Mono(2-ethylhexyl) phthalate modulates lipid accumulation and reproductive signaling in *Daphnia magna*

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Abstract
Mono(2-ethylhexyl) phthalate (MEHP) is a primary metabolite of di(2-ethylhexyl) phthalate (DEHP), which is widely used in industry as a plasticizer. Both DEHP and MEHP have been identified as endocrine disruptors affecting reproduction systems in natural aquatic environments. However, the effects of MEHP exposure on aquatic invertebrates such as *Daphnia magna* are still poorly understood. In the present study, lipid alterations caused by MEHP in *D. magna* were identified by analyzing lipid accumulation and nontarget metabolomics. In addition, reproductive endpoints were investigated. MEHP exposure under any conditions up to 2 mg/L was not associated with mortality of *D. magna*; yet, the number of lipid droplets and the adult female daphnids reproduction rates increased after 96 h of exposure and 21 days of exposure, respectively. MEHP also enhanced lipid metabolism, as evident from 283 potential lipid metabolites, including glycerolipids, glycerophospholipids, and sphingolipids, identified following 48 h of exposure. The MEHP-treated group exhibited significantly higher ecdysone receptor (EcR) and vitellogenin 2 (Vtg2) expression levels at 6 and 24 h. At 48 h, EcR and Vtg2 expression levels were downregulated in the 1 and 2 mg/L MEHP exposure groups. Our data reveal that the EcR pathway changes over MEHP exposure could be associated with lipid accumulation, owing to increased lipid levels and the subsequent increase in the reproduction of MEHP-exposed *D. magna*.

Keywords Mono(2-ethylhexyl) phthalate · Lipid accumulation · Reproduction · Nontarget metabolomics · Ecdysone receptor · *D. magna*

Introduction
Phthalates, used as plasticizers in a variety of consumer products, exert adverse effects and have raised serious concerns, because these agents are easily released into the environment (Staples et al. 1997). The most commonly used phthalate is di(2-ethylhexyl) phthalate (DEHP) (Kamrin 2009), which accounts for almost 80% of the world’s phthalate consumption. In Europe, DEHP use was banned in childcare products in 1999 because of its potentially harmful health effects ((EC)1999/815/EC 1999). DEHP is rapidly degraded by microorganisms (Chang et al. 2004; Jianlong et al. 1997; Liang et al. 2008; Singh et al. 2017), resulting in mono(2-ethylhexyl) phthalate (MEHP) as a primary metabolite (Baini et al. 2017; Frederiksen et al. 2007). Persistent detection of MEHP in the environment raises significant concerns for environmental health, given its potential risks (Baini et al. 2017).

*Daphnia magna* is a planktonic crustacean found in freshwater environments and has been globally used in ecotoxicological studies because it plays a central role in the food web as a predator of algae and as prey for fish (Campioli et al. 2011). Toxicity studies for other phthalate compounds, including DEHP, butyl benzyl phthalate (BBP), dibutyl phthalate (DBP), and diethyl phthalate (DEP), in Daphnia or other invertebrates such as *Chironomus riparius* were reported (Herrero et al. 2015, Herrero et al. 2017. Shen et al.
able grade. and chemicals used were of the highest commercially avail-
Fisher Scientific (Waltham, MA, USA). All other reagents
stand the adverse responses to MEHP exposure.
the present study aimed to investigate lipid metabolism, bio-
tation between lipid metabolism and poten-
Seyoum and Pradhan 1992). Therefore, it is worthwhile to under-
magna et al. 2011). Additionally, the dynamics of lipid storage in
accumulation because it promotes adipogenesis (Campioli
D. magna et al. 2016a). Hence, the formation of lipid droplets in D.
has been a subject of interest for the storage lipids accumulation because it promotes adipogenesis (Campioli
depend on the molt and reproductive cycle (Tessier
magnas known to regulate lipid metabolism and, subsequently, lipid accumulation (Jordão
d. magna depend on the molt and reproductive cycle (Tessier
and Goulden 1982). Therefore, it is worthwhile to under-
understand the relationship between lipid metabolism and poten-
tial adverse effects of MEHP in D. magna. In this direction, the present study aimed to investi-
gate lipid metabolism, biomarkers, and reproductive endpoints in D. magna to under-
stand the adverse responses to MEHP exposure.

