Asian Fisheries Science **31** (2018):284–296

ISSN 0116-6514
E-ISSN 2073-3720
https://doi.org/10.33997/j.afs.2018.31.04.004

Asian Fisheries Science
The Journal of the Asian Fisheries Society

Di-isononyl Phthalate (DINP) Impairs Reproduction in the Freshwater Fish, *Oreochromis mossambicus* (Peters 1852)

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Abstract

Phthalates, the plasticisers widely used to improve the characteristics of polyvinyl chloride products, are frequently released into the environment causing severe health hazards. Reproductive toxicity of phthalate plasticisers in aquatic organisms is of major concern. Therefore, the present study was aimed to examine the role of di-isononyl phthalate (DINP) on reproductive impairment in *Oreochromis mossambicus* (Peters 1852). DINP at 300 ppm (mg L^{-1}) concentration was exposed to fish for short-term (24, 48, 72 and 96 h) and long-term (7, 14, 30 and 60 days) durations. After the treatment period, the activities of superoxide dismutase, catalase, glutathione reductase and glutathione peroxidase were evaluated in the ovary and testis. The level of lipid peroxidation along with the steroidogenic enzyme activities namely, 3β- and 17β-hydroxysteroid dehydrogenase was also observed. A significant ($P < 0.05$) decrease in the activities of all antioxidant enzymes with a concomitant increase in the level of lipid peroxidation was noted in both ovary and testis. The inhibitory effect of DINP on antioxidant enzymes increases with time, indicating the induction of oxidative stress in gonads. Besides, the activities of steroidogenic enzymes decreased significantly ($P < 0.05$) demonstrating that DINP altered the normal ovarian and testicular steroidogenesis thereby impairs reproduction of fish.

Keywords: DINP, *Oreochromis mossambicus*, oxidative stress, ovary, testis

Introduction

Like other aquatic organisms, fish are exposed to a wide range of environmental contaminants throughout their existence. Fish are introduced to multiple types of pollution that enter through gill, skin and alimentary canal thereby causing serious consequences, which is a recent major concern in ecotoxicological research. Fish are an important source of nutrients to humans, so any harmful effects on the fish population may contribute to direct adverse effects on humans and long-term effects on the environment. Phthalates or esters of phthalic acid are one of the environmental contaminants extensively added to polyvinyl chloride (PVC) materials. The

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extensive use of PVC in a variety of applications including plastic products, automobiles, building and construction, healthcare products, electrical wires and cables, pipe and plumbing items and so on makes difficult to eliminate phthalate plasticisers from the surrounding environment.

Di-isononyl phthalate (DINP) is a high molecular weight plasticiser for PVC having 7-13 carbon atoms in the chemical backbone that provides increased permanency and durability. The DINP at 20,000 ppm concentration showed degeneration of meiotic spermatocytes and Sertoli cells in the testis and decreased corpora lutea in the ovary of rats thus confirming anti-androgenic activity (Masutomi et al. 2003). The DINP along with the primary and secondary metabolites are detected in several body fluids such as saliva, breast milk, serum and urine (Latini et al. 2009). In mice, oral exposure to DINP aggravated allergic contact dermatitis, which was positively regulated through nuclear factor-kB (Kang et al. 2016). The DINP also induced allergic asthma in mice by a different mechanism with the production of IL-4, IL-5, IgE and IgG1 by the reduction of IFN-γ and IgG2a thereby confirming the infiltration of inflammatory cells, goblet cell hyperplasia and expression of caspase-1 and caspase-3 genes in the lung tissue (Hwang et al. 2017). Thus, the mechanism of toxic effects of DINP was modulated through a different pathway in various animals.

In the aquatic environment, DINP has been detected to release from various sources like direct or indirect sewage discharge, surface water run-off and atmospheric deposition. Due to the high hydrophobicity property, DINP gets adsorbed into the sediments and also found associated with suspended particulate matters of surface water. Therefore, the sediments act as a long-term reservoir and may act as a potential environmental threat to aquatic organisms (Adeniyi et al. 2011). Adverse effects of DINP on growth and maturation of oocytes have been observed in zebrafish causing abnormal gonadal development and reproduction (Santangeli et al. 2017). The reproductive abnormality was further demonstrated in another experiment DINP exposed to environmentally relevant concentration changed the level of endocannabinoids system (ECS) by inducing changes in the genes coding for ECS receptors and enzymes, and also increased the activity of fatty acid amide hydrolase in zebrafish (Forner-Piguer et al. 2018). Therefore, the literature reviewed provide with the information that DINP can exert its toxic effects by different mechanisms.

