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Heavy metal stress induced hyperglycemia in blue swimmer crab, *Portunus pelagicus*

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Abstract

The study was to find out the effect of cadmium and mercury on levels of hemolymph glucose, crustacean hyperglycemic hormone (CHH) and hepatopancreas glycogen in the blue swimmer crab *Portunus pelagicus*. The experiments were performed in both intact and eyestalk ablated crabs. Quantification of CHH was done by the indirect ELISA with the aid of primary anti-*Carcinus maenas*-CHH antibody. Higher glucose concentration was observed on exposure to 8×10^{-6} of cadmium ((825.6±5.42) μg/mL) and 6×10^{-6} of mercury ((90.5±6.25) μg/mL) after 48 h and 24 h respectively. Higher level of hemolymph glucose was observed in eyestalk intact crabs on exposure to cadmium and mercury than eyestalk ablated crabs. Decrease in the levels of CHH was observed in both eyestalk intact and ablated crabs on heavy metal exposure. Decline of the hepatopancreas glycogen level was also witnessed with the exposure to heavy metal, which validated its utilization in the production of glucose. Thus this study brings to light, the variations in hemolymph glucose, CHH and hepatopancreas glycogen on heavy metal stress. These carbohydrate metabolites can be used as biomarkers in assessing heavy metal contamination in water bodies.

Key words: crustacean hyperglycemic hormone, cadmium, mercury, decapod crustaceans, blue swimmer crab

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1 Introduction

Heavy metals from farming as well as industrial activities cause environmental pollution of the marine ecosystem and can easily enter the food chain through the process of bioaccumulation, which causes extreme health complications towards human beings. It has been well documented that crustaceans have greater sensitivity towards heavy metals (Ahsanullah et al., 1981; Migliore and De Nicola Giudici, 1990). Heavy metals have major impact on the life cycle, reproduction, moulting stages and nutrition values of the crustaceans (Vernberg et al., 1974; Madsen and Shine, 1992; McGee et al., 1998). Heavy metals are not only harmful but also have some beneficial role in crustaceans. Copper is important for the functioning of hemocyanin and zinc is important component of many enzymes (Bryan, 1984; Rainbow, 1988). Trace amounts of heavy metals are absorbed by animals and stored as metabolically active forms, and are actively involved in the essential biochemical processes. Sometimes the heavy metals are detoxified into chemically inert forms and stored permanently or temporarily. The accumulation process of heavy metals may differ depending on the metals and species (Rainbow, 1988, 1997). Cadmium and mercury attracted increased awareness after the outbreak of Minimata and Itai-Itai diseases due to consumption of fish and other seafood contaminated with those heavy metals (Ui, 1972). These heavy metals are more harmful to the aquatic organism even at lower concentration because of their bioaccumulation, immutable and non-degradable properties (Reddy et al., 2011).

Mercury and cadmium have the ability to inhibit ovarian maturation in *Procambarus clarkii* (Reddy et al., 1997); additionally, cadmium can reduce the fecundity and hatching success in *P. clarkii* (Naqvi and Howell, 1993), and inhibits the molting process in the crab *Chasmagnathus granulate* (Moreno et al., 2003). Excess amount of copper can stop the growth of *Penaeus merguiensis* and *Penaeus monodon* (Ahsanullah and Ying, 1995). Mercury, cadmium and zinc are found to inhibit limb regeneration and molting in *Uca pugilator* and other fiddler crabs (Weis, 1978, 1980).

Cadmium distribution has been studied in different subcellular fractions of gill and hepatopancreas tissues of eastern oysters *Crassostrea virginica*. In both the tissue types, there was a significant accumulation of cadmium. Among the organelles, mitochondria were the main target for cadmium bioaccumulation in gills, whereas in hepatopancreas tissues, it was in lysosomes (Júdová, 2006). Chronic metal exposure (Zn, Cu, Cd) has been demonstrated to inhibit growth of sea cucumber with increased metal concentration (Li et al., 2016). Accumulation of heavy metals especially cadmium has been found to be 12 times higher in crustaceans in the Gulf of Khumbat than that of European community, three times higher than that of England standards and one time higher than that of FAO/WHO prescribed limits (Prakash Jebakumar et al., 2015).

