

Phthalate induced hormetic effect reveals susceptibility of gill compared to muscle tissue after depuration in commercially important fish (*Etroplus suratensis*)

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Abstract

Effect of Bis-2ethylhexyl phthalate (DEHP) on commercially important tropical fish pearl spot has not been demonstrated at environmental concentrations along with depuration. The species is estuarine, juvenile and difficult to maintain but widely consumed and well distributed in tropical estuaries. Antioxidant activity of SOD, CAT and GPx was enhanced on all exposure days for gill and muscle suggesting high oxidative stress, except on day 5. Detoxifying enzyme-GST behaved differentially in gill and muscle tissue after depuration, depicting inhibited activity of GST in gill leading to lipid peroxidation. However, the muscle tissue was able to recover from stress after 7 days of depuration with the help of detoxifying enzymes. Overcompensation of antioxidant activity was observed over disruption of homeostasis defining hormesis effect. Integrated biomarker (IBR) index depicted high toxicity during entire exposure time, but after depuration, gill could not mediate LPO at lowest concentration (10 µg/l) while muscle tissue recovered, suggesting persistence of stress in gill.

Keywords: DEHP, phthalate, oxidative stress, IBR, *Etroplus suratensis*.

1.Introduction

Phthalic Acid ester's (PAE's/ phthalates) have wide industrial applications in production of plastics, pesticides, cosmetics, solvents, adhesives and polymers etc. (Gomez-Hens and Aguilar-Caballos, 2003; Halden, 2010). This wide use is due to their properties such as high bonding and low degradability, concurrently these compounds tend to have high environmental impacts owing to toxicity due to wide distribution (Paluselli and Kim, 2020). Globally, on an average 6-8 million tons of Phthalate is utilised per annum (Net et al., 2015) wherein Di-(2-ethylhexyl) phthalate (DEHP) is the most common and frequently used additive in polymer industry. Around 5.5 million tons of DEHP is produced worldwide (Shen et al., 2019). DEHP, a predominant constituent used in plastic products as softener, can easily leach out and enter into environment as they are not chemically bound to the polymers (Andjelković et al., 2021; Erythropel et al., 2014; Wright et al., 2020) and are therefore the most dominant PAEs in fresh and marine waters (Hermabessiere et al., 2017; Li et al., 2016; Paluselli and Kim, 2020). DEHP in aquatic systems enter via various pathways such as atmospheric deposition, treated and untreated sewage, plastic product utilisation and disposal (Hermabessiere et al., 2017; Zhang et al., 2018) .

Global, measurement reveals high DEHP concentration in surface waters of Europe from Germany (97.8 µg/l), France (76 µg/l), Netherlands (5.6 µg/l) and Italy (6.4 µg/l) (Net et al., 2015). While in Asian waters it has also been reported with reasonably high values viz; yellow river- China (31.80 µg/l) (Net et al., 2015) whereas from India it was reported from Kaveri river (0.514 µg/l) (Selvaraj et al., 2015), Cochin estuary (nd-3.861 µg/l) and Thane creek area (19.8 µg/l) (Ramzi et al., 2020; Tiwari et al., 2019) . These ambient concentrations are of concern as high octanol-water partition coefficient along with hydrophobicity makes DEHP highly bioavailable to aquatic organisms (Vitali et al., 1997). USEPA (2014) has listed DEHP as priority pollutant which is known endocrine disruptor (Akhbarizadeh et al., 2021; Park et al., 2019) in invertebrates like protozoans, mollusks as well as in fishes and cetaceans as it has significant effect on reproduction (Andrady, 2011). Diverse effects have been noted in aquatic organisms on exposure to PAE's viz oxidative stress, necrosis, DNA damage, cardio edema, tail curvature and death etc. (Net et al., 2015).

High DEHP accumulation has been reported in freshwater bream (1900-3120 µg/kg dw) and marine flounder (40-70 µg/kg dw) (Vethaak and Belfroid, 2002). *Oryzias melastigma* on exposure to DEHP showed disrupted endocrinal functions leading to accelerated spawning and decreased egg production. Further, histological changes in sex organs led to decreased spermatozoa in male and increased atretic follicle in female (Chen et al., 2014). Biochemical response serves as an early warning system in

organisms exposed to pollution. To monitor environmental pollutants, oxidative stress has been a frequently used reliable proxy (Liu et al., 2021; Wang et al., 2020).

Etroplus suratensis is widely distributed tropical, euryhaline fish from coastal India and Sri Lanka (Joy et al., 2017). It is a good test model due to its economic importance, regular consumption wide distribution, availability, easy to rear and maintain in laboratory condition. Although widely distributed, sporadic information is available on biochemical effect of phthalate on *E. suratensis* (Rejitha et al., 2016).

Present study is first of its kind wherein ambient concentrations for DEHP from Indian region have been considered while giving depuration time to analyse stress recovery. Juveniles of any species are vulnerable to sudden change in the environment or external stimuli in the form of toxicant, DEHP in the present case. Further the test organism used is a native species and known delicacy of coastal India. Multiple biochemical or toxicity studies use high concentrations of a given compound to have clear result but mild concentrations as well have shown significant effects and many a times antagonistic ones (Xu et al., 2013). Recent literature suggests that studies are rare that depict the effect of depuration after spiking with DEHP. Therefore the present sub-acute experiment aims to understand the biochemical effect of DEHP concentrations that occur in the environment and most likely have adverse effects on juvenile estuarine fish (*E. suratensis*). The important aim was to explore the time and dose dependent biochemical/ enzymatic response in gill and muscle tissue of *E. suratensis* and decipher the effects of depuration along with integrated toxicity response. Interpreting biomarker response individually is quite a challenge, to overcome this difficulty it is imperative to integrate the individual biomarker responses (Devin et al., 2017). (Beliaeff and Burgeot, 2002) proposed integrated biomarker response (IBR) to resolve the constraints in evaluating biomarker individually.

2. Material and Methodology

2.1 Test Chemical

Bis-2ethylhexyl phthalate (DEHP, #80030, Sigma Aldrich, USA) of 99% purity was dissolved in acetone (analytical grade) to prepare a stock solution. Further working solutions of desired concentration of 10,15 and 20 µg/l with acetone as vehicle was prepared. On each exposure day, working solutions were sonicated for 5 minutes prior to addition into treatment groups.