As organisms are continuously facing change in the environmental conditions by the rise in temperature, exposure to pollutants, UV radiation and so on, there is a possibility for the change in redox potential of cells or tissues. Further, fish tissues are often rich in polyunsaturated fatty acids, so they are highly susceptible to oxidative stress. The balance between the pro-oxidant and antioxidant defence system is essential to quench the reactive oxygen species (ROS) formed as a result of pollutant exposure. Damage caused by ROS generation may also impair the reproductive potential of the organism as it induces lipid peroxidation that leads to damage of most macromolecules (Halliwell and Gutteridge 2015). Thus the present study was undertaken to investigate the role of oxidative stress as an indicator of the impairment of reproduction after DINP exposure for short-term and long-term durations in *Oreochromis mossambicus* (Peters 1852). In addition, the activities of steroidogenic enzymes were also evaluated to understand the effect of DINP in gonadal steroidogenesis.

Materials and Methods

Animal

Freshwater teleostean fish, *Oreochromis mossambicus* of 3.5 ± 0.75 g weight and 5.5 ± 1.5 cm length were collected from a fish farm, Safa Aquarium, Kozhikode, Kerala, India. Fish were transported to the laboratory with minimum stress and were acclimatised for 2 weeks before the experiments by providing a constant supply of dechlorinated water and good lighting system (12 h light:12 h dark). Physico-chemical features of the tap water such as water temperature (28 ± 2 °C), oxygen saturation of water (70 and 100 %), pH (6.5 to 7.5) was estimated according to the standardized procedures recommended by APHA guidelines (1998), and the same was maintained throughout the experiment in both control and treatment groups.

Chemicals

Diisononyl phthalate (DINP)-CAS No. 28553-12-0 of 99 % purity was obtained from Sigma Aldrich Chemical Co., USA. Malondialdehyde, NADPH, glutathione oxidised, thiobarbituric acid, pyrogallol, dehydroisoandrosterone and 1,4-androstenedione-3,17-dione were obtained from Himedia Research Laboratories, Mumbai, India. All other chemicals were of analytical grade and obtained from local commercial sources.

Experimental design

The DINP at 300 ppm concentration dissolved in propylene glycol was exposed for short-term (24, 48, 72 and 96 h) and long-term (7, 14, 30 and 60 days) durations. The selection of test concentration and vehicle to dissolve DINP was described in the previous report (Revathy and Chitra 2015). Propylene glycol exposed group was maintained as vehicle control and the group of fish not exposed to either DINP or propylene glycol was maintained as a negative control. Ten fish were retained in each treatment groups, and the health status of the animal was continuously monitored throughout the experiment.

Tissue preparation

At the end of every exposure period, fish were captured using small dip net and sacrificed by decapitation. Ovary and testis were carefully excised and cleaned from mucous and debris, weighed and 1 % (w/v) crude tissue homogenates were prepared in ice-cold normal saline using a motor-driven tissue homogeniser. The homogenates were centrifuged at 3000 rpm for 15 min at 4 ^{o}C and the supernatants collected were then used for the biochemical studies.

Biochemical analysis

Total protein concentration in the supernatant of tissues was determined by the method of Lowry et al. (1951). Activities antioxidant enzymes such as superoxide dismutase (Marklund and Marklund 1974), catalase (Claiborne 1985), glutathione reductase (Carlberg and Mannervik 1985) and glutathione peroxidase (Mohandas et al. 1984) along with the level of lipid peroxidation

(Ohkawa et al. 1979) were assayed in the supernatants of ovary and testis tissues. The activities of 3β-hydroxysteroid dehydrogenase and 17β-hydroxysteroid dehydrogenase were also measured in gonadal tissues (Bergmeyer 1974).

Statistical analysis

Statistical analyses were performed using one-way analysis of variance (ANOVA) followed by Duncan's Multiple Range test using statistical package SPSS 17.0. Differences were considered to be significant at $P < 0.05$ against the control groups. Data are presented as mean ± SD for ten animals per group and asterisks (*) in the figures denotes significant at $P < 0.05$ against the control groups. All biochemical estimations were carried out in triplicate.

Results

The DINP exposure showed significant ($P < 0.05$) reduction in the weight of ovary from 96 h of treatment onwards in a time-dependent manner when compared to the control groups (Fig. 1A). The weight of testis showed significant ($P < 0.05$) decrease from 72 h of DINP treatment up to 60 days in a time-dependent manner (Fig. 1B).