Hormones play an important role in the different physiologic-

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al process of crustaceans. The production and release of hormones may differ before and after exposure to the pollutants. In crustaceans, the most important response is hyperglycemia after exposure to the pollutants (Fingerman et al., 1996, 1998). Research findings suggest that this stress hyperglycemia is instigated by the release of crustacean hyperglycemic hormone (CHH) to the circulatory system (Reddy et al., 2011). Numerous studies on endocrine regulation in stalk-eyed decapod crustaceans have established the neurosecretory structures in the eyestalk as the most important component of neuroendocrine system of these animals (Dean and Vernberg, 1965). The hemolymph glucose concentration is controlled by CHH (Abramowitz et al., 1944) that is synthesized in and released from the X-organ sinus gland complex. Reddy et al. (1994) reported that cadmium induced hyperglycemia in the red swamp crayfish, *P. clarkii*. Cadmium and naphthalene induced hyperglycemia in the fiddler crab, *U. pugilator* as reported by Reddy et al. (1996). Lorenzon et al. (2000) showed evidence that sublethal heavy metal concentration cause a variation of blood glucose level mediated by eyestalk hormone in *P. elegans* within a 24 h exposure period.

The blue swimmer crab, *Portunus pelagicus* represents a valuable component of small-scale coastal fisheries in many countries in the tropics (Batoy et al., 1980; Joel et al., 1987; Kyomo, 1999; Mgaya et al., 1999). Its distribution outspreads from the southern Mediterranean Sea, the east coast of Africa and across the Indian Ocean to Japan and the western Pacific Ocean (Smith, 1982; Potter et al., 1983). In India, it distributes extensively throughout West Bengal, Andhra Pradesh, Tamil Nadu, Kerala, Karnataka and Maharashtra, and contributes about 30% of the total annual marine crab landings (Samuel et al., 2004). Blue swimmer crabs are found mainly within estuaries and inshore coastal waters. In estuaries, they live in mud, sand and seagrass habitats, and often buried in the sediment.

Palanichamy and Rajendran (2000) have studied heavy metal concentration in seawater and sediments of the Gulf of Mannar and Palk Bay, southeast coast of India. Levels of cadmium were in order of Arumuganeri > Tuticorin > Thondi > Mandapam. Govindasamy et al. (2011) have observed high accumulation of heavy metals in Thondi. The increase in urbanisation and industrialisation leading to an increase of marine discharges and therefore the total load of pollutants being delivered to the sea, has been postulated as the reason for increased heavy metal incidence at Thondi. The culturable bacterial diversity of marine sediments from the Palk Bay (Thondi coast) has been evaluated using biochemical analysis, 16S rRNA gene sequencing, and their potential for antibiotic production has been assessed. Members of Firmicutes, Actinobacteria, Alphaproteobacteria, Gammaproteobacteria and *Bacillus* species have been identified in the Thondi coast (Nithya and Karutha Pandian, 2010). The blue swimmer crab, *P. pelagicus* inhabiting in Thondi coast, is often exposed to varying environmental stresses and hence its metabolism and physiological activities are affected. These crabs are observed to accumulate cadmium in the hepatopancreas after being presented elevated levels of the metals Cd, Cu, Zn, As, Fe and Al via a food source, the mussel *Trychomya hirsuta*. Over 8 weeks, crabs were fed a controlled diet to determine the accumulation of metals and accumulation was detected after 4 weeks of feeding (McPherson and Brown, 2001). Hence, the present study was conducted, to determine the effect of cadmium and mercury on hemolymph glucose and CHH in the intact and eyestalk ablated crabs after 24 h and 48 h exposure to metals in the blue swimmer crab, *P. pelagicus*.

