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Research article

Di (2-ethylhexyl) phthalate effects on the growth, development, and reproduction of *Moina macrocopa* (Crustacea: Cladocera)

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ABSTRACT

Di (2-ethylhexyl) phthalate (DEHP) is used as a plasticizer in plastics. The effects of DEHP on terrestrial vertebrates have been extensively reported but the effects of DEHP contamination on aquatic ecosystems have not been thoroughly studied. Since water bodies are one of the main mediums through which DEHP is released worldwide, the impacts of DEHP contamination should be manifested in water fleas. Therefore, maternal *Moina macrocopa* were exposed to 1, 10, 100, and 1000 μg/L concentrations of DEHP. Changes in growth and reproduction were evaluated. The findings demonstrated that DEHP exposure did not have a negative impact on growth or the ability to reproduce. An analysis of the ovary yolk body (YB) demonstrated that the average size and number of yolk bodies (YBs) produced by *M. macrocopa* exposed to 1000 μg/L DEHP were not significantly different to the average size and number of YBs produced in blank control and solvent control conditions. These outcomes support the cellular pathology data gathered by other researchers. Nevertheless, when *M. macrocopa* was exposed to 1000 μg/L DEHP for five days, a significant increase in YB numbers was observed with changes in YB morphology. The critical cellular pathology of YB showed morphological abnormalities, including rod-shaped YBs, and YB density was higher than in the blank and solvent controls. Even though these results suggest that antioxidative stress can be induced by DEHP exposure, growth, and reproduction were not significantly different among exposed water fleas compared to fleas in the blank and solvent controls. The result was attributed to the antioxidant response of the water flea. In conclusion, the present study enhances our understanding of previous findings from risk assessments of DEHP contamination in aquatic ecosystems.

1. Introduction

Di (2-ethylhexyl) phthalate (DEHP) is frequently used to soften polyvinyl chloride but is one of the most toxic phthalates for the human organism [1–3]. Because phthalate plasticizers do not chemically bind to polyvinylchloride, DEHP can leach, migrate or

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evaporate into air, interior and exterior dirt and dust [4,5], rainwater [6], surface runoff, untreated and treated wastewater [7,8], municipal wastewater [9,10], wastewater sludge [11], soil, sediment [12–14], rivers and lakes [12,13,15–18]. The contamination of aquatic systems with DEHP has been reported in many countries. In German rivers, levels of DEHP between 0.33 and 97.8 μg/L were found [12]; in the Aire River of the United Kingdom, concentrations between 0.36 and 21.0 μg/L were found [15]; in the Klang River of Malaysia found levels were between 3.1 and 64.3 μg/L [16]; and in the Rieti District of Italy concentrations were detected between 0.7 and 31.2 μg/L [17]. In 14 rivers of Taiwan reported levels ranged from non-detectable concentrations to 18.5 μg/L [18]; in water samples from the deltas of major rivers discharging into the Gulf of Thailand detected concentrations up to 8.64 μg/L were reported [13]; and in water from the U-Tapao Canal, Songkhla, Thailand detected concentrations ranged from 1.28 to 5.28 μg/L [19].

In aquatic environments, DEHP is a persistent organic pollutant that has shown toxicity to numerous organisms [20–30]. The adverse effects of DEHP on human growth and reproduction led to its classification as an emerging endocrine disruptor and epigenetic toxicant [31]. Phthalates can bind to and activate human estrogen receptors (ER) *in vitro* [32] and exposure to high concentrations of DEHP disrupted spermatogenesis in adult zebrafish and reduced spawned oocytes [29]. In contrast to these adverse effects, DEHP has been reported to induce enzymatic antioxidant activities in aquatic organisms such as fish [33–37], the zooplankton *D. magna* [38], and the pearl oyster [39].

Although the toxicity of DEHP has been reported in many studies of aquatic vertebrates, few studies of aquatic organisms have dealt with sublethal DEHP concentrations and chronic exposure. DEHP can be degraded in a process that involves direct oxidation, hydroxylation, breaking of the ester link, and decarboxylation [40]. The susceptibility of aquatic organisms to DEHP toxicity can be tested in a variety of aquatic organisms. In Thailand, the water flea *Moina macrocopa* is an abundant indigenous freshwater macroinvertebrate. It is a species whose populations are significant contributors to aquatic food webs in epicontinental habitats [41]. Furthermore, *M. macrocopa* is a good animal model for an ecotoxicological study because it is sensitive to pollutants [42] and has a short life cycle of approximately seven days under optimal conditions [43]. Although phthalate esters in the environment can be reduced via biodegradation [8,11], the severity of DEHP contamination in tropical aquatic ecosystems has not yet been thoroughly studied. Thus, the findings of this study describe the sublethal effects of DEHP on *M. macrocopa* in conditions of long-term, continuous exposure. Multiple toxic endpoints were investigated, including effects on growth, development, and reproduction.

