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Sublethal Effect of Benzo[a]pyrene (B[a]P) on Oxidative Stress and Antioxidant Enzymes in the Mud Crab *Scylla serrata*

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Under various severe environmental conditions, marine organisms can suffer from oxidative stress. In the mud crab, the sub-lethal effect of benzo[a]pyrene (B[a]P) using environmentally realistic concentrations (10 ppb and 20 ppb) on oxidative stress and antioxidant defenses was studied. For oxidative stress, levels of Thiobarbituric Acid (TBARS) and carbonyl protein (CP) were determined. After 30 days of B[a]P exposure, the tissues contained considerably more CP and TBARS. The tissues of crab exposed to sublethal level of B[a]P for 30 days showed decrease in antioxidant defense enzymes, including superoxide dismutase (SOD) and catalase (CAT). Under B[a]P exposure, SOD and CAT activities were decreased considerably in the hepatopancreas. The glutathione *S* transferase (GST) increased in the hepatopancreas due to B[a]P exposure. The

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current study found a strong link between oxidative stress and antioxidant defenses in crab exposed to B[a]P. Overall, the results show that hepatopancreas is the most sensitive component to oxidative damage, implying that *Scylla serrata* could be employed as a bioindicator of organic pollution exposure by evaluating antioxidant enzyme activities.

Keywords: Scylla serrata; hepatopancreas; Benzo[a]pyrene; oxidative stress; antioxidant defense.

1. INTRODUCTION

The discharge of synthetic chemicals into aquatic environments by residential, agricultural, and industrial sectors has a detrimental effect on aquatic life [1]. Polycyclic aromatic hydrocarbons (PAHs) are produced when organic material burns partially. These compounds are produced by human activity and are found all over the world. Because of human activity PAHs are becoming more common in estuaries and coastal areas and these compounds can be found in marine sediment, water and the tissues of marine species and they can disrupt the regular functioning of marine organisms' lives [2]. According to Go`mez-Mendikute et al. [3], PAHs, especially B[a]P, are common environmental pollutants that should be taken very seriously because of their toxicity, mutagenicity and carcinogenicity.

The metabolism of PAHs could result in the production of reactive oxygen species (ROS) [4]. Among the main effects of ROS formation in biological systems is DNA damage, protein oxidation and enzyme deactivation [5]. The steady-state level of ROS is defined as the balance between production and breakdown; an imbalance favouring the former is called "oxidative stress" [6,7]. Elevated ROS production is thought to be a key factor in pollutant-mediated toxicity in aquatic animals [8]. Oxidative stress can cause damage and disruption to numerous cellular processes, ultimately resulting in cell death.

To mitigate the possible hazards of ROS, antioxidant defences-based molecules have been developed in the organisms and these enzymes including catalase (CAT), superoxide dismutase (SOD), and glutathione s transferase (GST) are an essential interacting line of defence against reactive oxygen species (ROS) and their attack products [6]. Since antioxidant enzymes are crucial for maintaining cell homeostasis, it has been suggested that the induction of these enzymes in a variety of marine organisms can serve as a biomarker for oxidative stress caused by contaminants, with each organism's response to pollutants being represented differently [9].

Whether it emanates from artificial or natural sources, PAH interacts with aquatic life. Many researchers have documented PAHs through toxicological studies and risk evaluations [10-13]. Conversely, the mechanisms underlying the toxicity of PAHs to aquatic organisms remain poorly known. Numerous studies have demonstrated that PAHs alter the antioxidant activity in crab and oxidative stress is a well-established cause of PAH toxicity [14].

In order to further characterize the changes occurring in numerous indicators of oxidative stress following B[a]P exposure in mud crab *Scylla serrata*, the objective of this work is to use a number of biomarkers to find particular and different patterns of responses of the species. Studies have been conducted on carbonyl protein and lipid peroxidation as markers of oxidative stress in the hepatopancreas. The activities of specific antioxidant response factors, such as glutathione s transferase, superoxide dismutase, and catalase, were measured in the hepatopancreas of crabs exposed to B[a]P in response to oxidative stress.

