

# **Biochemical markers of oxidative stress in *Perna viridis* exposed to mercury and temperature.**

**X. N. Verlecar, K.B. Jena, \*G.B.N Chainy**

National Institute of Oceanography, Dona Paula, Goa, India

\*Department of Zoology and Biotechnology, Utkal University, Bhubaneswar, India

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## **Abstract:**

Oxidative damage and antioxidant properties have been studied in *Perna viridis* subjected to short-term exposure to Hg along with temperature (72 hrs) and long-term temperature exposures (14days) as pollution biomarkers. The elevated thiobarbituric acid reactive substances (TBA-RS) levels observed in gills and digestive gland under exposure to Hg, individually and combined with temperature, as also long term temperature stress have been assigned to the oxidative damage resulting in lipid peroxidation (LPX). Increased activities of antioxidants such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione reductase (GR) and glutathione-S-transferase (GST) both in gills and digestive glands under long term exposures to temperatures are more prominent to heat rather than cold stress suggesting activation of physiological mechanism to scavenge the ROS produced during heat stress. Also decreased values of reduced glutathione (GSH) on long exposures to temperature stress indicate utilisation of this antioxidant, either to scavenge oxiradicals or act in combination with other enzymes, was more than its production capacity under heat stress. The results suggest that temperature variation does alter the active oxygen metabolism by modulating antioxidant enzyme activities, which can be used as biomarker to detect sublethal effects of pollution.

**Keywords:-** temperature, lipid peroxidation, *Perna viridis*, antioxidant enzymes and reduced glutathione.

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## **Corresponding Author:**

Dr. X. N. Verlecar

Biological Oceanography Division, National Institute of Oceanography

Dona-Paula, Goa-403004

E-mail : verlecar@nio.org

## **Introduction:**

Marine bivalve, especially the mollusks because of their sedentary habitat, are exposed to a variety of environmental stressors, such as heavy metals, pesticides, petroleum products and thermal releases especially in area where cluster of industries are located. Since cellular and biochemical responses to pollutants can be quickly detected, their use in pollution monitoring programme is gaining momentum [1-3]. Most studies reported so far are directed towards the effect of a single environmental factor on marine bivalves [4-5]. However in the areas receiving mixed effluents from various point and non-point sources, the studies on combined effect of two or more stresses would be a more practical approach. Thermal and nuclear power plants often generate heated effluents, which find their way in coastal waters. Similarly sewage and industrial out falls may give rise to metal contaminants of which Hg is considered to be most toxic.

Hg is mostly available in inorganic form [6]. UNEP [7] has reported that at many locations around the world, mercury is found at levels that adversely affect humans and wildlife. In India Chloroalkali plants are the major source for mercury release in the atmosphere and surface water. Besides, the coal-fired plants such as thermal power plants, steel industries, and cement plants, may also contribute to a significant extent to mercury pollution [8].

Under normal physiological condition, animals maintain a balance between generation and neutralization of reactive oxygen species (ROS). However when organisms are subjected to xenobiotic compounds, rate of production of ROS, such as superoxide anion radicals ( $O_2^{\bullet-}$ ), hydrogen peroxide ( $H_2O_2$ ), hydroxyl radicals ( $^{\bullet}OH$ ) and peroxy radical ( $ROO^{\bullet}$ ) exceeds their scavenging capacity [9]. All organisms have their own cellular antioxidative defence system, composed of both enzymatic as well as non-enzymatic components. Enzymatic pathway consists of SOD, CAT and GPX.  $O_2^{\bullet-}$  are dismutated by SOD to  $H_2O_2$  which is reduced to water and molecular oxygen by CAT or is neutralized by GPX, that catalyzes the reduction of  $H_2O_2$  to water and organic peroxide to alcohols using GSH as a source of reducing equivalent. GR regenerates GSH from oxidized glutathione (GSSG), which is a scavenger of ROS as well as a substrate for the other enzymes. GST conjugates xenobiotics with GSH for excretion. The non-enzymatic component consists of small organic molecules such as  $\beta$ -carotene, GSH,

vitamin E and vitamin C [9]. Some of these parameters could serve as stress indicators in animals exposed to environmental contaminants.

Since bivalves inhabit the intertidal region, they could be exposed to extreme temperature fluctuations with a change in body temperature as wide as 10-20°C within few minutes during summer low tides [10]. Moreover in the areas receiving industrial effluents these bivalves could be subjected to thermal as well as toxic metal stress. In this context, the bivalves could be the best representative model for studies on effect of stress on antioxidant defence mechanism [2].

