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RESEARCH ARTICLE

Are 20-hydroxyecdysone and related genes potential biomarkers of sublethal exposure to lipid-altering contaminants?

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Abstract

In *Daphnia magna*, 20-hydroecdysone (20E) is the main molting hormone and its metabolism is of interest to identify new biomarkers of exposure to contaminants. The present study aimed to (i) assess baseline levels of 20E and transcription levels of four related-genes (shade, neverland, ultraspiracle, and ecdysteroid receptor); and (ii) evaluate effects in *D. magna* after 21 days of exposure to fenarimol (anti-ecdysteroid) and a mixture of gemfibrozil and clofibric acid (lipid-lowering drugs) at sublethal concentrations. Endpoints included transcription of the target genes and quantification of 20E, mortality, and reproduction of daphnids. Baseline results showed that average responses were relatively similar and did not vary more than 2-fold. However, intra-day variation was generally high and could be explained by sampling individuals with slightly different stages of their development. Exposure tests indicated a significant decrease in daphnid reproduction following chronic exposure to a concentration of 565 µg/L of fenarimol. However, no difference was observed between the control and exposed groups for any of the investigated genes, nor for the levels of 20E after 21 days of exposure. Following exposition to gemfibrozil and clofibric acid at 1 µg/L, no changes were observed for the measured parameters. These results suggest that changes in transcription levels of the target genes and concentrations of 20E may not be sensitive endpoints that can be used as biomarkers of sublethal exposure to the target compounds in *D. magna*. Measuring multiple time points instead of a single measure as well as additional molecular endpoints obtained from transcriptomic and metabolomic studies could afford more insights on the changes occurring in exposed daphnids to lipid-altering compounds and identify efficient biomarkers of sublethal exposure.

Keywords Ecdysteroids · Gene transcription · Sublethal effects · Crustaceans · Ecotoxicology

Introduction

Currently, more than 350,000 chemicals and mixtures have been registered for production and use around the world (Wang et al. 2020). The high production volumes and mobility of compounds lead to their detection in surface waters worldwide at trace concentrations (ng/L to µg/L). Exposure and accumulation in aquatic species can also adversely impact ecosystems (Bradley et al. 2017; Hughes et al. 2013). Some of these compounds, such as pesticides, have been created to specifically affect the endocrine system of arthropods and thus are toxic to nontarget aquatic organisms such as crustaceans (Jansen et al. 2011; Mnif et al. 2011). Pharmaceuticals are also susceptible to causing subtle changes in nontarget species such as feminization and impacting the behavior of different aquatic species (Richmond et al. 2017).

Classic toxicity tests using endpoints such as survival, growth, or reproduction are usually not sensitive enough to detect the effects of these compounds at environmental concentrations (Daughton and Ternes 1999). However, adverse effects such as changes in behavior, metabolic profile, or gene transcription have been observed at sublethal levels (De Lange et al. 2006; Houde et al. 2013; Kovacevic et al. 2016; Wagner et al. 2017). While in-silico techniques based on

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molecular modeling and docking simulations (Hirano et al. 2020; Li et al. 2023) can be useful to predict interactions of contaminants with key enzymes, in-vivo studies are necessary to identify new biomarkers of sublethal effects in order to assess biological changes in aquatic organisms chronically exposed to low concentrations of contaminants. An interesting model to look for those biomarkers is *Daphnia magna.*

Daphnia is a genus of freshwater crustaceans widely used in ecotoxicology as model species to test the toxicity of chemicals an even wastewaters (Tonkes et al. 1999) because of its easy culture in the laboratory, small size, as well as its parthenogenetic (clonal) reproduction (Dodson and Hanazato 1995). Daphnids occupy a key role in lentic ecosystems as filter feeders and prey of insects and small fishes (Miner et al. 2012). Reproduction and development in daphnids are regulated by ecdysteroids, a group of hormones derived from cholesterol. 20-Hydroxyecdysone (20E) is the main molting hormone in crustaceans and other arthropods and is also involved in the reproduction process and embryonal development of daphnids (LeBlanc 2007). Levels of 20E increase and decrease between successive molts in a pulsative manner, inducing ecdysis through the activation of the ecdysone nuclear receptor (EcR) (Martin-Creuzburg et al. 2007; Song et al. 2017). 20E metabolism is regulated by a group of genes named the Halloween family. These genes encode for cytochrome P450 (CYP450) enzymes that regulate the biosynthesis of ecdysteroids from cholesterol (Fig. 1) (Rewitz and Gilbert 2008).

Given their importance for the survival and reproduction of *D. magna*, ecdysteroids are potential targets for biomarker studies. Only a few publications have investigated changes in concentrations of ecdysteroids in *D. magna* after exposure to contaminants. Bodar et al. (1990) reported an increase in ecdysteroid titers of 257% in adult females following exposure to 20 µg/L of cadmium for 8 days and used enzyme immunoassays to quantify ecdysteroids as ecdysone equivalents. They speculated that the observed effect of cadmium on ecdysteroids was the product of cadmium interference with metallo-enzymes involved in the molting process. Mu and LeBlanc (2002) exposed neonates to 497 µg/L of fenarimol, a fungicide principally used on ornamental plants and vegetables, and observed a diminution of around 26% in ecdysteroid levels in exposed neonates and an induced delay for the first and second molting in a dose-dependent manner. These effects were somewhat reverted with the co-administration of 20E, which demonstrated the anti-ecdysteroid activity of fenarimol.