2.2 Experimental animal and design

A single batch of reared, uniform sized *E. suratensis* (weight approximately 1g; length 1-2 cm) juvenile fish were procured from local hatchery. Fish were acclimatised to laboratory conditions for two weeks with continuous aeration and regular water exchange with a 12:12 hour photoperiod at $25 \pm 1^\circ\text{C}$, and

were fed *ad libitum*. A semi-static experiment was designed as recommended by international testing guidelines, OECD (1992). Fish were transferred to experimental tanks with clean water 48 hr prior to the experiment with 120 organisms (10 ind/5L tank) in glass tanks with continuous air supply (DO: 7.0 ± 0.5 mg/l, pH: 8.0-8.4, Salinity: 10%, Temp: $25 \pm 1^\circ\text{C}$). Experiment was performed in triplicates with a vehicle control (acetone, 0.002% v/v) and three treatment groups of DEHP (10, 15 and 20 $\mu\text{g/l}$). Organisms were exposed for seven consecutive days and after the seventh day exposure was curtailed and next seven days were used as depuration period. Five sampling points were selected each on day 1,3,5,7 and 14. On these designated sampling points, 2 ind/ tank/ treatment were sampled and pooled together (n = 4), and further excised for muscle and gill tissue, snap frozen in liquid nitrogen, stored at -20°C for further analysis.

2.3 Biochemical analysis

Frozen tissue samples were thawed, weighed and homogenized in cold phosphate buffer saline in (1:5 w/v) ratio. Further centrifuged at 10000 g, 4°C for 20 min. The supernatant was collected in aliquots and preserved at -20°C . Enzyme activity was analysed in triplicates using Superoxide dismutase (SOD) assay kit (OD 450 nm, #19160, Sigma Aldrich, USA), Catalase (CAT) assay kit (OD 520 nm, #CAT100, Sigma Aldrich, USA), Glutathione Peroxidase (GPx) assay kit (OD 340 nm, #CGP1 Sigma Aldrich, USA), glutathione S-Transferase (GST) assay kit (OD 340 nm, #CS040, Sigma Aldrich, USA) and Lipid Peroxidation (LPO) assay kit (OD 532 nm, #MAK085, Sigma Aldrich, USA) (Ahn et al., 2020; Giri et al., 2021) following the manufacturers protocol. BCA protein assay kit (#786-570, G-biosciences, USA) was employed to determine total protein content with bovine albumin serum as standard.

2.4 Integrated Biomarker response (IBR)

To enhance the understanding of combined effect and batteries of biomarker, a general stress index “Integrated biomarker response” (Beliaeff and Burgeot, 2002) was employed. The result of IBR was visualised in terms of star plots indicating level of stress.

2.5 Statistical analysis

Statistical analysis was employed using graphpad Prism 9, all the values were expressed as mean \pm standard deviation. Kolmogorov-Smirnov test of Normality was performed to assess normal distribution of data. Two way analysis of variance (Two way ANOVA) followed by Dunnett’s t test was conducted to determine significant difference within and between groups. $p < 0.05$, $p < 0.01$ and $p < 0.0001$ was considered as significant.

3. Results

3.1 Biochemical response in Gill

SOD activity in gill was noted to be statistically significant for tested concentrations (two-way ANOVA, $p < 0.0001$) and time ($p < 0.0001$), further the interaction effect of concentration against time was notably significant ($p < 0.0001$; Table: 1). In gill, SOD activity was induced significantly on day 1, 3 and 7 in all the treatment groups ($p < 0.0001$; Fig: 1a). A Dunnett's post hoc test unveiled significant pair-wise differences between all the concentrations when compared to control (Table: 2). Whereas, on day 5 all the treatment groups (10,15 and 20 $\mu\text{g/l}$) were observed with no change compared to control (Fig: 1a). Depuration day revealed inhibition in all treatments with highest concentration group inhibited distinctly compared to other groups ($p < 0.0001$; Fig: 1a). The effect of concentrations (two-way ANOVA, $p < 0.0001$) and time ($p < 0.0001$) on CAT activity was highly significant, additionally significant interaction between these variables was noted ($p < 0.0001$; Table: 1). CAT depicted significantly elevated activity on day 1,3 and 7 ($p < 0.05$ and $p < 0.0001$), additionally day 3 observed highly significant ($p = 0.0003$) activity in highest concentration compared to others (Table: 2). Whereas, day 5 displayed significant inhibition in activity ($p < 0.0001$). In addition, remarkably significant inhibition activity was observed on the depuration day 14, where lowest treatment group observed significantly inhibited activity ($p < 0.0001$; Fig: 1b).

Statistically significant activity of GPx was noted for both concentrations and time (two-way ANOVA, $p < 0.0001$), inciting significant interaction effect ($p < 0.0001$; Table: 1). GPx observed significant increase in activity at the beginning for 10 and 15 $\mu\text{g/l}$ concentrations (day 1, $p < 0.05$ and $P = 0.004$; Table: 2). In the mid phase significant inhibition was noted particularly on day 5 at 10 $\mu\text{g/l}$ ($p < 0.0001$). On the last day of exposure an increase in activity was observed for 15 $\mu\text{g/l}$ ($p < 0.0001$). Depuration day displayed significantly inhibited activity particularly in the lowest treatment group ($p < 0.0001$; Table: 2). The GST content was significantly affected by DEHP concentrations and time ($p < 0.0001$), prevailing significant interaction among concentration against time was displayed in the gill ($p < 0.0001$; Table: 1). GST activity was significantly elevated from day 1 to 5 of exposure period ($p < 0.0001$; Table: 2). However, no significant activity was noted on last day of exposure. Depuration was significantly inhibited for all the treatments ($p < 0.0001$; Table: 2). As depicted in Table 1, LPO activity was significantly affected by concentration and time ($p < 0.0001$; Table: 2), further affecting interaction significantly ($p < 0.0001$; Table: 1). LPO followed the similar trend that of first line of defence (SOD, CAT and GPx). Day 1,3 and 7 had highly significant elevated activity ($p < 0.0001$; Fig: 1e). Whereas depuration day 14 displayed significant induction in activity in all concentrations with highest activity in lowest concentration ($p < 0.001$; Table: 2).