Although so far several studies on oxidative stress and antioxidant defence system in marine bivalves have been reported, very few of these are directed to thermal stress as also combined effect of the two stresses. Such studies became important taking into consideration excessive loads of pollutants reaching the coastal environment, arising through cluster of industries along the industrial belts [11]. The present studies were therefore aimed to investigate oxidative stress responses to short term exposure of bivalves to combined effect of temperature and toxic metal Hg and changes in antioxidants to long term exposures of temperature stress as biomarkers of pollution. Bivalve mollusk *P.viridis*, which commonly occurs along the coastal waters, was used as the experimental specimen. Gills - the principal respiratory organ and digestive gland - the major tissue involved in metabolic functions, were selected to measure the oxidative stress responses in experimental specimen. Both these organs form the active sites for xenobiotic uptake and oxyradical-generation as well as enzyme biotransformation process [12].

### **Material and methods:-**

#### *Experimental animals:-*

Green-lipped mussels (*Perna viridis*), length 8-10 cm were brought in March from an unpolluted area of Bambolim beach Goa, India. This place is relatively far less polluted because of the absence of any industrial or major fishing activity around it. Mussels were transported to the laboratory in the polythene bags filled with natural habitat seawater. The ambient temperature at which mussels were collected was  $26 \pm 1^\circ\text{C}$ . Fouling organisms were

removed from the external part of the shells. Mussels were acclimatized in aerated seawater for 2-3 week. To avoid faecal contamination, the water was drained at every 24 hours the water was drained and filled with fresh sand filtered seawater. Animals were fed daily with culture algae (*Chlorella*).

*Experimental designs:-*

**Exp:-A:** The acclimatized animals were divided into six groups.

Group I: Animals in ambient temperatures ( $26 \pm 1^\circ\text{C}$ ) (served as control).

Group II: Animals in ambient temperature with  $\text{HgCl}_2$  ( $45 \mu\text{g} / \text{L}$ ).

Group III: Animals in cold –stress ( $20 \pm 1^\circ\text{C}$ ).

Group IV: Animals in cold –stress ( $20 \pm 1^\circ\text{C}$ ) with  $\text{HgCl}_2$  ( $45 \mu\text{g} / \text{L}$ ).

Group V: Animals in heat –stress ( $32 \pm 1^\circ\text{C}$ ).

Group VI: Animals in heat –stress ( $32 \pm 1^\circ\text{C}$ ) with  $\text{HgCl}_2$  ( $45 \mu\text{g} / \text{L}$ ).

The animals were sacrificed after 72hrs

**Exp: B:-**The acclimatized animals were divided into three groups.

Group I: Animals in ambient temperatures ( $26 \pm 1^\circ\text{C}$ ) (served as control).

Group II: Animals in cold–stress ( $20 \pm 1^\circ\text{C}$ ).

Group III: Animals in heat–stress ( $32 \pm 1^\circ\text{C}$ ).

The animals were sacrificed after 7 and 14 days of treatment.

The temperature range was selected based on our earlier observations, on the effect of heated effluent releases of the power plant operations [13]. Since the mussels are generally inhabit in the intertidal region, they are subjected to the effect of tidal rhythm of high and low tides of

around 6 to 7 hours duration. Hence the experimental specimens were exposed to temperature stress of around 6 to 8 hours a day. The light and dark regime was kept constant at 12h: 12h.

#### *Chemicals:-*

Thiobarbituric acid (TBA), bovine serum albumin (BSA), cumene-hydroperoxide, 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), were purchased from Sigma Chemical Co. USA. nicotinamide adenine dinucleotide reduced (NADH), nicotinamide adenine dinucleotide phosphate reduced (NADPH), GSSG, GSH, 1-chloro-2,4-dinitrobenzene (CDNB), dithiothreitol (DTT), butylated hydroxytoluene (BHT) and phenyl methyl sulphonyl fluoride (PMSF) were obtained from SISCO Research laboratory, India. All other chemicals were analar grade.

#### *Sample preparations:-*

The digestive gland was carefully excised, surface dried with filter paper, thoroughly washed with 50 mM phosphate buffer pH 7.4 and homogenized with 50 mM phosphate buffer pH 7.4 containing, 1 mM EDTA, 1 mM DTT, 0.15 M KCl, 0.01 % PMSF. Homogenization was carried out at 4 °C using 12-15 strokes of a motor driven Teflon Potter homogenizer and centrifuge at 10,000 x g for 20 min at 4°C. Supernatant was used for biochemical studies.

#### *Biochemical biomarkers:-*

LPX level was assayed by measurement of malondialdehyde (MDA), a decomposition product of polyunsaturated fatty acids. Hydroperoxides were determined by the TBA reaction as described by Ohkawa et al.[14]. The absorbance was read at 532 nm after removal of any flocculated material by centrifugation. The amount of TBARS formed was calculated by using an extinction coefficient of  $1.56 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$  and expressed as nmol TBARS formed per mg protein.

