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## Long-term exposure to environmental diclofenac concentrations impairs growth and induces molecular changes in *Lymnaea stagnalis* freshwater snails

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4

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10

11 Keywords: NSAID, Gastropod, Pharmaceutical, Metabolomics, Gene expression

12

13 Abstract: As pharmaceutical substances are highly used in human and veterinary medicine and  
14 subsequently released in the environment, they represent emerging contaminants in the aquatic  
15 compartment. Diclofenac (DCF) is one of the most commonly detected pharmaceuticals in water and  
16 little research has been focused on its long-term effects on freshwater invertebrates. In this study, we  
17 assessed the chronic impacts of DCF on the freshwater gastropod *Lymnaea stagnalis* using life history,  
18 behavioral and molecular approaches. These organisms were exposed from the embryo to the adult  
19 stage to three environmentally relevant DCF concentrations (0.1, 2 and 10 µg/L). The results indicated  
20 that DCF impaired shell growth and feeding behavior at the juvenile stage, yet no impacts on hatching,  
21 locomotion and response to light stress were noted. The molecular findings (metabolomics and  
22 transcriptomic) suggested that DCF may disturb the immune system, energy metabolism,  
23 osmoregulation and redox balance. In addition, prostaglandin synthesis could potentially be inhibited  
24 by DCF exposure. The molecular findings revealed signs of reproduction impairment but this trend was  
25 not confirmed by the physiological tests. Combined omics tools provided complementary information  
26 and enabled us to gain further insight into DCF effects in freshwater organisms.

27

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28 1. Introduction

29 Traces of pharmaceutical substances are regularly found in surface waters, with a lower detection  
30 threshold regarding the advances in analytical chemistry techniques (Ebele et al., 2017). A diverse  
31 range of pharmaceutical compounds, including nonsteroidal anti-inflammatory drugs (NSAIDs), have  
32 been identified in freshwaters (Santos et al., 2010). Diclofenac (DCF) is one of the most commonly  
33 detected NSAIDs compounds in the aquatic environment at reported concentrations ranging from a  
34 few to several  $\mu\text{g/L}$  (Sathishkumar et al., 2020). DCF is often prescribed for human and veterinary care  
35 to treat acute pain and symptoms induced by chronic inflammatory diseases (McGettigan and Henry,  
36 2013). When this compound is flushed into the wastewater sewage system, it may be partly removed  
37 via processing in conventional wastewater treatment plants (WWTPs), yet the removal efficiency is  
38 generally around 30% (Zhang et al., 2008). The presence of DCF—considered to be biologically active—  
39 in the aquatic environment has raised the issue of its potential impact on non-target organisms. Hence,  
40 DCF had been included for the 2015-2018 period in the list of priority substances identified under the  
41 EU Water Framework Directive (WFD) 2000/60/EC (surface water Watch List, European Commission,  
42 2015), in order to determine the risk it may pose to the aquatic environment.

43 DCF, like many pharmaceuticals, may have effects on non-target species exposed in aquatic  
44 environments. Toxic concerns regarding DCF have been studied in laboratories using model organisms  
45 (polychaetes, crustaceans, mollusks and fish), and the findings have highlighted that even at low  
46 concentrations in the  $\mu\text{g/L}$  range, continuous exposure to this compound contributes to the disruption  
47 of many physiological processes in model organisms, vertebrates (Zebrafish, Medaka) and  
48 invertebrates (Daphnias, Mediterranean mussel) (Świacka et al., 2020). The main reported impacts of  
49 NSAIDs exposure in aquatic organisms were reproductive and locomotive disorders, teratogenicity,  
50 oxidative stress, body deformation and genotoxicity (Świacka et al., 2020). Reproductive effects and  
51 teratogenicity of DCF on aquatic species were documented in aquatic invertebrates at concentrations  
52 in the  $\mu\text{g/L}$  to  $\text{mg/L}$  range (Fontes et al., 2018; Lee et al., 2011; Mohd Zanuri et al., 2017; Munari et al.,  
53 2016). Behavioral studies have shown an impact of NSAIDs on locomotion in freshwater crustacean

54 (Lange et al., 2009). At the molecular level, oxidative stress induction has been reported in both  
55 vertebrate and invertebrate species, with changes in enzyme activity or in the expression of genes  
56 involved in antioxidant and detoxification pathways (Fu et al., 2021; Gonzalez-Rey and Bebianno, 2014;  
57 Guiloski et al., 2017; Liu et al., 2017; Schmidt et al., 2014, 2011). Metabolic disorders have also been  
58 reported, with DCF having an impact on metabolic pathways of *Mytilus galloprovincialis* and *Hyalella*  
59 *azteca* (Bonnefille et al., 2018; Fu et al., 2021). Physiological and behavioral assessments are needed  
60 to understand the sublethal impacts of DCF on aquatic organisms, in addition to “omics” techniques,  
61 which are highly efficient tools for studying detoxification mechanisms. Furthermore, the impacts of  
62 DCF exposure on freshwater invertebrates at environmentally relevant concentrations has barely been  
63 explored to date. The reported effects of DCF on freshwater invertebrates at concentrations in the  
64 µg/L range included cyto- and genotoxicity in *D. magna* and *Dreissena polymorpha* (Liu et al., 2017;  
65 Parolini et al., 2011, 2009) and induction of an immune response in *Lymnaea stagnalis* (Boisseaux et  
66 al., 2017). These studies demonstrated a potential impact of DCF on lower organisms in the freshwater  
67 environment, but this has been less documented as no information is available on biochemical  
68 pathways and physiological processes.

69       The phylum Mollusca is a major constituent in aquatic ecosystems, and can represent 80% of the  
70 invertebrate biomass in some lentic systems (Mouthon, 1982). The latter are the ultimate recipient of  
71 many chemicals, which means that *L. stagnalis* snails are an ecological receptor of environmental  
72 contaminants (Amorim et al., 2019). Regarding the widespread presence of this snail in continental  
73 surface waters and their trophic position, it has been suggested that it could serve as an interesting  
74 model for ecotoxicological assessment (Amorim et al., 2019). This snail is a simultaneous  
75 hermaphrodite which can produce a high number of offspring, and it has thus been used as a functional  
76 tool in fertility and fecundity tests (Amorim et al., 2019). In addition, a standard test is available in  
77 which *L. stagnalis* is used as test organism for reproduction assessment in ecotoxicological studies  
78 (OECD, 2016). The genome of this organism has been sequenced (Coutellec et al., 2019), while the  
79 metabolome of *L. stagnalis* has been investigated by Tufi et al. (2015a and b).

80        Regarding the literature, we hypothesized that DCF could impair life history traits, as egg mass  
81 hatching, growth and reproduction, behaviors as feeding and locomotion, and could lead to oxidative  
82 stress at molecular level. The present study was conducted to assess the long-term impacts of relevant  
83 environmental concentrations of DCF (0.1, 2 and 10 µg/L) on *L. stagnalis* freshwater snails. These  
84 organisms were exposed throughout their life cycle (embryonic to adult stage) for a 128 days period  
85 using a combination of life history, behavior and molecular (transcriptomic and metabolomic)  
86 assessment approaches. Transcriptomic and metabolomic approaches were conducted using RNAseq  
87 and liquid chromatography combined with high resolution mass spectrometry. Non-targeted  
88 approaches were preferred to highlight possible undocumented effects of DCF at molecular level, as  
89 well as the preconceived hypotheses. This is the first time that DCF exposure of an aquatic invertebrate  
90 is conducted at environmental concentrations on such long period. The approach of coupling  
91 phenotypic and molecular measurements is as well a novelty and may allow us to improve our  
92 understanding of the biochemical pathways affected by DCF exposure and fill current gaps in the  
93 literature.

94

95 2. Materials and methods

96 2.1. Chemicals

97 Diclofenac (purity  $\geq 98\%$ , CAS number 15307-79-6) was purchased from Sigma-Aldrich (Steinheim,  
98 Germany). Stock solutions of DCF were prepared at 2 mg/L in water and stored at 4 °C in opaque glass  
99 bottles. Analytical grade solvent hydrochloric acid (37%) was obtained from PanReac (Barcelona,  
100 Spain), HPLC grade trifluoroacetic acid was from VWR Chemicals (Leuven, Belgium) and hyper-grade  
101 acetonitrile for LC/MS was from Merck (Molsheim, France). Pesticide analytical-grade solvents  
102 (methanol, dichloromethane and ethanol) and LC/MS grade solvents (water, acetonitrile, formic acid  
103 99%) were from Carlo Erba (Val de Reuil, France). Ultrapure water was generated by a Simplicity UV  
104 system from Millipore (Bedford, MA, USA), with a specific resistance of 18.2 M $\Omega$ .cm, at 25 °C. Analytical  
105 pure standards used for identification at level one (Sumner et al., 2007) were obtained from the four  
106 following suppliers: Sigma-Aldrich (now part of Merck), Santa Cruz Biotechnology, Toronto Research  
107 Chemicals and LGC Standards.

108

109 2.2. Test organisms

110 *Lymnaea stagnalis* (Linnaeus, 1758) were obtained from the IRSTEA Lyon laboratory (France).  
111 Adult genitors were acclimated in a controlled-temperature chamber (21 °C  $\pm$  1 °C) with a simulated  
112 natural summer photoperiod (16 h L: 8 h D) (Bohlken and Joosse, 1981). They were held in mineral  
113 water (calcium 45 mg/L, magnesium 4.2 mg/L, sodium 7.5 mg/L, potassium 4.5 mg/L, sulfate 9.5 mg/L,  
114 chloride 4.6 mg/L, nitrate <1 mg/L, bicarbonate 151.6 mg/L) and fed fresh organic lettuce 3  
115 times/week. The water was renewed weekly during acclimatation and exposure. For the exposure  
116 experiments, egg masses (average 80 eggs/egg mass) were collected from 50 non-exposed adult  
117 genitors.