2. MATERIALS AND METHODS

2.1 Collection and Maintenance of Mud Crab in Laboratory Condition

Mud crab *Scylla serrata* weighing 110-120 g were collected and transported to the laboratory from the sea mouth of the Pulicat Lake near Chennai, Tamil Nadu, India. The crab were kept in big glass aquaria filled with seawater (90 X 60 X 45 cm) and fed a fresh prawn. For a week, crab was acclimatized to laboratory settings and a feeding regimen by changing the water every day. Dissolved oxygen (5–6 mg/L), salinity (30±1 ppt), temperature (28±1^{o}C), and pH (8.1±0.1) were the tank holding conditions. A 14:10 h light:dark cycle was used to produce illumination.

2.2 Determination of Lethal, Median Lethal and Sublethal Concentration of B[a]P

An acute toxicity (96-hour) investigation was carried out to determine the lethal (LC_{100}), median lethal (LC_{50}), and sublethal (LC_0) values of B[a]P to *S. serrata* using the static renewal approach [15]. In acetone, 1 part per million (PPM) of B[a]P was produced as a stock solution (HPLC grade). From this stock solution, concentrations of 10, 20, 40, 80, 160 and 320 ppb were prepared. Ten crabs were placed into each concentration in 20 L glass aquaria with seawater, and the test solution was changed daily. No food was given during the 96-hour bioassay test period. Dead crabs were immediately taken out of the test medium. Duplicates of each concentration were performed. After 96 hours, the percentage mortality of crabs was recorded, and the percent mortality was calculated using probit analysis to determine the 96-hour LC_{50} value according to the method of Finney [16].

2.3 Chronic Exposure of Crab at Sublethal Concentration of B[a]P

Crabs were divided into four groups of ten specimens each to assess changes in biomarkers when crabs exposed to B[a]P. Group I were treated with normal seawater and treated as control. Crabs in Group II were raised in acetone treated water (which is used as substance dissolves B[a]P). Crabs in groups III to IV were exposed to seawater containing 10 and 20 ppb B[a]P, which were the sublethal values at which 0 percent mortality occurred after 96 hours. The studies were conducted in glass aquaria (90" X 60" X 45") with triplicate chambers for each concentration. During the experiment, the test solution and seawater were replaced daily, and the crab was fed with fresh prawns. Duration of the exposure was 40 days, and the sampling of crab was taken at every 20 days, after 40 days all the treated crab was maintained in normal seawater to check the depuration of toxicant and recovery of the biomarkers. The hepatopancreas was removed and placed under ice and stored at -20 ^oC until.

2.4 Biomarkers for Evaluating Toxicity of B[a]P

Using 2,4-dinitrophenylhydrazine (DNPH), the carbonyl protein (CP) content was determined in accordance with the methodology of Lushchak et al. [17]. With a molar extinction value of $10^3\ M^{-1}\ cm^{-1}$, CP was measured spectrophotometrically at 370 nm. The levels of CP in the guanidine chloride solution were determined using nano moles per unit protein. Devasagayam and Tarachand [18] method was used to calculate TBARS. The colour produced was measured at 532 nm, and the malondialdehyde (MDA) concentration of the sample was reported as nmol of MDA created/unit protein.

The method that was employed to determine CAT activity was Sinha [19]. Dichromate in acetic acid was heated in the presence of H_2O_2 to produce chromic acetate, with perchromic acid developing as an unstable intermediate. Colorimetric examination of chromatic acetate was carried out at 570 nm. Different times were given to the reaction to run before it was stopped with a dichromate acetic acid solution. To determine how much H_2O_2 remained, colorimetric measurements of chromic acetate were employed. It was measured how much H_2O_2 was utilised per minute per millilitre of protein. SOD activity was calculated following the method of Marklund and Marklund [20], which measured the amount of pyrogallol auto-oxidation inhibition at an alkaline pH. One unit of SOD activity is defined as the amount of enzyme that inhibits the oxidation reaction by 50% of its maximum inhibition. For GST activity at 37 °C, the spectrophotometric measurement of the GST activity of the fraction obtained with the substrate 1-chloro-2,4-dinitrobenzene (CDNB) was conducted by conjugating the acceptor substrate with glutathione [21]. The converted conjugate/min/mg protein was used to express the results.