Materials and methods

Experimental chemicals and reagents

MEHP (CAS No. 4376–20-9, 97%), potassium dichromate, dimethyl sulfoxide (DMSO), and formaldehyde were pur-
chased from Sigma-Aldrich (St. Louis, MO, USA). The
MEHP stock solution was prepared in DMSO at a concentra-
tion of 25 g/L and stored in a brown bottle with a Teflon cap
at −20 °C. BODIPY 505/515 was procured from Thermo
Fisher Scientific (Waltham, MA, USA). All other reagents
and chemicals used were of the highest commercially avail-
able grade.

D. magna maintenance

D. magna were hatched from ephippia obtained from Micro-
BioTests Inc. (Gent, Belgium) and maintained for more than
ten generations in M4 medium at 21 ± 1 °C. The photoperiod
was maintained at 16:8 h (light:dark) with a light intensity
between 1000 and 1500 lx. Ten adult female daphnids were
placed in a 2-L beaker filled with 1.5-L M4 medium. The
culture medium was changed twice per week. The daph-
nids were fed daily ad libitum with Chlorella vulgaris (ca.
1.5×10^8 cells/mL) and twice a week with a mixture of yeast,
cerophyll, and trout chow (YCT). C. vulgaris was supplied
by the Culture Collection of Algae at Cologne University,
Germany. The sensitivity of D. magna was regularly checked
according to ISO 6341 (ISO 2012), with potassium dichrom-
ate as a reference substance.

Lipid droplet staining

Neonates (<8 h) were placed in a 100-mL beaker containing
80 mL of MEHP (0, 1, and 2 mg/L) for 96 h (0.01% DMSO).
After exposure, the neonates were washed with ISO medium
and transferred into 2-mL microcentrifuge tubes. Then,
1 mg/L BODIPY 505/515 was added to the tube. The neo-
nes were kept for 30 min in the dark at 20 °C. Lipid-stained
daphnids were collected and washed with ISO medium and
then fixed with 2% formaldehyde. Images were obtained
with a ZEISS SteREO DiscoveryV8 microscope and ZEN
3.0 blue edition (ZEISS, Jena, Germany). The lipids were
quantified using ImageJ software (Schneider et al. 2012).
The daphnids were fed the same amount of C. vulgaris as
normal culture conditions during the test.

Gene transcription analysis

For reverse-transcriptase quantitative polymerase chain re-
tion (RT-qPCR), five neonates (<8 h) per well of a six-well
plate were exposed to MEHP for 6, 24, and 48 h. Fifteen D.
magna neonates were pooled into 1.5-mL centrifuge tubes
after exposure and washed with distilled water. Each pooled
neonate was collected in triplicates (n = 3). Neonates were
lysed in TRIzol reagent using a tissue homogenizer, and the
total RNA was isolated using a column-based kit (Qiagen,
Valencia, CA, USA). The cDNA was synthesized using a
high-capacity RNA-to-cDNA kit (Applied Biosystems, Fos-
ter City, CA, USA). Primers designs were based on previous
studies (Table S1). The RT-qPCR assay was conducted using
Fast SYBR™ Green Master Mix (Applied Biosystems) on
a 7500 FAST real-time PCR system (Applied Biosystems).
Reaction cycles were performed as follows: initial denatura-
tion for 2 min at 95 °C, 40 cycles of amplification at 95 °C
for 5 s, and 60 °C for 1 min. D. magna actin was used as an
endogenous control for normalization (Cui et al. 2017; Herr-
ero et al. 2017; Xu et al. 2019). Gene expression was calcu-
ated using the 2−ΔΔCt method (Schmittgen and Livak 2008).

