used for quantitation. Finally, data acquisition and processing were performed using Analyst software (version 1.7.1.). The calibration procedure is provided in the supplementary material (S1.0).

#### 2.4.1. Solid phase extraction (SPE)

The following SPE method using Strata-X 33 μm Polymeric Reverse Phase (60 mg/3 mL) cartridge (Phenomenex, CA, USA), was developed

for this study. The method was applied to two matrices of interest: reverse osmosis-filtered dechlorinated tap water conditioned with the test organism (as the exposure medium matrix) and hemolymph samples derived from the test organism. Extractions of both matrices were performed at zero (blank), low and high analyte concentration levels in duplicate analyzed in 3 separate assays across several days. For the hemolymph matrix, acetonitrile was added to samples for protein precipitation at room temperature for 20 min. Thereafter, the mixture was centrifuged at 4500 x g for 15 min. The supernatant was carefully transferred into HPLC vials and evaporated to dryness at 40°C under a stream of Nitrogen. The residue was reconstituted in 2 mL of methanol and diluted in 10 mL of milli-Q water to which the IS (single high concentration level) had been added. The resulting samples were then applied to Strata X SPE cartridges previously conditioned with 2 mL methanol and 2 mL of milli-Q water. After extraction, sorbent clean-up was performed with 2 mL of 5 % methanol in water. The cartridges were then dried under vacuum (-20 inHg for 20 mins), and eluted with 2 mL of methanol under gravity. The eluate was evaporated to dryness under nitrogen at 40°C, and reconstituted in 500 μL methanol and milli-Q water (50:50, v/v). For the exposure medium matrix, samples (50 mL) were fortified with the analyte and the IS, and thereafter applied to the cartridges previously conditioned with 2 mL of methanol followed by 2 mL of mill-Q water. Sample extraction, sorbent clean-up and drying, elution, nitrogen blowdown and extract reconstitution, then proceeded as described for the hemolymph matrix. The method validation data are provided in the supplementary material (S2.0).

### 2.5. Statistical analysis

The data are presented as mean ± standard deviation (SD) of replicate values. Using the residuals, data were tested for normality with both normality tests (Kolmogorov-Smirnov and Shapiro-Wilk) and normality plots (Q-Q plots and histogram), while homogeneity of variance was evaluated with Lavene's test. When normality assumption was met, a one-way ANOVA, followed by Duncan's multiple range test (where the overall F statistic of ANOVA was significant), was performed. Otherwise, a Kruskal-Wallis test was used (number of eggs per mass). Pairwise comparison of morphometric data at baseline and after exposure was done using paired t-test. Multivariate analysis between the test chemical and biological responses was performed using principal component analysis (PCA). Values were considered significantly different at $P < 0.05$. All statistical tests of significance were carried out using SPSS (110 IBM, version 20), while PCA and graphical presentation of all data, were carried out using GraphPad Prism (version10.1.2).

## 3. Results

### 3.1. Test validity criteria

Dissolved oxygen (DO) content of the exposure medium before renewal was above 60 % of air saturation value (5.8±0.7 mg/L), while the average water temperature was 26.1±0.7°C. The values are presented in Table S1 (supplementary material). The pH, ammonia, nitrite and nitrate (evaluated using a colorimetric test kit) were within the normal range (OECD, 2010). There was no mortality in the control group throughout the study. In the treatment group, however, 1.1 % mortality was observed during exposure (i.e.1 snail in the 100 ng/L treatment group). A constant photoperiod of 16:8 L: D (with a short transition period) was maintained throughout the study.

### 3.2. AMI levels in water and hemolymph

The measured levels of AMI in the exposure system (stability test), freshly prepared exposure medium, old exposure medium at renewal, and in the hemolymph of exposed *B. glabrata* are presented in Table 1.

AMI concentrations in water samples left in glass beakers under exposure conditions (AMI stability test) and in samples of freshly prepared exposure medium (stored in LDPE bottles) were within ± 20 % of the nominal concentrations. At renewal, however, concentrations in the exposure medium were less than 80 % of the nominal, and the measured concentrations at renewal were reported as effective exposure concentrations (OECD, 2010). Furthermore, the measured concentrations of AMI in method validation samples, including accuracy, precision and LLOQ are presented in Tables S2-3. Method development parameters and method validation description are also provided (see the supplementary material). All method development and validation parameters were within acceptable limits.

