Mono(2-ethylhexyl) Phthalate Modulates the Lipid Accumulation and Reproductive Signaling in *Daphnia Magna*

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Abstract

Mono(2-ethylhexyl)-phthalate (MEHP) is the primary metabolite of di(2-ethylhexyl)-phthalate (DEHP), which is widely used in industry as a plasticizer. Previous studies showed that both DEHP and MEHP have been found in not only human urine samples but also natural aquatic environments, and well documented as toxicants for reproduction and endocrine disruptors, However, the effects of MEHP exposure on aquatic organisms, including invertebrates such as *Daphnia magna (D. magna)*, are still scarce. In the present study, the lipid alterations caused by MEHP in *D. magna* have been identified by analyzing lipid accumulation and nontarget metabolomics. Additionally, the expressions of reproduction were investigated. Ecotoxicologically relevant concentrations (1 to 2 mg/L) and exposure time ranges (24 h to 21 days) have been tested. MEHP resulted in no mortality to *D. magna* with all exposure conditions, but the number of lipid droplets increased after 96 h of exposure and enhanced reproduction of female adult daphnids was observed in the 21-day of exposure. Besides, we observed that MEHP enhanced lipid metabolism with the identification of 283 potential lipid metabolites, including glycerolipids, glycerophospholipids, and sphingolipids, following 48 h of exposure. MEHP treated group exhibited significantly higher expression of ecdysone receptor (EcR) and vitellogenin 2 (Vtg2) at 6 h and 24 h. Meanwhile at 48 h, EcR and Vtg2 were downregulated in 1 and 2 mg/L MEHP exposure. Our data show that the changes in EcR pathway with different exposure time could be associated with the lipid accumulation due to lipids increment subsequently to increased reproduction in MEHP-exposed *D. magna*.

1. Introduction

Phthalates are used in a variety of products as plasticizers, but their adverse effects on the environment are of concern due to their easy release into the environment from products (Staples et al. 1997). The most widely used phthalate is di(2-ethylhexyl) phthalate (DEHP) (Kamrin 2009), which accounts for almost 80% of phthalate consumption in the world. In Europe, it was first banned for DEHP use in childcare products in 1999 due to its potentially harmful health effects ((EC)1999/815/EC 1999). Regardless of this restriction in DEHP usage, DEHP ubiquitously exists in many different environments (Fromme et al. 2002, Maradonna et al. 2013). DEHP is rapidly degraded by microorganisms (Chang et al. 2004, Jianlong et al. 1997, Liang et al. 2008, Singh et al. 2017), resulting in the presence of mono(2-ethylhexyl) phthalate (MEHP) as its primary metabolite in the environment (Baini et al. 2017, Frederiksen et al. 2007). Continuous observations of the natural environment and the toxicity of MEHP raise concerns about the potential risks present in the environment (Baini et al. 2017).

MEHP is known to have adverse effects on the endocrine and reproductive systems of environmental organisms as increasing the estradiol level of male marine medaka, reducing the egg fertilization rates of marine medaka and reducing the egg diameter of zebrafish (Ye et al. 2014, Zhu et al. 2016). Moreover, MEHP has been reported as a ligand of peroxisome proliferator-activated receptor-alpha (PPAR-α) and peroxisome proliferator-gamma (PPAR-γ) (Lovekamp-Swan et al. 2003), MEHP induces lipid accumulation by activating PPAR-γ in vertebrates (Zhang et al. 2019).

Daphnia magna(D.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). Most of the works with *D. magna* have been relied only on the phenotypic changes, such as immobilization, heartbeat, and reproduction rate, which resulted in limited insights into the mechanistic toxicity (Seyoum and Pradhan 2019). Regarding the regulation of lipid metabolism in *D. magna*, the ecdysone receptor (EcR), the retinoid X receptor (RXR) and the methyl farnesoate hormone receptors (MfR) are known to regulate lipid accumulation in this organism (Jordão et al. 2016a). Significantly, the formation of lipid droplets in *D. magna* has been a subject of interest among accumulation of storage lipids because the MEHP is known to promote adipogenesis (Campioli et al. 2011). Additionally, the dynamics of lipid storage in *D. magna* depend on the molt and reproductive cycle (Tessier and Goulden 1982). Therefore, it is worthwhile to understand the relationship between lipid metabolism and the adverse effects of MEHP on *D. magna*. In accordance, our research aimed to integrate the lipid metabolism, biomarkers, and reproductive endpoints to understand the comprehensive adverse responses in *D. magna* after MEHP exposure.

2. Materials And Methods

2.1 Experimental chemicals and reagents

MEHP (CAS No. 4376-20-9, 97%), potassium dichromate, DMSO and formaldehyde were purchased from Sigma Aldrich (St. Louis, MO, USA). The stock solution of MEHP was prepared in DMSO and kept in a brown bottle with a Teflon cap at − 20°C until the experiment was performed. BODIPY 505/515 was purchased from Thermo Fisher Scientific (Waltham, MA, USA). All other reagents and chemicals were of the highest grade commercially available.