118

119

120

121 2.3. Diclofenac exposure

122 Exposure was conducted at three nominal DCF concentrations: 0.1, 2 and 10 µg/L, prepared weekly  
123 in containers by diluting the stock solution in mineral water. For control and each exposure  
124 concentration (0.1, 2 and 10 DCF µg/L), 15 egg masses were randomly placed in three containers (Fig.  
125 S-1). *L. stagnalis* snails were exposed throughout their life cycle, from the embryonic to the adult stage.  
126 At the beginning, static DCF exposure was performed in 1 L glass beakers with 300 mL of exposure  
127 medium. After hatching, snails (randomly selected) from a beaker were divided in 3 beakers with a  
128 total of 9 beakers/treatment concentration (40 snails/beaker; 360 snails/condition). At 7 weeks post-  
129 hatch (wph), snails were randomly assigned to two 30 L aquaria with 20 L of exposure medium,  
130 containing 150 snails each (300 snails/condition). *L. stagnalis* were fed boiled organic lettuce until 4  
131 wph and were then fed fresh organic lettuce (Hoefnagel and Verberk, 2017). Dead snails were removed  
132 every week and a final mortality rate was calculated at 14 wph. Snail mortality at 14 wph ranged from  
133 12.5 to 22.5%, with no significant effects ( $p>0.05$ ) of DCF treatment on mortality (Table 1). Chemical  
134 analyses of DCF in water are described in the Supplementary data, Section 1. The Mann-Whitney U-  
135 test was used to assess differences between DCF concentrations at day 0 and at day 7. DCF  
136 concentrations measured after renewal of the medium (day 0) were close to the nominal  
137 concentration (See Supplementary data, Table S-1). There were no statistical differences between days  
138 0 and 7 for the 3 DCF concentrations (Mann-Whitney U-test,  $p>0.05$ ).

139

140 2.4. Life history traits and behavioral test conditions

141 2.4.1. Life history traits

142 Egg mass hatching was monitored daily between days 8 and 18 after egg laying, with 12 egg masses  
143 monitored per condition. Egg masses with undeveloped embryos were discarded. Shell size was  
144 determined for the entire population ( $n=300$ /condition) using a caliper to measure the distance  
145 between the shell aperture and apex at 7, 10 and 14 wph.

146           Reproduction tests were performed at 16 wph on 6 replicates of 5 individuals in 1 L beakers with  
147 800 mL of exposure medium (30 snails/condition). Animals were chosen that had a homogeneous shell  
148 size ( $21.5 \pm 1.5$  mm) and they were allowed to acclimate for 3 days. This test was conducted over a 28  
149 day period. Water was renewed weekly and egg masses were removed and counted daily. Shell sizes  
150 were measured at the end of the test. Endpoints were the number of egg masses/snail, number of  
151 eggs/egg mass and number of eggs/snail.

152

#### 153           2.4.2. Behavioral test conditions

154           Snail shell sizes (mm) and wet weight (mg) were noted before behavioral tests. Snails were gently  
155 dried on a paper towel before the wet weight measurements. Food intake was determined via feeding  
156 tests on randomly chosen snails at 8, 11 and 15 wph. Feeding tests were conducted on snails placed in  
157 individual exposure systems (32 snails/condition) with standardized lettuce disks (300 mm dia., 28.26  
158 cm<sup>2</sup> area). Pictures were taken of the remaining lettuce (iPhone 6, Apple) on the top of the aquaria,  
159 while respecting same distance between the aquaria and the camera lense at each shot. The images  
160 were processed with Photoshop CS6 software according to the method described by Lebreton et al.  
161 (2021) and analyzed using ImajeJ software (National Institute of Health, version 1.52a). The consumed  
162 lettuce area after 6 h of feeding was calculated and normalized by snail wet weight, as described by  
163 Niyogi et al. (2014).

164           Locomotion behavior was monitored in 24 randomly sampled adult snails per condition at 16 wph.  
165 Snails were monitored for 2 h in petri dishes (140 mm dia.) containing 150 mL of exposure medium  
166 using EthoVision XT behavior software (Noldus, Wageningen, Netherlands) with a camera (Basler, acA  
167 1300-60 gm, lens Kowa LMVZ4411). The average distance moved (in mm) in 2 h normalized by the snail  
168 wet weight (mg) was calculated. The stress response test was performed on the same individuals and  
169 under the same conditions as used in the locomotion tests. 24 animals per condition were filmed for  
170 15 min with the light on, followed by 15 min in the dark, and 15 min with the light on. The average

171 distance moved (mm) in each light condition (15 min), normalized by the snail wet weight (mg) was  
172 calculated.

### 173 2.4.3. Statistical analyses

174 Statistical analyses were performed with R. Studio 1.3.1056 software using a one-way analysis of  
175 variance (ANOVA), following a Shapiro-Wilk and a Bartlett test to confirm that the data met the  
176 parametric test assumptions. Differences were further analyzed with a multiple comparison Tukey  
177 test. A multiple comparison test after a non-parametric Kruskal-Wallis test (pgirmess R package) was  
178 used for data that had not fulfilled the normality and homoscedasticity assumptions.

179

## 180 2.5. Metabolomics study

### 181 2.5.1. Tissue sample preparation

182 At 16 weeks post-hatch (wph), snails were anaesthetized by cold and dissected: 4 reproductive  
183 apparatuses (preputium, prostate, oviduct, seminal vesicles, albumen gland, oothecal gland and  
184 mucipareous gland) and 4 heads (cut just behind the tentacles, with cerebral ganglia inside) were  
185 pooled and placed in cryotubes and directly frozen in liquid nitrogen, before being stored at -80 °C.  
186 This resulted in 10 pools of 4 organs per condition and per organ. Snails were chosen with a 20-25 mm  
187 shell size after sexual maturity.

188 Samples were lyophilized for 3 days and crushed with a metal ball until a fine powder was obtained.  
189 Metabolomics studies were performed on control, 0.1 µg/L and 10 µg/L exposed snails. Considering  
190 the low mass of lyophilized organs, only pools containing sufficient tissue were used for the  
191 metabolomics approach.

192 15 mg dry weight ( $\pm 0.20$  mg) of pooled reproductive apparatus ( $n=8$  at 0.1 µg/L and  $n=9$  at 0 and  
193 10 µg/L) were extracted with a biphasic mixture of methanol/dichloromethane/water (16:16:13 v/v/v)  
194 as described by Bonnefille et al. (2018) and Dumas et al. (2020). Samples were mixed with 120 µL of  
195 methanol and 37.5 µL of water and vortexed for 45 s. 120 µL of dichloromethane and 60 µL of water  
196 were added and then vortexed again for 45 s. After a 15 min rest at 4 °C, samples were centrifuged

2000 g; 15 min; 4 °C). 40 µL of supernatant (polar phase) were collected in a glass tube and evaporated to dryness under a nitrogen stream. Samples were then reconstituted with 150 µL of water/acetonitrile (95:5; v/v) and filtered into vials with modified polyethersulfone membrane (VWR, cut off at 10 kDa) by centrifugation (10 min, 10 000 g). A quality control (QC) was prepared by pooling 40 µL of each sample extract included in the experiment. 12 mg dry weight ( $\pm 0.20$  mg) of the pooled heads (n=9 at 0.1 µg/L and n=10 at 0 and 10 µg/L) were extracted using the same protocol.

203

#### 204 2.5.2. Data acquisition

205 The reproductive apparatus injections were randomly performed on an Exactive Orbitrap LC-HRMS  
206 (Thermo Fisher Scientific) equipped with a heated electrospray ionization source (HESI). For LC  
207 separation, a reverse phase pentafluorophenylpropyl (PFPP) analytical column (100 x 2.1 mm; 3 µm  
208 particle size; Sigma Aldrich) was used. The LC mobile phases were water (A) and acetonitrile (B), both  
209 modified with 0.1% formic acid. Each sample (10 µL) was loaded onto the column with full loop  
210 injection. The flow rate was 200 µL/min according to the following gradient system (A/B): 95/5 from 0  
211 to 3 min, 60/40 at 8 min, 50/50 at 9 min, 30/70 at 13 min, 5/95 from 15 to 18 min, return to initial  
212 conditions at 21 min followed by a re-equilibration period (95/5) for 7 min (total run time, 28 min). The  
213 Exactive HRMS was tuned to a mass resolution of 25,000 (FWHM, m/z 200) with a mass spectrum range  
214 of 50-750 m/z. Samples were analyzed simultaneously in both positive and negative electrospray  
215 ionization modes (ESI+ and ESI-). The analyses were completed with a spray voltage of 3.35 |kV| a  
216 sheath gas flow rate of 55, auxiliary gas flow rate of 10, a capillary voltage of 43 V, a tube lens voltage  
217 of 90 V, and a skimmer voltage of 21 V, while the capillary temperature was 300 °C and the heater  
218 temperature was 250 °C.

219 The pooled head injections were randomly performed on the Q Exactive Focus Orbitrap LC-HRMS  
220 (Thermo Fisher Scientific), equipped with a heated electrospray ionization source (HESI). The LC  
221 conditions were the same as described for the reproductive apparatus. The Exactive HRMS was tuned  
222 to a mass resolution of 35,000 (FWHM, m/z 200) with a mass spectrum range of 50-750 m/z. Samples

223 were analyzed simultaneously in both positive and negative electrospray ionization modes (ESI+ and  
224 ESI-) and acquired with the following parameters: spray voltage at 3.35 [kV], sheath gas flow rate of  
225 55, aux gas flow rate of 10, S-lens RF level of 50, while the capillary temperature was 300 °C and the  
226 heater temperature was 250 °C.

227

### 228 2.5.3. Quality control

229 A quality control (QC) corresponding to a pool of each sample extract, one per organ, was injected  
230 several times at the beginning of the analytical sequence to equilibrate the column, then QC injections  
231 were repeated every 7 sample injections in order to assess analytical repeatability and sensitivity of  
232 the acquisitions. The relative standard deviation (RSD) was calculated for each feature detected in the  
233 replicated QC injections. The analytical repeatability characterized by >70% of the signals with a RSD <  
234 30% was considered acceptable (Want et al., 2010).

