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Role of Di-(2-Ethylhexyl) Phthalate on the Antioxidant Status in Ovary and Testes of the Fish, *Oreochromis mossambicus* (Peters, 1852)

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Abstract
Di-(2-ethylhexyl) phthalate (DEHP) is one of the endocrine disrupting chemicals that possess estrogenic properties. The aim of the present study was to evaluate the reproductive toxicity of DEHP that are mediated through the antioxidant defense system in ovary and testes of the freshwater fish, *Oreochromis mossambicus*. DEHP at 60 ppm concentration was exposed to fish for short-term (24, 48, 72 and 96 h) and long-term (7, 14, 30 and 60 days) durations along with control groups. Activities of antioxidant enzymes such as superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase were measured in the ovary and testis. The level of lipid peroxidation and the activities of steroidogenic enzymes namely 3β-hydroxysteroid dehydrogenase and 17β-hydroxysteroid dehydrogenase were also analysed. The results when compared to the control tissues indicated that DEHP-exposure significantly (P<0.05) decreased the activities of antioxidant enzymes with concomitant significant (P<0.05) increase in the level of lipid peroxidation in both ovary and testis. The activities of steroidogenic enzymes showed significant (P<0.05) reduction in ovary and testis after DEHP exposure. The results suggest that DEHP exposure disturbed the pro-oxidant and antioxidant balance that preferentially affected the reproductive functions of the fish. The study provides early warning indicators for the ecological risk assessment to monitor plasticizer pollution in aquatic ecosystems.

Keywords
DEHP, *Oreochromis mossambicus*, testis, ovary, antioxidant enzymes, steroidogenic enzymes.

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INTRODUCTION
Increases in the anthropogenic activities on ecosystems are evident in the form of global climatic changes and associated environmental problems. Hence, the assessment of the impacts of pollutants on ecology and physiology of organisms is very much essential. Over past few decades, plastic derivatives gained considerable attention as one of the most widespread contaminants. Phthalates that belong to a family of plasticizers are widely used as softener to

increase the flexibility of many polyvinyl products including food wrappers, paints, automobile upholstery, cosmetics, medical devices, inks and so on. Global demand for phthalate plasticizers is forecasted to increase towards 9.75 billion tonnes in 2024 whereas the estimated production rate of di-(2-ethylhexyl) phthalate (DEHP), the most frequently used plasticizers in 2016 is 3.07 million tonnes [1]. The European Commission has addressed the possible health effects of DEHP in rats, mice, hamsters, ferrets, marmosets and humans. Endocrine disrupting properties of DEHP and its metabolites has been proved by their reproductive and developmental toxicity in mammalian and piscine models [2-4].

Endocrine disruptors disturb the activity of endocrine system by interacting with estrogen or androgen receptors and functions agonistic or antagonistic against the endogenous hormones [5]. One of the preliminary studies revealed that some common phthalates such as di-n-butyl phthalate (DnBP), di(2-ethylhexyl) phthalate (DEHP), butyl benzyl phthalate (BBP) and their metabolites are estrogenic and exhibited adverse reproductive effects rather than other organ toxicity [6]. The exact mechanisms of reproductive toxicity have not been exactly sorted out so far but some reports have stated the possible activation of peroxisome proliferator-activated receptor-α (PPARα), which can alter testosterone biosynthesis through inducing changes in the gene expression of the associated enzymes and this could be due to the anti-androgenic effect [7].

Another mechanism by which environmental contaminants exert reproductive toxicity is by the production of reactive oxygen species (ROS) that are regulated by an antioxidant system, under normal physiological conditions [8]. Pollutants in the aquatic environment exert pro-oxidant effects in fish population; therefore, oxidative stress is often used as biomarker in detecting the toxic effects of environmental contaminants. Endogenous antioxidant system functions as first line of defense mechanism to scavenge the free radicals formed. Thus the levels of antioxidant enzymes have been extensively used as an early warning indicator of aquatic pollution and protect the cells or tissues against the potential toxic effects of pollutants [9]. ROS contributes to a wide range of pathologies including reproductive impairment such as modifications in sperm functions, disruption in gametogenesis, steroidogenesis, and vitellogenesis, development of tumour, decline in fecundity and infertility.

