QUANTIFICATION OF CRUSTACEAN HYPERGLYCEMIC HORMONE AND ITS HEMOLYMPH METABOLITES: FOLLOWING VARIOUS STRESSES IN THE BLUE SWIMMER CRAB PORTUNUS PELAGICUS

UNIVERSITY GRANTS COMMISSION

MAJOR RESEARCH PROJECT

LIFE SCIENCES
(AQUACULTURE)

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FINAL PROJECT REPORT
(February 2011 to January 2014)

Submitted By

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FINAL REPORT OF THE WORK DONE ON THE MAJOR RESEARCH PROJECT

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2.	Name and Address of the Institution	Dept. of Oceanography and Coastal Area Studies, School of Marine Sciences, Alagappa University, Thondi Campus, Thondi – 623 409, Tamilnadu, India.
3.	UGC Approval No. and Date	F. No.39-566/2010 (SR) Dated: 12.01.2011
4.	Date of Implementation	1st February 2011
5.	Tenure of the Project	February 2011 to January 2014
6.	Total Grant Allocated	Rs. 9,12,300.00
7.	Total Grant Received	Rs. 9,09,212.00
8.	Final Expenditure	Rs. 9,09,212.00
9.	Title of the Project	Quantification of Crustacean hyperglycemic hormone and its hemolymph metabolites: following various stresses in the blue swimmer crab *Portunus pelagicus*
10.	Whether Objectives were achieved **The following objectives were achieved:** • Evaluation of hemolymph proteins by gel electrophoresis • Identification and Molecular weight determination of crustacean hyperglycemic hormone by western immunoblotting • Exposure of crabs to thermal and heavy metal stresses and studying the modulatory effects of stresses on hemolymph CHH, glucose and hepatopancreas glycogen levels	

11.	Achievements from the Project The results of this research have demonstrated that measurements of hemolymph CHH (and selected circulating metabolites) to be useful in monitoring a variety of stress responses in *P. pelagicus*.	
12.	Summary of the findings The present study has been successful in immunochemically detecting CHH with molecular weight ~74 kDa in the hemolymph of *P. pelagicus*. Furthermore, the study demonstrated that environmental stresses such as temperature and heavy metal exposure resulted in elevations in the carbohydrate metabolite levels. The increase of CHH level in response to thermal stress during the hours of recovery was observed which may be related either to the hypoxic conditions existing in warm seawater or to increased general metabolism at higher temperatures. Sublethal heavy metal concentrations caused a variation of blood glucose levels mediated by eyestalk hormone in *P. pelagicus* within a 24-h exposure period. An insignificant increase in the glucose concentration in eyestalk ablated crabs was observed suggesting other sites of CHH synthesis and release other than the sinus gland. Furthermore, an invariable decrease in CHH level was observed which could be related to the complete removal of the main source of neurohormones secretion, the X-organ sinus gland. Therefore the differential effect on the glycemia stress response proves to be a generalized and predictable sublethal reaction that can be used as a quantitative physiological biomarker for water quality monitoring assessment. The results of the study gives a better understanding of the relationship between tolerance mechanisms against environmental stress in this species helping for more efficient control of such mortality and its higher degree of adaptability to thermal and heavy metal regimes. This could lead to a potential of widened farming locations and increased yield in present locations.	
13.	Contribution to the Society The study has provided a better understanding of the relationship between tolerance mechanisms against environmental stress in this species leading to the possibility of more efficient control of mortality and its higher degree of adaptability to thermal and salinity regimes. This could lead to a potential of widened farming locations and increased yield in present locations.	
14.	Whether any Ph.D ~~Enrolled~~ /Produced out of the Project	Ph.D Produced - One
15.	No. of Publications out of the Project	Three

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DETAILED REPORT

The differences in the ecological regimes of the Bay and Gulf waters seem to have profound influence on the distribution and biology of the marine biota. Activities such as canal widening and deepening for maintenance could affect the soil sedimentation and may cause or accelerate erosion. Disposal of dredged spoil will have serious impacts thereby stressing the fauna and flora of the Palk Bay-Gulf of Mannar region. Because of industrial and agricultural activities, aquatic environments are contaminated with various kinds of pollutants, many of which can interfere with hormonal signaling. Therefore suitable biological indicators are required for the monitoring of aquatic stresses and other impacts on the survival and establishment of indigenous flora and fauna.

Neuropeptides are an important class of chemical messengers that modulate nervous system response. These signaling molecules are involved in initiation, modulation, and regulation of many physiological processes. Often times these peptides need to be secreted into circulating fluids to exert their hormonal effects on distant organs that participate in a variety of functions such as food intake, pain sensing, stress response, and molting, among many others. These peptide hormones have been implicated in regulation of physiological processes, including the control of heart function, hemolymph circulation, and digestion, to name a few. Monitoring these released neuropeptides is therefore essential for identification of bioactive neuropeptides and serves as an important step towards understanding their functions.

Hyperglycemia as a response to various kinds of stress is well documented in decapods crustaceans. Regulation of hemolymph glucose is mediated by the release of the crustacean hyperglycemic hormone (CHH) that is synthesized in the eyestalk X-organ and stored prior to release from the sinus gland. Since CHH is essentially an adaptive hormone, it is proposed that any perceived stressful change in the external environment will be immediately accounted for release of CHH, to meet increased energy demands following stress. *Portunus pelagicus* a commercially important marine crab species and a targeted species for both pond and recirculating

aquaculture in the Thondi Coast region is quite often exposed and senstive to environmental stresses. Hence, the Research project was intended to find out the effect of various stresses faced by the blue swimmer crabs of Thondi Coast in terms of the levels of CHH proving it to be a bioindicator.

(I) EVALUATION OF HEMOLYMPH PROTEINS BY GEL ELECTROPHORESIS

Molting is a characteristic feature in the growth of all arthropods to permit periodic body growth by shedding away the old exoskeleton and forming a new cuticle in its place. Most crustaceans are generally heavy-armored by intense calcification of the cuticle, making the process of molting most conspicuous and cumbersome. Obviously, the molting in several adult crustaceans is a protracted event in order for the animal to digest the inner cuticle and secrete a new cuticle, before the old cuticle could be shed away. The classical work of Drach and Tchernigovtzeff (1967) made it possible to recognize three major molt stages such as intermolt, premolt and postmolt, which are further subdivided into several substages. The pattern of changes, an increase in blood glucose and protein level during premolt, a decrease following the molt and more or less rapid return to the intermolt level, has been reported (Telford, 1974).

Hemolymph serves as an abundant sample source that can be collected without sacrificing the animals. Thus, the analysis of hemolymph neuropeptides profiles offers a great opportunity to monitor peptide secretion changes under different physiological states or in response to different functional manipulations with the usage of the same animal. Consequently, developing effective and highly sensitive methods to examine peptide hormones in circulating hemolymph is an important step toward peptide functional studies.

Most of the data available for the hemolymph protein content in decapod Crustacea (Leone, 1953; Florkin, 1960) were obtained from hemolymph collected during unspecified stages of the molt cycle. Changes in concentration of hemolymph protein occurred rapidly at premolt in *Fenneropenaeus indicus*. Hemolymph protein concentration in *Penaeus duorarum* was maximal in the premolt stage; the lowest values were encountered in the postmolt individuals. Similar observations have been reported for *Panulirus argus* (Travis, 1955), *Carcinus maenas* (Robertson, 1960),

Orconectes limosus (Andrews, 1967) and *Cragon vulgaris* (Djangmah, 1970). The higher premolt hemolymph protein levels are generally thought to be due to resorption from the chitin-protein complex of the old exoskeleton (Passano, 1960). The decreased protein concentration in the postmolt stage results from hemolymph dilution by water that enters at molt (Travis, 1955; Robertson, 1960); however, protein utilization in connection with chitin formation has also been accounted for part of this low concentration (Travis, 1957; Passano, 1960). The present objective aims to quantify and to determine the molecular weight of the hemolymph proteins during various molting stages in *P. pelagicus.*

❖ **Collection and Maintenance of crabs**

Adult crabs, *Portunus pelagicus*, were obtained from commercial fisherman from Thondi Coastal area, Thondi (Figs. 1a – 1d). The crabs were transported to the laboratory in aerated plastic containers and acclimatized for a week in continuously aerated and filtered seawater at about 35 ± 2 ppt salinity. Feeding was stopped 24 hrs before the start of the experiments and no food was given during experimentation. Crabs are being maintained in the laboratory throughout the year (Fig. 2).

❖ **Molt staging**

The molting stages of the crab were studied as per the methodology of Drach and Tchernigovtzeff (1967) based on setogenesis and epidermal retraction. The results of the study present stages A, B, C, D (D_0, D_1, $D_{2\text{-}3}$) and E (Table 1). In the early postmolt stage A, the cuticle is thin and wrinkled. The epidermal tissue appears to be the spongy structures. The epicuticle is thin and exocuticle is complete but unhardened. And the setal lumen is filled with setal matrix and the beginning of the internal cone formation occurs (Figs. 3a & 3b). In the late postmolt stage B, the internal cone formation is completed. Completion of the exoskeleton begins by the secretion of the endocuticle. The cuticle becomes more rigid and the epidermal tissues begin to concentrate along the inner surface of the cuticle. The formation of ramii takes place and the endocuticle has thicker lamellae and grows to a thickness more than the exocuticle (Figs. 3c & 3d).

In the intermolt stage C, the formation of cuticle is completed. Tubule-like structure is seen in the cuticle. The endite is thick and hard. Epidermal retraction does

not take place. The setae are empty and setal cones are present. The cuticular tubes are clearly visible below the setal cones (Figs. 3e & 3f). In the early premolt D_0, the onset of the separation of the cuticle and epidermis occurs. The retraction of the epidermis from the cuticle i.e., apolysis begins slightly which is visible at the setal bases (Figs. 3g & 3h). During the intermediate premolt D_1, further epidermal invagination takes place which leads to the enlargement of the epidermis and initiates the formation of the new setae and appendages (Figs. 3i & 3j). During the late premolt D_{2-3}, the thin cuticle is secreted on the epidermal surface, the gap formation between the cuticle and epidermis expands largely and the new setae arise from the cuticle region (Fig.3k). The maximum epidermal retraction is noticed in this stage. The new setae are completely formed and it is folded under the old epidermis (Fig. 3k). During stage E (ecadmiumysis), the exuvae were completely shed down and the setogenesis is completed. This period is a very short period which lasts for less than an hour. And it occurs during night time in the dark (Fig.3l).

- **Separation and Quantification of hemolymph proteins during the molting stages**

Quantification of hemolymph proteins was carried out during the molting stages. Hemolymph samples drawn from 5 crabs of each molt stage and were subjected to protein quantification as per the methodology of Bradford (1976) involving the binding of Coomassie Brilliant Blue G-250 thereby causing a shift in the absorption maximum of the dye from 465-595 nm and it is the increase in absorption at 595 nm which is monitored. Proteins were quantified by using the calibration curve prepared with different concentrations of Bouvine Serum Albumin. Prior to protein quantification, the hemolymph was centrifuged at 10000 rpm for 10 min. at 4^{o}C and the supernatant was stored for quantification.

Increase in the hemolymph proteins was witnessed during intermolt stage C (333.63±1.22 µg/µl). A gradual decrease to 314.95±2.13 µg/µl was observed thereafter till late premolt stage D_{2-3} followed by a steady increase in its level during postmolt stage B (320.26±3.25 µg/µl) (Fig. 4). The increase in the level of hemolymph proteins during intermolt stage may be due to the internal tissue growth and the metabolic activities that take place during this stage. The premolt stages D_0 to D_{2-3} are characterized by epidermal retraction and slowing down of the metabolic

activities thereby resulting in the decrease of the level of hemolymph proteins till apolysis. Steady increase in the hemolymph proteins during postmolt stages A and B could be due to the initial growth and development of the new epidermis. Thus, for studies on molecular weight determination, hemolymph samples were taken only from intermolt crabs and for further CHH experimentation, only intermolt C stage crabs will be chosen.

❖ **Molecular weight determination of hemolymph proteins**

Separation of the hemolymph proteins was achieved by SDS-PAGE (Sodium dodecyl sulphate – Polyacrylamide Gel Electrophoresis) and the molecular weight of the proteins thus separated were referred with a standard protein molecular weight marker (BSA). Hemolymph samples were mixed with loading buffer (200 mM Tris-HCl pH 6.8, 4% (w/v) SDS, 25% glycerol, 0.04 % (w/v) bromphenol blue, 12% β-mercaptoethanol) at the ratio of 1:2, then boiled for 5 min and subjected to 10% SDS-PAGE. The electrophoresis was performed at constant voltage of 100V at room temperature. The protein bands were stained with Coomassie Brilliant Blue R-250. Upon staining, intense bands were found in the range of 55 kDa to 95 kDa (Fig. 5).

Summary:

Increase in the hemolymph proteins was witnessed during intermolt stage C (333.63±1.22 µg/µl). A gradual decrease to 314.95±2.13 µg/µl was observed thereafter till late premolt stage D_{2-3} followed by a steady increase in its level during postmolt stages A and B (320.26±3.25 µg/µl). The increase in the level of hemolymph proteins during intermolt stage may be due to the internal tissue growth and the **metabolic activities** that take place during this stage. The premolt stages D_0 to D_{2-3} are characterized by epidermal retraction and slowing down of the metabolic activities thereby resulting in the decrease of the level of hemolymph proteins till apolysis. Steady increase in the hemolymph proteins during postmolt stages A and B could be due to the initial growth and development of the new epidermis. Thus, **for studies on molecular weight determination, hemolymph samples were taken only from intermolt crabs and for further CHH experimentation, only intermolt C stage crabs were chosen**. SDS-PAGE revealed hemolymph proteins in the molecular weight range of 66 kDa to 80 kDa.

(II) IDENTIFICATION AND MOLECULAR WEIGHT DETERMINATION OF CRUSTACEAN HYPERGLYCEMIC HORMONE BY WESTERN IMMUNOBLOTTING

Previous research on the chemical nature of CHH has focused on the isolation, characterization, and physiological effects of CHH material in the sinus gland. In this neurohemal organ, the agent causing hyperglycemia in various species of decapod crustaceans has been described primarily as a neuropeptide with a molecular weight of around 7000 Da (Kleinholz, 1985). Kegel *et al.* (1989) described the amino acid sequence of the CHH (8524 Da) from the crab *Carcinus maenus*. Reports have shown that the sinus gland of the lobster *Homarus americanus* has several CHH and CHH-like molecular forms (Tensen *et al.*, 1989). Furthermore, limited research on the chemical nature of newly synthesized CHH points to the presence of a prohormone or precursor in the perikarya (Stuenkel, 1983; Van Wormhoudt *et al.*, 1984a, b; Kallen *et al.*, 1986).

