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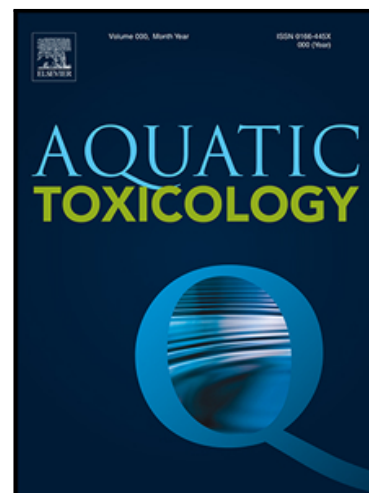
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Journal Pre-proof

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Highlights

- Diclofenac (DCF) exposure induces multigenerational effects in *Lymnaea stagnalis*.
- Growth is affected in the F1 and F2 generations.
- The impact of exposure differs between generations.
- DCF causes inhibition of reproductive and feeding parameters in the F1 generation.
- DCF stimulates hatching, growth, egg-laying and feeding in the F2 generation.

Journal Pre-proof

Multigenerational responses in the *Lymnaea stagnalis* freshwater gastropod exposed to diclofenac at environmental concentrations

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Abstract: Over the last decade, there has been increased concern about the occurrence of diclofenac (DCF) in aquatic ecosystems. Living organisms could be exposed to this “pseudo-persistent” pharmaceutical for more than one generation. In this multigenerational study, we assessed the DCF impact at environmentally relevant concentrations on the life history and behavioral parameters of two offspring generations (F1 and F2) of the *Lymnaea stagnalis* freshwater gastropod. Snail growth was affected by DCF in the F1 generation, with increased shell sizes of juveniles exposed to 0.1 $\mu\text{g.L}^{-1}$ concentration and a decreased shell size at 2 and 10 $\mu\text{g.L}^{-1}$. DCF also lowered food intake, enhanced locomotion activity and reduced the number of eggs/egg mass in the F1 generation. For the F2 generation, shorter time to hatch, faster growth, increased food intake and production of more egg masses/snail were induced by DCF exposure at 10 $\mu\text{g.L}^{-1}$. Over time, DCF exposure led to maximization of *L. stagnalis* reproductive function. These results show that multigenerational studies are crucial to reveal adaptive responses to chronic contaminant exposure, which are not observable after short-term exposure.

Keywords: NSAID, pharmaceutical, mollusk, sublethal effects, behavior, life history

1. Introduction

The presence of pharmaceuticals in the environment has become an important issue in ecotoxicology in recent years. Moreover, this problem is likely to worsen over time as the aging population grows, leading to increased drug consumption (Pereira et al., 2020). Non-steroidal anti-inflammatory drugs (NSAIDs) represent one of the most frequently detected pharmaceutical classes in the environment (Fekadu et al., 2019). Among them, diclofenac (DCF) was included in the previous Watch List published under the EU Water Framework Directive 2000/60/EC (European Commission, 2015) and has shown an acute toxicity potential to aquatic organisms (Cleuvers, 2003; Haap et al., 2008; Lee et al., 2011). Acuña et al. (2015) reported a median concentrations of $21 \pm 722 \text{ ng.L}^{-1}$ DCF in surface water after analysis of 142 publications from 38 countries. The highest concentrations were observed in river directly receiving wastewater treatment plants effluent (e.g., $1.2 \text{ }\mu\text{g.L}^{-1}$ in Germany) (Ternes, 1998) or untreated domestic and industrial effluents (e.g., $8.5 \text{ }\mu\text{g.L}^{-1}$ in Pakistan) (Scheurell et al., 2009). DCF chronic impact was subsequently assessed at low concentrations on aquatic species, showing reproduction disruption and teratogenic properties on fish in the 0.6 to $100 \text{ }\mu\text{g.L}^{-1}$ concentration range (Yokota et al., 2018, 2017; Zhang et al., 2020). Few studies have been conducted on the reproductive function of mollusks at environmental concentrations ($< 10 \text{ }\mu\text{g.L}^{-1}$), while the mechanism of action of DCF in humans leads to the decreased levels of prostaglandins (Gan, 2010), lipid compounds involved in invertebrate reproduction (Kunigelis and Saleuddin, 1986; Osada and Nomura, 1990; Stanley-Samuelson, 1994). Furthermore, prostaglandin levels also appeared to decrease in aquatic invertebrates after DCF exposure (Bouly et al., 2022; Courant et al., 2018; Fu et al., 2021). A study on marine invertebrates (starfish, sea urchins and polychaete worms) demonstrated that DCF reduced sperm motility at concentrations below $1 \text{ }\mu\text{g.L}^{-1}$ (Mohd Zanuri et al., 2017). Regarding the teratogenic impact, Munari et al. (2016) highlighted the induction of abnormal *Ruditapes philippinarum* larval development by DCF alone at $0.5 \text{ }\mu\text{g.L}^{-1}$ or combined with water acidification, with induction of shell abnormalities. On a behavioral level, locomotion was impaired in the freshwater crustacean *Bryocamptus pygmaeus* after 72 h exposure to $50 \text{ }\mu\text{g.L}^{-1}$ DCF, with a

reduction in swimming speed, swimming activity, exploration ability and thigmotaxis, along with increased swimming path tortuosity (Di Cicco et al., 2021). Feeding behavior was shown to be impacted in *D. magna* with decreased ingestion rates after DCF exposure at $100 \mu\text{g.L}^{-1}$ concentration (Nkoom et al., 2019). With regard to previous studies, there is a severe lack of information on DCF effects at environmental concentrations on the aquatic organism behavior (feeding, locomotion, response to light stress), although these aspects are crucial for the species' survival.

DCF is a “pseudo-persistent” contaminant in the aquatic environment due to its continuous release (Acuña et al., 2015). Acute and chronic exposure studies provide information on contaminant effects on organisms, yet they are more likely exposed for more than one generation in freshwater environments. Life cycle and multigenerational studies are therefore essential to more accurately assess the risk posed by xenobiotics in aquatic habitats. Organisms could develop tolerance or sensitivity in subsequent generations, depending on mechanisms involved in the exposure response (Kim et al., 2012; Pinto et al., 2021). Multigenerational effects of DCF have been assessed in daphnids, revealing a delay in the age of the first reproduction in F0 and F2 generations and an increase in neonate body length in F1 and F5 generations after exposure to $0.36 \mu\text{g.L}^{-1}$ DCF (Dietrich et al., 2010). However, *D. magna* seems less sensitive than other species to pharmaceutical pollutants (Batucan et al., 2021). The insensitivity of this model has already been reported for other contaminants, such as neonicotinoid insecticides, leading to underestimation of the environmental risk posed by those substances (Sánchez-Bayo et al., 2016). It would therefore be essential to propose new model species to gain insight into pharmaceutical impact on the ecosystems.

