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**Low concentrations of oxazepam induce feeding and molecular changes in**  
***Radix balthica* juveniles**

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## Abstract

Psychotropics, especially benzodiazepines, are commonly prescribed worldwide. Poorly eliminated at wastewater treatment plants, they belong to a group of emerging contaminants. Due to their interaction with the GABA<sub>A</sub> receptor, they may affect the function of the nervous system of non-target organisms, such as aquatic organisms. The toxicity of oxazepam, a very frequently detected benzodiazepine in continental freshwater, has been largely studied in aquatic vertebrates over the last decade. However, its effects on freshwater non-vertebrates have received much less attention. We aimed to evaluate the long-term effects of oxazepam on the juvenile stage of a freshwater gastropod widespread in Europe, *Radix balthica*. Juveniles were exposed for a month to environmentally-relevant concentrations of oxazepam found in rivers (0.8 µg/L) and effluents (10 µg/L). Three main physiological functions were studied: feeding, growth, and locomotion. Additionally, gene expression analysis was performed to provide insights into toxicity mechanisms. There was a strong short-term activation of the feeding rate at low concentration, whereas the high dose resulted in long-term inhibition of food intake. A significant decrease in mortality rate was observed in juveniles exposed to the lowest dose. Shell growth and locomotor activity did not appear to be affected by oxazepam. Transcriptomic analysis revealed global over-expression of genes involved in the nervous regulation of the feeding, digestive, and locomotion systems after oxazepam exposure. The molecular analysis also revealed a possible interference of animal manipulation with the molecular effects induced by oxazepam exposure. Overall, these results improve our understanding of the effects of the psychoactive drug oxazepam on an aquatic mollusc gastropod.

**Keywords :** pond snail, oxazepam, benzodiazepine, feeding rate, gene expression

## 1. Introduction

Psychoactive drugs, such as antidepressants, antiepileptics, and anxiolytics are widely prescribed and consumed. Conventional wastewater treatment plants (WWTPs) are unable to completely eliminate these compounds and they persist in the environment (Cunha et al., 2017; Hummel et al., 2006). These molecules have thus been considered to be emerging contaminants over the last several years. Among psychotropic drugs, benzodiazepines are generally used as nervous-system inhibitors to treat anxiety, insomnia, and epilepsy (Argyropoulos and Nutt, 1999). Indeed, they are agonists of gamma-aminobutyric acid (GABA) that bind to GABA<sub>A</sub> receptors, keeping chloride-ion channels open and leading to membrane hyperpolarization of neural cells.

Among benzodiazepines, oxazepam is ranked among the 20 molecules showing the highest hazardous quotient (Orias and Perrodin, 2014). Indeed, it is a metabolite of other benzodiazepines (diazepam, nordazepam, and lorazepam) that are among the most highly consumed worldwide (Kosjek et al., 2012). Moreover, oxazepam is very poorly removed by WWTPs, and is frequently present at detectable levels (Cunha et al., 2017; Fick et al., 2017). It is commonly measured at concentrations of hundreds of ng/L in WWTP effluents (Calisto and Esteves, 2009; Cunha et al., 2017) and tens of ng/L in rivers (Fick et al., 2017; Hummel et al., 2006), but a recent study reported its presence at concentrations of up to 7.4 µg/L in WWTP effluents and 0.8 µg/L in a French river (Mazzitelli et al., 2018). Studies have also highlighted its resistance to photodegradation and its persistence in lake sediments in an unchanged form for at least 40 years (Calisto et al., 2011; Klaminder et al., 2015). Despite its relative hydrophilicity (Log K<sub>ow</sub> = 2.31), oxazepam has been found at non-negligible concentrations in sediments downstream from WWTPs in France (9.7-12 ng/g) (Aminot et al., 2015). Oxazepam thus requires monitoring because of its persistence in the environment, with high levels of bioaccumulation in aquatic organisms (Lagesson et al., 2016), and potential long-term effects on aquatic fauna.

Despite these data, ecotoxicological studies are scarce and most concern the bioaccumulation and impact of oxazepam on the development and behaviour of various species of fish. In fish, the highest concentrations in adults have been found in the brain, plasma, and liver (Huerta et al., 2016), whereas bioaccumulation is weak in embryos (Granger Joly de Boissel et al., 2017). Heynen et al. (2016) showed that the bioconcentration negatively correlates with the weight of perch. In terms of its impact, oxazepam exposure on Japanese medaka and Eurasian perch causes spinal and cardiac

malformations of embryos, delayed growth, and decreased mortality (Granger Joly de Boissel et al., 2017; Klaminder et al., 2014). It also has effects on behaviour, causing erratic swimming in medaka embryos, stimulating post-hatching and adult activity, increasing boldness and feeding rate in perch and roach, and disrupting migration behaviour in salmon (Brodin et al., 2017, 2013; Granger Joly de Boissel et al., 2017; Hellström et al., 2016; Klaminder et al., 2014; Saaristo et al., 2019). Nevertheless, Sundin et al. (2019) showed that these effects can be alleviated after a depuration phase.

Knowledge of the toxicity of oxazepam for invertebrates is limited. Garcia-Galan et al. (2017) reported a decrease of the mortality in the crustacean *Gammarus fossarum*, with a high level of bioaccumulation, and an increase in activity and distance travelled has been observed in the crayfish (*Procambarus virginalis*) (Kubec et al., 2019).

Because of their benthic habitat, primary consumer status, and widespread presence in continental surface waters (Seddon et al., 2014), the use of freshwater gastropods as a reference organism in ecotoxicology has been suggested (Amorim et al., 2019; Tallarico, 2016). The ecotoxicological response of these organisms is beginning to be understood, especially after heavy-metal and pesticide exposure (Bouétard et al., 2013; Cœurdassier et al., 2003; Coutellec et al., 2013; Das and Khangarot, 2011; Gupta and Singh, 2011). The effects of pharmaceuticals, in particular psychoactive drugs, are however not well documented. Pond snails are particularly amenable to studying the effects of exposure to psychoactive drugs, as their behaviour and nervous system are well characterized (Benjamin, 2012; Kodirov, 2011; Syed and Winlow, 1991), providing a great advantage for the understanding of toxicity mechanisms. The knowledge of such toxicity mechanisms can now be studied in more detail as the *de novo* transcriptome of *Radix balthica* has been obtained from juveniles exposed to oxazepam (Mazzitelli et al., 2017).

In this context, it is relevant to advance studies of oxazepam adverse effects on the freshwater gastropod *R. balthica*, which we have used over the last few years as a laboratory model to evaluate the effect of various psychoactive drugs (Mazzitelli et al., 2017). We aimed to evaluate the toxicity of oxazepam on the juvenile stage of *R. balthica* and gain insight into the mechanisms of toxicity by combining complementary physiological, behavioral and molecular approaches. The physiological and behavioral approaches focused on three important endpoints: feeding, growth, and locomotion, and the molecular approach targeted genes involved in these three functions, as well as other systems,

such as detoxification. This study is one of the first long-term analyses of both the physiological and molecular aspects of oxazepam exposure on a freshwater invertebrate.