Kallen *et al.* (1990) have pointed to substances of high molecular weight in the hemolymph that show strong affinity for hyperglycemic factors from the sinus gland. Purification of sinus gland extract by gel filtration has always resulted not only in the purification of a hyperglycemic factor with a molecular weight of around 6500 Da, but also in immunological and biological activity in the void volume. Moreover, the bioactivity of released CHH was far higher than that of the CHH stored in the sinus gland. The high bioactivity of the CHH in the hemolymph has been suggested to be caused by binding of the release factor to a carrier-protein.

Western Immunoblotting

In the present study, western immunoblotting of the hemolymph proteins of *P. pelagicus* was done by electrophoretically transferring the gel on to a nitrocellulose membrane (pore size - 0.45µm) for 1.15 hr at 30 Volt in refrigerator. After protein transfer, blots were blocked with 2% BSA in washing buffer (10mM phosphate buffered saline, pH-7.4 and 0.1% Tween 20) for overnight at 4˚C. Blots were washed with washing buffer for 15 min. They were then incubated with primary antibody (Anti-*Carcinus maenas*-CHH (kindly gifted by **Dr. Heinrich Dircksen,** Stockholm University, Sweden), dilution 1:10000 in blocking buffer) for 2 hrs at room temperature. After wash with washing buffer, membranes were incubated with

secondary antibody (Anti-Rabbit IgG-Peroxidase (Sigma Aldrich), dilution 1:500 in blocking buffer) for 2 hrs at room temperature. Again the blots were washed with washing buffer and stained with 3,3'-diaminobenzidine-4 for the colour development. Finally, water was added to stop the reaction. All the incubations were done at room temperature. Results of the study cleared showed immunodetectable CHH-like proteins with molecular weight ~74 kDa (Fig. 6).

(III) THERMAL STRESS AND ITS MODULATORY EFFECT ON HEMOLYMPH CHH, GLUCOSE AND HEPATOPANCREAS GLYCOGEN LEVELS

Crustaceans exhibit many kinds of stress responses when their controlled physiological organization is stretched out beyond their normal range. Temperature stress is a significant physiological and ecological factor. In evolution, those species apparently are more successful that better cope with the physiological effects of stress, i.e. respond with less expense of energy (Henkel and Hofmann, 2008). The ability of organisms to tolerate temperature stress depends on the thermal history of their habitat (Feder and Hofmann, 1999). The temperature of habitat water has a drastic influence on the behavioural, physiological and biochemical mechanisms of crustaceans. This stress provides stimulus for the induction of stress proteins/heat shock proteins (Hsps) which help protect the organisms and repair the cellular damage. Hsps are formed as a response to increased requirement for descriptive and the correct refolding of protein aggregates which spontaneously form denatured polypeptide chains. This phenomenon known as the thermotolerance is acquired normally when the temperature raises 5-100°C above the ambient temperature of the organism. With the non-heat shock protein suppressed at elevated temperature, the hsps are synthesized for several hours post heat shock. Among the four major hsp families of 90, 70, 60,16-24 kDa molecular weight, the hsp70 family is the best studied in marine organisms. It acts by preventing the denaturation of protein and holding them in the state of folding or assembly to facilitate repair.

Direct demonstration of CHH release as a response to stressful conditions had to await methods for determination of circulating CHH in the hemolymph. Radioimmuno assays for CHHs of *Carcinus maenas, Orconectes limosus* and *Cancer*

pagurus have been developed and proved sensitive enough to detect CHH in relatively small hemolymph samples (Keller, 1988; Webster, 1996). These assays have been used to measure CHH levels in *O. limosus* hemolymph under different experimental conditions (Keller and Orth, 1990). In addition, an Enzyme linked immunosorbent assays, which proved to be of higher sensitivity than the RIA, has been developed for crab CHHs and lobster CHHs (Keller *et al.*, 1994; Chang *et al.*, 1998).

The present study focuses on the effect of thermal stress on carbohydrate metabolism in terms of levels of hemolymph glucose, hepatopancreas glycogen and Crustacean hyperglyemic hormone (CHH) paving way to better understand the hormone level responses to different temperature stress in *P. pelagicus.* The present study would provide answers to the questions viz. (i) At what temperature does *Portunus pelagicus* respond physiologically to thermal stress? (ii) Thermaltolerance of the crabs as witnessed during the recovery periods and (iii) Suggest whether the intertidal crab may be well adapted to life in the high intertidal zone and have the plasticity to acclimate to higher temperatures.

Heat Shock Treatments

The intermolt crabs were divided into two experimental groups consisting of 10 crabs each for stress treatments and the crabs were maintained at the ambient temperature (28°C) which served as the control:

Experimental group 1: This experimental group was exposed to 26°C for 3 hrs

Experimental group 2: This experimental group was exposed to 30°C for 3 hrs

Immediately after thermal incubation, all the experimental crabs were placed in the sea water with ambient temperature (28°C). At 0, 3, 6, 9 and 12 hrs after thermal incubation, hemolymph samples will be collected from the crabs to study the thermal recovery.

Hemolymph samples were drawn from the crabs after 0, 3, 6, 9, 12 hrs of thermal incubation through the arthroidal membrane of the pereiopods by using disposable insulin syringes. Immediately the hemolymph samples were stored in -20^{o}C for further experiment. Hepatopancreas of the crabs was also removed during the recovery period for glycogen analysis.

Effect of thermal stress on hemolymph glucose

Determination of glucose was achieved by using the glucose oxidase method (Tietz *et al.,* 1976) in a multiwall format. Before assay, collected hemolymph samples were centrifuged at 1000 rpm for 10 min at room temperature to drive CFH (Cell free hemolymph). Ten microlitre of CFH was mixed with 1000µl glucose reagent and the resultant colour complex was read at 492 nm spectrophotometrically.

Decrease in the levels of hemolymph glucose was observed on exposure to 26ºC (166.6±2.30 µg/ml) and 30ºC (80.0±1.23 µg/ml) when compared to the control at 28ºC ambient temperature (299.9±3.56 µg/ml) ($P<0.05$). Both the experimental groups (26ºC and 30ºC) experienced an increase in hemolymph glucose level till 3 hrs of recovery period (200.02±1.12 µg/ml and 346.6±1.16 µg/ml). These levels decreased till 9 hrs of recovery period and reached its maximum by 12 hrs in both the experimental groups. Crabs of the experimental group II (30ºC) showed faster and higher glucose recovery when compared to the experimental group I (26ºC) (Fig. 7, $t<0.01$).

Effect of thermal stress on hepatopancreas glycogen

Estimation of glycogen was done as per the methodology of Carroll *et al.* (1956) method. Hundred micrograms of hepatopancreas was homogenized with 1ml of 80% ethanol. The homogenate was centrifuged at 1000 rpm for 5 min at room temperature. Supernatants were mixed with 5ml of 5% TCA and centrifuged at 1000 rpm for 5 min. Hundred microlitre of the supernatant was mixed with 1ml of anthrone reagent, boiled for 15min. and the optical density was measured at 630nm spectrophotometrically.

Significant decrease in the level of hepatopancreas glycogen was observed in both the experimental groups I (1.238±0.35 µg/mg) and II (0.508±0.33 µg/mg) when compared to the control (2.075±0.32 µg/mg) ($P<0.01$). Gradual increase in the levels was observed through the recovery periods. A maximum increase of 2.177±0.36 µg/mg at 9 hrs in experimental group I and 0.941±1.16 µg/mg at 6 hrs in experimental group II was observed. Among the experimental groups studied, crabs exposed to 30ºC (experimental group II) experienced heavy and significant depletion in the level of hepatopancreas glycogen (Fig. 8, $t<0.001$).

Effect of thermal stress on hemolymph CHH

Hemolymph CHH levels in the hemolymph were determined using indirect ELISA (Levenson *et al.*, 1999). Quantification of CHH was carried out with HPLC-purified CHH standard from the crayfish *Orconectes limosus* (0.3-20 fmol) and *Carcinus maenas* CHH primary antibody raised in rabbit. Antibodies were mixed with the blocking buffer to obtain the desired dilutions.

Cell free hemolymph samples (CFH) collected at different recovery time periods i.e. 0, 3, 6, 9 and 12 hrs were mixed 1:1 v/v with coating buffer (0.2M Sodium carbonate-bi carbonate buffer, pH-9.4) (Lee *et al.,* 2001; Santhoshi *et al.,* 2008) and 100µl was loaded in each well and incubated at 4°C overnight. The sample was discarded and the wells were washed with washing buffer (10mM Phosphate Buffered Saline, pH-7.4 and 0.1% Tween 20). The wells were blocked with 100µl of blocking buffer (10mM PBS, 0.1% Tween 20, 2% BSA) for 2 hrs at room temperature. After washing, the wells were incubated with primary antibody (Anti-*Carcinus maenas*-CHH, 1:10000 in blocking buffer) for 2h at room temperature. Wells were then incubated with secondary antibody (Anti-Rabbit IgG-Peroxidase (Sigma Aldrich), 1:500 in blocking buffer) for 2 hrs at room temperature. Hundred microlitre of the substrate TMB (1 mg Tetramethyl benzoate, 1 ml DMSO, 9 ml 50 mM Phosphate Citrate buffer, 2 µl 30% Hydrogen peroxide) was added into each well to initiate the enzymatic reaction and incubated for 10 to 30 min at 37°C. The reaction was stopped with 2M H_2SO_4 after the required colour development was attained. Absorbance at 450 nm was measured in an automated ELISA plate reader (Cyberlab, USA).

Variations in the level of CHH were in correlation with the levels of hemolymph glucose and hepatopancreas glycogen. Both the experimental groups suffered decrease in hemolymph CHH 9.24±1.10 fmol/ml (26ºC) and 5.39±0.75 fmol/ml (30ºC) when compared to the control (11.55±2.11 fmol/ml) (28ºC) ($P<0.05$). A maximum CHH level of 17.82±0.63 fmol/ml (experimental group I) and 23.98±3.24 fmol/ml (experimental group II) was observed at 12 hrs of recovery. Crabs exposed to 30ºC (experimental group II) showed significant decrease in CHH level during incubation and increase during recovery periods when compared to experimental group II (26ºC) ($P<0.01$) (Fig. 9).

In the present study, a temperature of 28±2ºC for the stress exposure experiments was chosen as it marks an extreme situation that occasionally arises in the native environments of the test species, *P. pelagicus* and thus can be expected to produce significant physiological results and ensure the survival of the test individuals. The increase of CHH in response to thermal stress may be related either to the hypoxic conditions existing in warm seawater or to increased general metabolism at higher temperatures. Results obtained demonstrated that the highest and lowest temperature tested influenced crab biological responses, and indicated that *P. pelagicus* modulated its cellular and biochemical parameters in order to cope with temperature.

(IV) HEAVY METAL STRESS AND ITS MODULATORY EFFECT ON HEMOLYMPH CHH, GLUCOSE AND HEPATOPANCREAS GLYCOGEN LEVELS

The impact of heavy metals on crustaceans is a matter of ongoing concern (Fingerman *et al.,* 1996). The toxicity induced by a pollutant could be the result of interference by the compound or one of its metabolites with biochemical events involved in the homeostatic control of a physiological process (Brouwer *et al*. 1990). In crustaceans, physiological processes are often coordinated by hormones. Changes in hormone levels are expected to occur soon after exposure to a pollutant. Therefore, it follows that biosentinel parameters of toxicity can be identified by looking for alterations and modifications in endocrine patterns (Fingerman *et al*. 1996, 1998).

In studies with crustaceans, Clare *et al.* (1992) and Weis *et al.* (1992) observed inhibitory effects of metals on molting and regeneration of limbs, which are neuroendocrine-mediated processes, and suggested the use of these processes as biomarkers for developmental, physiological, and endocrinological effects of pollutants on crustaceans. Shrimps and crabs have been well established as bioindicators for monitoring the concentration of heavy metals (Kargin *et al.*, 2001; Vazquez *et al.*, 2001). It is plausible that one of the general effects of heavy metals may be to this family of eyestalk peptides.

Hyperglycemia is a typical sublethal response of aquatic organisms to heavy metals (Haux *et al.* 1986). In crustaceans, the medulla terminalis X-organ-sinus gland neuroendocrine complex in the eyestalk is the source of the crustacean hyperglycemic

hormone (CHH). The role of CHH in pollutant-induced blood glucose changes has only recently begun to be studied. Reddy et al (1994) provided evidence that CHH mediates cadmium-induced hyperglycemia in the red swamp crayfish, *Procambarus clarkii*. Earlier studies (Fingerman and Fingerman 1978; Staub and Fingerman 1984) showed that, like cadmium, both a PCB, Aroclor 1242, and naphthalene induced black pigment aggregation in *Uca pugilator.*

Cadmium is a biologically non-essential heavy metal that occurs naturally and is released from various sources (Webster, 1998; Vogiatzis and Loubourdis, 1997). The cadmium contamination in surface and groundwater is believed to be of non-point source as a result of agricultural and urban run-off. Concentrations of cadmium measured in environmental water samples vary considerably from 1 ppb to over 400 ppb in contaminated sites (Thorton, 1992). Cadmium has been shown to alter the structure and to cause morphological changes of varying severity in different organs of eel (Lemaire-Gony and Lemaire, 1992). Hazardous effect of cadmium is well recognized with Itai–Itai episode caused due to biomagnifications of this metal along aquatic food chains (Giordana *et al.*, 1991). Cadmium causes impairment of reproductive activity and disrupts endocrine function in fish population exposed to environmentally relevant concentrations of cadmium (Hatakeyma and Yasuno, 1981).

The accumulation of cadmium has also been described in different tissues of crustaceans (Zanders and Rojas, 1996). Naqvi and Howell (1993), using the red swamp crayfish, *Procambarus clarkii,* reported that cadmium exposure reduces fecundity and hatching success. The effects of heavy metals have revealed that cadmium inhibits synthesis of the hyperglycemic hormone and black pigment-dispersing hormone in the eyestalks of *U. pugilator* (Reddy and Fingerman, 1995; Reddy *et al.,* 1996). Sarojini *et al.* (1995) suggested that naphthalene inhibits release, but not the synthesis, of GSH from neuroendocrine cells in *P. clarkii.*

Bigi *et al.* (1996) reported cadmium accumulation in the gills and carapace of an estuarine crab *Chasmagnathus granulata*. Weis *et al.* (1989) indicated that cadmium increases secretion of CHH and MIH from the sinus gland in the eye stalk. Moreno *et al.* (2003) reported the molting inhibitory effect of cadmium in crab, *Chasmagnathus granulata.* Therefore, molting is also inhibited in crustaceans. Cadmium is also known to induce metallothionenin production in the gills and hepatopancreas of different invertebrates exposed to metals (Roesijadi, 1981; Carpene

and Yallapragada, 1993; Canli *et al.*, 1997). Therefore, gill and hepatopancreas have been shown to be vulnerable to trace metal exposure, with ultra structural changes (Gilles and Pequeux 1983). Soegianto *et al.* (1999) also observed such effects in *Penaeus japonicus* exposed to copper and cadmium in solution at relatively high concentrations.