Toxicity of phthalates are also evidenced by increased Fas ligand expression in Sertoli cells that initiated apoptosis in germ cells thereby affecting the growth and functioning of spermatocytes and oocytes [10]. Phthalate-induced reduction in the activity and expression of superoxide dismutase enzyme in antral follicle are known to affect the growth and performance of oocyte [10]. Although several investigations focussed on the endocrine-disrupting effects of DEHP it is critical to understand the mechanism of reproductive toxicity mediated through ROS generation. The present study was aimed to assess the toxic effects of DEHP on the antioxidant status and also focused to evaluate the role in steroidogenic pathway in testes and ovary of the freshwater fish, *Oreochromis mossambicus*.

MATERIALS AND METHODS

Collection and maintenance of animal

Freshwater fish, *Oreochromis mossambicus* weighing 3.5 ± 0.75 g and length 5.5 ± 1.5 cm were collected from a fish farm, Safa Aquarium, Kozhikode, Kerala, India. Fish were acclimatized for two weeks in laboratory conditions in 40 L cement tanks with dechlorinated water and good lighting system containing ten specimens in each tank. Each tank was covered with nylon mesh, tightly tied at the top to prevent the escape of fish from the container. Optimal conditions were maintained throughout the experiment as recommended in APHA guidelines [11]. Accordingly, standard water temperature (28 ± 2°C), oxygen saturation of water (70 and 100 %) and pH (6.5 to 7.5) was retained in both control and experimental tanks.

Chemicals

Di-(2-ethylhexyl) phthalate (DEHP; CAS No. 117817) of 99.7% purity was obtained from Sigma Aldrich chemical Co., USA. Malondialdehyde, NADPH, glutathione oxidized, thiobarbituric acid, pyrogallol, horseradish peroxidase, dehydroisoandrosterone and 1,4-androstenedine-3,17-dione were obtained from Himedia Laboratories, Mumbai, India. All other chemicals of analytical grade were obtained from local commercial sources.

Preparation of test solution and grouping of fish

Propylene glycol (1 M) was used as vehicle control to dissolve DEHP, and the concentration of 60 ppm was selected as test dose based on the maximum solubility [12]. Fish were randomly divided into four groups maintaining 10 animals in each group as follows:

Group 1: Control group (vehicle and toxicant-free)
Group 2: Vehicle control group (propylene glycol)

Group 3: Short-term treatment group (DEHP-60 ppm concentration exposed for 24, 48, 72 and 96 h)
Group 4: Long-term treatment group (DEHP-60 ppm concentration exposed for 7, 14, 30 and 60 days)
The health status of fish was continuously monitored during the experiment period, where no mortality was observed. The handling of test organism conforms to the policy of animal care as prescribed in Animal Welfare Board of India.

Preparation of tissue samples

At the end of every exposure period, fish were captured gently using small dip net with least disturbances, weighed and killed by decapitation. Ovary and testes were carefully excised and cleaned from mucous and debris, weighed and 1% (w/v) crude tissue homogenates were prepared in ice-cold saline using a motor driven tissue homogenizer. The homogenates were centrifuged at 1000 *g* for 15 min at 4°C and the supernatants obtained were then used for the biochemical studies.

Biochemical analysis

Total protein concentration in the tissues of ovary and testes was determined by the method of Lowry et al. [13]. Activities of antioxidant enzymes such as superoxide dismutase [14], catalase [15], glutathione reductase [16] and glutathione peroxidase [17] and the level of lipid peroxidation [18] were assayed. The activities of 3β- and 17β-hydroxysteroid dehydrogenases were measured by the method of Bergmeyer [19].

Statistical analysis

Mean values obtained were analyzed for statistical significance ($P \leq 0.05$) using one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range as Post-hoc test with the Statistical Package for Social Sciences (SPSS, version 17.0). Data are presented as Mean ± Standard Deviation for ten animals per group and asterisks (*) in the Figures denotes significance at $P \leq 0.05$ against the control groups. All biochemical estimations were carried out in triplicate.

