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Antioxidant responses in *Mesopodopsis zeylanica* at varying salinity to detect mercury influence in culture ponds

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Abstract: Antioxidant responses in the estuarine mysid, *Mesopodopsis zeylanica*, were used to assess mercury (Hg) metal contamination in relation to salinity. Natural mysids acclimatized at 5 psu were subjected to laboratory exposures of 5, 15, and 25 psu salinity singly and under the sublethal Hg concentration of 5 µg/L (one-fifth of 24 h LC_{50} value). Lipid peroxidation in the test species increased with salinity variation from 5 to 15 psu and retained the same up to 25 psu with no change after Hg addition. CAT increased significantly from 5 to 15 psu before Hg influence, and GST increased after addition of the metal. However, a reduction in both these enzymes is evident at higher salinities (25 psu) with or without Hg. The energy cost involved in adjusting ionic concentration with hemolymph during salinity deviation from an isotonic environment could be a cause for the reduction of antioxidants and LPX accumulation at 25 psu. Insignificant changes after Hg addition, however, indicate that Hg^{2+} free ions did not produce much toxicity to the cellular system. The results suggest that *M. zeylanica* is comfortable scavenging oxyradicals within the salinity range of 5–15 psu to protect itself from anthropogenic contaminants as evidenced by elevated enzyme activity.

Key words: *Mesopodopsis zeylanica* (mysid), salinity, mercury, lipid peroxidation, antioxidant enzyme

Introduction

Biochemical changes in organisms exposed to environmental contaminants are generally regarded as more sensitive measures of potential impact than the accumulation of these contaminants in body tissues. This is because biochemical components offer more complete and biologically relevant information regarding the possible impact of contaminants on the health of organisms at the initial stage (1). In dynamic systems such as estuarine or coastal waters the environment is influenced by various abiotic factors such as salinity, temperature, and dissolved oxygen. These are likely to modify the impact of contaminants on the organisms present.

Mercury (Hg) is highly toxic among metals, even at relatively low concentrations especially when found at metabolically active sites. Elemental, inorganic, and organic forms of mercury exhibit toxicological characteristics including neurotoxicity, nephrotoxicity, and gastrointestinal toxicity with ulceration and hemorrhage (2). Exposure of catfish to mercuric chloride ($HgCl_2$, 0.20 mg/L) for up to 30 days resulted in significant increases in lipid peroxidation in the liver, brain, and muscle (3). Hence, the presence of this metal in the natural environment may be of great concern to aquatic organisms. In our studies of green mussel (*Perna viridis*) exposed to sublethal Hg concentrations there was a sharp rise in

lipid peroxidation, protein carbonyl, DNA damage, and antioxidants such as glutathione, superoxide dismutase, catalase, and others. This indicates the importance of these parameters as a measure of metal-induced oxidative stress (4). Further exposure of these mussels to heat or cold stress under sublethal Hg content also resulted in increased lipid peroxidation. This reaction was more pronounced at higher temperatures, indicating the influence of abiotic factors in altering the metal stress response (5). Salinity is actively involved in the osmoregulation systems of animals and plays a dominant role in estuarine conditions. In addition, salinity determines the chemical speciation of Hg compounds in aquatic media (6); this is because chloride ions are among this metal's main inorganic ligands in solution. Therefore, numerous toxicity studies are focused on measuring the responses of various metals under the influence of salinity; however, very few are concerned with Hg.

Laporte et al. observed that Hg accumulation in the gills of shore crab (*Carcinus maenas*) is favored under low salinity conditions (7). Verslycke et al. evaluated the influence of changing salinity (from 5 to 25 psu) on the acute toxicity of metal mixtures on the European estuarine mysid *Neomysis integer* (8). These authors observed that the salinity effect was strongest for cadmium and lead, probably due to complexation with chloride; however, in the case of Hg it was almost negligible. McKenney and Costlow observed a progressive reduction in the survival capacity of estuarine xanthid larvae of the crab *Rhithropanopeus harrisii* under Hg–salinity interaction (9). Most of these studies relate to the influence of Hg and other trace metals on physiological changes under varying salinities in marine animals including mysids. However, limited information is available on the antioxidant responses to Hg toxicity, particularly in relation to mysid shrimp.