3.2 Biochemical response in Muscle

A highly significant effect of concentrations (two-way ANOVA, $p < 0.0001$) and time ($p < 0.0001$) was observed on SOD activity, further stimulating interaction effect ($p < 0.0001$; Table: 1) of these in muscle. SOD activity in muscle tissue was noted to be significantly high on day 1, 3 and 7 ($p < 0.0001$; Table: 2) in all treatment groups. However, depuration day (day 14) observed inhibition in activity at lowest ($p < 0.05$) and highest ($p < 0.0001$) treatment groups (Table: 2). According to Table 1, concentration of DEHP (two-way ANOVA, $p < 0.0001$) and time ($p < 0.0001$) of exposure revealed a statistically significant effect on CAT activity. Additionally, enhancing the interaction effect between these variables in muscle tissue. CAT showed a steady increase in activity on day 1 to 3. On day 1 significantly elevated levels were noted as compared to control in all the treatment groups ($p < 0.0001$; Fig: 2b), these levels further increased on day 3 ($p < 0.0001$). whereas significant inhibition of activity was observed on day 5 ($p < 0.0001$). On last day of exposure an induction in activity was noted in all treatment groups ($p < 0.05$ and $p < 0.001$; Table: 2). CAT activity notably inhibited on day 14 for all the treatment groups ($p < 0.0001$; Table: 2). Although, a statistically significant effect of concentration (two-way ANOVA, $p < 0.0001$) and time ($p = 0.0001$) was depicted by GPx activity, the interaction effect of these variables was observed to be less significant ($p = 0.01$; Table: 2) compared to other biochemical markers. GPx depicted similar trend that of CAT, at the beginning, significant induction was observed on day 1 and 3 ($p < 0.05$ and $p < 0.0001$; Table: 2). Day 5, 7 and 14 noted no significant changes. The effect of concentration (two-way ANOVA, $p < 0.0001$) and time ($p < 0.0001$) on GST activity was noted to be statistically significant, further signifying significant ($p < 0.0001$; Table: 1) interaction effect of concentration against time. GST activity noted significant increase on day 1 ($p = 0.006$) in lowest concentration group. While, day 3 depicted highly significant induced activity in all treatment groups ($p < 0.001$; Table: 2). Day 5 observed inhibition only in highest concentration treatment group ($p < 0.05$; Table: 2). Last exposure day 7 denoted highly significant induction of GST activity in lowest treatment ($p = 0.0008$; Table: 2) group. While, day 14 had no significant activity. In the muscle tissue LPO activity was significantly affected by concentration (two-way ANOVA, $p < 0.0001$) and time ($p < 0.0001$; Table: 1). Additionally, stimulating significant interaction ($p < 0.0001$; Table: 2) among the variables. LPO in muscle was significantly elevated on day 1, 3 and 7 ($p < 0.0001$; Table: 2). whereas day 5 observed highly significant inhibition activity ($p < 0.0001$; Table: 2). Depuration day observed no significant changes in activity of LPO.

3.3 Integrated Biomarker Response

In the present study IBR index was employed to understand cumulative effect of five biochemical markers (SOD, CAT, GPx, GST and LPO) on gill and muscle tissue of juvenile *E. suratensis*, exposed

to three concentration gradient of DEHP (10, 15 and 20 µg/l). IBR index values were calculated for all the five biomarkers in each treatment group. All the results were formulated in the form of star plots. Higher values depict elevated toxicity to the organism.

3.3.1 Gill

A differential response of biomarkers was recorded for the IBR values at individual concentrations over the period of exposure and depuration. Day 1 and 3 depicted highest IBR values (17.1 and 16.2) at highest concentration (20 µg/l) (Fig: 3f). With highest IBR value of (19.9) at 10 µg/l treatment group on day 7 was observed to be significantly different from the other treatments. Most significant activities in this treatment were of SOD, GST and LPO. The lowest IBR value (2.6) was noted on depuration day 14 for the lowest concentration group (10 µg/l). On exposure days 1, 3 and 7 all the biomarkers were activated which was in accord with enzyme activity results (Fig: 3f).

3.3.2 Muscle

Muscle reflected dissimilar response of IBR activation compared to gill. IBR values were found to be high for all biomarkers at all exposure days except for day 5. Unlike gill, highest IBR value (17) was noted for day 1 at highest exposure concentration. CAT, GST and LPO activities were the most significant ones at (20 µg/l) exposure group. Day 1 and 3 depicted almost similar IBR value (11.1 and 11.6) for (10 µg/l) treatment group (Fig: 4f).

3.3.3 Gill vs Muscle

IBR index followed the similar trend of biomarker activation throughout the experiment in gill and muscle. Although, the trend of activation was similar, it differed in level of magnitude of activity. Gills clearly depicted higher activation rate compared to muscle on all exposure days along with day of depuration. The highest IBR value (57.1) for gill was noted on day 7, while muscle depicted day 1 to be highest IBR (37.2) (Fig: 5).

4. Discussion

Multibiomarker approach is a reliable tool in monitoring the response of specific organisms to organic contaminants and toxins (Zheng et al., 2022). The most susceptible are the early developmental stages of any organism specifically to organic compounds. Among the organic compounds, DEHP is one of the most widely used plasticizers with high volume production resulting in ubiquitous global presence, and therefore a threat to marine environment and its organisms (Wang et al., 2018). DEHP is known to affect the early stages of many organisms (Mathieu-Denoncourt et al., 2016; Yang et al., 2018) and is a major endocrine disruptor significantly influencing life history traits (Mathieu-Denoncourt et al.,

2016; Wang et al., 2018). Although, *Etroplus suratensis* (Pearl Spot) is an important and widely consumed fish from the Indian coastal waters, the effects of DEHP on this estuarine fish is yet to be understood, specifically the impacts on muscle and gill tissue of juveniles.

4.1 DEHP induced oxidative stress in *E. suratensis*

To understand the effects of low dose response in any organism it is imperative to test both dose (concentration gradient) at multiple time points (Calabrese, 2001). Numerous studies on the exposure of DEHP on fish have demonstrated accumulation in different organs and tissue. Marine medaka embryos exposed to different DEHP concentrations revealed accumulation in the range of 0.99-28.97 $\mu\text{g/g}$ (Ye et al., 2016), responsible for endocrine disruption. Another study on the same species showed accumulation of metabolites (Ye et al., 2014) after DEHP exposure. DEHP accumulated in the gonads of marine bivalve (*M. coruscus*) upto 1 mg/kg at 40 $\mu\text{g/l}$ concentration (Gu et al., 2021), altered SOD and CAT activity while GSH was induced to eliminate ROS stress. Another study on bivalve (*M. galloprovincialis*) observed DEHP accumulation in hepatopancreas from 0.03- 105.93 $\mu\text{g/g}$, disrupting antioxidant activity of SOD and CAT (Andreyeva et al., 2023).