Invertebrates represent 95% of existing species, and they play a crucial role in the functioning and health of aquatic ecosystems (Segner et al., 2003). *Lymnaea stagnalis* is a freshwater gastropod which was recently suggested to be a powerful ecotoxicology research model (Amorim et al., 2019). This benthic organism is widely distributed in the northern hemisphere and—like other freshwater gastropods—plays a crucial role in the balance of some aquatic ecosystems (Covich et al., 1999). In addition, this snail has a simple and adaptable behavioral repertoire that could facilitate ecological

risk assessment (Aonuma et al., 2020; Dobranskyte et al., 2006; Niyogi et al., 2014; Svigruha et al., 2021). An OECD guideline proposes *L. stagnalis* as model organism for reproductive assessment in ecotoxicology studies (OECD, 2016), thereby making it the first aquatic non-arthropod invertebrate model organism to be recognised in environmental risk assessments. This snail is a simultaneous hermaphrodite that produces transparent egg masses, which facilitates monitoring of embryo development in the eggs. Furthermore, its short 3-month life cycle is convenient for multigenerational laboratory studies (Amorim et al., 2019).

In a previous study, this model was successfully used to investigate the DCF impact on freshwater snails at adult life stages after full life cycle exposure, combining physiological, behavioral and molecular observations (Bouly et al., 2022). While the organisms appeared to be impacted at the molecular level, there was little effect on life-history traits and behavior of the parental generation. Based on the molecular impacts on the parental generation, we hypothesized that DCF may induce growth, feeding, locomotion and reproductive disorders in further generations. The aim of this study was to assess DCF effects at environmental concentrations (0.1, 2 and 10 $\mu\text{g}\cdot\text{L}^{-1}$) on the life history traits and behaviour of subsequent F1 and F2 generations. We thus measured hatching success of egg masses, juvenile growth and egg production of adult snails. Behavioral endpoints (locomotion, feeding rate, response to light stress) were monitored in the adult stage to assess the consequences of full life cycle xenobiotic exposure on adult populations.

2. Materials and methods

2.1. Test organisms

Lymnaea stagnalis (Linnaeus, 1758) freshwater snails were from Biochemistry and Toxicology of Bioactive Substances (BTSB, EA 7417, Albi) laboratory cultures that had been started in 2019 with organisms obtained from the IRSTEA Lyon laboratory (France). They were maintained in a controlled-temperature chamber ($21\text{ }^{\circ}\text{C} \pm 1\text{ }^{\circ}\text{C}$) under a simulated natural photoperiod (16 h L/8 h D) and fed fresh organic lettuce 3 times/week. They were held in mineral water (calcium $45\text{ mg}\cdot\text{L}^{-1}$, magnesium

4.2 mg.L⁻¹, sodium 7.5 mg.L⁻¹, potassium 4.5 mg.L⁻¹, sulfate 9.5 mg.L⁻¹, chloride 4.6 mg.L⁻¹, nitrate <1 mg.L⁻¹, bicarbonate 151.6 mg.L⁻¹), with weekly renewal.

2.2. Chemicals

Diclofenac (DCF, purity ≥98%, CAS number 15307-79-6) was purchased from Sigma-Aldrich (Steinheim, Germany). Stock solutions of DCF were prepared at 2 mg.L⁻¹ in water and stored at 4 °C in opaque glass bottles. The stock solution concentrations were then measured as described in section 2.4. Ultrapure water was generated by a Purelab Classic system from Elga LabWater (Lane End, UK) with a specific resistance of 18.2 MΩ.cm at 25 °C. Analytical grade hydrochloric acid (37%) solvent was obtained from PanReac (Barcelona, Spain), HPLC grade trifluoro acetic acid was from VWR Chemicals (Leuven, Belgium), while hyper-grade acetonitrile for LC/MS was from Merck (Molsheim, France).

2.3. Exposure conditions

Snail exposure was conducted at three nominal DCF concentrations: 0.1 (low), 2 (medium) and 10 (high) µg.L⁻¹. Stock solutions were prepared in containers, diluted in mineral water and changed weekly. The exposure and tests conditions of the snail parental generation (F0) were detailed in Bouly et al. (2022). Briefly, the F0 generation was exposed at 0, 0.1, 2 and 10 µg.L⁻¹ DCF from the first day of the embryo to the adult stage (16 weeks post-hatch, wph). The materials and methods described here only concern the F1 and F2 offspring generations (Fig. 1). F1 and F2 were started by collecting egg masses from previous generations (control, low, medium and high groups) at 16 wph over a maximum 7-day period. Egg masses were exposed in glass beakers in a 300 mL volume of exposure medium (3 replicates of 5 egg masses/condition). After hatching, young snails were randomly divided in 5 glass beakers containing 40 snails (200 snails/condition), in a 300 mL volume of exposure medium that was renewed weekly. At 8 weeks post-hatch (wph), individuals were randomly distributed in three 20 L-aquaria of 40 snails (120 snails/condition) in a 5 L volume of

exposure medium. In the F1 generation, due to technical issues, the low treatment was renewed with new egg masses and the tests were split between the low treatment and the medium and high treatments. Therefore, there were two controls (Ctrl_{Low} and Ctrl_{Medium, High}) for this generation at the juvenile and adult stages, and in the hatching test for the F2 generation to allow comparison with appropriate controls, carried out at the same time and under the same conditions. After hatching, control offspring were randomly mixed to obtain a single control for the F2 generation tests (Fig. 1). Snails were fed fresh organic lettuce three times/week. Dead snails were removed every week and a final survival rate was assessed at 16 wph.