The activities of antioxidant enzymes such as superoxide dismutase (Figs. 2A and 3A), catalase (Figs. 2B and 3B), glutathione reductase (Figs. 2C and 3C), and glutathione peroxidase (Figs. 2D and 3D) showed significant ($P < 0.05$) decrease in the tissues of both ovary and testis. However, there was a significant ($P < 0.05$) increase in the level of lipid peroxidation (Figs.2E and 3E) in ovary and testis in a time-dependent manner when compared to the control groups.

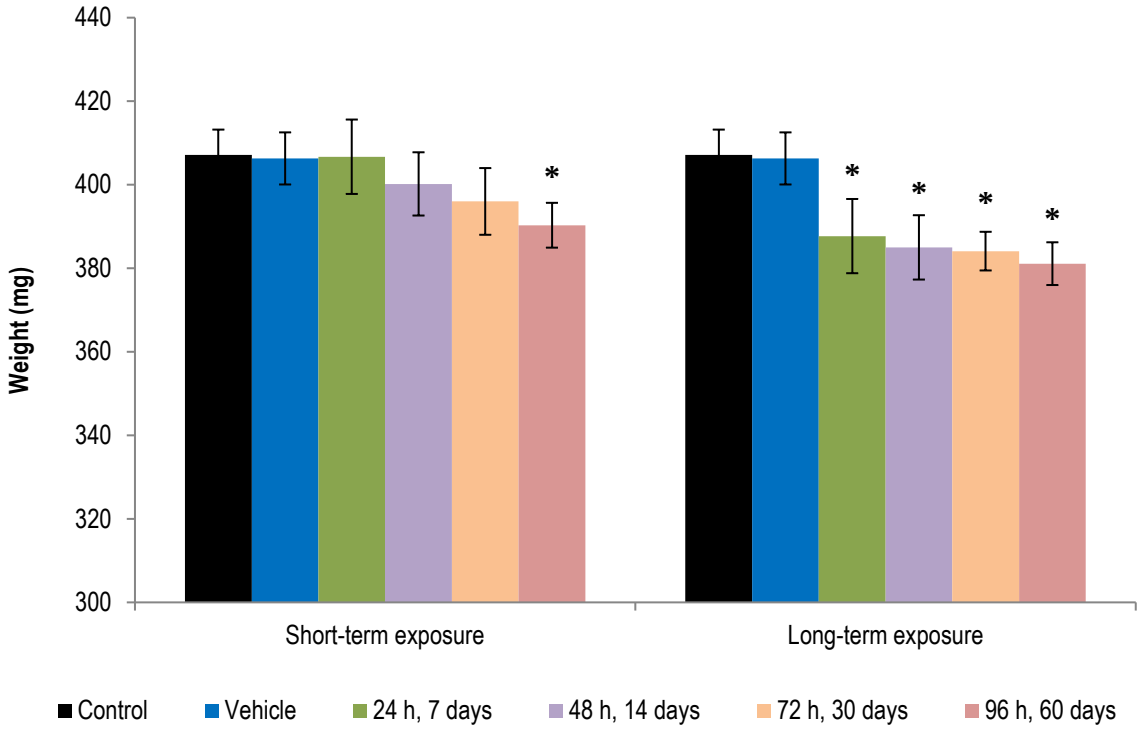

Fig. 1A. Effect of di-isononyl phthalate on the weight of ovary in *Oreochromis mossambicus.* * denotes significant difference at $P < 0.05$ against the control group.