2.2 D. magna maintenance

D. magna were hatched from ephippia purchased from MicroBioTests Inc. (Gent, Belgium) and maintained for more than 10 generations in our laboratory with M4 medium at 21 ± 1°C. The photoperiod was kept at 16:8 h (light: dark) with a light intensity between 1000 and 1500 lux. Ten adult females were placed in a 2 L beaker filled with 1.5 L M4 medium. The culture medium was changed twice a week. The daphnids were fed daily *ad libitum* with *Chlorella vulgaris* (ca. 1.5×10^8 cells/mL) and a mixture of yeast, cerophyll and trout chow (YCT) twice a week. *C. vulgaris* were purchased from the Culture Collection of Algae at Cologne University, Germany. The sensitivity of *D. magna* was regularly checked according to ISO 6341 (2012), including potassium dichromate as a reference substance.

2.3 Lipid droplet staining

Neonates (< 8 h) were placed in a 100 mL beaker containing 80 mL MEHP (0, 1, and 2 mg/L) for 96 h (DMSO 0.01%). After exposure, the neonates were washed with ISO medium and transferred into 2 mL microcentrifuge tubes. Then, 1 mg/L BODIPY 505/515 (company, place) was added to the tube, and the neonates were kept for 30 min in the dark at room temperature. Lipid-stained daphnids were collected

and washed with ISO medium. They were fixed with 2% formaldehyde. Images were taken with a ZEISS SteREO DiscoveryV8 microscope and ZEN 3.0 blue edition (ZEISS, Jena, Germany). The lipids were quantified with ImageJ (Schneider et al. 2012). During the test, the daphnids were fed the same amount of *Chlorella vulgaris* as in the regular culture conditions.

2.4 Reproduction toxicity test

Ten replicates of each concentration were used for the chronic test according to the OECD Test Guideline 211 (OECD 2012). Neonates (< 24 h) were placed in 100 mL beakers filled with 80 mL of each concentration. Test solutions (DMSO 0.008%) were diluted from the stock solution. Media were changed every two days. Newly born neonates were counted every day and removed from the media. The DMSO content was the same in all tested samples. Dissolved oxygen, total hardness, and pH were measured before and after 48 h of exposure.

2.5 Gene transcription analysis

MEHP exposure for qRT-PCR was carried in 6 well plate. *D. magna* neonates (< 8 h) were exposed with 5 daphnia in each well for 6, 24, and 48 h. After exposure, fifteen *D. magna* neonates were pooled into 1.5 mL centrifuge tubes and washed with distilled water. Neonates were lysed in TRIzol reagent using tissue homogenizer, and total RNA was isolated using a column-based kit (Qiagen, Valencia, CA, USA). cDNA was synthesized using a high-capacity RNA-to-cDNA kit (Applied Biosystems, Foster City, CA, USA). Primers were designed from several works in the literature (Table S1). qRT-PCR assay was conducted using Fast SYBR™ Green Master Mix (Applied Biosystem) on a 7500 FAST real-time PCR system (Applied Biosystems).

2.6 Nontarget metabolomics

Nontarget metabolomics using quadrupole time-of-flight (Q-TOF) high-resolution mass spectrometry (HRMS) was conducted to define the reproduction-related changes in MEHP treatment. Fifteen neonates (< 8 h) were placed in a 500 mL beaker containing 300 mL MEHP test solutions for 48 h (DMSO 0.01%). After exposure, the neonates were washed with ISO medium and transferred into 2 mL microcentrifuge tubes. Fifteen daphnids were homogenized in a Tissue Lyser LT with LC-MS grade methanol. Then, the homogenates were centrifuged at 10,000 × g for 20 min, and the supernatant was collected. All of the samples were analyzed using a Triple TOF 6600 + QTOF mass spectrometer (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 by reversed-phase (RP) and hydrophilic interaction chromatography (HILIC) mode with both positive and negative polarity were used. RP separation was performed via a Waters ACQUITY UPLC™ BEH column (100 × 2.1 mm, 1.7 µm, Milford, MA, USA) using a gradient mobile phase A as 5 mM ammonium acetate and 0.05% formic acid in water and mobile phase B as 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% and it 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). The HILIC column was operated at a flow rate of 0.3 mL/min using mobile phase A water:ACN 1:1, v/v (A) containing 5 mM ammonium acetate and 0.05% formic acid in and mobile phase B water:ACN 5:95, v/v containing 5 mM ammonium acetate and 0.05% formic acid. After sample injection, the column was kept for 2 min at 92% B followed by gradient elution from 2–18 min (92 to 0% B). The column was returned to the initial condition from 18–18.01 min and re-equilibrated for 11.99 min at 92% B before injection of the next sample. The autosampler was operated at 5°C, and the column oven was operated at 50°C. The system was run by Analyst TF Software (version 1.8.1, AB Sciex). All data were acquired in the scan range from m/z 100 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 5 samples.

2.7 Data statistics and lipid enrichment analysis

Data of lipid droplet staining, reproduction test and gene transcription analysis were presented as means ± standard deviation (SD). Data were analyzed with unpaired Student's *t*-tests for two-group comparisons, and P values < 0.05 were considered to indicate statistical significance unless indicated otherwise

The 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). Additionally, a principal component analysis was performed to visualize any patterns and groupings, and a partial least-squares-discriminant analysis (PLS-DA) was conducted to determine separate influential variables between the treatment and control groups based on their variable importance in projection (VIP). In addition, multivariate analysis can be overly optimistic for assigned peaks (Rodríguez-Pérez et al. 2018, Tapp and Kemsley 2009), so the differences in assigned peaks in the nontarget metabolomics were analyzed by the Kruskal–Wallis nonparametric test in XCMS. After sorting of the significant peaks, the peaks were reviewed by SCIEX-OS to confirm an accurate mass (± 10 ppm) and its MS/MS spectrum. The Human Database (HMDB, (Wishart et al. 2013), the Metlin Database (Smith et al. 2005), MASS BANK (Horai et al. 2010), and LIPID MAPS (Fahy et al. 2007) were used to identify metabolites that could be potential markers.