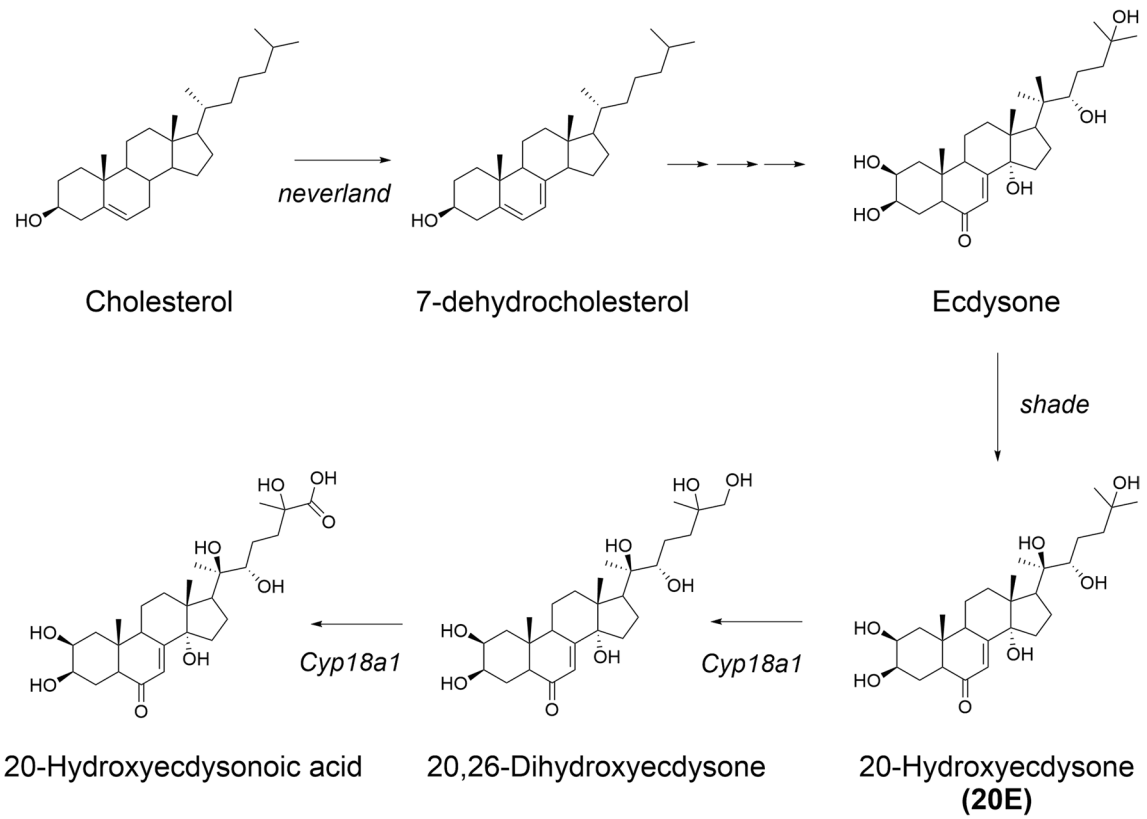

Fig. 1 Biosynthesis of 20E from cholesterol in *D. magna*. Associated genes are presented in italics. Multiple steps have been omitted between 7-dehydrocholesterol and ecdysone synthesis to simplify the figure. Image adapted from LaFont et al. (2012) and Song et al. (2017)

Baseline assessment of both key metabolites and transcription of genes taking part in the synthesis of those metabolites and can help to differentiate natural fluctuations from responses induced by exposure to exogenous compounds. Therefore, in order to understand the effects of contaminants on the metabolism of 20E, it is important to determine first baseline levels of 20E as well as of genes associated with its synthesis and activity.

At the gene level, baseline information of Halloween genes transcription was evaluated during normal growth in daphnids by Sumiya et al. (2014). The transcription level of the evaluated genes fluctuated between 2- and 3-fold during an 80-h period, equivalent to the duration of molting in adult daphnids. At the metabolite level, two studies have reported ecdysteroid baseline levels in adult *D. magna* during a single molt cycle (Martin-Creuzburg et al. 2007; Sumiya et al. 2016). In those studies, the authors used immunoassay-based techniques to quantify ecdysteroids, and they both observed an increase in basal levels of ecdysteroids between ≈ 30 to 50 h after ecdysis followed by return to basal levels ≈ 40 h later. However, reported values by these studies diverge. Martin-Creuzburg et al. (2007) used a radioimmunoassay technique and observed that free ecdysteroids increased from ≈ 5 to 10 pg per individual at the end of the first molt to a maximum of ≈ 2 to 50 pg/individual 38–42 h later. Then ecdysteroids declined back to basal levels before the second molt. Sumiya et al. (2016) used enzyme-linked immunosorbent assay to measure ecdysteroids but maximum levels were only about ≈ 2.4 fg/ind. These differences may be due to the distinct techniques and sampling protocols employed. To the author's knowledge, the only study that has quantified 20E in *D. magna* is the work of Venne et al. (2016). The authors of that paper used liquid chromatography-triple quadrupole mass spectrometry (LC-QqQMS) to quantify 20E in adult daphnids (19 ± 8 pg/ind). However, only one measure was performed. Therefore, up to now, the baseline of 20E in multiple molt cycles of *D. magna* is unknown.

The working hypothesis of the present work was that changes in levels of 20E and/or changes in the transcription of 20E-related genes (shade, neverland, ultraspiracle, and ecdysteroid receptor) would be observed in *D. magna* after exposure to low concentrations of lipid-altering organic contaminants. Fenarimol, a fungicide demethylation inhibitor and known ecdysteroid inhibitor, as well as a mixture of gemfibrozil and clofibric acid, two lipid-lowering molecules commonly found in environmental waters were chosen as target compounds. Thus, the present study aimed to (i) assess the baseline of 20E and transcription of the genes associated with 20E regulation in *D. magna* over a 21-day period; and (ii) evaluate the effects following a 21-day exposure to the target compounds on the concentration of 20E, expression of target genes, and life history parameters (i.e., fertility and mortality).

Materials and methods

Reagents and chemicals

Standards of 20E (catalog number: SC-202407A, > 98% purity) and makisterone A (SC-202218A, > 95% purity) were obtained from Santa Cruz Biotech (Dallas, TX). Makisterone A is non-endogenous ecdysteroid with 28 carbon atoms that differs from all 27 carbon molting hormones like ecdysone and 20E by having a methyl group at the C-24 position. This compound responds similarly to 20E during extraction and LC-QqQMS analysis; it was therefore used as an internal standard for 20E quantification. Additional purification of makisterone A to remove 20E and ecdysone impurities was done following the method described by Venne et al. (2016). Water, methanol (MeOH), acetonitrile (ACN), methyl *tert*-butyl ether (MTBE), formic acid (FA), and acetic acid (AA) were LC or LC-MS grade and were purchased from Thermo Fisher Scientific (Waltham, MA). The derivatization reagent hydroxylamine hydrochloride (159417-100G, > 99%), fenarimol (45484-250MG, ≥ 99%), its internal standard nuarimol (31116, ≥ 99%), clofibric acid (90323-100MG, ≥ 99%), and gemfibrozil (91823-100MG, ≥ 98.5%) were purchased from Sigma-Aldrich (St-Louis, MO). Deuterated standards, clofibric acid-d3 (D-6005, 98%), and gemfibrozil-d6 (D-6144, 99%) were purchased from CDN Isotopes (Pointe-Claire, QC, Canada). Stock solutions of 20E were prepared at 0.1 mg/mL in MeOH and working solutions, prepared in 1% FA in MeOH and stored at – 20 °C. The aqueous solution of hydroxylamine hydrochloride solution (100 mg/mL) was prepared before each derivatization. The main properties of the target compounds are found in Table S1 (Supplementary Information).