2. Materials and methods

2.1. DEHP preparation

Di (2-ethylhexyl) phthalate (DEHP) (purity >98%) was purchased from Sigma–Aldrich, St. Louis, MO, USA. A stock solution of DEHP was prepared in 0.1 v/v dimethyl sulfoxide (DMSO) (Sigma–Aldrich, St. Louis, MO, USA).

2.2. Culture condition

A single clone of *M. macrocopa* was used in all the experiments. The *M. macrocopa* breeding stock was maintained as a pure parthenogenetic culture in a 250 mL beaker of culture medium which contained freshwater (pH 7–8; total hardness above 140 mg $CaCO_3$/L; dissolved oxygen concentration >3 mg/L). The culture medium was adapted from a formula of the Pathum Thani Inland Aquaculture Research and Development Center, Department of Fisheries. One liter of culture medium of *M. macrocopa* contained 0.4 g/L urea fertilizer (46–0–0), 0.4 g/L ammonium phosphate fertilizer (16–20–0), 0.6 g/L $CaOH_2$, and 1.2 mL of monosodium glutamate effluent. *M. macrocopa* was nourished with fresh *Chlorella* sp. at a density of approximately 1.2×10^5–1.4×10^5 cells/mL of *Chlorella* sp. The incubation temperature was 28 ± 5 °C, and the photoperiod was 12L:12D with an average light intensity of 3.6 MJ/m^2/day. Healthy *M. macrocopa* neonates (at around 24 h) were exposed to treatments in 250 mL beakers filled with 50 mL of culture medium.

2.3. Sublethal toxicity test (chronic exposure)

M. macrocopa neonates were exposed from 24 h to postnatal day 8 to four sublethal concentrations of DEHP in culture medium. In this study, the diluent was 0.1%v/v DMSO, which is considered to be safe for almost all cells [44]. The maximum LC_{50} value for *M. macrocopa*, based on the results of 24 h DEHP exposure, was reported to be 4410 μg/L [45]. Environmental freshwater DEHP concentrations were reported to range from non-detected to 97.8 μg/L [12,13,15–18]. DEHP concentrations in municipal wastewater were reported to range from 1.74 to 182 μg/L [9,10]. From the above data, the four sublethal concentrations of DEHP applied to *M. macrocopa* were 1, 10, 100 and 1000 μg/L. To obtain these concentrations, DEHP was diluted in 0.1% v/v DMSO. Two control groups were applied: a blank control of 0 μg/L DEHP and a solvent control of 0.1% v/v DMSO. Treatments and controls were evaluated using three replicates, each with three *M. macrocopa* neonates per treatment in 250 mL glass beakers containing 50 mL of test solution. The temperature and photoperiod conditions were similar to the culture conditions. The adverse effects of long-term exposure to DEHP on the growth and reproduction of *M. macrocopa* were assessed daily for approximately eight days after exposure or to the end of the lifespan.

2.3.1. Effect of DEHP on M. macrocopa growth

M. macrocopa neonates tested in 0, 1, 10, 100, 1000 μg/L concentrations of DEHP. At least three replicates were used to test growth. Each replicate was repeated with three *M. macrocopa* per concentration level (Fig. 1). The total number of samples per treatment was n ~18. Neonates were tested in 250 mL glass beakers containing 50 mL of test solution and the exposure condition was similar to the

culture condition. Growth was assessed from body length at the mature stage. The body length of individuals was observed 48 h after exposure by capturing images under an optical microscope with a 5x objective lens (Motic, China) fitted with an eyepiece camera (Xenon TC3100, China). The captured images were then analyzed with the ToupView 3.7 image software to determine body length from head to tail.