These phytoplankton-feeding mysids are an important food for fish and prawn populations (10,11). Mysids are often exposed naturally to a wide range of salinity and are a potentially good organism for testing contaminant sensitivity (12). Due to the limited life span of this species, it is preferred for use in short-term bioassay tests. For these reasons *Mesopodopsis zeylanica*, a mysid species commonly associated with the coastal waters of Goa, was chosen as biological model for the present experiment. This is probably the first attempt to evaluate the antioxidant response to Hg in this invertebrate crustacean. As salinity is the most effective parameter for coastal waters, the influence of changing salinity also forms an integral part of this study. The present investigation focuses on antioxidant responses such as LPX, CAT, and GST activities with reference to toxic impact of Hg on a typical mysid species, *Mesopodopsis zeylanica*, at varying salinity. Due to limited availability of natural mysid specimens, few parameters could be analyzed. Nonetheless this preliminary investigation could be applied to understanding biochemical changes in mysids reared to feed prawn farms.

Materials and methods

Field specimen collection and laboratory exposures

M. zeylanica were collected from a fish farm situated at Old Goa alongside the Mandovi estuary. Salinity variation in the farm ranges from 5 to 25 psu during the year. At the time of sampling the observed salinity was 5 psu. The specimens were transported to the laboratory immediately (within 30 min) in 10 L plastic bags filled with natural water from the collection site under continuous aeration. The organisms were transferred to a 70 L well-aerated tank. The acclimatization of specimens continued for 3 days at a salinity of 5 psu. The specimens were fed with old hatched artemia during the acclimatization phase. During the experimental phase the mysid specimens were exposed to 3 different salinities: 5, 15, and 25 psu with and without sublethal Hg concentrations. The sublethal Hg concentration used was 5 µg/L (free Hg ion activity). This represents one-fifth of 24 h LC_{50} Hg level for the same mysid specimen. The exposure experiments under different salinities continued for over 24 h. Juveniles of approximately the same size (7 mm) were collected from the stocking tank and randomly transferred to 10 L experimental glass aquaria. The experiments continued under controlled temperature conditions (25 °C). Each experimental tank contained 200 individuals. During the exposure period animals were fed with old nauplii to prevent cannibalism, and dead specimens were removed periodically.

Biochemical analysis

After the exposure period live mysids were removed, blotted on filter paper, and thoroughly washed with 50 mM phosphate buffer (pH 7.4). These specimens were homogenized with 50 mM phosphate buffer (pH 7.4) containing 1 mM EDTA, 1 mM dithiothreitol (DTT), 0.15 M KCl, and 0.01% (w/v) PMSF. Homogenization was carried out at 4 °C using 12–15 strokes of a motor driven Teflon Potter homogenizer and centrifuged at 10,000 rpm for 10 min. The supernatant was used for biochemical analysis. LPX level was assayed by the measurement of malondialdehyde (MDA), a decomposition product of polyunsaturated fatty acids, and hydroperoxides were determined by the TBA reaction, as described by Ohkawa et al. (13). Absorbance was read at 532 nm after removal of any fluctuated material by centrifugation. The amount of thiobarbituric acid reactive substances (TBARS) formed was calculated using an extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} and expressed as nmol TBARS formed per milligram of protein. CAT activity was determined according to Aebi (1974) by monitoring the decrease in absorbance of H_2O_2 at 240 nm, and enzyme activity was expressed as nkat per milligram of protein (1 katal = 1 mol s^{-1}) (14). GST activity was measured as suggested by Habig et al. using CDNB as a substrate. The change in absorbance was recorded at 340 nm, and enzyme activity was expressed as nmol CDNB conjugate formed per minute per milligram of protein using molar extinction (15). Protein content was estimated by the Folin–Phenol method of Lowry et al. using bovine serum albumin as a standard (16).

Metal analysis in water, sediments, and biota

Metal analysis in water was achieved through pre-concentration of the metals by chelating the metals, back extraction to inorganic solution, and metal analysis by atomic absorption spectrophotometer (AAS) (A Analyst 300, Perkin Elmer, USA). Sediment and tissue metals were analyzed by acid digestion of part of a dry sample and measurement of dissolved metals by AAS.

Statistical analysis

Analytical results are expressed as mean and standard deviation (±SD). Changes in biochemical parameters were tested using ANOVA and post hoc tests (Newman–Keuls) to discriminate between means of values. Differences were considered statistically significant at $P < 0.05$.