235

### 236 2.5.4. Data processing

237 Data processing was identical on the reproductive apparatus and head samples. Raw data were  
238 converted into mzXML files with MSConvert freeware (ProteoWizard 3.0, Holman et al., 2014). A multi-  
239 step data processing strategy was applied, as described in Dumas et al. (2020). ESI+ and ESI-  
240 acquisitions were separately processed using the XCMS package (Smith et al., 2006) in the R  
241 environment. First, peak alignment was performed with peak grouping and retention time alignment  
242 to ensure that peaks for the same ion [ $m_i/z_i$ ] in all samples were detected at identical retention times  
243 ( $rt_i$ ). Secondly, the abundance of each feature was reflected by the peak integration, and feature  
244 abundances were compared between two groups based on a Welsh t-test, showing statistically  
245 significant changes among the detected peaks. Thirdly, XCMS generate a table containing peak  
246 information and feature abundances in all samples. XCMS parameters were applied as follows for ESI-  
247 and ESI+:  $m/z$  interval was set at 0.01 and 0.002, the signal-to-noise ratio threshold was set at 10, the  
248 group bandwidth was set at 8, and the minimum fraction was set at 0.5. Raw data were visually

249 inspected and features with a non-Gaussian chromatographic peak and with a RSD > 20% were  
250 discarded from the dataset.

251

#### 252 2.5.5. Statistical analysis

253 A Welch t-test was applied for the univariate statistical analysis to compare the exposed groups  
254 (0.1 and 10 µg/L DCF) to the control group. Features with a p-value lower than 0.1 and an abundance  
255 ratio between exposed and control snails higher than 20% were selected. The rationale for choosing  
256 such a p-value threshold was based on: i) the low number of samples per group, which may lead to a  
257 low statistical power, and ii) the opportunity to reveal a biological modulation trend due to the  
258 exposure. Indeed, a trend ( $p < 0.1$ ) toward down- or up-modulation of several metabolites from the  
259 same pathway (highlighting a disruption in the biochemical cascade) may be more biologically relevant  
260 than a single marker metabolite ( $p < 0.05$ ). Even if the latter is statistically more powerful, a single  
261 metabolite is however not strong enough to accurately reveal an effect.

262 Multivariate statistical analyses were performed with MetaboAnalyst 5.0 online software using the  
263 MetaboAnalystR package. Datasets were log transformed and Pareto-scaled before principal  
264 component analysis (PCA) to assess clustering between samples.

265

#### 266 2.5.6. Metabolite annotation and identification

267 Metabolite annotation was performed based on the two confirmation levels described by Sumner  
268 et al. (2007), i.e. level 1 corresponds to annotation confirmed by injection of the analytical standard  
269 on the same analytical platform under the same conditions (validation based on both the accurate  
270 mass and retention time); while level 2 is characterized by metabolites putatively annotated on the  
271 basis of public databases. Here metabolite annotation was performed by mass-matching with 0.002  
272 Da precision using the online Human Metabolome Database tool (HMDB; <http://www.hmdb.ca>).

273 Identified and annotated metabolites were assigned to metabolic pathways according to the Kyoto  
274 Encyclopedia of Genes and Genomes (KEGG; <http://www.kegg.jp>) and HMDB.

275

## 276 2.6. Transcriptomic

### 277 2.6.1. Tissue sample preparation and RNA extraction

278 At 17 wph, snails ( $22.5 \pm 1.5$  mm) were anaesthetized by cold and dissected. The same organs  
279 (reproductive apparatus and heads) were sampled as in the metabolomics study. Samples were frozen  
280 in 1 mL of TRIzol Reagent at  $-80$  °C. Before RNA extraction, samples were crushed and centrifuged for  
281 15 min at  $4$  °C and  $3000$  g speed and lysates were collected. RNA extraction was performed as  
282 described by Lebreton et al. (2021).  $200$  µL of chloroform isoamyl alcohol was added to each sample,  
283 and the samples were then vortexed for 15 s and centrifuged for 15 min at  $4$  °C,  $12000$  g. Supernatants  
284 (aqueous phase) were collected in an Rnase-free tube, 96% ethanol (v/v) was added and the samples  
285 were purified on spin columns (Miniprep Kit, Quiagen) with centrifugation ( $8000$  g, 1 min,  $21$  °C).  
286 Samples were washed twice with  $450$  µL sodium acetate 3M then once with  $320$  µL 70% ethanol. RNA  
287 was eluted with  $30$  µL of water and centrifuged for 2 min at  $8000$  g and treated with amplification  
288 grade DNase I (Sigma Aldrich) according to the manufacturer's instructions.

289

### 290 2.6.2. cDNA library preparation and Illumina sequencing

291 RNAseq analysis was carried out on 3 pools of 2 reproductive apparatus and on 3 pools of 2  
292 heads, on control,  $0.1$  µg/L and  $10$  µg/L exposed organisms. RNA concentration and quality were  
293 assessed using an Agilent 2100 Bio-analyzer (Agilent Technologies, Santa Clara, CA, USA) and library  
294 preparation and sequencing was performed via the GeT-PlaGe GenoToul platform (INRAE, France).  
295 Before fragmentation, oligo d(T) beads were used to purify poly(A) mRNA from total RNA. RNA libraries  
296 were sequenced on the Illumina NovaSeq6000 platform on 2 lines.

297

### 298 2.6.3. RNA-seq data analyses

299 Data preprocessing, reference genome mapping (Coutellec et al., 2019) and transcript  
300 quantification were performed with the Nextflow v20.01.0 workflow manager (Di Tommaso et al.,

2017; Lataretu and Hölzer, 2020). After log transformation and normalization of counting data with the TMM method (trimmed mean of M-values normalization method) (Robinson and Oshlack, 2010), a differential analysis was performed using a generalized linear model (GLM) (false discovery rate adjusted p-value < 0.05; fold change >2), using the EdgeR package in the R environment. Differentially expressed genes (DEGs) were annotated on the Uniprot (Swiss-Prot) database, followed by a second annotation on non-referenced proteins from the Uniprot TrEMBL database. Conserved regions of non-annotated sequences were determined using the PROSITE database (de Castro et al., 2006). Gene ontology (GO) enrichment was performed using QuickGo for GO annotation.

309

### 310 3. Results

#### 311 3.1. Effects of DCF exposure on life history and behavior parameters

312 No impacts on hatching rates of *Lymnaea stagnalis* egg masses exposed to DCF were observed  
313 (Fig. 1A). The shell size measurements during exposure revealed an impact of DCF on growth (Fig. 1B).  
314 Shell sizes were larger in all treatment groups at 7 wph (p <0.001), and at 10 wph (0.1 µg/L: p <0.05; 2  
315 and 10 µg/L: p <0.001) compared to controls. This effect was no longer observed at 14 wph. Over the  
316 28 day reproduction test, control snails produced an average of 3.83 egg masses/snail, 43.92 eggs/egg  
317 mass and 168.73 eggs/snail (Table 1). Hatching was evaluated, showing no differences between control  
318 and exposed egg mass (results not shown). The reproduction parameters did not significantly differ in  
319 DCF exposed organisms. No mortality was observed during the reproduction test (28 days).

320 Regarding the feeding test, lettuce area consumption was lower in the 2 µg/L treatment group (p  
321 <0.01) at 8 wph, while no significant differences were found in the 0.1 and 10 µg/L treatment groups,  
322 even though we did observe a downwards trend (Fig. 1C). Feeding was no longer impacted at 11 and  
323 15 wph. During the locomotion test, the average distance travelled by control snails—normalized  
324 according to the wet weight—was 27.07 mm/mg (Table 1), and the distance travelled by exposed snails  
325 did not differ significantly from control. Locomotion in light stress conditions did not show any impact

326 of DCF exposure (Fig.1D): regardless the treatment group, there was a reduction of activity in dark  
 327 conditions, with an increase after the lightning was turned on.

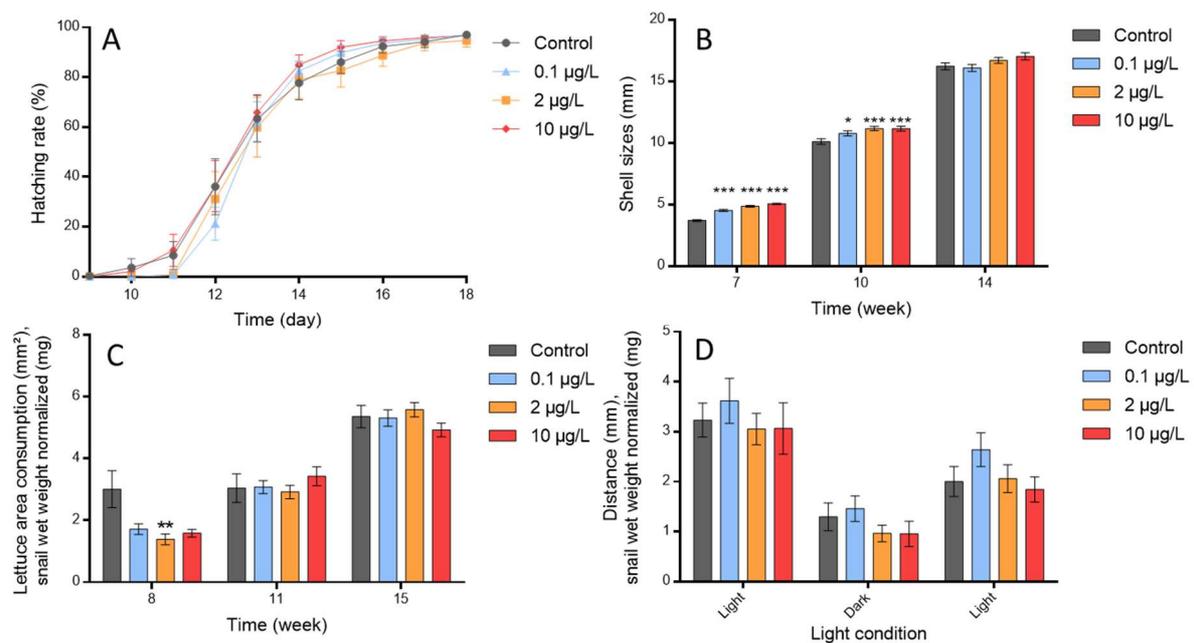
328

329 Table 1 –Summary of mean diclofenac effects on mortality, locomotion and reproduction in *Lymnaea*  
 330 *stagnalis*

DCF nominal concentration	Control	0.1 µg/L	2 µg/L	10 µg/L
Mortality at 14 wph ± SD <sup>a</sup>	19.3 ± 3.0%	23.7 ± 2.5%	12.5 ± 0.5%	22.5 ± 4.8%
Reproduction test				
Egg masses/snail <sup>b</sup> (SEM)	3.83 (0.29)	3.83 (0.48)	3.57 (0.08)	3.03 (0.25)
Eggs/snail <sup>b</sup> (SEM)	168.73 (9.40)	172.67 (18.94)	149.47 (8.95)	133.00 (9.09)
Eggs/egg mass <sup>a</sup> (SEM)	43.92 (1.04)	45.53 (1.36)	41.82 (1.85)	44.26 (2.20)
Locomotion test				
Distance travelled in 2 h (mm), normalized with the snail wet weight (mg) <sup>a</sup> (SEM)	27.07 (2.85)	24.85 (2.48)	22.76 (2.86)	25.55 (3.99)

331 <sup>a</sup>Kruskal-Wallis, p >0.05 in all treatment conditions; <sup>b</sup>ANOVA, p >0.05 in all treatment conditions, SD: standard  
 332 deviation, SEM: standard error of the mean.