2.3.2. Effect of DEHP on *M. macrocopa* reproduction

M. macrocopa reproduction was tested in 0, 1, 10, 100, and 1000 μg/L concentrations of DEHP. At least three replicates were used. Each replicate was repeated with three *M. macrocopa* per treatment (n ~ 18 per treatment) (Fig. 1). Neonates were tested singly in 250 mL glass beakers containing 50 mL of test solution. The exposure condition was similar to the culture condition. Individuals were exposed to DEHP for their whole lifespan. Monitored reproduction characteristics included the time in days to the first reproduction (initial time of reproduction), and the total number of neonates per female (offspring/female). Once reproduction commenced, brood size was observed every day, and the mother was separated from neonates. This procedure was repeated until the mother died.

2.3.3. Cellular pathology

The maximum DEHP concentration of 1000 μg/L was used in this study of 24 h-neonates cultivated singly in 250 mL glass beakers containing 50 mL of test solution. A blank control and solvent control (0.1%v/v DMSO) were included. The exposure condition was similar to the culture condition. Individuals were exposed to DEHP for their whole lifespan. To avoid premature mortality and cell abnormalities due to premature mortality from DEHP exposure, *M. macrocopa* was sampled for observation of cellular pathology after five days of exposure. Specimens were preserved in 2.5% glutaraldehyde solution and prepared for observation of cellular pathology according to the procedure reported by the Department of Tropical Pathology, Faculty of Tropical Medicine, Mahidol University, Thailand. Cellular pathology of the maternal (P0) ovary was examined under a transmission electron microscope (TEM, Hitachi HT-7700) at the Department of Tropical Pathology, Faculty of Tropical Medicine, Mahidol University. The ultrastructure of the maternal ovary was photographed. There were at least three replicate samples in each group. In the maternal ovary, the yolk body (YB) was clearly visible in TEM images. Ovaries were photographed under an optical microscope with a 5x objective lens (Motic, China) fitted with an eyepiece camera (Xenon TC3100, China). The average size of YBs was determined from the YB surface area by analyzing captured images with ToupView 3.7 software. The surface area (A) of YBs was calculated by multiplying π by a (the semi-major axis) and b (the semi-minor axis) ($A = \pi ab$).

2.4. Data analysis

All data are expressed as means with standard deviation. After analyzing the data using Boxplot, outliers or incorrectly observed specimens were eliminated. The statistical differences were evaluated by one-way ANOVA followed by Duncan's test and the significance level was 0.05. The Kolmogorov-Smirnov test and Shapiro-Wilk test were applied for normal distribution, and homogeneity of variance was assessed by the Levene test. One-way ANOVA and post hoc were used to test significant differences among the groups. SPSS software was used for the statistical comparison.

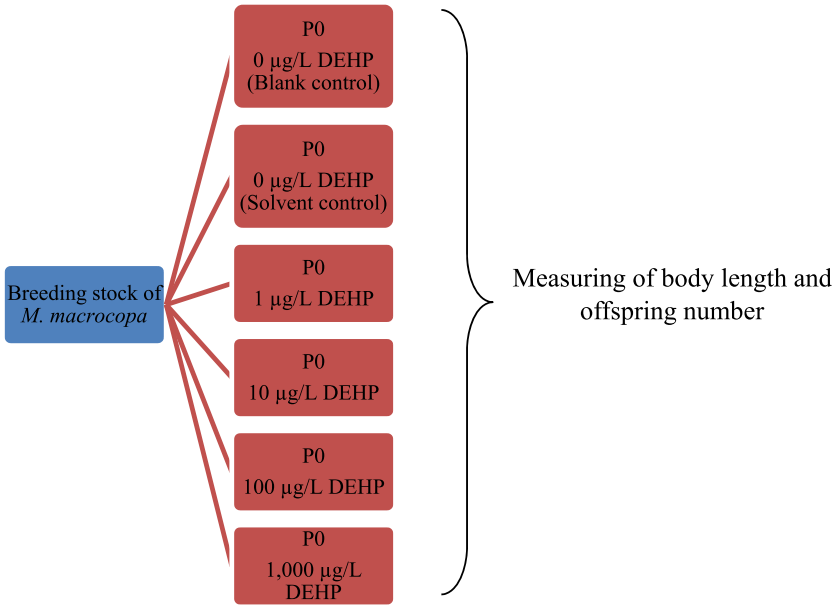

Fig. 1. Experimental design of the growth and reproductive assay of *M. macrocopa* exposed to DEHP. P0 (parental generation) was continuously exposed to different concentrations of DEHP until death.