Hydroperoxides were determined spectrophotometrically according to the method of ferrous oxidation with xylenol orange (FOX1) [15]. Hydro peroxides oxidize ferrous to ferric ions selectively in dilute acid and the resultant ferric ions can be determined by using ferric-sensitive dyes as an indirect measure of hydro peroxide concentration. Xylenol orange binds

ferric ions with high selectivity to produce a coloured (blue-purple) complex. The absorbance was read at 560 nm after removal of any flocculated material by centrifugation. The signal was read against an H<sub>2</sub>O<sub>2</sub> standard curve.

The activity of total SOD determined by the method of Paoletti et al. [16]. The EDTA / MnCl<sub>2</sub>, β-mercaptoethanol and oxygen was used to generate O<sub>2</sub><sup>•-</sup>. This anion produced the oxidation of NADH. The SOD of the sample removed the O<sub>2</sub><sup>•-</sup> and brought an inhibition of NADH oxidation. One unit of SOD activity was defined as 50 % inhibition of oxidation process. The absorbance was recorded at 340 nm and expressed as U / mg of protein. CAT activity was determined according to Aebi [17] by monitoring decrease in absorbance of H<sub>2</sub>O<sub>2</sub> at 240 nm and enzyme activity was expressed as nkat / mg protein. The activity of GPX was determined by the method of Pagilia and Valentine [18]. GPX catalyses the oxidation of GSH to GSSG in the presence of H<sub>2</sub>O<sub>2</sub>. This reaction was coupled to NADPH oxidation in presence of exogenous GR to maintain GSH concentration. Total GPX activity was measured taking cumene-hydroperoxide as the substrate. The absorbance was recorded at 340 nm. Enzyme activity was expressed as nmol NADPH oxidized / min/ mg proteins using a molar extinction coefficient of 6.22 mM<sup>-1</sup> cm<sup>-1</sup>. The activity of GR was assayed following the rate of NADPH oxidation at 340 in the presence of GSSG [19]. Enzyme activity was expressed as nmol NADPH oxidized / min/ mg proteins using a molar extinction coefficient of 6.22 mM<sup>-1</sup> cm<sup>-1</sup>. GST activity was measured according to Habig et al.[20], using CDNB as a substrate. The change in absorbance was recorded at 340 nm and enzyme activity was expressed as nmol CDNB conjugate formed / min / mg protein using a molar extinction coefficient of 9.6 mM<sup>-1</sup> cm<sup>-1</sup>. GSH was measured according to Ellman, [21] using DTNB and the formation of thiol anion was measured at 412 nm.

Protein content was estimated by Folin-Phenol method of Lowry et al. [22], using bovine serum albumin as a standard.

#### *Statistical analysis:*

Results are expressed as mean ± standard deviation (SD). Difference among the means of control and treatment was analyzed by Student's t-test. Differences were considered statistically significant when p<0.05.

## **Results:-**

### *TBA-RS:-*

It can be seen from Fig-1 that mussels exposed to temperature stress individually or combined with Hg, both showed higher TBA-RS level in digestive gland as well as gills compared to control group after 72hrs. However the tissue specific response to TBA-RS production could be observed only when mussels were subjected to temperature stress alone for longer duration of 7 and 14 days (Fig-2 A & B). In digestive gland the increased TBA-RS was seen both on 7<sup>th</sup> and 14<sup>th</sup> day for heat as well as cold exposures. However the TBA-RS concentration was much lower on 14<sup>th</sup> day in cold exposed mussels than on 7<sup>th</sup> day. In case of gills the significant increase in TBA-RS was seen only in heat stressed mussels, the value being much higher on 14<sup>th</sup> day than the 7<sup>th</sup> day.

### *SOD and CAT:-*

Activities of SOD & CAT were almost similar (Fig-2 C-F). SOD was not much affected by cold stress but increased significantly at higher temperatures in relation to control both in digestive gland as well as gills during entire exposure period (Fig-2 C & D). Although the behavior of CAT in gills was almost similar to SOD (Fig-2 D & F), in the digestive gland the increased activity could only be seen on 14<sup>th</sup> day for both cold and heat stress (Fig-2 E).

### *GPX, GR and GST:-*

These three enzymes showed almost similar behaviour in gills but in digestive gland the GR was seen to be more active than the other two enzymes (Fig-3 A-F). In digestive gland the increased activity of these three enzymes were seen on 14 days for heat stress (Fig-3 A, C & E). Significant activity of GR also seen on 7<sup>th</sup> day for both heat and cold stress (Fig-3 C). However not much changes was seen in responses of GPX and GST on 7<sup>th</sup> day in relation to control. In case of gills all the three enzyme showed the increased activity both on 7<sup>th</sup> and 14<sup>th</sup> day, for heat and cold stress, the values remaining being much higher for heat stress on 14<sup>th</sup> day (Fig-3 B, D & F).