## 2. Materials and methods

### 2.1. Test organisms

*Radix balthica* (Linnaeus, 1758) genitors were sampled from the bank of the Garonne river (France) (GPS coordinates: 44.139933, 0.758923, pH: 7.9, water temperature: 15.3°C). We placed 75 genitors in each 50 L aquarium. These individuals were allowed to acclimate for a period of two weeks in a controlled-temperature chamber ( $21 \pm 1^\circ\text{C}$ ), in ISO 6341: 2012 water (64 mg/L  $\text{NaHCO}_3$ ; 294 mg/L  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ; 63 mg/L  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ; 6 mg/L KCl) (OECD, 2016a). Egg masses were collected with a blade and placed in glass jars until hatching. We then placed 25 to 30 newly hatched juveniles in each of six glass jars containing 200 mL ISO water. The jars were incubated under a 16 h light:8 h dark photoperiod, at a constant temperature of  $21 \pm 1^\circ\text{C}$  for two months. The water was renewed weekly and the juveniles were fed boiled organic lettuce *ad libitum* for better assimilation.

### 2.2. Experimental design

Oxazepam (CAS No 604-75-1, Log KoW: 2.31, solubility in water: 20 mg/L (Yalkowsk and He, 2003)) was purchased from Alsachim (Illkirch Graffenstaden, France, purity > 98%). A 1 mg/L stock solution was prepared in ISO 6341 water, sonicated for 1 h, filtered on a 0.47  $\mu\text{m}$  PTFE filter, the concentration measured, and solution stored at  $4^\circ\text{C}$ . For each concentration, 40 juveniles (two months old) were placed in an exposure system that allowed individual monitoring throughout the experiment. The exposure system consisted of a plexiglas slab mounted on feet and drilled with holes. Tubes with a grid at the bottom were inserted into each hole. Each individual had 57  $\text{cm}^3$  of volume available. Each system was covered with a wire mesh to avoid escape of the animals. The exposure system was then partially submerged in an aquarium containing 16 L ISO 6431 water supplied with bubbling air on both sides of the aquarium. Three experimental conditions were assessed, a control (ISO water) and two oxazepam concentrations, consisting of a 'low' (0.8  $\mu\text{g/L}$ ) and 'high' (10  $\mu\text{g/L}$ ) concentration. At the beginning of the experiment, snails of similar size were evenly distributed between concentrations (control:  $4.4 \pm 0.5$  mm, 0.8  $\mu\text{g/L}$ :  $4.6 \pm 0.6$  mm, 10  $\mu\text{g/L}$ :  $4.5 \pm 0.5$  mm). Individuals were exposed for 28 days in a light/dark cycle of 16/8 h at  $21 \pm 1^\circ\text{C}$  and the culture medium was entirely renewed weekly. Water was sampled to assess the stability of the oxazepam over time. General parameters of

the water (temperature, pH, conductivity) were monitored twice a week. Twice a week, mortality was assessed and the shell length was measured from the aperture to the apex, with a calliper ( $\pm 0.03$  mm). The growth rate was calculated as the weekly gain in length (in mm).

### 2.3. Chemical analysis

For each concentration, four water samples were taken (two samples after two weeks and two samples after three weeks of exposure) immediately after renewal of the medium (day 0) and four samples were collected at the same time points but after seven days of exposure in the aquarium (day 7). The samples were stored at  $-20^{\circ}\text{C}$  until analysis. For each sample, 200 mL acidified water (pH 2) was extracted on a SPE Strata C18-E column, 1 g/6 mL (Phenomenex, Le Pecq, France). Columns were then rinsed with 4 mL deionized water (pH 2) and dried for 4 h in a laboratory oven at  $45^{\circ}\text{C}$ . Components were eluted with 4 mL hyper-grade methanol for LC/MS (Merk, Molsheim, France). The extract was then dried with a stream of nitrogen and reconstituted with 200  $\mu\text{L}$  methanol. Analysis was performed with a Spectra system (Thermo Fisher Scientific, Courtaboeuf, France) equipped with a Spectra system UV8000 detector (Thermo Fisher Scientific, Courtaboeuf, France) and a Venusil XBP 5  $\mu\text{m}$  C18 (4.6 x 250 mm) capillary column (Agela Technologies, Torrance, USA). The solvents were A) water + 0.05% trifluoroacetic acid (TFA) and B) ACN + 0.05% TFA and the elution gradient was (t in min): t0, B = 30%; t11, B = 100%; t13, B = 100%, t13.5, B = 30%, t18, B = 30%. The column oven temperature was set to  $40^{\circ}\text{C}$ , the injection volume was 20  $\mu\text{L}$ , and the flow rate was 1 mL/min. The working UV wavelength was 230 nm. Data analyses were performed using ChromQuest 5.0. For the quantification of oxazepam concentrations, a nine-point calibration curve, extending over the 3 ng/mL to 5  $\mu\text{g/mL}$  range, was established in triplicate, with oxazepam dissolved in methanol (external standard method). For the evaluation of oxazepam recovery after SPE extraction, we extracted and analyzed ISO water samples containing the same concentrations of oxazepam as used on the calibration curve (from 3 ng/mL to 5  $\mu\text{g/mL}$ ). Extraction efficiency was calculated by comparing areas for the solutions in methanol and the solutions after extraction. The limit of quantification (LOQ) and the limit of detection (LOD) were determined by the signal-to-noise (S/N) approach. The instrumental detection limit for oxazepam was calculated as the lowest concentration giving a S/N ratio of at least 3, whereas the quantification limit was calculated as the lowest concentration giving a S/N ratio of at least 9 (three times the LOD). Instrumental stability was checked regularly, by injecting a concentration corresponding to a point on the calibration curve, in triplicate.

#### **2.4. Feeding rate**

Each organism was individually fed twice a week with a boiled organic standardized lettuce disc (16 mm diameter). The remaining food disc was photographed ( $\alpha$ 3000, Sony) twice a week and renewed. All images were rescaled and treated with Adobe Photoshop CS6 software by adjusting the colour range until obtaining a black and white image. Images were then analysed using ImageJ software (National Institutes of Health, version 1.8.0) and the consumed area of each lettuce disc calculated. Results are presented as the weekly surface of lettuce consumed (in cm<sup>2</sup>) relative to the volume of the individual (cubic shell length, in mm<sup>3</sup>).

#### **2.5. Locomotion**

Locomotor behaviour was monitored twice a week and on the same individuals for each concentration (n = 9 to 12, depending on treatment) at a fixed time (early afternoon) during the four weeks of the experiment. Each individual was removed from the exposure system and placed on its foot in a Petri dish (54 mm diameter) containing 10 mL of the water used for the exposure. Eight-minute videos were immediately recorded with a camera ( $\alpha$ 3000, Sony). Videos were analysed using an Ethovision image analysis system (Noldus, Wageningen, Netherlands). The average distance moved (in cm) during 8 min was calculated.