The lipid enrichment analysis in ranking mode was performed using the Lipid Ontology (LION) enrichment analysis (Molenaar et al. 2019). Excluding unidentified names in LION and overlapped names with multiple injections, 206 lipid metabolites were selected and identified. This feature selection based on one-way ANOVA F-test is used to determine the ranked input identifiers. The peaks intensities were normalized by percentage, Kolmogorov–Smirnov (K-S) test setting was two-tailed, graph is created combined up-and downregulated metabolites as bar chart.

3. Results

3.1 Changes in lipid storage and analysis of lipid metabolism

Lipid droplet is first visualized using the fluorescent dye BODIPY 505/515. MEHP induced the lipid droplet formation in *D. magna* at 1 mg/L and 2 mg/L which are ecotoxicologically relevant concentrations based on the classification, labelling a packaging (CLP) regulation for acute and chronic aquatic toxicity for crustacean ((EC)1272/2008) (Fig. 1). Assessment of lipid metabolites was analyzed for the changes in lipid droplet formation after exposure to MEHP through nontarget metabolomics. Supplementary figure S1 shows multiple variabilities from each injection in the analysis of these metabolites between positive and negative methods with both reversed phase (RP) and HILIC columns. The partial least squares-discriminant analysis (PLS-DA) method was applied for all detected metabolites in the different treatment groups, including a control group. In this PLS-DA score plot, despite the observation of slightly overlapping points between the control group and QC in negative RP and HILIC modes, the data points for both treatment groups in all injections were clearly separated from the corresponding control groups. The calculated VIP scores and t-test *p* values were used to determine specific metabolites regarding significant changes in the MEHP exposure of the daphnids. For loading plots of dominant peaks, with the selection of a VIP score of higher than 1, and *p* value, calculated by the Kruskal–Wallis nonparametric test in the online XCMS, of less than 0.05 were selected.

3.2 Identification of differentially regulated metabolites

The significant changes in the lipid metabolites were observed after MEHP exposure, including phospholipids, glycerols, and ceramides presented as radar plots in Fig. 2. All of the identified peaks and intensities are denoted in supplementary data 1. Amongst lipid metabolites, Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were identified as typical fragmentations in positive mode 86.10 (C5H12N) and 184.07 (C5H15NPO4Na), and negative mode 168.04 (C4H11O4NP) (, Ostrowski et al. 2005, Pi et al. 2016). Monoacylglycerols were identificated as its typical fragments (fatty acid - H2O + H, Gao et al. 2016). The typical fragmentations were indicated in supplementary data 1.

A total of 283 lipid metabolites were identified and significantly changed after MEHP exposure of *D. magna*, from which 130 and 153 peaks were corresponded to the positive and negative mode, respectively. Changes in the peaks for the treatment group were quantified through relative normalization against the control group and are summarized in the supplementary data 1. Briefly, the average increments in all lipid metabolites were calculated as 72.49 ± 18.59 and 78.27 ± 12.91% for the treatment of 1 mg/L and 2 mg/L MEHP, respectively. Amongst tetrapyrrole like metabolites including biliverdin, bilirubin, phaeophoribide, harderoporphyrin, and coproporphyrin were significantly decreased in MEHP exposure (1 mg/L to control, $p < 0.05$; 2 mg/L to control, $p < 0.05$; Student's t-test). Using LION enrichment analysis in ranking mode comparisons between all three group peak intensities normalization by percentage using a one-way ANOVA F-test, the results are summarized in Fig. 3. In addition, the comparison between control and 2 mg/L of MEHP exposure were presented in Fig. 3B. The 2 mg/L of

MEHP exposure was decreased negative intrinsic curvature. In addition, 2 mg/L of MEHP exposure increased low lateral diffusion. The detailed results of enrichment analysis were represented in supplementary data 2.

Ponsteride A and juvenile hormone III, major biomarkers from the mass peaks for reproduction in crustacean ecdysteroids and juvenile hormones 20-hydroxyecysteroid, were identified by previous reports (Gao et al. 2016, Miyakawa et al. 2018, Nakagawa and Sonobe 2016). However, the peaks corresponding to these biomarkers with identical fragmentation (Destrez et al. 2009, Miyashita et al. 2011) were not detected in our results.