2 Materials and methods

2.1 *Collection and maintenance of crabs*

Healthy adult blue swimmer crabs, *P. pelagicus*, were caught from the Thondi coast, Thondi (9°45′N, 79°04′E) with the carapace length of (10±1) cm and (80±5) g wet weight. The crabs were cautiously transported to the laboratory in aerated plastic troughs and introduced into the tank containing pre-aerated filtered sea water and acclimatized for a week at about 34±2 salinity and room temperature ((30±2)°C). During the period, the crabs were fed with oyster (*Crassostrea madrasensis*) meat twice a day. The unconsumed meat and other debris particles were removed by siphoning. The water was removed and fresh sea water was introduced daily.

2.2 *Heavy metal stress*

Crabs were gathered and segregated into groups for different heavy metals treatment. Each group contains ten crabs for heavy metal stress. Heavy metals like mercury (Hg) and cadmium (Cd) are actively involved in bioaccumulation and transferable through the food chain process.

Heavy metal stock solution:

Mercury and cadmium stock solutions ($1\ 000\times10^{-6}$) were prepared by dissolving their respective salts namely, mercury chloride and cadmium chloride.

Experimental setup:

Experimental group 1: the intact crabs exposed to mercury (8×10^{-6});

Experimental group 2: the eyestalk ablated crabs exposed to mercury (8×10^{-6});

Experimental group 3: the intact crabs exposed to cadmium (8×10^{-6});

Experimental group 4: the eyestalk crabs exposed to cadmium (8×10^{-6});

Experimental group 5: this group served as control.

2.3 *Collection of hemolymph and hepatopancreas*

Hemolymph and hepatopancreas were sampled from Groups 1 and 2 crabs at 0th, 12th, 24th, 36th and 48th h, samples from Groups 3 and 4 were collected at 0th, 12th and 24th h. Before sample collection, crabs were anaesthetised on ice for 5 min and the hemolymph (~2 mL/crab) was collected from the arthrodial membrane of swimmeret and stored at –20°C. The hemolymph collected from the experimental crabs during different hours of exposure was centrifuged at 5 000 r/min for 5 min to get cell free hemolymph (CFH) and was used to estimate hemolymph glucose and CHH levels.

Determination of glucose was done by glucose oxidase method (Tietz et al., 1976) in a multiwall format. Estimation of total glycogen in hepatopancreas was done according to the methodology of Carroll et al. (1956).

2.4 *Quantification of hemolymph CHH by indirect ELISA*

Quantification of CHH in the hemolymph was carried out as per the methodology of Levenson et al. (1999). The CFH were mixed 1:1 (v/v) with coating buffer (0.2 mol/L sodium carbonate-bi carbonate buffer, pH 9.4) and 100 μL was loaded in each well. The plate was incubated at 4°C overnight. After washing with washing buffer (10 mmol/L PBS, pH 7.4 and 0.1% Tween 20) the plate was blocked with 100 μL of blocking buffer (10 mmol/L PBS, 0.1% Tween 20, 2% BSA) for 2 h at room temperature. After washing, the plate was incubated with anti-*Carcinus maenas*-CHH (dilution 1:10 000 in blocking buffer) for 2 h at room tem-

perature. Plate was then washed and incubated with the secondary antibody, anti-rabbit IgG peroxidase (Sigma, A4914) (dilution 1:500 in blocking buffer) for 2 h at room temperature. Again, the plate was washed and 100 μL of TMB substrate was added into each well to initiate the enzymatic reaction. The plate was incubated in dark for 10–30 min at 37°C. The reaction was stopped by adding 2 mol/L H_2SO_4. Finally, multiwell plates were read at 450 nm on ELISA-reader (Cyberlab Inc., USA).

3 Results

3.1 *Effect of cadmium*

Preliminary experiments revealed higher hemolymph glucose level ((824.9±40) μg/mL) in the crabs when exposed to 8×10^{-6} of cadmium chloride after 48 h than other concentrations (Fig. 1). This concentration was chosen as the test concentration for hemolymph glucose, hepatopancreas glycogen and hemolymph CHH studies over increasing time periods.