#### **2.6. RNA extraction and RT-qPCR**

At the end of the locomotion and feeding-rate experiments (day 28), the shells of 30 animals per concentration (including animals used for the locomotion assays for each concentration, hereafter referred to as 'followed juveniles', and animals not used for the locomotion assays, hereafter referred to as 'non-followed juveniles') were removed, and the entire soft bodies of the individuals were dried on absorbent paper and weighed (wet weight).

Total RNA was extracted using an adapted protocol from Yaffe et al. (2012). Tissue was crushed in 500 mL TRIzol Reagent (Ambion) and centrifuged at 3,000 x g for 15 min at 4°C. The supernatant was recovered, chloroform/isoamylalcohol (24:1) (Sigma Aldrich) added (1/5 v), and the tube shaken strongly. After centrifugation (12,000 x g, 15 min, 4°C), 96% ethanol was added to the supernatant (v/v) and the mixture loaded onto Quick Plasmid Mini Columns (Pure Link, Invitrogen). The columns were then centrifuged (8,000 x g, 1 min, 21°C) and washed twice with 450  $\mu$ L 3M sodium acetate to dissolve the polysaccharides and low-molecular-weight RNA. Finally, the columns were washed with



70% ethanol, centrifuged twice (15,000 x g, 2 min, 21°C), and the RNA eluted by adding 20 µL DEPC water and centrifuging at 8,000 x g for 2 min at 21°C. RNA was treated with amplification grade DNase I (Sigma Aldrich) following the manufacturer's instructions. Treated RNA was quantified by spectrometry (Nanodrop 2000, Thermo Scientific, USA). Reverse transcription was carried out with 200 U/µL M-MLV reverse transcriptase (Invitrogen, USA) according to the manufacturer's instructions on 300 ng total RNA.

Specific primers were designed using Primer 3 software (v 4.1.0) (Table S1, Supplementary Information). The PCR efficiency of the primer pairs was estimated by serial dilution. Efficiencies were considered to be good if they were between 90 and 110%. *Tubulin* and *Ribosomal Protein L18* (RPL18) were selected as reference genes.

High-throughput real-time quantitative PCR was performed using the Biomark microfluidic system from Fluidigm, according to the Get-PlaGe (GenoToul, Toulouse, France) protocol, in which every sample-gene combination is quantified using a 96.96 Dynamic Array™ IFCs (BMK-M-96.96, Fluidigm). First, 6.5 ng of each cDNA was initially pre-amplified (10 min 95°C activation and 14 PCR cycles of 15 sec at 95°C and 4 min at 60°C) with PreAmp Master Mix (100-5581, Fluidigm) and a pool containing all primers targeting all genes (200 nM). Pre-amplified samples were diluted 1/5 after exonuclease treatment (M02935, NEB). To prepare samples for loading onto the IFC, a mix was prepared, consisting of 440 µL 2X TaqMan Master Mix (Applied Biosystem, 4369016), 44 µL 20X DNA Binding Dye Sample Loading Reagent (100-7609, Fluidigm), 44 µl 20X Evagreen (31000, Biotium), and 132 µL TE, and 6 µL was dispensed into each well of a 96-well assay plate. Two microliters of pre-amplified and diluted cDNA sample were added to each well and the plate briefly vortexed and centrifuged. For the assays, 5 µL of Assay (5 µM each primer in primer-mix (2X assay loading reagent (100-7611, Fluidigm) and Tris EDTA)) was dispensed into each detector inlet of the 96.96 IFC. Following priming of the IFC in the IFC Controller HX, 5 µL of the cDNA sample + reagent mix and 5 µl of Assay were dispensed to each Sample Inlet of the 96.96 IFC. After loading the assays and samples into the IFC in the IFC Controller HX, the IFC was transferred to the BioMark and PCR performed using the following thermal protocol: thermal mix of 50°C, 2 min; 70°C, 30 min; 25°C, 10 min; hot start at 50°C, 2 min; 95°C, 10 min; PCR of 35 cycles of 95°C, 15 sec; 60°C, 60 sec, and melting analysis (60°C, 30 sec; 95°C, 1°C/3 sec). Results were analysed using Fluidigm real-time PCR analysis software v.4.1.3. Accurate amplification of the target amplicon was checked by performing a melting

curve. The geometric mean of the two reference genes (*Tubulin* and *Ribosomal Protein L18*) was used to calculate fold changes using the  $2^{-(\Delta\Delta Ct)}$  method where the  $\Delta\Delta Ct$  for one individual X of the control or exposed group is:  $((Ct_{target,indX} - GeoAvg[Ct_{ref1,ref2indX}]) - (AvgCt_{target,controlgroup} - GeoAvg[Ct_{ref1,ref2,controlgroup}]))$ . For each target gene, the mean fold-change in expression of the control or exposed group (Table 2) is the mean fold-changes calculated for each individual of the corresponding control or exposed group (Livak and Schmittgen, 2001).

## 2.7. Statistics

All statistical analysis were performed and graphs were plotted with R 4.0.0 software. In order to take into account pseudoreplicates, linear mixed-effects models were performed (after checking for the normal distribution of residuals) with the *lme4* R package (treatment and time as fixed factors and individuals as a random factor) for feeding rate (square root transformation), growth rate, shell length and distance travelled data. If a significant difference was detected between treatments or times, the least square means post hoc method with Bonferroni correction (*emmeans* R package) was used to assess the difference. If a significant difference between the interaction treatment with time was observed, a multiple comparison was performed with the same post hoc test (least square means post hoc test with Bonferroni correction). For the analysis of gene expression data, ANOVA was used if the assumptions of normality and homoscedasticity were fulfilled. Otherwise, the Kruskal-Wallis test followed by the Kruskal Wallis multiple comparison test (*pgirmess* R package) was performed. Survival rate has been analyzed using Kaplan-Meier log-rang test with Bonferroni correction. Differences were considered significant if  $p < 0.05$ . Results are shown as the mean  $\pm$  SE, unless otherwise indicated.

## 3. Results

### 3.1. Water concentrations

Quantitative HPLC analysis showed that the measured concentrations of oxazepam after the renewal of the exposure medium (day 0) were very close to the nominal concentrations (Table 1). After seven days (day 7), the oxazepam concentration was stable for the 0.8  $\mu\text{g/L}$  concentration and decreased by more than 30% for the 10  $\mu\text{g/L}$  concentration.

Table 1. Water concentrations of oxazepam (mean (SD),  $n = 2$ ) used to expose juvenile pond snails after the renewal of the medium (day 0) and after a week (day 7).

			1
	<b>0.8 µg/L</b>	<b>10 µg/L</b>	2
			3
day 0	0.92 (0.09)	10.16 (0.13)	4
day 7	0.90 (0.12)	7.12 (0.57)	5
			6

### 3.2. Impact of oxazepam exposure on survival rate

The survival rate was measured twice a week until the end of experiment for each treatment (Figure 1). The survival rate of the group exposed to the low concentration of oxazepam was significantly higher (97.5%, as one individual died) than that in the control group (80%, as eight individuals died;  $p = 0.014$ ,  $\alpha = 1.7\%$ ).