3. Results

3.1. Effect of DEHP exposure on M. macrocopa reproduction

Three independent biological replicates were performed with at least three *M.macrocopa* in each group. The time to the first production of neonates (initial time) after the parental generation (P0) was exposed to each treatment was recorded. There was no difference in the initial time of reproduction between the solvent control and blank control (Table 1). In addition, the initial time of reproduction was not significantly different between DEHP treatments and the solvent control. The average initial time of reproduction was one day in every treatment and the average number of neonates was 20–22 per female.

The brood size of the P0 was counted as total progeny. To measure brood size, three independent biological replicates were performed with at least three *M.macrocopa* in each group (Fig. 2). There was no significant difference in brood size between the blank control and solvent control, and the number of offspring in the solvent control was not significantly different from the number of offspring in each DEHP treatment. The average numbers of neonates produced by the P0 of blank control, solvent control, 1, 10, 100, and 1000 μg/L DEHP treatments were 21, 20, 22, 21, 20, and 20 per female, respectively.

3.2. Growth of M. macrocopa exposed to DEHP

The survival rate in both controls and all DEHP treatments was 100% (Table 2). No significant difference was observed in the body length of P0 *M. macrocopa* in the blank and solvent controls (Fig. 3). Furthermore, no significant difference in the body length of P0 *M. macrocopa* was observed between DEHP treatments (1, 10, 100, and 1000 μg/L) and the solvent control (0.1% v/v DMSO) (Fig. 3).

3.3. Cellular pathology

Ovarian cortex tissue ultrastructures were studied in P0 *M. macrocopa* in both controls and in the 1000 μg/L DEHP treatment. The cellular pathology of P0 ovaries was observed under a light microscope (Fig. 4 (A)). Once the point of the ovarian cortex was located, it was observed under the transmission electron microscope (TEM) (Fig. 4 (B, C, D, E)). P0 *M. macrocopa* exhibited normal growth in both controls and 1000 μg/L DEHP treatment (Fig. 4 B, C, D). (Fig. 4). However, P0 *M. macrocopa* in the 1000 μg/L DEHP treatment exhibited abnormal YBs found as rod-shaped oval or irregular cells with indistinct membranes (Fig. 4 (E)).

3.4. Yolk body (YB) size in ovarian cortex tissue of M. macrocopa exposed to DEHP

The average size of the YB in ovarian cortex tissue of P0 *M. macrocopa* exposed to 1000 μg/L DEHP was determined by analyzing TEM images (Hitachi HT-7700) with ToupView 3.7 software. YB size was calculated from the YB surface area. The average sizes of YB in blank control, solvent control, and the 1000 μg/L DEHP treatment were 234.32 ± 70.87, 201.65 ± 66.46, and 195.80 ± 68.65 nm^2, respectively (Table 3). The average size of YB in the 1000 μg/L DEHP exposure was the smallest but was not significantly different from the average size of YB in the solvent control (0.1%v/v DMSO).

4. Discussion

Numerous environmental compartments can be contaminated with DEHP. This study observed whether DEHP contamination in water causes adverse effects on the aquatic organism *M. macrocopa.* If the numbers of *M. macrocopa* decrease, numerous other species will be affected, such as the next level in the food chain. Prolonged exposure of *M. macrocopa* to 1, 10, 100, 1000 μg/L concentrations of DEHP revealed changes in demographic responses not only in growth variables but also in reproductive parameters.

In this study, a blank control and solvent control were used. The solvent control was 0.1 v/v DMSO, which was used as the solvent for DEHP treatments. No significant decrease in growth or reproduction was found between *M. macrocopa* in the solvent control and *M. macrocopa* exposed to 1, 10, 100, and 1000 μg/LDEHP. Furthermore, *M. macrocopa* was not affected by 0.1 v/v DMSO compared with the blank control. Previous studies [21,23,26–31] reported adverse effects on the growth and reproduction of freshwater *M. macrocopa* due to DEHP, acute toxicity due to dibutyl phthalate [46], and chronic effects on survival and reproduction due to butyl benzyl phthalate (two successive generations of the cladoceran) [47].

Table 1
Reproductive parameters of the maternal generation *M. macrocopa* (P0) exposed to various concentrations of DEHP with normal diets.

Concentration (μg/L)	The initial time of reproduction[a] (day)	The average number of neonates (neonates per female)
Blank control	1 ± 0	21
Solvent control	1 ± 0	20
1	1 ± 0	22
10	1 ± 0	21
100	1 ± 0	20
1000	1 ± 0	20

[a] Initial time of reproduction was the time to the first production of neonates (24 h after hatching) after exposure of the P0 to each treatment.