2.5 Statistical Analysis

SPSS version 20.0 software were used to analyse the statistical difference. To summarise, six crabs were collected in duplicate for every group, and the mean standard error of six individuals per group was reported as the outcome. The data's homogeneity and normalcy were evaluated using Bartlett's test (Snedecor and Cochran, 1983). A one-way analysis of variance (ANOVA) was performed to determine whether there were any differences between the groups because all of the data was normally distributed. A p-value of less than 0.05 was used to determine significance. The Tukey's multiple comparison post hoc tests was used to ascertain the statistical difference between each treatment group.

3. RESULTS

During an acute B[a]P exposure, survivability of crabs was recorded. Survivability decreased as the concentration of B[a]P in the exposure media increased. At 10 and 20 ppb of B[a]P 100 percent survival was recorded and at 40 ppb of B[a]P about 85% of crabs survived after 96 h exposure, however at 80 ppb only 55% of crabs survived and no crab survived at 640 ppb.

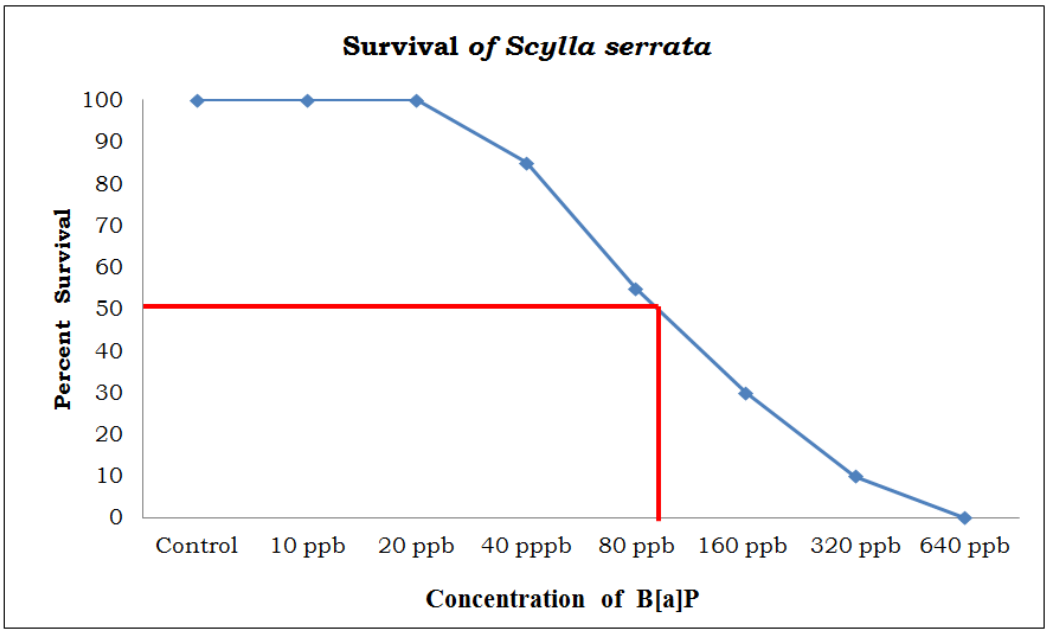

Fig. 1. Percent survival when the crabs exposed to different concentration of B[a]P