Daphnia magna culture

D. magna parent stock originated from ephippia acquired from EBPI Canada (Burlington, ON) and maintained in the laboratory in synthetic Moderately Hard Reconstituted Water (Environment Canada 1990). Cultures were kept at 20 ± 1 °C with a 16-h light:8-h dark photoperiod and were renewed every 2 months using neonates from 3rd to 5th broods. Daphnids were fed every second day with 2 mL of green algae *Raphidocellis subcapitata* (≈ 3.85×10^5 cells/mL). Microalgae were cultured in Bold Modified Basal Freshwater medium from Sigma-Aldrich under the same laboratory conditions described above for *D. magna*. Algae were regularly harvested while still in the exponential growth phase and inoculated in fresh medium. All experiments were initiated with neonates (>

24 h old), born between the 3rd and 5th broods, derived from a healthy parent stock.

Baseline levels of 20E and transcription of target genes

The first experiment aimed to evaluate the gene transcription of target genes and 20E levels over a 21-day period, the duration of standardized chronic toxicity tests for *D. magna*. During this experiment, daphnids were maintained under the conditions described above. Every second day, starting from day 9 (organisms at maturity, size needed for LC-QqQMS analysis) until day 21, three replicates of 15 daphnids were sampled for 20E quantification and stored in MeOH at − 80 °C until analysis. Five replicates of one daphnid each were also collected for gene transcription analysis and stored in trizol at − 80 °C until analysis.

Chronic exposure to fenarimol, gemfibrozil, and clofibric acid

Three different exposure tests were performed to evaluate if changes in levels of 20E would be observed in *D. magna* after exposure to low concentrations of lipid-altering organic contaminants. For test N° 1, gemfibrozil and clofibric acid were used at a concentration of 1 µg/L each which is of the same order of magnitude as the maximum reported environmental concentrations in surface waters reported so far for these two compounds (Ebele et al. 2017). For tests N° 2 and N° 3, two concentrations of fenarimol (113 µg/L and 565 µg/L) corresponding to the no observed adverse effect concentration (NOAEC) and the lowest observed effect concentration (LOEC) for reduced fertility in *D. magna*, respectively, were employed. Thus, the employed exposure concentrations were selected to reflect environmental levels (test N° 1), as well as reported sublethal concentrations (test N° 2 and N° 3) that are representative of worst-case exposure scenarios such as contaminated discharges due to runoff events near agricultural fields (Lefrancq et al. 2017).

In all assays, neonates (< 24 h) were exposed for 21 days to the contaminant following the OECD guidelines (OECD 2008). Tests were performed thrice using 10 replicate groups (5 control, 5 exposed) of 25 daphnids each. Temperature was kept at 20 ± 1 °C using an incubator, light intensity was 2000 ± 70 lx and a 16-h light:8-h dark photoperiod was maintained. On day 1 of the tests, neonates from the control groups were transferred in 2 L beakers filled with culture medium to which 40 µL of MeOH was added. Neonates from the exposed groups were transferred to 2 L beakers filled with culture medium containing 40 µL of MeOH containing the test compound. Medium was renewed 3 times a week. When performing these renewals, daphnids were sorted by size using a series of sieves, according to a standard protocol used by the Ministry of the Environment of Quebec, Canada (Centre d'expertise analytique environnementale du Québec 2011). Adults were collected on a 900 µm sieve, juveniles on a 560 µm sieve, and neonates on a 300 µm sieve. Offspring (juveniles and neonates) were counted and then eliminated; only adults were transferred to the renewed solutions. At each media renewal, survival was determined by counting and averaging number of non-immobilized adults and reproduction was determined by counting and averaging the number of offspring per adult. Dormant eggs or males were never observed throughout the experiments, which indicated that experimental conditions were adequate to support a healthy population of *D. magna*. Daphnids were fed with 2 mL of a *Raphidocelis subcapitata* algae solution at every media renewal and the number of offspring and mortality was recorded. A maximum of 18.9% of mortality was observed in the exposure experiment, thus respecting the sublethality criteria guidelines of the OECD (2008). Detailed mortality curves can be found in Figs. S1, S2 and S3 (Supplementary Information). At the end of the exposure period, *D. magna* adults were collected in MeOH 1% FA in MeOH or trizol for 20E levels and gene transcription analysis, respectively, and stored at – 80 °C until analysis.

Quantification of 20E using liquid chromatography-triple quadrupole mass spectrometry

20E was extracted and quantified from whole daphnids (15–25 individuals) following the method developed by Venne et al. (2016) with minor modifications. Briefly, *D. magna* were sorted by size and adult individuals (> 900 µm) were collected on a tissue strainer before being washed with deionized water (18 MΩ). Adult daphnids were homogenized with a mortar and a pestle and sonicated in an ultrasonic bath for 15 min in a volume of 1 mL of 1% FA in MeOH inside a 1.5 mL Eppendorf tube. Next, an aliquot of 800 µL was transferred in a glass tube with 50 µL of internal standard solution (makisterone A) and the solvent was evaporated under a gentle flow of $N_{2(g)}$. 20E and makisterone A were then derivatized to their oxime analogues with 1 mL of a hydroxylamine hydrochloride solution (100 mg/mL). A liquid-liquid extraction with 2 × 1.5 mL of MTBE was carried out with a vortex mixer. The test tubes were then placed at – 20 °C until the water froze, and the organic layer (unfrozen) containing the derivatized analyte and its internal standard was transferred to a test tube and evaporated to dryness. Finally, 250 µL of MeOH were added and the samples were transferred to vials for analysis.

The 20E concentration in daphnid extracts was quantified by LC-QqQMS using an Acquity UPLC system coupled to a Xevo TQ-S micro triple quadrupole mass spectrometer, both from Waters (Billerica, MA). The method was adapted

from Venne et al. (2016). Briefly, the compounds were separated on a reversed-phase column Acquity UPLC Cortecs C18+ from Waters of dimensions 50 × 2.1 mm and 1.6 µm particle size. The mobile phase was composed of eluent A (H_2O containing 0.1% *v/v* of AA) and eluent B (mixture of MeOH-ACN 3:2 *v/v*, containing 0.1% *v/v* of AA). The elution gradient started with 5% of B, increasing to 55% in 7.9 min, rising immediately to 100% of B and hold for 2 min, then back to initial conditions for column re-equilibration (2.1 min). The sample injection volume was set to 10 µL. Electrospray in the positive mode was used as ionization source and the mass spectrometer was operated in the multiple reaction monitoring (MRM) mode. MRM transitions used were *m/z* 478.3 → *m/z* 316.3 for 20E oxime and *m/z* 492.2 → *m/z* 316.1 for makisterone A oxime. The concentration of 20E was reported as the average mass per adult individual. The total number of *D. magna* used and the number of pooled groups varied among the tests due to differences in the mortality of daphnids at the end of the 21-day period.