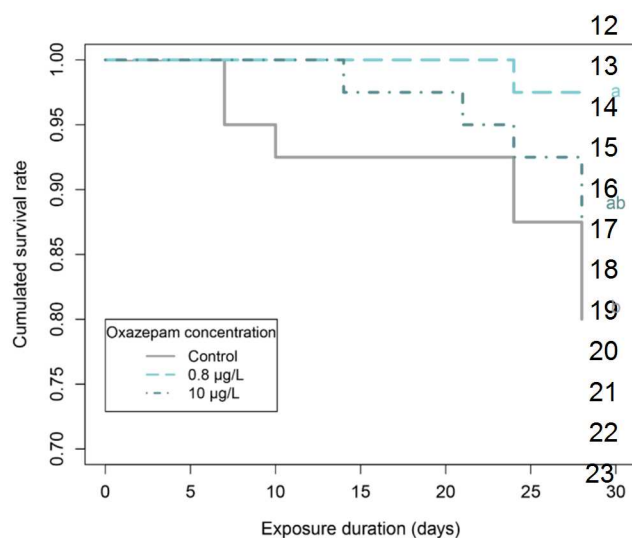


Figure 1. Survival rate throughout the experiment ( $n = 40$  individuals per concentration). Letters indicate significant differences ( $p < 0.05$ ) between treatments.

### 3.3. Impact of oxazepam exposure on feeding rate

The feeding rate of individuals presented a significant interaction between treatment and time ( $F = 4.101$ ,  $p = 0.00056$ ). Non-exposed individuals presented a constant feeding rate, except during the fourth week where the feeding rate decreased relative to the first week. At low concentration (0.8 µg/L), the gastropods consumed more lettuce than controls during the first week of the experiment:  $11.9 \pm 0.9 \text{ cm}^2/\text{mm}^3 \cdot 10^3$  of organism versus  $6.5 \pm 0.7 \text{ cm}^2/\text{mm}^3 \cdot 10^3$  (Figure 2). The difference decreased thereafter until the feeding rates were equivalent to control. In contrast, individuals exposed to the high dose of oxazepam (10 µg/L) showed significant long-term decrease of feeding relative to that of the controls from the second week ( $p < 0.0001$ ) until the end of exposure (third week:  $p =$

0.0017, fourth week:  $p < 0.0001$ ). Non-normalized data (consumed surface in  $\text{cm}^2$ ) showed very similar results (Figure S1, Supplementary Information).

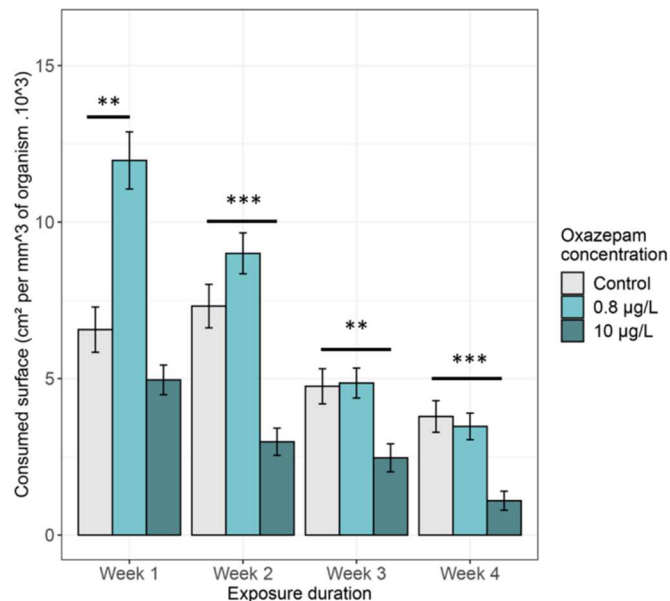
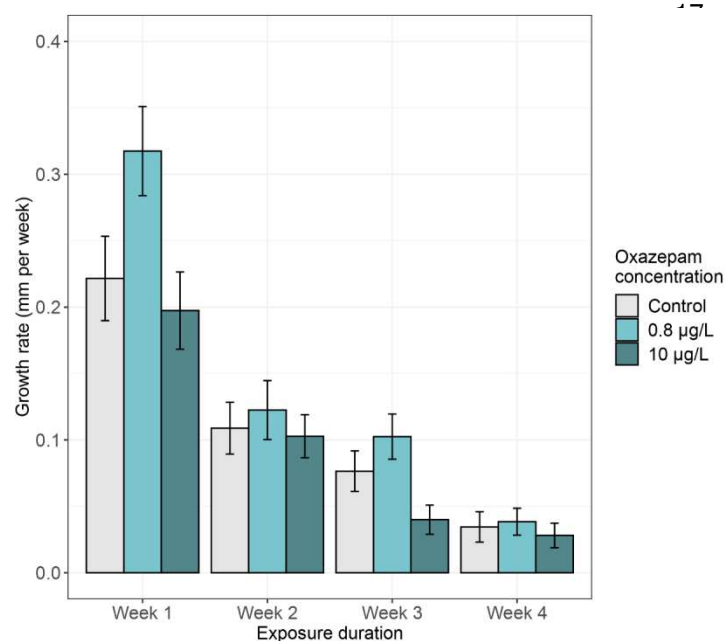


Figure 2. Food consumption (relative to the size of organism) for each week of oxazepam exposure (mean,  $n = 40$ ). Error bars represent  $\pm$  SE of the mean. Asterisks indicate significant differences between conditions for each week.  $**p < 0.01$ ,  $***p < 0.001$ .

### 3.4. Impact of oxazepam exposure on shell growth and wet weight

Shell growth rate appeared to follow the same pattern as food consumption data, with a decrease throughout the experiment for all concentrations ( $F = 57.13$ ,  $p < 2.2 \cdot 10^{-16}$ ). (Figure 3). There was also a significant effect of treatments ( $F = 6.92$ ,  $p = 0.001$ ) due to the difference between low and high doses ( $p = 0.0007$ ). No treatment by time interaction was observed ( $F = 1.77$ ,  $p = 0.1027$ ). The crude data (shell length over time) were presented in Figure S2 (Supplementary Information). In addition, the wet weight of organisms at the end of the experiment (day 28) did not differ significantly between non-



exposed and exposed individuals (data not shown).

Figure 3. *Growth rate of the experimental organisms (mm of shell per week) for each treatment (mean, n = 40). Error bars represent  $\pm$  SE of the mean.*

### 3.5. Impact of oxazepam exposure on locomotion

Locomotion assays performed on the same individuals throughout exposure did not showed any treatment effect ( $F = 0.001$ ,  $p = 0.998$ ) but there was a significant effect of time ( $F = 8.77$ ,  $p = 2.038 \cdot 10^{-10}$ ). Results showed that the distance travelled by non-exposed individuals dropped from  $197.0 \pm 21.7$  mm on day 0 to  $64.6 \pm 13$  mm on day 28 (Figure 4), with a significant difference between day 0 and days 14 to 28 ( $p < 0.005$ ). The distance travelled by individuals exposed to high concentration also showed an important decrease, with a significant difference between day 0 and days 3, 21, 24 and 28 ( $p < 0.05$ ). By contrast, the distance travelled seemed more stable over time in low exposure group, as only days 17 and 28 were statistically significant from day 0 ( $p < 0.05$ ).