The first defence response against a stress generated by Reactive Oxygen Species (ROS) is by elimination of the same (ROS). For any given toxicant in the body, the initial response is by SOD that acts on superoxide anion radicals (O_2^-) that are transformed into H_2O_2 as final product. DEHP triggered an imbalance in homeostasis in gill and muscle of *E. suratensis* increasing the (O_2^-) radical thereby inducing SOD spike of inverted 'U' shape curve over the duration of 14 days (Fig: 1a). As a result of hormetic response, a significant induction in SOD activity was observed initially (i.e. till day 3) in both gill and muscle (Fig: 1a). Progressively on day 5 inhibition was observed which could be the result of overwhelming activity of SOD in response to excessive generation of (O_2^-) radicals where SOD failed to eliminate excessive superoxide radicals (Liu et al., 2017; Ma et al., 2017).

To cope with excessive stress generated by ROS, the activity of SOD increased on last day of exposure for gill and muscle (Fig: 1a). Highest concentration group (20 $\mu\text{g/l}$) exhibited significant SOD inhibition in both gill and muscle (Fig 1 and 2a) at the end of depuration period. SOD activity was in synergy with increase in DEHP concentration, signifying "dose effect" relationship (Li et al., 2022). Inhibited SOD activity resulted, could be due to accumulation of excessive ROS which in turn may lead to total shut down of antioxidant system following reduction in certain enzyme functions (Butterfield et al., 1998; Li et al., 2022).

Although belonging to category of antioxidant enzymes, SOD is also the source for hydrogen peroxide, thus H_2O_2 scavenging enzymes follow after SOD activity (Regoli and Giuliani, 2014). CAT actively

scavenges on H_2O_2 by transforming it into H_2O and O_2 . CAT was significantly activated on days 1 and 3 in muscle and gill (Fig: 1 & 2b), synergistically followed the SOD activation and inhibition trends (Fig: 1b). Thus, to maintain homeostasis CAT scavenges the H_2O_2 produced by SOD as byproduct (Ge et al., 2015). On day 5 there was a significant CAT inhibition in both gills and muscle followed by activation on last day of exposure (Fig: 1 and 2b). Where lowest concentration (10 $\mu g/l$) noted significant inhibition in gill while muscle tissue depicted inhibition at 15 $\mu g/l$ (Fig: 1 and 2b). The cause of inhibition would be excessive accumulation of ROS and was relative to the inhibition activity of SOD (Song et al., 2022). Notably, CAT activity was significantly inhibited at end of depuration day in gill and muscle. This eventually enhanced the stress causing imbalance in elimination and production rate of ROS, leading to suppression in CAT activity (Wang et al., 2018).

If the excessive H_2O_2 still remain in the system, GPx being the second line of defense reduces hydrogen peroxide radicals, to H_2O . Inverted 'U' shape activity curve defined the response of GPx as well in gill and muscle tissue (Fig: 1 and 2c). Much similar to SOD the GPx activity was of lower magnitude as target ROS (i.e. H_2O_2) is shared by CAT as well (Halliwell and Gutteridge, 2015; Regoli and Giuliani, 2014).

In synchrony with CAT, GPx as well was involved in decomposition of H_2O_2 in gill and muscle, although no inverse relationship to SOD was observed. Sequence of detoxification could be the reason for synergistic effect adopted by SOD and followed by CAT and GPx. GST binds through glutathione conjugation of numerous electrophiles generated by toxicant protecting cell against environmental contaminants via phase-II biotransformation.

Activation-inactivation trend was observed in gill tissue whereas muscle denoted gradual increase in activity throughout the experiment period. A significant increase in activation of GST was noted on day 5 in gill tissue, while day 7 denoted minimal activity (Fig: 1 and 2d). This result corroborated to activity of first and second line of defence (SOD, CAT and GPx), where significant inhibition ($p < 0.05$, $p < 0.0001$) was observed on day 5 while denoting significant induction on day 7 ($p < 0.0001$). Increased GST activity on day 5 suggests significant activation resulting in overcompensation leading to failure of detoxification (Samanta et al., 2014) and activation of stress on day 7 of exposure period. At the end of depuration period significant inhibition was observed in lowest concentration of DEHP in gill (Fig: 2a). Thus it can be concluded that excessive stress generated by DEHP persists in gill tissue even after depuration.

Gradual increase in GST was noted in muscle compared to activation and inactivation in the gill during the entire experimental period. Whereas on the depuration day there was an increase in activity for

highest concentration in muscle, while lowest DEHP levels depicted homeostasis (Fig: 2d). The activation of GST throughout the exposure time depicts excessive stress generated by ROS during exposure period in muscle. However at depuration, GST was able to detoxify the DEHP generated stress in muscle tissue. Thus, role of GST varied among the target tissue. Although a high scavenging rate was observed in gill, GST failed in detoxification of ROS at depuration (Fig: 2d). While in muscle it could handle the stress and maintain homeostasis. In conclusion, GST may be beneficial or non-beneficial depending upon the target tissue in fish (Samanta et al., 2014).

Ultimately the remainder of free radicals (mainly $\text{OH}\cdot$) attack PUFA (polyunsaturated fatty acid) causing oxidative damage that results in lipid peroxidation (LPO). Changes in receptors and enzyme functions as well as damage to biological membrane (Toroser et al., 2007) can be caused due to hydroxyl radicals ($\text{OH}\cdot$). Interestingly, Peroxidation was observed in gill and muscle over exposure period, but significant variations were noted at the end of depuration for both the target tissue (Fig: 1 and 2e). LPO was high in all three concentrations compared to control group and increased with time and concentrations (Fig: 1e). On the last day of exposure, peroxidation was observed to be significantly high in all treatment groups in gill tissue with highest in 10 $\mu\text{g/l}$ conc. (Fig: 1e) and similar trend was noted on depuration day. Therefore we postulate that DEHP exposure in gill impedes antioxidant enzyme's capacity to timely eradicate ROS and/or completely. Thus the excessive ROS will continuously target PUFA in biological membranes further inflicting lipid peroxidation and cell damage. Thus it can be inferred that peroxidation in gill was consistent with activity of ROS, as DEHP has continuous detrimental effects on gill tissue. Peroxidation/ ROS was observed to be minimal at the latent stage in zebrafish and earthworm due to high concentrations of DEHP used for exposure (Li et al., 2022; Ma et al., 2017). While lipid peroxidation in muscle followed consistent trend with respect to the antioxidant enzyme activity (Fig: 2e). On depuration day, unlike gill, LPO activity in muscle tissue indicated homeostasis as a result of increasing activity of GST throughout the experiment. Similarly, inhibition in detoxification level triggered activation of LPO content in *Oryzias melastigma* exposed to DEHP (Yin et al., 2021). Thus it can be inferred that muscle tissue copes with stress generated by excessive ROS induced by DEHP for several days, if depuration of certain days is provided at all the concentrations.