### *GSH*:-

The behaviour of GSH was somewhat different as compared to the activities displayed by the antioxidant enzymes. Although some increase in GSH could be observed on 7<sup>th</sup> day in heat exposed mussels, this antioxidant surprisingly decreased substantially on 14<sup>th</sup> day both in gills and digestive gland. The concentration of this antioxidant however remained almost unaffected under cold stress (Fig-3 G & H).

### **Discussion:-**

Interactions between abiotic and biotic factors are more common in the aquatic environments. Among abiotic factors, thermal stress is a physiological disorder in animals, which directly affects the metabolism, resulting in the accumulation of ROS [23-25]. The oxidative stress results from altered balance between ROS production and neutralization [9]. LPX which is a marker of oxidative stress can be measured directly by HPLC method [26] or indirectly by TBARS formation [14, 27]. The TBARS method could overestimate the actual levels of MDA due to potential reactions with  $\beta$ -unsaturated aldehydes, cyclic peroxides, and other contaminants [27]. However it is considered as an effective measure of oxidative stress since several other thiobarbituric acid reactive aldehydes are also product of LPX [28]. In the present studies TBARS induction has been measured to represent the level of LPX in the experimental animals. As a validation model to this technique hydroperoxides (FOX1) were analysed in control animals and compared with TBARS curve. The result indicated in Figure-4 showed that the trend of variation of the hydro peroxides and TBARS is almost similar, indicating that the observed TBARS technique could be representative of LPX levels in the test species. It is clear from the Figure 1 and 2 A & B that the combined effect of short term exposure (72hr) of mussels to Hg and temperature stresses together to TBA-RS production was much higher than that displayed by temperature alone (Fig-2 A & B), even at longer duration. The stress effect was more prominent, at higher temperature than the lower, which could be due to the generation of ROS under combined effect of Hg and temperature. Similar results on increase in MDA in the gills and the digestive gland of mussels *Mytilus edulis* exposed for 21 days either to Cd or Hg have been reported by Geret et al.[5]. Our recent laboratory studies have also indicated that long-term exposure of mussels to sub-lethal Hg concentrations can



induce LPX as well as protein carbonyl up to a maximum of 90 % and 64 % respectively in gills in relation to control.

Since temperature is one of the fundamental factors influencing physiological state of animals such as growth and mortality [25], the induction of antioxidants under temperature stress as seen in the present study may be indicative of adaptive behaviour in mussels to temperature changes. While TBARS production by heat stress was seen in both the tissues, the effective response to cold stress could be seen only in digestive gland rather than gills (Fig-2 A & B). In *P. viridis* the digestive gland being the main organ involved in metabolic functions, the stress evoked from temperature changes may directly influence the metabolic pathway resulting in either increased production and accumulation of ROS. Our results that higher temperatures are effective in TBARS formation in gills could be compared with those reported by Parihar and Dubey [29] for catfish *H. fossilis* exposed to temperature stress from 25 to 37°C. These authors suggested that the higher temperatures enhanced the phagocytic activity in gills resulting in  $O_2^{\bullet-}$  production and subsequent generation of LPX.

The present results on antioxidant defence system indicate that SOD, CAT, GPX, GR, GST activities and GSH levels express both in digestive gland as well as gills, with some tissue specific and stress specific responses. Parallel changes in SOD and CAT seen in gills and digestive gland (Fig-2 C-F) suggest that these two enzymes act interdependently to scavenge ROS. SOD being a primary scavenger of  $O_2^{\bullet-}$  generated during stress condition, the induction of this enzyme during heat stress may indicate that it helps in inhibiting the oxygen radical accumulation. This is achieved by increased dismutation of  $O_2^{\bullet-}$  to  $H_2O_2$ , thereby reducing the oxidative damage to cells in gills and digestive gland. In support of these results Parihar et al.[30] have also reported increased SOD activity in gills of *H. fossilis* on short-term temperature enhancement.

Similarly higher activities of the  $H_2O_2$  scavenging enzymes such as CAT and GPX, shown in digestive gland and gill tissues (Fig 2-(E) and (F), 3 (A) and (B)), suggest excess  $H_2O_2$  production as a result of heat stress. Effective induction of these enzymes in cells and tissues would help to clear the peroxides accumulated under stress. Our results are in good agreement with those of Parihar et al.[30] and Khessiba et al.[31] on antioxidant responses of