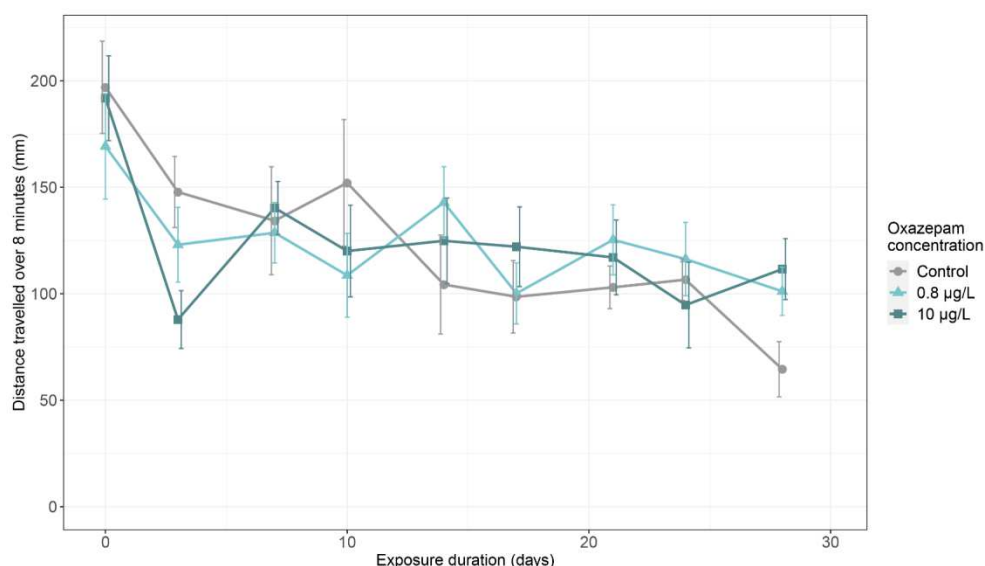


Figure 4. *Sum of the distance travelled (mm) by juveniles placed in Petri dishes for 8 min for each concentration (n = 9 to 12 individuals per concentration). Points and error bars indicate the mean and the SE of the mean, respectively.*

### 3.6. Transcriptomic analysis

We carried out gene expression analysis on individuals exposed to oxazepam for 28 days, including individuals used in both the behaviour and growth/feeding experiments (referred here as ‘followed juveniles’) and individuals used only in growth/feeding tests (‘non-followed juveniles’). Real-time PCR analysis showed a large difference between the two groups but the Mann-Whitney U test side by side comparison did not showed significant difference between expression level of controls (Table S2 Supplementary Information). Most of the genes in the ‘non-followed juveniles’ group were under-expressed, especially at the highest concentration of oxazepam. In total, 26 of the 85 selected genes were significantly under-expressed relative to the control and only one gene was over-expressed (Table 2). Eight additional genes tended to be under-expressed ( $p < 0.1$ ). Most of the genes differentially expressed (DE) after oxazepam exposure are involved in the nervous system, more precisely, in the GABA, serotonin, and dopamine pathways (*GABA receptors*, *glutamate receptor*, *serotonin receptor*, *serotonin/dopamine transporter*, *tyrosine hydroxylase*, *tryptophan hydroxylase*) or encode neuropeptides and their receptors (*LIPs*, *MIP*, *MIP receptor*) and components of the protein kinase pathways (*PKA*, *PKG*, *calmodulin*). Some marker genes of ciliary and muscular structure were also under-expressed (*tektin 2*, *myosin regulatory light chain A*, *tropomyosin*), as well as genes encoding digestive enzymes (*lysosomal and non-lysosomal glucosyl ceramidases*, *fructose biphosphate aldolase*, *alpha glucosidase*). Finally, only one gene involved in detoxification was downregulated (*SOD Cu*) and the cell communication function (Notch pathway) was also affected (*notch*, *furin*, *presenilin*). The soma ferritin gene was the only gene upregulated following oxazepam exposure.

By contrast to the results obtained for the ‘non-followed juveniles’ group, 12 of the 85 selected genes were significantly over-expressed relative to the control in the ‘followed juveniles’ group (for one or both treatments), seven genes tended to be overexpressed ( $p < 0.1$ ), and only one was underexpressed (*Cyp450 3E4*) (Table 3). The overexpressed genes include those of the nervous system, with genes encoding neuropeptides (*pedal peptide*, *myomodulin*, *pro-neuropeptide Y*), the digestive system, with genes encoding enzymes involved in carbohydrate metabolism (*endoglucanase*, *alpha amylase*, *neutral alpha glucosidase*, *fructose biphosphate aldolase*), and the detoxification process, with the genes encoding *SOD*, *catalase*, and *Cyp450 49a1*. The gene encoding soma ferritin was also strongly overexpressed, which was the only gene similarly expressed in both

groups. For a better visualisation, a pie chart representing the part of the genes differentially expressed in each group and the part of genes non-differentially expressed is presented in Figure S3 (Supplementary Information).

Table 2. Fold changes of genes after oxazepam exposure at 0.8 or 10 µg/L of juveniles not followed for locomotion after 28 days. Genes were classified according to their function (nervous system, muscle and ciliary structure, digestive system, detoxification, and cell communication). Results are presented as mean fold changes ( $\pm$  SE),  $n = 9$  per concentration. Significance versus control:  $+p < 0.1$ ,  $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$ . The colour code from beige to dark orange indicates upregulated genes and from light blue to dark blue downregulated genes.