333



334 Figure 1 – Effects of diclofenac on life history and behavioral endpoints in *Lymnaea stagnalis*. Hatching rate of egg masses  
 335 between days 9 and 18 (A), Snail shell sizes (mm) at 7, 10 and 14 weeks post-hatch (wph) (B), Lettuce area consumption  
 336 normalized according to the wet weight of the snails (mm<sup>2</sup>/mg) at 8, 11 and 15 wph (C), Distance travelled normalized  
 337 according to the wet weight of the snails (mm/mg) in response to light stress (15 min in light followed by 15 min in dark and  
 338 15 min in light) (D). Error bars represent ± SE of the mean. Asterisks indicate significant differences between conditions  
 339 (control vs treatment groups; Kruskal-Wallis). \*p <0.05, \*\*p <0.01, \*\*\*p <0.001.

341

342

### 343 3.2. Metabolomics results

#### 344 3.2.1. Fingerprint overview

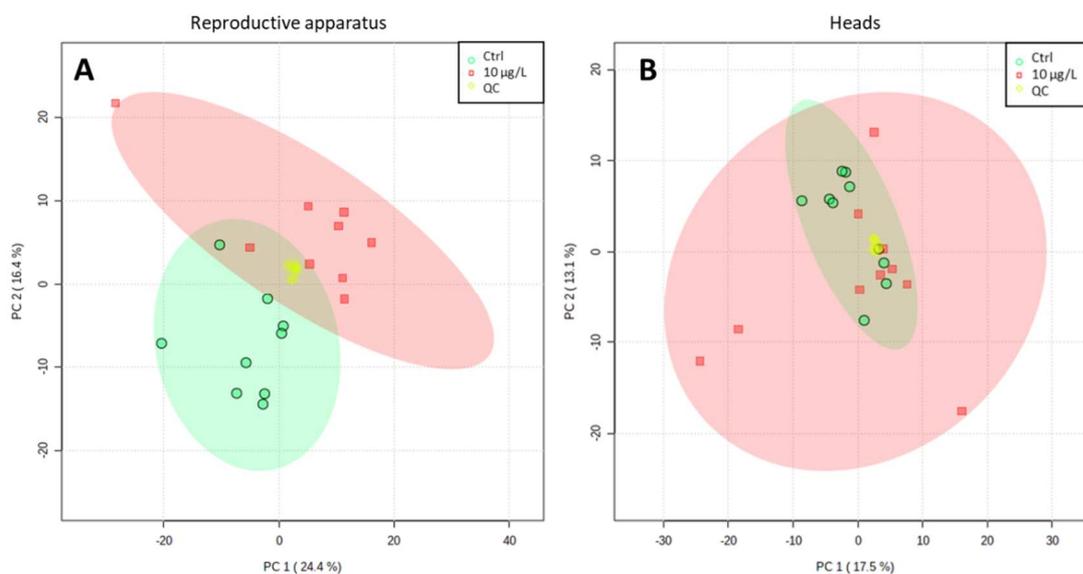
345 In both organs, more features were detected in positive (ESI+) (8643 in reproductive apparatus  
346 samples and 5218 in heads samples) than in negative (ESI-) electrospray ionization mode (4196 and  
347 3908). Regarding the reproductive apparatus fingerprints, the percentages of ions with RSD < 30% in  
348 ESI- and ESI+ were 96% and 83%, respectively, thus confirming the analytical repeatability according  
349 to Want et al. (2010). Considering the heads fingerprints, the analytical repeatability was also  
350 acceptable with 78% of ions detected in ESI- and 69% of those detected in ESI+ with RSD QC <30%.

351 Features were analytically selected based on RSD <20%, a retention time (Rt) in the 65-1260 s  
352 range and on visual inspection of the extracted ion chromatograms. For both organs, repeatedly  
353 injected QC samples were grouped on the PCA score plot (Fig. 2A and 2B) based on selected features.

354

355

356



357  
358 Figure 2 - PCA score plot of metabolic fingerprints from: A) reproductive apparatus and B) heads of control (green circle) and  
359 10 µg/L DCF exposed (red square) snails; QC are represented with yellow diamonds. Data were log transformed and Pareto  
360 scaled. Ellipses are 95% confidence intervals.

361

### 362 3.2.2. *Lymnaea stagnalis* metabolome

363 Annotations were performed based on an internal database consisting of 282 compounds in order  
364 to acquire further information on *Lymnaea stagnalis* metabolomes, as previously explored by Tufi et  
365 al. (2015). Among these metabolites, 87 and 70 were confirmed (level 1 annotation according to  
366 Sumner et al. (2007) in reproductive apparatus and head samples, respectively. Most of the identified

367 metabolites were derived from amino acid metabolism (37 and 30), while nucleotide (11 and 10) and  
368 carbohydrate metabolisms (18 and 12) were the other main forms represented.

369 28 metabolites were identified as specific to the reproductive apparatus and 11 metabolites were  
370 only identified in head samples (see Supplementary data, Table S-2.). Among the common metabolites,  
371 differences in abundance were observed between organs.

372

### 373 3.2.3. Molecular effects of DCF exposure

374 In both organs, as already mentioned, QC clustering on the PCA score plots revealed that the  
375 analytical variability was controlled. The biological variability of individuals appeared to be higher than  
376 the analytical variability. Metabolic fingerprints from the reproductive apparatus of control and 10  
377  $\mu\text{g/L}$  exposed snails showed a clustering pattern between control and exposed snails (Fig. 2A).  
378 Conversely, the PCA score plot obtained from head samples did not show a clear separation between  
379 control and DCF exposed snails (Fig. 2B), indicating that DCF exposure had a greater signature for the  
380 reproductive apparatus metabolome.

381 The univariate statistical results (Welch t-test) showed 595 modulated ions at 0.1  $\mu\text{g/L}$  DCF ( $p < 0.1$ ,  
382 abundance variation  $> 20\%$ ) and 1227 modulated ions at 10  $\mu\text{g/L}$  DCF in the reproductive apparatus. In  
383 head samples, univariate analysis provided 625 modulated ions at 0.1  $\mu\text{g/L}$  DCF ( $p < 0.1$ , abundance  
384 variation  $> 20\%$ ) and 908 modulated ions at 10  $\mu\text{g/L}$ . The number of modulated ions was higher in 10  
385  $\mu\text{g/L}$  exposed snails than in 0.1  $\mu\text{g/L}$  DCF exposed snails, which seemed to show a concentration-effect  
386 relationship of DCF exposure.

387

### 388 3.2.4. Metabolite modulation and pathway analysis

389 Identified and putatively annotated metabolites of interest ( $p < 0.1$ ; RSD  $< 20\%$ ; amplitude  
390 difference  $> 20\%$ ) in the reproductive apparatus and head samples are reported in Tables 2 and 3,  
391 respectively. A shift between the theoretical and observed masses of low molecular weight compounds  
392 was noticed in ESI- for reproductive apparatus injections, sometimes with a deviation of around 12

393 ppm (Table 2). However, this mass shift was also observed when pure analytical standards were  
394 injected. This mass shift was not observed for head samples since different analytical devices were  
395 used. This could also explain the differences in retention times observed for the same metabolite  
396 detected in both organs.

397 Thirteen metabolites from purine metabolism were significantly modulated by DCF exposure in  
398 reproductive apparatus or head samples. For example, adenine was observed in ESI+ and in ESI- and  
399 was up-modulated in the reproductive apparatus at 10 µg/L (+35%; p=0.072 in ESI+; +36%; p=0.078 in  
400 ESI-) and in head samples at 0.1 µg/L (+51%; p=0.004 in ESI+; +46%; p=0.01 in ESI-). Other Identified  
401 modulated metabolites were from pyrimidine metabolism (e.g. inosine, uridine), amino acid  
402 metabolism (e.g. homogentisic acid, tryptophan, serine, asparagine, proline) and other metabolisms  
403 (e.g. nicotinic acid, pyridoxal, carnitine). Modulation of prostaglandin related compounds was also  
404 demonstrated with level 2 annotations. Note that in the reproductive apparatus, the annotated  
405 prostaglandin signals did not correspond to prostaglandin D<sub>2</sub>, E<sub>2</sub> or F<sub>2α</sub>. These compounds were  
406 identified at other retention times (and were not modulated).