Furthermore, biochemical parameters are the best indicators of stress situations caused by heavy metals. The biochemical constituents such as total protein and lipid were excessively utilized in the exposed aquatic organisms probably degraded the biochemical content to generate required energy in an attempt to challenge the toxic effects of cadmium in aquatic organisms (Mohan Raj, 2007). The loss of protein content in hemolymph, hepatopancreas and ovary is due to excessive proteolysis to overcome the metabolic stress (Nataraj *et al.*, 1985; Kasturi *et al.*, 1997). Significant reduction in protein, lipid and carbohydrate was observed in the fish, Mystus guilia (James *et al.*, 1995; Prasantananda *et al.*, 2000). The difference between intact and eyestalkless animals has been reported by Reddy *et al*. (1994) for *P. clarkii* exposed to cadmium and in *U. pugilator* exposed to cadmium and naphthalene (Reddy *et al*. 1996) and also for *P. elegans* injected with lipopolysaccharide as well (Lorenzon *et al*. 1997).

These apparent different modes of action of cadmium on the metabolism and physiology of crustaceans led to the investigation of the heavy metal–mediated stress response affecting glucose homeostasis and its eyestalk hormone control in both eyestalk intact and eyestalk ablated *Portunus pelagicus,* an eurythermal and euryhaline crustacean species widespread along the coastal areas of South India.

Bilateral eyestalk ablation

Eyestalk ablation is a classical operation in crustacean endocrinology; it removes the X-organ and sinus gland, the neurohormonal organ containing the neuron endings of neurosecretory system, which is the secretory and release site of an array of hormones, including the hyperglycemic hormone. Crabs were individually taken and the right eye was extirpated first by cutting at the end of eyestalk using fine surgical scissors. The crabs were held outside water for half a minute for the hemolymph to clot and gently released into the trough. To avoid stress, after two hours, the left eye was also ablated with fine scissors and released gently. Thus, bilateral eyestalk ablation was performed atleast 24 hrs prior to the experiments in order to enable the mobilization of circulating CHH.

Heavy metal exposure

Stock solutions of the heavy metal (cadmium chloride) were prepared freshly and stored at room temperature. Final concentrations of 10, 8, 6, 4 and 2 ppm were prepared from the stock solution prior to experimentation. Healthy intermolt female crabs (eyestalk intact and eyestalk ablated) were segregated into five groups with each group having 2 crabs. Crabs were washed with metal free water and wiped with blotting paper before introduction into the tanks with varying concentration of the heavy metal upto 48 hrs. At the end of the experiment, hemolymph and hepatopancreas were collected for further analyses. Independent experiments were carried out in duplicate for each concentration of the metal.

Preliminary experiments revealed higher hemolymph glucose level (824.9±40 μg/ml) in the crabs exposed to 8 ppm of cadmium chloride than other concentrations (Fig. 10). This concentration was chosen as the test concentration for hemolymph glucose, hepatopancreas glycogen and hemolymph CHH studies under various time periods.

Effect of heavy metal stress on hemolymph glucose

Determination of glucose was achieved by using the glucose oxidase method (Tietz *et al.,* 1976) in a multiwall format. Before assay, collected hemolymph samples were centrifuged at 1000 rpm for 10 min at room temperature to drive CFH (Cell free hemolymph). Ten microlitre of CFH was mixed with 1000μl glucose reagent and the resultant colour complex was read at 492 nm spectrophotometrically.

Fluctuating variations in the hemolymph glucose level was observed on exposure to 8 ppm cadmium chloride over the 48 hrs time period (Fig. 11). Decrease in the level of glucose was observed at 12 hrs of exposure in both eyestalk intact (113.3±10 μg/ml) and eyestalk ablated crabs (73.3±3 μg/ml) from 0 hrs of exposure. A gradual increase was observed thereafter and a significant elevated level of hemolymph glucose was observed on 48 hrs exposure (740±20 μg/ml) in eyestalk intact crabs and at 24 hrs exposure (320±10 μg/ml) in eyestalk ablated crabs ($t<0.01$).

Effect of heavy metal stress on hepatopancreas glycogen

Estimation of glycogen was done as per the methodology of Carroll *et al.* (1956) method. Hundred micrograms of hepatopancreas was homogenized with 1ml of 80% ethanol. The homogenate was centrifuged at 1000 rpm for 5 min at room temperature. Supernatants were mixed with 5ml of 5% TCA and centrifuged at 1000 rpm for 5 min. Hundred microlitre of the supernatant was mixed with 1ml of anthrone reagent, boiled for 15min. and the optical density was measured at 630nm spectrophotometrically.

Insignificant variations in the hepatopancreas glycogen level were observed between eyestalk intact and ablated crabs during the period of exposure (Fig. 12). Variations did not follow a definite trend of increase or decrease. Increase in the hepatopancreas glycogen levels was observed during 24 hrs of cadmium exposure in both eyestalk intact (1.7±0.4 μg/mg) and eyestalk ablated (1.633±0.12 μg/mg) crabs ($t<0.05$).

Effect of heavy metal stress on hemolymph CHH

Hemolymph CHH levels in the hemolymph were determined using indirect ELISA (Levenson *et al.*, 1999). Quantification of CHH was carried out with HPLC-purified CHH standard from the crayfish *Orconectes limosus* (0.3-20 fmol) and *Carcinus maenas* CHH primary antibody raised in rabbit as described in the previous section.

Significant variations were observed in the hemolymph level of CHH in both eyestalk intact and ablated crabs (Fig. 13). Higher level of hemolymph CHH was observed in the eyestalk ablated crabs during 36 hrs of cadmium exposure (33±3.4 fmol/ml) when compared to eyestalk intact crabs (15.84±3.2 fmol/ml). Decrease in the level of hemolymph CHH was observed from 0 hrs (25.63±2.3 fmol/ml) to 48 hrs (16.83±4.9 fmol/ml) of cadmium exposure in eyestalk intact crabs ($P<0.001$). A gradual but insignificant decrease was observed in eyestalk ablated crabs from 0 hrs (31.57±4.6 fmol/ml) to 48 hrs (24.53±2.5 fmol/ml) of cadmium exposure ($P<0.05$).

Studies on the effect of heavy metal stress (cadmium chloride) in both eyestalk intact and eyestalk ablated crabs revealed significant variations in hemolymph glucose and CHH levels. Cadmium chloride exposure had altogether lowered the hemolymph glucose and CHH levels in eyestalk intact and eyestalk ablated crabs. The decrease in

the level of CHH in eyestalk ablated crabs suggests the effect of cadmium on the circulating CHH levels as well as on the other CHH sources in the crab. Eyestalkless animals are the most sensitive to heavy metals, and the result suggests a resistance homeostatic reaction mediated by neurohormones in the intact crabs. Eyestalkless animals exposed cadmium did not show a relevant hyperglycemic effect except for a slight increase after 24 hrs. This further proves the significant involvement of the eyestalk neuroendocrine centers while the residual response can be interpreted as a peripheral mechanism. It is elicited either by non-eyestalk based neuroendocrine control or directly affected by the action of the heavy metal or of messengers released by their action on the target tissues.

Summary of the findings

The present study has been successful in immunochemically detecting CHH with molecular weight ~74 kDa in the hemolymph of *P. pelagicus.* Furthermore, the study demonstrated that environmental stresses such as temperature and heavy metal exposure resulted in elevations in the carbohydrate metabolite levels. The increase of CHH level in response to thermal stress during the hours of recovery was observed which may be related either to the hypoxic conditions existing in warm seawater or to increased general metabolism at higher temperatures. Sublethal heavy metal concentrations caused a variation of blood glucose levels mediated by eyestalk hormone in *P. pelagicus* within a 24-h exposure period. An insignificant increase in the glucose concentration in eyestalk ablated crabs was observed suggesting other sites of CHH synthesis and release other than the sinus gland. Furthermore, an invariable decrease in CHH level was observed which could be related to the complete removal of the main source of neurohormones secretion, the X-organ sinus gland. Therefore the differential effect on the glycemia stress response proves to be a generalized and predictable sublethal reaction that can be used as a quantitative physiological biomarker for water quality monitoring assessment. The results of the study gives a better understanding of the relationship between tolerance mechanisms against environmental stress in this species helping for more efficient control of such mortality and its higher degree of adaptability to thermal and heavy metal regimes. This could lead to a potential of widened farming locations and increased yield in present locations.

Table 1 Molt cycle stages in *P. pelagicus*

Moult stages	Diagnostic characters
Postmolt	
Stage A	Freshly molted crabs and extremely quiescent; cuticle soft and pliable. Swimmeret soft and transparent, setae thin walled; granular protoplasmic matrix.
Stage B	Carapace hardens; swimmeret hard and rigid with the development of cuticular nodes.
Intermolt	
Stage C	Exoskeleton remains hard; setal lumen becomes narrow; setal wall translucent; setal cone formation
Premolt	
Stage D_0	Beginning of epidermal retraction (apolysis); protoplasmic invagination in the site of future setae resulting in scalloped epidermis
Stage D_1	Exoskeleton remains brittle; retracted zone between old cuticle and epidermis widens; the tip of new setae is either within the setal groove or protrudes into the retracted zone; new setae clearly visible
Stage D_{2-3}	Epidermal retraction continues; fully developed new setae appear in the matrix as tube-in tube structures.
Moulting	
Stage E	Crab inactive; appearance of suture at intersegmental membrane of the carapace; rejection of the old cuticle.

Fig. 1a & 1b Dorsal view of Male and Female Blue swimmer crab, *Portunus pelagicus*

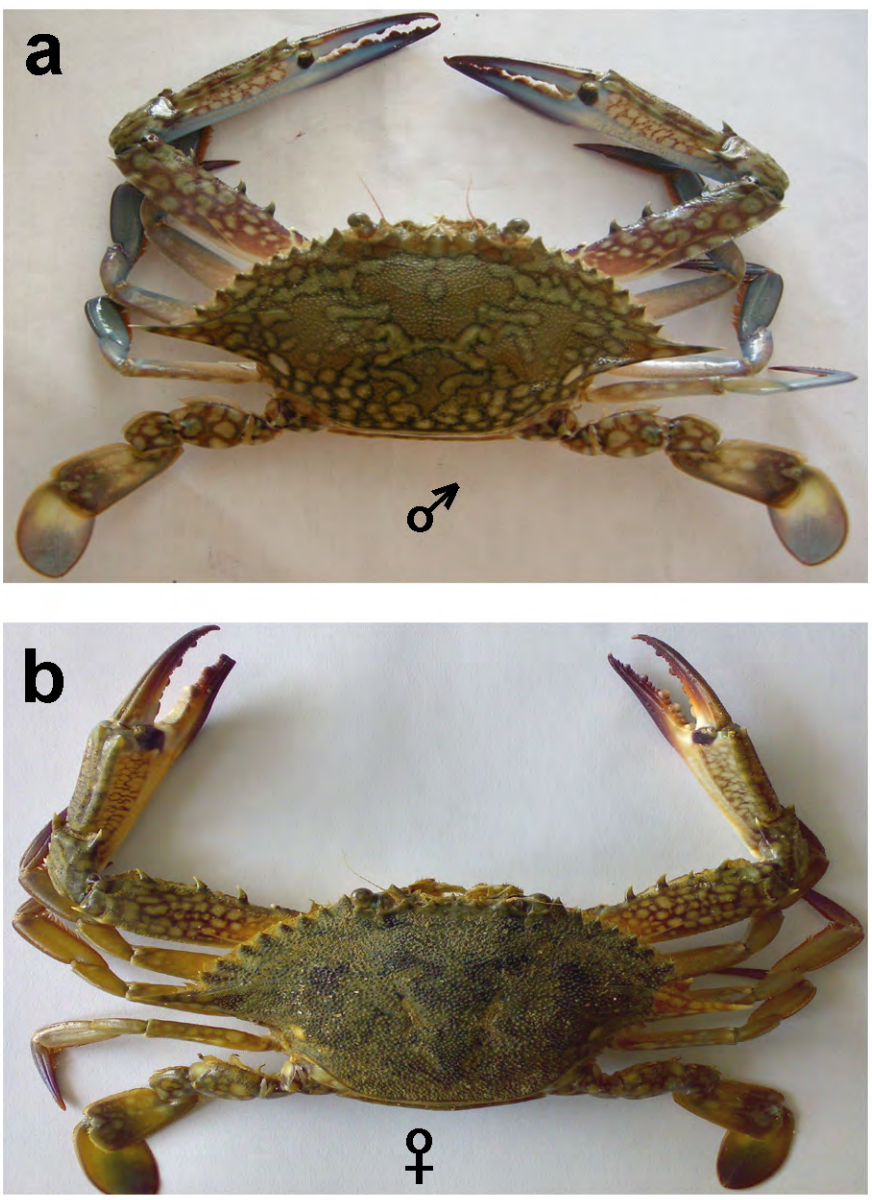
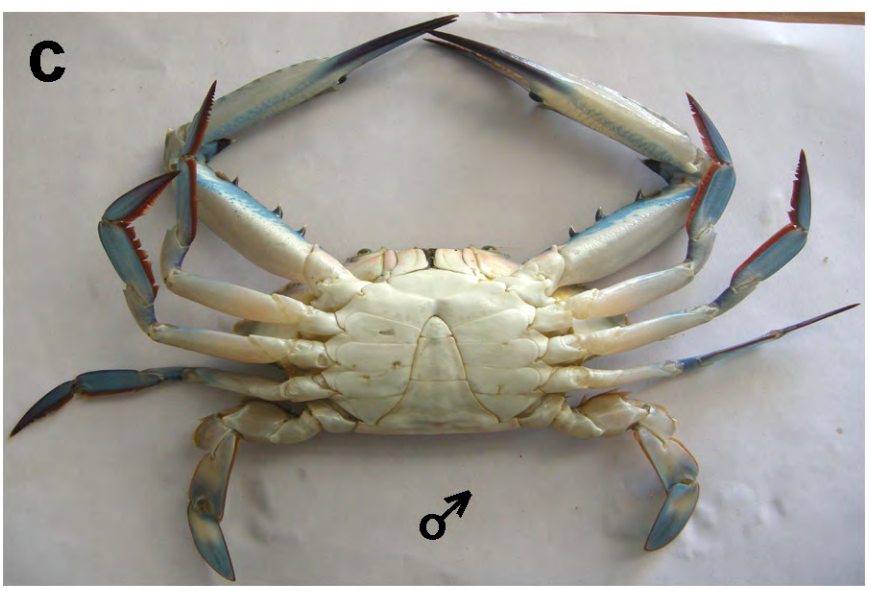