### 3.3. The fish plasma model

The predicted levels of AMI in *B. glabrata* hemolymph using the FPM are shown in Table 2**.** Interestingly**,** measured hemolymph levels of AMI in *B. glabrata* agreed well with the predicted levels in fish plasma. Furthermore, the measured hemolymph levels of AMI at the effective exposure concentration of the 1000 ng/L treatment group were also consistent with the measured plasma level of AMI in fish at comparable exposure concentrations.

### 3.4. Effects of AMI exposure on feeding, reproduction, behaviors and growth

The effect of exposure of *B. glabrata* to AMI on feeding is shown in Fig. 2. Food intake in the 1000 ng/L treatment group was significantly lower ($p < 0.001$) than the control and other treatment groups in the first week of exposure, and although this reduced food consumption pattern in the 1000 ng/L exposure group continued throughout the remainder of the exposure period, the difference was not statistically significant. However, the total food consumed in the 1000 ng/L group over the entire exposure period (Fig. S4) was significantly lower ($p=0.004$) than the total food consumed in the control and other treatment groups.

The effects of exposure on the cumulative number of egg masses produced per snail, cumulative number of eggs per mass per snail and

**Table 1**
Measured AMI levels in the exposure medium and heamolymph of *B. glabrata*. Heamolymph samples were collected at the end of the exposure period.

| | Measured AMI concentrations | | | |
|---|---|---|---|---|
| | Water (ng/L) | | | |
| Test concentrations (ng/L, nominal) | [r]Stability test (48hrs, n=2) | Exposure (Freshly prepared medium, n=2) | Exposure (Renewal, n=4) | Hemolymph (ng/mL) |
| Control | <DL | <DL | <DL | <DL |
| 10 | 11.9 ±0.5 | 10.9±0.1 | < DL | < DL |
| 100 | 89.15±26.7 | na | na | < DL |
| 500 | 602.0 ±5.7 | na | na | <LLOQ |
| 1000 | 1120.0±56.6 | 827±9.9 | 312.5±15.9 | 0.74±0.56 |

DL, Detection limit; LLOQ, Lower limit of quantitation; na, not analyzed; hemolymph was analyzed as pooled replicate samples (n=3 replicates, 6 snails per replicate).
[r]AMI stability test under exposure conditions was performed without the snail and feed. All values are presented as mean±SD.

**Table 2**
Predicted and measured hemolymph/plasma levels of AMI in *B. glabrata* and *Rutilus rutilus* using the fish plasma model.

| | | | | Hemolymph/plasma levels (ng/mL) | | | |
|---|---|---|---|---|---|---|---|
| | | | | Predicted | Measured | | |
| Organism | Drug | $H_TPC$ (ng/mL) | MEC (ng/L) Mean±SD | $F_{SS}PC$/ $M_{SS}HC$ | Hemolymph/ Plasma levels Mean ±SD | Range | Reference |
| Mollusc | AMI | 50–300[a] | 312.5±15.9 | 2.75 | 0.74±0.56 | 0.34–1.13 | The present study |
| Fish | AMI | 50–300[a] | 374±44 | [b]3.29 | 1.5±0.5 | 0.90–2.2 | David et al. (2018) |

AMI, amitriptyline; $H_TPC$, Human therapeutic plasma concentrations; MEC, Measured exposure concentration, $F_{SS}PC$, fish steady state plasma concentration; $M_{SS}HC$, mollusc steady state hemolymph concentration; [a](David et al., 2018); [b]This value was not reported by the authors, was calculated in the present study using $logD^{7.4}$; 2.50 was used as $logD^{7.4}$ of AMI (Tsopelas et al., 2015); $logD^{7.4}$, ionization corrected $logK_{OW}$ at a pH of 7.4.

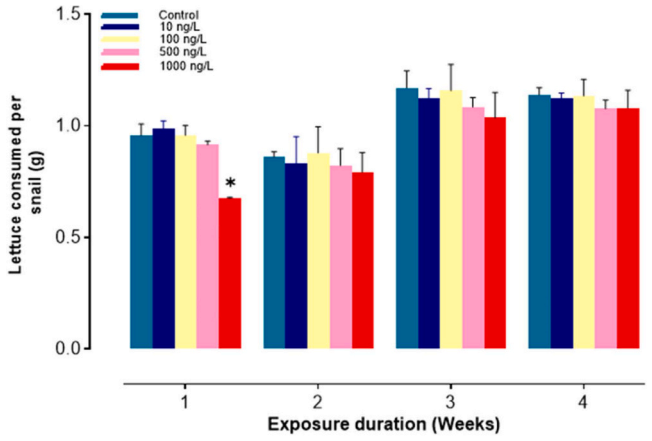


**Fig. 2.** Food consumption per week per snail (*B. glabrata*) exposed to AMI. All values represent mean±SD (n = 3 replicates; 6 snails per replicate). Asterisk indicates statistically significant difference for each week.