Reproduction toxicity test

The reproduction test was performed according to the OECD
Test Guideline 211 (OECD 2012). Ten neonates per con-
centration were used for the reproduction toxicity test. The
average offspring counts per adult female D. magna were
determined. Each neonate (<24 h) was placed in a 100-
mL beaker filled with 80 mL of different concentrations
of MEHP test solutions. The test solutions (0.008% DMSO
in ISO medium) were diluted from the stock solution. The
DMSO content was the same in all tested samples. The
media were changed every 2 days. Neonates were counted
daily and removed from the media. Temperature, pH, dissolved oxygen, and total hardness were measured weekly during 21 days of exposure, and the values are presented in Table S2.

Nontarget metabolomics

The nontarget metabolomics approach was conducted using quadrupole time-of-flight (Q-TOF) high-resolution mass spectrometry (HRMS) to evaluate reproduction-related changes after MEHP treatment. Fifteen neonates (<8 h) were placed in a 500-mL beaker with 300 mL MEHP test solutions for 48 h (0.01% DMSO). Then, the neonates were washed with ISO medium and transferred into 2-mL microcentrifuge tubes. Fifteen daphnids were homogenized using a Tissue Lyser LT with methanol. The homogenates were centrifuged at 10,000 × g for 20 min, and the supernatant was collected. All samples were analyzed using a Triple TOF 6600 + QTOF HRMS (AB Sciex, Framingham, MA, USA) coupled with an IonDrive Turbo spray electrospray ionization (ESI) source and an Exion HPLC system (AB Sciex). The autosampler was operated at 4 °C, and the column oven was operated at 40 °C. The injection volume was 5 μL. Four different injections using two different chromatographic separations, reverse-phase (RP) and hydrophilic interaction chromatography (HILIC) mode, with both positive and negative polarities, were used. A separation performed via a Waters ACQUITY UPLC™ BEH C18 column (100 × 2.1 mm, 1.7 μm, Milford, MA, USA) and mobile phase A (5 mM ammonium acetate and 0.05% formic acid in water) and mobile phase B (5 mM ammonium acetate and 0.05% formic acid) in water/acetonitrile (5:95) at a flow rate of 0.4 mL/min. The initial composition of the mobile phase B was 5%, which was maintained for 2 min, increased to 20% in 4 min, increased to 100% until 22.5 min, and then maintained for 4.5 min; it was then re-equilibrated to the initial condition for 2 min.

HILIC separation was performed using a Luna NH2 column (150 × 2.1 mm, 3 μm, Phenomenex, Torrance, CA, USA) and operated at a flow rate of 0.3 mL/min using mobile phase A (water:acetonitrile, 1:1 [v/v] containing 5 mM ammonium acetate and 0.05% formic acid) and mobile phase B (water:acetonitrile, 5:95 [v/v] containing 5 mM ammonium acetate and 0.05% formic acid). After sample injection, the column was maintained for 2 min at 92% B and then subjected to gradient elution from 2 to 18 min (92 to 0% B). The column was then returned to the initial condition for the next 0.01 min and re-equilibrated for 11.99 min at 92% B before injection of the following sample. The autosampler and the column oven were operated at 5 °C and 50 °C, respectively. The system was run using Analyst TF software (version 1.8.1, AB Sciex). All data were acquired in the scan range from m/z 50 to 1000 in a TOF information-dependent acquisition (IDA) MS2 mode scan. MS/MS was run with a mass range from m/z 50 to 1000, and a positive/negative calibration solution for the ESI source was used to correct the mass during the analysis for every five samples.

Data statistics and lipid enrichment analysis

Data of lipid droplet staining, gene transcription analysis, and reproduction test are presented as mean ± standard deviation (SD). Data were analyzed with unpaired Student’s t-test, and the normality of data was confirmed by the Shapiro–Wilk test using GraphPad Prism 8.4.3 (alpha = 0.05). P values less than 0.05 indicate statistical significance unless stated otherwise.