a
♂
b
♀

Fig. 1c & 1d Ventral view of Male and Female Blue swimmer crab, *Portunus pelagicus* showing variation in the abdominal flap


c
♂

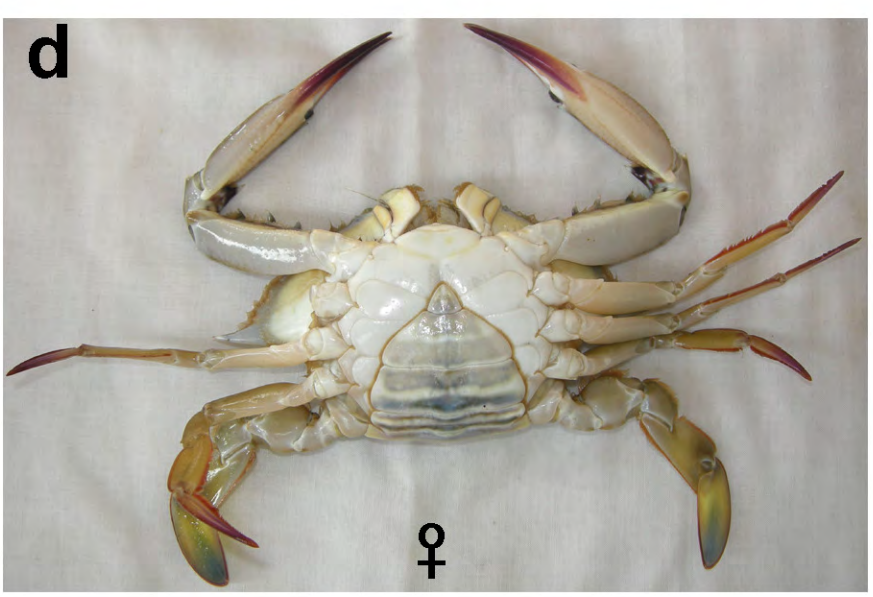

d
♀

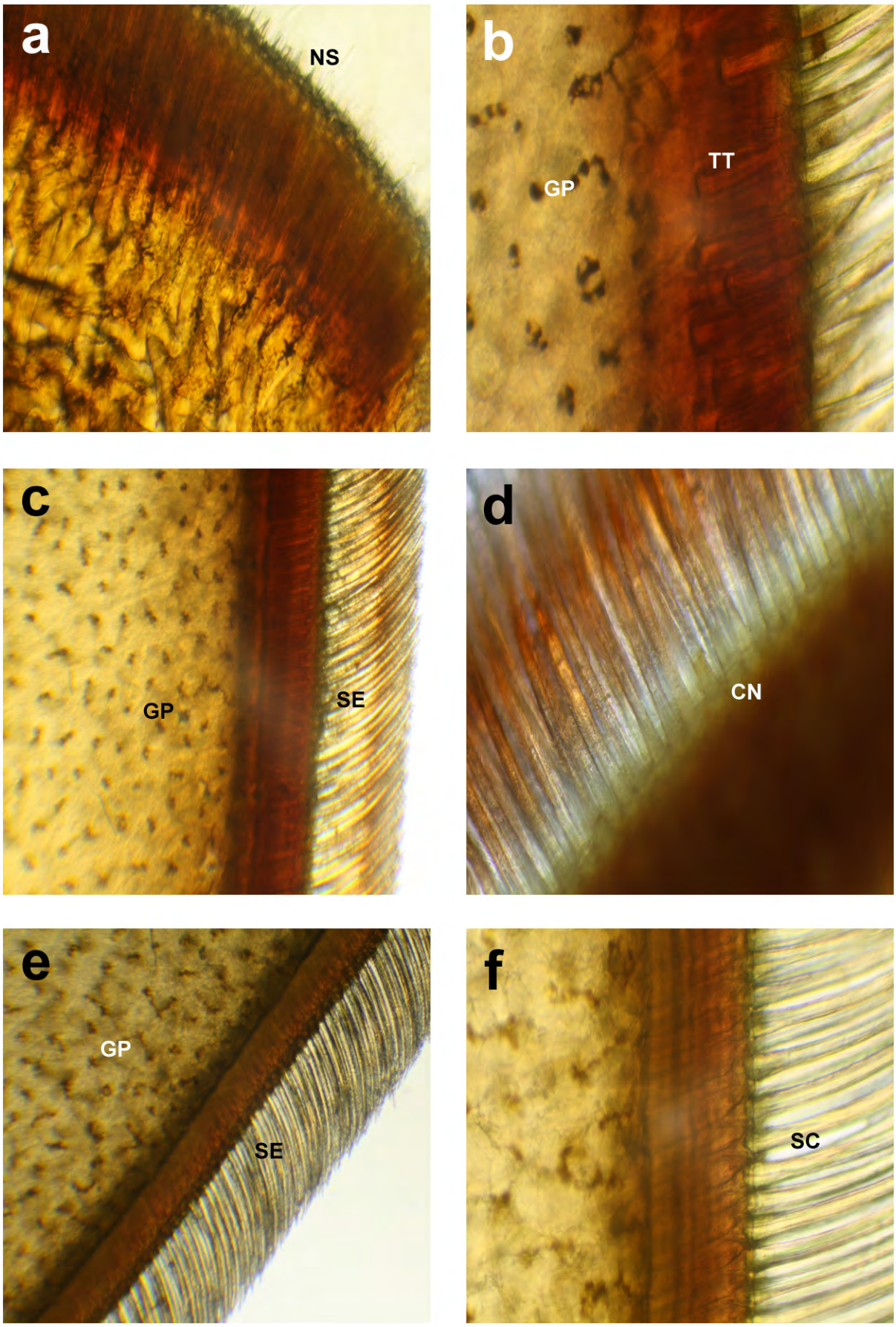
Fig. 2 Experimental setup showing crabs of various molting stages under laboratory conditions

Figs. 3a-3l Photomicrographs showing swimmeret setogenesis in *Portunus pelagicus*

3a & 3b Postmoult stage A showing the granular protoplasmic matrix (GP), new setae (NS) and tube-in-tube (TT) structure. X 50, X100

3c & 3d Postmoult stage B showing setae (SE) and cuticular nodes (CN). X 50, X100

3e & 3f Intermoult stage C with distinct setal cone (SC) formation under varying magnifications. X 50, X 100


a
NS
b
GP
TT
c
GP
SE
d
CN
e
GP
SE
f
SC

Figs. 3g & 3h Premoult stage D_0 showing epidermal retraction (ER) and scalloped epidermis (SD). X 50, X 100

Figs. 3i & 3j Premoult stage D_1 with further epidermal retraction (ER) and setal articulation (SA). X 50, X 100

Fig. 3k & 3l Premoult stage D_{2-3} showing complete epidermal retraction (ER), new setae (NS) formation and old cuticle (OC). X 50, X 100

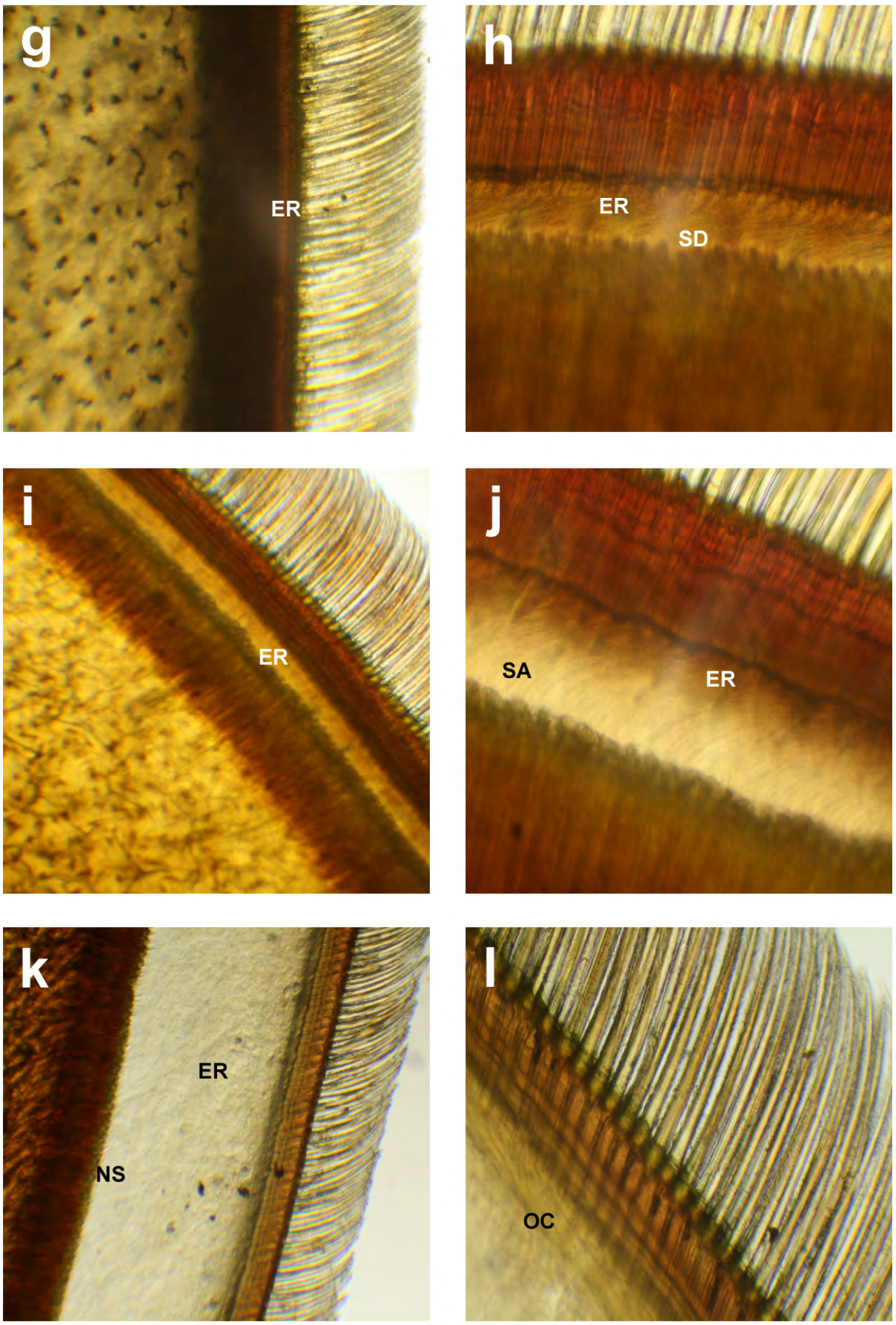

g
ER
h
ER
SD
i
ER
j
SA
ER
k
ER
NS
l
OC

Fig. 4 **Hemolymph protein levels during molting stages in *P. pelagicus***

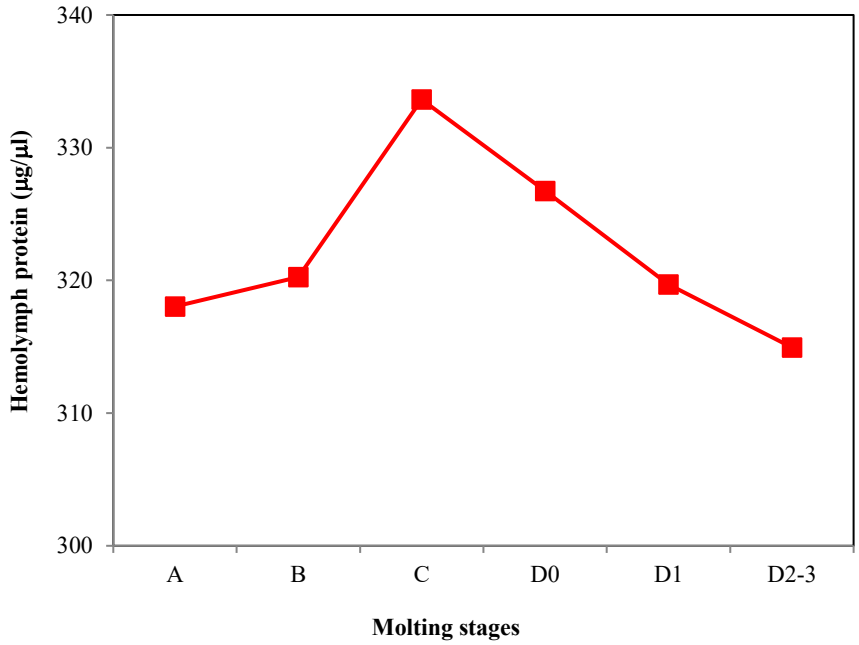
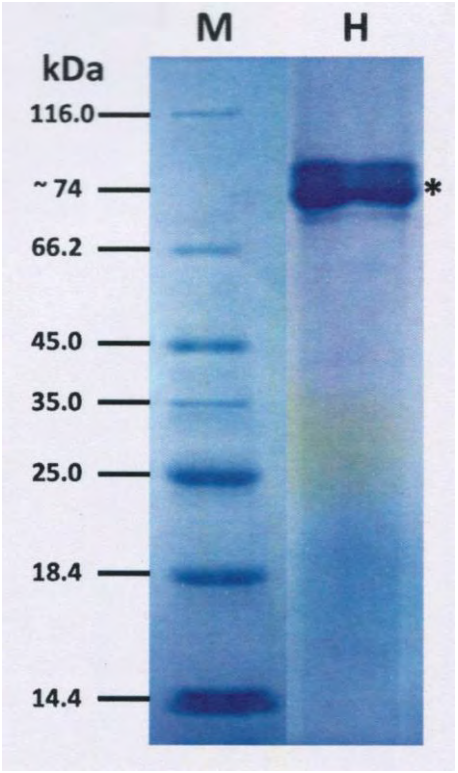
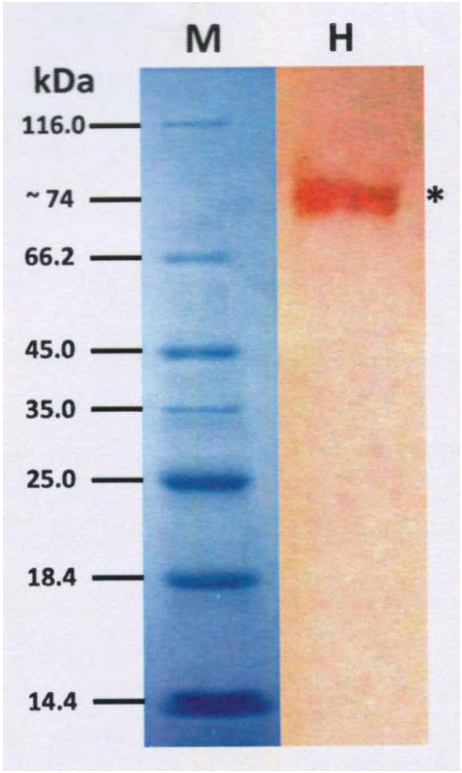