Table 2 – Metabolites modulated by all-life exposure to diclofenac (0.1 µg/L and 10 µg/L) in the *L. stagnalis* reproductive apparatus, considering metabolites with p < 0.1 (Welch)

Metabolism pathway	Metabolite	Rt (min)	Molecular ion species	Calculated mass	Observed mass	Mass difference	RSD <sup>b</sup> %	Control vs 0.1 µg/L DCF		Control vs 10 µg/L		Annotation level <sup>a</sup>
								Difference	p-value	Difference	p-value	
Purine metabolism	<i>Adenine<sup>d</sup></i>	2.96	[M+H] <sup>+</sup>	136.0618	136.0612	4	2.3	↗+27	0.19043	↗+35	0.07161	1
		2.96	[M-H] <sup>-</sup>	134.0472	134.0460	9	2.3	↗+25	0.19724	↗+36	0.07752	1
	<i>Hypoxanthine<sup>d</sup></i>	1.7	[M-H] <sup>-</sup>	135.0312	135.0300	9	5.0	↘-21	0.51012	↘-120	0.00652	1
	<i>Inosine<sup>d</sup></i>	1.85	[M+H] <sup>+</sup>	269.0880	269.0869	4	18.7	↗+117	0.01286	↗+417	0.00003	1
		1.85	[M-H] <sup>-</sup>	267.0735	267.0732	1	9.7	↗+50	0.01531	↗+283	0.00002	1
	<i>Xanthylic acid</i>	1.42	[M-H20-H] <sup>-</sup>	345.0236	345.0230	2	1.4	/	0.16339	↗+28	0.01359	2
	<i>Glycineamideribotide</i>	1.89	[M-H20-H] <sup>-</sup>	267.0382	267.0376	2	1.9	↗+804	0.12544	↗+2967	0.00106	2
	<i>5-Aminoimidazole</i>	1.17	[M+K] <sup>+</sup>	122.0115	122.0118	2	1.2	↗+38	0.02578	/	0.69402	2
	<i>Guanosine diphosphate</i>	1.87	[M+NH4] <sup>+</sup>	461.0582	461.0585	1	1.9	↗+184	0.00940	↗+594	0.00000	2
	<i>2,8-Dihydroxyadenine</i>	1.4	[M-H] <sup>-</sup>	166.0370	166.0362	5	1.4	↘-358	0.03486	↘-502	0.02672	2
Pyrimidine metabolism	<i>Uridine<sup>d</sup></i>	1.68	[M+H] <sup>+</sup>	245.0768	245.0770	1	17.4	↘-112	0.04780	↘-926	0.00182	1
		1.68	[M-H] <sup>-</sup>	243.0622	243.0618	2	17.6	↘-65	0.04699	↘-199	0.00128	1
	<i>dTDP</i>	1.7	[M+Na] <sup>+</sup>	425.0122	425.0139	4	8.4	↗+219	0.08565	↗+437	0.00002	2
	<i>Ureidopropionic acid<sup>d</sup></i>	1.3	[M-H] <sup>-</sup>	131.0462	131.0449	10	2.3	↘-37	0.07699	↘-36	0.10885	2
	<i>Malonate semialdehyde</i>	1.62	[M+NH4] <sup>+</sup>	106.0499	106.0493	5	12.1	↗+27	0.14667	↗+30	0.09752	2
	<i>dUDP</i>	1.57	[M+NH4] <sup>+</sup>	406.0411	406.0406	1	36.7	↗+892	0.22192	↗+2292	0.04428	2
Nicotinate and nicotinamide metabolism	<i>Fumaric acid<sup>c</sup></i>	2.09	[M-H] <sup>-</sup>	115.0037	115.0023	12	3.3	/	0.38747	↗+35	0.04793	1
	<i>Niacinamide</i>	2.5	[M+H] <sup>+</sup>	123.0553	123.0547	4	0.8	↘-64	0.10946	↘-106	0.02464	1
	<i>Nicotinic acid<sup>d</sup></i>	1.88	[M-H] <sup>-</sup>	122.0248	122.0234	11	7.3	↗+58	0.09457	↗+180	0.00023	1
	<i>Nicotinate β-D-ribose nucleotide</i>	1.68	[M+FA-H] <sup>-</sup>	380.0394	380.0408	4	6.1	↗+25	0.26771	↗+59	0.00033	2
	<i>L-Glutamic acid<sup>cd</sup></i>	1.45	[M-H] <sup>-</sup>	146.0459	146.0446	9	3.0	/	0.33169	↗+31	0.02782	1
Alanine, aspartate and glutamate metabolism	<i>L-Asparagine</i>	1.3	[M-H] <sup>-</sup>	131.0462	131.0449	10	2.3	↘-37	0.07699	↘-36	0.10885	2
	<i>Oxoglutaric acid</i>	1.41	[M+FA-H] <sup>-</sup>	191.0197	191.0188	5	4.3	↘-88	0.02393	↘-46	0.13508	2
	<i>3,4-Dihydroxybenzeneacetic acid</i>	6.75	[M-H] <sup>-</sup>	167.0350	167.0338	7	2.3	↗+27	0.27351	↗+43	0.02196	1
Tyrosine metabolism	<i>3-Methoxy-4-hydroxyphenylglycolaldehyde</i>	6.7	[M-H] <sup>-</sup>	181.0506	181.0496	6	6.7	↗+29	0.33137	↗+46	0.04674	2
	<i>Genticic acid</i>	8.75	[M-H] <sup>-</sup>	153.0193	153.0181	8	8.8	↘-32	0.07542	/	0.54458	2
	<i>L-Serine<sup>d</sup></i>	1.32	[M+H] <sup>+</sup>	106.0499	106.0496	3	3.7	/	0.32445	↗+21	0.08634	1
Glycine, serine and threonine metabolism		1.32	[M-H] <sup>-</sup>	104.0353	104.0341	12	2.2	/	0.46713	↗+15	0.06769	1
	<i>L-Citrulline<sup>d</sup></i>	1.42	[M+H] <sup>+</sup>	176.1030	176.1022	5	4.7	/	0.76830	↗+26	0.06141	1
Arginine biosynthesis		1.42	[M-H] <sup>-</sup>	174.0884	174.0875	5	3.8	/	0.77729	↗+27	0.06558	1
	<i>L-Tryptophan<sup>cd</sup></i>	9.75	[M+H] <sup>+</sup>	205.0972	205.0965	3	2.2	/	0.66712	↗+49	0.04903	1
Tryptophan metabolism		9.75	[M-H] <sup>-</sup>	203.0826	203.0819	3	2.4	/	0.55885	↗+73	0.03970	1
	<i>Serotonin</i>	9.01	[M+H] <sup>+</sup>	177.1022	177.1016	3	1.8	↘-30	0.07720	/	0.94585	1
Pentose phosphate pathway	<i>Gluconic acid<sup>d</sup></i>	1.11	[M-H] <sup>-</sup>	195.0510	195.0501	5	3.1	↗+26	0.19525	↗+52	0.00091	1
Vitamin B6 metabolism	<i>Pyridoxal</i>	3.71	[M+H] <sup>+</sup>	168.0655	168.0647	5	8.1	/	0.72666	↗+31	0.04869	1
		3.71	[M-H] <sup>-</sup>	166.0499	166.0499	0	7.4	/	0.79280	↗+50	0.02824	1
osmoprotective compound	<i>Proline betaine</i>	1.64	[M+H] <sup>+</sup>	144.1019	144.1012	5	7.4	↘-34	0.04894	↘-23	0.07403	1
β-oxidation of very long chain fatty acids	<i>L-Acetylcarnitine</i>	6.14	[M+H] <sup>+</sup>	204.1230	204.1223	3	3.0	↘-55	0.02844	↗+18	0.68700	1
		6.14	[M+FA-H] <sup>-</sup>	248.1140	248.1136	2	5.2	↘-58	0.05417	↗+28	0.62174	1
Biosynthesis of ophthalmic acid	<i>L-Carnitine</i>	2.63	[M+FA-H] <sup>-</sup>	206.1034	206.1027	3	2.9	↘-35	0.01147	/	0.72889	1
	<i>L-α-Aminobutyric acid</i>	1.67	[M+H] <sup>+</sup>	104.0706	104.0703	3	1.0	↘-59	0.00671	↘-158	0.00010	1
Glycerophospholipid metabolism	<i>Glycerophosphorylcholine</i>	1.1	[M+H] <sup>+</sup>	258.1101	258.1090	4	1.7	↘-25	0.12672	↗+32	0.06284	1
Amino sugar and nucleotide sugar	<i>N-Acetyl-D-glucosamine<sup>d</sup></i>	1.1	[M+Cl] <sup>-</sup>	256.0593	256.0591	1	7.4	/	0.67266	↗+23	0.09529	1
Arachidonic acid metabolism	<i>PGF<sub>3α</sub> or PGH<sub>2</sub> or PGI<sub>2</sub> or TX<sub>A2</sub></i>	11.82	[M-H] <sup>-</sup>	351.2177	351.2176	0	4.8	↘-49	0.09289	↘-38	0.13354	2
		11.40	[M-H] <sup>-</sup>	351.2177	351.2176	0	6.9	↘-26	0.03135	/	0.86811	2
		12.50	[M-H] <sup>-</sup>	351.2177	351.2177	0	5.9	↘-42	0.01339	↘-40	0.00262	2
	<i>PGD<sub>1</sub> or PGE<sub>1</sub> or PGF<sub>2α</sub> or PGH<sub>1</sub></i>	11.73	[M-H] <sup>-</sup>	353.2333	353.2334	0	1.1	↘-30	0.08544	/	0.87493	2
	<i>PGA<sub>2</sub> or PGB<sub>2</sub> or PGJ<sub>2</sub> or PGC<sub>2</sub></i>	12.66	[M-H] <sup>-</sup>	333.2071	333.2072	0	5.7	↘-34	0.09132	/	0.75044	2

408

Metabolites shown in italics had a p-value between 0.05 and 0.1 or were analytically less restrictive (RSD >20%) and may represent biologically relevant metabolites. PG: prostaglandin, TX: thromboxane <sup>a</sup>Based on the work of Sumner et al. (2007);

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<sup>b</sup> RSD : Relative standard deviation calculated for each feature detected in the quality control sample injected throughout the analytical sequence (analytical variability); <sup>c</sup> Belongs to several metabolic pathways; <sup>d</sup> Also modulated in head samples

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Table 3 - Metabolites modulated by all-life exposure to diclofenac (0.1 µg/L and 10 µg/L) in *L. stagnalis* head samples, considering metabolites with p < 0.1 (Welch)