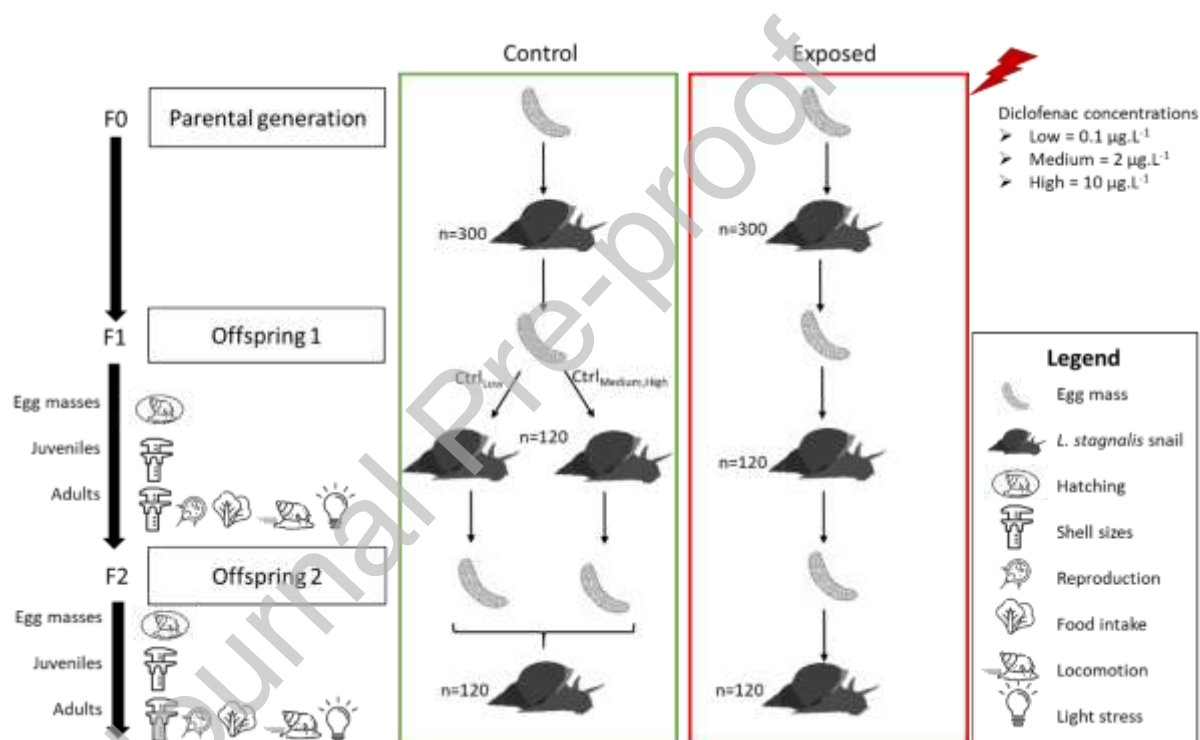


Figure 1 - Diclofenac exposure of *L. stagnalis* for 3 generations and tests performed at each life stage. Tests conducted on the F0 generation were detailed in our previous publication. Juvenile parameters were monitored between 7 and 8 weeks post-hatch (wph), while adult parameters were determined between 15 and 20 wph, depending on the parameter assessed.

2.4. DCF analysis in water

The water chemical analysis was as described in Bouly et al. (2022). For each treatment concentration, six water samples were randomly taken after water renewal (T0) and six were taken at day 7, just before water renewal (T7). The samples were stored at -20 °C until analysis. For each sample, 200 mL HCl 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 with HCl (pH 2) and dried for 4 h in a laboratory oven at 45 °C. Components were eluted with 4 mL acetonitrile. The extracts were then dried under a nitrogen stream and reconstituted with 200 µL acetonitrile. HPLC 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 µm C18 (4.6 × 250 mm) capillary column (Agela Technologies, Torrance, USA). The solvents were: A) water + 0.05% trifluoroacetic acid (TFA), and B) ACN + 0.05 % TFA. The elution gradient was as follows (t in min): t₀, B = 30 %; t₁₁, B = 100 %; t₁₃, B = 100 %, t_{13.5}, B = 30 %, t₁₈, B = 30 %. The column oven temperature was set at 40 °C, with a 20 µL injection volume and 1 mL/min flow rate. The working UV wavelength was 275 nm. Data analyses were performed using ChromQuest 5.0. For DCF concentration quantification, 2 calibration curves were plotted in triplicate, with DCF dissolved in acetonitrile (external standard method). The first one was a 4 point-calibration curve spanning the 0.1-1 mg.L⁻¹ range, and the second one was a 5 point-calibration curve spanning the 1-10 mg.L⁻¹ range. Measurements were obtained in 100X concentrated extracts, and the concentrations in water samples were then calculated, while taking the extraction recoveries into account. Stock solution DCF concentrations were measured using the same analytical method. The limit of quantification (LOQ) was 0.1 mg.L⁻¹ and the limit of detection (LOD) was 0.03 mg.L⁻¹. Instrumental stability was checked regularly in triplicate by injecting a concentration corresponding to one point on the calibration curve.

2.5. Life history traits

F1 and F2 egg masses were collected from aquaria and placed in glass petri dishes (50 mm dia.) in 20 mL of exposure medium. Egg mass hatching was monitored daily between days 8 and 18 after egg laying, with 16 egg masses/condition. Egg masses with undeveloped embryos were discarded. Hatching success (%) and time to hatch were determined for each egg mass.

Shell sizes were determined for the entire population using a caliper to measure the distance between the shell aperture and apex at 8 (juvenile stage) (n=200) and 16 wph (adult stage) (n=120).

The reproduction test was adapted from the OECD reproduction test (OECD, 2016). It was performed at 16 wph on 6 replicates of 5 individuals in 1 L beakers containing 800 mL of exposure medium (30 snails/condition). The organisms were selected after sexual maturity, with homogeneous shell sizes of 24.5 ± 3.5 mm. They were collected from the exposure aquaria and were allowed to acclimate for 3 days in beakers filled with exposure medium. This test was conducted over a 28-day period. Water was renewed weekly and egg masses were removed and counted daily. Shell sizes were measured at the beginning and end of the test. Endpoints were the number of egg masses/snails and the number of eggs/egg mass. To better highlight the impact of DCF exposure between generations, we plotted the growth test results and reproductive parameters normalized to the mean control value.

2.6. Behavioral endpoints

Snails from F1 and F2 generations selected for behavioral tests were randomly taken under the shell size range of 24 ± 3 mm. Feeding and locomotion endpoints were normalized to the mean control level on the plot so as to facilitate comparison between generations.