Quality control (QC) samples were used in order to determine the deviation percentage of the method and thereby verify the accuracy and precision of the measured concentrations. Three concentrations of QC samples were used: low (≈ 100 pg/mL), medium (≈ 300 pg/mL), and high (≈ 500 pg/mL). Matrix effects correction was done by using extracts of frozen daphnids at 50 mg/mL in 1% FA in MeOH. These extracts were used to prepare QC samples. Results were considered acceptable if the QC samples were within ± 20% of the expected value.

Chemical stability

The chemical stability of fenarimol, gemfibrozil, and clofibric acid was evaluated during the tests between two medium renewals. Nuarimol, gemfibrozil-d_6, and clofibric acid-d_4 were used as internal standards. Aliquots of 50 mL were sampled immediately after and before media renewal at three different moments during the testing. Extraction was performed using Strata-X solid-phase extraction cartridges (polymeric reversed phase with a particle diameter of 33 µm, 200 mg of bed mass, and 6 mL of volume) from Phenomenex (Torrance, CA). Quantification was carried out using the same LC-QqQMS system described previously. The entire protocol is detailed in the Supplementary Information.

RNA extraction

Total RNA was extracted from single *D. magna* using a Trizol-RNeasy Plus Mini Kit hybrid protocol (Ponton et al. 2011). Briefly, daphnids were homogenized in 500 µL of trizol with a micro pestle before being sonicated for 5 min. A volume of 200 µL of chloroform was added and the tubes were centrifuged at 10,000 g for 18 min at 4 °C. The top layer was then transferred on a RNeasy Plus Mini column from Qiagen Canada (Montreal, QC) and the manufacturer's instructions were followed from that point (Quiagen 2020). Extractions were performed on 10 independent biological replicates for exposures to 113 µg/L and 15 replicates for the 565 µg/L treatment with fenarimol.

RNA was quantified with a NanoDrop ND-000 spectrophotometer from Thermo Fisher Scientific (Waltham, MA). All samples had a 260 nm/280 nm ratio > 1.8 and a concentration > 54 ng/µL. Chloroform (HPLC, 99%) and ethanol (98%) were purchased from Sigma-Aldrich Canada (Oakville, ON). Random hexamer 5′-NNN NNN-3′ made for L. Gaudreau (IDT, lot 213632751) was used for reverse transcription in addition to dNTP mix (10 mM) from KAPA Biosystems (Cape Town, South Africa), Moloney Murine Leukemia Virus (M-Mulv reverse transcriptase, 200,000 U/mL, lot 12R091118), and 10×M-MulV RT Buffer (lot 081618) from QIAGEN and sterile water (Molecular grade) from Wisent (St-Bruno, QC). Advanced qPCR Mastermix (lot 800431) from Wisent, 96-well plates (Low profile, Clear) from Axygen (Union City, CA), and sealing tapes (optically clear) from Sarstead (Newton, NC) were used for qPCR reaction.

Real-time quantitative PCR

Real-time quantitative PCR (RT-qPCR) analyses were conducted on four selected transcripts of the target genes shade (*shd*), neverland (*nvd*), ecdysteroid receptor (*ecr*), and ultraspiracle (*usp*) and normalized with a combination of the most suitable reference genes cyclophilin (*cyc*), tubulin α (*tuba*), ubiquitin (*ubi*), elongation factor 2 (*eef2*), and glyceraldehyde 3-phosphate dehydrogenase (*gapdh*). Genes *ubi*, *eef2*, and *gapdh* were used for the baseline assessment experiment. Genes *ubi*, *tuba*, and *eef2* were used for test N° 2 (exposure to 113 µg/L of fenarimol). Genes *ubi*, *tuba*, and *gapdh* were used for test N° 3 (exposure to 565 µg/L of fenarimol). Primer-specific efficiencies and sequences are listed in Table S2 (Supplementary Information) along with sequences.

Total RNA (300 ng) was reverse transcribed using M-Mulv reverse transcriptase according to the manufacturer's instructions (Qiagen 2023). After dilution of the cDNA samples (1/8 dilution), analyses were then carried out on a CFX96 Connect real-time PCR detection system from Bio-Rad (Hercules, CA) using Advanced qPCR Mastermix with a final concentration of 400 nM for each primer in a total reaction volume of 10 µL. The qPCR conditions were as follows: 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s, 60 °C for 15 s, and 68 °C for 15 s. Each reaction was run in technical triplicate and the mean of all independent biological replicates was calculated. All results were normalized using mRNA level of the reference genes recommended by

geNorm depending on the exposition test. Relative expression values were calculated by the qBase relative quantification software (Hellemans et al. 2007). Microcapillary gel electrophoresis (Fig. S4. Supplementary Information) and melt curve analysis were performed on amplicons to verify the specificity of the amplification using a 2100 Bioanalyzer from Agilent (Santa Clara, CA) and CFX96 Connect real-time PCR detection system.

Data analysis

Two-sample *t* tests ($\alpha = 0.05$) using Microsoft Excel 365 were employed to evaluate significant differences between exposed and control samples for survival, number of offspring, and 20E levels observed. *F* test were also performed to compare variances between the two groups. For the baseline analysis of 20E levels and the transcription of target genes, analysis of variance (ANOVA) tests ($\alpha = 0.05$) were performed between time points using OriginPro version 2023. Before ANOVA tests, data normality and homoscedasticity were verified using Kolmogorov-Smirnov's test and Levene's test, respectively. When one of these conditions were not respected, a Kruskal-Wallis ANOVA test was used instead. Tukey's and Dunn's post-hoc tests were used to determine which groups were different for ANOVA and Kruskal-Wallis tests, respectively. Graph Pad Prism version 6 was used to plot the results of all assays.