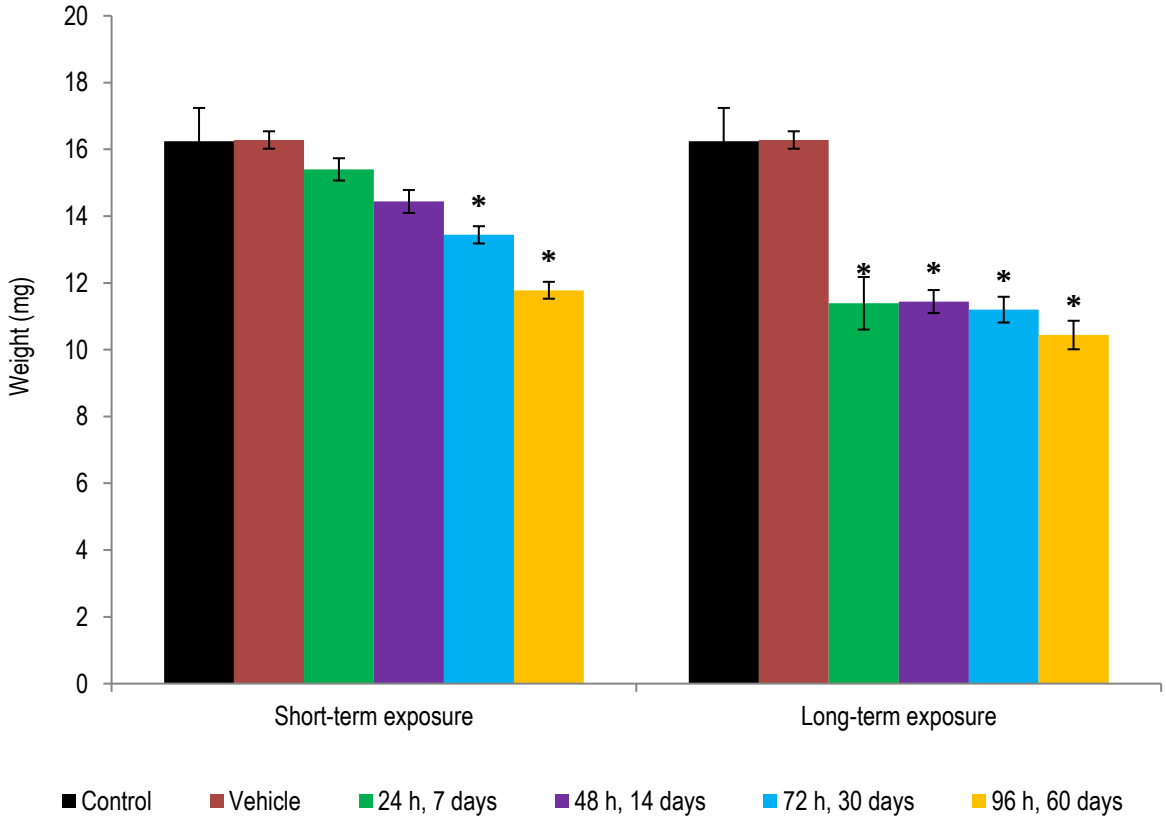

Fig. 1B. Effect of di-isononyl phthalate on the weight of testis in *Oreochromis mossambicus.* * denotes significant difference at $P < 0.05$ against the control group.