Fig. 5. Electrophoretic pattern of hemolymph proteins on a 10% denaturing gel



M – Molecular Weight Marker
H – Hemolymph

Fig. 6. Western Immunoblotting with CHH antibody showing positive reactivity



M – Molecular Weight Marker
H – Hemolymph

Fig. 7 **Thermotolerance of hemolymph glucose levels in *P. pelagicus***

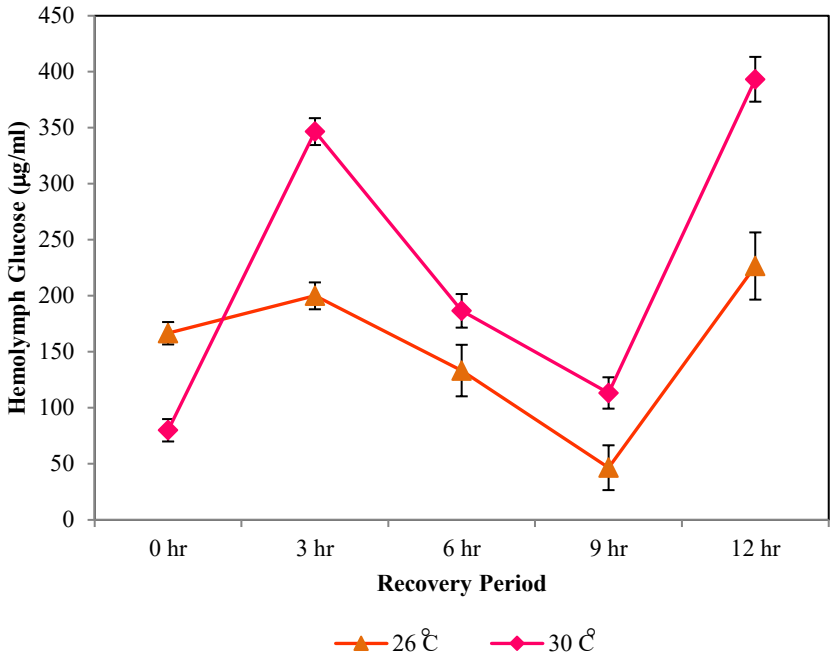


Fig. 8 **Thermotolerance of hepatopancreas glycogen levels in *P. pelagicus***

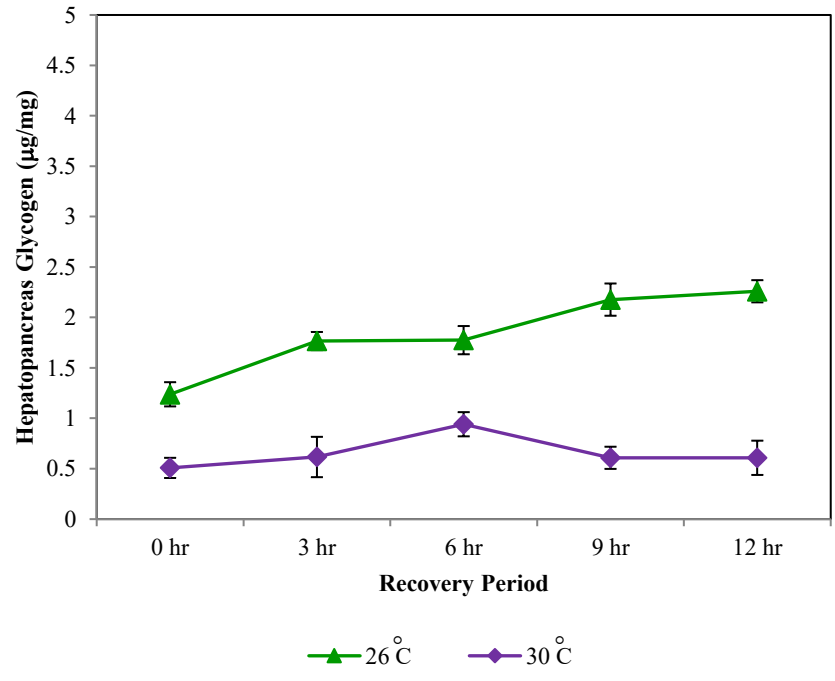


Fig. 9 **Thermotolerance of hemolymph CHH levels in *P. pelagicus***

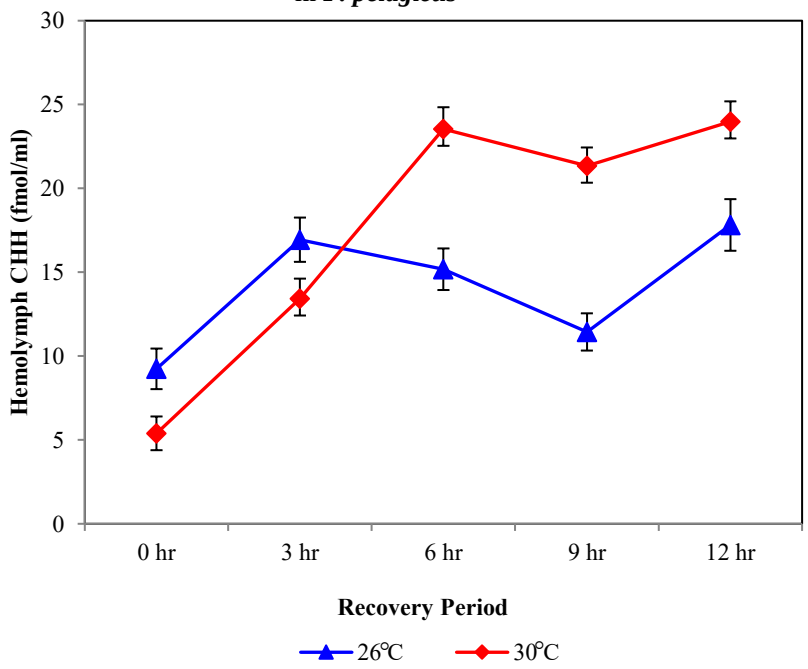


Fig. 10 **Effect of varying cadmium concentrations on hemolymph glucose level in *P. pelagicus***

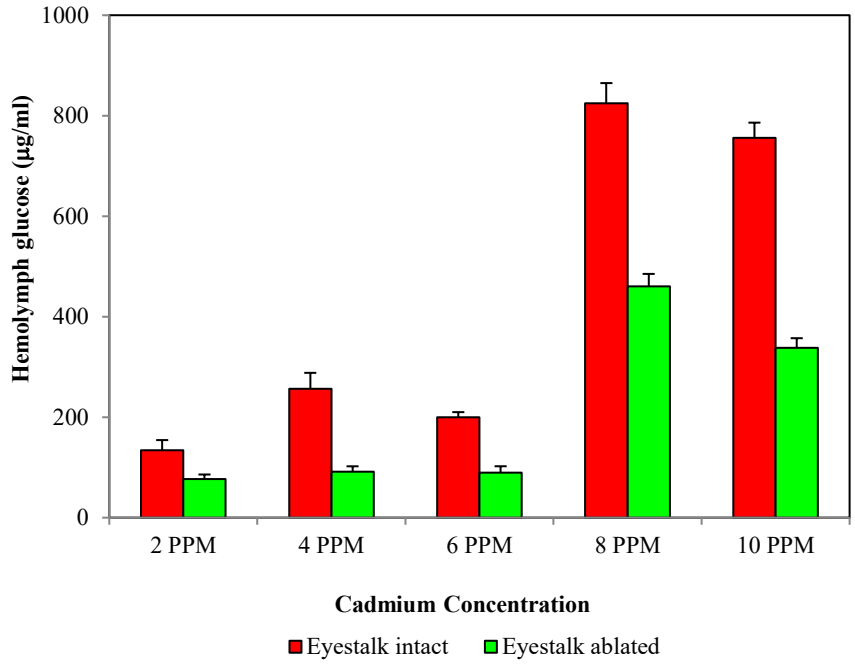


Fig. 11 **Variations in the level of hemolymph glucose on cadmium exposure in *P. pelagicus***

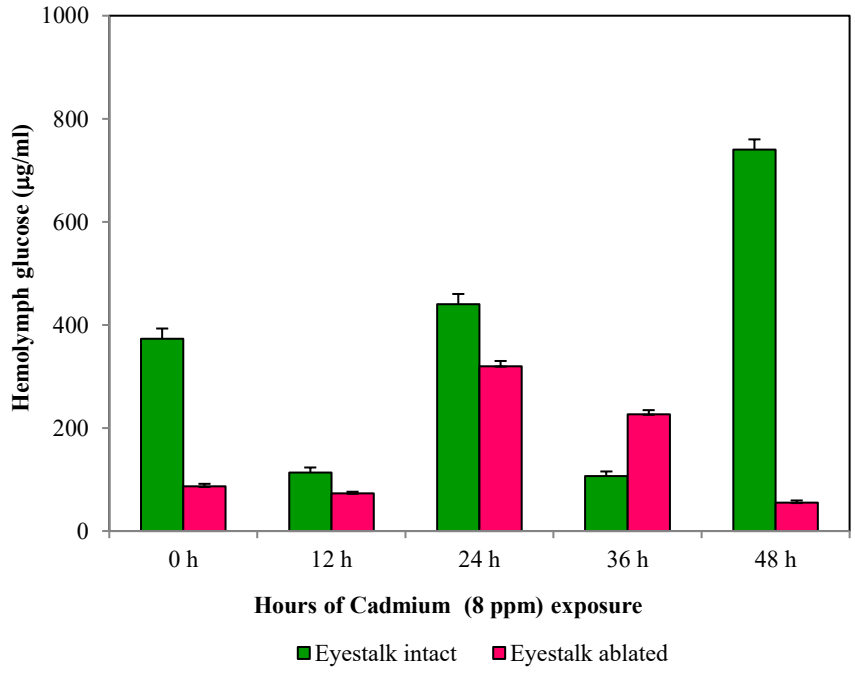


Fig. 12 **Variations in the level of hepatopancreas glycogen on cadmium exposure in *P. pelagicus***

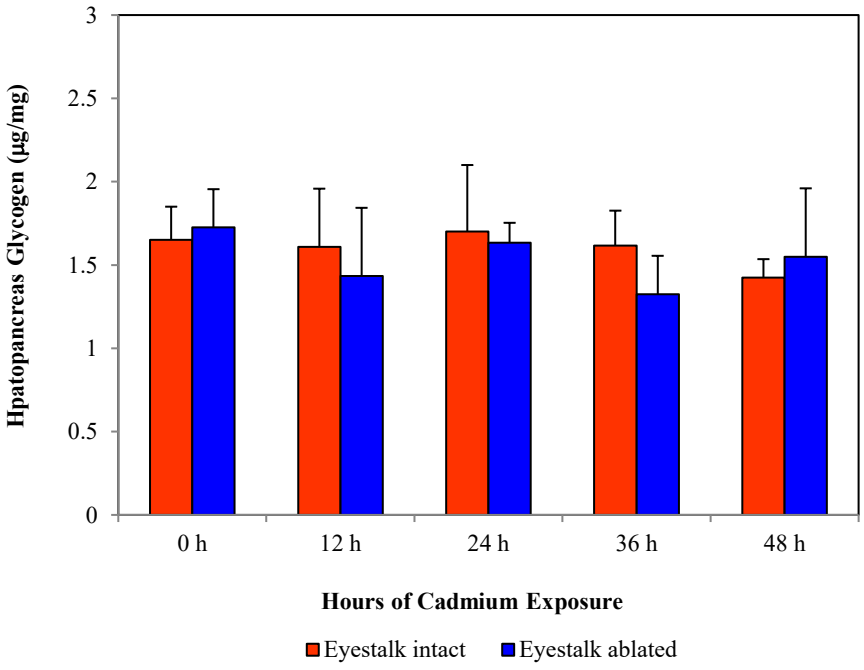
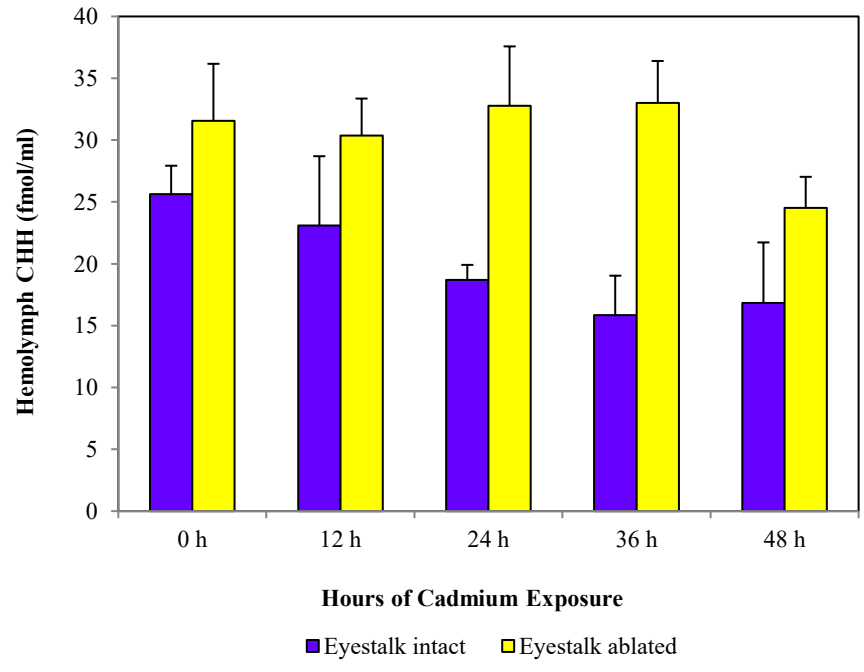


Fig. 13 **Variations in the level of hemolymph CHH on cadmium exposure in *P. pelagicus***



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Biochemical and Microbiological Evaluation of Raw and Processed Meat with a Note on Bioluminescent Bacteria in the Blue Swimmer Crab, *Portunus pelagicus*

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Abstract: Studies were put forth to elucidate variations in the biochemical constituents of raw and processed meat of the blue swimmer crab, *Portunus pelagicus* of the Thondi coast following standard procedures. Owing to the importance of micro-organisms in the aquaculture scenario of *Portunus sp.*, the present study was also aimed at the evaluation of the microbial colonies harvesting the raw and processed meat and also to enumerate possible bioluminescent bacteria from the carapace scrap and hemolymph of *P.pelagicus.* Results of the biochemical analysis showed clear variations among the parameters studied in the samples. Total protein content was present in a higher proportion than compared to all other constituents in both the samples, followed by total free sugars, phospholipids and total lipids. Almost a two-fold level of total protein was observed in raw meat than processed meat. Processing has been found to have a greater impact on the total free sugars with a four-fold decrease as recorded in the processed meat. Total lipids were also influenced by processing as with a significant variation between raw and processed meat. As an exception, only slight decrease of phospholipids was observed on processing in the present study. Morphological examinations suggested that the microbial colonies may belong to the genus *Enterobacteriaceae, Pseudomonas, Aeromonas, Alcaligenes, Chromobacterium, Vibrio* sp. Biochemical tests showed variable results with none of the isolates showing positive to methyl red and lipid hydrolysis test suggesting the presence of the genus *Brucella, Pseudomonas, Klebsiella, Escherichia, Salmonella, Vibrio*, etc. Luminescent colonies were observed as a bright green colour fluorescent light. The emission of light lasted even after 6 hours of incubation and lasted for a maximum of 3 to 4 hours. The colonies remained sandal white in colour and mucoidal. Results of the morphological tests suggest that the microbial colonies may be either *Vibrio* or *Pseudomonas*. Positivity was observed on performing the citrate utilization test, triple sugar iron agar test, starch hydrolysis test and catalase test suggesting that the bioluminescent bacteria would be *Pseudomonas sp*.