Results

Baseline of target genes and levels of 20E

Transcription of the target genes (*shd*, *nvd*, *ecr*, and *usp*) as well as 20E levels were evaluated starting from day 9 of the daphnids' life until day 21 to assess temporal variability during normal development. Most of the transcription of the targeted genes was relatively stable with little to no change over the duration of the observations (Fig. 2). A slight but significant increase at day 19 compared to days 9, 11, and 15 was observed for the transcription levels of *ecr* (Tukey's test, $p < 0.05$) and at day 17 compared to day 15 for *usp* (Dunn's test, $p = 0.0084$). Transcripts of *shd* were the most stable over time with no significant difference ($p > 0.05$) at any day and *nvd* was the most active gene in terms of fluctuation. The relative transcription levels of the latter closely followed the concentrations of 20E (Fig. 3). The gene *nvd* is responsible for the 7-dehydrogenase, the enzyme catalyzing the first step of ecdysteroid biosynthesis (Song et al. 2017). The transcription analysis results thus suggest that the retroaction in response to 20E levels controlling ecdysteroids production mainly affects this step (7-dehydrogenation of cholesterol).

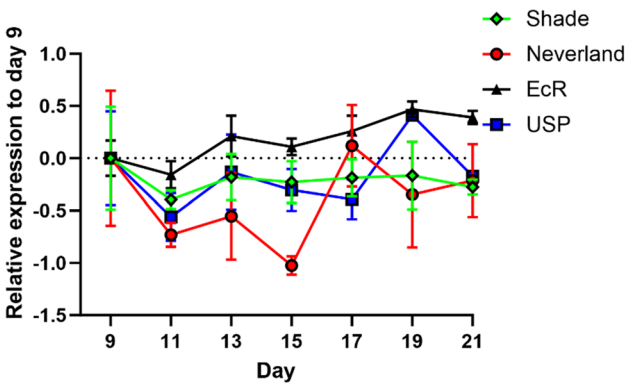

Fig. 2 Relative gene transcription of *shd*, *nvd*, *ecr*, and *usp* in daphnids for a 12-day period ($n = 5$). Error bars represent 95% confidence interval limits. Data was normally distributed according to Kolmogorov-Smirnov's test (for all genes $p > 0.1$) and the variance was homogeneous according to Levene's test only for *ecr* ($p = 0.3952$)

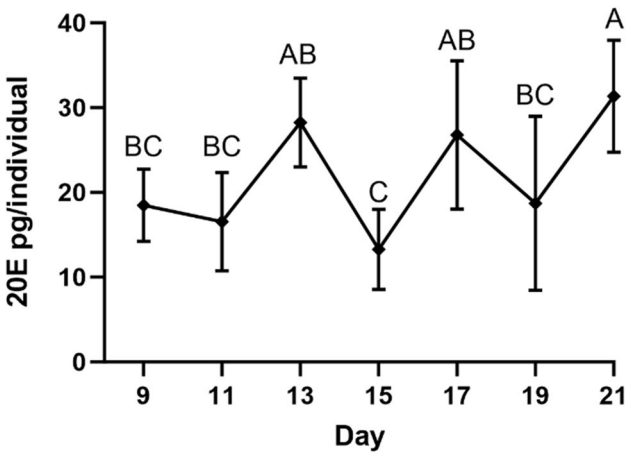

Fig. 3 20E baseline in daphnids over a 12-day period ($n = 3$ per time point). Error bars represent ± 1 standard error. Means that do not share the same letter were significantly different ($p < 0.001$) according to Tukey's post-hoc test. Data was normally distributed according to Kolmogorov-Smirnov's test ($p = 1.0$) and the variance was homogeneous according to Levene's test ($p = 0.1477$)

Figure 3 shows the 20E levels in *D. magna* at different time points. Significantly different ($p < 0.05$) levels of 20E were seen depending on the age of the individuals and the concentration of 20E per daphnid oscillated about every 4 days since day 11 which seems to correspond to the molting cycle duration of *D. magna* in light and temperature conditions employed (Ebert 2005). However, contrary to previous reports (Martin-Creuzburg et al. 2007; Sumiya et al. 2016), a clear ecdysteroid peak (up to ≈ 50 times the basal level) is absent in Fig. 3.

In the present study, the maximum difference between 20E concentrations measured was only 18 pg/ind and the relative standard deviation was between ± 20 and ± 50%. This observation could be explained by the desynchronization of

the molting between sampled daphnids and differences in sampling frequency. Indeed, despite the authors' efforts to control culture experimental conditions (feeding, temperature, photoperiod, culture medium, etc.), molting cycles of daphnids could not be synchronized and it was hypothesized that small differences in their age (a few hours) could result in significant different levels of 20E at the time of sampling. According to Martin-Creuzburg et al. (2007), 20E concentrations could vary by a factor as high as 50× in less than 36 h. At this point, it cannot be ruled out that other uncontrolled experimental parameters affected the rhythm of the daphnids molting cycles which, like other rhythmic behaviors, could be influenced by numerous environmental cues (Häfker and Tessmar-Raible 2020).

Survival

Daphnids reached adulthood, defined in the present study as a size > 900 µm, between days 7 and 10. Survival was over 80% for all groups in all tests, thus respecting the sublethality criteria of the OECD. According to Figs. S1 to S3 (Supplementary Information), no significant difference ($p > 0.05$) in the survival was observed between exposed and control groups in test N° 1 (gemfibrozil and clofibric acid at 1 µg/L each), but a significant difference in survival was observed for tests N° 2 and N° 3 (fenarimol at NOAEC and LOEC, respectively) after 21 days. This was surprising for the lowest concentration of fenarimol, but the survival rate was still over 80%.

Reproduction

In test N° 1 (gemfibrozil and clofibric acid each at 1 µg/L), only at day 10, a significant difference ($p = 0.0218$) was observed on the number of offspring (Fig. 4a). Results from daphnids exposed to fenarimol (test N° 2: 113 µg/L and N° 3: 565 µg/L) indicated that only the highest concentration used in latter test caused a consistent decrease in reproduction (Fig. 4b and c). Indeed, in test N° 3, a significant diminution in the number of offspring per adult was observed compared to controls in three consecutive days: 17 ($p = 0.0020$), 19 ($p = 0.0495$), and 21 ($p = 0.0013$).

Ecdysteroid levels

For all tests, experiments showed that the level of 20E did not differ significantly ($p > 0.05$) between controls and organisms exposed (Fig. 5). For the gemfibrozil and clofibric acid exposure (Fig. 5a), control and exposed groups had 20E mean levels of 21 ± 6.1 pg/ind and 16 ± 6.0 pg/ind, respectively. In test N° 2 (Fig. 5b), the values observed for control and exposed individuals were 20 ± 17 pg/ind and 9.8 ± 9.1 pg/ind, and for test N° 3 (Fig. 5c), 35 ± 24 pg/ind and 24.0 ± 9.7 pg/ind.