fishes *H. fossilis* and mussels *M.galloprovincialis*, exposed to thermal stress. Consequently, the activation of antioxidant defence is adjustable to a certain optimum level depending on the physiological status of the test species [32]. While no measurement of  $O_2^{\bullet-}$ ,  $H_2O_2$ ,  $\cdot OH$  or other ROS markers were made in this study, it is understood that elevated enzymatic activities of SOD, CAT and GPX are indicators of oxidative stress [9]. While there are no earlier reports on thermal stress on GR induction in *P.viridis*, increased activity seen presently (Fig-3 C & D) suggest its important role in maintaining the thiol status of the cell. As GR is involved in regenerating GSH from GSSG, its induction becomes highly essential under all stress conditions i.e. cold or heat which is seen in the present results. Increased GR activity has also been observed in fishes exposed to temperature [31] and hence GR could essentially be an indicator of oxidative stress. The activities of GST and GPX as pro-oxidant have been studied in many animals [32,33]. Since both the enzymes are involved in detoxification of hydro peroxides, the increased activity of these two enzymes during heat stress in present experiment suggest that their protective mechanism towards cell is active both in gills as well as in digestive gland. Over the experimental period, several enzymes demonstrated an interdependent response to cope against stress condition. These data may be interpreted as possible protection by antioxidant and detoxifying enzymes, which could be sensitive to ROS generated during thermal stress.

GSH importantly contributes as a primary nucleophile in numerous protective detoxification reactions as also in the maintenance of cellular redox status [34]. Possibly, the temperature stress activates increased level of total glutathione initially, which serves as a compensatory mechanism, to allow mussels to maintain constant GSH / GSSG ratio despite heat induced oxidative stress. Notably this level surprisingly decreased at longer exposure to heat in both the tissues. This mechanism of decreased GSH at longer exposure to temperature in both the tissues is difficult to explain and would need further detailed investigations. The instant depletion of GSH level would probably be seem to enhance the risk of oxidative stress in digestive gland and gills (Fig-3 G & H), and induced membrane damage as evidenced by high accumulation of TBA-RS in the present study (Fig-2 A & B).

It can be inferred from the present results that although oxidative stress evidenced both at cold and heat stress either individually or in conjunction with Hg, the responses of

antioxidants appear to be strongly manifested at higher temperature rather than cold stress. Moreover the response of digestive gland are more sharp than gills during cold stress to measure oxidative damage (TBA-RS formation) making it a better representative tissue for biomarker studies.

### **Conclusion:-**

- Marine bivalves *Perna viridis* inhabiting the intertidal coastal waters have been chosen to study oxidative stress responses in terms of TBA-RS content, arising from changes in temperature combined with the Hg pollution.
- Increased TBA-RS content associated with both heat and cold stress in conjunction with sub lethal Hg levels indicates that oxidation of lipids takes place in digestive gland as well as gills after 72 hr exposure, which could be representative of an emerging picture of potential ecological impacts on mussel population
- The other tissue specific responses to long term exposure to heat or cold by these mussels to TBA-RS and antioxidants such as SOD, CAT, GPX, GR, GST and GSH indicate interdependency of these antioxidants to scavenge the reactive oxygen radicals.
- The result indicates that both the organs gills and digestive glands are active in generation of oxidative stress and antioxidant responses which can be used as biomarkers of pollution. Compared to gills oxidative stress responses are well marked in digestive gland even for cold stress making it an ideal tissue for biomarker studies in *P.viridis*.