RESULTS AND DISCUSSION

Phthalates or phthalic acid esters are widely used as industrial additives in the manufacture of many polyvinyl chloride (PVC) products. Currently, the high production rate and continuous release into the environment creates potential ecological risk of di-(2-ethylhexyl) phthalate (DEHP) into the aquatic ecosystem, which is of increasing concern to researchers. Like other anthropogenic contaminants, DEHP can also enter into the aquatic compartments from variety of direct or indirect sources including direct discharge, accidental spillage, rainwater run-off, atmospheric deposition etc. [20]. The present study provides evidence that soluble concentration of DEHP (60 ppm) interfere with the reproductive function thereby alert risk for the aquatic population exposed to phthalates as pollutants. Exposure to DEHP showed significant ($P<0.05$) decrease in the weight of ovary after 48 h in time-dependent manner whereas the weight of testes decreased significantly ($P<0.05$) only after 30 and 60 days of treatment when compared to the control groups (Figs. 1A and 1B). The decrease in ovo-testes weight could be due to the reduction in cellular proliferation of gonads influenced by the estrogenic potency of DEHP.

There are several mechanisms by which DEHP exert reproductive toxicity and the present study evaluated the role of toxicant on the antioxidant status in ovary and testes of the freshwater fish, *Oreochromis mossambicus*. Fish are generally equipped with series of antioxidant enzymes that enable the potential to scavenge the free radicals generated as a result of toxicant exposure. In order to maintain the pro-oxidant-antioxidant balance, the reactive oxygen species (ROS) such as molecular and singlet oxygen, hydrogen peroxide, hydroxyl radical, superoxide anion and their derivatives must be continuously generated and eliminated [21]. Exposure to environmental contaminants could lead to disturbance in the redox status resulting in oxidative stress [22]. In the present study, DEHP exposure caused significant ($P<0.05$) decrease in the activities of antioxidant enzymes such as superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase in ovary and testes (Figs. 2 and 3). However, there was a significant ($P<0.05$) increase in level of lipid peroxidation in both ovary and testes (Figs.2 and 3) in a time-dependent manner when compared to the control groups.

Superoxide dismutase and catalase enzymes play important roles in protecting the cell against the potential toxic effects of environmental pollutants [23]. Superoxide dismutase catalyzes the dismutation of the superoxide ion to hydrogen peroxide and oxygen molecule. Later hydrogen peroxide produced is converted into water by the action of catalase and glutathione peroxidase. Reduction in the activities of antioxidant enzymes reveals the failure of antioxidant defense system to scavenge the free radicals, which was evidenced by the increase in the level of lipid peroxidation. The generation of oxygen free radicals plays an important role in reproductive impairment thereby causing ovarian and testicular dysfunction and sex reversal in fish population [24]. In fish, lipids are considered as an important energy source for reproduction and the

constituents of lipids are more prominent in gonads than the other tissues [25]. The results suggested that the reduction in the activities of antioxidant enzymes and increase in the level of lipid peroxidation altered the pro-oxidant/ antioxidant balance in gonads of DEHP exposed fish. Thus the reproductive toxicity of DEHP may be due to induction of oxidative stress in ovary and testes.

One of the previous studies have reported that exposure of DEHP at 5000 mg/ kg body weight to male zebrafish, *Danio rerio* has been shown to disrupt spermatogenesis and reduced the number of spermatozoa by the altered activities of antioxidant enzymes and induction of oxidative stress [26]. In the present study, DEHP significantly elevated the level of lipid peroxidation in both ovary and testes in time-dependent manner when compared with the corresponding control groups (Figs. 2 and 3). This could be due to the oxidative degradation of membrane polyunsaturated fatty acids (PUFA) into lipid peroxides as a result of DEHP exposure. Testis and ovary are more prone to oxidative stress as its membrane is rich in PUFA and the impairment in the activities of antioxidant enzymes in gonads reflects oxidative stress.

Two major key-enzymes involved in ovarian and testicular steroidogeneis includes 3β- and 17β-hydroxysteroid dehydrogenase enzymes. The formation of progesterone from pregnenolone is catalyzed by 3β-hydroxysteroid dehydrogenase (3β-HSD) enzyme whereas the conversion of estradiol through aromatization of androstenedione to estrone occurs in the presence of 17β-hydroxysteroid dehydrogenase (17β-HSD) enzyme [27]. In the present study, DEHP exposure decreased the activities of 3β-HSD and 17β-HSD in ovary and testes in all treatment groups (Fig. 4). Similarly, female carp when exposed to 20.5 mg/ L concentration of DEHP for 48 h has been shown to reduce the activities of 3β-HSD and 17β-HSD [28]. The inhibition of steroidogenic enzyme activities suggests the reproductive dysfunction in fish, which is the clear indication of reduction in spawning frequency and cumulative fecundity in female and decline in sperm functions in male fish. Thus the study is the relevant bio indicator for DEHP-induced reproductive dysfunction and can be used for identifying risk assessment in exposed fish population.