2.6.1. Feeding test

Feeding tests were conducted on snails placed in individual exposure systems at 16 wph (32 snails/condition) with standardized lettuce disks (300 mm dia., 28.26 cm² area). After 6 h, pictures were taken from the top of the aquaria of the remaining lettuce (Mi 9 SE, Xiaomi), while respecting same distance between the aquaria and the camera lens at each shot. The images were processed with Photoshop CS6 software according to the method described by Lebreton et al. (2021) and analyzed using ImajeJ software (National Institute of Health, version 1.52a). The consumed lettuce area after 6 h of feeding normalized to the snail shell size was calculated.

2.6.2. Locomotion test

Locomotion behavior was monitored at 17 wph as described in Bouly et al. (2022). Twenty-four snails/condition were monitored for 2 h in petri dishes (140 mm dia.) containing 150 mL of exposure medium using EthoVision XT behavior software (Noldus, Wageningen, Netherlands) with a camera (Basler, acA 1300-60 gm, Kowa LMVZ4411 lens). The average distance moved (in mm) in 2 h was normalized to the snail shell size.

2.6.3. Light stress test

The stress response test was performed on the same individuals and under the same conditions as used in the locomotion tests. Twenty-four animals per condition were filmed for 15 min with the light on, followed by 15 min in the dark, and 15 min with the light on. The average distance moved (mm) in each light condition (15 min), normalized to the snail shell size (mm), was calculated.

2.7. Statistical analysis

The data were analyzed using GraphPad Prism (Version 6.01 for Windows, GraphPad Software, San Diego, California, USA) and R Studio Version 1.3.1056 with R Version 4.1.0. In all experiments, assumptions for the parametric tests (normality and homoscedasticity) were assessed and not met. Consequently, shell sizes, feeding rate, distance moved, number of egg masses/snail, number of eggs/egg mass and hatching success were analyzed using a multiple comparison test after a non-parametric Kruskal-Wallis test (pgirmess R package) or using a Mann-Whitney test. Time to hatch was analyzed—after rounding the hatching percentage to the decimal point—via a log-rank survival analysis followed by a pairwise Mann-Whitney test.

3. Results

3.1. Exposure conditions and survival

In the F1 and F2 generation, survival rates ranged between 67.1 and 74.9% in controls, and 59.8 and 73.5% in treatments (Table S-1). This was considered acceptable regarding the long exposure time. Indeed, Reátegui-Zirena et al. (2017) reported an 80% survival rate in *L. stagnalis* control snails after a 56-day experiment, i.e. half as long as in the present study. DCF concentrations at day 0 were confirmed to be within a reasonable nominal range (maximum of 25% deviation from the nominal value) (Table S-2). After 7 days, DCF concentrations were stable for the 0.1 $\mu\text{g.L}^{-1}$ (low group) and 10 $\mu\text{g.L}^{-1}$ (high group) concentrations and decreased by more than 30% for the 2 $\mu\text{g.L}^{-1}$ concentration (medium group).

3.2. Life history traits

3.2.1. Hatching success and time-to-hatch

Hatching success was not affected by DCF exposure in the F1 and F2 generations (Kruskal-Wallis test, $p > 0.05$). Considering the time to hatch, there were no differences between control and exposed egg masses for the F1 generation, yet there were statistically significant differences for the F2 generation (Fig. 2). Egg masses that emerge as the F2 generation and which were exposed to 10 $\mu\text{g.L}^{-1}$ (high group) took shorter to hatch compared to control (Mann-Whitney test, $p < 0.05$).

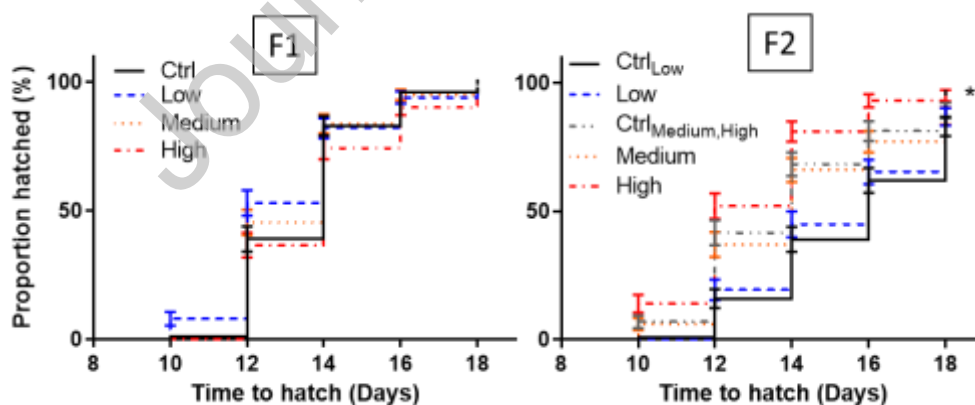


Figure 2—Time to hatch of eggs from control or DCF exposed snails and emerging as the F1 (left) and F2 (right) generations. Bars represent standard error. * $p < 0.05$ compared to control; Mann-Whitney test.

3.2.2. Growth

DCF exposure affected the shell sizes of juvenile snails in both offspring generations (Fig. 3A).

Figure 3 represents the shell sizes of the exposed groups normalized to the mean control value, thereby highlighting the size modulations relative to the control in each generation. Mean control values and standard deviations were reported in the Supplementary data (Table S-3). Juvenile snails from the low group had a longer shell size in both generations F1 (Mann-Whitney test, $p < 0.001$) and F2 (Kruskal-Wallis test, $p < 0.001$). The medium and high groups from the F1 generation had shorter shells at the juvenile stage (Kruskal-Wallis test, $p < 0.001$), while those from the F2 generation had longer shells compared to the control (Kruskal-Wallis test, $p < 0.001$). At the adult stage, there were no longer differences in shell size of exposed snails compared to control for both generations (Fig. 3B).