cumulative number of eggs per snail are shown in Fig. 3A-C**.** The lowest numbers of egg masses and eggs were laid by *B. glabrata* in the 1000 ng/L treatment group, while egg masses with the lowest number of eggs per mass were laid in 500 ng/L group, followed by 1000 ng/L group. They were, however, not significantly different from control and other treatment groups.

Locomotor behaviour patterns in *B. glabrata* in the control and all treatment groups are shown in Fig. 4A-C**.** Mating behaviours, substrate-dependent locomotion and 'passive' movement through the layers of the water column in all the treatment groups were generally consistent with the control. Showing similar levels of aggregation and intromission, the snails were mainly resident in the bottom layer with occasional transition to the upper layer.

Total body weight (TBW) and shell diameter (SD) of *B. glabrata* at baseline (day 0) and after exposure (day 28) are presented in Fig. S5. While there was a slight decrease in TBW (1000 ng/L), SD increased marginally (500 and 1000 ng/L), and these differences were not statistically significant (paired t-test: TBW, $p$=0.167; SD, $p$=0.568).

### 3.5. Principal component analysis (PCA)

Apical effect response variables (feeding, egg masses, eggs per mass, eggs, total body weight and shell diameter) and the test chemical (AMI) were subjected to multivariate analysis (PCA) in order to understand (i) relative effect size in the responses of apical effect variables, and (ii) whether primary effect responses in any of the apical effect variables could account for responses seen in other apical effect variables. PCA extracted two principal components (PC1 and 2) that accounted for 96.18 % of the total variance. PC1 accounted for 68.33 % of the total variance, while PC2 accounted for 27.85 %. The biplot loading (Fig. 5) shows that all response variables, except shell diameter, were negatively correlated with AMI, with food intake being the most negatively correlated (-0.995), followed by eggs (-0.944) and body weight (-0.826).

## 4. Discussion

The present study is the first report on the effects of AMI on feeding, reproduction and mating behavior in molluscs to our knowledge. The measured hemolymph levels of AMI in *B. glabrata* were quite variable, and ranged from below LLOQ (500 ng/L nominal exposure) to 0.74 ±0.56 ng/mL (1000 ng/L nominal exposure). These seemingly low hemolymph levels of AMI may be due to a number of factors. Firstly, a large amount of AMI may have been absorbed from the exposure medium by the uneaten feed and feacal matter present in the medium during exposure as the highest test concentration was only 31.25 % of the nominal at renewal. All test concentrations of AMI measured in the exposure system were found to be stable over a period of two days in the absence of the snails, feed and feacal matter (Table 1). Secondly, as the partition of pharmaceuticals into body tissues tends to be generally higher than their corresponding plasma levels (David et al., 2018; Robert et al., 2017), sequestration into body tissues may therefore, in part, also explain the measured low hemolymph levels of AMI in *B. glabrata.* Finally, as AMI usually undergoes extensive metabolism in humans (Breyer-Pfaff, 2004), metabolic conversion of the parent compound may further account for the measured hemolymph level of AMI in the present study. Interestingly, however, a similar level of AMI (1.5 ±0.5 ng/mL) has been reported in the plasma of Roach (*Rutilus rutilus*) exposed in a laboratory to an effluent containing 374 ±44 ng/L of AMI in a flow through system (David et al., 2018). Indeed, the exposure concentration at renewal (312.5±15.9 ng/L) in our study, which is comparable to the one reported for *R. rutilus* (374 ±44 ng/L), may explain the measured hemolymph levels of AMI in *B. glabrata*. Building on this insight, it is critical to understand how the measured hemolymph levels of AMI compare with its predicted levels in fish using the FPM; and whether *B. glabrata* is responsive to this low hemolymph levels of AMI through the apical endpoints.