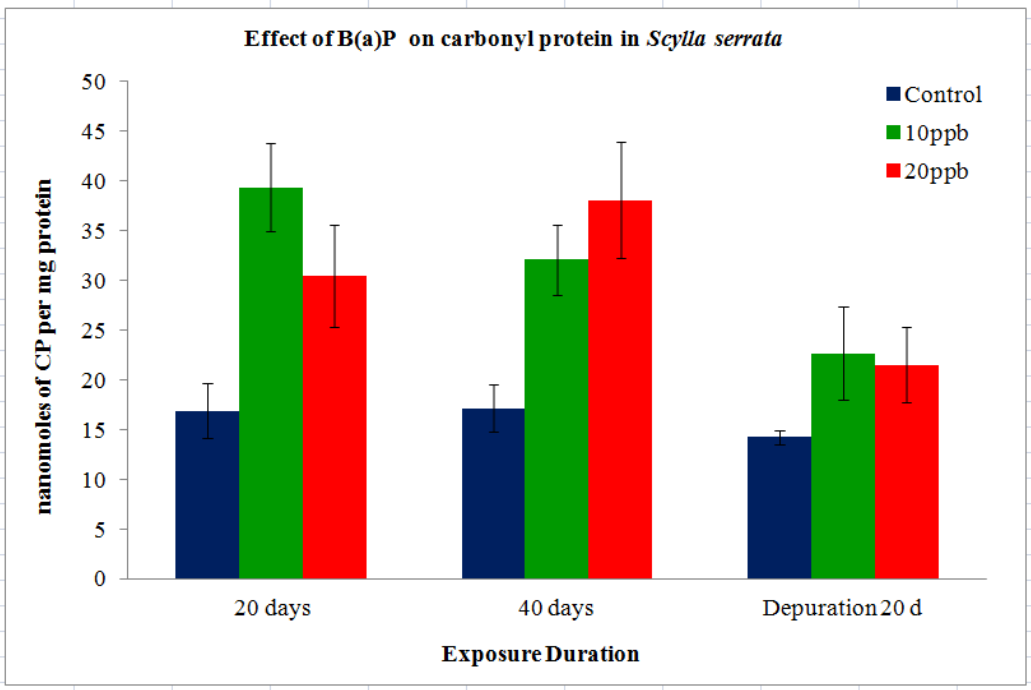

Fig. 2. Carbonyl Protein content in crabs exposed to different concentration of B[a]P for 40 d and followed by the depuration of 20 d. Values are expressed mean ± S.D.