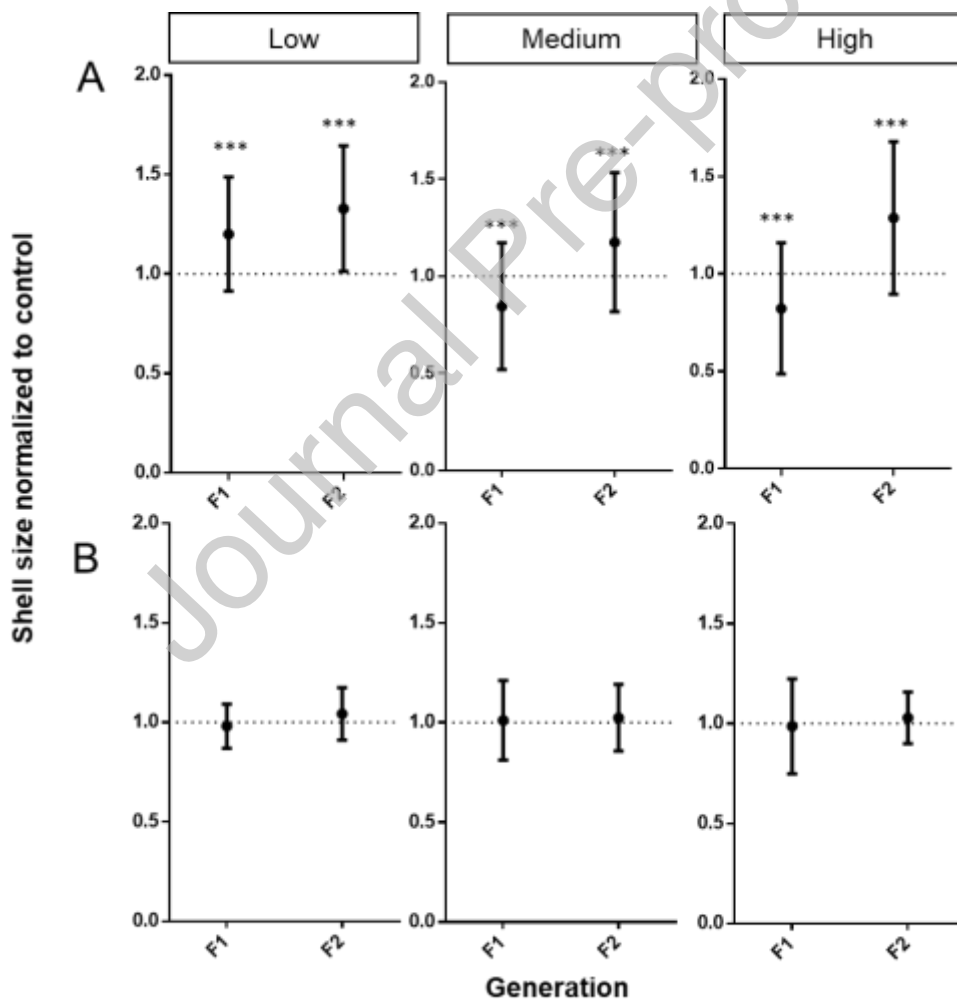


Figure 3- Shell size (mm) of A) juveniles (8 wph) and B) adults snails (16 wph) exposed to DCF normalized to the control mean shell size for F1 and F2 generations. Bars represent standard deviation. The dotted lines at 1 represent control level. *** $p < 0.001$ compared to control; Kruskal-Wallis test.

3.2.3. 28-day reproduction test

Reproductive endpoints for both F1 and F2 generations were impacted by DCF exposure. In the F1 generation, DCF caused a significant decrease in the number of eggs/egg mass in the medium group (Kruskal-Wallis test, $p < 0.05$), with a decreasing trend also noted in the high group (Kruskal-Wallis test, $p < 0.1$) (Fig. 4A). The number of egg masses/snail was impacted by DCF exposure, with a significant increase in the high group (Kruskal-Wallis test, $p < 0.01$) for the F2 generation (Fig. 4B). Although there were no significant differences in shell size at the beginning of the test (Kruskal-Wallis test, $p > 0.05$), at the end of the 28-day reproduction test, shell sizes in the F1 low treatment and in the F2 low and high treatment were significantly smaller than control (Kruskal-Wallis test, $p < 0.05$, data not shown).

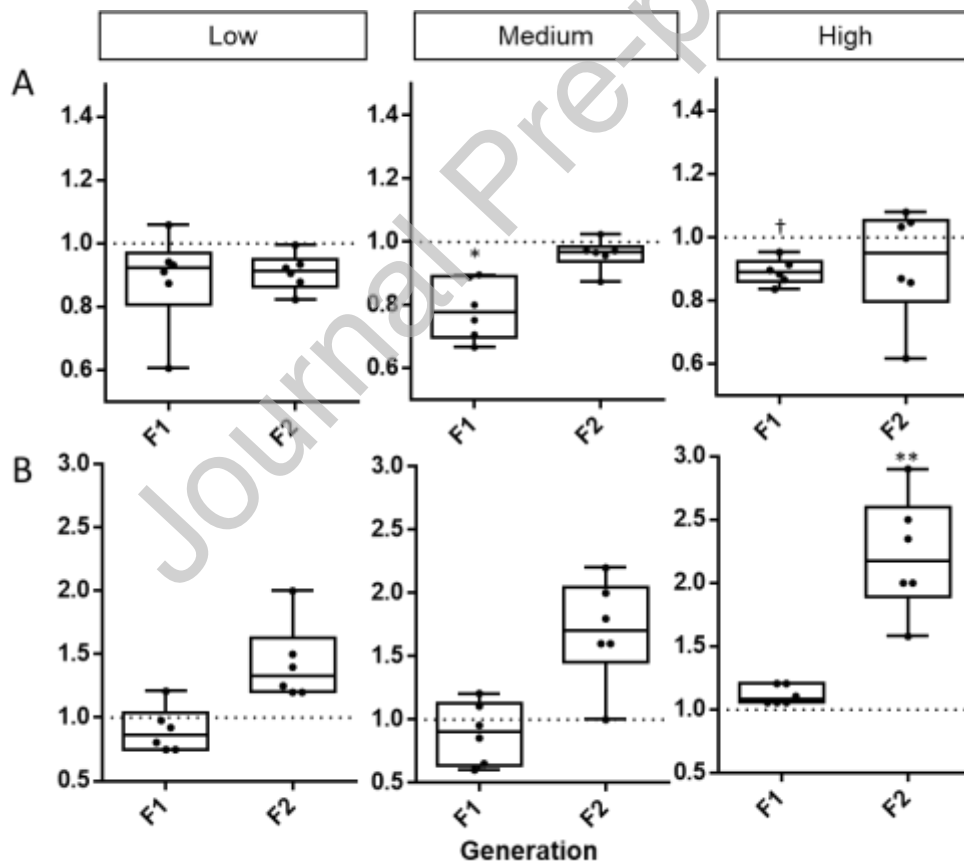


Figure 4 – Reproductive parameters in the low, medium and high DCF exposure groups, F1 and F2 generations. A) Number of eggs/egg mass normalized to the control mean eggs/egg mass and B) number of egg masses/snail exposed to DCF normalized to the control mean egg masses/snail. The dotted lines at 1 represent the control level. ** $p < 0.01$; * $p < 0.05$; † < 0.1 compared to control; Kruskal-Wallis test.