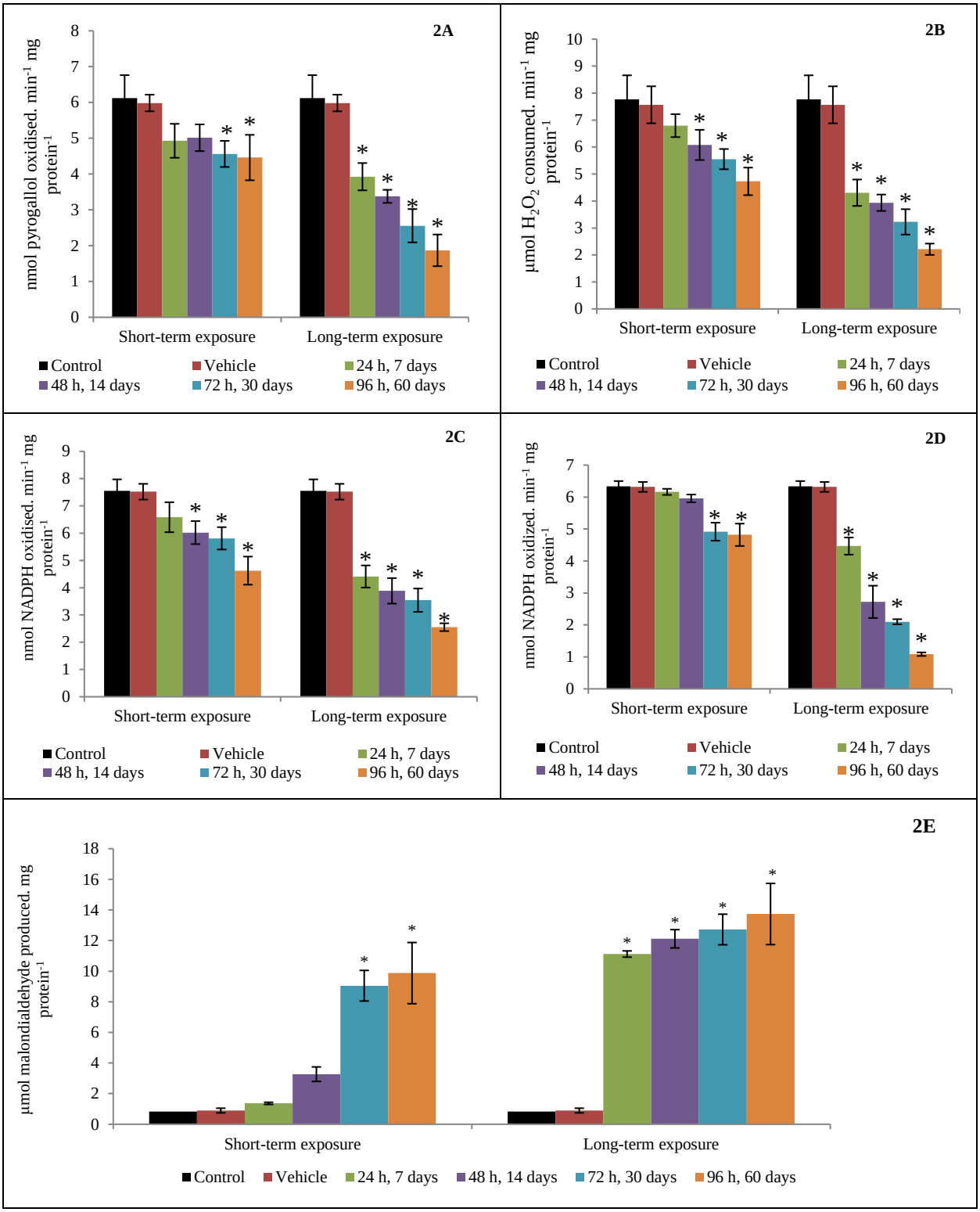

Fig. 2. Effect of di-isononyl phthalate on the activities of A) Superoxide dismutase; B) Catalase; C) Glutathione reductase; D) Glutathione peroxidase; E) Level of lipid peroxidation in the ovary of *Oreochromis mossambicus.* * denotes significant difference at $P < 0.05$ against the control group.