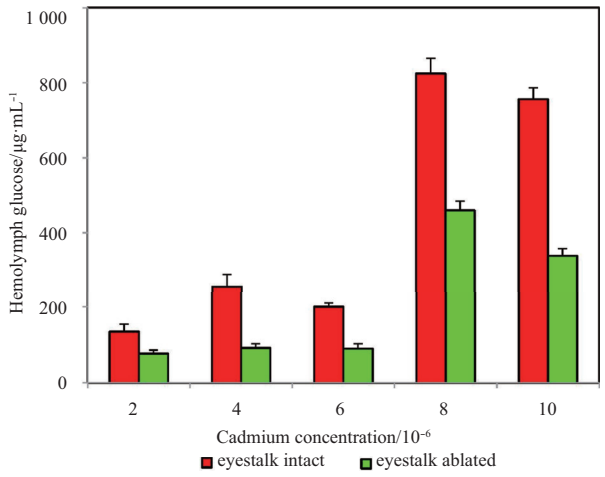

Fig. 1. Effect of varying cadmium concentrations on hemolymph glucose level in *P. pelagicus*.

3.2 *Effect of cadmium on hemolymph glucose*

Fluctuating variations in the hemolymph glucose level was observed on exposure to 8×10^{-6} cadmium chloride over the 48 h time period (Fig. 1). A significant increase in the hemolymph glucose level was observed at 24 h ((440±7.20) μg/mL) and 48 h ((825.6±5.42) μg/mL) of cadmium exposure in eyestalk intact crabs when compared to the 0 h of exposure ((373±5.23) μg/mL) (t<0.001). A sharp decline was observed between the two hours, i.e., during 12 h and 36 h.

The first peak in glucose level of (320±7.21) μg/mL was observed after 24 h of cadmium exposure compared with (86.6±5.24) μg/mL at 0 h of exposure in eyestalk ablated crabs. A decline in its level was observed thereafter at 36 h ((226.6±8.32) μg/mL) and then increased to its maximum around 48 h ((459.2±4.88) μg/mL) (t<0.01). Comparison between the two experimental groups of crabs showed higher level of hemolymph glucose in eyestalk intact crabs at 24 h period of exposure (P<0.01) (Fig. 2).

3.3 *Effect of cadmium on hepatopancreas glycogen*

Insignificant variations in the hepatopancreas glycogen level were observed between eyestalk intact and ablated crabs during the period of exposure (P>0.05) (Fig. 3). Variations did not follow

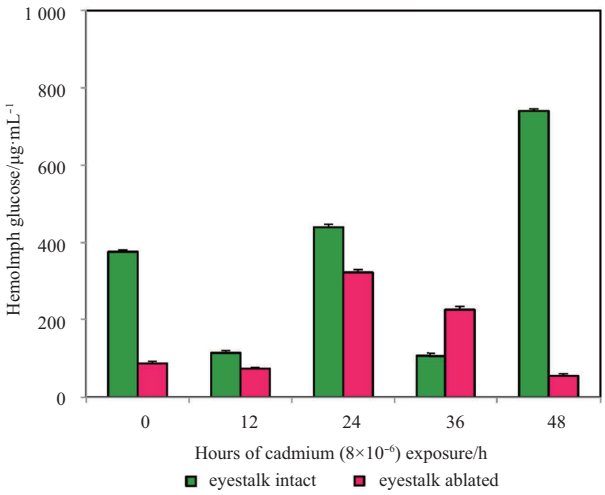

Fig. 2. Variations in the level of hemolymph glucose on cadmium exposure in *P. pelagicus*.

a definite trend of increase or decrease. The hepatopancreas glycogen level was (1.55±0.2) μg/mg at 0 h of exposure, without drastic changes during the later hours of cadmium exposure till 48 h ((1.425±0.11) μg/mg) (t>0.05). Though a notable decrease in the glycogen level of (1.433±0.11) μg/mg and (1.325±0.23) μg/mg were observed during 12 h and 36 h of cadmium exposure in eyestalk ablated crabs, the levels were set back to those of 0 h of exposure ((1.725±0.23) μg/mg) at 48 h (t<0.05).