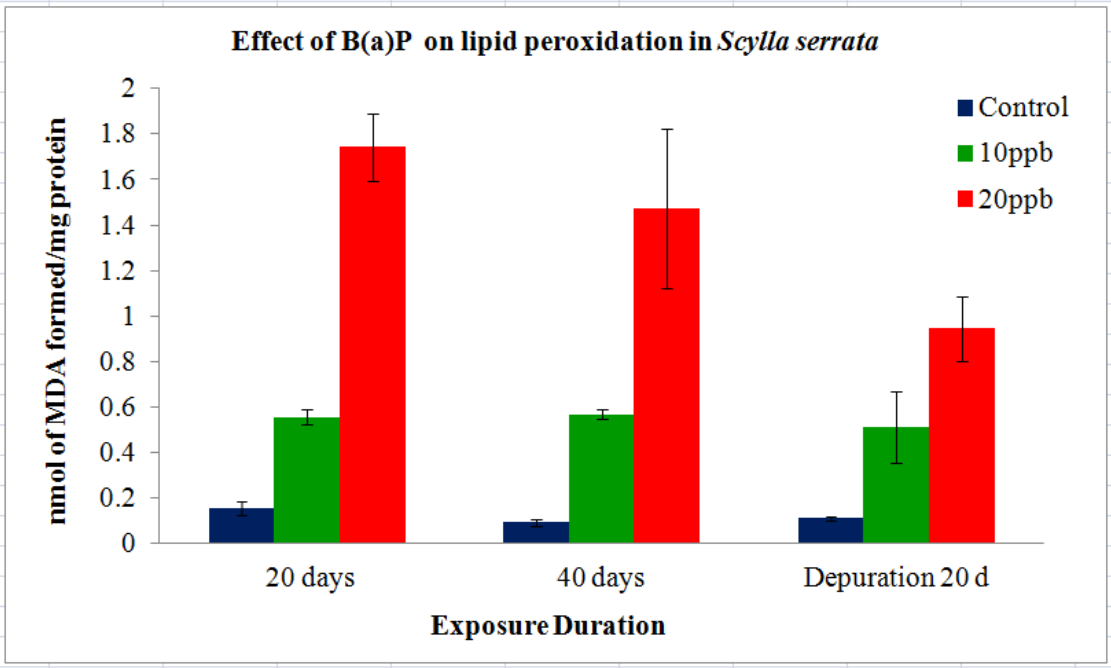

Fig. 3. Lipid peroxidation level in the crabs exposed to different concentration of B[a]P for 40 d and followed by the depuration of 20 d. Values are expressed mean ± S.D

After 96 hours of exposure, no mortality was found at 10 and 20 ppb, hence these concentrations were used as sublethal concentration (Fig. 1). A long-term exposure was carried out at these concentration for 40 days. Furthermore, since there is no significant difference between the solvent control and normal seawater control during the biochemical analysis, the normal control data were not used for any comparison. Solvent control was represented as control.

3.1 Measure of Oxidative Stress

Carbonyl protein is to measure the oxidative stress formed in the crab due to B[a]P exposure. The level of Carbonyl protein varied among the different concentration of the B[a]P after 20 and 40 days of exposure (Fig. 2). Carbonyl protein was present predominantly in hepatopancreas of crab exposed to the highest B[a]P (20 ppb) concentration. The carbonyl protein content in hepatopancreas was greatest after 40 days of B[a]P exposure, the increase in carbonyl protein was concentration dependent for long time exposure. The depuration of the crab at normal seawater shows the carbonyl protein level decreased but not significantly.

Exposure to B[a]P resulted in statistically significant oxidative stress as determined by concentrations of LPO in hepatopancreas of crabs (Fig. 3). LPO of hepatopancreas was greater in crabs exposed to B[a]P after 40 d. The magnitudes of LPO in hepatopancreas were greater in crabs exposed at 20 ppb. All the two exposed concentration showed statistically significant. The increase in LPO level was concentration dependent and the depuration of crab reduced the LPO level which is not so significant compared to the respective control (Fig. 3).

3.2 Measure of Antioxidant Response

The primary antioxidant and associated enzymes such as CAT, SOD and GST in the hepatopancreas of crabs changed after 40 d of B[a]P exposure when compared to control (Figs. 4-6). SOD activities decreased significantly when the crabs exposed to the highest concentration of B[a]P (20 ppb). The level of SOD in control crabs was greater after 40 days of exposure compared with other exposure group and the same decreased nearly 50% at the highest concentration of B[a]P exposure (Fig. 4). The decrease in SOD levels were concentration dependent and the depuration exposure shows that the SOD level increased.

Similar decreasing trend was observed for CAT activity when the crabs were exposed to B[a]P for 40 days. The lowest tested concentration (ppb) of B[a]P showed very meagre change with respective control group, however the highest concentration decreases the CAT activity predominantly in the hepatopancreas of crabs when compared to control group (Fig. 4). Depuration of crab recovered the CAT activity in the hepatopancrease activity.

GST activity is to measure the oxidative stress as well as the antioxidant response formed in the crab due to B[a]P exposure. The level of GST activity varied among the different concentration of the B[a]P after 20 and 40 days of exposure (Fig 2). GST activity was present predominantly in hepatopancreas of crab exposed to the highest B[a]P (20 ppb) concentration. The GST activity in hepatopancreas was greatest after 40 days of B[a]P exposure, the increase in GST activity was concentration dependent for long time exposure. The depuration of the crab at normal seawater shows GST level decreased significantly only when the crab exposed to 10 ppb.