The predicted mollusc steady state hemolymph concentration ($M_{SS}HC$) of AMI (2.75 ng/mL) using the FPM agreed quite well with the measured hemolymph level of AMI (0.74±0.56 ng/mL) at 312.5 ±15.9 ng/L exposure concentration. By definition, the predicted level (2.75 ng/mL) is the expected theoretical plasma level of AMI at steady state in fish using the FPM, or in the present study, the expected theoretical hemolymph levels of AMI at steady state in molluscs, if the FPM holds true for molluscs, both at the exposure concentrations. Note that comparable differences in measured versus predicted levels of AMI in plasma of *R. rutilus* exposed to a slightly higher concentration of AMI (Table 2) have also been observed (David et al., 2018). In *B. glabrata*, the predicted hemolymph level of AMI using the FPM was only 3.7-fold higher than the mean value of the measured hemolymph levels, while in *R. rutilus*, the predicted plasma level of AMI was 2.2-fold higher than its measured plasma levels, probably reflecting the slightly higher exposure concentration when compared to the 3.7-fold difference in

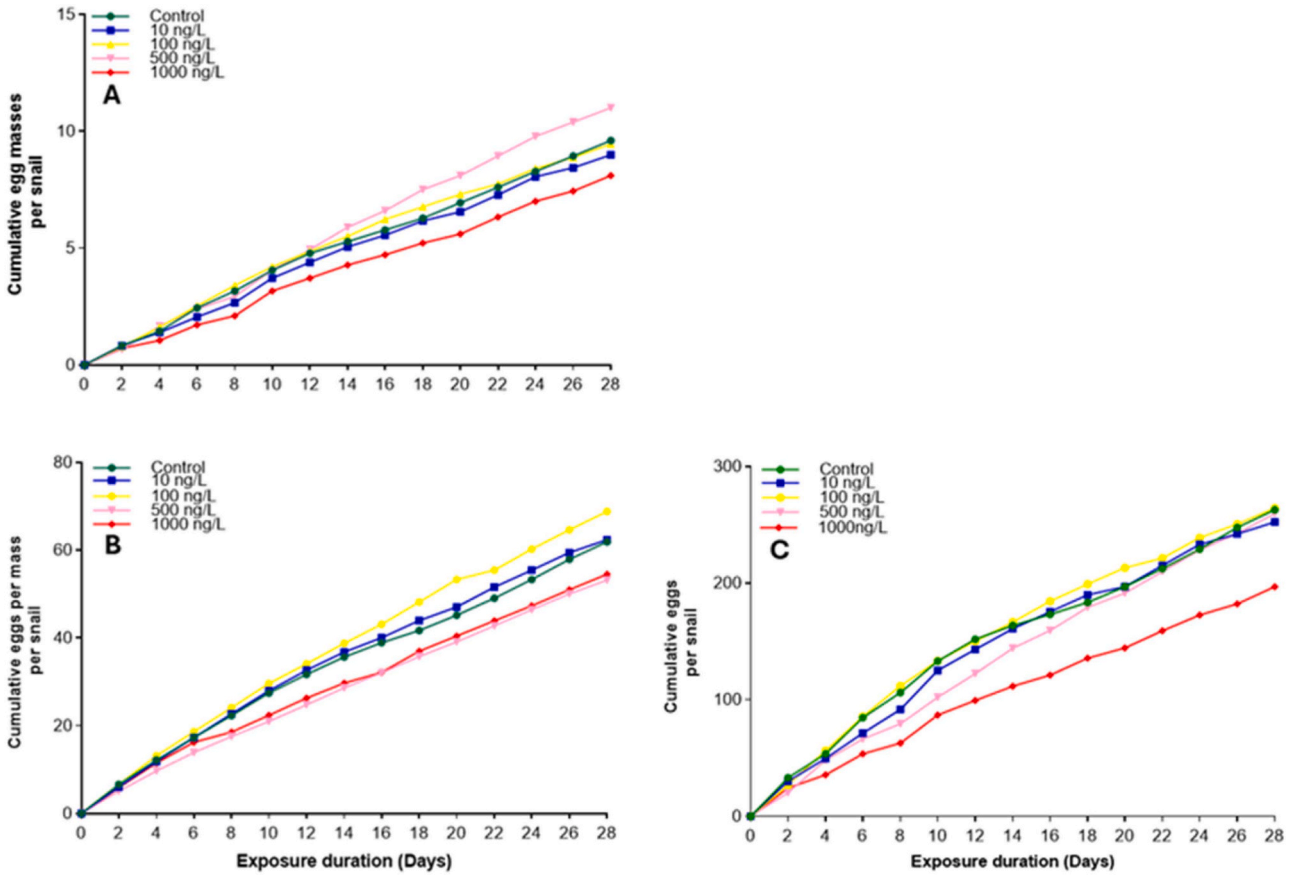


**Fig. 3.** Effects of AMI on reproductive potential of *B. glabrata* over the period of exposure (A) cumulative number of egg masses laid per snail (9.6 ±1.2, 9.0 ±0.8, 9.5 ±1.6, 11.0 ±3.1 and 8.1 ±2.2); (B) cumulative number of eggs per mass laid per snail (61.9 ±1.1, 62.4 ±6.1, 68.8 ±17.9, 53.1 ±5.2 and 54.5 ±4.3); and (C) cumulative number of eggs laid per snail (262.9 ±27.7, 252.3 ±21.7, 264.3 ±50.8, 259.5 ±81.2 and 196.9 ±55.7). Respective cumulative values (mean±SD) from control to the highest test concentrations on day 28 are presented in parenthesis. (n = 3 replicates; 6 snails per replicate).

*B. glabrata.* Again, the agreement is easily appreciated from the overlapping range of the hemolymph and plasma levels of AMI respectively in *B. glabrata and R. rutilus* (Table 2). This observation clearly shows that the estimation of hemolymph bioconcentration factor of AMI in *B. glabrata*, using Eq. (3), is consistent with the estimation of plasma bioconcentration factor of AMI in fish, with the same equation. It further suggests that possible difference in the sensitivity of molluscs and fish to AMI (at least, at the tested concentration) is not a function of pharmacokinetic differences that together affect the internal (plasma/hemolymph) concentration of AMI. The observed applicability of the fish plasma model to molluscs as seen in the present study may be attributable to (i) shared blood plasma and respiratory surface physiology (see Section 2.3.4 -fish plasma model), and (ii) the fact that AMI is an ionizable pharmaceutical (basic) which has a $logD_{7.4}$ (ionization-corrected $logK_{OW}$ at a physiological pH of 7.4) of 2.50 (Tsopelas et al., 2015), with low partition coefficient (used in the present study), as against $logK_{OW}$ of 4.92 (Giebułtowicz and Nałecz-Jawecki, 2014), and therefore minimizes overestimation in model prediction (Nallani et al., 2016). Importantly, these findings show that the FPM (a powerful tool used to identify and prioritize pharmaceuticals with potential to exert drug target-mediated effects at environmental levels in fish) can also be applied to freshwater molluscs at least for ionizable pharmaceuticals. Furthermore, while the measured hemolymph level of AMI in *B. glabrata* in the present study is up to 67–405-fold less than the $H_TPC$ of AMI, *B. glabrata* exhibited intrinsic sensitivity to this low hemolymph level of AMI, with apical responses. Apical effects were, however, not investigated in the *R. rutilus* exposed to AMI (David et al., 2018).

The observed statistically significant disruption of food intake in the highest exposure concentration group in the first week of exposure may have been caused by increased hemolymph levels of AMI immediately following the commencement of exposure (dosing) in the first week of exposure before subsequent decrease with repeated dosing (Krause et al., 2021). This initial surge in hemolymph levels of AMI may have inactivated monoamine receptor subtypes, including serotonin receptors, that regulate feeding in molluscs. In gastropod molluscs, rhythmic feeding movement patterns, including those that culminate in biting and swallowing, are controlled by a network of neurons, the feeding central pattern generators (CPG), in the buccal ganglia. Serotonin and dopamine have been shown to initiate and modulate the activity of the feeding CPG as neurohormones through their receptors in the buccal area including the radula (Alexeeva et al., 1998; Hernádi et al., 2008). It is possible that AMI-induced inactivation of monoamine receptors that mediate feeding may have also continued at lower hemolymph levels of AMI that is expected with repeated dosing after the first week of exposure, but with less receptor inactivation. This might explain why the reduced weekly food intake in the 1000 ng/L treatment group from the second week to the end of exposure was not statistically significant, but the total food consumed in this group was significantly lower than those of the control and other treatment groups over the entire period of exposure. It is noteworthy that, overall, the highest food intake was observed in the control. Furthermore, the observed marginal decrease in TBW in the 1000 ng/L group compared to other treatment groups and the control may be explained by the disruption of feeding in this group as food intake provides energy necessary for growth. The absence of marked increase in SD in the control is not surprising considering the age of the test organism at the start of exposure. On the other hand, however, the slight increase in SD in the 500 and 1000 ng/L treatment groups compared to control and other treatment groups could