Fig. 1 Effect of DEHP on the organ weights in the fish, *Oreochromis mossambicus*

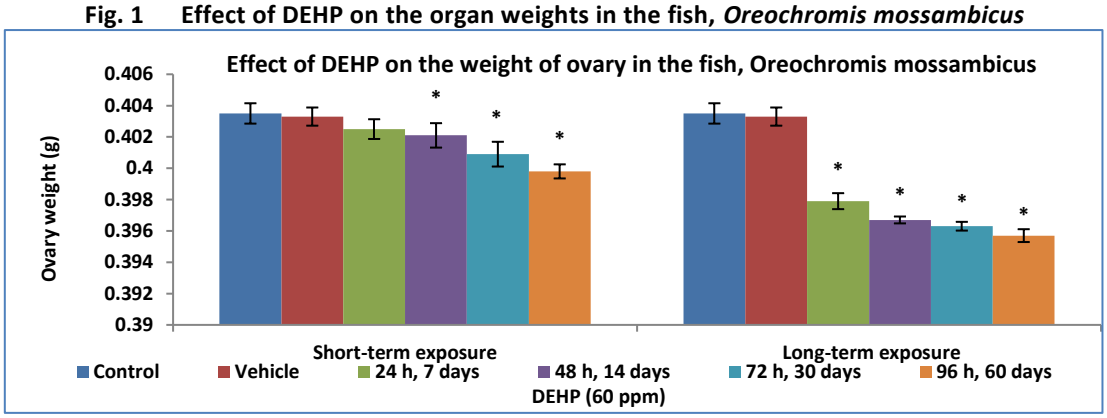
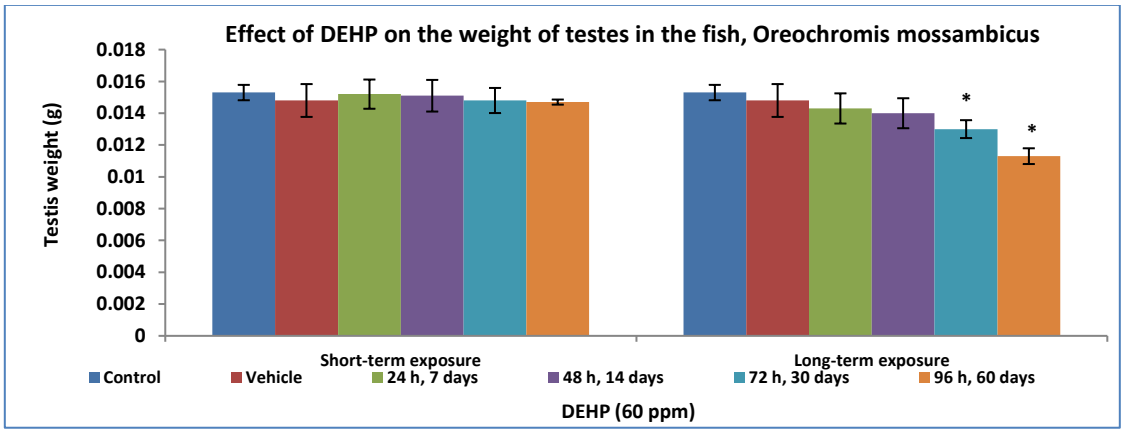


Fig 2: Effect of DEHP on the activities of antioxidant enzymes in the ovary of the fish, *Oreochromis mossambicus*