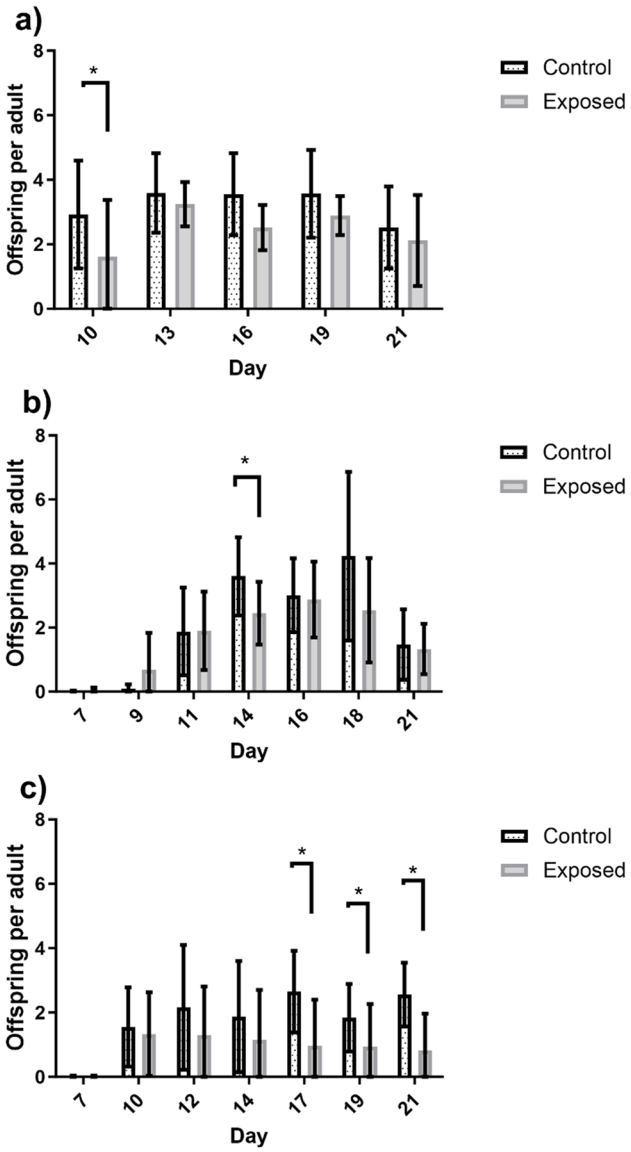

Fig. 4 Offspring production per daphnid exposed for 21 days to **a** 1 µg/L of gemfibrozil and clofibric acid ($n = 10$), **b** 113 µg/L ($n = 10$), and **c** 565 µg/L ($n = 15$) of fenarimol. Error bars represent ± 1 standard error. Asterisks (*) indicate statistically significant differences compared to controls ($p < 0.05$)

Transcription of targeted genes

Genes linked to ecdysteroid metabolism such as *shd*, *nvd*, *usp*, and *ecr* (Goodman and Cusson 2012) were monitored to evaluate if exposure to fenarimol at sublethal levels (test N° 2 and N° 3) during 21 days could change their transcription levels. The results of these experiments are shown in Fig. 6a and b.

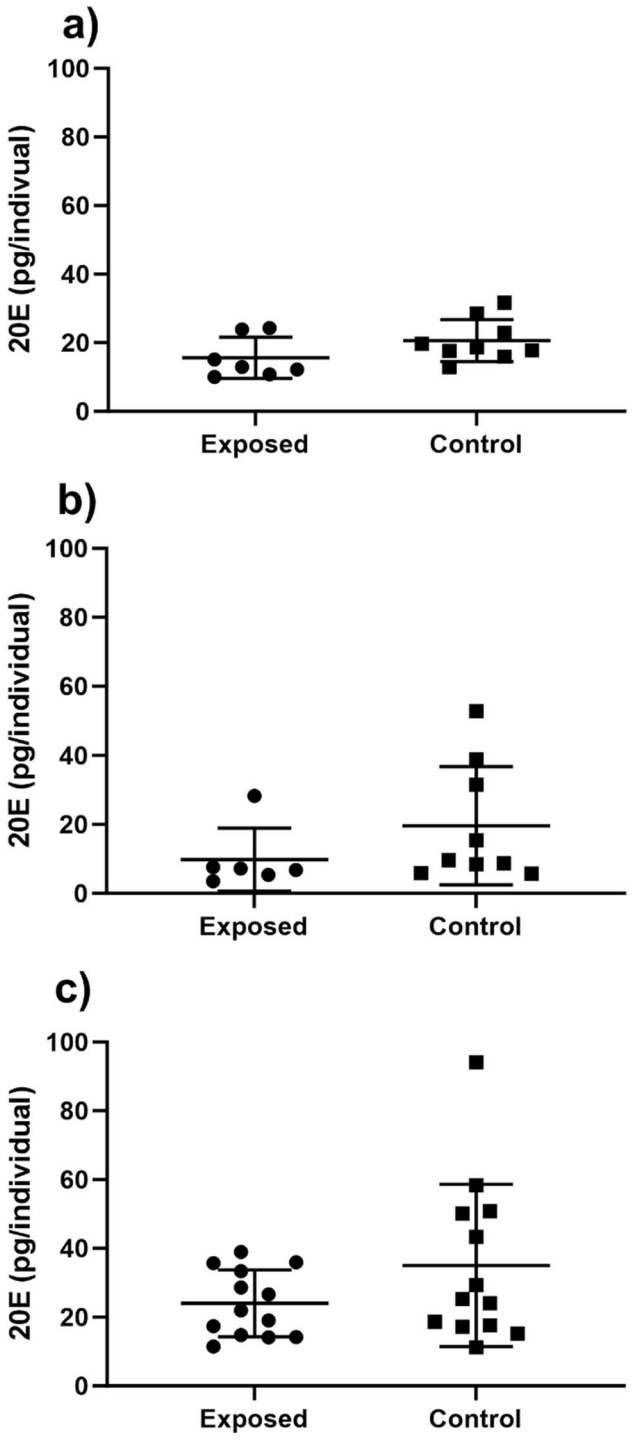

Fig. 5 Concentration of 20E (pg/individual) in daphnids exposed for 21 days to **a** 1 µg/L of gemfibrozil and clofibric acid ($n = 10$), **b** 113 µg/L ($n = 6$ for exposed group and $n = 9$ for control group), and **c** 565 µg/L ($n = 13$ for both groups) of fenarimol. Error bars represent ± 1 standard error. Asterisks (*) indicate statistically significant differences compared to controls ($p < 0.05$)