Function	Gene name	Control	0.8 µg/L	10 µg/L
Nervous system	GABA B1 subunit receptor	1.042 ( $\pm$ 0.106)	0.775 ( $\pm$ 0.058)	0.674 ( $\pm$ 0.072) *
	GABA A beta subunit receptor	1.045 ( $\pm$ 0.108)	0.782 ( $\pm$ 0.087)	0.602 ( $\pm$ 0.109) *
	Glutamate receptor	1.089 ( $\pm$ 0.148)	0.854 ( $\pm$ 0.087)	0.609 ( $\pm$ 0.090) *
	Serotonin receptor	1.077 ( $\pm$ 0.139)	0.646 ( $\pm$ 0.078)	0.597 ( $\pm$ 0.110) *
	Tyrosine hydroxylase	1.080 ( $\pm$ 0.134)	0.779 ( $\pm$ 0.085) +	0.582 ( $\pm$ 0.086) *
	Serotonin/dopamine transporter	1.097 ( $\pm$ 0.132)	0.882 ( $\pm$ 0.122)	0.563 ( $\pm$ 0.069) ***
	Tryptophan hydroxylase	1.039 ( $\pm$ 0.101)	0.778 ( $\pm$ 0.099) +	0.693 ( $\pm$ 0.085) *
	cAMP-dependent protein kinase	1.017 ( $\pm$ 0.067)	0.817 ( $\pm$ 0.054)	0.562 ( $\pm$ 0.051) ***
	Calmodulin	1.008 ( $\pm$ 0.043)	0.940 ( $\pm$ 0.067)	0.699 ( $\pm$ 0.065) *
	Neurocalcin	1.014 ( $\pm$ 0.060)	0.768 ( $\pm$ 0.043) *	0.605 ( $\pm$ 0.092) ***
	Nitric oxide synthase	1.074 ( $\pm$ 0.147)	1.063 ( $\pm$ 0.077)	0.744 ( $\pm$ 0.072) +
	cGMP protein kinase	1.031 ( $\pm$ 0.090)	0.864 ( $\pm$ 0.070)	0.704 ( $\pm$ 0.076) *
	Small cardiopeptide	1.028 ( $\pm$ 0.088)	0.776 ( $\pm$ 0.107) +	0.790 ( $\pm$ 0.129) +
	Buccalin	1.075 ( $\pm$ 0.150)	0.916 ( $\pm$ 0.126)	0.699 ( $\pm$ 0.099) +
	LFRFamide	1.024 ( $\pm$ 0.081)	0.904 ( $\pm$ 0.068)	0.818 ( $\pm$ 0.120) +
	Lymnaea inhibitory peptides	1.052 ( $\pm$ 0.124)	0.814 ( $\pm$ 0.089)	0.649 ( $\pm$ 0.076) *
	Molluscan insulin-related peptide	1.021 ( $\pm$ 0.070)	0.436 ( $\pm$ 0.384) *	0.834 ( $\pm$ 0.258)
	Molluscan insulin-related peptide receptor	1.028 ( $\pm$ 0.089)	0.827 ( $\pm$ 0.074)	0.737 ( $\pm$ 0.056) *
Muscle / ciliary structure	Tektin 1	1.044 ( $\pm$ 0.110)	0.856 ( $\pm$ 0.080)	0.701 ( $\pm$ 0.097) +
	Tektin 2	1.040 ( $\pm$ 0.107)	0.841 ( $\pm$ 0.086)	0.619 ( $\pm$ 0.097) *
	Axonemal dynein light chain	1.033 ( $\pm$ 0.094)	0.878 ( $\pm$ 0.057)	0.762 ( $\pm$ 0.071) +
	Myosin heavy chain	1.053 ( $\pm$ 0.111)	0.942 ( $\pm$ 0.112)	0.656 ( $\pm$ 0.132) +
	Myosin regulatory light chain A	1.031 ( $\pm$ 0.083)	0.818 ( $\pm$ 0.100)	0.589 ( $\pm$ 0.097) *
	Tropomyosin	1.032 ( $\pm$ 0.085)	0.644 ( $\pm$ 0.067) *	0.659 ( $\pm$ 0.133) *
Digestive system	Lysosomal glucosyl ceramidase	1.013 ( $\pm$ 0.058)	0.875 ( $\pm$ 0.069)	0.658 ( $\pm$ 0.061) **
	Non-lysosomal glucosyl ceramidase	1.058 ( $\pm$ 0.133)	0.976 ( $\pm$ 0.101)	0.658 ( $\pm$ 0.094) *
	Fructose-bisphosphate aldolase	1.033 ( $\pm$ 0.089)	0.806 ( $\pm$ 0.062)	0.646 ( $\pm$ 0.075) *
	Alpha glucosidase	1.038 ( $\pm$ 0.105)	0.825 ( $\pm$ 0.084)	0.626 ( $\pm$ 0.069) ***
Cell communication	NOTCH	1.048 ( $\pm$ 0.105)	0.871 ( $\pm$ 0.047)	0.793 ( $\pm$ 0.070) *
	Furin	1.013 ( $\pm$ 0.060)	0.890 ( $\pm$ 0.091)	0.694 ( $\pm$ 0.073) **
	Presenilin	1.013 ( $\pm$ 0.058)	0.981 ( $\pm$ 0.076)	0.794 ( $\pm$ 0.051) *
Detoxification	Soma ferritin	1.213 ( $\pm$ 0.329)	1.910 ( $\pm$ 0.214)	4.235 ( $\pm$ 0.613) ***
	Superoxide dismutase Cu	1.035 ( $\pm$ 0.100)	0.943 ( $\pm$ 0.064)	0.773 ( $\pm$ 0.059) *
	Glutathione synthetase	1.047 ( $\pm$ 0.116)	0.869 ( $\pm$ 0.133)	0.733 ( $\pm$ 0.115) +

Table 3. Fold changes of genes after oxazepam exposure at 0.8 or 10 µg/L of juveniles followed for locomotion after 28 days. Genes were classified according to their function (nervous system, muscle and ciliary structure, digestive system, and detoxification). Results are presented as mean fold changes ( $\pm$  SE),  $n = 9$  per concentration. Significance versus control: + $p < 0.1$  \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ . The colour code from beige to dark orange indicates upregulated genes and from light blue to dark blue downregulated genes.

Function	Gene name	Control	0.8 µg/L	10 µg/L
Nervous system	Pedal peptide	1.084 ( $\pm$ 0.144)	1.809 ( $\pm$ 0.216) *	1.271 ( $\pm$ 0.213)
	FMRFamide	1.088 ( $\pm$ 0.149)	1.523 ( $\pm$ 0.200) *	0.946 ( $\pm$ 0.156)
	Acetylcholine binding protein	1.146 ( $\pm$ 0.196)	1.747 ( $\pm$ 0.187) *	1.485 ( $\pm$ 0.254)
	Myomodulin	1.065 ( $\pm$ 0.120)	1.382 ( $\pm$ 0.156)	1.853 ( $\pm$ 0.191) *
	Pro-neuropeptide Y	1.013 ( $\pm$ 0.054)	1.409 ( $\pm$ 0.178)	1.693 ( $\pm$ 0.191) *
	Cerebrin prohormone	1.044 ( $\pm$ 0.104)	1.495 ( $\pm$ 0.193)	1.883 ( $\pm$ 0.291) *
	Calmodulin	1.088 ( $\pm$ 0.141)	1.105 ( $\pm$ 0.051)	1.520 ( $\pm$ 0.092) *
	Schistosomin	1.126 ( $\pm$ 0.199)	1.499 ( $\pm$ 0.190)	2.022 ( $\pm$ 0.407) *
Muscle / ciliary structure	Axonemal dynein light chain	1.071 ( $\pm$ 0.126)	1.535 ( $\pm$ 0.131) *	1.692 ( $\pm$ 0.159) *
	Tropomyosin	1.164 ( $\pm$ 0.194)	1.594 ( $\pm$ 0.166) *	1.259 ( $\pm$ 0.217)
Digestive system	Endoglucanase isoform 1	1.256 ( $\pm$ 0.214)	2.398 ( $\pm$ 0.426)	4.165 ( $\pm$ 0.657) *
	Endoglucanase isoform 2	1.292 ( $\pm$ 0.286)	2.137 ( $\pm$ 0.422)	2.597 ( $\pm$ 0.470) *
	Alpha amylase	1.394 ( $\pm$ 0.336)	2.227 ( $\pm$ 0.651)	4.018 ( $\pm$ 0.710) *
	Fructose-bisphosphate aldolase	1.076 ( $\pm$ 0.130)	1.432 ( $\pm$ 0.116) *	1.497 ( $\pm$ 0.097) *
	Neutral alpha-glucosidase	1.033 ( $\pm$ 0.091)	1.217 ( $\pm$ 0.061) *	1.065 ( $\pm$ 0.090)
Detoxification	Soma ferritin	1.058 ( $\pm$ 0.121)	0.975 ( $\pm$ 0.091)	3.956 ( $\pm$ 0.640) ***
	Superoxide dismutase Mn	1.021 ( $\pm$ 0.075)	1.236 ( $\pm$ 0.100) *	1.196 ( $\pm$ 0.090)
	Catalase	1.135 ( $\pm$ 0.220)	1.070 ( $\pm$ 0.118)	1.678 ( $\pm$ 0.171) *
	Cytochrome p450 49a1	1.197 ( $\pm$ 0.246)	1.685 ( $\pm$ 0.328)	2.168 ( $\pm$ 0.278) *
	Cytochrome p450 3E4	1.437 ( $\pm$ 0.476)	1.012 ( $\pm$ 0.247)	0.208 ( $\pm$ 0.080) ***