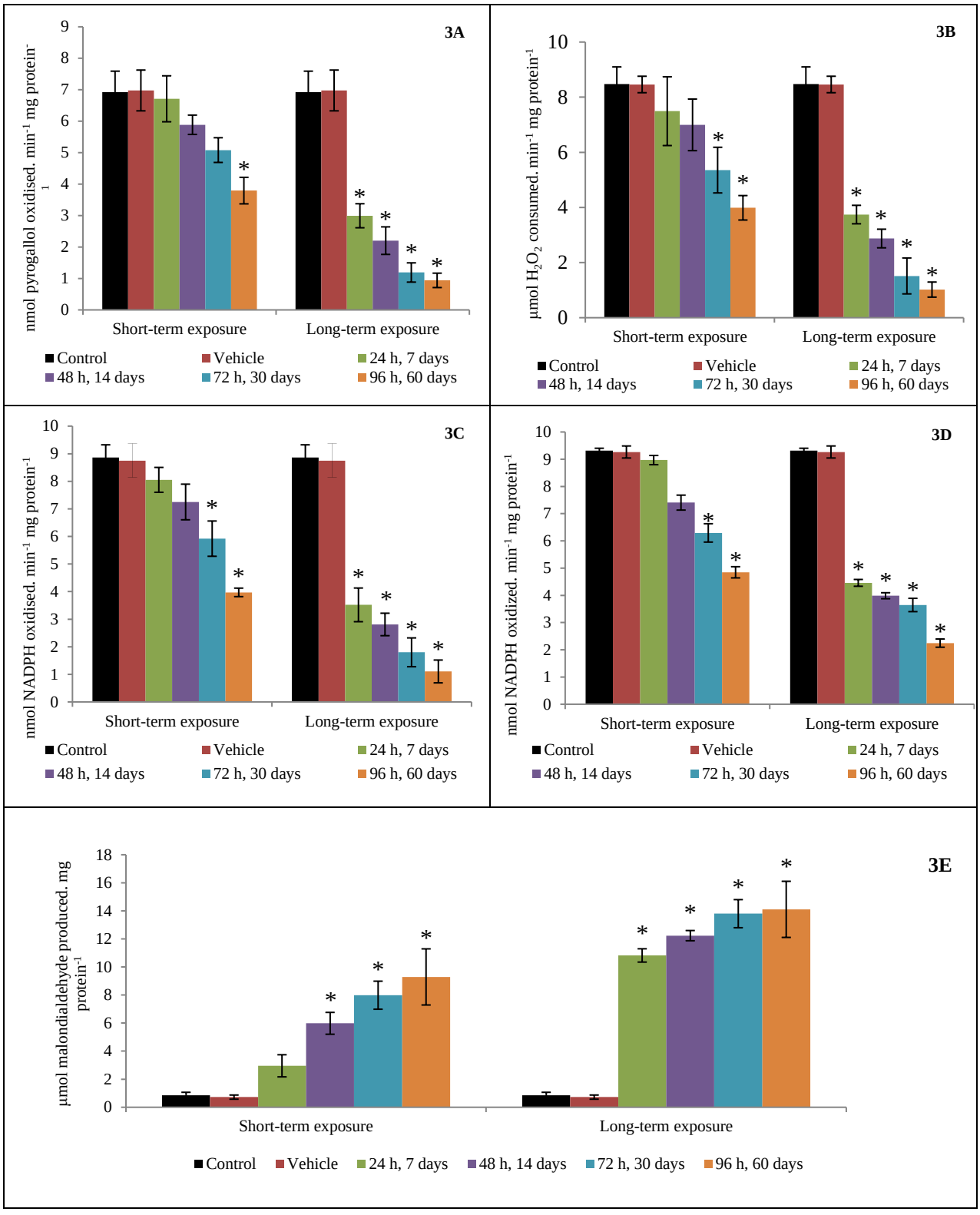

Fig. 3. Effect of di-isononyl phthalate on the activities of A) Superoxide dismutase; B) Catalase; C) Glutathione reductase; D) Glutathione peroxidase; E) Level of lipid peroxidation in the testis of *Oreochromis mossambicus.* * denotes significant difference at $P < 0.05$ against the control group.

The activities of 3β-hydroxysteroid dehydrogenase (Figs. 4A and 4C) and 17β-hydroxysteroid dehydrogenase (Figs. 4B and 4D) showed significant ($P < 0.05$) decrease in testis and ovary after short-term and long-term exposure.