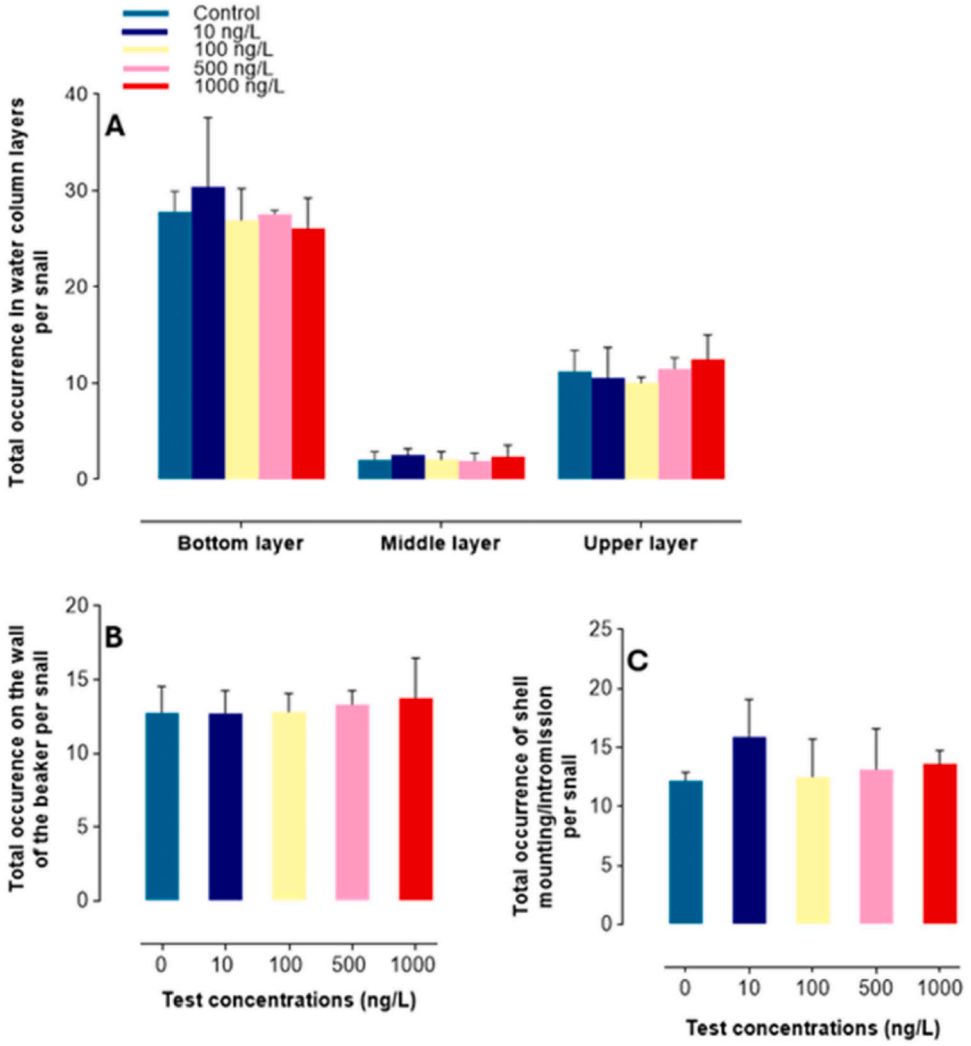


**Fig. 4.** Effects of exposure of *B. glabrata* to AMI on locomotor behaviours (A) total number of occurrences in the water column layers per snail. (B) total number of occurrences on the wall of the beaker per snail. (C) total number of occurrences of shell mounting/intromission per snail. All values represent mean±SD (n = 3 replicates; 6 snails per replicate).

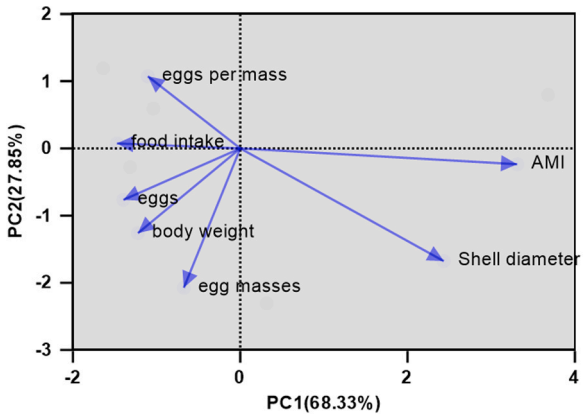


**Fig. 5.** Principal component analysis (PCA) of apical endpoint variables and AMI. PC 1 is a weighted contrast of AMI and shell diameter (positive coefficients) with the other variables, while PC 2 is a weighted contrast of eggs per mass and food intake (positive coefficients) with the other variables. AMI is the primary variable on PC 1 that accounted for 68.33 % of the total response variance.