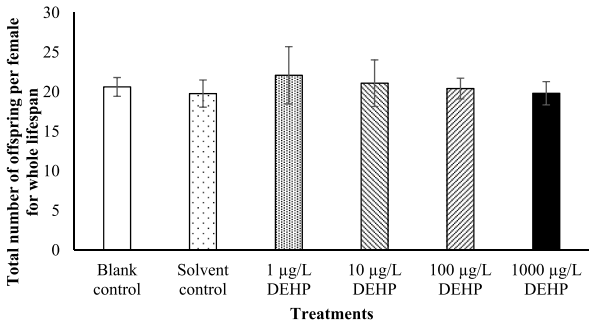

Fig. 2. Cumulative numbers of neonates produced by *M. macrocopa* exposed to 1, 10, 100, and 1000 µg/L DEHP with normal diets. Error bars represent standard deviations. ANOVA and post hoc test were used to identify significant differences between treatments and solvent control (0.1% v/v DMSO). n = 12.

Table 2
The survival rate of maternal *M. macrocopa* (P0).

Chemical concentration (µg/L)	Survival rate (%)
Blank control	100
Solvent control	100
1	100
10	100
100	100
1000	100

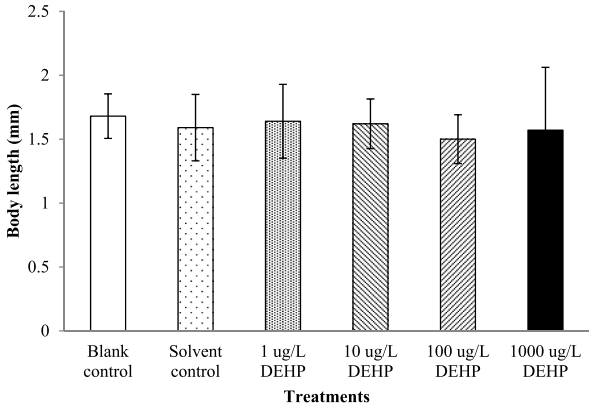

Fig. 3. Body length of maternal *M. macocopa* (P0) exposed to 1, 10, 100, and 1000 µg/L DEHP with normal diets. Body length was measured by analyzing photomicrographs with ToupView 3.7 image software. Error bars show standard deviations. ANOVA and post hoc test were used to identify significant differences between treatments and solvent control (0.1% v/v DMSO).

Differences between the findings of the present study and previous DEHP toxicity studies suggest that the antioxidant properties of DEHP resolved the critical situation. This outcome was consistent with studies that found that DEHP might stimulate enzymatic antioxidant activity (antioxidant defense) in aquatic organisms, including several types of *D. magna* [38], fish [33–37], the pearl oyster [39] and harlequin fly *Chironomus riparius* [48].

Furthermore, our observation of cell pathology after DEHP exposure for five days showed that yolk bodies were smaller in the 1000 µg/L DEHP treatment than in the solvent control, but they were not significantly different. Nevertheless, there was a significant increase in YB numbers in the 1000 µg/L DEHP treatment compared with the solvent control and YB density was also higher in the 1000 µg/L DEHP treatment than in the solvent control. Moreover, the critical cellular pathology of YB in the 1000 µg/L DEHP treatment showed some abnormal morphologies, such as rod-shaped YB. The abnormal YB morphology from prolonged DEHP exposure was similar to yolk sac abnormalities observed in zebrafish [49].

The results showed that DEHP exposure did not cause significant differences in body length and offspring number, but some features at the cellular level were different between DEHP treatments and the solvent control. Since DEHP exposure protects against oxidative stress [33–39], and DEHP can be degraded by a degradation process [40], we hypothesized that differences would be found in YB density, size and shape between DEHP treatments and the solvent control. However, it is unlikely that DEHP can affect