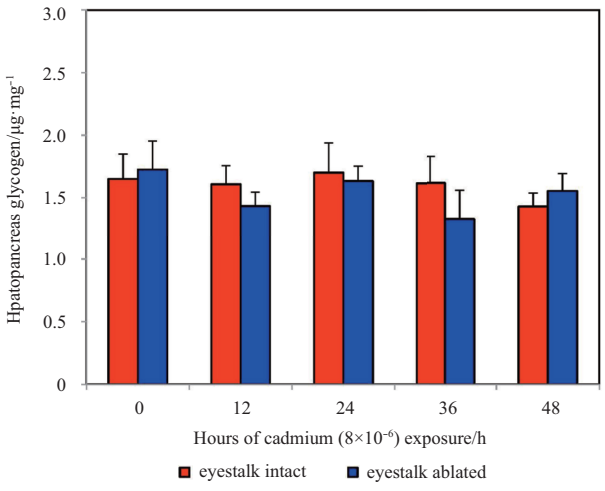

Fig. 3. Variations in the level of hepatopancreas glycogen on cadmium exposure in *P. pelagicus*.

3.4 *Effect of cadmium on hemolymph CHH*

Significant variations were observed in the hemolymph level of CHH in both eyestalk intact and ablated crabs (Fig. 4). Higher level of hemolymph CHH was observed in the eyestalk intact crabs after 36 h of cadmium exposure ((33.02±1.44) fmol/mL) when compared to eyestalk ablated crabs ((15.84±1.27) fmol/mL). Decrease in the level of hemolymph CHH was observed from 0 h ((25.63±2.32) fmol/mL) to 48 h ((16.83±2.14) fmol/mL) of cadmium exposure in eyestalk ablated crabs (P<0.001). Variations noted in the hemolymph CHH levels of eyestalk intact crabs were vague and did not follow a steady pattern of increase or decrease. Increase in the CHH levels to (32.78±2.85) fmol/mL

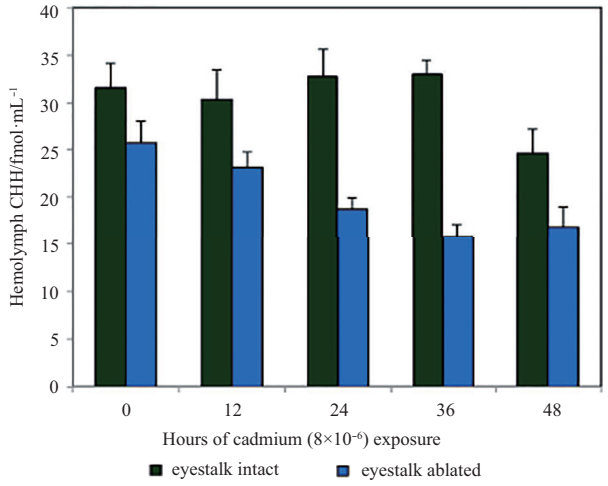

Fig. 4. Variations in the level of hemolymph CHH on cadmium exposure in *P. pelagicus*.

was observed after 24 h of cadmium exposure and further increased after 36 h of exposure (P<0.05).

3.5 *Effect of mercury*

Crabs were pushed to heavy stress on 36 h and 48 h of mercury exposure and mortality was recorded after 24 h of exposure (Fig. 5). An elevated glucose level of (817.7±40) μg/mL was observed after 24 h of exposure in the crabs when exposed to 6×10^{-6} of mercuric chloride than other concentrations. Hence, 6×10^{-6} was tested over increasing time periods in terms of hemolymph glucose, hepatopancreas glycogen and hemolymph CHH.