Data analysis was performed using XCMS (Huan et al. 2017). MetaboAnalyst (version 4.0) was used for multivariate statistical analyses, and all variables were Pareto-scaled (Chong et al. 2019). In addition, a principal component analysis was performed to visualize any patterns and groupings; a partial least-squares discriminant analysis (PLS-DA) was carried out to determine separate influential variables between the treatment and control groups based on their variable importance in projection (VIP). Because multivariate analysis can be overly optimistic for assigned peaks (Rodríguez-Pérez et al. 2018; Tapp and Kemsley 2009), the differences in assigned peaks in the nontarget metabolomics were analyzed using the Kruskal–Wallis nonparametric test in XCMS. After sorting the significant peaks, the peaks were reviewed by SCIEX-OS to confirm the accurate mass (± 10 ppm) and MS/MS spectrum. The MS spectrum databases such as Human Metabolome Database (HMDB; Wishart et al. 2013), Metlin Database (Smith et al. 2005), MASS BANK (Horai et al. 2010), and LIPID MAPS (Fahy et al. 2007) were used to identify metabolites as potential biomarkers.

Lipid enrichment analysis in ranking mode was performed using lipid ontology (LION) enrichment analysis (Molenaar et al. 2019). First, 206 lipid metabolites were selected, excluding unidentified names in LION and overlapping names with multiple injections. Then, the ranked input identifiers were determined by using the feature selection based on a one-way analysis of variance (ANOVA) F-test. Next, the peak intensities were normalized by percentage, and the Kolmogorov–Smirnov (K-S) test setting was twotailed. Finally, a graph was generated by combining upregulated and downregulated metabolites as a bar chart.

Results

Changes in lipid storage and analysis of lipid metabolism

Lipid droplets were visualized using the fluorescent dye BODIPY 505/515. MEHP induced lipid droplet formation
in *D. magna* at 1 and 2 mg/L, which are ecotoxicologically
relevant concentrations for acute and chronic aquatic toxicity
in crustaceans based on the classification, labeling, and
packaging (CLP) regulation (1272/2008/EC 2008) (Fig. 1).

We assessed lipid metabolites by nontarget metabolomics
to analyze changes in lipid droplet formation after exposure
to MEHP. Supplementary Figure S1 shows multiple vari-
abilities in analyzing these metabolites from each injection.

**Fig. 1** Alteration in lipid droplets of *D. magna*. **A** Individual
daphnids were observed under a fluorescence microscope
following a 96-h exposure to MEHP. **B** BODIPY fluorescence intensity of lipid droplets
quantified from microscopy images (*****$P < 0.001$); $n = 5$
between the positive and negative modes with both RP and HILIC columns. The PLS-DA method was applied to all detected metabolites in different treatment groups, including the control group. The data points for both treatment groups in all injections were separated from the corresponding control groups, except slightly overlapping points between the control group and quality control (QC) in negative RP and HILIC modes in the PLS-DA score plot. The calculated VIP scores and \( t \)-test \( P \) values were used to determine specific metabolites with significant changes following exposure of daphnids to MEHP. For loading plots of dominant peaks, we selected a VIP score higher than 1 and \( P < 0.05 \) calculated by the Kruskal–Wallis nonparametric test in the online XCMS.

Identification of differentially regulated metabolites and effects on the lipid contents

Significant changes in the lipid metabolites were observed after MEHP exposure, as shown in the heat maps in Fig. 2. All identified peaks and intensities are denoted in Supplementary Data 1. Among lipid metabolites, phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were identified from the typical fragmentations resulting in the fragments of \( m/z \) 86.10 (C5H12N) and 184.07 (C5H15NPO4Na) in the positive-ion mode and 168.04 (C4H11O4NP) in the negative-ion mode (Gao et al. 2016; Ostrowski et al. 2005; Pi et al. 2016), respectively. In addition, Monoacylglycerol

![Fig. 2](image-url)
(MG) was identified as a typical fragment (fatty acid — H₂O + H; Gao et al. 2016). Typical fragmentations are indicated in Supplementary Data 1.