3.3. Behavioral endpoints

Feeding behavior was impacted by DCF exposure in opposite ways depending on the generation monitored. In the F1 generation, there was a significant decrease in lettuce area consumption in F1 snails in the low (Mann-Whitney test, $p < 0.01$) and medium (Kruskal-Wallis test, $p < 0.05$) groups compared to the control (Fig. 5A). In the F2 generation, there was a significant increase ($p < 0.05$) in lettuce area consumption in the high group. Regarding the locomotion behaviour, DCF affected the distance moved in 2 h in the medium (Kruskal-Wallis test, $p < 0.05$) and high (Kruskal-Wallis test, $p < 0.01$) groups in the F1 generation, with a greater distance moved compared to the control (Fig. 5B). DCF exposure did not alter the response to light stress in any of the monitored generations (Fig. S-1).

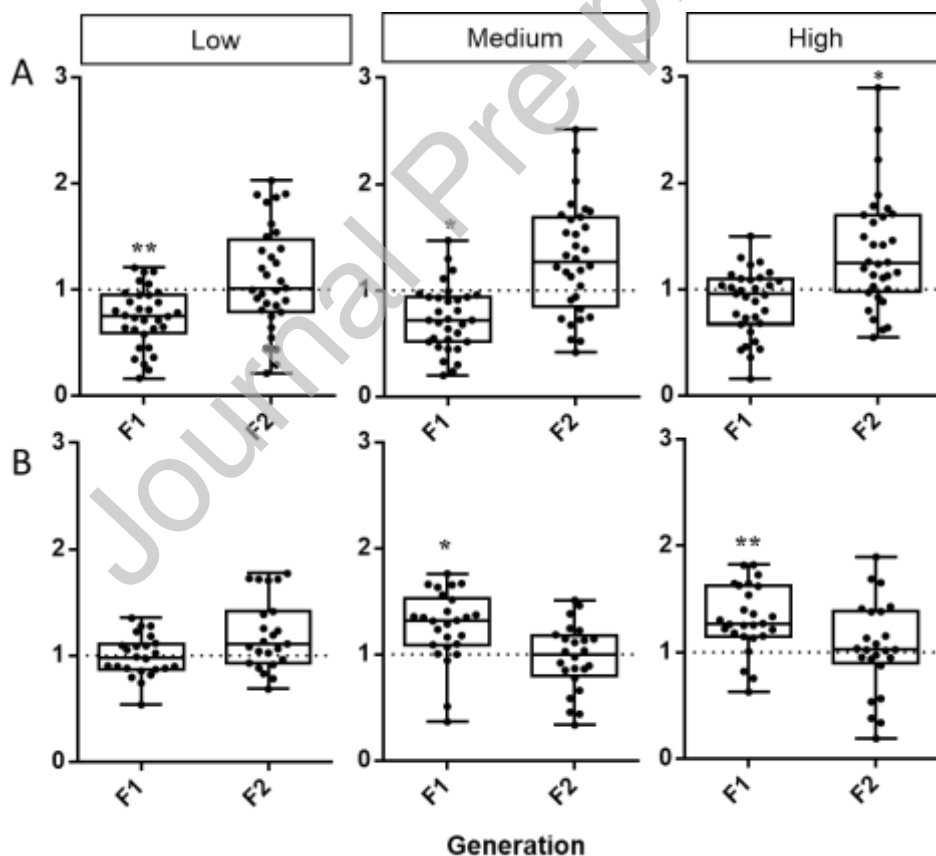


Figure 5 – Feeding and locomotion endpoints in the low, medium and high DCF exposure groups for F1 and F2 generations. A) Lettuce area consumption in 6 h normalized to the snail shell sizes and to the control mean lettuce area consumption. B) Distance moved in 2 h normalized to the snail shell sizes and to the control mean level. The dotted lines at 1 represent the control level. ** $p < 0.01$; * $p < 0.05$ compared to control; Kruskal-Wallis test.

4. Discussion

Standard ecotoxicity tests usually focus on acute or short-term exposure to contaminants, and few full life cycle or multigenerational studies have been reported (Fent et al., 2006; Philippe et al., 2017). Pharmaceuticals are released continuously in the aquatic environment and represent chronic toxicity drivers for living organisms (Fent et al., 2006; Gworek et al., 2019). DCF has been described as a “pseudo-persistent” compound that is more likely to impact freshwater species with short or medium life cycles for more than one generation (Acuña et al., 2015). In order to contribute to the assessment of DCF impacts on the aquatic environment, we conducted a multigenerational study on a freshwater gastropod at environmental exposure concentrations in close to realistic conditions. Regarding the exposed parental generation, the only DCF impact on life-history traits was faster juvenile snail growth (Bouly et al., 2022). A higher DCF incidence was noted in subsequent generations (i.e. impact on time-to-hatch, reproductive parameters, feeding and locomotion), indicating an increase in snail sensitivity to DCF.

The primary impact of DCF exposure on life-history traits was growth impairment. The highest shell length in juveniles of the F1 generation exposed to $0.1 \mu\text{g.L}^{-1}$, and those of the F2 generation exposed to 0.1, 2 and $10 \mu\text{g.L}^{-1}$ suggest faster growth, before reaching a steady state at the adult stage. This showed a lower growth rate in exposed snails at the adult stage compared to control. Therefore, growth was slower in the F1 generation exposed to 2 and $10 \mu\text{g.L}^{-1}$ DCF. An impact on shell growth was also noted in the DCF-exposed parental generation, with a higher shell size in exposed juvenile snails compared to control (Bouly et al., 2022). This faster growth suggested an hormetic response, characterized by the stimulation of functions at low toxicant concentration with an inhibitory response at high concentration (Calabrese, 2008). Hormesis is due to an adaptive response of an organism to low concentrations of environmental stressors (Nassour et al., 2021). Hormetic effects on growth have already been reported in response to xenobiotic exposure in the *Physella acuta* gastropod, with growth enhanced after exposure to low concentrations (0.5 to 1.0

$\mu\text{g.L}^{-1}$) of the antibacterial agent triclosan, and retarded growth at $5 \mu\text{g.L}^{-1}$ concentration and above (Brown et al., 2012). Regarding multigenerational NSAIDs exposure, two studies performed on daphnids have shown a stimulatory effect of NSAIDs on growth. Larger sizes were demonstrated in F1 and F5 generations exposed to $0.5 \mu\text{g.L}^{-1}$ DCF (Dietrich et al., 2010) and higher growth rates in the F5 generation continually exposed to the NSAID ibuprofen at $4 \mu\text{g.L}^{-1}$ concentration (Grzesiuk et al., 2020).