4.2 DEHP induced Integrated responses

As it is difficult to draw conclusions depending on single biomarker the cumulative effects of DEHP, can be deciphered by IBR (Integrated Biomarker Response). IBR values of antioxidant enzymes (SOD, CAT and GPx) for gill responded actively on 1, 3 and 7 days of exposure, which corroborated with enzyme activity (Fig: 3). GST and LPO index was on greater side compared to baseline values, which

signifies induction in lipid peroxidation and damage to gill induced by DEHP. Similar variations in IBR were displayed on day 1, 3 and 14, relative to DEHP concentrations. Last day of exposure exhibited an inverse trend of IBR for higher DEHP concentrations as compared to earlier time points. Maximum IBR values for 10 and 20 µg/l in gill tissue were observed at first and last day of exposure respectively (Fig: 3a and d). Depuration day experienced slightly higher IBR values compared to baseline in 10 µg/l concentration for LPO index (Fig: 3d) indicating persistence of lipid peroxidation in gill after 7 day of depuration. Thus it can be concluded that a biphasic dose response was exhibited by gill tissue upon exposure to DEHP concentration (Calabrese et al., 2016).

IBR index followed similar trend of activation that of gill in muscle tissue. All the biomarkers significantly responded at the exposure period of 7 days, especially at 20 µg/l concentrations. Suggesting DEHP exposure stimulated antioxidant enzyme system to counter stress. LPO and GST values signified lipid peroxidation and tissue damage due to failure of antioxidant system and accumulation of residual ROS in muscle. High IBR values (17 and 11.6) were observed initially (till day 3) for 20 and 10 µg/l respectively (Fig: 4f). In spite of high IBR at commencement of experiment, depuration values were well below baseline IBR. This confirms the individual enzymatic results wherein muscle counter and adopt to stress posed by DEHP efficiently after depuration time of a week.

To understand the holistic picture of DEHP induced feedback by target tissue, IBR values between gill and muscle were compared. Wherein gill clearly depicts excessive response compared to muscle (Fig: 5). Day 7 depicted highest IBR value in gill (57.1) were for day 7 and in muscle for day 1 (37.2; Fig: 5) suggesting DEHP induced toxicity varied according to mode of exposure and target tissue. Depuration day values differed for gills and muscle, where gill depicted higher IBR than baseline values and muscle showed near to baseline values. Thus, all modes of analysis point to the inference that gill remain stressed and may require much longer duration to mitigate negative effects of DEHP unlike muscle.

Present study follows the “Hormesis” effect which defines the biphasic dose response to chemical exposure. Low dose exposure induces hormesis wherein induction of antioxidant enzymes is observed to compensate the damage caused and further helps in protecting adaptive processes. Hormesis can be activated through direct stimulation or due to imbalance in homeostasis through overcompensation (Calabrese et al., 2016).

5. Conclusion

Present study, is leading investigation unraveling the effect of DEHP on gill and muscle tissue of estuarine fish *E. suratensis* post depuration period of seven days. Results suggest oxidative stress was

produced in both the target tissues largely on all exposure days, indicating elevated production of ROS in gill and muscle. Whereas it differed on depuration day suggesting exposure pathway plays an imperative role in induction of oxidative stress depending upon contact and susceptibility of target tissue. Lipid peroxidation depicted lipid membrane damage in gill tissue after depuration while muscle was able to eradicate the stress after receiving depuration period. IBR results corroborated with antioxidant enzyme activity. Where highest toxicity was observed in gill tissue compared to muscle. Present study implies that low concentration of DEHP has detrimental effects on early developmental stages of estuarine fishes.

Credit Author Statement

Aniket Desai: Conceptualization, Formal analysis, writing-original draft preparation, investigation, data curation, review and editing; **Faruq Pathan:** Formal analysis, writing-review and editing, investigation; **Ranjana Yadav:** writing-review and editing, **Deepa Yogi:** writing- review and editing, **Mandar Nanajkar:** Conceptualization, data curation, writing- review and editing, editing and project supervision.

Declaration of Competing Interest

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.

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Table 1: Two-Way ANOVA: Effects of DEHP concentrations and time in Gill and muscle tissue of *E.suratensis* affecting biochemical biomarkers. Significant values are highlighted in bold ($p < 0.05$).

		Concentrations	Time	Concentrations \times Time
Biochemical markers in gills	SOD	F (4, 40) = 35.15 P < 0.0001	F (3, 40) = 64.63 P < 0.0001	F (12, 40) = 29.56 P < 0.0001
	CAT	F (4, 40) = 226.3 P < 0.0001	F (3, 40) = 14.66 P < 0.0001	F (12, 40) = 81.45 P < 0.0001
	GPx	F (4, 40) = 15.97 P < 0.0001	F (3, 40) = 9.631 P < 0.0001	F (12, 40) = 8.330 P < 0.0001
	GST	F (4, 40) = 415.5 P < 0.0001	F (3, 40) = 161.0 P < 0.0001	F (12, 40) = 141.1 P < 0.0001
	LPO	F (4, 40) = 82.85 P < 0.0001	F (3, 40) = 93.46 P < 0.0001	F (12, 40) = 43.05 P < 0.0001
Biochemical markers in muscle	SOD	F (4, 40) = 110.6 P < 0.0001	F (3, 40) = 245.3 P < 0.0001	F (12, 40) = 104.5 P < 0.0001
	CAT	F (4, 40) = 160.9 P < 0.0001	F (3, 40) = 25.95 P < 0.0001	F (12, 40) = 64.51 P < 0.0001
	GPx	F (4, 40) = 11.02 P < 0.0001	F (3, 40) = 8.988 P = 0.0001	F (12, 40) = 2.519 P = 0.0142
	GST	F (4, 40) = 255.8 P < 0.0001	F (3, 40) = 10.32 P < 0.0001	F (12, 40) = 5.452 P < 0.0001
	LPO	F (4, 40) = 2406 P < 0.0001	F (3, 40) = 210.2 P < 0.0001	F (12, 40) = 279.8 P < 0.0001

Table 2: Dunnett's t test (post hoc): Differences in effects of DEHP concentrations against time in Gill and muscle tissue of *E.suratensis* affecting biochemical biomarkers. "ns" represents not significant.