3.4 Changes in gene expression associated with reproduction

The gene expression profiles were represented in Fig. 4. The exposure of *D. magna* to 1 mg/L of MEHP was able to induce the up-regulated mRNA expressions of ecdysone receptor alpha (EcR-A) and ecdysone receptor beta (EcR-B) in both 6 and 24 h, meanwhile MEHP resulted in the down-regulated EcR-A and EcR-B in 48 h (Fig. 4A and B). Although 2 mg/L MEHP exposure has no significant alteration in EcR-B, we assumed that the hormonal receptor needed to mediate the hormonal alteration at least for 48 h. Likewise, 1 mg/L MEHP exposure induced the higher expression level of vitellogenin 2 (Vtg2) in 6 and 24 h but resulted in a dramatic decreased in 48 h exposure (Fig. 4C). No changes in chitinase level was observed in 48 h even in up-regulated in 2 mg/L MEHP at 24 h (Fig. 4D). Juvenile hormone esterase (JHE) and superoxide dismutase (SOD) in levels were not significantly changed in 6 and 24 h (Fig. 4E and F). However, it revealed the decreased JHE level in 48 h MEHP exposure. The total number of neonates per adult female significantly increased in the 21-day test (Table 1). Initial offspring started at 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, 2 mg/L of MEHP. It showed no prominent toxic effects in the 6 and 24 h acute exposures but increased population levels at 21-day for the concentrations investigated (1 and 2 mg/L), with identical effects in both 6 and 24 h continuous exposure experiments as an agonist for EcR A/B, JHE and Vtg2.

Table 1
Number of neonates per adult *D. magna* after the long-term test.

Day	Control	1 mg/L	2 mg/L
9	15	45	32
10	38	62	58
11	64	25	61
12	16	0	0
13	70	106	100
14	26	27	113
15	28	15	0
16	78	38	0
17	40	86	127
18	63	47	68
19	0	0	0
20	101	132	50
21	70	35	144
Total number	609	618	753
Average	60.9 ± 2.60	61.8 ± 2.86	75.3 ± 6.87 ***

There is no offspring during from day 1 to 8. Asterisks indicate significant differences between control group and MEHP exposure group (***, $p < 0.001$). $n = 10$.

4. Discussion

After exposure to MEHP, the lipid metabolism and accumulation and the reproduction rate were significantly increased in *D. magna*. On the one hand, this result is consistent with a previous study in which the treatment with 1 μM DEHP increased lipid accumulation and induced a 1.5-fold increase in neonates per female adult *D. magna* in a 30-day test (Seyoum and Pradhan 2019). On the other hand, post-spawning female daphnids exposed to 1 μM DEHP during the first egg provisioning stage (72 h) contained fewer lipid droplets than the control group (Jordão et al. 2016b). This might suggest that the daphnids favored lipid accumulation for reproduction because phthalates promoted the transfer of accumulated lipids from adults to neonates, resulting in a positive clutch size correlated with lipid storage (Fuertes et al. 2020, Tessier and Goulden 1982). Since MEHP is the major metabolite of DEHP, it is reasonable to speculate that MEHP increased reproduction via the promotion of lipid metabolism.

Our results showed the accumulation of lipid droplets (Fig. 1) and increased lipid metabolites (Fig. 2) in *D. magna* after MEHP exposure. These are also in agreement with the previous report that phthalates prominently alter lipid metabolisms (Seyoum and Pradhan 2019). According to another study, the MEHP exposure significantly increased fatty acids, glycerols, and PCs in villous cytotrophoblasts, while lower levels of several lipid subclasses were observed together (Shoaito et al. 2019). In addition, the DEHP treatment resulted in the increased level of phospholipid compositions (e.g., phosphatidic acid, phosphatidylethanolamine, and PC) in rats (Yanagita et al. 1987) and upregulated the expression of lipid metabolism genes in *Caenorhabditis elegans* (Pradhan et al. 2018).

We also observed the decreased heme-related tetrapyrrole like metabolites. Regarding these results, it is notable that the pheophorbide b is a degradation product of chlorophyll from *C. vulgaris*, used as a food for daphnids (Smith 1984). The decreased pheophorbide b in the MEHP-treated group can be explained by an increased ingestion rate, resulting in the syntheses of lipid metabolites. In another study by Wibe et al., the increased feeding motivation of sticklebacks was observed after exposure to butyl benzyl phthalate for 31 days. This altered feeding behavior might be a physiological compensatory response against environmental stresses, as treatment group became increasingly sticklebacks starved compared to the control group (Wibe et al. 2004). Additionally, the exposure to DEHP was shown to downregulate two hemoglobin genes in *Chironomus tentans* (Lee et al. 2006) and also downregulate the expression of the CYP4G gene in *C. riparius* (Herrero et al. 2017). Since the cytochrome P450 (CYP) contains heme and CYP4G is involved in detoxification processes (Bernabò et al. 2017, Martínez-Paz et al. 2012), the suppressed expression of heme-related genes in our study suggests that these genes may be affected by phthalates.