In the study of Grzesiuk et al. (2020), those increased growth rates were combined with reproductive impairment, possibly due to reinvestment of resources from reproduction to growth. This was not highlighted in the present study where, conversely, growth seemed to be positively correlated with reproductive function. In the F1 generation, the number of eggs/egg mass was decreased in $2 \mu\text{g.L}^{-1}$ DCF exposed snails compared to the control, where growth of the juveniles was significantly slower at the same exposure concentration. The size of the eggs was not recorded in our study but could also have been an interesting endpoint to record in order to better understand the strategy of the gastropod to handle stress. However, the number of egg masses laid per snail was not impacted in this condition. A reduced number of eggs/egg mass was already observed in freshwater gastropod in response to the exposure to a contaminant, as in the *Radix balthica* freshwater gastropod exposed to a low concentration ($0.8 \mu\text{g.L}^{-1}$) of the oxazepam pharmaceutical (Lebreton et al., 2021a) and in *L. stagnalis* exposed to cadmium (25 to $400 \mu\text{g.L}^{-1}$) (Reátegui-Zirena et al., 2017). This impact could be an adaptive response to stress in *L. stagnalis*, aiming to preserve energy for other functions.

DCF exposure decreased the snail feeding behaviour in the F1 generation exposed to 0.1 and $2 \mu\text{g.L}^{-1}$. A negative impact of DCF on feeding behaviour was also shown in previous study performed at high level of exposure. Feeding activity was impaired in *D. magna* exposed for 5 h to DCF at $100 \mu\text{g.L}^{-1}$ concentration (Nkoom et al., 2019) and time to eat had increased in medaka fish after 5 to 9 days exposure to 1.0mg.L^{-1} DCF (Nassef et al., 2010). Impairment of feeding activity following long-term DCF exposure may impact other key organism functions and could lead to population declines in the

natural environment. In the parental generation, modulation in glutamate levels and gene expression of nitric oxide synthase has been reported (Bouly et al., 2022), both of which are involved in the control of *L. stagnalis* feeding behavior (Brierley et al., 1997; Kobayashi et al., 2000). If this disruption at the molecular level also occurs in subsequent generations, it could lead to altered behavior. The observations performed on the F1 generation (slower growth, production of less eggs/egg mass, lower food consumption) may be the indication of an energy conservation strategy in *L. stagnalis* after chronic exposure to DCF (Sokolova et al., 2012). These mechanisms aimed at reducing the energetic expenditure in the expectation of finding more favorable environmental conditions.

On the contrary, snail locomotion behavior showed an activity increase in the F1 generation after exposure to 2 and 10 $\mu\text{g.L}^{-1}$ DCF. A biphasic impact of NSAIDs exposure on locomotion and ventilation was highlighted in *Gammarus pulex* crustaceans exposed to ibuprofen (De Lange et al., 2009, 2006). Increased ventilation linked with decreased locomotion was observed at 1 to 100 ng.L^{-1} pharmaceutical concentrations. On the contrary, ventilation was lower and locomotion was higher in control and in gammarids exposed to 1 $\mu\text{g.L}^{-1}$ to 1 mg.L^{-1} ibuprofen concentrations (De Lange et al., 2009). The DCF impact observed on locomotion in our study is in line with the study of De Lange et al. (2009), as an increase locomotion was observed at concentrations in the range at 2 and 10 $\mu\text{g.L}^{-1}$ DCF. The need for increased ventilation in freshwater gastropods has different consequences than in gammarids because they are able to breathe air at the surface. Ventilation of *L. stagnalis* after DCF exposure was not assessed here, but in *R. balthica*, at $> 0.9 \mu\text{g.L}^{-1}$ DCF these freshwater gastropods switched from aquatic respiration to air breathing in order to regulate their metabolic needs (Morozov et al., 2021). In *L. stagnalis* snails, coordination between respiration and locomotion has been reported, which could be due to stimulation of similar neuronal mechanisms (Vorontsov et al., 2003). DCF exposure may increase energy requirements leading to increased locomotion and respiration in *L. stagnalis*.

The impact of DCF on the F2 generation showed a different pattern compared to the F1 generation, with mostly stimulation. Indeed, there was an increase in the number of egg

masses/snail in the F2 generation exposed to 2 and 10 $\mu\text{g.L}^{-1}$ DCF compared to the control. Similarly, in daphnids exposed to 0.5 $\mu\text{g.L}^{-1}$ DCF, more neonates were noted in the F1 exposed generation than in control (Dietrich et al., 2010). An increase in the number of egg masses laid per snail under stress conditions seems to be regularly observed in freshwater gastropods (Reátegui-Zirena et al., 2017; Sánchez-Argüello et al., 2009; Suski et al., 2012). This situation may reflect fecundity compensation, where animals offset impending survival effects by reaching reproductive maturity sooner and increasing offspring production (Suski et al., 2012; Żbikowska et al., 2006). When focusing on the high treatment of the F2 generation, there was a shorter time-to-hatch, faster growth, increased food intake and production of more egg masses/snail. These observations supported the fecundity compensation hypothesis. Indeed, body size (and therefore shell size) is related to fecundity in *L. stagnalis*, and faster growth could lead to earlier and increased reproduction (Montagne-Wajer et al., 2007). Shorter time-to-hatch and increased feeding are processes required to rapidly reach reproductive body size. For the reproductive assessment, snails were randomly selected under a defined shell size range at the beginning of the test. But interestingly, mean exposed snail shell sizes of 0.1 and 10 $\mu\text{g.L}^{-1}$ were lower than that of control at the end the 28-day test, indicating that the growth rate of the treated snails was slower than control during this period, which correspond to the adult stage. In *L. stagnalis*, a trade-off between growth, female (egg production) and male (sperm and seminal fluid production) functions has been demonstrated (Koene and Maat, 2006). Exposure to stress in great pond snails for several generations could result in them reaching reproductive size and sexual maturity earlier and then reallocating resources for reproduction from the growth and maintenance budget. This strategy is known in snails in response to parasite infection and could be followed by early host death (Żbikowska et al., 2006).