Time	Interaction	Gill					Muscle				
		SOD	CAT	GPx	GST	LPO	SOD	CAT	GPx	GST	LPO
DAY 1	Control vs. 10 µg/l	<0.0001	<0.0001	0.0175	<0.0001	<0.0001	<0.0001	<0.0001	0.0246	0.0066	<0.0001
	Control vs. 15 µg/l	<0.0001	<0.0001	0.0048	<0.0001	<0.0001	<0.0001	<0.0001	0.3638	ns	0.0001
	Control vs. 20 µg/l	<0.0001	<0.0001	ns	<0.0001	<0.0001	<0.0001	<0.0001	0.0021	ns	<0.0001
DAY 3	Control vs. 10 µg/l	<0.0001	0.0109	ns	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0008	<0.0001
	Control vs. 15 µg/l	<0.0001	0.0262	ns	<0.0001	<0.0001	<0.0001	<0.0001	0.0546	<0.0001	<0.0001
	Control vs. 20 µg/l	<0.0001	0.0003	ns	<0.0001	<0.0001	<0.0001	0.0004	0.002	0.0021	<0.0001
DAY 5	Control vs. 10 µg/l	ns	<0.0001	<0.0001	<0.0001	<0.0001	ns	<0.0001	ns	ns	<0.0001
	Control vs. 15 µg/l	ns	<0.0001	0.0085	<0.0001	0.0177	<0.0001	<0.0001	ns	ns	<0.0001
	Control vs. 20 µg/l	ns	<0.0001	0.0034	<0.0001	0.0002	ns	<0.0001	ns	0.0135	<0.0001
DAY 7	Control vs. 10 µg/l	<0.0001	<0.0001	ns	ns	<0.0001	<0.0001	0.0102	ns	0.0008	<0.0001
	Control vs. 15 µg/l	<0.0001	<0.0001	<0.0001	ns	0.0139	<0.0001	0.0037	ns	ns	<0.0001
	Control vs. 20 µg/l	<0.0001	<0.0001	ns	ns	0.0001	<0.0001	0.0011	ns	0.034	<0.0001
DAY 14	Control vs. 10 µg/l	0.0103	<0.0001	<0.0001	<0.0001	<0.0001	0.0296	<0.0001	ns	ns	ns
	Control vs. 15 µg/l	0.0019	<0.0001	ns	<0.0001	<0.0001	ns	<0.0001	ns	ns	ns
	Control vs. 20 µg/l	<0.0001	<0.0001	ns	<0.0001	<0.0001	<0.0001	<0.0001	ns	ns	ns

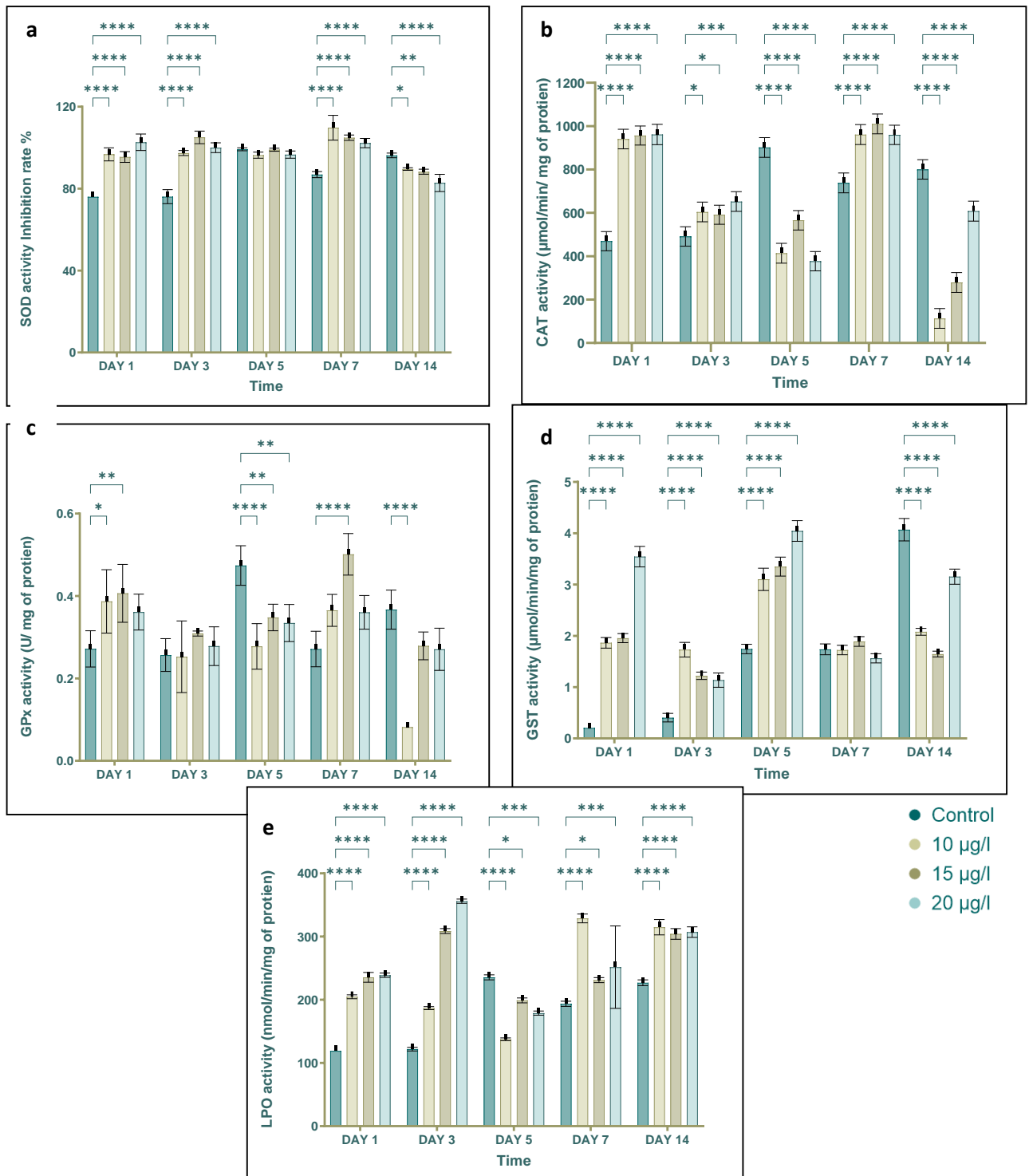


Fig1: Enzyme activity induced by DEHP (a) SOD, (b) CAT, (c) GPx, (d) GST and (e) LPO on gill of *E. suratensis*. * indicates significant difference with respect to control ($p < 0.05, 0.001$ and 0.0001).