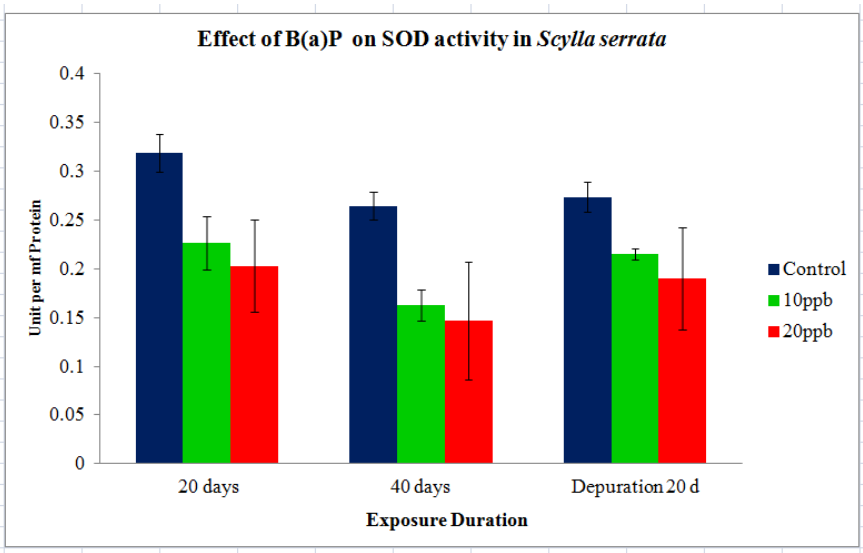

Fig. 4. Super oxide dismutase activity in the crabs exposed to different concentration of B[a]P for 40 d and followed by the depuration of 20 d. Values are expressed mean ± S.D

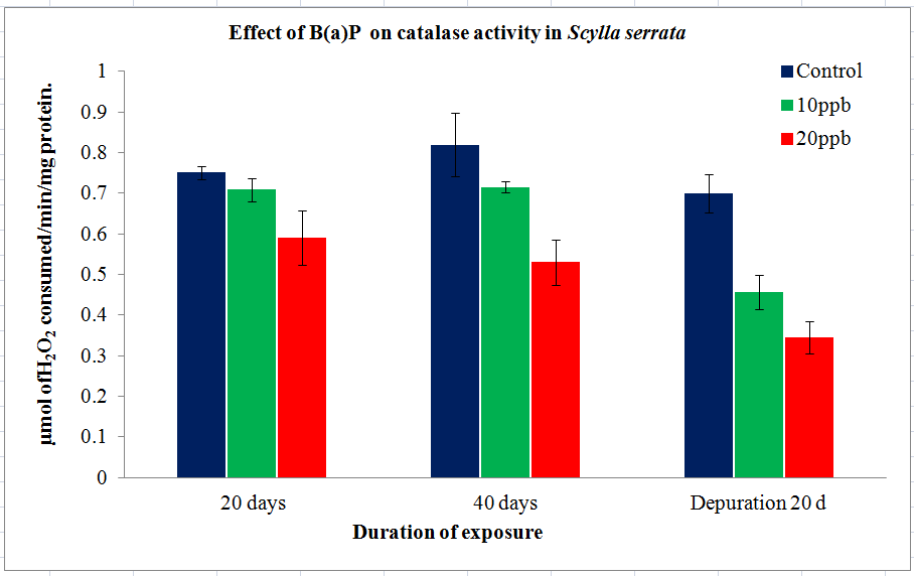

Fig. 5. Catalase activity in the crabs exposed to different concentration of B[a]P for 40 d and followed by the depuration of 20 d. Values are expressed mean ± S.D