These observed effect on the snail physiology might have been the consequence of modifications occurred at the molecular level. The impact of DCF on the F0 generation was assessed at the molecular level using metabolomics and transcriptomic approaches, revealing 293 differentially expressed genes and 56 modulated metabolites in exposed snails compared to control (Bouly et al.,

2022). In the parental generation, the demonstrated molecular impacts of DCF on reproductive function did not have an impact on snail egg laying, which was found to be disturbed in the F1 and F2 generations. The ovulation prohormone was found to be under-expressed in *L. stagnalis* heads after full life cycle exposure to $10 \mu\text{g.L}^{-1}$ DCF (Bouly et al., 2022). This compound was then cleaved in various substances, including cerebral neurosecretory caudodorsal peptides (CDCP) and the ovulation hormone (CDCH). Those neuropeptides are well-known to have a function in the regulation of *L. stagnalis* female sexual behavior (Hermann et al., 1997). This modification could be an early biomarker of disturbed reproductive parameters in the offspring generations. In addition, the gene coding for the FMRFamide receptor was over-expressed in heads of 0.1 and $10 \mu\text{g/L}$ exposed snails (Bouly et al., 2022). In *L. stagnalis*, FMRFamide is involved in male reproductive behavior (De Boer et al., 1996) and in motoneuron activity (Kyriakides and McCrohan, 1989). In the parental generation, down-modulation of serotonin levels was noted in the reproductive apparatus of snails exposed to $0.1 \mu\text{g.L}^{-1}$ DCF along with up-modulation of its precursor L-tryptophan in the heads of snails exposed to 0.1 and $10 \mu\text{g.L}^{-1}$ DCF, showing serotonin synthesis disruption (Bouly et al., 2022). Serotonin is involved in the transmission of non-genetic instructive signal from mother to progeny in *L. stagnalis* and can modulate juvenile growth, feeding and locomotion behaviors in great pond snail offspring (Ivashkin et al., 2015). The modulations of serotonin metabolism of the F0 generation may then have an influence of the development and behavior of subsequent generations, on which a stronger impact of DCF exposure was observed. Those overall observations suggest that molecular tools could reveal premature xenobiotic effects that are only expressed at the physiological level after multigenerational exposure. These findings should require further studies to explore the possible link between molecular disturbance of the parental generation and the differences in the offspring life history traits and behaviors.

The multigenerational response to toxic compounds generally leads to resistance mechanisms or enhanced toxicity (Dietrich et al., 2010; Salice et al., 2009; Schür et al., 2020; Silva et al., 2017). Like vertebrates, the response to toxicant exposure in freshwater invertebrates could differ between

species and depending on the specific mode of action of the substances (Alonso et al., 2009; Hickey, 1989; van Wezel et al., 1995). In the present study, the impact of DCF on snails oscillated between stimulation and inhibition of processes, with greater effects observed in subsequent generations. Indeed, in the parental DCF-exposed generation, juvenile growth was faster but other life-history traits (hatching, reproduction) and behavioral responses (feeding, locomotion and response to light stress) in adult snails remained unchanged (Bouly et al., 2022). The greater impact of NSAIDs on offspring was also highlighted in *D. magna* continually exposed to ibuprofen at low concentrations ($4 \mu\text{g}\cdot\text{L}^{-1}$), with greater embryo deformation noted in the F5 generation (Grzesiuk et al., 2020). In the present study, disrupted processes (growth, egg-laying, feeding, locomotion) may be the expression of toxic impact of DCF on the organisms. Trade-offs could be altered to induce resistance, resulting in stimulation or inhibition of specific functions. Little is known with regard to *L. stagnalis*, but such adaptive responses over successive generations have been demonstrated in *Biomphalaria glabrata* gastropods, with a genetic adaptation in offspring to increase cadmium tolerance in snails whose progenitors were exposed (Salice et al., 2010). Irregularity in organism responses between generations has also been shown in daphnids exposed to environmental propranolol concentrations (Jeon et al., 2013). In our study the results suggest that the response strategy to multigenerational exposure to DCF in the great pond snail varied across generation. The F1 generation appeared to have adopted an energy conservation strategy, with a decrease in basal activities. On the contrary, the F2 generation likely switched to a fecundity compensation strategy to promote species survival. This shift could be a consequence of maintaining the stressor in the environment. It would have been interesting to continue the experiment on subsequent generations to observe if this stress response is sustained over time.

5. Conclusion

DCF exposure was demonstrated to have a greater impact on subsequent generations (F1 and F2) compared to parental generation, with oscillation between process stimulation and inhibition. This could indicate hormesis with disruption of functional trade-offs over time. Growth was affected

in the F1 generation, with impacts contrasting according to the exposure concentration. In addition, food intake was lowered, locomotion activity was enhanced and snail egg masses contained fewer eggs, which could be the consequence of energy conservation mechanisms. In the F2 generation, a stimulation was demonstrated in snails exposed to $10 \mu\text{g.L}^{-1}$ DCF, with a shorter time to hatch, faster growth, increased food intake and production of more egg masses/snail. This could be a reaction to contaminant-induced stress geared towards maximizing the reproductive function. DCF exposure did not impact the light stress response of snails over both generations. Those results highlighted a potential impact of DCF on freshwater gastropod populations after long-term exposure, as the disrupted functions of the organism (growth, egg-laying, feeding, locomotion) are crucial for survival. The increased sensitivity of *L. stagnalis* offspring to the contaminant highlights the need for studies conducted over several generations.

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Lucie Bouly: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft, Writing – Review & editing. **Caroline Vignet:** Methodology, Supervision, Writing - review & editing. **Jean-Luc Carayon:** Investigation, Resources. **Jean-Michel Malgouyres:** Resources. **Hélène Fenet:** Conceptualization, Supervision, Writing - review & editing, Funding acquisition. **Florence Gélet:** Conceptualization, Supervision, Writing - review & editing, Funding acquisition.

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