### **Acknowledgements**

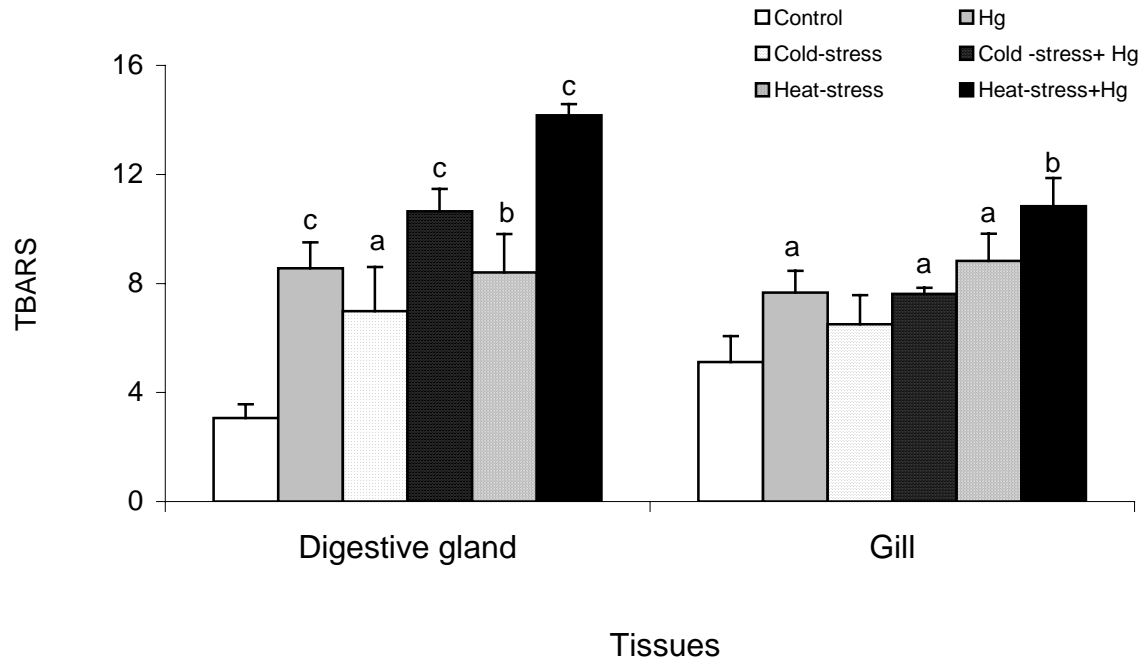
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