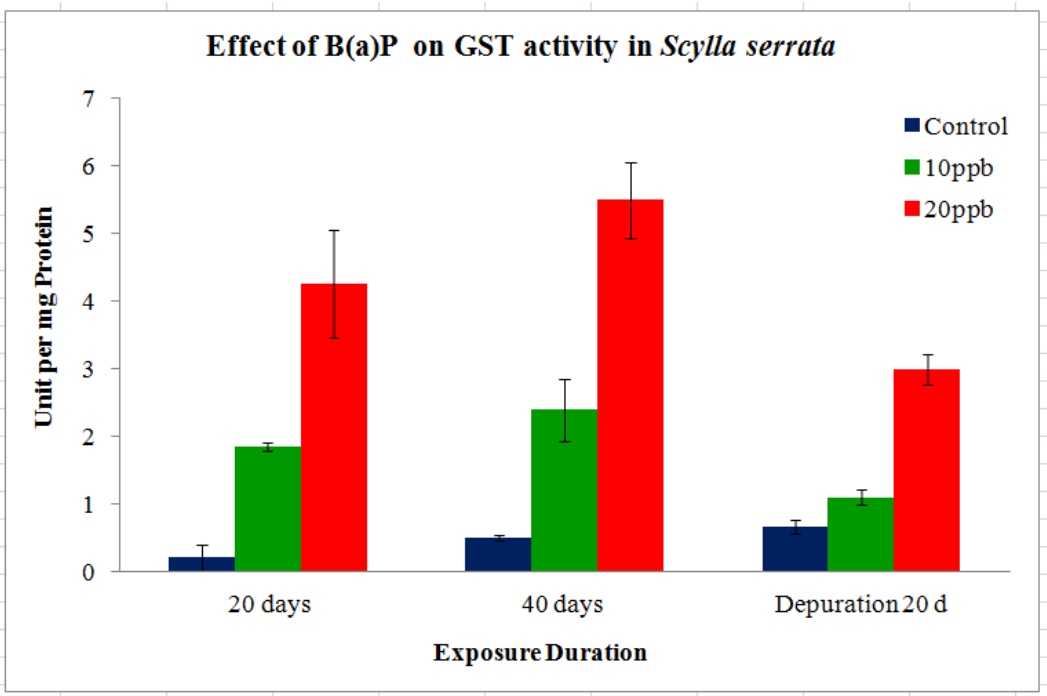

Fig. 6. Glutathione s transferase activity in the crabs exposed to different concentration of B[a]P for 40 d and followed by the depuration of 20 d. Values are expressed mean ± S.D

4. DISCUSSION

The chosen organic pollutant, B[a]P, has been documented in the Chennai coastal region. The environmental concentrations of B[a]P in the surface water in the local coastal area were approximately 2-4 ppb. The concentrations utilised in this study are likely near environmental relevant levels, and the higher concentrations in the tested animals may be due to bioaccumulation. The current study clearly shows that the sublethal concentration of B[a]P that crabs are exposed to affects both antioxidant and oxidative stress parameters in their hepatopancreas.

Protein oxidation is caused by carbonylation of proteins. A protein undergoes an irreversible conformational shift and produces carbonyl when it is harmed by ROS due to toxicant exposure [22] and such activity will lead to a decrease in enzyme catalytic activity, which will cause protein degradation [23]. The results of the current analysis indicate that protein carbonyl levels of 10–15 nmol carbonyl/mg protein are higher than those observed in mammals under normal, non-disease settings, which are in the range of 1-4 nmol carbonyl/mg protein [24].

Aggregation, cross-linking, and reduced solubility can stabilise highly oxidised proteins [25]. An increase is perhaps the most severe impact that could occur. It is necessary to ascertain the degree to which such levels are not anticipated to have an adverse effect on crab survival, nevertheless. Numerous studies have demonstrated that LPO levels can increase in a range of tissues of organisms subjected to different environmental conditions [26]. A dose depended on B[a]P concentration is correlated with an increased amount of LPO in the hepatopancreas of crabs. It has been demonstrated that malonaldehyde, an LPO by product, is highly reactive and a major mediator of DNA damage.