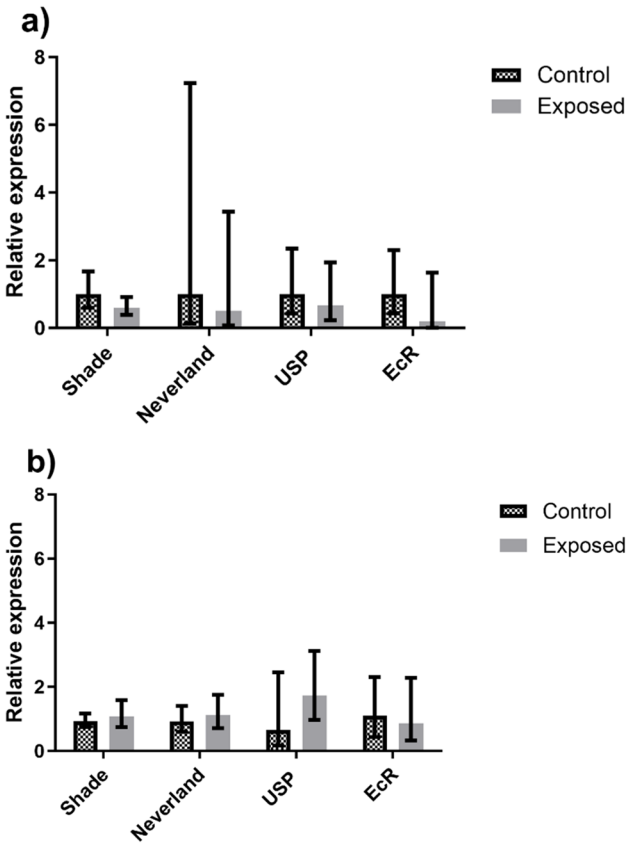

Fig. 6 Relative gene transcription of *shd*, *nvd*, *usp*, and *ecr* in daphnids exposed for 21 days to **a** 113 µg/L ($n = 8$) and **b** 565 µg/L ($n = 7$) of fenarimol. Error bars represent 95% confidence interval limits. Asterisks (*) indicate statistically significant differences compared to controls ($p < 0.05$)

As shown in Fig. 6a and b, expression of any gene at the end of the experiments was not affected at the fenarimol NOAEC (113 µg/L) or LOEC (565 µg/L). These results agree with the 20E data presented in Fig. 5. In Fig. 6a, we can see that *nev* is the most variable gene in those observed. Since *nev* regulates the first step of the ecdysteroids biosynthesis from cholesterol, it is possible that this gene is more up and down regulated, initializing and stopping the biosynthesis when needed, thus explaining these variations in transcription levels. However, it not yet clear why such high variability is only observed at the lowest concentration of fenarimol tested.

Discussion

Baseline of target genes and levels of 20E

Results reported by Sumiya et al. (2014) for *D. magna* were similar to the transcription levels measured in the present study for *shd*, *nvd*, *usp*, and *ecr* across an intermolt sampling

period, with variation ranging between 2 and 3 times the fold change depending on the time point. The relative stability observed here for gene transcription during the normal growth of daphnids could be due to the developmental stage of daphnids sampled and the selection of genes. Many other genes and their isoforms are responsible for the regulation of ecdysteroids. For example, besides *shd* and *nvd* other genes of the Halloween family (spook, spookier, disembodied, and phantom) are implicated in the metabolic cascade that biosynthetizes 20E (Song et al. 2017). A time course transcriptomic analysis during the growth period would provide optimal information for the selection of the genes and periods to follow during exposures. When looking at the transcription levels of Halloween genes during normal development in other species, lower differences could be observed between time points. Indeed, for *shd* and *nvd*, two genes also measured in the present study, the relative transcription levels in the moth *Plutella xylostella* oscillated between 0.02 and 0.2 (Peng et al. 2019). The same study also observed differences in gene expression of Halloween genes in *P. xylostella* according to the developmental stage of individuals and the tissues sampled. These results indicate that even if very slight or non-significant changes in the transcription levels of these genes are observed, developmental changes (growth, reproduction) can still occur.

Finding the right moment for sampling gene transcription levels is crucial, especially for rapidly modulated transcripts. By following several genes of the same family throughout a time-course interval, a better picture of the metabolism regulated by those genes can be drawn. A pulsating pattern was observed during the normal growth of the daphnids, and the basal transcription levels were assessed. However, more genes should be studied, ideally following one another in the metabolic pathway of the studied way. As for the sampling interval, a much shorter time period could give a better picture of the transcription levels across time and will help in correlating these transcription levels with the corresponding metabolites such as ecdysteroids.

The results in Fig. 3 were obtained with pooled individuals (15–25 daphnids). Therefore, daphnids are no longer synchronized by day 9, and as a result, differences in 20E concentration as a function of time are much more subtle and the variability of each measure is higher. Regarding sampling frequency, 7 measurements were done within a 12-day period, while Martin-Creuzburg et al. (2007) performed more than 20 measurements within a 3-day period. Finally, while it is not possible to compare the concentrations observed since previous studies used immunoassay-based techniques rather than mass spectrometry, the reported concentrations herein were about an order of magnitude lower than those reported by Martin-Creuzburg et al. (2007) but about 10,000 times higher than those reported by Sumiya et al. (2016). Besides the techniques employed, those differences can be explained by the sampling of individuals at different stages of their molting cycles.

Reproduction

The results from tests N° 1 (gemfibrozil and clofibric acid each at 1 µg/L) (Fig. 4a) are in agreement with a previous study that showed that chronic exposure (30 days) to 1 µg/L of clofibric acid did not affect the reproduction of *D. magna* (Flaherty and Dodson 2005). Steinkey et al. (2018) also reported that daphnids exposed to low concentrations of gemfibrozil (0.05 µg/L) produced broods at an earlier age and had larger broods than control individuals, but such effect was only observed when food availability was high. The authors also observed that organisms were larger and had higher lipid energy reserves for which mechanisms of action were unexplained by the authors.

Concerning tests N° 2 (fenarimol 113 µg/L, Fig. 4b) and N° 3 (fenarimol 565 µg/L, Fig. 4c), similar observations were also made by Mu and LeBlanc (2002) for *D. magna* exposed for 21 days to similar concentrations of fenarimol. A reduction in the number of offspring in aquatic invertebrates has been attributed to impairment of energy supply and demand or to endocrine disrupting effects (Barata et al. 2004). Under normal conditions, individuals use energy for their basal metabolism, growth, and reproduction. In the case of chemical exposure, a larger amount of the assimilated energy can be used to cope with the stressor and to maintain or compensate basal metabolism, leaving less energy available for growth and reproduction (Sokolova 2013). Therefore, to survive in test N° 3, exposed daphnids had to diminish the production of their offspring.