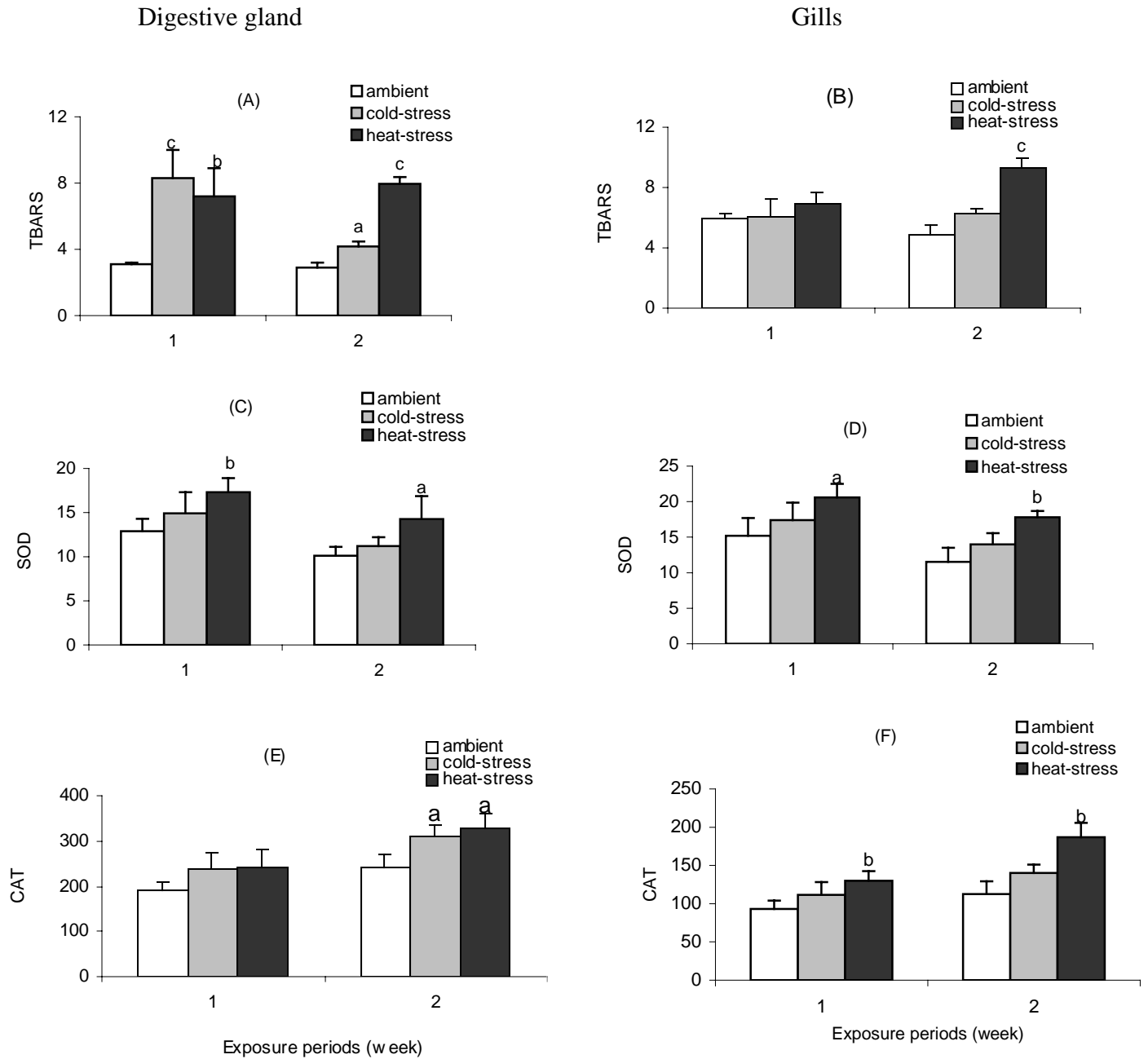
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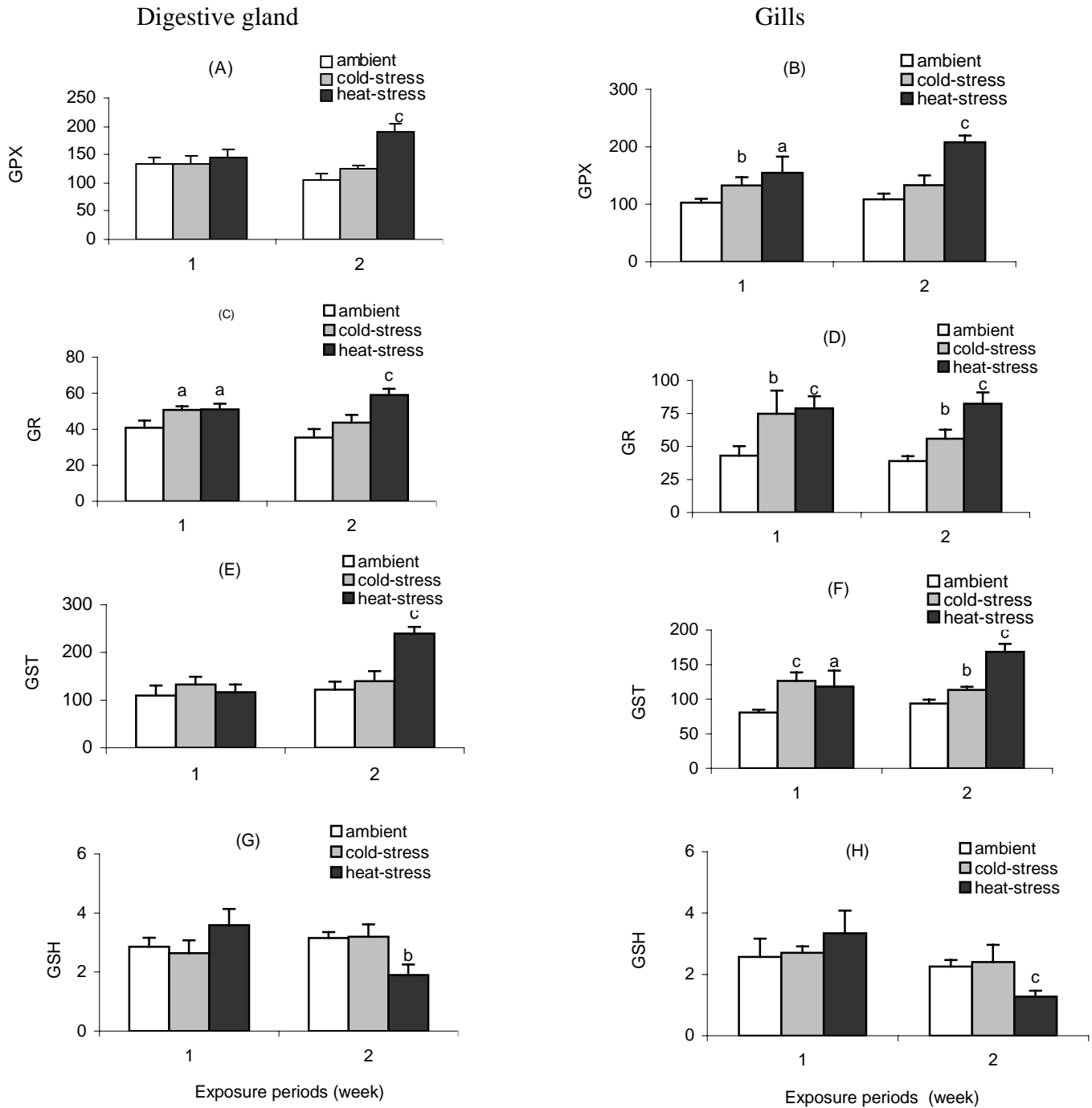