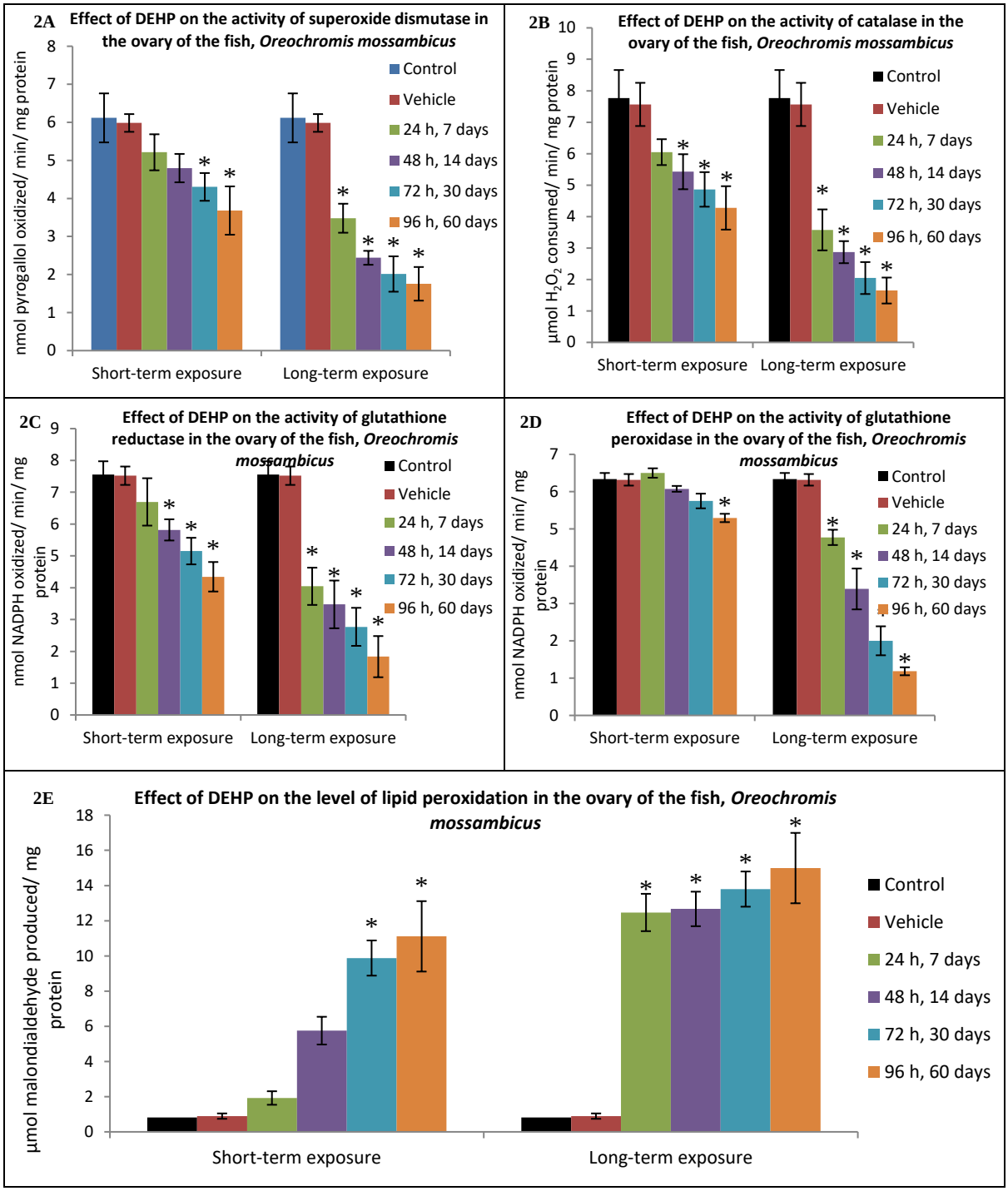

2A
Effect of DEHP on the activity of superoxide dismutase in the ovary of the fish, Oreochromis mossambicus
nmol pyrogallol oxidized/ min/ mg protein
Control
Vehicle
24 h, 7 days
48 h, 14 days
72 h, 30 days
96 h, 60 days
Short-term exposure
Long-term exposure
2B
Effect of DEHP on the activity of catalase in the ovary of the fish, Oreochromis mossambicus
µmol H2O2 consumed/ min/ mg protein
2C
Effect of DEHP on the activity of glutathione reductase in the ovary of the fish, Oreochromis mossambicus
nmol NADPH oxidized/ min/ mg protein
2D
Effect of DEHP on the activity of glutathione peroxidase in the ovary of the fish, Oreochromis mossambicus
2E
Effect of DEHP on the level of lipid peroxidation in the ovary of the fish, Oreochromis mossambicus
µmol malondialdehyde produced/ mg protein

Fig. 3: **Effect of DEHP on the activities of antioxidant enzymes in the testis of the fish, *Oreochromis mossambicus***

