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Exposure to DEHP and MEHP from hatching to adulthood causes reproductive dysfunction and endocrine disruption in marine medaka (*Oryzias melastigma*)

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ABSTRACT

Concern has increased regarding the adverse effects of di-(2-ethylhexyl)-phthalate (DEHP) on reproduction. However, limited information is available on the effects of DEHP in marine organisms. The aim of the present study was to examine whether long-term exposure to DEHP and its active metabolite mono-(2-ethylhexyl)-phthalate (MEHP) disrupts endocrine function in marine medaka (*Oryzias melastigma*). Marine medaka larvae were exposed to either DEHP (0.1 and 0.5 mg/L) or MEHP (0.1 and 0.5 mg/L) for 6 months, and the effects on reproduction, sex steroid hormones, liver vitellogenin (VTG), gonad histology and the expression of genes involved in the hypothalamic-pituitary-gonad (HPG) axis were investigated. Exposure to DEHP, but not MEHP, from hatching to adulthood accelerated the start of spawning and decreased the egg production of exposed females. Moreover, exposure to both DEHP and MEHP resulted in a reduction in the fertilization rate of oocytes spawned by untreated females paired with treated males. A significant increase in plasma 17β-estradiol (E2) along with a significant decrease in testosterone (T)/E2 ratios was observed in males, which was accompanied by the upregulation of *ldlr*, *star*, *cyp17a1*, *17βhsd*, and *cyp19a* transcription in the testis. Increased concentrations of T and E2 were observed in females, which was consistent with the upregulation of *ldlr*. The expression of brain *gnrhr2*, *fshβ*, *cyp19b* and steroid hormone receptor genes also corresponded well with hormonal and reproductive changes. The liver VTG level was significantly increased after DEHP and MEHP exposure in males. DEHP induced histological changes in the testes and ovaries: the testes displayed a reduced number of spermatozoa, and the ovaries displayed an increased number of atretic follicles. In addition, the tissue concentrations of MEHP, MEHHP and MEOHP in DEHP-exposed groups were much higher than those in MEHP-exposed groups, and there were no dose- or sex-specific effects. Thus, DEHP exerts more obvious toxic effects compared with MEHP. There were some commonalities in the toxic effects and molecular mechanisms of DEHP and MEHP, suggesting that some of the toxic effects of DEHP may be induced by both DEHP itself and DEHP metabolites (including MEHP). Taken together, these results indicate that exposure to DEHP and MEHP from hatching to adulthood causes endocrine disruption with sex-specific effects in marine medaka, with males being more sensitive than females.

1. Introduction

Di-(2-ethylhexyl)-phthalate (DEHP) has been widely used as a plastic softener in manufacturing of various products composed of polyvinyl chloride (Koo and Lee, 2004). DEHP was reported to reach a level of up to 98 μg/L in surface water, 182 μg/L in wastewater and 154 mg/kg (wet weight [wt]) in wastewater treatment sludge (Stales et al., 1997). DEHP is globally ubiquitous in marine environments. The reported concentrations of phthalate esters (PAEs) in surface marine water, surface marine sediment and marine organisms were 0–300 μg/L, 3 μg/g and 4.07 ng/g, respectively (Liu et al., 2009). In the North Sea, the reported DEHP concentrations were up to 6.6 ng/L in water and 3390 μg/kg (dry wt) in surface sediments (Ebinghaus and Xie, 2006; Tong and Chung, 2003). In the Gulf of Mexico, an average DEHP concentration of 5 ng/g was observed

Abbreviations: arα, androgen receptor α; BSI, brain somatic index; cyp3a, cytochrome P450 3a; cyp11b, cytochrome P450 11b; cyp17a1, cytochrome P450 17α-hydroxylase 1; cyp19, cytochrome P450 19; cyp21a, CYP450 21-α-hydroxylase; DEHP, di-(2-ethylhexyl)-phthalate; DMSO, dimethyl sulfoxide; er, estrogen receptor; E2, 17β-estradiol; fsh, follicle-stimulating hormone; ghr, growth hormone receptor; gnrhr2, gonadotropin-releasing hormone receptor 2; gr, glucocorticoid receptor; GSI, gonadal somatic index; HPG, hypothalamic-pituitary-gonad; hsd, hydroxysteroid dehydrogenase; igf1r, insulin-like growth-factor-1 receptor; *K*, condition factor; ldlr, low density lipoprotein receptor; MEHP, mono-(2-ethylhexyl)-phthalate; MEOHP, mono-(2-ethyl-5-oxohexyl)-phthalate; MEHHP, mono-(2-ethyl-5-hydroxyhexyl)-phthalate; nr5a2, nuclear receptor subfamily-5 group a member 2; ppar, peroxisome proliferator-activated receptor; qRT-PCR, real-time quantitative reverse transcriptase polymerase chainreaction; star, steroidogenic acute regulatory protein; T, testosterone; VTG, vitellogenin.