A total of 283 identified lipid metabolites were significantly changed after exposure of *D. magna* to MEHP: 130 in the positive-ion mode and 153 in the negative-ion mode. To enumerate, the composing metabolites were as follows: 59 PE, 40 PC, 19 phosphatic acids (PA), 23 phosphoserines (PS), 14 ceramides (CE), 15 phosphoglycerol (PG), 8 phosphoinositols (PI), 16 lysophosphatidylcholines (LPC), 11 lysophosphatidylethanolamines (LPE), 41 diacylglycerols (DG), 8 MG, 9 sphingomyelins (SM), and 3 fatty acid estolides. Changes in the detected peaks for the treatment group were quantified through relative normalization against the control group and are summarized in Supplementary Data 1. Briefly, the average increments in all lipid metabolites were calculated as 72.49% ± 18.59% and 78.27% ± 12.91% for 1 and 2 mg/L MEHP treatment groups, respectively. Among tetrapyrrrole-like metabolites, biliverdin, bilirubin, phaeophoribide, harderoporphyrin, and coproporphyrin decreased significantly following MEHP exposure (1 mg/L to control, *P* < 0.05; 2 mg/L to control, *P* < 0.05; Student’s *t*-test). Figure 3 displays the results of the LION enrichment analysis performed between all three groups. Specifically, Figure 3B compares the control and 2 mg/L MEHP exposure groups. Exposure to 2 mg/L MEHP led to a decrease in the negative intrinsic curvature and an increase in the low lateral diffusion. The detailed results of the enrichment analysis are provided in Supplementary Data 2.

Ponsteride A and juvenile hormone III, major biomarkers from the mass peaks for reproduction in the crustacean, and ecdysteroids and juvenile hormones 20-hydroxyecystereoid were reported in previous studies (Gao et al. 2016; Miyakawa et al. 2018; Nakagawa and Sonobe 2016). However, the peaks corresponding to these biomarkers
Fig. 4 Comparison of expression levels of reproductive genes and SOD gene in *D. magna* after 6, 24, and 48 h of MEHP exposure. Data are expressed as mean ± SD of three repeated experiments. Asterisks indicate significant different between control and MEHP exposure groups (*P* < 0.05; **P** < 0.01)
with identical fragmentation patterns (Destrez et al. 2009; Miyashita et al. 2011) were not detected.

Changes in gene expression associated with reproduction

Figure 4 displays the gene expression profiles representing the mRNA levels encoding six genes. The exposure to 1 mg/L MEHP at 6 and 24 h upregulated ecdysone receptor A (EcR-A) and ecdysone receptor B (EcR-B); conversely, it downregulated the expressions at 48 h (Fig. 4A, B). Although 2 mg/L MEHP exposure induced no significant alteration in EcR-B during the 24 h exposure time, we assumed that the hormonal receptor might mediate alterations at least for 48 h. Likewise, 1 mg/L MEHP induced vitellogenin 2 (Vtg2) expression at 6 and 24 h but dramatically decreased Vtg2 level after 48 h (Fig. 4C). Chitinase levels increased in the 2 mg/L MEHP treatment group at 24 h but did not change after 48 h (Fig. 4D). Juvenile hormone esterase (JHE) and superoxide dismutase (SOD) levels did not significantly change at 6 and 24 h (Fig. 4E and F). However, the JHE level decreased after 48 h of MEHP exposure. MEHP exerted similar effects at both 6 and 24 h of continuous exposure as an agonist for EcR-A/B, JHE, and Vtg2. The total number of neonates per adult female significantly increased after exposure to 2 mg/L MEHP for 21 days compared to the control group (Fig. 5). The initial offspring started during the same period for all test groups. In the reproduction toxicity test, all D. magna did not show any cytotoxic response up to the highest concentration of 2 mg/L MEHP.