Results and discussion

Environmental contamination and oxidative stress

Trace levels of Hg can deleteriously interfere with physiological as well as biochemical activities of estuarine animals as a result of cellular oxidative stress (5). This stress is generated in cells through an increase in the production of ROS as superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^\bullet$). The antioxidant system modifies highly reactive oxygen species to the less reactive intermediate, which no longer poses a threat to cell functions. Although Hg is present in trace concentrations, a localized increase in the water column or sediment may occur due to direct or indirect anthropogenic discharges. The chloralkali and steel industries and power plants are the major contributors of aquatic Hg contamination in India (17,18). The main antioxidant enzymes that detoxify harmful ROS, or xenobiotics, are superoxide dismutase (SOD), catalase (CAT), and the glutathione-dependent enzymes glutathione peroxidase (GPX) and glutathione S-transferase (GST). In addition to anthropogenic inputs such as trace Hg, deviation in abiotic factors may act as an environmental stress in aquatic invertebrates, imposing high metabolic activity (19) and generating ROS (4,5). The Hg contents in water, sediment, and mysids from the sampling site were sufficiently low up to 90 ng L^{-1}, 0.14 mg kg^{-1}, and 0.05 μg g^{-1}, respectively. Similarly, the cadmium and lead contents were low in water up to 0.035 μg L^{-1} and 0.31 μg L^{-1} and in sediment up to 21.5 mg kg^{-1} and 15.35 mg kg^{-1}, respectively.

LPX response to salinity and Hg

Results from exposure of *M. zeylanica* to varying salinities show a significant increase in thiobarbituric acid reactive substances (TBARS) at salinities of 15 and 25 psu compared to 5 psu. The addition of a sublethal Hg concentration produced no significant changes in the results (Figure 1a, $P < 0.05$). Elevated lipid peroxidation values, following a rise in salinity from 10 to 35 psu, for gills and abdominal muscles of mud crab (*Scylla serrata*) have been reported (20). Enhanced lipid peroxidation with gradation

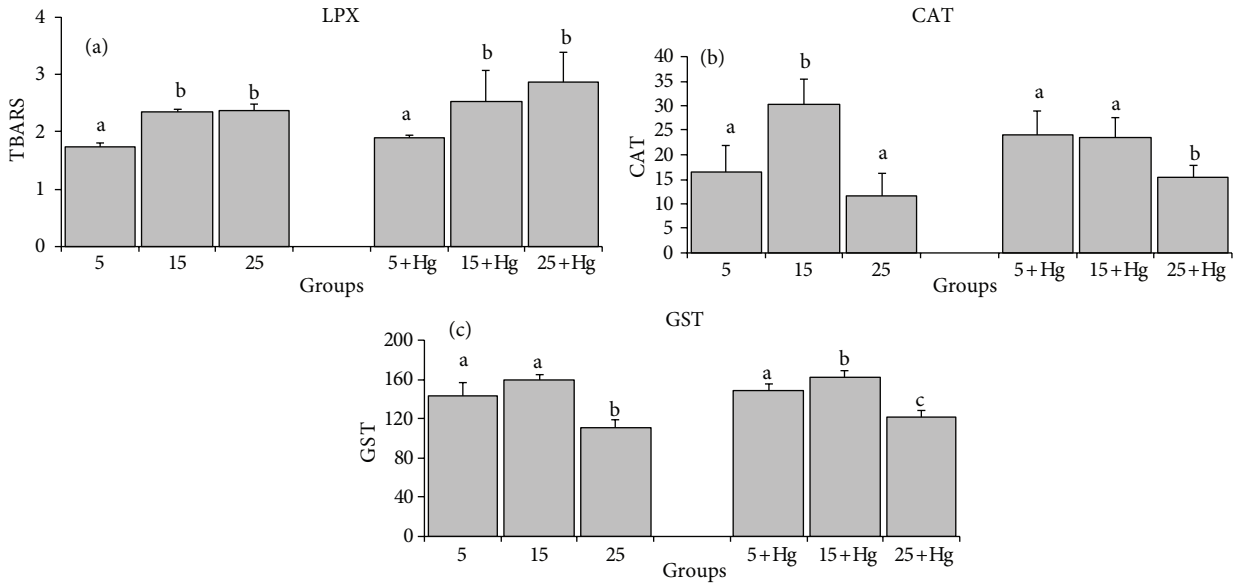

Figure 1. (a) Lipid peroxidation (nmol TBARS per milligram of protein), (b) catalase (nkat per milligram of protein), and (c) S-transferase (nmol CDNB conjugate formed per minute per milligram of protein) activity after 24 h exposure of *Mesopodopsis zeylanica* to different salinities before and after addition of Hg. Data expressed as mean ± SD (n = 3). P values denote significant difference between control and exposed specimen: $^{a}P < 0.05$, $^{b}P < 0.01$, and $^{c}P < 0.001$.

in salinity has also been shown for larvae of oyster (*Crassostrea gigas*) during a 24 h period (21). Elevated lipid peroxidation values were reported in oyster (*Crassostrea gigas*) and mussel (*Mytilus edulis*) exposed to Hg (22). Our experiments on short- and long-term Hg exposure in marine bivalves (*Perna viridis*) have also shown increased lipid peroxidation (4,5).