MEHP can be further 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) with the hydroxylation of carbon side chains (Ito et al. 2014, Nakajima et al. 2015). Of these, the carbon side chain of metabolites can be further cleaved to phthalic acid by *O*-dealkylation (Choi et al. 2012). For example, the cleavage of MEHP can generate a fatty alchol, 6-methyloctan-3-ol (C9H20O). In addition, 2cx-MMHP and MECPP result in the formation of 3-methyl octanoic acid (C9H18O2) and anteisononanoic acid (C9H18O2), respectively. These lipid metabolites are used as the suppliers of lipid precursors, which might be enhanced lipid metabolism and accumulation. The LION enrichment analysis revealed the downregulation of fatty acid with less than 2 double bonds and with 18 carbons or less in MEHP exposure (Fig. 3A). Previous study showed that exposure of phthalates (diethylhexyl phthalate, dibutyl phthalate and diethyl phthalate) in *D. magna* altered expression level involved with lipid related protein genes and lead the lipid accumulation (Seyoum and Pradhan 2019). Also, the expression levels of retinoid X receptor (RXR), ultraspiracle protein and nuclear HR96 receptor genes, which were involved with ecdysteroid signaling pathways, were regulated by fatty acids (Bonneton and Laudet 2012, Fuertes et al. 2019). These results suggest the possibility of increasing usage of less than two double bonds in fatty acid. The usage of fatty acid with 18 carbons or less carbons numbers usage were possibly increased, even under increment of other lipids including phospholipids and glycerol. In addition, the MEHP

exposure at the concentration of 2 mg/mL showed a downregulation of the negative intrinsic curvature of membrane which was inverted cone shape of a head of lipid head groups (Zelnik et al. 2020). The membrane curvature is also affected by the lipid composition rearrangement and membrane into a bilayer (Ashery et al. 2014, Destrez et al. 2009, Furber et al. 2009). Lateral diffusion of proteins is interrelated to controlling dynamics and functions in cell membranes (Ronchi et al. 2008), and increased low lateral diffusion also correlated with membrane fluidity (Ballweg et al. 2020). In addition, Lateral diffusion of proteins can be affected by their binding interaction with other lipid containing membranes and cytoskeletal proteins (Alenghat and Golan 2013). However, the mechanistic relationships between lipid metabolism and physiological changes such as fecundity and hatching and cellular responses by the MEHP exposure are intricate, thus more studies are needed to explore further effects of MEHP metabolites in lipid metabolism of *D. magna*. It was reported that butyl benzyl phthalate (BBP) upregulated the expression level of the ecdysone receptor (EcR) gene in *Chironomus 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 in 24 h exposure, and EcR gene was downregulated in 48 h from 0.001 µg/L to 1 µg/L BBP in *C. riparius* larvae (Herrero et al. 2015). In our study, EcR gene was upregulated in both 6 h and 24 h, indicating that upregulated EcR gene expression after MEHP exposure can affect the major mechanism of the lipid accumulation and lipid metabolism as BBP (Herrero et al. 2015). In contrast, EcR, Vtg2 and JHE gene expressions were downregulated at 48 h exposure in the present study. These effects were agreed with the previous BBP study that the time-dependent response of the EcR could repress the transcription in after 48 h treatment (Herrero et al. 2015).

Vtg2 can serve as a potential biomarker in endocrine disruption of phthalates in aquatic organisms as it is more responsive to xenobiotics than Vtg1 (Hannas et al. 2011). For example, the expression level of Vtg1 was not affected by three phthalates, DEHP, diethyl phthalate (DEP), and dibutyl phthalate (DBP), in contrary to that of Vtg2 was downregulated by DEP and DBP after 24 h of exposure (Seyoum and Pradhan 2019). An increased number of neonates per adult female could be attributed to the altered expression of Vtg2. When fenoxycarb (juvenile hormone analog) was applied and significantly downregulated the expression of vitellogenin, a reduced total number of offspring per adult female was observed (Kim et al. 2011). In the present study, Vtg2 was up-regulated in MEHP exposure of 24 h. However, Vtg2 and JHE expression were down repressed by 1 or 2 mg/L MEHP treatment at 48 h. Although these repressions might be followed by rapid compensative and feedback regulation, future study should be focused on time-dependent regulation of EcR and JHE signal to reproduction change.

5. Conclusion

The MEHP exposure to *D. magna* increased lipid droplets, various lipid metabolites, EcR and Vtg2 expression. In addition, the increased lipid storage was correlated with the increased reproduction rate after MEHP exposure for three weeks. This result demonstrated that the phthalates could induce lipid accumulation in daphnids, similar to mammals, which would further cause population changes and consequently affect ecological systems. Further research is required to clarify how phthalates modulate

the lipid accumulation associated with the EcR and Vtg2 pathway to understanding the underlying molecular mechanisms responses to MEHP exposure.

6. Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

Datasets generated during the present study are not publicly available but are available from the corresponding author on reasonable request. Materials are not applicable.

Authors' contributions

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

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Competing interests

The authors declare that there is no conflict of interest.