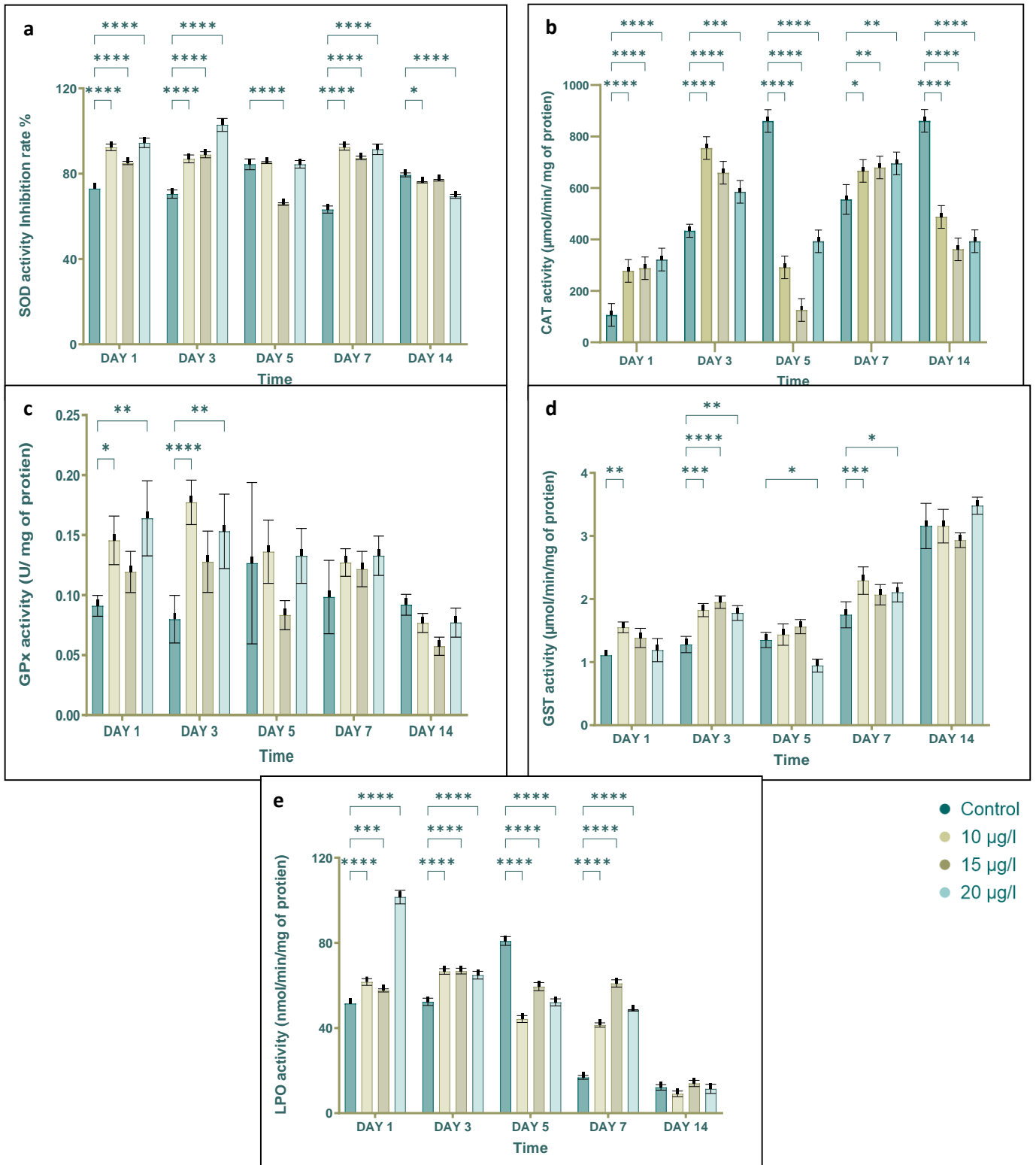


Fig2: Enzyme activity induced by DEHP (a) SOD, (b) CAT, (c) GPx, (d) GST and (e) LPO on muscle of *E. suratensis*. * indicates significant difference with respect to control ($p < 0.05, 0.001$ and 0.0001).

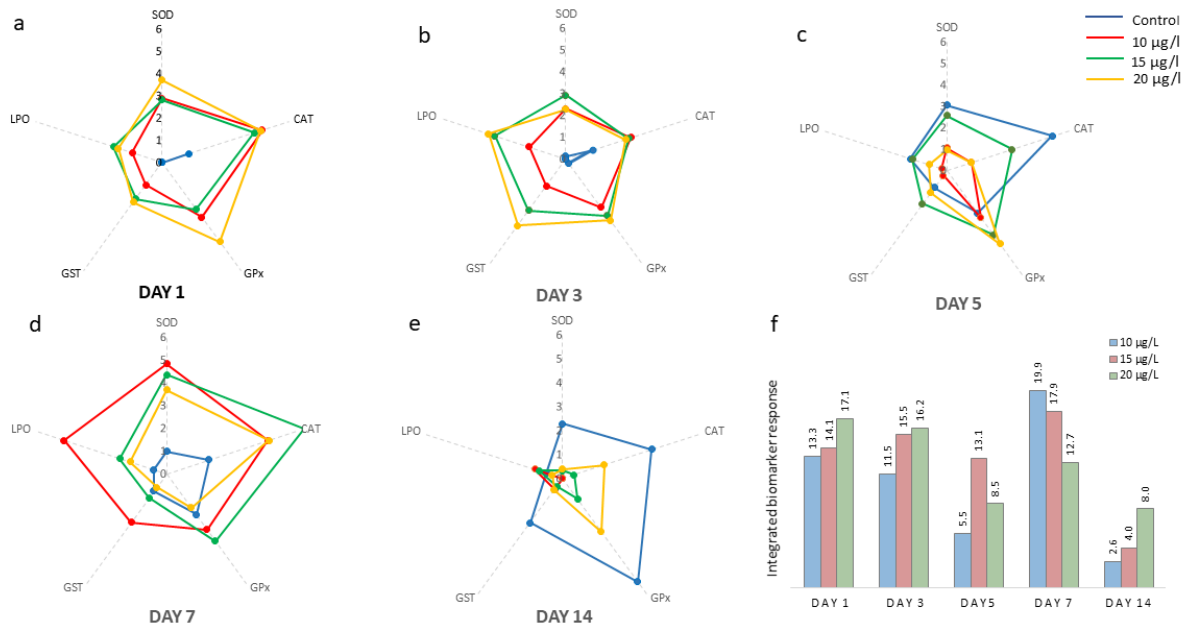


Fig 3: Star plots depicting distinct biochemical responses to SOD, CAT, GPx, GST and LPO in *E. suratensis* gill tissue (a-e), and IBR index bar chart for different DEHP concentrations and time period in gill tissue (f).