be suggestive of treatment-induced adaptative shell growth or thickening in which metabolic materials are initially stored up in the shell (Pascoal et al., 2012; Pinkina et al., 2022), and then used up subsequently if disruption in food intake is prolonged (Porcel et al., 1996). Note that the position of the SD on PC1 (Fig. 5) was particularly influenced by its association with AMI. Importantly, as food resources in the natural environment are limited and also affected by seasonal variations, chronic disruption of feeding at critical life stages may result in significant population-level effects in wildlife by affecting survival and recruitment (Stroud et al., 2019). It is noteworthy that the PCA clearly reveals that feeding was the most impacted apical endpoint of exposure of *B. glabrata* to environmental levels of AMI (Fig. 5).

Although not statistically significant, the reduced reproductive output in the 500 and 1000 ng/L treatment groups compared to control also indicates exposure-induced disruption of reproduction in *B. glabrata.* The apparent variation in the observed reproductive effects (fecundity) can be explained by the physiology of both egg production and packaging in *B. glabrata*. In this species, while eggs are laid as egg masses (i.e. collections of eggs that are packed in gelatinous materials and capsules secreted respectively by muciparous and oothecal glands), they are initially processed individually (Boyle and Yoshino, 2002). The eggs undergo fertilization, perivitelline coating and membrane encapsulation individually in different parts of the hermaphroditic duct

(carrefour region, albumin gland and membrane gland, respectively) before they are eventually packed and released as egg masses (Hathaway et al., 2010). Varying degrees of disruptions to these processes (as would be expected from different exposure concentrations of AMI) may therefore differently affect oocyte maturation and release, the number of eggs that are available for fertilization, the number of fertilized eggs that get packed into egg masses, and how often egg masses are available for release. Expectedly, the total number of egg masses laid, and the total number of eggs produced, were lowest in the 1000 ng/L treatment group throughout the period of exposure, while the lowest eggs per mass occurred in the 500 ng/L (followed by the 1000 ng/L) group. Cumulative egg production was also low in the 500 ng/L group from day 6 to day 16 of exposure compared to control and the low treatment groups (10 and 100 ng/L). The increased rate of egg mass production over the latter course of exposure in the 500 ng/L group compensated for the effects of AMI on fecundity overall (Fig. 4C). Furthermore, although the disruption of reproductive output was not statistically significant, chronic or lifetime exposure of freshwater gastropods to AMI could affect reproductive success with potential to impact population growth. This is further corroborated by PCA which reveals that the total number of eggs per snail (the actual measure of reproductive potential) was the most negatively impacted of the three fecundity parameters evaluated in the present study (Fig. 5). Besides, as the concentration of AMI in the 1000 ng/L group was only 31.25 % of the nominal at renewal, and environmental levels of AMI up to 3.35 μg/L have been reported after the commencement of the present study (Perez et al., 2022), our findings confirm that environmental levels of AMI disrupt reproduction in freshwater molluscs. Furthermore, as serotonin is known to enhance oocyte maturation and egg laying in *B. glabrata* (Manger et al., 1996), and inhibition of serotonin reuptake transporters would be expected to increase tissue levels of serotonin, the binding of AMI to serotonin reuptake transporters therefore does not explain the reduction in the number of egg masses and eggs observed in *B. glabrata* in the present study. Additionally, although feeding has been shown to affect reproduction in molluscs (Vianey-liaud, 1984), the reduced feeding rate in AMI-exposed snails in the present study does not also fully explain the disruption in reproduction as there was no corresponding drop in physical activity (locomotor behaviour) in any of the treatment groups compared to control. Therefore, it is possible that AMI acted in the present study by antagonistic binding to monoamine tissue-specific receptor subtypes, including serotonin receptors, that regulate oocyte maturation and ovulation in *B. glabrata.* Indeed, serotonin has been shown to mediate oocyte maturation, oocyte release from ovarian tissue and oviposition in molluscs (Manger et al., 1996; Muschamp and Fong, 2001), and the serotonin receptors mediating these physiological processes have been pharmacologically characterized on oocyte membrane in a number of bivalve molluscs (Tanabe et al., 2006). Furthermore, the absence of statistically significant difference, or the occurrence of only mild disruption of reproductive output, across the entire period of exposure compared to the observed statistically significant disruption in food intake, may be attributable to differences in the sensitivity of the serotonin receptor subtypes that regulate feeding and reproduction in *B. glabrata* as monoamines mediate multiple physiological processes through various tissue-specific receptors (Barbas et al., 2002).