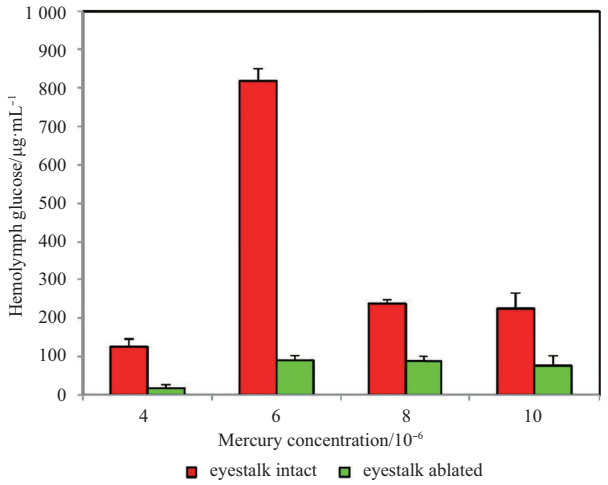

Fig. 5. Effect of varying mercury concentrations on hemolymph glucose level in *P. pelagicus*.

3.6 *Effect of mercury on hemolymph glucose*

Highly significant increase in hemolymph glucose level to (817±11.78) μg/mL was observed on 24 h of mercury exposure when compared to 0 h of exposure ((182.21±9.14) μg/mL) in eyestalk intact crabs (t<0.000 1) (Fig. 6). Eyestalk ablated crabs showed less significant variations in the hemolymph glucose level on mercury exposure. The hemolymph glucose level of (26.21±8.24) μg/mL observed at 0 h of exposure and increased insignificantly to (45.13±5.23) μg/mL during 12 h of exposure and reached (90.5±6.25) μg/mL at 24 h of mercury exposure (t<0.05).

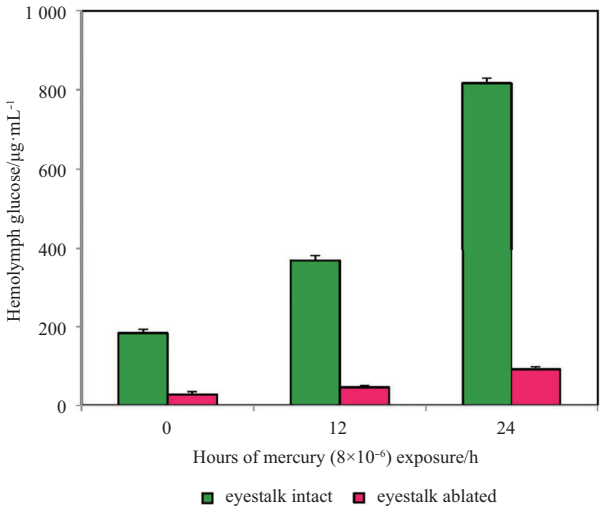

Fig. 6. Variations in the level of hemolymph glucose on mercury exposure in *P. pelagicus*.

3.7 *Effect of mercury on hepatopancreas glycogen*

Insignificant variations were observed in the level of hepatopancreas glycogen between eyestalk intact and eyestalk ablated crabs (P>0.05) (Fig. 7). The hepatopancreas glycogen reserves were observed to decrease gradually from 0 h ((1.24±0.12) μg/mg), through 12 h ((0.683±0.11) μg/mg) to 24 h ((0.437±0.08) μg/mg) of mercury exposure in eyestalk intact crabs (t<0.05). A similar trend was observed in eyestalk ablated crabs with the level of glycogen being comparatively lower. Higher level of hepatopancreas glycogen of (0.78±0.13) μg/mg was observed at 0 h of exposure which declined to (0.371±0.10) μg/mg at 24 h of exposure (t>0.05).