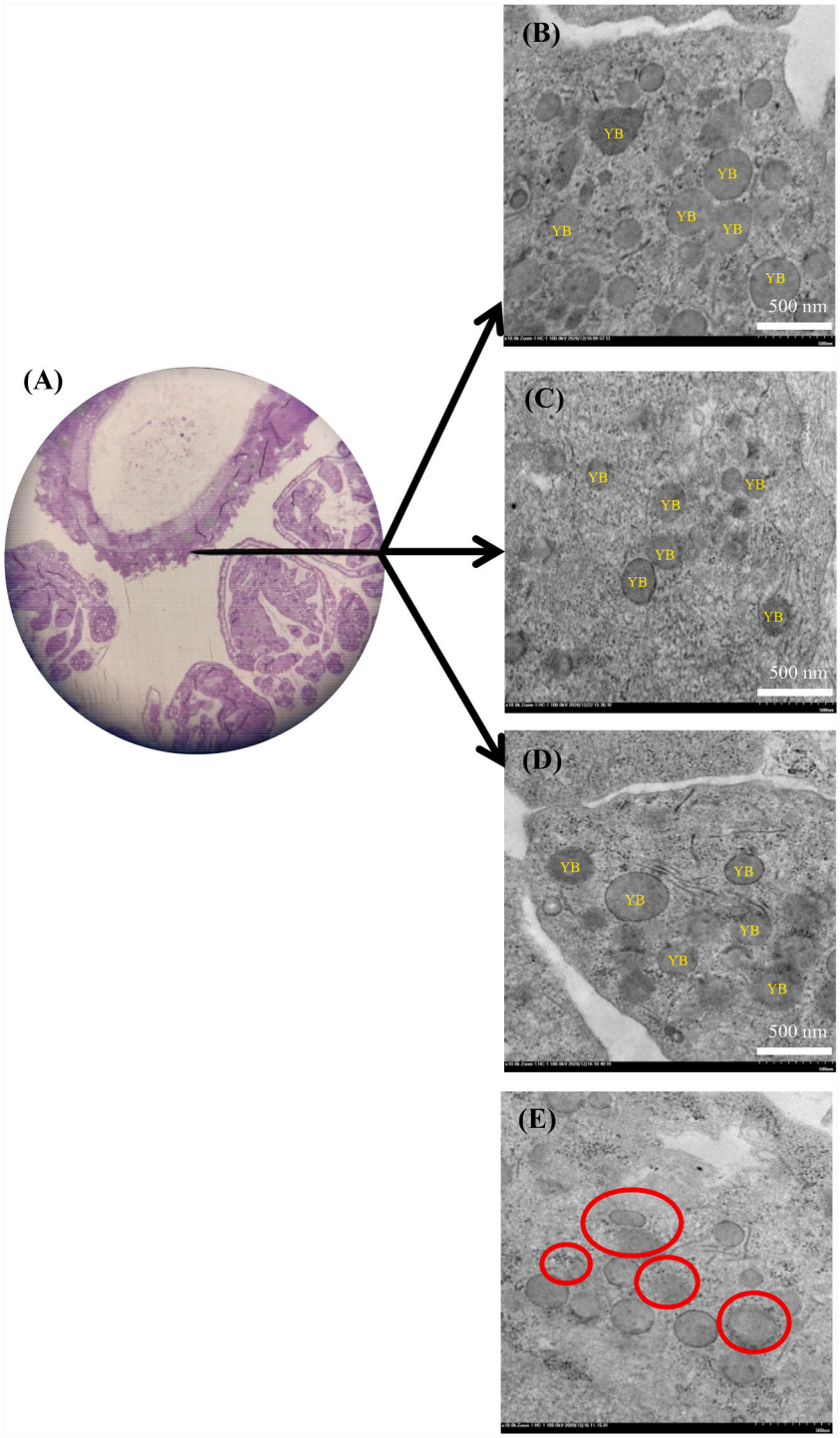

Fig. 4. Cellular pathology of ovarian tissue in maternal *M. macrocopa* (P0). The sections were obtained from a normal adult ovarian cortex showing normal round or oval yolk bodies. **(A)**: A photomicrograph (40x) of a normal ovarian cortex. Ultrastructures of ovarian tissue were studied under the transmission electron microscope (TEM) after *M. macrocopa* was exposed for 5 days continuously to a blank control **(B)**, a solvent control **(C)**, and 1000 μg/L DEHP **(D)**. Yolk bodies with irregular shapes and unclear membranes were found in the 1000 μg/L DEHP treatment **(E)**. Scale bar: 500 nm.

Table 3
Average size of yolk body in ovarian cortex tissue of adult *M. macrocopa*.