Discussion

To evaluate the potential toxicity and its mechanism of toxicity of MEHP, lipid staining, nontargeted metabolomics, and reproduction-related gene expressions were examined. After exposure to MEHP, lipid metabolism, lipid accumulation, and reproduction rates significantly increased in D. magna. This result is consistent with a previous study where the treatment with 1 μM DEHP increased lipid accumulation and induced a 1.5-fold increase in neonates per adult female D. magna in a 30-day test (Seyoum and Pradhan 2019). As MEHP is a major metabolite of DEHP, it is reasonable to speculate that it increased reproduction by promoting lipid metabolism. On the contrary, post-spawning female daphnids exposed to 1 μM DEHP during the first egg provisioning stage (72 h) were shown to have fewer lipid droplets than the control group in another study (Jordão et al. 2016b). Thus, one may infer that daphnids favored lipid accumulation for reproduction because phthalates promoted the transfer of accumulated lipids from adults to neonates, resulting in a positive clutch size that correlated with lipid storage (Fuertes et al. 2020; Tessier and Goulden 1982).

Our results demonstrated the accumulation of lipid droplets (Fig. 1) and increased lipid metabolites (Fig. 2) in D. magna after MEHP exposure. These results also agree with a previous report where phthalates prominently altered lipid metabolism in D. magna (Seyoum and Pradhan 2019). In addition, DEHP treatment increased the phospholipid composition (e.g., phosphatidic acid, PE, and PC) in rats (Yanagita et al. 1987) and upregulated the expression of lipid metabolism-related genes in Caenorhabditis elegans (Pradhan et al. 2018).

We observed decreased levels of heme-related tetrapyrrole-like metabolites. Notably, pheophorbide b, also used as daphnids food, is a product of chlorophyll degradation from C. vulgaris (Smith 1984). The decreased pheophorbide b level in the MEHP-treated group can be explained by the increase in ingestion rate, which promoted lipid metabolite synthesis. In another study (Wibe et al. 2004), increased feeding of sticklebacks was observed after exposure to butyl benzyl phthalate (BBP) for 31 days. This altered feeding behavior might be a physiological compensatory response against environmental stress, as the treatment group became increasingly starved as compared to the control group. In addition, the exposure to DEHP was shown to downregulate two hemoglobin genes in Chironomus tentans (Lee et al. 2006) as well as the expression of the cytochrome P450 (CYP) 4G gene, which plays a major role in inducible
metabolizers of xenobiatics in in crustacean (Herrero et al. 2017). As CYP contains heme and CYP4G is involved in detoxification (Bernabò et al. 2017; Martínez-Paz et al. 2012), the suppressed levels of heme-related genes, including hemoglobin genes and CYP4G, in our study suggest that their expression may be affected by phthalates.

MEHP can be metabolized to CYP-mediated mono(2-ethyl-5-hydroxyhexyl) phthalate (5-OH MEHP), mono(2-ethyl-6-hydroxyhexyl) phthalate (6-OH MEHP), 5 oxo-MEHP (MEOHP), mono(2-carboxymethyl-hexyl) phthalate (2cx-MMHP), and mono(2-ethyl-5-carboxypentyl) phthalate (MECPP) following hydroxylation of the carbon side chains (Ito et al. 2014; Nakajima et al. 2015). The carbon side chain of metabolites can be further cleaved to phthalic acid by O-dealkylation (Choi et al. 2012). For instance, the cleavage of MEHP can generate fatty alcohol, 6-methyloctan-3-ol (C9H20O). In addition, 42cx-MMHP and MECPP can produce 3-methyl octanoic acid (C9H18O2) and 6-methyloctanoic acid (C9H18O2), respectively. These lipid metabolites are used as the suppliers of lipids precursors, potentially enhancing lipid metabolism and accumulation. The LION enrichment analysis revealed the downregulation in fatty acids with less than two double bonds and with 18 carbons or less after MEHP exposure (Fig. 3A). A previous study showed that the exposure to phthalates (diethylhexyl phthalate, dibutyl phthalate [DBP], and diethyl phthalate [DEP]) in D. magna altered the expression levels of lipid-related protein genes and consequently enhanced lipid accumulation (Seyoum and Pradhan 2019). In addition, the expression levels of RXR, ultraspiracle protein, and nuclear HR96 receptor genes, which are involved in ecdysteroid signaling pathways, are regulated by fatty acids (Bonneton and Laudet 2012; Fuertes et al. 2019). These results suggest the possibility of increasing the usage of less than two double bonds in fatty acids. The usage of fatty acids with 18 or fewer carbons was possibly increased, even in the presence of other lipids, including phospholipids and glycerol. In addition, MEHP exposure at a concentration of 2 mg/mL downregulated the negative intrinsic curvature of the membrane, which was an inverted cone shape of the head of lipid head groups (Zelnik et al. 2020). The membrane curvature is also affected by rearranging the lipid composition and membrane into a bilayer (Ashery et al. 2014; Destrez et al. 2009; Furber et al. 2009). However, the mechanistic relationships between lipid metabolism and physiological changes such as fecundity, hatching, and cellular responses to MEHP exposure are intricate; thus, more studies are warranted to explore the effects of MEHP metabolites on lipid metabolism in D. magna.