The poisoning effects of Hg are higher in dilute media than at elevated salinity in osmoregulating euryhaline crab species (23). This is because Hg directly affects the ionic permeability properties of their epithelium membranes. Laporte et al. indicates that bioaccumulation of free ion Hg^{2+} and the cationic species $HgCl^{+}$ increases markedly at low salinity in shore crab (*Carcinus maenas*) during inorganic Hg ($HgCl_2$) contamination (7), whereas Hg at higher salinities reduced developmental rates of estuarine xanthid crab megalopa (*Rhithropanopeus harrisii*) (9). Similarly, increasing salinity did not have much effect on Hg toxicity on European estuarine mysid (*Neomysis integer*) at 5 and 25 psu salinities due to the low free ion (Hg^{2+}) presence at both salinities (8). These observations suggest that the behavior of cells to changing salinities varies according to the osmoregulatory status of the individual species.

Estuarine and coastal waters along the Indian subcontinent are largely influenced by seasonal monsoon precipitation, which lowers salinity in downstream estuarine regions to 5 psu and below. Under reduced fresh water inputs this amount builds up slowly over the post-monsoon season and reaches almost 30–32 psu by pre-monsoon (24,25). Along the west coast of India the estuaries have a long hinterland connection (50 km or more). Estuarine mysids are subjected to salinity changes that act as a stress to generate oxyradicals. Our earlier experiments on *Perna viridis* showed elevated H_2O_2 and LPX associated with Hg exposure (4,5). Thus, enhancement in lipid peroxidation in *M. zeylanica* at higher salinities (15 psu and above) indicates a stressful situation. Similarly, insignificant changes in lipid peroxidation after Hg addition may indicate low availability of free ion Hg^{2+}, resulting in little damage to the cellular system (8).

Catalase response

Induction of SOD, CAT, and glutathione-related antioxidant enzymes has been identified as an indicator of a protective mechanism in animal cells against oxidative injury caused by O_2^{-} and other ROS such as OH^{-}. Increased CAT activity (up to 46%) in

salinity exposures from 5 to 15 psu, consistent with LPX, shows the antioxidant enzyme response to overcome the oxidative stress of changing salinities in *M. zeylanica* (Figure 1b). However, increase in salinity to 25 psu decreased the CAT values at par with 5 psu. The addition of Hg maintained similar CAT values from 5 to 15 psu with a significant reduction at 25 psu.

In a chain reaction to dismutate ROS, the 2 main enzymes involved in the removal of H_2O_2 formed by the action of superoxide dismutase on O_2^- are CAT and GPX. Accordingly, the highest CAT activity is observed at 15 psu salinity rather than at 5 or 25 psu. In support of these results a significant increase in CAT and LPX has been recorded in ark shell (*Scapharca broughtonii*) in relation to changes in environmental parameters such as salinity and temperature (26). Similarly, reduction in CAT activity associated with H_2O_2 accumulation at high salinities of 35 psu has also been observed in mud crab (*Scylla serrata*) (20). Insignificant changes after Hg addition, however, indicate that Hg^{2+} free ions did not produce much toxicity to the cellular system (22).

GST response

GST is a phase II detoxifying enzyme. It conjugates xenobiotics with reduced glutathione for excretion and detoxifies a variety of reactive intermediates and oxyradicals, thereby preventing formation of any hydrogen peroxide from these compounds (27). GST activity in the present experiments appears to increase, although insignificantly, in salinity from 5 to 15 psu. The rise in GST values from 5 to 15 psu is significant after the addition of Hg. However, a further increase in salinity to 25 psu reduces GST content significantly, even before Hg addition (Figure 1c). This decline in GST values at 25 psu salinity is in line with CAT values (Figure 1b).