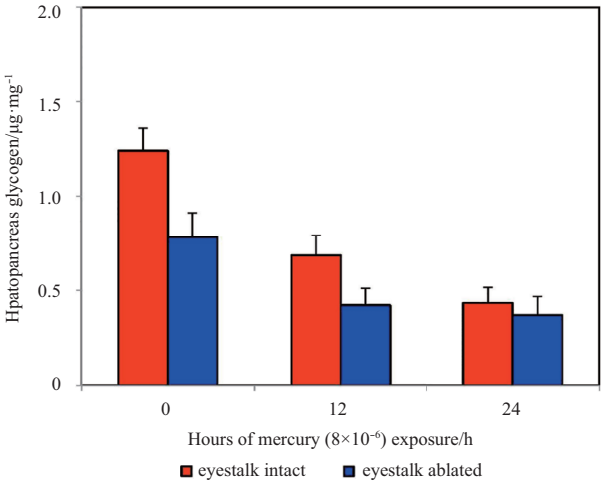

Fig. 7. Variations in the level of hepatopancreas glycogen on mercury exposure in *P. pelagicus*.

3.8 *Effect of mercury on hemolymph CHH*

Mercury exposure decreased the hemolymph CHH levels and proved its effect on the hormone in the eyestalk intact and ablated crabs, with the eyestalk intact crabs showing higher levels of CHH (t<0.05). In eyestalk intact crabs, the CHH level was (33.18±0.99) fmol/mL at 0 h of exposure and decreased significantly to (14.21±1.12) fmol/mL around 12 h of exposure. This level

increased to (21.57±1.54) fmol/mL at 24 h of exposure after which mortality of the crab was observed. The hemolymph CHH levels decreased during the early hours of exposure from 0 h ((27.11±1.28) fmol/mL) till 12 h ((10.21±1.94) fmol/mL) and increased slightly to (14.79±2.01) fmol/mL at 24 h of exposure in eyestalk ablated crabs (Fig. 8).

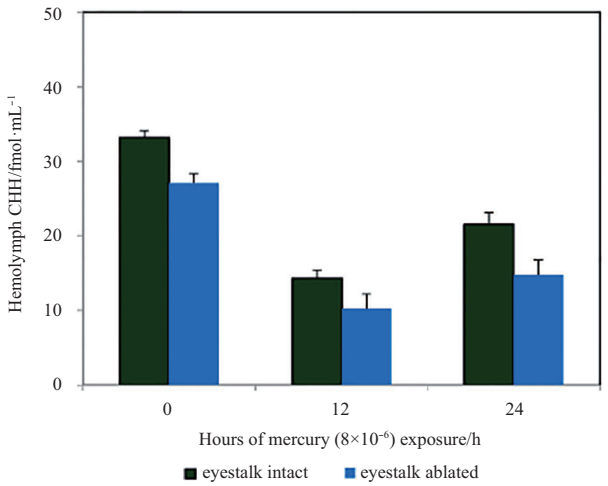

Fig. 8. Variations in the level of hemolymph CHH on mercury exposure in *P. pelagicus*.

4 Discussion

This paper aims to investigate the relationship between heavy metal stress and the release of crustacean hyperglycemic hormone from the x-organ sinus gland into the hemolymph, and thereby glycemic response in the blue swimmer crab *P. pelagicus*. Furthermore, the present study validates the use of a cross reactivity of the antibody, anti-*Carcinus maenas*-CHH to access the CHH in the hemolymph of *P. pelagicus*, with the focuses on quantification of the variation in glucose, glycogen and CHH after exposure with different heavy metals.