BBP was shown to upregulate the expression level of the EcR gene in C. riparius as an ecdysone hormone agonist, while DEHP downregulated the expression of the EcR gene (Planelló et al. 2011). In addition, BBP exposure increased EcR expression at 100 µg/L after 24 h but downregulated EcR expression at 48 h and 0.001 to 1 µg/L concentration in C. riparius larvae (Herrero et al. 2015). In our study, the EcR gene expression was upregulated at both 6 and 24 h. Thus, the upregulated EcR gene expression after MEHP exposure can affect the central mechanism of lipid accumulation and lipid metabolism (Herrero et al. 2015). In contrast, EcR, Vtg2, and JHE gene expressions were downregulated after 48 h of exposure in the present study. These observations are in line with those reported in a previous BBP study where the time-dependent response of EcR could repress transcription after 48 h of treatment (Herrero et al. 2015). The treatment of 20-hydroxyecdysone also upregulated the EcR-A in 24 h but repressed in 48 h (Hannas and LeBlanc 2010). The hypothesis of EcR-A, EcR-B, and JHE gene regulation in crustaceans is relatively quickly activated in 24 h and then get feedback downregulation in the long term.

Vtg2 can serve as a potential biomarker for endocrine disruption by phthalates in aquatic organisms, as it is more responsive to xenobiatics than Vtg1 (Hannas et al. 2011). For instance, the expression of Vtg1 was not affected by the phthalates DEHP, DEP, and DBP; in contrast, that of Vtg2, which DEP and DBP downregulated after 24 h of exposure (Seyoum and Pradhan 2019). The increased number of neonates per adult female could be attributed to the altered expression of Vtg2. Fenoxycarb (juvenile hormone analog) application significantly downregulated the expression of vitellogenin and reduced the total number of offspring per adult female (Kim et al. 2011). In the present study, Vtg2 expression was upregulated following MEHP exposure for 24 h. However, Vtg2 and JHE were downregulated by 1 or 2 mg/L MEHP treatment at 48 h. Although this repression might be followed by rapid compensative and feedback regulation, future studies should focus on the time-dependent regulation of EcR and JHE signaling in reproduction.

**Conclusion**

Exposure of D. magna to MEHP increased lipid droplets and various lipid metabolites and upregulated EcR and Vtg2 expression. In addition, MEHP exposure for 3 weeks increased reproductions. This aspect of the research suggests that phthalates could induce lipid accumulation in daphnids, similar to mammals. Further research is warranted to clarify how phthalates modulate the lipid accumulation associated with the EcR and Vtg2 pathways to understand the molecular mechanism underlying the effect of MEHP exposure.
Data Availability.

Datasets generated during the present study are not publicly available but are available from the corresponding author upon reasonable request. These materials are not applicable.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11356-022-19701-1.

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Author contribution HC, YS, SB, BS, and CSR performed the experimental studies and data analysis. SB and CSR established a non-target metabolomic method. HC, YS, and CSR drafted the manuscript. SB, BS, and YJK contributed to the data analysis, interpretation, and manuscript finalization. YJK supervised the study and finalized the manuscript. All authors have read and approved the final manuscript carefully.

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Declarations

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

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