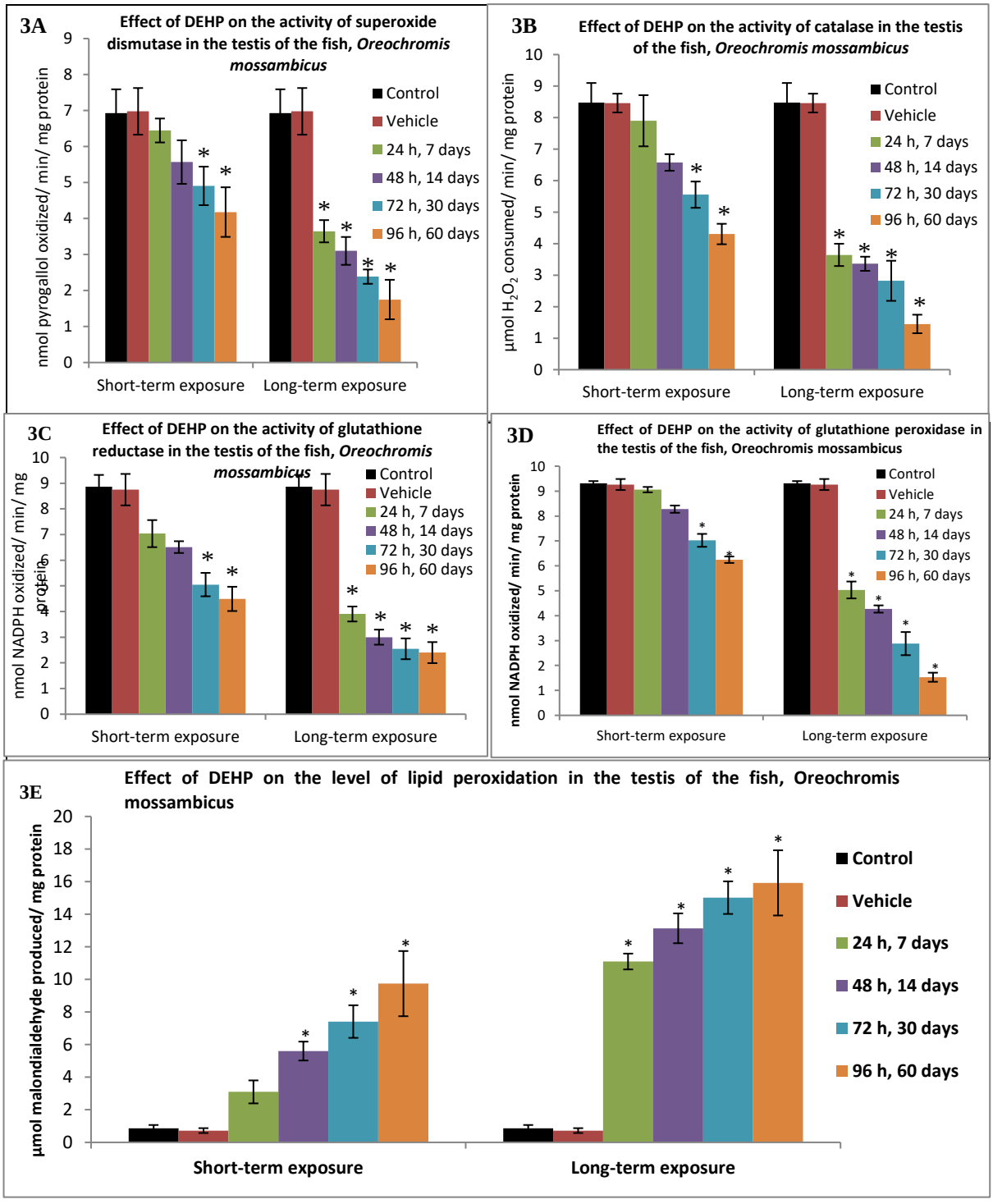
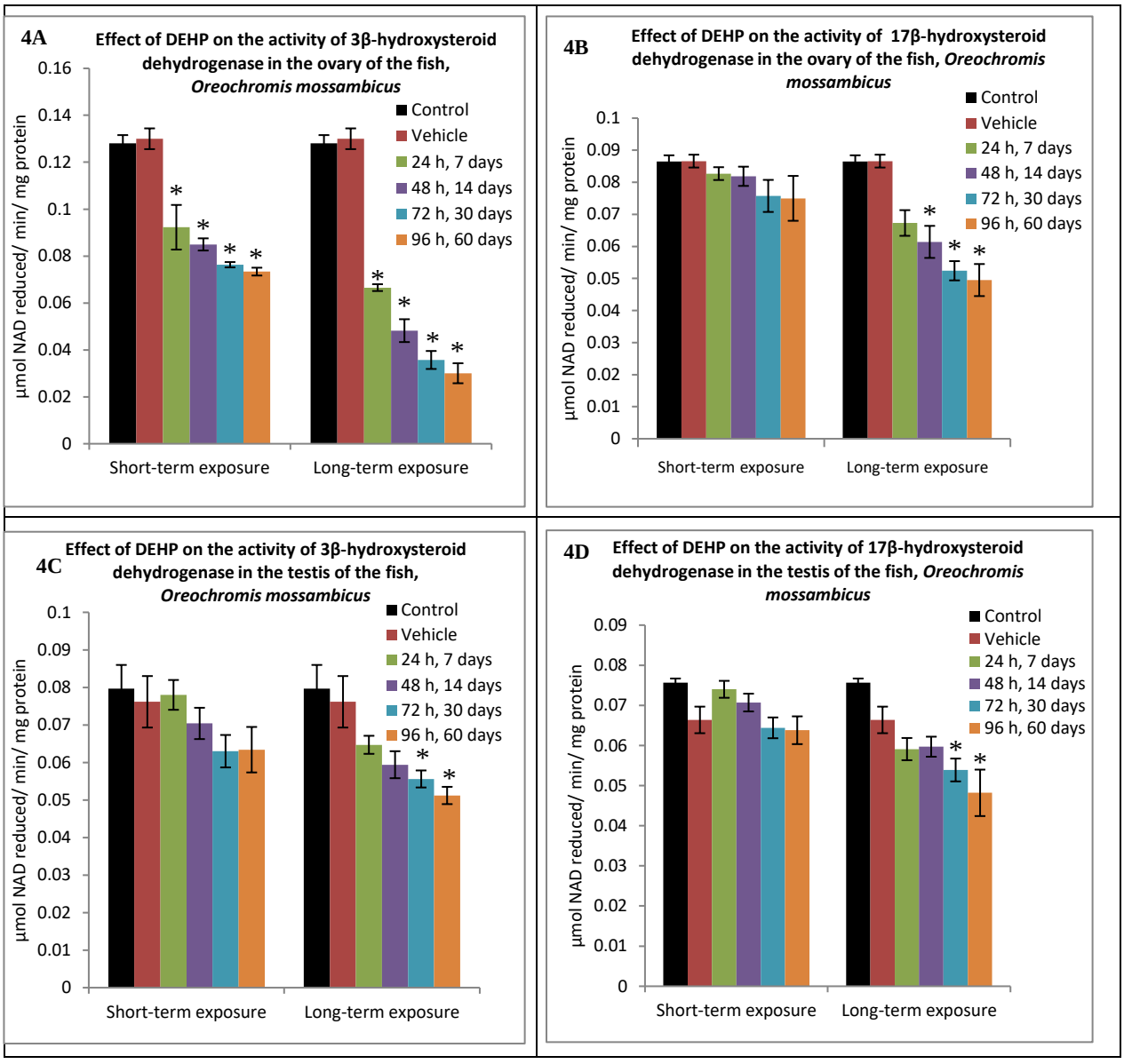


Fig. 4: Effect of DEHP on the activities of 3β-hydroxysteroid dehydrogenase and 17β-hydroxysteroid dehydrogenase in the ovary and testis of the fish, *Oreochromis mossambicus*



CONCLUSION

Aquatic organisms possess well-defined antioxidant defensive mechanism to eliminate the generated reactive oxygen species. As a negative consequence, DEHP exposure induced oxidative stress by decreasing the activities of antioxidant enzymes in ovary and testis of the fish, *Oreochromis mossambicus*. Reproductive toxicity of DEHP was further evident by reduction in gonadal steroidogenic enzyme activities. Thus the soluble concentration of DEHP in natural environment could cause remarkable reproductive impairment that ultimately affects the fish population.

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