Changes in GST activity with steady CAT values in oyster larvae (*Crassostrea gigas*) have been reported for salinities from 25 to 35 psu at 20 °C (21). Wild mussels (*Mytilus galloprovincialis*) from the coast of Spain have also shown increased GST and glutathione-related enzymes in tissues against metal contamination, while environmental parameters influenced gill tissue enzymes (28). Increased CAT and GST activities in response to Hg and environmental parameters were reported in the tissues of different mussels such as *Perna viridis* and *Mytella guyanensis* and crab (*Carcinus maenas*) (29–31). These observations suggest that the influence of salinity and Hg on antioxidant response varies by species. CAT and GST are both involved in the detoxification of hydroperoxides. Hence, their increased activity at 15 psu suggests that cell protective mechanism activation is at its peak at this salinity and is reduced with increased salinity, maintaining similar behavior even under Hg stress.

Relation of LPX with catalase and GST

The overall efficiency of the enzymes in scavenging oxyradicals resulting from salinity change or Hg toxicity is well-reflected in the regression analysis of LPX against CAT and GST (Figures 2a–d). The positive correlation between LPX and CAT under changing salinities (Figure 2a) suggests that CAT maintains sufficient capacity to remove reactive oxygen produced by salinity stress. However, their negative correlation under Hg influence (Figure 2b) indicates the inefficiency of CAT in scavenging oxyradicals produced by salinity stress and Hg toxicity, which results in LPX increase. The negative correlation of LPX with GST before and after Hg addition (Figures 2c, d) indicates the limitations of GST activity to remove hydroperoxides or detoxicate the Hg metal.

As hyperosmoregulators at low salinities, euryhaline crabs actively pump ions from sea water into hemolymph (32). Accordingly, *Scylla serrata* shows higher energy utilization and oxygen consumption at low salinities (10 psu), enhancing the production of superoxide radicals (20). Conversely, reduced oxygen consumption in the hyper-ionic state of 35 psu, as opposed to the acclimated 17 psu, also elevated ROS generation (20). Accordingly, the oxidative stress terms of LPX generation in *M. zeylanica* may reflect the energy utilization status required for oxygen consumption at lower salinities (5 and 15 psu) or maintenance of the oxygen-diminished state at higher salinity (25 psu). Based on respiratory rate Erk et al. suggested that isosmotic and hypo-osmotic (5 psu) media are optimal environments for *Neomysis integer* (33). Likewise, hyper-osmotic medium is suboptimal in the test species, which rarely occurs in waters of more than 18 psu. A reduction in non-protein-SH levels in gills at