An elevation in the glucose concentration was observed in the hemolymph of *P. pelagicus* following exposure to cadmium chloride and mercury chloride. Higher glucose concentration was observed on exposure to 8×10^{-6} of cadmium and 6×10^{-6} of mercury after 48 h and 24 h respectively. Insignificant increase in glucose level was observed in the eyestalk ablated individuals. Cadmium exposed eyestalk ablated crabs showed gradual increase in glucose until 36 h and sudden decrease till 48 h. Mercury exposed eyestalk ablated crabs also showed the pattern of gradual increase in glucose level until the end of the experiment. Cadmium exposed intact crabs showed higher glucose during different exposure time. Lorenzon et al. (2000) showed that the higher concentrations of mercury, cadmium and lead elicited no hyperglycemia in 24 h, while the intermediate sub lethal concentration of these metals produced a significant hyperglycemic response in the shrimp *Palaemon elegans*. Studies demonstrate that heavy metals induce hyperglycemic response in the fresh water crab *Barytelphusa cunicularis* (Machale et al., 1989), *Oziotelphusa senex senex*, the crayfish *P. clarkii*, the fiddler crab *U. pugilator* (Reddy et al., 1994, 1996, 2011) and in the shrimp *P. elegans* (Lorenzon et al., 2000). Variations in the glucose level of intact and eyestalk-ablated individuals have been reported in *U. pugilator* and in *O. senex senex* (Reddy et al., 1996, 2011) on exposure to cadmium and mercury.

Hyperglycaemia results from the mobilization of glycogen in target tissues (e.g., midgut glands and abdominal muscles), due to the activation of phosphorylase and the inhibition of glycogen synthase via the CHH (Sedlmeier, 1982). Stentiford et al. (2001) found significantly lower levels of hepatopancreatic glycogen in lobsters *Nephrops norvegicus* after infected with dinoflagellate parasite (*Hematodinium* sp.). CHH-injected crayfish (*Cherax quadricarinatus*) showed increased hemolymphatic levels of glucose, in accordance with a significant utilization of glycogen reserves from the hepatopancreas (Prymaczok et al., 2016). It has been reported that the CHH levels increased instantly after exposure to stressful conditions such as emersion, hypoxia, salinity and thermal stress (Chang et al., 1998; Chung and Zmora, 2008; Webster, 1996), which leads to the modulation of glucose levels in hemolymph through utilization of glycogen stored in hepatopancreas and muscle (Santos and Keller, 1993; Santos et al., 2001; Sedlmeier, 1985).

In our study, variations of glycogen did not follow a definite trend of increase or decrease after exposure to cadmium. The hepatopancreas glycogen level was higher at 0 h of exposure, did not experience drastic changes during the later hours of cadmium exposure till 48 h. Though a notable decrease in the glycogen level was observed at 12 h and 36 h of cadmium exposure in eyestalk ablated crabs, the levels at 48 h were back to those of 0 h of exposure. Whereas, mercury exposed crabs showed insignificant differences in glycogen levels between eyestalk intact and eyestalk ablated crabs. Significant decrease of glycogen levels was observed during the exposure period from 0 h to 24 h.

In the present study, more substantial hyperglycemic response in *P. pelagicus* was observed at LC_{50} levels of cadmium and mercury exposure than higher concentrations in 24 h. Lorenzon (2005) also reported that intermediate sublethal concentration of cadmium, mercury and lead showed significant hyperglycemic response than higher concentrations during 24 h incubation period. Heavy metals like cadmium, mercury and lead have been reported to cause hyperglycemia in the freshwater prawn, *Macrobrachium kistenensis*, the crab, *Barytelphusa canicularis* (Nagabhushanam and Kulkarni, 1981; Machale et al., 1989) and *Scylla serrata* (Reddy and Bhagyalakshmi, 1994). Lorenzon et al. (2004) found that massive release of CHH from the eyestalk into hemolymph of *P. elegans* after exposure to copper. Moreover, cadmium chloride has been observed to induce hyperglycemia in intact crayfish *P. clarkii*, but not in eyestalk ablated crabs (*U. pugilator*), suggesting a CHH mediated response (Reddy et al., 1996). The two heavy metals did not elicit significant hyperglycemic response in eyestalk ablated crabs which clearly indicates the involvement of optic ganglia. The relationship between toxicant exposure and release of the CHH was confirmed by variation in hemolymph glucose (Lorenzon et al., 2005).

In conclusion, the present study demonstrated different stress effect of heavy metals on glycemic control system in blue swimmer crab and the regulation of glycemic response. Base evidence of heavy metals interference on control of this mechanism and analysis of these metabolites could lead to better understanding and assessment of the environment quality.

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