**Fig 1:-** Combined effects of mercury and temperature on lipid peroxidation (nmol TBARS / mg proteins), in digestive gland and gills of *P.viridis*. Data are expressed as mean  $\pm$  SD (n=3). <sup>a</sup>p<0.05. <sup>b</sup>p<0.01 and <sup>c</sup>p<0.001 in comparison to respective control.

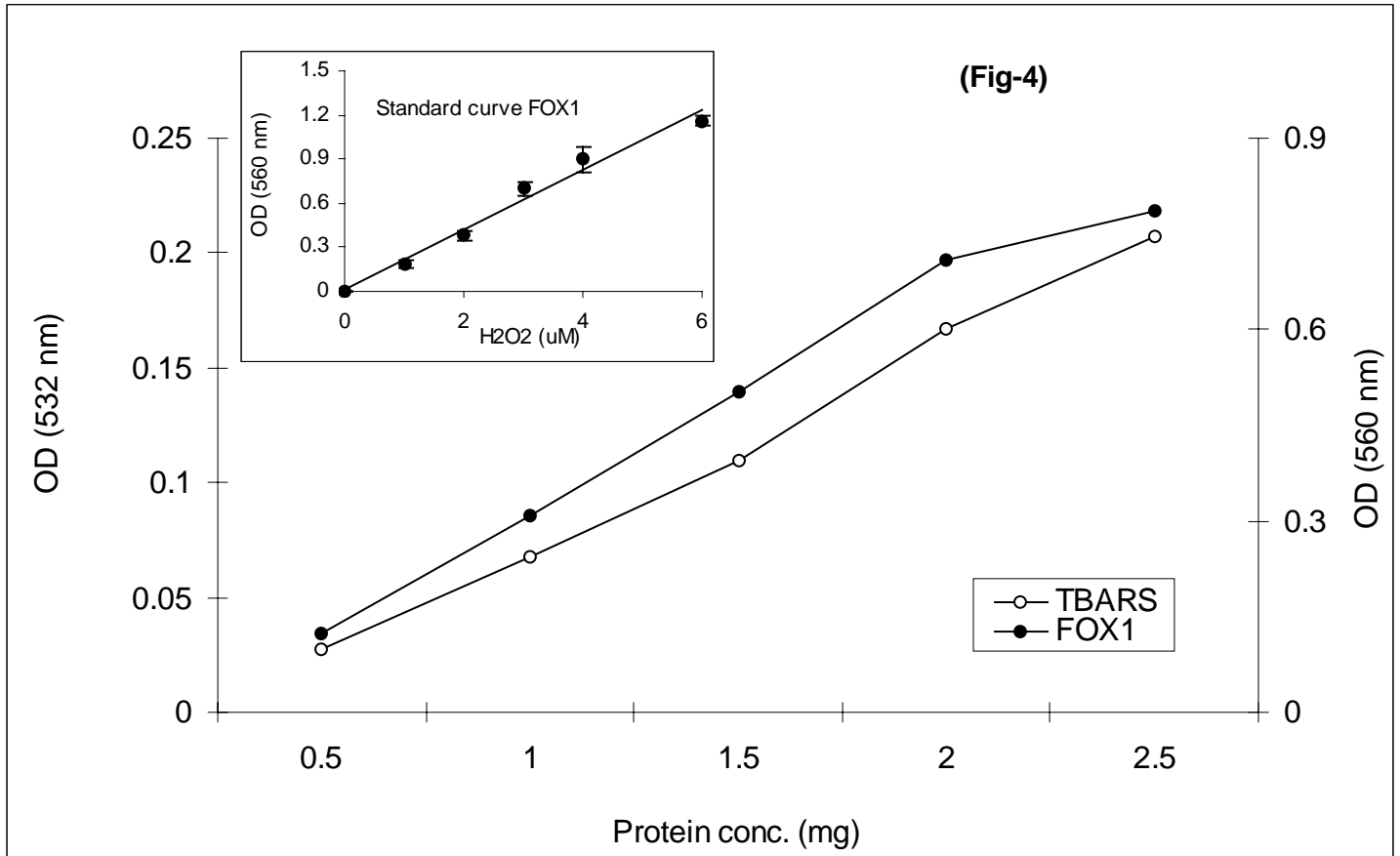


**Fig-2:** Effect of temperature on lipid peroxidation ( nmol TBARS / mg proteins), superoxide dismutase activity (Unit/mg proteins), catalase activity (nkat / mg proteins) in digestive gland and gills of *P.viridis*. Data are expressed as mean  $\pm$  SD (n=4).<sup>a</sup>p<0.05 .<sup>b</sup>p<0.01 and <sup>c</sup>p<0.001 in comparison to respective control.





**Fig-3:-** Effect of temperature on glutathione peroxidase activity (nmol NADPH oxidized / min/ mg proteins), glutathione reductase activity (nmol NADPH oxidized / min/ mg proteins), GST activity (nmol CDNB conjugate formed / min/ mg proteins) and reduced glutathione level ( $\mu\text{mol} / \text{gm wt. tissues}$ ) of digestive gland and gills of *P.viridis*. Data are expressed as mean  $\pm$  SD (n=4). <sup>a</sup>p<0.05 .<sup>b</sup>p<0.01 and <sup>c</sup>p<0.001 in comparison to respective control.



**Fig-4** :-Typical absorbance curve of TBARS (OD at 532 nm) and FOX1 (OD at 560 nm) in control samples of *Perna viridis*. Data represents mean and SD (n=10). In the box: calibration curve for FOX1 in the concentration range of 0-6 μM H<sub>2</sub>O<sub>2</sub>.