Control vs 0.1 µg/L

Control vs 10 µg/L

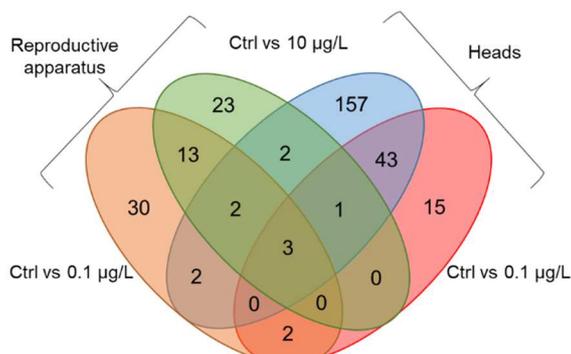
Metabolism pathway	Metabolite	Rt	Molecular	Calculated	Observed	Mass	RSD <sup>b</sup>	Difference	p-value	Difference	p-value	Annotation	
Purine metabolism	Adenine <sup>d</sup>	3.19	[M+H] <sup>+</sup>	136.06180	136.0617	1	7.6	↗+51	0.00372	/	0.38727	1	
		3.19	[M-H] <sup>-</sup>	134.04720	134.0472	0	5.3	↗+46	0.01005	/	0.39161	1	
	Adenosine	3.51	[M+H] <sup>+</sup>	268.10400	268.1039	0	7.5	↗+24	0.0162	↗+21	0.22691	1	
		3.51	[M+FA-H] <sup>-</sup>	312.09500	312.0949	0	4.5	↗+29	0.00843	↗+23	0.24314	1	
	Guanosine	2.12	[M+H] <sup>+</sup>	284.0989	284.0987	1	3.2	↗+37	0.00034	↗+21	0.08812	1	
	Hypoxanthine <sup>d</sup>	1.95	[M+H] <sup>+</sup>	137.0458	137.0457	0	7.7	↗+45	0.00736	/	0.33596	1	
	Inosine <sup>d</sup>	1.95	[M-H] <sup>-</sup>	267.0735	267.0736	0	3.4	↗+50	0.00588	↗+31	0.03136	1	
	Xanthine	1.94	[M+H] <sup>+</sup>	153.0407	153.0406	1	11	↘-88	0.06298	↘-99	0.03721	1	
		1.94	[M-H] <sup>-</sup>	151.0261	151.0261	0	4.2	↘-51	0.06557	↘-42	0.07069	1	
	<i>2,8-Dihydroxyadenine</i>	1.73	[M-H] <sup>-</sup>	<i>166.0370</i>	<i>166.0369</i>	<i>1</i>	6.1	↘-163	<i>0.06958</i>	↘-167	<i>0.06381</i>	2	
	Xanthosine	2.28	[M-H] <sup>-</sup>	283.0684	283.0686	1	8.4	↘-142	0.01039	↘-24	0.46168	2	
	Pyrimidine metabolism	Uridine <sup>d</sup>	1.95	[M-H] <sup>-</sup>	243.0623	243.0622	0	5.1	↗+25	0.00494	/	0.37070	1
Cytidine		2.91	[M+H] <sup>+</sup>	244.0928	244.0927	0	4.3	↗+41	0.01819	↘-30	0.12257	1	
		2.91	[M-H] <sup>-</sup>	242.0782	242.0781	0	4.3	↗+49	0.04395	↘-38	0.14024	1	
Ureidopropionic acid <sup>d</sup>		1.46	[M-H] <sup>-</sup>	131.0462	131.0462	0	12.9	↘-46	0.05965	/	0.35690	2	
Nicotinate and nicotinamide metabolism	Nicotinic acid <sup>d</sup>	1.46	[M+H] <sup>+</sup>	133.0608	133.0606	1	17.8	↘-69	0.02494	↘-24	0.34294	2	
		2.01	[M+H] <sup>+</sup>	124.0393	124.0393	0	3.3	↗+52	0.00337	↗+42	0.00133	1	
		2.01	[M-H] <sup>-</sup>	122.0248	122.0248	0	7.2	↗+46	0.00653	↗+40	0.00107	1	
Alanine, aspartate and glutamate metabolism	L-Glutamic acid <sup>cd</sup>	1.6	[M-H] <sup>-</sup>	146.0459	146.0458	1	1.5	↗+21	0.02930	/	0.27777	1	
	Tyrosine metabolism	Homogentisic acid	4.56	[M-H] <sup>-</sup>	<i>167.0350</i>	<i>167.0349</i>	<i>0</i>	5.4	↗+34	<i>0.06904</i>	↗+23	<i>0.09372</i>	1
Tyrosine metabolism	Gentisate aldehyde	9.65	[M-H] <sup>-</sup>	137.0244	137.0244	0	3.4	↗+27	0.01961	/	0.35400	2	
		9.65	[M+FA-H] <sup>-</sup>	183.0299	183.0299	0	10.4	↗+30	0.02526	/	0.32830	2	
	Gentisic acid	10.3	[M-H] <sup>-</sup>	153.0193	153.0193	0	1.9	/	0.36408	↘-41	0.00796	2	
	<i>Vanillylmandelic acid</i>	12.43	[M-H] <sup>-</sup>	<i>197.0455</i>	<i>197.0454</i>	<i>1</i>	2.7	/	<i>0.48055</i>	↘-26	<i>0.08766</i>	2	
	<i>or 3-(3,4-Dihydroxyphenyl)lactic acid</i>												
	<i>or 3,4-Dihydroxyphenylacetaldehyde</i>												
Glycine, serine and threonine metabolism	Norepinephrine sulfate	8.65	[M+FA-H] <sup>-</sup>	294.0289	294.0299	3	6.5	↘-34	0.00243	↘-24	0.01711	2	
	L-Serine <sup>cd</sup>	1.47	[M-H] <sup>-</sup>	104.0353	104.0354	1	2.1	↗+22	0.00548	/	0.01387	1	
	Glyceric acid	1.32	[M-H] <sup>-</sup>	105.0193	105.0194	0	5.9	/	0.00006	↗+28	0.00003	1	
Tryptophan metabolism	L-Tryptophan <sup>cd</sup>	11.88	[M+H] <sup>+</sup>	205.0972	205.0973	0	4.7	↗+25	0.02574	↗+28	0.00190	1	
		11.88	[M-H] <sup>-</sup>	203.0826	203.0826	0	4.9	↗+24	0.05390	↗+32	0.00160	1	
Histidine metabolism	L-Histidine	2.4	[M+H] <sup>+</sup>	156.0768	156.0767	1	24.3	↗+47	0.01210	↗+20	0.15527	1	
		2.4	[M-H] <sup>-</sup>	154.0622	154.0621	1	26.2	↗+48	0.02397	/	0.24212	1	
Arginine and proline metabolism	L-Proline	1.62	[M+H] <sup>+</sup>	116.0706	116.0705	1	5.1	↗+21	0.04707	/	0.03020	1	
		<i>4-Hydroxyproline</i>	1.38	[M+H] <sup>+</sup>	<i>132.0655</i>	<i>132.0654</i>	<i>1</i>	4.2	↗+35	<i>0.07762</i>	/	<i>0.4035</i>	1
			1.38	[M-H] <sup>-</sup>	<i>130.051</i>	<i>130.0509</i>	<i>1</i>	2.5	↗+37	<i>0.05626</i>	/	<i>0.90283</i>	1
Arginine biosynthesis	L-citrulline <sup>d</sup>	1.6	[M+H] <sup>+</sup>	176.1030	176.1028	1	5.8	↗+37	0.09130	↗+30	0.06979	1	
		1.6	[M-H] <sup>-</sup>	174.0884	174.0884	0	11.3	↗+23	0.13837	↗+25	0.01563	1	
Phenylalanine metabolism	L-Phenylalanine <sup>c</sup>	8.7	[M+H] <sup>+</sup>	166.0863	166.0862	1	3.6	↗+31	0.00680	↗+24	0.01869	1	
Pentose phosphate pathway	Gluconic acid <sup>d</sup>	1.24	[M-H] <sup>-</sup>	195.0510	195.0511	0	2.7	↗+56	0.00216	↗+42	0.04436	1	
Glutathione metabolism	<i>Cysteinylglutathione disulfide</i>	1.62	[M-H] <sup>-</sup>	<i>425.0806</i>	<i>425.0806</i>	<i>0</i>	9	↘-33	<i>0.08854</i>	↘-37	<i>0.05260</i>	1	
Amino sugar and nucleotide sugar metabolism	N-Acetylmannosamine or N-acetyl-D-Glucosamine <sup>de</sup>	1.3	[M+FA-H] <sup>-</sup>	266.0881	266.0882	0	2.1	↗+40	0.00132	/	0.06104	1	
Metabolite of GABA (lactam cyclization)	Pyrrrolidin-2-one	2.04	[M+H] <sup>+</sup>	86.0600	86.0599	2	15.6	↗+36	0.01932	↗+48	0.00135	1	
Arachidonic acid metabolism	<i>PGD<sub>2</sub>/PGE<sub>2</sub></i> <sup>e</sup>	13.3	[M-H] <sup>-</sup>	<i>351.2177</i>	<i>351.2177</i>	<i>0</i>	9.4	/	<i>0.26705</i>	↘-22	<i>0.09678</i>	1	
		14.95	[M-H] <sup>-</sup>	351.2177	351.2177	0	25.7	↘-50	0.03245	↘-21	0.23267	2	
		14.68	[M-H] <sup>-</sup>	<i>367.2126</i>	<i>367.2128</i>	<i>0</i>	27.6	↗+61	<i>0.05890</i>	↗+52	<i>0.08206</i>	2	

412 Metabolites shown in italics had a p-value between 0.05 and 0.1 and may represent biologically relevant metabolites. PG: prostaglandin, TX: thromboxane <sup>a</sup> Based on the study of Sumner et al. (2007); <sup>b</sup> RSD: relative standard deviation calculated for  
413 each feature detected in the quality control sample injected throughout the analytical sequence (analytical variability); <sup>c</sup> Belongs to several metabolic pathways; <sup>d</sup> Also modulated in the reproductive apparatus; <sup>e</sup> Compounds could not be separated  
414 in liquid chromatography;

### 415 3.3. Transcriptomic results

416 The RNAseq analyses generated 150 bp paired-end reads, for a total of 1,893,106,342 reads.  
417 Differential analyses between the control and treated snails revealed 78 and 227 DEGs in reproductive  
418 apparatus and head samples, respectively (FDR-adjusted  $p < 0.05$ ;  $FC > 2$ ). Amongst all DEGs (293), 168  
419 (57.3%) were successfully annotated on referenced proteins, 55 (18.8%) were annotated as  
420 unreviewed proteins and 70 (23.9%) remained unknown or uncharacterized. Within the unreviewed  
421 and unknown sequences, 28 presented conserved domains. Exhaustive lists of DEGs with their relative  
422 fold changes, p-values and corresponding annotations are reported in the Supplementary data, Table  
423 S-4 and S-5 for the reproductive apparatus and head samples, respectively. Conserved domains of  
424 unreviewed and unknown DEGs are reported in the Supplementary data, Table S-6. In the reproductive  
425 apparatus samples, the number of DEGs at 0.1  $\mu\text{g/L}$  (52 DEGs) and 10  $\mu\text{g/L}$  (44 DEGs) of DCF was nearly  
426 the same, with 18 DEGs common to both conditions. In the head samples, our analysis revealed more  
427 than 200 DEGs at 10  $\mu\text{g/L}$  of DCF and 64 DEGs at 0.1  $\mu\text{g/L}$ , with 47 DEGs common to both conditions  
428 (Fig. 3). Twelve DEGs (4% of the total DEGs) were common to the reproductive apparatus and head  
429 samples. Among these DEGs, 11 were successfully annotated. They coded for nitric oxide synthase  
430 (NOS), protein sneaky (SNKY), dynein heavy chain 1 (DNAH1), dorsal-ventral patterning tolloid-like  
431 protein 1 (TLL1), angiotensin-converting enzyme (ACE), thyrostimulin alpha-2 subunit, an Ig-like  
432 domain-containing protein, a calponin-homology (CH) domain-containing protein, barrier-to-  
433 autointegration factor (BAF), ubiquitin-60S ribosomal protein L40 and peroxisomal biogenesis factor 3  
434 (Table S-4 and S-5).