Key words: Protein · Free sugars · Lipids · Carapace · Bioluminescence

INTRODUCTION

Aquaculture, also known as aqua farming, is the farming of aquatic organisms such as fish, crustaceans, molluscs and aquatic plants. Crabs rank third among the edible marine crustaceans of India by virtue of their importance as an esteemed gourmet and the value of fishery they support [1]. The increased demand for the crabs in different markets and the depletion of resources along the coast has necessitated an urgent need for promoting crab culture in India. India is fast developing in crab fishery and there is a vast scope for the crab meat due to its delicacy and nutritional richness. In India, the coastal belt from Tuticorin to Mallipattinam has been proven as the strongest potential of edible sea crabs [2].

Among the commercially important crabs, the genus *Scylla* ranks first followed by the members of the family *Portunidae* [3-6]. The blue swimmer crab, *Portunus pelagicus* is the most important commercial species of the Indo-Pacific region. Other species of commercial value are *P. sanguinolentus, Charybdis feriatus, C. lucifera* and *Podopthalmus vigil*. The fishery of *P. pelagicus* along the South East coast was facing fishing pressure due to increasing demand for the species in the export market.

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The annual marine crab landings in India have steadily increased from 20,000 to 48,380 tonnes during 1977-2005, of which *P. pelagicus* contribute about 30% [7,8].

Biochemical studies are very important for studying the nutritional aspects of the animal. The biochemical constituents in animals are known to vary with season [9], size of the animal, stage of maturity, temperature and availability of food etc. [10]. Protein is very much essential for the sustenance of life and growth and hence it is present in largest quantity. It is the most prominent biochemical component of crustaceans from eggs to adult and is strikingly dominant in younger phases. The protein content in crab was reported to be higher in case of hard shell crabs [11]. Crabs are good sources of food including protein source for marine lives as well as for human. Nutritional quality of the crab proteins were compared vary favorable than that of muscle meat of mutton, chicken, duck and fish [12].

Carbohydrates constitute only a minor percentage in case of total biochemical composition. In fishery products, glucosides are found in abundance, the majority of which consists of glycogen. Traces of glucose, fructose, sucrose and other mono and disaccharides have also been reported. Lipids are highly efficient as sources of energy and they contain more than twice the energy of proteins and carbohydrates [13]. In crustaceans, lipids are not only the principal organic reserve and source of metabolic energy, but also indispensable in maintaining cellular integrity. Lipids acts as major food reserve along with protein and are subjected to periodic fluctuation influenced by environmental variables like temperature [14].

Marine invertebrates are constantly exposed to high concentration of microorganisms [15]. In crustaceans, the defence system against microbes rests largely on cellular activities performed by haemocytes such as adhesions, phagocytosis, encapsulation, nodule formation and melanisation. The multimeric coagulation and phenoloxidase systems are also considered to be important defence as part of the immune systems include agglutinins, hemolysins, lysozyme and antimicrobial factors. Crabs and shrimps have been implicated in *Vibrio parahaemolyticus* food poisoning [16, 17], Cholera [18], Salmonellosis [19], Shigellosis [20] and Yersinia food infection [17]. Deaths from staphylococcal food poisoning have been reported [21]. The report also asserted that the offending organism, *Staphylococcus aureus* grow rapidly and produces enterotoxins between 66°F and 99°F (20°C and 37°C) and that the staphylococcal enterotoxins are highly resistant to heat. Bergdoll [22,21] reported that the normal temperature used in cooking will not destroy the toxins and foods containing staphylococcal enterotoxins and they usually look and taste normal.

Bioluminescence is the chemical emission of light by organisms. It is a widespread but randomly distributed natural phenomenon, occurring more commonly among animals than plants [16]. Bioluminescence has been reported among microorganisms and has been used as an index of various characteristics, including ability to utilize organic compounds [23, 24]. Breitung *et al.* [23] have shown the bioremediation of 2, 4 and 6-trinitrotoluene (TNT)-contaminated soils by two different aerated compost systems using the inhibition of bioluminescence of *Vibrio fsicheri* as an index of the toxicity of the mineralized TNT. Ninety percent of deep sea marine lives are estimated to produce bioluminescence in one form or another. Most marine light-emission belongs in the blue and green light spectrum, the wavelengths that can transmit through the seawater most easily. However, certain loose-jawed fishes emit red and infrared light and the genus *Tomopteris* emits yellow bioluminescence. Four genera of bacteria (*Vibrio, Photobacterium, Alteromonas* and *Xenorhabdus*) naturally fall under this category [25].

In general, bioluminescence involves the combination of two types of substances in a light-producing reaction. One is a luciferin, or a light-producing substance. The other is a luciferase, or an enzyme that catalyzes the reaction. In some cases, the luciferin is a protein known as a photoprotein and the light-making process requires a charged ion to activate the reaction. Neurological, mechanical, chemical or as yet undiscovered triggers can start the reactions that create light. Often, the process requires the presence of other substances, like oxygen or adenosine triphosphate (ATP). ATP is a molecule that stores and transports energy in most living organisms, including the human body. The luciferin-luciferase reaction can also create byproducts like oxyluciferin and water. All luminous bacteria share this reaction, but their control systems differ. *In vitro* the reaction emits blue-green light with a maximum at 485-495 nm, yet the light from live *Vibrio fischeri* is bluer (max. at 475 nm) because a blue-fluorescent protein (or lumazine protein) accepts energy from the reaction and emits light at its own characteristic wavelength. The present investigation is aimed to enumerate the variations in the biochemical constituents of raw and processed crab meat together with the identification of possible bacteria following standard microbiological assays. Furthermore, studies were also putforth to unravel the presence of bioluminescent bacteria in the crab carapace scrap.

MATERIAL AND METHODS

Collection and Maintenance of Crab: Adult blue swimmer crab, *Portunus pelagicus*, were caught from the Thondi coast, Thondi (9° 45′N 79° 04′ E) (Fig. 1a and 1b). The crabs were transported to the laboratory in aerated plastic troughs. They were weighed and acclimatized for a week in tanks containing 10-15cm of stand at the bottom at about 34±2 ppt salinity and at room temperature (30±2°C). During the period, the crabs were fed with oyster (*Crossostrea 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.

Collection of Raw Crab Meat: The live crabs were taken and their surface was cleansed with distilled water to remove any debris particles present at the surface (Fig. 1c) and the edible meat portion of the crab was collected aseptically using sterilized scissors and forceps. One gram of the collected meat was weighed and was taken for further analyses.

Collection of Processed Crab Meat: The processed crabs were collected from the processing plant at Thondi. The live crabs were collected by the fishermen from the coast and brought to the processing plant. In the processing plant, the crabs were weighed and boiled at 100°C for 20 minutes. After heat processing, the crabs were packed in sterilized bags and packed in the ice boxes (Fig. 1d). From that processing plant, the boiled crabs were collected in sterilized cover and brought to the laboratory for biochemical analysis.

Quantification of Biochemical Constituents: The biochemical composition of raw and processed meat was estimated following standard procedures.

Estimation of Total Proteins: Estimation of total proteins was done as per the methodology of Bradford [26]. The colour developed was measured spectrophotometrically at 595 nm using a UV-Visible Spectrophotometer, Labomed Inc. USA.

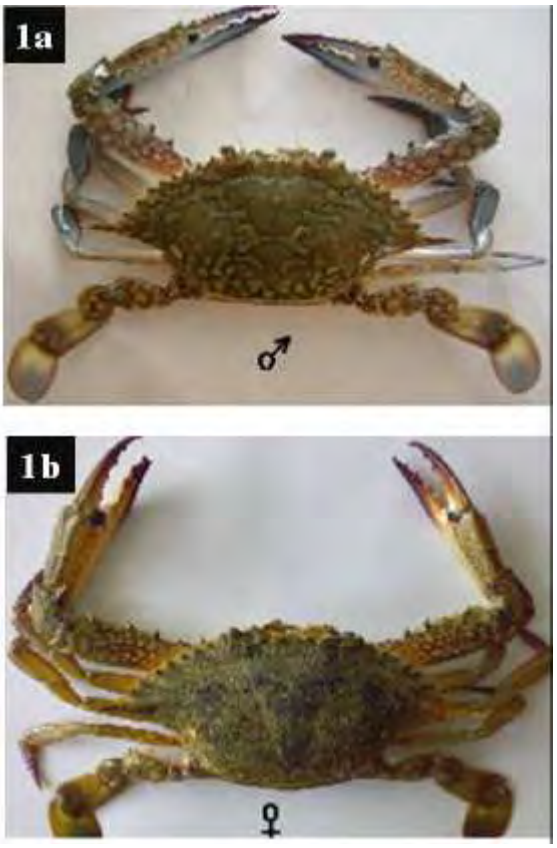

Fig. 1a,b: Dorsal view of Male and Female Blue swimmer crab, *Portunus pelagicus*

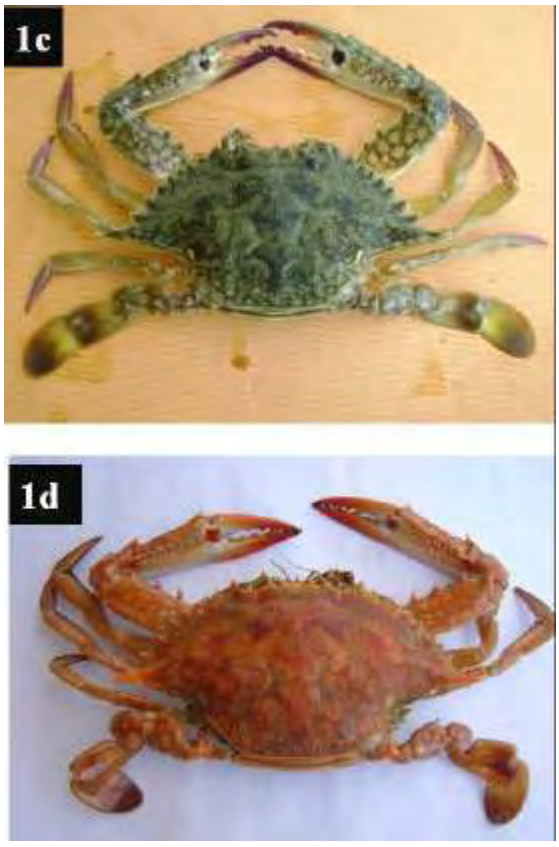

Fig. 1c,d: Raw and processed blue swimmer crab, *P. pelagicus*

Estimation of Total Free Sugars: Estimation of total free sugars was done according to the methodology of Roe [27]. The resultant colour complex was measured spectrophotometrically at 620 nm.

Estimation of Total Lipids: Estimation of total lipids was done according to the methodology of Barnes and Blackstock [28]. Extraction of lipids from sample was done following the procedure of Folch *et al.* [29]. The mixture was allowed to stand for 30min. and the colour developed was read at 520 nm.

Estimation of Phospholipids: Estimation of phospholipids was carried out as per the methodology of Rouser *et al.* [30]. The colour complex developed was read spectrophotometrically at 700nm.

Microbiological Analysis

Microbial Assay of Raw and Processed Meat

Collection of Processed Meat: The processed blue swimmer crab, *P. pelagicus* was collected from the processing plant at Thondi. The live crabs were collected by the fishermen from the coast and brought to the processing plant. In the processing plant, the crabs were weighed and boiled at 100°C for 20 minutes. After heat processing, the crabs were packed in the ice boxes. From that processing plant, the boiled crabs were collected in sterilized cover and brought to the laboratory for microbial analyses.

The live and processed crabs were taken and their surface was cleansed with distilled water to remove any debris particles present at the surface. The edible meat portion of the crab was collected aseptically using sterilized scissors and forceps. One gram of the meat was weighed and was taken for analysis. Then the serial dilution was performed by adding one gram of the meat in 9 ml of distilled water. And from that 1 ml was taken and serially diluted which was considered as 10^{-9} and from that dilution 1 ml was taken and serially diluted upto 10^{-1}. Then from the dilutions, 1 ml of the sample was transferred to the nutrient agar plates by using pour plate method. After 24 hours of incubation at 37°C, the plates were observed for colony morphology and each different colony were streaked in separate nutrient agar plates in order to get discrete pure culture colonies. The plates were then utilized for studying the morphological and biochemical characteristics of the microorganisms.