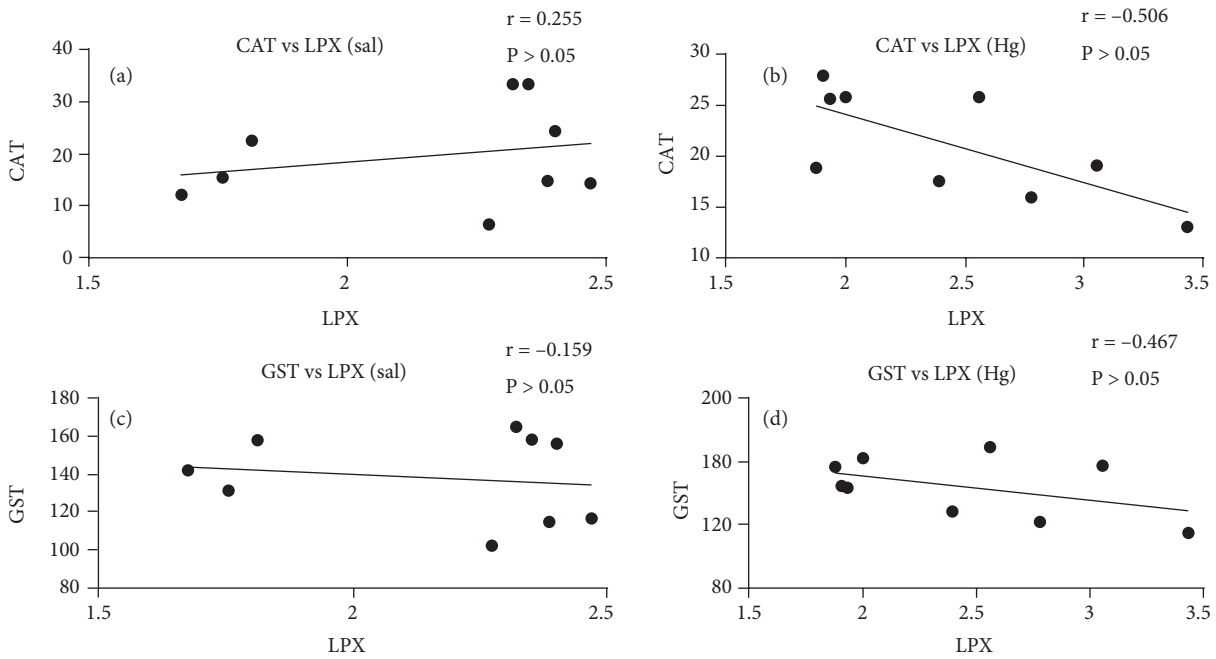

Figure 2. Correlation between (a, b) CAT and LPX and (c, d) GST and LPX at different salinities before and after Hg addition.

high salinity (35 psu) accompanied by elevated LPX has been reported in mud crab (*Scylla serrata*) (20). This is assigned to removal of hydrogen peroxides and other lipoperoxides by the GST enzyme during exposures to high salinity.

Hg is a non-redox metal, unable to associate with Fenton-type reactions. It has a high affinity to glutathione (GSH), which is the primary intracellular antioxidant agent, and can bind and cause irreversible excretion of GSH; this leads to the depletion of GSH and an increase in TBARS (34). It has been reported that Hg(II) at low concentrations depletes mitochondrial GSH and enhances H_2O_2 formation in rat kidney mitochondria under conditions of impaired respiratory chain electron transport (35). The increased H_2O_2 formation by Hg(II) may lead to oxidative tissue damage, such as lipid peroxidation, observed as Hg-induced nephrotoxicity.

The antioxidant defense mechanism in *M. zeylanica* is not yet well understood. However, the comparison of results in other marine species reveals that the deviation of crustaceans from their isotonic environment involves an energy cost as they adjust ionic concentrations with hemolymph. This could also limit the oxidative defense mechanism, thereby reducing the effective stimulation of antioxidants and the accumulation of LPX (36,37). This probably explains the behavior of CAT and GST in our experiments, which showed decreased values at hyperosmotic salinity (25 psu) and maintained optimum values at isosmotic levels of 15 psu, even after the addition of Hg (Figures 1 a–c).

Salinity tolerance and Hg stress

Laboratory studies of salinity tolerance specific to *M. zeylanica* are unavailable. However, observations in the same genus (*Mesopodopsis orientalis*), as reported by Bhattacharya indicated that the best salinity range for survival in juveniles ranged between 15–21 psu (38). Full strength seawater (35 psu) was not favorable to juvenile survival, and poor survival was recorded in salinities below 15 psu with 1.75 psu being lethal. This seems to be applicable to *M. zeylanica* as the annual distribution of both these species in Cochin backwaters reaches peak distribution during the monsoon (June–September) when salinities range from 1 to 20 psu with an average value below 10 psu (11,39). In addition, both *M. zeylanica* and *M. orientalis* prevail in Goa waters almost concomitantly, showing their abundance in particular in secluded areas, fish ponds, and estuarine regions (personal

observations). Fockedey et al. showed that sexual maturation in the European brackish water mysid (*Neomysis integer*) was only possible in the narrower range of 5–15 psu, although the survival range was much larger (40). Li et al. compared SOD and CAT activity in the hepatopancreas with biochemicals in the hemolymph of shrimp (*Litopenaeus vannamei*) at different salinities (3 psu with reference to salinities of 17 and 35 psu) (41). It was inferred that the increased oxyradical scavenging capacity of low salinity stress assured a healthy status for shrimp farmed at 3 psu rather than the higher salinities. The natural *M. zeylanica* population is abundant during monsoon when the average salinity is below 10 psu rather than during the post-monsoon or pre-monsoon periods when salinity rises to 20 psu and above. Similarly, although acclimatized at a natural ambient level of 5 psu, the antioxidant content in our experimental mysids remained considerably low at 25 psu rather than at salinities of 5 or 15 psu, with or without Hg influence (Figures 1b, c). This suggests that the animals are in a more comfortable position to scavenge oxyradicals and withstand metal toxicity at lower salinities (5–15 psu). Thus, based on the optimum efficiency of antioxidant defense in this species, maintaining their culture at a lower salinity range (5–15 psu) is recommended. This could hold true for prawn farming practices at locations near industrialized sites where occasional trace metal contamination could be encountered from accidental effluent leakages.

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