435 The main biological functions of the DEGs were controlling development, the immune response,  
436 reproduction, oxidative stress and neurogenesis (Table 4). All DEGs and their functions are reported in  
437 the Supplementary data, Table S-7. Some were involved in many functions as ACE, which is associated  
438 with the immune response and reproduction in invertebrates (Salzet et al., 2001).



439 Figure 3- Venn diagram reporting the number of differentially expressed genes (GLM, FDR<0.05) for reproductive apparatus  
 440 and head samples with comparisons among control and exposed groups.  
 441

442 Table 4 – Main functions impacted by DCF exposure according to the observed DEGs (over-expressed (↗) and under-  
 443 expressed (↘)). Single DEGs could be involved in various functions.  
 444

Function	Number of DEGs (percentage of total DEGs)	Reproductive apparatus		Heads	
		Ctrl vs 0.1 µg/L	Ctrl vs 10 µg/L	Ctrl vs 0.1 µg/L	Ctrl vs 10 µg/L
Control of development	6 (2.0%)	2↗ 1↘	1↗	2↗ 2↘	3↗ 1↘
Immune response	17 (5.8%)	0	1↗	2↗ 2↘	10↗ 6↘
Reproduction	13 (4.4%)	4↗	5↗	3↗	7↗ 3↘
Oxidative stress	5 (1.7%)	0	1↗ 1↘	1↘	3↘
Neurogenesis	7 (2.4%)	0	0	1↗ 3↘	1↗ 6↘

445  
 446 3.4. Discussion DCF effects on *L. stagnalis*: life history and behavior endpoints

447 Phenotypic effects of DCF were observed mostly on juvenile snails, with an increase in shell size  
 448 and a decrease in feeding rate in exposed animals. To our knowledge, this is the first time that a growth  
 449 increase has been noted after DCF exposure. An opposite trend has been reported, with a decrease in  
 450 growth rate in amphibian larvae at DCF concentrations from 125 to 2000 µg/L, and in marine organisms  
 451 as mussel and shrimp at 1 and 900 µg/L DCF, respectively (Ericson et al., 2010; González-Ortegón et  
 452 al., 2013; Peltzer et al., 2019). The growth increase could be due to hormesis, where low  
 453 concentrations of a contaminant could lead to a compensatory response in individuals, while high  
 454 concentrations could inhibit processes and cause detrimental effects (Calabrese, 2008). Indeed, the  
 455 reduction in growth reported in other species was generally observed at higher exposure  
 456 concentrations, which seems consistent with a hormetic effect. Therefore, a study conducted on  
 457 *Daphnia magna* exposed to the NSAID ibuprofen at low concentrations (4 µg/L) showed faster growth  
 458 rates on the sixth generation exposed (Grzesiuk et al., 2020). This could indicate that a greater share

459 of energy was allocated to growth in exposed organisms than in control. The altered feeding behavior  
460 observed in this study was similar to that observed in medaka fish after exposure to 1.0 mg/L DCF  
461 (Nassef et al., 2010) with an increased eating time. Unfortunately, transcriptomics and metabolomics  
462 studies were not conducted on the juvenile life stage, and molecular information obtained at the adult  
463 stage may not explain these observed impacts.

464

465 3.5. DCF effects on *L. stagnalis*: molecular endpoints

466 3.5.1. *Lymnaea stagnalis* metabolome

467 The metabolome of *L. stagnalis* was little explored in the past. Tufi et al. (2015a, 2015b)  
468 reported information on albumen gland, central nervous system (CNS) and digestive gland metabolic  
469 profiles in *L. stagnalis*. 35 metabolites common to those reported by Tufi and 58 metabolites not  
470 reported so far were identified in this study (Table S-2). Interestingly, pyruvate which was previously  
471 observed only in the albumen gland, was specifically highlighted in the reproductive apparatus in this  
472 study. In mammals, pyruvate was shown to be produced by follicular cells and have a role in oocyte  
473 maturation and aging inhibition (Donahue and Stern, 1968; Liu et al., 2009; Rieger and Loskutoff, 1994).  
474 This compound thus might be involved in *L. stagnalis* reproductive functions. Other differences in  
475 observed metabolites could be explained by differences in methodological protocols and organs  
476 explored.

477

478 3.5.2. Hypotheses on biological effects of DCF

479 Among the metabolite alterations observed in this study, similarities with the findings of  
480 previous studies conducted on NSAIDs were observed. Serine and asparagine modulation was noted  
481 in *D. magna* exposed to ibuprofen (Kovacevic et al., 2016) and the tyrosin pathway was impacted in  
482 this study, as already demonstrated in mussels (Bonnefille et al., 2018). However, tryptophan  
483 metabolism seemed to be less altered in this study than in previous studies in mussels (Bonnefille et  
484 al., 2018). Osmoregulation was hypothesized to be an impaired function in mussels after DCF exposure

485 (Bonnefille et al., 2018). Osmoregulation in freshwater mollusks is an important process with regard  
486 to their survival, as the salinity level in their body is higher than in the external medium (Oglesby,  
487 1981). The free amino acid content was shown to be involved in osmoregulation in freshwater  
488 organisms (Hanson and Dietz, 2011; Matsushima et al., 1989). Here, amino acid levels were increased  
489 overall (tryptophan, serine, histidine, proline, phenylalanine, glutamic acid), which could have been  
490 the result of pressure modifications. In addition, proline betaine, i.e. a metabolite involved in the  
491 osmotic balance in marine organisms (Pierce and Rowland, 1988; Shinagawa et al., 1992), was  
492 decreased in the *L. stagnalis* reproductive apparatus in this study. Other compounds such as  
493 prostaglandin E and serotonin (also modulated in this study) were demonstrated to be involved in  
494 osmoregulation in freshwater bivalves (Evans, 2008; Ruggeri and Thoroughgood, 1985). To our  
495 knowledge, no studies on the osmoregulatory function of these compounds have been conducted on  
496 freshwater gastropods. Eades and Waring (2010) reported that environmental DCF concentrations (10  
497 and 100 ng/L) have impacts on the hemolymph osmolality and osmolarity capacity in *Carcinus maenas*  
498 crabs. All of this information lead us to formulate the hypothesis of an alteration of osmoregulatory  
499 processes in *L. stagnalis* following DCF exposure.

500         Regarding the potential mode of action of DCF in great pond snails, the arachidonic acid  
501 pathway—with modulation of prostaglandin related signals—was found to be disturbed in the present  
502 study. Prostaglandin levels were already found to be down-modulated in marine invertebrates after  
503 DCF exposure (Courant et al., 2018; Fu et al., 2021), suggesting that DCF inhibits prostaglandin  
504 synthesis as its mode of action in humans. Indeed, in humans, the mechanism of action of DCF is the  
505 inhibition of prostaglandin production *via* the inhibition of cyclooxygenase (COX) (Gan, 2010).  
506 Prostaglandins have been recognized as being involved in egg production in freshwater snails (Kunigelis  
507 and Saleuddin, 1986) and in reproduction in invertebrates (Stanley-Samuels, 1994; Wongprasert et  
508 al., 2006). Interestingly, the impact of DCF on prostaglandin-related metabolites was higher at the  
509 lowest concentration in the reproductive apparatus. In addition, *prostaglandin reductase 1* was shown  
510 to be specifically upregulated in this organ at the lowest concentration. This enzyme is responsible for

511 the biological inactivation of prostaglandins, and contributes to ovarian development in giant tiger  
512 prawns, which are marine invertebrates (Prasertlux et al., 2011). Moreover, serotonin was down  
513 modulated in this specific organ at 0.1 µg/L concentration. The serotonergic system is involved in  
514 oviposition and the transmission of non-genetic information from mother to progeny in *Lymnaea*  
515 *stagnalis* (Ivashkin et al., 2015). It is also involved in egg laying in *Biomphalaria glabrata* freshwater  
516 snails (Muschamp and Fong, 2001). Prostaglandin and serotonin modulation could therefore have  
517 possibly resulted in reproduction impairment in our model. Several genes coding for proteins involved  
518 in reproduction were also differentially expressed, e.g. serine protease inhibitor, signal transducers,  
519 transcription activator, DNAH1, ACE, fibrous sheath interacting protein, ovulation prohormone taking  
520 part in various processes such as oogenesis, sperm motility and spermatogenesis. These genes  
521 involved in reproduction were identified in both organs, which is not surprising regarding the neuronal  
522 control of reproduction behavior. However, no effects of DCF on egg laying were demonstrated in this  
523 study. These molecular modulations may not be substantial enough to induce physiological effects.  
524 Sperm counting and motility were not assessed but could represent an interesting endpoint for further  
525 assessment of the detected negative effects of DCF on semen quality in mammals and marine  
526 invertebrates (Banihani, 2021; Mohd Zanuri et al., 2017). DCF has a similar mode of action (MoA) in  
527 mollusks and in humans regarding its impact on prostaglandin levels, including alteration of COX  
528 activation, while prostaglandin production is the main MoA for NSAIDs. However, another hypothetic  
529 pathway in humans that could explain the analgesic effects of DCF is activation of the L-arginine–nitric  
530 oxide–cGMP pathway (Gan, 2010). In our study, nitric oxide synthase (NOS) upregulation (all  
531 concentrations and organs) was observed. NOS is an enzyme that catalyzes nitric oxide (NO)  
532 production from L-arginine. NO levels were not measured, but L-citrulline—another product of this  
533 reaction—was found to be upmodulated in both organs. NOS overexpression and enhanced citrulline  
534 levels suggest that NO levels could also be increased by DCF exposure, and that DCF may activate a  
535 pathway in *L. stagnalis* that is similar to the L-arginine–NO–cGMP pathway in humans. In mollusks, it  
536 has been demonstrated that the NO-cGMP pathway has a function in mediating chemosensory

537 activation of feeding (Elphick et al., 1995) and could have been involved in the altered feeding behavior  
538 of young snails in this study.