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7. References

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Figures

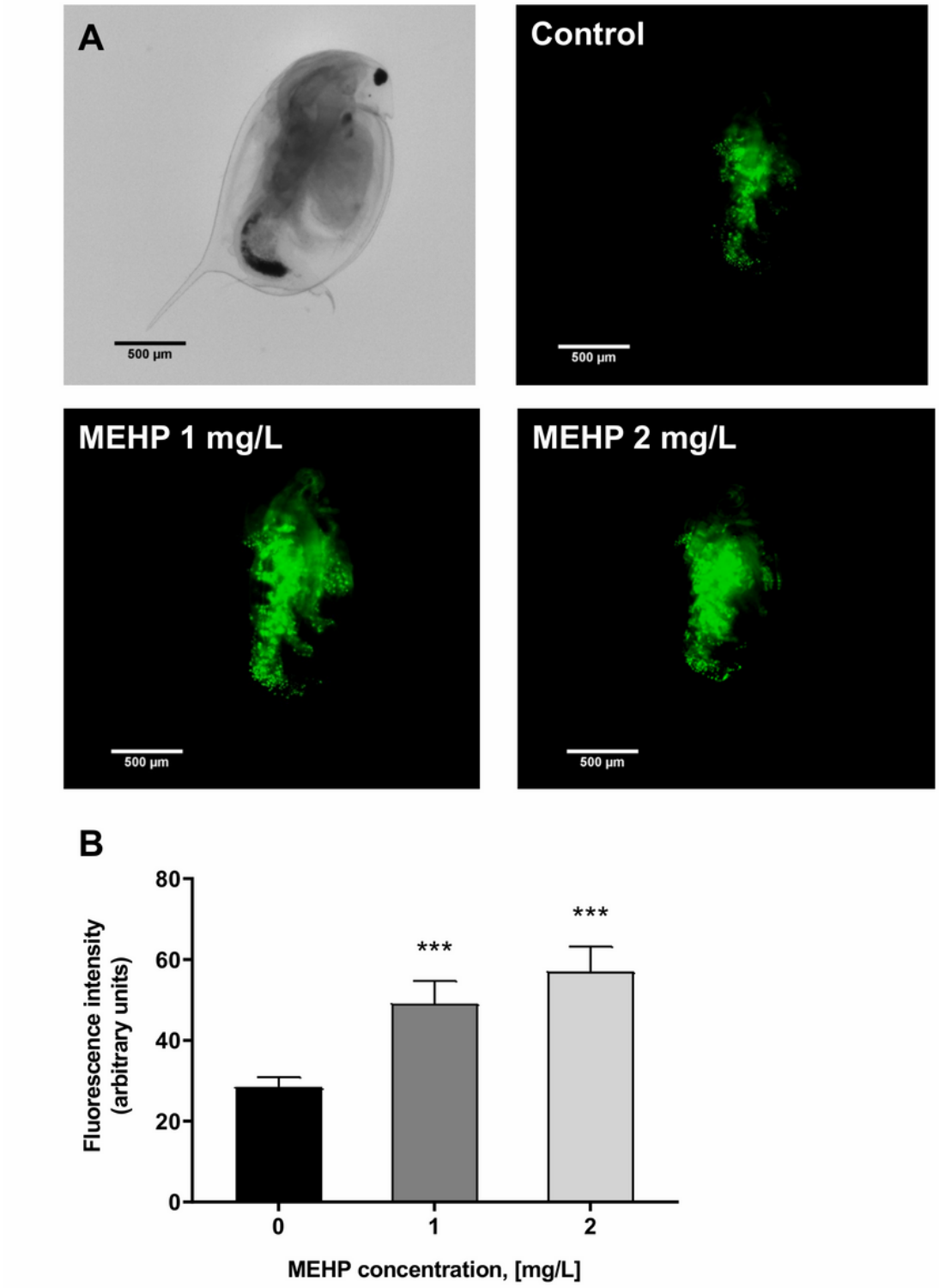

Figure 1

The alteration of lipid droplets in D. magna. (A) Individual daphnids were observed under a fluorescence microscope following 96 h exposure to MEHP. (B) BODIPY fluorescence intensity of lipid droplets, quantified from the microscopy images. (***, $p < 0.001$). n = 5.