Bioluminescent Bacteria

Preparation of Carapace Scrap and Serial Dilution: The cultured crabs were taken and cleaned with distilled water to remove the adhering debris particles. With the help of a sterile scalpel, the exoskeleton was scrapped gently and approximately one gram was serially diluted and used for plating. Nine millilitre of distilled water was taken in 9 (1, 2, 3, 4, 5, 6, 7, 8 and 9) test tubes. All of them were sterilized by autoclaving at 121°C for 15 minutes at 15 lbs pressure. The scrapped carapace was inoculated into the 9 ml of distilled water and mixed well. Then with a sterile pipette, 1ml from the first test tube was aseptically transferred to the second tube. The dilutions were made up to 10^{-9}.

Collection of Hemolymph and Serial Dilution: The live crabs were kept on ice for a short period until completion of the hemolymph sampling. Hemolymph samples were drawn through the arthroidal membrane of the periopods with the help of a fresh disposable syringe. Approximately 5 ml of hemolymph was collected from each crab. The hemolymph was inoculated by pour plate method where 1 ml of sample was poured into a sterile petriplate, after which the medium was poured. The serially diluted carapace scrap was inoculated by spread plate method where 0.1 ml of sample was introduced into the agar plate and spread evenly with the help of an L-rod. The hemolymph and carapace samples were inoculated in BOSS medium(Sodium chloride 30g, peptone 10g, beef extract 3g, agar 15g and glycerol 3g and pH adjusted to 7.3) and they were incubated at 28°C for 24-36 hours. After incubation period the plates were observed in the complete dark for luminescent colonies.

Microbial Identification Tests: These tests were carried out to identify the isolated microorganisms at the genus level on the basis of their morphological and biochemical characterization. Morphological tests such as Gram's staining and motility test (Hanging Drop Method) were performed to differentiate the bacteria as Gram positive, Gram negative bacteria, motile and non-motile colonies. Biochemical tests such as indole test, methyl red test, utilization test, triple sugar iron agar test, protein hydrolysis, lipid hydrolysis, starch hydrolysis and catalase test were carried out.

Statistical Analysis: The results of biochemical analysis were subjected to Analysis of Variance (ANOVA) to reveal whether the variations in biochemical constituents of the raw and processed meat of *P. pelagicus* were statistically significant.

RESULTS

Biochemical Constituents: The results of the present study revealed significant variations in the biochemical constituents of raw and processed meat of the blue swimmer crab, *Portunus pelagicus*, as summarized in Table 1 and Fig. 2.

Total Protein: The total protein content was higher both in raw and processed meat when compared to the free sugars, lipids and phospholipid levels. Significantly higher level of protein was observed in raw meat (127.34±4.24 µg/g) when compared to processed meat (70.47±4.41 µg/g) (P<0.001).

Total Free Sugars: Total free sugars form the second largest biochemical component to be present in raw meat and third in processed meat. The raw meat showed a significant increase of total free sugars (98.50±3.78_µg/g) when compared to that of processed meat (21.74±4.34 µg/g) (P<0.001).

Total Lipids: The level of total lipids rank the lowest among the quantified biochemical constituents studied in the samples. A higher amount o 45.74±2.00 µg/g was observed in raw meat when compared to the lipid level of 16.82±2.28 µg/g in processed meat (P<0.01).

Phospholipids: Phospholipids stand the thirst largest biochemical constituent in raw meat whereas in processed meat it remains the second largest component. Insignificant variation in the levels of phospholipids was observed between raw meat (77.27±4.49 µg/g) and processed meat (72.58±2.84 µg/g) as compared to other constituents.

Thus, the results of the present study show higher levels of biochemical constituents in the raw meat and significant decrease in the levels after processing.

Microbial Analysis of Raw and Processed Meat: After 24 hours of incubation, the plates were observed for the colony formation and totally 9 morphologically different bacteria were isolated from both raw and processed crab meat. Those colonies were marked as 1, 2…9 in order to perform the biochemical tests to identify their genus (Table 2).

Table 1: Biochemical composition of raw and processed meat of *P. pelagicus*

Biochemical composition	Raw Meat(µg/g)	Processed Meat (µg/g)
Protein	127.34 ± 4.25	70.47 ± 4.41
Free Sugars	98.50 ± 3.78	21.74 ± 4.34
Lipid	45.74 ± 2.00	16.82 ± 2.28
Phospholipids	77.27 ± 4.49	72.58 ± 2.84

Table 2: Morphological and biochemical characteristics of raw and processed meat of *P. pelagicus*

Experiments	1	2	3	4	5	6	7	8	9
Morphological tests									
Gram Staining	-	-	-	-	-	-	-	-	-
Motility Test	-	+	-	-	-	+	+	-	-
Biochemical Tests									
Indole Test	-	-	-	-	-	-	+	-	+
Methyl Red	-	-	-	-	-	-	-	-	-
Citrate Utilization test	+	-	+	-	+	+	+	+	+
Triple sugar iron agar test	+	+	-	-	+	-	+	-	-
Protein hydrolysis	+	+	-	-	-	-	-	-	-
Lipid hydrolysis	-	-	-	-	-	-	-	-	-
Starch hydrolysis	+	-	-	-	-	+	-	-	-
Catalase test	+	+	+	+	-	+	-	+	-

+ = Positive -= Negative

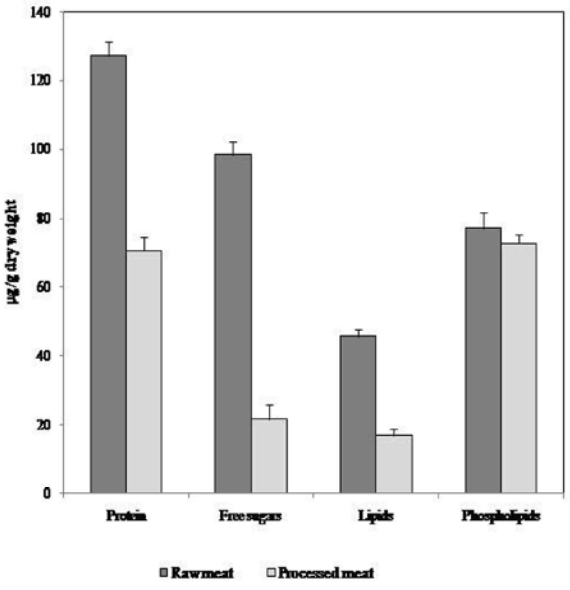

Fig. 2: Biochemical composition of raw and processed meat of *P. pelagicus*

Morphological Tests

Gram Staining: The isolated colonies appeared as pink colour when it was visualized under microscope and it was found to be rod shaped. It clearly showed that the bacteria falls under the category of Gram negative, whose morphological structure was determined as rods. The gram negative rods may belong to the genus, *Enterobacteriaceae, Pseudomonas, Aeromonas, Alcaligenes, Chromobacterium, Vibrio*, etc.

Motility Test: The isolated microorganisms were subjected to motility test. The isolates 2, 7, 8 showed motility whereas the isolates 1, 3, 4, 5, 6, 9 were immotile. The organisms which showed motility may belong to the genus *Aeromonas, Escherichia, Alkaligenes, Serratia, Salmonella, Vibrio, Pseudomonas*, etc. And the immotile organisms may belong to the genus *Corynebacterium, Mycoplasma, Brucella, Fusobacterium*, etc.

Biochemical Tests: Biochemical tests showed variable results with none of the isolates showing positive to methyl red and lipid hydrolysis test suggesting the presence of the genus *Brucella, Pseudomonas, Klebsiella, Escherichia, Salmonella, Vibrio*, etc. Maximum number of the colonies showed positivity to citrate utilization test indicating the possibility to the presence of the genus *Chromobacter, Azospirillum, Klebsiella, Citrobacter, Enterobacter, Serratia*, etc. As 3 microbial isolates showed negative result to catalase test, the genus *Eschericia, Salmonella, Shigella, Yersinia*, etc may be present in the samples tested. The isolate 6 from the fresh meat sample showed positivity to citrate utilization test, starch hydrolysis test and catalase test possibly proposing the presence of the genus *Chromobacterium, Azospirillum, Pseudomonas, Serratia*. The isolate 9 showed positivity only to citrate utilization test.

Microbial Analysis of Bioluminescent Bacteria: Bioluminescent colonies were observed after 5 days of incubation as a dark band along the entire diameter of a petriplate in which the hemolymph was inoculated and there was no bioluminescent colonies in the carapace scrap inoculated plates. The colonies emitted a bright green colour fluorescent light (Figs. 3a and 3b), whereas the isolated pure cultures were able to emit light after 6 hours of incubation and it lasts for maximum of 3 to 4 hours, after that the capability to emit light gradually decreased and almost lost after 24 hours.

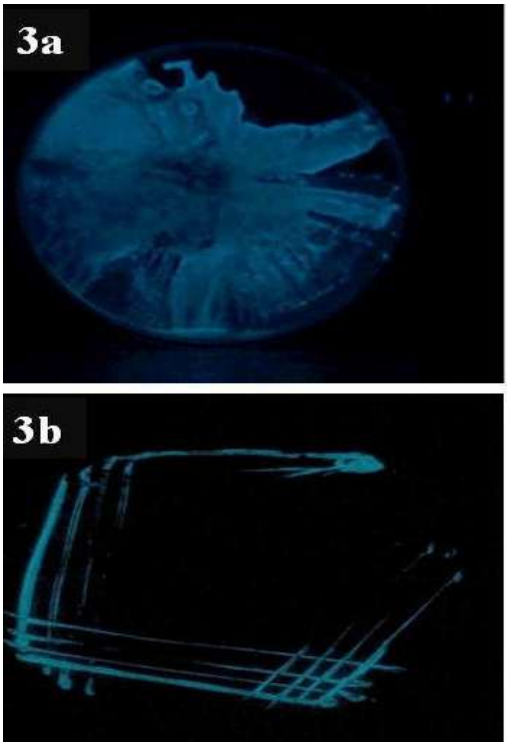

Fig. 3ab: Photographs showing the isolated luminescent colonies from the carapace scrap of *P. pelagicus*

Table 3: Morphological and Biochemical characteristics of the isolated bioluminescent bacteria

Experiment	Control	Sample
Morphological Tests		
Gram's staining		-
Motility		+
Biochemical Tests		
Indole test	-	-
Methyl red test	-	-
Citrate utilization test	-	+
Triple sugar iron test	-	+
Protein hydrolysis	-	-
Lipid hydrolysis	-	-
Starch hydrolysis	-	+
Catalase test	-	+

+= Positive -= Negative

However, these luminescent colonies were able to regain their capacity to emit light when they were transferred to a fresh medium. The colonies were sandal white in colour and mucoidal and the results for morphological and biochemical tests were tabulated in Table 3.

Morphological Tests

Gram Staining: The isolated colony appeared as pink colour when it was visualized under microscope and it was found to be rod shaped. It clearly shows that the bacteria falls under the category of Gram negative, whose morphological structure was determined as rods. The gram negative rods may belong to the genus, *Enterobacteriaceae, Pseudomonas, Aeromonas, Alcaligenes, Chromobacterium, Vibrio*, etc.

Motility Test: The movement of the micro organism was clearly noticed under the microscope, thus these microbes use flagella for their locomotion and they are motile in nature. The organism may belong to the genus *Aeromonas, Escherichia, Alkaligenes, Serratia, Salmonella, Vibrio, Pseudomonas*, etc.

Biochemical Tests: The bacterial isolate showed positivity with citrate utilization test, triple sugar iron agar test, starch hydrolysis test and catalase test. Negativity was observed with protein hydrolysis test, lipid hydrolysis test, indole test and methyl red test. The results of the above biochemical tests suggest that the bioluminescent bacteria would be *Pseudomonas sp*.

DISCUSSION

Accumulation of energy reserves in species dependent upon unstable food resources has been reported by several authors [31, 32, 33]. *Portunus pelagicus* occurs in large numbers along the coasts of Palk Bay and Gulf of Mannar. Even though the protein content is less in crabs than in fishes (8.3-23.8%) they form a well established food. Proximate chemical composition, energy content and metabolic rates of a large number of pelagic crustaceans and fishes have been studied intemperate and subtropical latitudes [34, 35, 36,37, 38, 39, 40]. Many authors have revealed that mesopelagic species show variability in proximate composition as a function of depth of occurrence and as a function of regional productivity. Depth and productivity both affect food availability and thus influence chemical composition. In particular, lipid and protein content (% wet weight) both decline and as a result water concentration increases with increasing depth of occurrence [38]. Moreover, mesopelagic crustaceans and fishes living at greater depths have much lower metabolic rates than shallower-living pelagic species; the ammonia excretion and oxygen consumption rates decline with increasing depth [41, 37, 42, 38]. It is worth noting that these physiological and biochemical changes have been attributed to factors correlated to depth, aside from the possible influence of temperature [43, 44] or hydrostatic pressure [45].

All the biochemical constituents observed in the present study show a massive level of decline in the processed meat when compared to raw meat. The values of protein in the present study are in agreement with that of Zamir *et al*. [46] who have determined the deteriorative changes in the nutritive quality of crab meat during storage at refrigerator temperature (7±2) for the period of one week. The results indicate a significant increase ($p = 0.001$) in pH water, TMA, TVB while total protein salt soluble protein and total lipid contents were significantly decreased ($p<0.001$) as compared to fresh tissue and recommended that the quality of crab meat is acceptable upto one day of storage at refrigerator temperature. Balasubramanian and Suseelan [47] observed the protein values in *C. smithii* to vary from 59.8 to 71% on a dry matter basis. In *S. serrata*, the protein content of the body meat and claw meat was 20.11% and 18.54% respectively [48].

Carbohydrates constitute only a minor percentage of total biochemical composition in the present study. The previous studies suggest that the carbohydrate in the muscle varied from 0.3 to 0.63% in *P. vigil* [49], 2.4 to 3.4% in *C. smithii* [47], 0.17% in body meat, 0.24% in claw meat of *S. serrata* [48] and 0.44 to 0.73% in *P. sanguinolentus* [50]. The results of Alva and Pascal [51] and Diaz and Nakagawa [52] indicate that dietary carbohydrate can influence the proximate composition of prawn while the studies of Soundarapandian and Ananthan [10] indicate that dietary carbohydrate has no effect on body carbohydrate of *M. malcolmsonii*. Lipids are highly efficient sources of energy in a way that they contain more than twice the energy of carbohydrates and proteins [13]. Kannupandi *et al*. [53] also reported that the utilization of lipid was greater than protein in *S.brockii.* In the present study, lipid content of the hard shell crab (2.41%) was higher than soft shell crabs (1.50%).