539 NOS and NO are also related to immunity processes in mollusks (Ottaviani, 2006). Sixteen DEGs  
540 involved in the immune system response were detected in this study, particularly with regard to innate  
541 immune responses (Table 4). *L. stagnalis* has a quasi-specific innate-like immune system, with  
542 circulating immunocompetent cells called hemocytes (Rowley and Powell, 2007). Boisseaux et al.  
543 (2017) reported that DCF has an impact on the hemocyte immunocapacity and efficiency in *L. stagnalis*  
544 after a 3 days exposure, which is characteristic of an inflammatory response. Inflammation was shown  
545 to be a conservative mechanism in mollusks and involved in the innate immune response (Barcia and  
546 Ramos-Martínez, 2008; Ottaviani et al., 1995; Rosani et al., 2015). In addition, DEGs coding for  
547 uncharacterized proteins were found to present conservative domains with an immunity function, like  
548 ankyrin repeat region profiles (GSLYST00003453001) (Picone et al., 2015), C-type lectin domain  
549 signature and profile (GSLYST00005882001) (Gorbushin and Borisova, 2015) and TNF family signature  
550 and profile (GSLYST00007116001) (Zhang et al., 2008). Our metabolomics results were in line with  
551 published results on inflammation since nicotinate and nicotinamide metabolism were disrupted.  
552 These compounds have been recognized to have anti-inflammatory properties in mammals (Godin et  
553 al., 2012). Furthermore, pyridoxal—the precursor of the pyridoxal 5'-phosphate (active form of vitamin  
554 B6)—is upmodulated after DCF exposure. This metabolite is also involved in inflammatory processes  
555 (Bird, 2018). Modifications in metabolites that have a function in inflammation lead us to hypothesize  
556 a modulation of the inflammatory balance in *L. stagnalis* after DCF exposure. The immune response is  
557 a high energy-demanding process and long-term impacts can lead to major trade-offs (Coyne, 2011).  
558 In addition, under stress conditions, the energy metabolism demand can be enhanced to ensure  
559 maintenance of the organism and other functions such as reproduction, growth or locomotion  
560 (Sokolova et al., 2012). Decreased carnitine and acetylcarnitine levels were observed in this study. They  
561 are known to be involved in fatty acid transport through the mitochondrial membrane and to play a  
562 key role in fatty acid  $\beta$ -oxidation, which in turn leads to ATP production. The carnitine shuttle pathway

563 was also affected by DCF exposure in *H. azteca*, a freshwater crustacean, suggesting that DCF alters  
564 fatty acid  $\beta$ -oxidation in various species (Fu et al., 2021). Besides, levels of adenine and adenosine were  
565 increased and they may subsequently be used for ATP production. Those results suggest overactivation  
566 of energy metabolism to meet the energy demand. Faster juvenile snail growth could be an impact of  
567 this imbalance of energy modulation. Furthermore, we observed 6 DEGs involved in the control of  
568 development, including organ development and dorsal and ventral pattern formation. However,  
569 transcriptomic and metabolomics studies were conducted at the adult stage and a study focused on  
570 the juvenile stage could enhance our understanding of the molecular mechanisms modulated at this  
571 specific life stage.

572 Purine and pyrimidine metabolisms were particularly impacted here, i.e. mainly the  
573 modulation of nucleic base (adenine, hypoxanthine and xanthine) and nucleoside (adenosine,  
574 guanosine, inosine, uridine and cytidine) levels. Although adenine and adenosine can be used to  
575 produce ATP, nucleic bases and nucleosides first and foremost support DNA synthesis, and  
576 modulations in the levels of these compounds could impact RNA and DNA repair and turnover. These  
577 purine and pyrimidine modulations have already been found in non-target species exposed to  
578 xenobiotics (Dumas et al., 2020; Sotto et al., 2017; Tufi et al., 2015b). In the present study, we noted  
579 that guanosine was specifically upmodulated in head samples, which suggested another potential  
580 function of this compound. Guanosine has been shown to be neuroprotective against excitotoxicity  
581 induced by glutamate, with the promotion of astrocyte glutamate uptake (Dal-Cim et al., 2019; dos  
582 Santos Frizzo et al., 2003). Indeed, glutamate has neurotoxic properties in vertebrates, mediated by  
583 overactivation of glutamatergic receptors (Granzotto et al., 2020). Glutamate was upmodulated in our  
584 study and the increased guanosine levels in head samples could be a response to glutamate toxicity in  
585 the CNS. To our knowledge, this is the first time that this effect has been reported in gastropoda.  
586 Moreover, 7 DEGs involved in neurogenesis were specifically identified in head samples, mainly with  
587 underexpression of those genes (Table 4). Neurogenesis disturbed processes could also be a  
588 consequence of the potential neurotoxic effect induced by DCF exposure.

589 We previously assumed that glutamic acid may have neurotoxic effects. Moreover, glutamic acid  
590 up-modulation may also be a response against oxidative stress, as already observed by Salyha and  
591 Salyha (2018) in rats. Regarding DEGs, five of them code for enzymes involved in oxidative stress and  
592 antioxidant responses, i.e. superoxide dismutase (SOD), peroxidase (PXDN), dual oxidase 2 (DUOX2)  
593 and chorion peroxidase. The genes encoding these enzymes were mostly underexpressed in our study,  
594 and levels of cysteineglutathione disulfide, which is produced from glutathione (Bellomo et al., 1987),  
595 were decreased in head samples. These changes in enzyme, cysteineglutathione disulfide and glutamic  
596 acid levels in *L. stagnalis* after long-term DCF exposure suggest that this exposure cause a redox  
597 imbalance. Interestingly, Gonzalez-Rey and Bebianno (2014) documented enhancement of SOD and  
598 catalase activity in mussels after 3 days of exposure, followed by decreased activity after 15 days of  
599 exposure. Since we conducted a long-term study, the underexpression of genes encoding antioxidant  
600 enzymes that we noted was in line with published results. The redox imbalance may be caused by an  
601 antioxidant response activated to counteract enhanced ROS levels. This oxidative stress caused by DCF  
602 exposure was previously highlighted in several non-target species (fish, mussels) exposed to low DCF  
603 concentrations and it seems to be a common response to NSAIDs exposure (Ajima et al., 2021;  
604 Gonzalez-Rey and Bebianno, 2014; Trombini et al., 2019).

605

## 606 CONCLUSION

607 *L. stagnalis* snails exposed to environmentally relevant DCF concentrations were affected during  
608 growth, with an increase in shell size in exposed snails and a decrease in feeding rate. No evidence of  
609 an impact was observed on other life history and behavioral parameters (hatching, locomotion,  
610 response to stress, reproduction). DCF seems to have similar molecular targets in mollusks and  
611 humans, with prostaglandins level modifications. Other molecular results suggest that DCF induces  
612 neurotoxicity and an oxidative stress response, along with energy metabolism modulation. Although  
613 no effects were observed on reproduction parameters explored in this study, we noted an impact on

614 this function at the molecular level. Moreover, functions like osmoregulation and immunity could be  
615 impaired by DCF exposure, and further studies are needed to clarify this issue.

616

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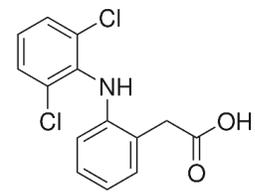
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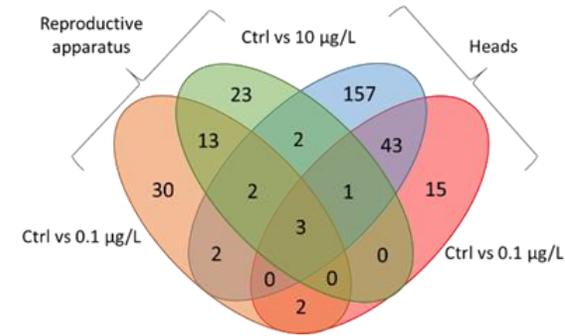
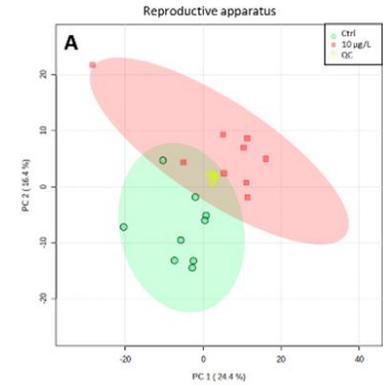
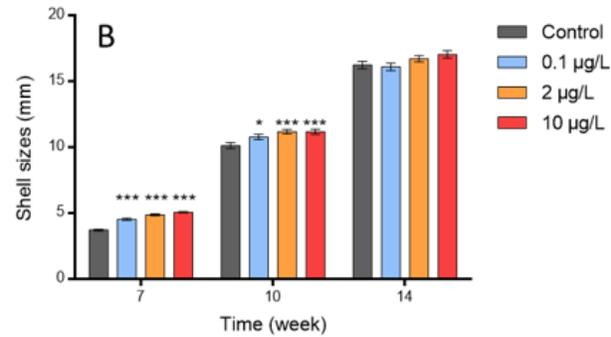
Diclofenac

*Lymnaea stagnalis*

Life history traits  
Behavior

Metabolomics

Transcriptomic



**Modifications of**

- Shell growth
- Feeding behavior
- Prostaglandins levels

**Modifications at molecular level involved in**

- Oxidative stress
- Neurotoxicity
- Immune response
- Energy metabolism
- Osmoregulation