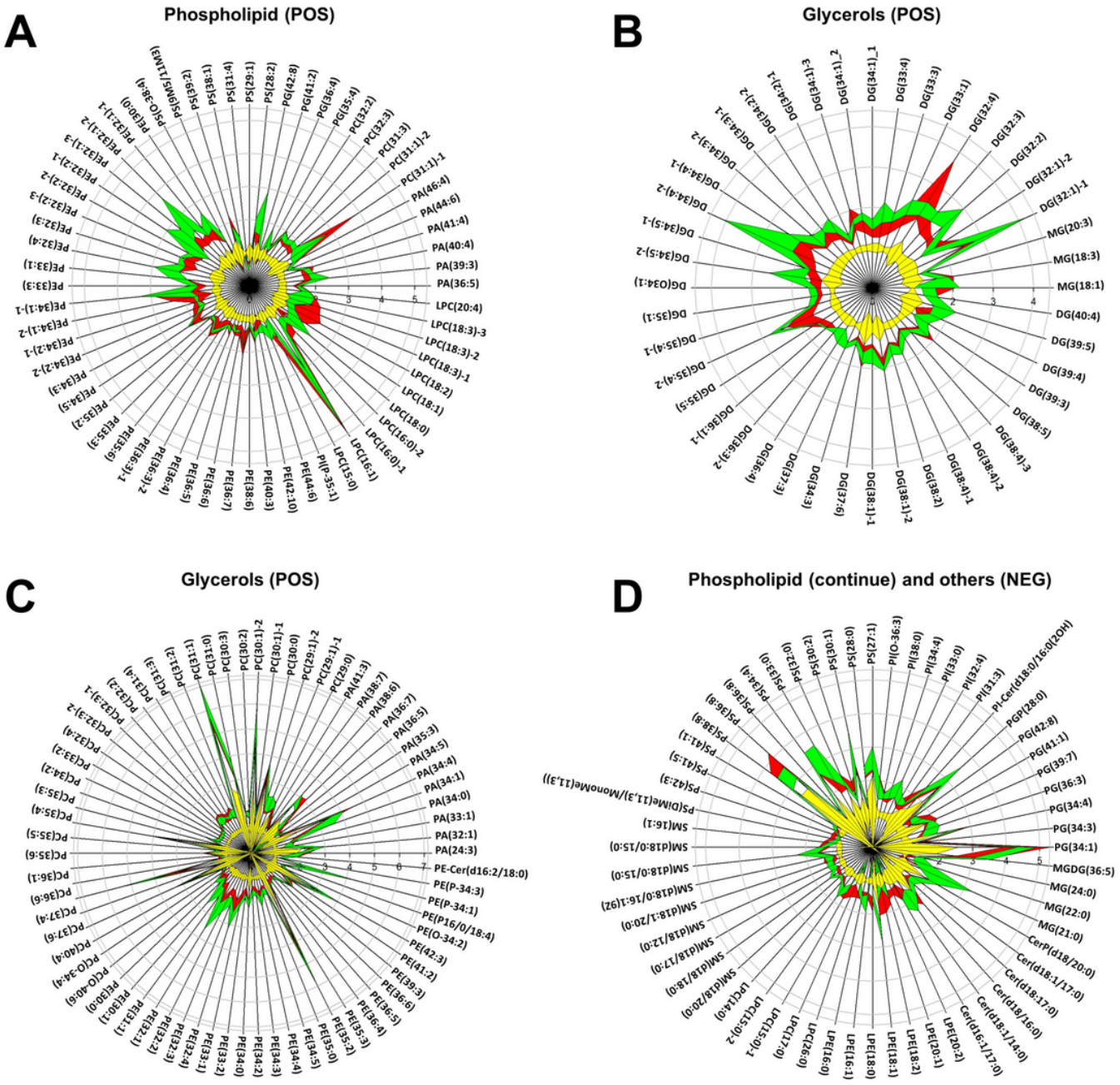

Figure 2

Radar plot to summerize the lipid metabolites analysis for the neonates collected from the 48 h exposures to 1 mg/L and 2 mg/L of MEHP. The yellow indicates the control, and the red and green indicates the 1 mg/L and 2 mg/L of MEHP treatment, respectively. Data separately presented in (A) phospholipids in a positive mode, (B) glycerols in a positive mode, (C) and (D) phospholipids and other lipids in a negative mode. The data were shown by mean ± SD compared to control group.