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in marine biota (mainly fish) (Giam et al., 1978). Because DEHP is ubiquitous in the environment, it is expected that its metabolites are also ubiquitous. Mono-(2-ethylhexyl)-phthalate (MEHP) was detected at concentrations of 0.010–1.30 μg/L in the Tama River in Tokyo (Suzuki et al., 2001). In Canada, the MEHP concentrations were 45.49–57.2 ng/L, 3.30–6.72 ng/g wet wt, 0.39–1.13 ng/g wet wt, 0.24–1.1 ng/g wet wt and 0.33–0.84 ng/g dry wt in sea water, blue mussels, Dungeness crab, white spotted greenling and sediments, respectively (Blair et al., 2009). The wide distribution of DEHP and MEHP in marine environments has aroused great concern for aquatic organisms.

DEHP has been extensively characterized as a developmental and reproductive toxicant in many aquatic toxicological studies. DEHP was found to retard the oocyte development of female Japanese medaka (*Oryzias latipes*) (Kim et al., 2002). Recently, DEHP was shown to significantly impair oogenesis and embryo production in female zebrafish (Carnevali et al., 2010). Meanwhile, DEHP disrupted spermatogenesis with a consequent decrease in the ability of male zebrafish (*Danio rerio*) to fertilize oocytes (Uren-Webster et al., 2010). Exposure to DEHP during the fry stage affected the normal maturation of medaka with a reduction in the gonadal somatic index (GSI) and body weight (Chikae et al., 2004b). DEHP also negatively affected Japanese medaka embryos: mortality was increased, body weight was reduced and the sex ratio was distorted (Chikae et al., 2004a). DEHP showed estrogenic potency in both male and female hepatocyte cultures (Maradonna et al., 2013). In addition, DEHP modulated the transcription profiles of genes involved in steroidogenesis and altered plasma sex hormone levels in several freshwater fish species, such as the Chinese rare minnow (*Gobiocypris rarus*) (Wang et al., 2013), carp (*Cyprinus carpio*) (Thibaut and Porte, 2004) and fathead minnows (*Pimephales promelas*) (Crago and Klaper, 2012). Moreover, because pollutant metabolites have been reported to be more toxic than their precursors, we focused attention on MEHP, a primary hydrolyzed metabolite of DEHP, which could alter reproductive functions and exert distinct effects on reproductive organs in rodent animals (Inada et al., 2012; Muczynski et al., 2012). The metabolism of DEHP involves hydrolysis to MEHP and subsequent oxidation to mono-(2-ethyl-5-hydroxyhexyl)-phthalate (MEHHP) and mono-(2-ethyl-5-oxohexyl)-phthalate (MEOHP) (Monfort et al., 2010). MEHP was reported to be readily absorbed, and a majority of MEHP (more than 80%) was quickly excreted in 24 h (Chu et al., 1978). Only a small portion of MEHP was metabolized to MEHHP and MEOHP, whereas 44% of DEHP was metabolized to MEHP, MEOHP and MEHHP in humans (Koch et al., 2004). DEHP and its main metabolites – MEHP, MEOHP and MEHHP – were identified as anti-androgenic chemicals (Stroheker et al., 2005) in an in vitro study. However, few data are available on the effect of MEHP on aquatic organisms, especially marine fish.