Ecdysteroid levels

Little information is known on the impact of lipid-lowering molecules on the production of ecdysteroids in *D. magna*. The present results show that no effect is observed for this parameter in daphnids exposed for 21 days to a mixture of gemfibrozil and clofibric acid at a concentration of 1 µg/L (Fig. 5a) as well as for the two fenarimol exposure assays (113 µg/L and 565 µg/L, Fig. 5b and c, respectively). While the results of the exposure to 113 µg/L of fenarimol could be explained by a concentration too low to have an effect on the metabolism of ecdysteroids, the results for the exposition at 565 µg/L are contrary to those obtained by Mu and Leblanc (2004). In that study, the authors exposed *D. magna* neonates during the first intermolt period (approximatively during 25 h) to fenarimol at a similar concentration (497 µg/L) than the experiments presented here. This exposure caused a diminution of around 26% in ecdysteroid levels and induced a delay for the first and second molt. However, the discrepancies observed between their study and

the present study can be explained by several factors such a difference in methods of quantification of ecdysteroids, the age of the daphnids used as well as the time of exposure. Another hypothesis explaining the similar 20E levels in exposed organisms is the transfer of 20E to embryos. Since ecdysteroids are transferred to neonates during embryogenesis (Subramoniam 2000), and that a lower number of neonates was produced in exposed daphnids, the total 20E present in the colony could have been lower in the exposed groups compared to controls if neonates had been included in the measurements. Unfortunately, the present method used was not sensitive enough to quantify 20E levels in neonates (Venne et al. 2016). When a lower number of neonates are produced, a lower quantity of ecdysteroids and overall resources are needed by the daphnids compared to daphnids producing many neonates. Finally, it could also be possible that cholesterol 7-dehydrogenation was indeed inhibited by exposure to fenarimol, but that the inhibition did not affect the end of the line concentrations of 20E as the organisms were able to cope by other mechanisms. Thus, cholesterol and 7-dehydrocholesterol levels should be assessed and compared between the control and exposed groups to verify this hypothesis.

Transcription of targeted genes

Since fenarimol targets the 7-dehydrogenation step in the biosynthesis of ecdysteroids (LeBlanc 2007), an increase in transcription of *nvd* would have been expected as this gene encodes for the 7-dehydrogenase enzyme. It is important to note that a single time point (day 21) was measured in control and exposed groups. Therefore, it is possible that differences in transcription levels could have been occurring earlier in time. Soetaert et al. (2007) reported changes in gene expression (cuticula proteins, proteases related genes) in *D. magna* following exposure to fenarimol for 96 h at a concentration of 1 mg/L using cDNA microarray. When Soetaert et al. (2007) used a concentration similar to the present study (500 µg/L), no changes were observed except for an unknown transcript. These results combined with the ones from the present study suggest that fenarimol may reduce fecundity in *D. magna* by first delaying molting and development as reported by Hassold and Backhaus (2009); but that *shd* and *nvd* genes are not involved in these changes.

Gene expression is a dynamic process where changes can occur within hours (Storey et al. 2005). When changes follow a transient manner, the expression of affected transcripts returns to pre-response levels (Bendjilali et al. 2017). Therefore, finding the right window for the measurements is essential. Only a few transcripts were evaluated in this study; it could be interesting to use techniques with a wider approach such as RNA-sequencing.

Conclusion

The present study aimed to assess the natural baseline of 20E and the transcription of four genes that regulate ecdysteroids in *D. magna* over a 21-day period, and to evaluate the chronic effects of fenarimol and a mixture of gemfibrozil and clofibric acid on multiple levels of biological responses.

Baseline measurement of 20E and transcription levels of *shd*, *nvd*, *usp*, and *ecr* indicated concentration of 20E oscillating between 13 and 31 pg/individuals and transcription levels between 1 and − 1 relative to day 9 (first measurement). The hypothesis of the present work that levels of 20E and the transcription of 20E-related genes would be affected by exposure to lipid-altering compounds could not be proved. Although a diminution in number of offspring was observed consistently in the exposition to 565 µg/L of fenarimol, no differences were observed for the molecular assays tested here, i.e., concentration of 20E and transcription levels of *shd*, *nvd*, *usp*, and *ecr*. Other pathways regulating growth and reproduction, such as the juvenoid hormones (Goodman and Cusson 2012), might be affected and would need to be studied to understand what lies behind this observation. Results suggest that concentrations of 20E and gene transcription quantified in daphnids observed in this work might be too variable when using a single punctual measure to discern effects due to exposure to sublethal concentrations of the target compounds. Therefore, at least for the compounds tested, changes in levels of 20E and/or changes in the transcription of 20E-related genes cannot be used as biomarkers of exposure. Nevertheless, it is not yet clear if the same outcome could be extrapolated to other inorganic or organic contaminants or by modifying the experimental conditions.

Identifying the molting stage of the daphnids is demanding and difficult to synchronize between individuals when exposed for several days. Therefore, another molecule, produced in a more stable way, could be used to normalize the ecdysteroids levels before statistical analysis. Measuring multiple time points instead of a single measure as well as additional molecular endpoints obtained from transcriptomic and metabolomic studies could also afford more insights on the changes occurring in exposed daphnids. Additionally, studying the role of neonates on 20E levels can help understand differences observed with previous studies. Indeed, measuring these parameters sooner in time could offer more insights on the adaptation of the daphnids to lipid-altering compounds on *D. magna*. In this way, it may be possible to obtain a more comprehensive view of the effects of stressors having common modes of action.

Additional experiments are required to help explain the lowered fertility observed in *D. magna* when exposed

to an ecdysteroid synthesis inhibitor such as fenarimol. Future work could employ untargeted transcriptomic and lipidomic to evaluate changes in the lipid profile in whole daphnid extracts following exposure to other organic contaminants of interest. Such an approach could offer a better chance of identifying impacted metabolites and lead to a better understanding of mechanisms of toxicity. Moreover, it would be interesting to determine how daphnids adapt to exposure to different organic contaminants in the long term through their whole life cycle and multigenerational experiments since exposure periods of 21 days or less might be too short to clearly observe more gradual effects on survival or reproduction.

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Data availability Available upon reasonable request.

Declarations

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