#### 4. Discussion

The aim of this study was to evaluate the effects induced by the anxiolytic drug oxazepam on the metabolic pathways associated with growth, locomotory and feeding behavior in the freshwater snail *Radix balthica* and gain insight into the mechanisms of toxicity using a transcriptomic approach. We observed a strong dose-dependant effect on the feeding rate, and a decrease of mortality at low dose. Gene expression analysis highlighted a large difference in the effects induced at the molecular level between individuals used in both the locomotion and growth/feeding experiments ('followed juveniles') and those only used for the growth/feeding tests ('non-followed juveniles'), suggesting that manipulation of the animals may have interfered with the molecular effects induced by oxazepam exposure.



Mortality rate was quite high in control, but nevertheless conformed to OCDE guidelines for ecotoxicological studies on freshwater gastropods, recommending a mortality of no more than 20% over 28 days in control conditions for such studies (OECD, 2016a, 2016b). Moreover, these guidelines were made for adult gastropods, whereas the present study worked with juveniles and it is known that juvenile mortality may be higher than that of adults. Additionally, other studies have reported a high mortality rate for *Radix balthica* juveniles not exposed to contaminants (Hallgren et al., 2012; López-Doval et al., 2019). When exposed to low dose of oxazepam, the mortality rate significantly decreased. Other studies also observed a decrease in the mortality of vertebrates (newly-hatched and adult Eurasian Perch *Perca fluviatilis*) and invertebrates (the crustacean *Gammarus fossarum*) exposed to an equivalent dose of oxazepam (1 µg/L) (Garcia-Galan et al., 2017; Klaminder et al., 2014). This may be explained by the fact that oxazepam may reduce the stress induced by isolation and handling. Indeed, confinement and handling are known to induce stress in fish (Rotllant et al., 2001; Sharpe et al., 1998) and crustaceans (Aparicio-Simón et al., 2010; Fotedar and Evans, 2011). Our results suggest that such stress may also occur for molluscs under our experimental maintenance conditions.

The feeding assays revealed short-term stimulation of food intake at the low concentration. These results are in accordance with those of the study of Brodin et al. (2013), who observed that perch exposed to similar doses of oxazepam (1.8 µg/L) started feeding earlier than controls after seven days. Benzodiazepines have been known to increase food intake by mammals for decades (Bainbridge, 1968; Brown et al., 1976) and have been characterized as appetite-enhancing molecules (Cooper, 1980). In molluscs, feeding neural networks have been well described, especially for the gastropod *Lymnaea stagnalis* (Benjamin, 2012; Cropper et al., 2004) and the implication of the neurotransmitter GABA in the regulation of feeding has been studied (Romanova et al., 1996). Indeed, injection of GABA into the isolated central nervous system excited motor neurons of the cerebral ganglion involved in the protraction of tentacles and strongly stimulated the feeding rhythm generator in the buccal ganglion (Arshavsky et al., 1993, 1991). The same study hypothesized that activation of the tentacle motor neurons was mediated by the GABA<sub>A</sub> receptor, whereas the feeding rhythm generator was mediated by the GABA<sub>B</sub> receptor. Moreover, a recent study demonstrated that the feeding activity of the Prussian Carp *Carasius gibelio* is under control of the GABA<sub>A</sub> receptor and that a benzodiazepine (phenazepam) clearly stimulates feeding behaviour in a GABA-dependent manner (Snigirov and Sylantyev, 2018). It is thus conceivable that the stimulation of feeding rate observed in

1 *R. balthica* after one week of exposure to oxazepam was linked to its interaction with the GABA<sub>A</sub>  
2 receptor. Interestingly, exposure to a higher dose (10 µg/L) induced long-term inhibition of the feeding  
3 rate. This is the first observation of long-term inhibition of food intake after exposure to  
4 benzodiazepines. These results contradict those of Belal and Assem (2011), who showed long-term  
5 stimulation of the feeding rate of fish after a diet contaminated with 12 mg oxazepam per 100 g food.  
6 Further studies are needed to confirm such long-term inhibition of food intake on other molluscan  
7 species.

8 Growth and weight monitoring revealed no significant effects of oxazepam exposure. These results  
9 are consistent with those of Lagesson et al. (2018), who showed no effect of oxazepam on fish growth  
10 after long-term exposure. However, the kinetics of growth are linked to feeding rate. The lack of a  
11 significant relationship between the food intake data and that for growth/weight can be explained by  
12 the fact that only part of the energy available after digestion is allocated to growth. The rest may be  
13 allocated to other processes, such as detoxification (Petitjean et al., 2019; Sokolova et al., 2012).  
14 However, in this case, because of the lower feeding rate in the presence of 10 µg/L oxazepam and the  
15 higher energy demand for detoxification, we would have expected to observe a decrease in growth at  
16 this concentration. Alternatively, growth rate naturally decreases with increasing age. Indeed,  
17 Wullschleger and Jokela (2002) described the growth curve of *Lymnaea peregra* juveniles (presently  
18 *Radix balthica*) and showed a reduction of growth rate seven weeks after hatching. The present study  
19 begun from the age of 8 weeks and continue to the age of 12 weeks. This phenomenon could thus  
20 explain the reduction in the growth rate observed in our experiment.

21 The locomotion assays have not showed any effect of oxazepam exposure on the locomotor activity.  
22 Contrasting results have been published for oxazepam, with some authors reporting a stimulatory  
23 effect on the activity of fish (Brodin et al., 2017, 2014) and crayfish (Kubec et al., 2019), which was not  
24 confirmed by another recent study (Cerveny et al., 2020). It should be noted that, in the  
25 aforementioned studies, activity was assessed only once, after one week of exposure. By contrast, we  
26 performed repeated measurements on the same individuals, twice weekly for four weeks, and memory  
27 processes may therefore have been involved.