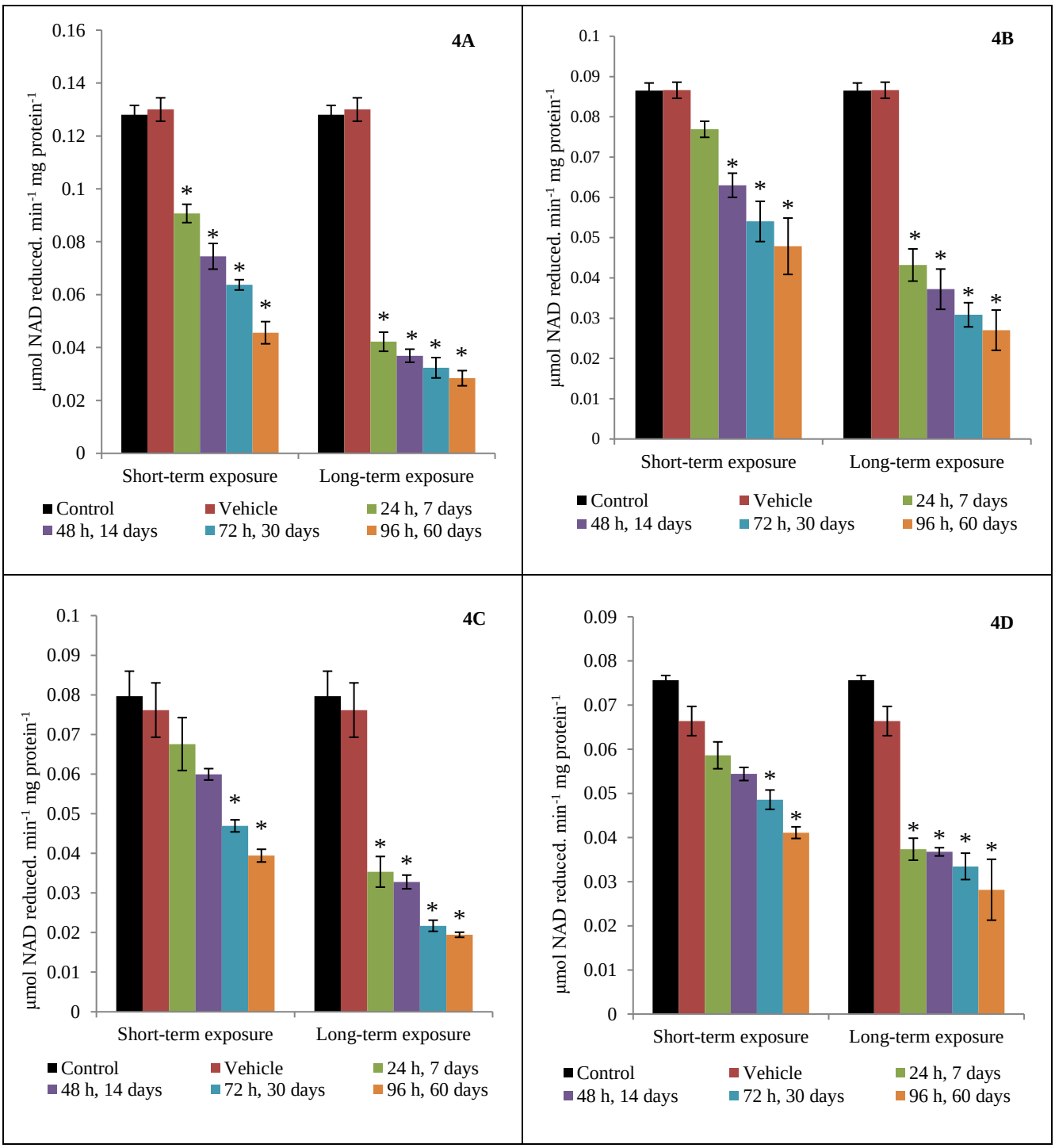

Fig. 4. Effect of di-isononyl phthalate on the activities of A) 3β-hydroxysteroid dehydrogenase; B) 17β-hydroxysteroid dehydrogenase in the ovary; and C) 3β-hydroxysteroid dehydrogenase; D) 17β-hydroxysteroid dehydrogenase in the testis of *Oreochromis mossambicus.* * denotes significant difference at $P < 0.05$ against the control group.

Discussion

Phthalates are ubiquitous in the environment that exerts toxic action in human male reproduction by inhibiting the synthesis of testosterone from Leydig cells and showing anti-androgenic activity at receptor level (Specht et al. 2014). Di-isononyl phthalate (DINP) is commonly used phthalate plasticisers in polyvinyl chloride (PVC) applications. The DINP is an

isomeric mixture, which exists in different forms such as DINP-1, 2 and 3, based on the length of carbons in alkyl chains. The toxicological properties of DINPs differ based on the physico-chemical features, where European Union risk assessment in 1995 stopped the manufacture of DINP-3. In the present study, DINP-2 (CAS 28553-12-0) used is n-butene based phthalate consisting exclusively of C9 chains, produced by esterification of phthalic anhydride with isononyl alcohol in a closed system (EC 2003). DINP has been shown to possess anti-androgenic effects in rats as evidenced by an alteration in reproductive development, causing sexual dimorphic behaviour and masculinization (Boberg et al. 2011). In zebrafish, exposure to DINP adversely affected the reproduction of fish by a shift in fecundity, oocyte growth and maturation (Santangeli et al. 2017).

Reproduction is an important characteristic of an organism, which not only helps in the survival but also helps in the successful continuity of the species or race. There are several environmental, physical and genetic factors that influence the growth, development and reproduction of animals. A number of reproductive impairments such as reduction in gonad size, alteration in the circulating steroids, reduction in gamete production, steroidogenesis, and spawning success in females, and a decline in sperm viability, motility and concentration in males are influenced by reactive oxygen species (ROS) generated by toxicant exposure. The present study was performed to analyse the effects of DINP on the reproductive impairment in *O. mossambicus.* The environmental concentration of DINP in the aquatic environment is known to range between 1.2 and 9.7 μg L^{-1} (EC 2003). In the current research, the concentration of 300 ppm was selected based on the solubility limit in the vehicle solvent, propylene glycol (Revathy and Chitra 2015). In ecotoxicological studies, fish are considered as an appropriate model because it is indicator species sensitive to changes in the aquatic environment. Moreover, the toxicants are transferred easily to humans through the food chain as it occupies in the higher trophic levels of the food web.

Exposure to DINP showed a significant reduction in the weight of ovary after 96 h of short-term exposure and all treatment groups of long-term exposure and this indicate the reduction in the number of oocyte production or atresia in the ovum, which was evident in our previous findings (Revathy and Chitra 2016). The weight of testis also showed a significant decrease in 72 and 96 h of short-term DINP exposure and in all long-term treatment groups in a time-dependent manner, and this could be due to pathological lesions and anti-androgenic effects of DINP (Revathy and Chitra 2016).