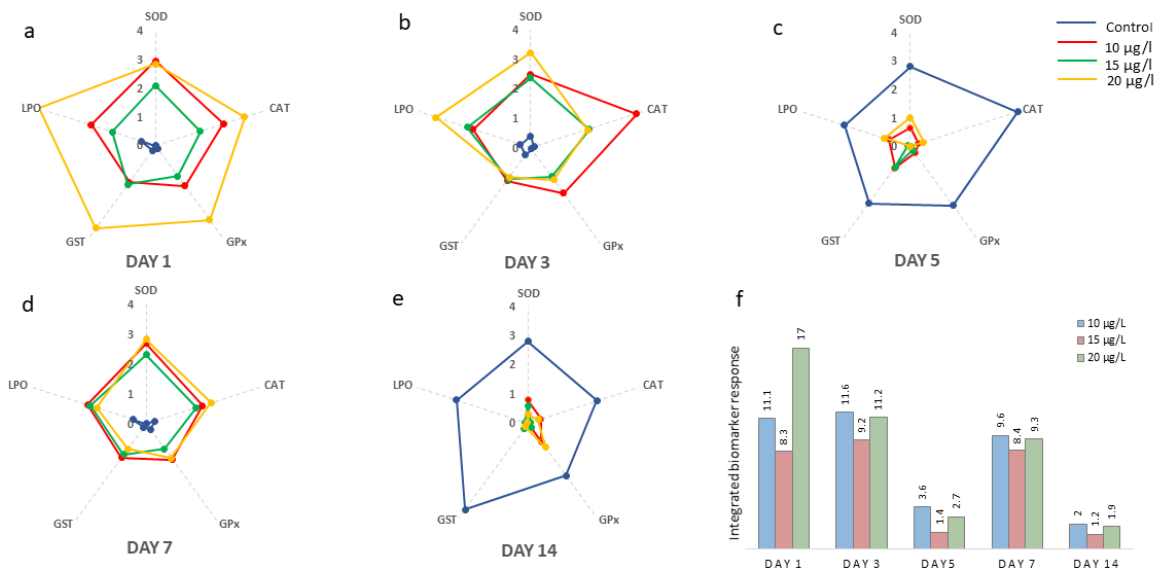


Fig 4: Star plots depicting distinct biochemical responses to SOD, CAT, GPx, GST and LPO in *E. suratensis* muscle tissue (a-e), and IBR index bar chart for different DEHP concentrations and time period in muscle tissue (f).

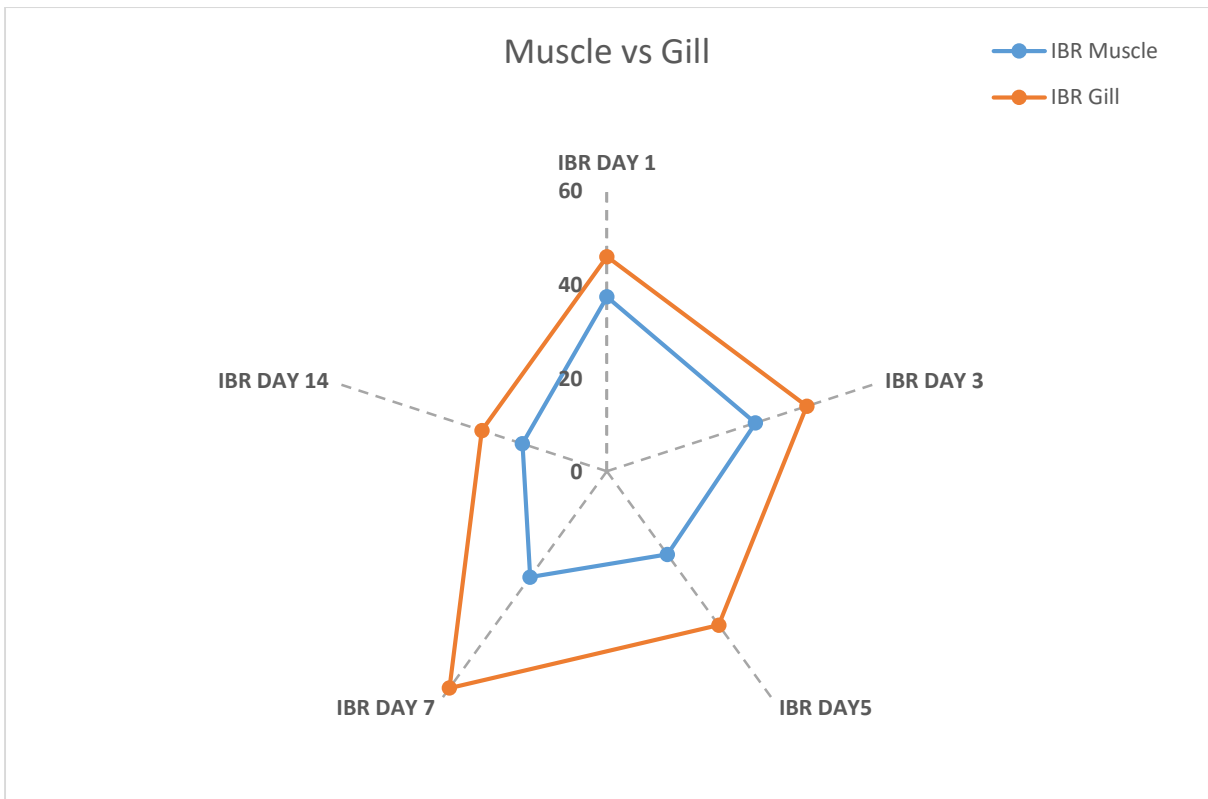


Fig 5: IBR index star plot for DEHP at different time period in gill and muscle tissue.