28 The gene expression analysis revealed highly different responses between the two groups (juveniles  
29 followed and not followed for locomotion), whereas the physiological results (mortality, feeding rate,

1 growth rate) did not show such differences (data not shown). 'Non-followed' juveniles exposed to  
2 oxazepam for 28 days showed global downregulation of the targeted genes. This was especially true  
3 at the high concentration, but a dose-effect was sometimes apparent, with under-expression at 0.8  
4 µg/L also. Among the DE genes, a large proportion are linked to neurotransmission, both in the central  
5 and peripheral systems. Receptors and transporters of 'classical' neurotransmitters (GABA, glutamate,  
6 serotonin, dopamine) were affected, as well as precursors of catecholamines, serotonin, and nitric  
7 oxide. Several studies have focused on the effects of benzodiazepine exposure on these  
8 neurotransmitters in invertebrates but the results are still unclear (Gagné et al., 2010; Rivetti et al.,  
9 2019). These neurotransmitters are involved in numerous molluscan functions, such as locomotion,  
10 feeding behaviour, and chemoreception (Brown et al., 2018; Elliott and Susswein, 2002; Matsuo et al.,  
11 2009; Miller, 2019; Pavlova, 2001). The transcriptomic responses also showed under-expression of  
12 genes encoding neuropeptides (*LIPs*, and *MIPs*), shown to be peripheral neurotransmitters in  
13 gastropods involved in the regulation of food intake, metabolism and growth (Dogterom, 1980;  
14 Geraerts, 1976; Li et al., 1996). Although we did not observe significant growth effects during the  
15 month of exposure, it is possible that these DE genes correspond to a precursor response. Cyclic  
16 nucleotide-signalling pathways appear to be affected by oxazepam exposure, with the under-  
17 expression of genes known to interact with the cAMP and cGMP pathways (*cAMP-dependant protein*  
18 *kinase*, *cGMP-dependant protein kinase*, *calmodulin*, *neurocalcin*). The cAMP signalling pathway is  
19 involved in many functions (smooth muscle and heart cell contraction, gills and gonad function, and  
20 metabolic regulation) (Fabbri and Capuzzo, 2010) and it has been demonstrated that the NO-cGMP  
21 pathway, mediated by calcium/calmodulin, plays a role in chemosensory activation of feeding (Elphick  
22 et al., 1995). Other classes of genes affected by oxazepam exposure are those that play a role in  
23 ciliary (*tektin 2*) and muscular (*myosin regulatory light chain A*, *tropomyosin*) structures. There are no  
24 data on the effects of benzodiazepine on these functions in non-vertebrates, but it has been shown  
25 that quail oviduct ciliogenesis is inhibited by diazepam and medazepam (Boisvieux-Ulrich et al., 1987).  
26 Several genes involved in the digestive system, especially carbohydrate metabolism, were also  
27 affected. To date, there is no clear evidence of an interaction between benzodiazepine and digestive  
28 enzymes. However, modifications of the nervous system suggest neuroendocrine regulation of the  
29 digestive system. Finally, cellular communication genes of the Notch signalling pathway were  
30 differentially expressed, in the same way as those of *R. balthica* embryos exposed to oxazepam, as

1 reported by Mazzitelli et al. (2017). The detoxification pathway appeared to be only slightly affected,  
2 except for the strong overexpression of the soma ferritin gene, known to act in detoxification and iron  
3 storage.

4 Interestingly, **individuals** similarly exposed to oxazepam but removed from culture to perform the  
5 locomotion assay showed a very different pattern of DE genes than that observed for the 'non-  
6 followed' group. Most were overexpressed and mostly different from those DE in the 'non-followed'  
7 group. The expression of genes specifically involved in the stimulation of ciliary beating (*pedal peptide*)  
8 and feeding behavior (*myomodulin*, *pro-neuropeptide Y*, *cerebrin*) (Chamorro et al., 2002; Hall and  
9 Lloyd, 1990; Kellett et al., 1996; Li et al., 2001) were elevated at the low and high doses. These  
10 observations suggest activation of these functions, especially of the feeding system, which is coherent  
11 with the over-expression of genes encoding digestive enzymes, such as endoglucanases (cellulose  
12 decomposition), and enzymes of carbohydrate metabolism. Finally, unlike the 'followed' juveniles, the  
13 detoxification process appeared to be globally stimulated, except for one gene, *cytochrome p450 3A4*,  
14 involved in benzodiazepine metabolism (Olsen et al., 2016), which was downregulated.

15 Despite the significant difference, the levels of expression of DE genes were close to those of the  
16 controls, at levels of about -35% for underexpressed genes and + 90% for over-expressed genes.  
17 These levels of expression are similar to those reported in an RNA-seq analysis of differential  
18 expression in *Radix balthica* embryos exposed to oxazepam (Mazzitelli et al., 2017), confirming that  
19 oxazepam induces a small change in gene expression levels in *Radix balthica*.

20 The difference in transcriptomic response between the followed and non-followed individuals is  
21 potentially interesting, but the underlying mechanisms remain unknown. To our knowledge, this is the  
22 first study to describe a coupled effect of psychotropic drug exposure and locomotor activity (or  
23 repeated manipulation) on gene expression. This finding is particularly interesting because all the non-  
24 exposed individuals, whether or not they were followed, had similar levels of gene expression,  
25 indicating that locomotor activity alone has no impact on gene expression.

26 These transcriptomic results suggest that, under classical conditions of exposure, oxazepam affects  
27 nervous system transmission linked to displacement and feeding activity. It may, therefore, induce  
28 molecular disturbances in the feeding systems of exposed individuals studied in locomotion assays. It

1 is thus possible that repeated activity or stress induced by recurrent manipulations disturbs the original  
2 molecular response in animals exposed to oxazepam. Moreover, these differences between the two  
3 groups were only observed at the level of gene expression, not the physiological level, suggesting a  
4 precursor response of gene expression.

## 5 6 **5. Conclusion**

7 We describe, for the first time, the effects of long-term oxazepam exposure on a freshwater gastropod  
8 mollusk. We observed both increases and decreases in feeding rate, depending on the concentration  
9 of oxazepam. Exposure to high concentrations, corresponding to those found in effluent, could have a  
10 negative impact on *Radix balthica* populations in the long time, by decreasing feeding rate, potentially  
11 leading to a decline in energy reserves. However, we found no significant effect on growth, and we  
12 cannot therefore draw any firm conclusions on the impact of oxazepam on this function. Another key  
13 finding is the strong apparent effect of activity or manipulation on the transcriptomic response. Care is  
14 therefore required when comparing groups, particularly in transcriptomic analysis. Further studies on  
15 the impact of oxazepam on reproduction and embryonic development will be performed, to evaluate  
16 the toxicity of this molecule at other stages in the life cycle of this species.

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