SOD is an important antioxidant enzyme and the first to scavenge superoxide radicals (O_2^-), whereas CAT oversees detoxifying large amounts of H_2O_2 generated because of the SOD catalyzed process. The reduced SOD and CAT activity seen in this study could be due to ROS produced during PAH metabolism. The increased formation of superoxide anion radical, which has been shown to impede CAT activity, can also be blamed for the decrease in CAT [27].

The Haber-Weiss reaction states that when hydroxyl radicals are produced by coupled oxygen radicals and H_2O_2, increases in TBARS and which indirectly decrease the level of antioxidant levels in crab hepatopancreas are offset by these increased levels of oxidative stress indicators, which reduces oxidative stress. Lower concentrations of antioxidant enzymes in the hepatopancreas, such as SOD, CAT, may be an indication of the animal's adaptive reaction to oxidative stress brought on by exposure to B[a]P. Glutathione S transferase is widely distributed in the hepatopancreas as a result of oxidative stress brought on by B[a]P. Elevated glutathione consumption as a ROS scavenger or direct interactions with PAH chemicals may be the cause of glutathione s transferase levels. The current study's induction of GST activity in crabs exposed to B[a]P for 30 days shows that the crabs are stressed because of B[a]P. GSH is a crucial cellular antioxidant that aids in defending cell constituents against damage caused by ROS and RNS. Therefore, in crabs exposed to B[a]P, inhibition or depletion of such antioxidant defences may increase the vulnerability of the crabs to lipid peroxidation. Moreover, after 40 days of exposure to B[a]P, there was a noteworthy reduction in GST activity accompanied by an equivalent rise in MDA levels.

The impact of xenobiotic exposure on antioxidant enzyme activity in marine invertebrates has been the subject of several studies [28,29,30,31]. According to these studies, as time or dosage is increased, antioxidant enzymes can rise in low toxicant concentrations but can also decline or even be inhibited. Our study's findings showed that the antioxidant enzyme activity in the crabs' hepatopancreas dropped at all concentrations, pointing to a rise in the production of oxygen free radicals (O_2^- and H_2O_2). The studied hepatopancreas antioxidant enzyme activity varied greatly as well, suggesting that B[a]P has a variety of physiological responses and roles. Both the reduction of hydrogen peroxide to water and the reduction of lipids or hydroperoxides to alcohols are catalysed by CAT activity. The biotransformation enzyme GST has a role in defending DNA and lipids against peroxidation products and oxidative damage [32]. Crab's vulnerability to some xenobiotics can be influenced by GST [33].

During last two decades a significant increase in the research studying the effects of xenobiotic

stress in aquatic organisms, especially in species that are farmed for human consumption. It is true that researching how aquatic creatures respond to stress, especially when it comes to oxidative stress (antioxidant defence mechanisms), can yield important insights on how well the environment is doing. Our results demonstrate that oxidative stress and the modulation of antioxidant and associated enzymes like SOD, and CAT occur in the hepatopancreas of crab following 30 continuous exposures to a sublethal amount of B[a]P. Therefore, the mud crab could be used as a bioindicator to measure PAH pollution by using biomarkers evaluated in hepatopancreas.

5. CONCLUSION

Host's health may be shown by measuring the function of the hepatopancreas and this organ in marine invertebrate found large amount of detoxification enzymes. This study's findings lead us to the conclusion that crab exposed to B[a]P greatly raises reactive oxygen species in crabs. This, in turn, may have an indirect effect on antioxidant scavenging capabilities, as evidenced by a considerable rise in all oxidative stress indicators. Overall, the results support the view that B[a]P is the primary cause of oxidative stress in the hepatopancreas of crabs.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

Author(s) hereby declare that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc) and text-to-image generators have been used during writing or editing of manuscript.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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