There is increase research on the role of reactive oxygen species generated by environmental contaminants in the reproductive impairment of organisms. Environmental toxicants imbalance the activities of antioxidant enzymes and reactive oxygen species generation that results in cellular damage, thus the failure of an antioxidant defence system to eliminate or neutralise the excess ROS may lead to oxidative stress (Isik and Celik 2008). Exposure to DINP showed a significant decrease in the activity of superoxide dismutase in the tissues of both ovary and testis when compared to the control groups. Superoxide dismutase represents the first line of defence enzyme against the ROS in biological systems. Free radicals such as superoxide radical or singlet oxygen radical generated in tissues by the exposure to toxicants are catalytically converted to hydrogen

peroxide and molecular oxygen by the enzyme, superoxide dismutase (Phaniendra et al. 2015). The decrease in the activity of superoxide dismutase reflects the failure of the enzyme to scavenge the free radicals in gonadal tissues. Hydrogen peroxide thus generated is toxic to cells or tissues and are readily converted to deleterious hydroxyl radical in the presence of ferrous ion through Fenton reaction (Ercal et al. 2001). The accumulation of the toxic hydrogen peroxide is prevented by the enzyme, catalase by breaking down to water and molecular oxygen. However, DINP exposure decreased the activity of catalase in ovary and testis thereby indicating failure of the enzyme to curtail free-radical induced damage.

The activities of glutathione reductase and glutathione peroxidase also showed a significant reduction in gonadal tissues in a time-dependent manner. Glutathione peroxidase is an important intracellular enzyme found abundant in mitochondria and sometimes in the cytosol that breaks down hydrogen peroxides to water (Takahashi et al. 1987). Besides, glutathione peroxidase is a crucial enzyme to inhibit the lipid peroxidation process, which is one of the principal indicators of oxidative stress (Falfushynska and Stolyar 2009). The results coincide with the increase in the level of lipid peroxidation in tissues of ovary and testis in a time-dependent manner. Lipid peroxidation is a free radical-mediated chain of reactions, and when once initiated may result in oxidative deterioration of polyunsaturated lipids (Halliwell and Gutteridge 1984). Thus, DINP exposure resulted in the loss of membrane integrity in ovary and testis and subsequently resulted in oxidative damage. Our previous study also reported that DINP induced oxidative stress in gill, liver and muscle tissues of *O. mossambicus* (Revathy and Chitra 2018).

Phthalates were earlier considered only as environmental contaminants with no specific biological activity. However, some reports have documented that phthalates possess weak estrogenic property *in vitro* (Zacharewski et al. 1998) and anti-androgenic property in rodents (Lee and Koo 2007). Phthalates and their metabolites have been shown to interfere with normal steroidogenesis by a reduction in the expression of steroidogenic enzyme activities (Moody et al. 2013). The key steroidogenic enzymes include 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-hydroxysteroid dehydrogenase (3β-HSD). The role of the 3β-HSD enzyme is to catalyse the biosynthesis of pregnenolone to progesterone, 17α-hydroxypregnenolone to 17α-hydroxyprogesterone, dehydroepiandrosterone to androstenedione, androstenediol to testosterone, androstadienol to androstadienone (Simard et al. 2005). While 17β-HSD is a group of alcohol oxidoreductases involved to catalyse the reduction of 17-ketosteroids and dehydrogenation of 17β-hydroxysteroids in steroidogenesis and steroid metabolism (Moeller and Adamski 2009). In the present study, DINP exposure decreased the activities of 3β-HSD and 17β-HSD in both ovary and testis indicating the down-regulation of steroidogenesis, which could ultimately lead to a decline in the production of sex hormones. One of the phthalates, di-n-butyl phthalate has been shown to downregulate several steroidogenic genes including steroid acute regulatory protein and the activity of 3β-HSD in fetal testes of male rats (Lehmann et al. 2004). Inhibition of estradiol or testosterone production is known to cause several reproductive abnormalities including a reduction in fecundity and spawning rate in females, and a decline in sperm count, sperm viability, motility and increased sperm DNA damage in males. The study demonstrates the toxic reproductive effects of DINP by its negative impact on steroidogenic enzyme activities and the induction of oxidative damage in both testis and ovary of *O. mossambicus*.

Conclusion

Di-isononyl phthalate at a concentration within the solubility range, when exposed over a period of time could potentially affect the population of fish and other aquatic organisms, which become a threat to our valuable fishery resources and also disturbs human health status on consumption of the exposed fish.

Acknowledgements

Authors gratefully acknowledge the UGC-SAP, Govt. of India for providing equipment and infrastructure for carrying out this study.

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Received: 19/08/2018; Accepted: 22/12/2018; (AFSJ-2018-0072)