Despite the many studies dealing with the harmful effects of DEHP in aquatic environments, studies examining DEHP-induced developmental and reproductive disruption have mostly concentrated on several signals and some phenotypic effects; the mechanisms affecting reproduction in aquatic organisms, and especially marine organisms, are not yet fully understood. The hypothalamic-pituitary-gonad (HPG) axis in fish has been a useful tool for ecotoxicological research to evaluate endocrine disrupting effects and investigate the underlying molecular mechanisms in the aquatic environment (He et al., 2012). An investigation of the effects of DEHP on the pathways of the HPG axis is critical to achieving a mechanistic understanding of the chemical disruption of reproduction and development. Aquatic toxicological investigations of DEHP were mostly limited to freshwater species; to our knowledge, the effect of DEHP on marine fish has not been reported. However, salinity has been shown to influence the toxic effects of chemicals in aquatic organisms by changing the characteristics of the chemicals or the physiological characteristics of the aquatic organisms (Jeon et al., 2010; Zanette et al., 2011). Thus, the effect of a given chemical on marine fish is likely to be different from its effect on freshwater fish. Therefore, a marine fish model for assessing the toxicity of DEHP in the marine environment is urgently needed. The marine medaka, *Oryzias melastigma*, has many experimental advantages, such as its small size, ease of culture and short generation time. Additionally, this fish has several promising characteristics, such as its susceptibility to chemicals and its ability to adapt to a wide range of salinities, that have made it a potential marine model fish that can be utilized in diverse fields of fish biology and ecotoxicology (Chen et al., 2009, 2011; Kong et al., 2008).

The current study aims to (1) analyze the endocrine disrupting effects induced by DEHP and MEHP in marine medaka; (2) determine whether some of the toxic effects of DEHP were due to DEHP metabolites; and (3) explain the underling mechanism of endocrine disruption. We studied the effects of DEHP and MEHP exposure on fecundity, spawning time, sex ratio, gonadal growth and spawning. In addition, plasma testosterone (T) and 17β-estradiol (E2) concentrations were measured to assess changes in hormone production. Liver vitellogenin (VTG) production and gonadal histopathology were also analyzed as biomarkers for reproduction disorders. Moreover, DEHP and MEHP metabolites were determined in fish tissue at the end of the exposure period. Finally, a qRT-PCR (real-time quantitative reverse transcriptase polymerase chain reaction) array was utilized to investigate the mechanistic basis of the endocrine-disrupting effects of DEHP and MEHP by examining the transcriptional responses of key genes along the HPG axis of male and female marine medaka.

2. Materials and methods

2.1. Chemicals

DEHP was obtained from Supelco (Bellefonte, PA, USA). MEOHP ($^{13}C_4$, 99%), MEOHP unlabeled, MEHHP ($^{13}C_4$, 99%), MEHHP unlabeled, MEHP ($^{13}C_4$, 99%), and MEHP unlabeled were obtained from Cambridge Isotope Laboratories (Andover, MA, USA). DEHP and MEHP were dissolved in dimethyl sulfoxide (DMSO, Sigma–Aldrich Corp. St. Louis, MO. USA), and the final DMSO concentration in the exposure water was 0.1% (v/v).

2.2. Fish maintenance, exposure and sampling

During breeding, *O. melastigma* were raised in artificial seawater at a salinity level of 30‰ and subjected to standard laboratory conditions of 28 ± 1 °C on a 14:10 light/dark photoperiod in a recirculation system. The fish were fed with freshly hatched *Artemia nauplii* twice daily at 9:00 am and 3:00 pm, respectively. Embryos were collected from the abdomens of healthy females, and the larvae hatched within one week were used for the subsequent exposure experiment.

Marine medaka larvae hatched within 1 week were exposed to vehicle control (DMSO at a final concentration of 0.1% [v/v] in water), 0.1 mg/L of DEHP, 0.5 mg/L of DEHP, 0.1 mg/L of MEHP or 0.5 mg/L of MEHP for 6 months. Nominal concentration was used in all cases; three replicates were established for each treatment. 50 larvae were assigned to each replicate (3 L of exposure medium) for one month, and then 2 month in 6 L of exposure medium, finally the remaining fish were transferred to a 10 L of exposure medium. The temperature, photoperiod and feeding regimes for each group were the same as those used for brood stocks. The solvent control and exposure seawater were completely renewed three times per week in all treatment groups. During the exposure, the time of starting spawning of approximately 70% of the females was investigated