	Size (sq nm)	SD	Sampling size
Blank control	234.32	70.87	36
Solvent control	201.65	66.46	32
1000 μg/L DEHP	195.80	68.65	37

vitellogenin and yolk body formation if antioxidant mechanisms are stimulated. Therefore, this study does not indicate the presence of defective growth, development or reproduction of *M. macrocopa* during prolonged exposure to DEHP.

Long-term DEHP exposure in *Chironomus riparius* [48] affected biomarkers which indicated the upregulation of *C. riparius* metallothionein mRNA, and the level of vitellogenin mRNA was significantly increased. Metallothionein is a superfamily of cysteine-rich proteins related to metal metabolism, detoxification of heavy metals, and immune responses such as protection against ionizing radiation and antioxidant defenses [50]. Vitellogenin is a precursor protein of egg yolk vitellin that supplies energy reserves in oviparous vertebrates and invertebrates [51]. The results of the present study showed that no change occurred in the growth and reproduction of *M. macrocopa* exposed to 1, 10, 100, and 1000 μg/L DEHP (indicated as a maximum level of DEHP contamination in the environment). As a result of the findings above, which were related to antioxidant mechanisms and the DEHP degradation process, various hypotheses were inferred to explain these mechanisms. The hypotheses included firstly DEHP absorption, secondly, environmental concentration, and thirdly, xenobiotic metabolism. Firstly, DEHP was not absorbed by *M. macrocopa* through the ingestion of water. Secondly, because the applied DEHP concentrations from 1 to 1000 μg/L were lower than the LC_{50} of *M. macrocopa* (4410 μg/L) [45], the results did not show significant data. Finally, monoester metabolites produced after DEHP exposure, such as mono-2-ethylhexyl phthalate, are less toxic than DEHP [2], the adverse effects of continuous exposure to DEHP did not appear.

From the results of this study, we can assess the biotic potential and environmental resistance of *M. macrocopa*. We investigated the relationship between DEHP exposure and oxidative stress in *M. macrocopa* and the data provided useful information for understanding the environmental resistance of *M. macrocopa* to antioxidant properties.

5. Conclusion

The results of this study informed the sub-lethal effects on *M. macrocopa* of chronic exposure to DEHP. Effects of exposure on growth and reproduction were investigated. The effects on the growth and reproduction of *M. macrocopa* exposed to DEHP at the amount found in the environment were not significant.

For the above discussion about the antioxidant mechanism, this study can help assess the biotic potential and environmental resistance of *M. macrocopa*. Furthermore, this study implied a correlation between DEHP exposure and oxidative stress in *M. macrocopa*. These data provided useful information for understanding the environmental resistance to antioxidant properties.

Ethical statement

M. macrocopa is an invertebrate and not included in the list for consideration of Animal Ethics Committees, Animal for Science Act, 2015. Therefore, licenses or permits were not required for this study.

Data availability statement

Muenpo, Chutchawan (2023), "Data in Brief_Heliyon", Mendeley Data, V1, https://doi.org/10.17632/yf5j9s28ts.1.

CRediT authorship contribution statement

Amornrat Chaikritsadakarn: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Banchong Witthayawirasak:** Funding acquisition, Conceptualization. **Dudsadee Muenhor:** Validation. **Ronald D. DeLaune:** Validation. **Chutchawan Muenpo:** Validation, Supervision, Methodology.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Amornrat Chaikritsadakarn (Ph.D. student) reports financial support was provided by the Research Program of Municipal Solid Waste and Hazardous Waste Management under the grant of Center of Excellence on Hazardous Substance Management (HSM), Bangkok, Thailand with grant number HSM-PJ-CT-17-02. Asst. Prof. Dr. Chutchawan Muenpo reports equipment, drugs, or supplies and writing assistance were provided by Prince of Songkla University, Thailand. Asst. Prof. Dr. Dudsadee Muenhor reports administrative support, article publishing charges, and statistical analysis were provided by Prince of Songkla University, Thailand. Associate Prof. Dr. Banchong Witthayawirasak reports administrative support, equipment, drugs, or supplies, and statistical analysis were provided by Prince of Songkla University, Thailand. Professor Ronald D. DeLaune reports writing assistance was provided by Louisiana

State University, Baton Rouge, LA 70803, USA. Asst. Prof. Chutchawan Muenpo reports a relationship with Prince of Songkla University, Thailand that includes: employment. Dr. Chutchawan Muenpo has patent No patent issued to No patent. 1. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper. 2. We do not have patent in this experiment.

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