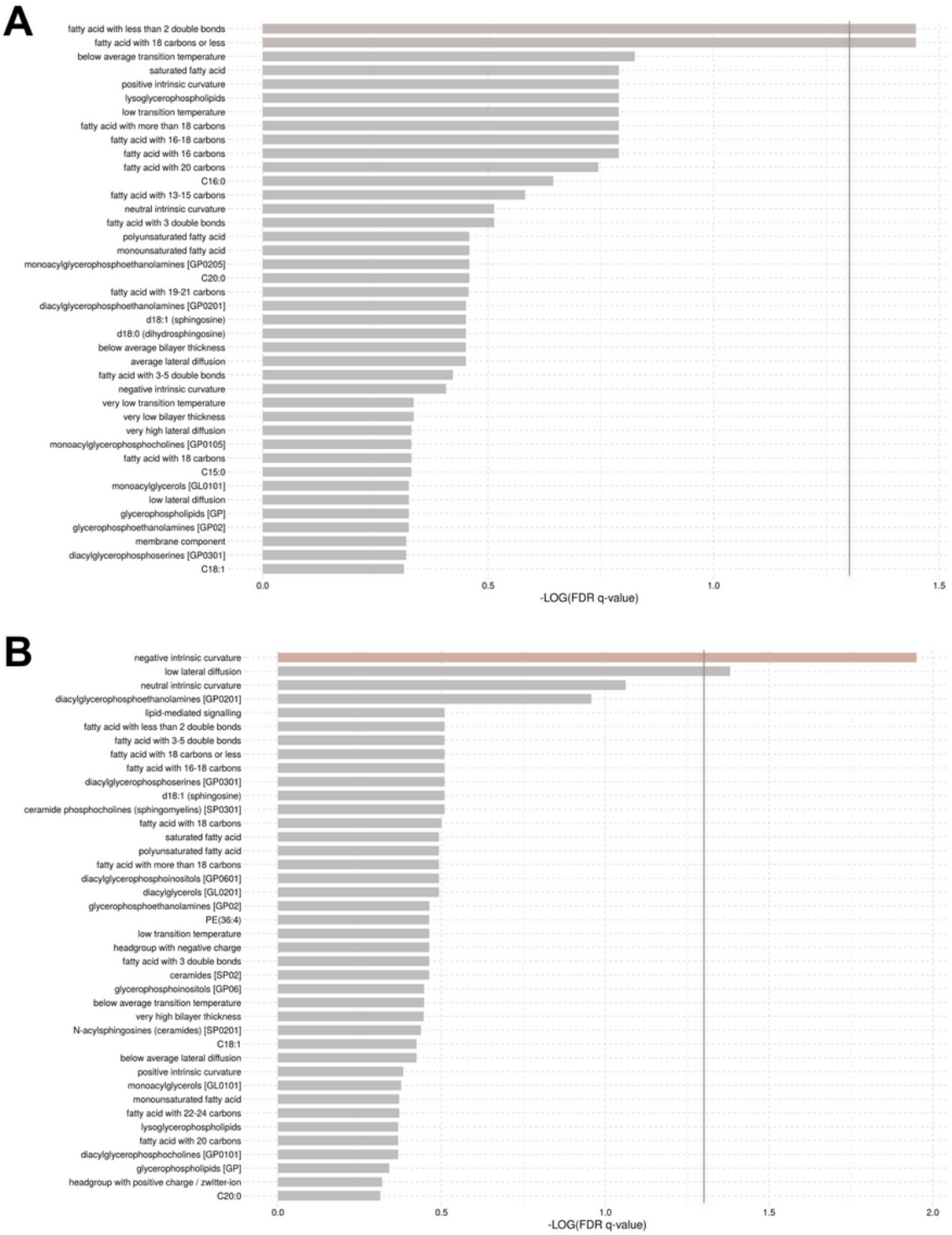

Figure 3

Enriched lipid ontology (LION) enrichment analysis, (A) in ranking mode of comparisons of control, 1 mg/L of MEHP and 2 mg/L of MEHP exposure groups via one-way ANOVA F-test. (B) comparison between control and 2 mg/L of MEHP exposure groups via t-test. Gray vertical line indicates the cut-off value of significant enrichments ($q < 0.05$). Specific enrichment analysis results and LION term lipid associations are provided in the Supplementary Data 2.

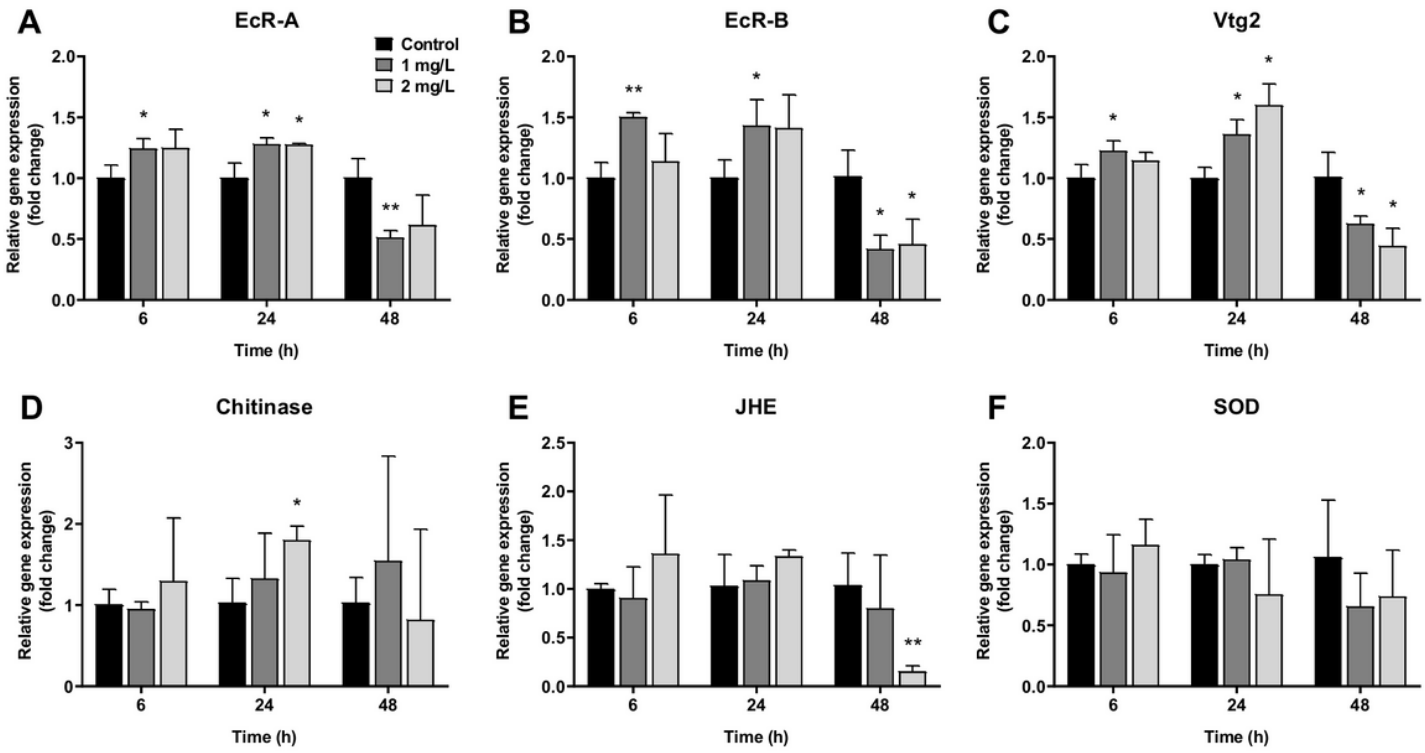

Figure 4

Comparisons of expression levels of reproductive genes and SOD gene in D. magna after 6, 24 and 48 h of MEHP exposures. Data are expressed as mean ± SD of three repeated experiments. Asterisks indicate significant different between control group and MEHP exposure group (*, $p < 0.05$; **, $p < 0.01$).

Supplementary Files

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