AMI had no apparent effects on mating and locomotion behaviours in *B. glabrata* in any of treatment groups compared to the control. It is probable, therefore, that concentrations higher than the ones tested in the present study would be required to disrupt locomotor behaviours in *B. glabrata.* Indeed, studies on aquatic molluscs that reported effects of AMI on locomotor behaviors used concentrations several order of magnitude higher than environmentally relevant levels used in the present study. In the marine mud snail, *Ilyanassa obsoleta,* exposure to AMI at 31.3 μg/L did not result in effects on righting time, but righting time was affected from 156 μg/L to 3.13 mg/L of AMI (Fong et al., 2019). Similarly, exposure of the freshwater snail, *Leptoxis carinata*, to 3.13 and 156 μg/L of AMI did not result in effects on righting time, whereas from 234 μg/L to 3.13 mg/L of AMI, righting time was affected (Fong et al., 2019). In many freshwater pulmonate gastropods, networks of neurons, including serotonergic neurons, innervating the pedal sole ciliary cells, control locomotor activity of the ciliated sole epithelium to produce locomotion (Delgado et al., 2012; Deliagina and Orlovsky, 1990; Longley and Peterman, 2013). Interestingly, locomotor activities in these cells have been shown to be mediated by serotonin receptors (Longley and Peterman, 2013). The absence of effects on locomotor behaviours in *B. glabrata* (compared to feeding and reproduction) suggests that hemolymph levels of AMI were below the effect threshold of monoamine receptor subtypes controlling locomotion in *B. glabrata*. Furthermore, the observed distribution pattern was consistent with a previous report on the normal behaviour of *B. glabrata* in water column (Corr et al., 1984). The highest density occurred in the bottom layer, and may be due to the presence of settled food. The lowest level of occupancy was observed in the middle layer, which was used as a transition layer between the bottom and the upper layers of the beakers (Fig. 3A). The level of occupancy observed in the upper layer may have been associated with atmospheric oxygen acquisition, which is typical for pulmonate gastropods.

## 5. Conclusion

We investigated the effects of AMI on feeding, reproduction and key complex ecologically relevant locomotor behaviours for the first time in molluscs. The study demonstrates that environmental levels of AMI disrupt feeding and reproduction in molluscs at particularly low hemolymph levels of AMI. As the highest test concentration was only 312.5 ±15.9 ng/L at renewal, and environmental level of AMI up to 3.35 μg/L has recently been reported, these findings further show that environmental levels of AMI may induce higher magnitude of feeding and reproductive disruption than observed in the present study in molluscs. The demonstrated agreement between the predicted plasma level of AMI in fish and its measured hemolymph levels in *B. glabrata* and plasma levels in *R. rutilus* reveals the applicability of the FPM to molluscs for pharmaceutical hazard identification. The particularly strong negative association of food intake and number of eggs with low hemolymph levels of AMI suggests that hemolymph levels of AMI may be a useful indicator for biomonitoring feeding and reproductive disruptions in wild populations of freshwater gastropods. Further studies on the effects of AMI on feeding and reproduction, including the elucidation of the mode of action, in other freshwater gastropod species are needed to urgently provide a comprehensive insight into the effects of tricyclic ADDs on freshwater gastropods. Overall, our study provides novel critical data needed to inform ecological risk assessment of environmental tricyclic ADDs, and the development of proactive methods for improved wastewater treatment and disposal of the pharmaceuticals, for the protection of freshwater biodiversity.

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## CRediT authorship contribution statement

**Maurice E. Imiuwa**: Writing - Review & Editing, Writing - Original Draft, Visualization, Validation, Resources, Project administration, Methodology, Investigation, Formal analysis, Data Curation, Conceptualization. **Alice Baynes**: Writing - Review & Editing, Supervision, Methodology, Conceptualization. **Rakesh Kanda**: Writing - Review & Editing, Validation, Resources, Methodology. **Edwin J. Routledge**: Writing - Review & Editing, Validation, Supervision, Resources, Project administration, Methodology, Conceptualization.

## 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.

## Data availability

Data will be made available on request.

## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2024.116656.

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