The microbiological studies have revealed the presence of a broad range of gram negative rod-like bacteria in both the raw and processed meat. The indigenous pathogenic *Vibrio* species has been reported to be far less hosted by the crabs of cold waters than shellfish from temperate waters [54,55]. Continued studies are needed to assure the safety of crabs and to establish adequate test procedures for assessing the probability of contamination with potential human pathogen. As in the present study, Reid *et al*. [56] have reported the

prevalence, pathogenesis and occurrence of pandemic *Vibrio parahaemolyticus* in clinical samples and seafood in China, thereby re-emphasizing the public health significance of this pathogen. It is advised that crabs when harvested from the lagoons or other sources be frozen at temperature of between 0ºC and subzero since toxin production by the pathogens especially *Staphylococcus aureus* will be stalled [21,22]. The presence of potentially pathogenic bacteria in seawater associated with seafood in this in case crabs, can have serious ecological, public health epidemiological implications. Consequently, consumption of raw and partially cooked *Portunus* sp. would pose serious danger to consumers of this protein and calcium-rich sea food.

Luminescent bacteria occur in the intestinal tracts of marine animals [57, 58] and may be associated with luminous faecal pellets [59]. Lesions on the chitinous exoskeleton of crustaceans can be caused by bacteria [60]. An overall pattern of worldwide distribution of luminous bacteria with respect to temperature and salinity has been reported [61,62, 58, 63]. The present study has revealed that the presence of bioluminescent bacteria in the blue swimmer crab *P. pelagicus.* There are a large number of luminescent species of bacteria which are distributed among various genera, including the *Pseudomonas* and *Vibrio*, as well as subgenus *Photobacterium* [64]. The two luminous species which have been used most extensively for physiological and biochemical studies are *P.phosphoreum* and *Achromobacter fischeri* (*Bacteriumphosphorescens indigenus*), which are marine forms. Among the factors which have been found to influence bacterial luminescence are salt concentration, amino acids, carbon sources and molecular oxygen. The work of Farghaly [65] has demonstrated that there is a rather critical optimum of salt concentrations for bacterial luminescence and growth.

For example, "Microtox" for water quality/toxicity testing employs the bioluminescent marine bacteria *Vibrio fischeri*. When this organism is challenged by a toxin, the respiration pathway is disrupted, resulting in a decrease in bioluminescent intensity.

CONCLUSION

The results of the present study reveals the possible impact of the processing technique on the important biochemical constituents and seeking way towards alternative in the processing technique without much altering the biochemistry of the meat in rear future. The study also implicates the invasion of microbes on processing; and the fresh meat almost remains less invaded by microbes necessitating an indepth study upto the species level as an initial step towards the eradication of microbes during processing and to enlighten and deplenish the possible factors favoring their growth. Further studies of the bioluminescent bacteria upto the species level would throw light on their efficient use in commercial aquaculture.

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Effect of Temperature on the Biochemical Constituents of the Blue Swimmer Crab *Portunus pelagicus*

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Abstract: Heat shock refers to a short exposure of an organism or cells to a near lethal high temperature that result in an increase in tolerance to a subsequent exposure at a lethal temperature (thermotolerance). The present study emphasizes on the variations in the levels of hemolymph biochemical constituents on exposure to varying water temperatures and their recovery periods in the blue swimmer crab, *Portunus pelagicus*. Results of the present study on the level of proteins reveal it to be the immediate metabolites for help under heat and cold stress. Higher temperature of 30°C was found to have pronounce effect on the protein level (4.09±0.45 g/ml). The decline in the protein level during the initial hours of recovery demonstrates the effective utilization of proteins to overcome the heat stress. Only minor variations in the level of total free sugars were observed with increase after heat stress and decrease after cold stress. No prominent loss or restoration of the total free sugars was observed during the recovery process. This suggests neither the utilization of the free sugars nor their production during the heat and cold stress. During both the water treatments (30°C and 26°C), significant increase in the level of lipids was observed, viz., 16.2±0.03 g/ml and 12.6±0.44 g/ml, respectively. Though there was a decline in the level of total lipids during the early hours of recovery, its level was even higher than the normal crabs after 10 hrs of recovery period (14±0.005 g/ml (30°C) and 22.7±0.89 g/ml (26°C). From the results it becomes clear that proteins and lipids are the two important biochemical constituents that come into action to overcome stressful conditions that generally prevail in the natural habitat of the test animal, *P. pelagicus*. Data provided by this study will help to model temperature-dependent growth in the field and assist in designing the best possible temperatures and diets for crabs.

Key words: Thermotolerance · Heat shock treatment · Biochemical constituents · Recovery period

INTRODUCTION

Temperature stress provokes energy-demanding responses at the cellular level, which eventually may reduce the organism's competition and reproduction abilities [1, 2]. Hence, temperature stress is a significant physiological and ecological factor. In evolution, those species apparently are more successful that better cope with the physiological effects of stress, i.e. respond with less expense of energy [3]. The ability of organisms to tolerate temperature stress depends on the thermal history of their habitat [4].

In the intertidal zone of the Indo pacific, body temperatures of sessile marine organisms can reach 35°C for an extended time during low tide, resulting in potential physiological stress. Eurythermal crustaceans survive seasonal, diurnal or tidal changes in environmental temperature by developing several capacity and resistance adaptations to change their behaviour, physiology, growth and metabolism. In temperature climates, marked physiological differences have been observed between summer and winter crayfish with seasonal changes in haemocyanin oxygen affinity and changes in the relationship between pH and temperature [5].

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The temperature of habitat water has a drastic influence on the behavioural, physiological and biochemical mechanisms of crustaceans. This stress provides stimulus for the induction of stress proteins/heat shock proteins (Hsps) which help protect the organisms and repair the cellular damage. Hsps are formed as a response to increased requirement for descriptive and the correct refolding of protein aggregates which spontaneously form denatured polypeptide chains. This phenomenon known as the thermotolerance is acquired normally when the temperature raises 5-100°C above the ambient temperature of the organism. With the non-heat shock protein suppressed at elevated temperature, the hsps are synthesized for several hours post heat shock. Among the four major hsp families of 90, 70, 60,16-24 kDa molecular weight, the hsp70 family is the best studied in marine organisms. It acts by preventing the denaturation of protein and holding them in the state of folding or assembly to facilitate repair.

Much of research carried out on thermal impact has focused on marine benthic communities, which are suitable group for detecting the effects of different kinds of pollutant, due to their limited ability to escape. Some organisms, inhabiting the intertidal zone have emerged in recent years as potential harbingers of beach pollution. Alterations brought about in a marine environment by discharge of heated effluents may vary greatly as a function of the quantity of heat discharged and of the ecological and biological features of the study environments.

It is now well established that animals acclimated to low temperatures show a higher metabolic rate than animals acclimated to high temperatures. A moderate change in ambient temperature can lead to vital physiological and biochemical adjustments in ectotherms, one of which is a change in fatty acid composition. When temperature decreases, the composition of membrane lipids (phospholipid fatty acids) is expected to become more unsaturated to be able to maintain homeoviscosity. Although different in function, storage lipids (triacylglycerol fatty acids) are expected to respond to temperature changes in a similar way. Age-specific differences, however, could influence this temperature response between different life stages. This has been studied extensively by many workers [6-11]. Laboratory acclimation to low and high temperatures has been demonstrated in many animals [11,12]. The effect of salinity changes on temperature tolerance have been studied less extensively the work of Brockema [13] on the shrimp *Crangon crangon*, McLeese [14] on lobster, *Homarus americanus* require special mention.

Variation of 19 free amino acids (FAA) in serum and hepatopancreas, glucose in serum and glycogen in hepatopancreas was investigated in white shrimp, *Litopenaeus vannamei* (IBW 5.0±0.3g) when water temperature dropped from 28 to 13°C within 4days, in order to provide basic data for biochemical changes of the species during cold stress [15]. Van Dooremalen *et al.* [16] have investigated the effect of repeated temperature fluctuations on fatty acid composition and thermal tolerance on exposing the springtail *Orchesella cincta* to two constant temperatures of 5 and 20°C and a continuously fluctuating treatment between 5 and 20°C every 2 days. Fatty acid composition differed significantly between constant low and high temperatures. As expected, animals were most cold tolerant in the low temperature treatment, while heat tolerance was highest under high temperature. Recently, Tu *et al.* [17] have studied the combined effects of temperature and salinity on some oxidative stress biomarkers as well as on acetylcholinesterase activity (AChE) in hepatopancreas, gills and muscle of black tiger shrimp (*Penaeus monodon*). A combination of three temperatures (24, 29 and 34°C), two salinities (15 and 25 ppt) and the absence or presence of 0.1 µg L(-1) deltamethrin was applied on shrimp during 4 d under laboratory conditions. Lipid peroxidation level (LPO) and glutathione S-transferase activity (GST) were not affected by combined effect of temperature, salinity and deltamethrin in any of the studied tissues.

Matozzo *et al.* [18] have evaluated the effects of temperature on cellular and biochemical parameters of the crab *Carcinus aestuarii*.Stoner *et al.* [19] have undertaken experiments to explore how temperature mediates growth and energy allocation in newly metamorphosed juveniles of the red king crab, Paralithodes camtschaticus. Overall growth increased as an exponential function of temperature, with slightly higher growth rates observed in populations than for isolated individuals. Growth records for individuals revealed an inverse exponential relationship between water temperature and intermolt period. There was also a small increase in growth increment from juvenile stage 1 to stage 2 with increasing temperature that appeared to be linear.

The blue swimmer crab, *Portunus pelagicus* (Crustacea: Decapoda: Brachyura) is a dominant intertidal species utilized throughout the indo-pacific region and is a particularly important species of Palk bay. It has high nutritional value and delicious taste and hence their requirements of capture and cultivation of this species are constantly increasing. This species experiences varying

and increasing temperature levels as it resides in an higher intertidal zone of Thondi coast. Thus a better understanding of the relationship between tolerance mechanisms against temperature in this species in laboratory condition may give rise to the possibility of more efficient control of their mortality and its higher degree of adaptability to thermal regimes. These studies would be highly helpful to the local fishermen of Palk Bay who are entirely dependent on the crab fishery for their daily life.

The present study would provide answers to the questions viz. (i) At what temperature does *Portunus pelagicus* respond physiologically to thermal stress. (ii) Does the induction temperature elicit the level of biochemical constituents and (iii) Suggest whether the intertidal crab may be well adapted to life in the high intertidal zone and have the plasticity to acclimate to higher temperatures.

MATERIAL AND METHODS

Collection and Maintenance: Adult blue swimmer crabs, *Portunus pelagicus,* were caught from the Thondi Coast, Thondi (9°45'N 79°04'E). The crabs were transported to the laboratory in aerated plastic troughs. They were weighed and acclimatized for a week in tanks containing 10-15 cm of sand at the bottom at about 34±2 ppt salinity and at room temperature (30±2° C). During the period, the crabs were fed with oyster (*Crassostrea madrasensus*) 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.

Analysis of Intermolt Stage: Setal development of *P. pelagicus* was observed on the basis of the epidermal retraction observed at the posterior median part of the swimmeret. Molt stages were determined using morphological changes of the seta as described by [20] using light microscope (Optika B-350, Italy) and only intermolt crabs were chosen for the present study.

Heat Shock Treatments: The intermolt crabs were divided into two experimental groups consisting of 10 crabs each for stress treatments and the crabs were maintained at the ambient temperature (28°C) which served as the control:

Experimental Group 1: This experimental group was exposed to 26°C for 2 hrs

Experimental Group 2: This experimental group was exposed to 30°C for 2 hrs.

Immediately after thermal incubation, all the experimental crabs were placed in the sea water with ambient temperature (28°C). At 4, 6, 8 and 10 hrs after thermal incubation was terminated, hemolymph samples will be collected from the crabs.

Hemolymph Sampling: Haemolymph samples were drawn from the crabs during the various molt stages, through the arthrodial membrane of the pereiopods by using disposable syringes. Approximately 5 ml was obtained from each crab. The collected haemolymph was stored in separate vials under-20°C until further use.

Quantification of Biochemical Constituents: The biochemical composition of the hemolymph in the experimental groups and during the hours of recovery was studied following standard biochemical procedures. Estimation of total proteins was done as per the methodology of Bradford [21]. Estimation of total free sugars was done according to the methodology of Roe [22]. Estimation of total lipids was done according to the methodology of Barnes and Blackstock [23]. Extraction of lipids from sample was done following the procedure of Folch *et al*. [24].

Data Analysis: The results of the present study enumerating the effect of varying temperatures on the biochemical constituents viz., total proteins, total free sugars and total lipids were subjected to T-Test and Two-way ANOVA to test whether the variations among and between them are significant.

RESULTS

Heat Shock Response of *P. pelagicus*: The blue swimmer crab, *P. pelagicus,* on exposure to varying temperatures revealed significant variations in the biochemical constituents. Together, the crab showed immense recovery of its metabolites within few hours of incubation at the ambient temperature.

Total Proteins: The protein level in the hemolymph tend to increase when the water temperature was increased to 30°C (4.07±0.45 g/ml) and decreased with decrease in water temperature to 26°C (2.8±0.002 g/ml) when compared to the control ($t<0.1$, $t<0.05$) (Fig.1). During the initial hours of recovery, the protein level dropped to 1.5±0.37 g/ml (4 hrs) and gradually rose to 5.63±0.34 g/ml after 10 hrs, in the experimental group I (30°C). Crabs of experiment group II (26°C) had a recovery in the protein level until 6 hrs (5.0±0.34 g/ml) and dropped back to

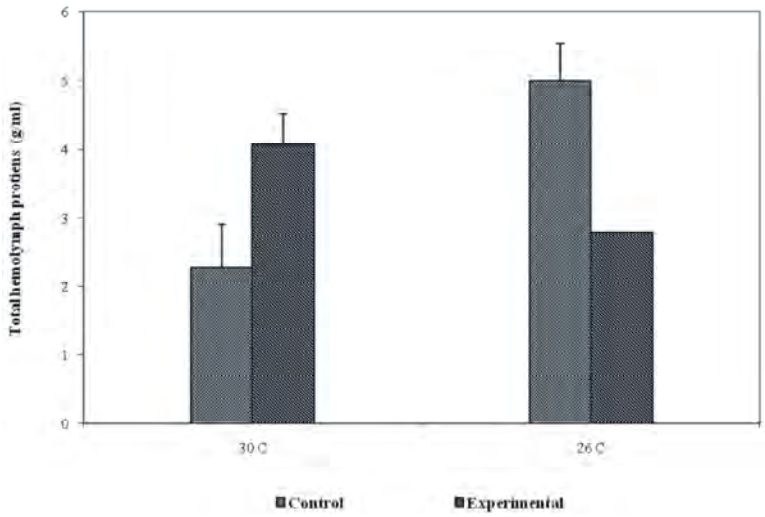

Fig. 1: Variations in the level of total proteins of *P. pelagicus* on exposure to varying temperatures

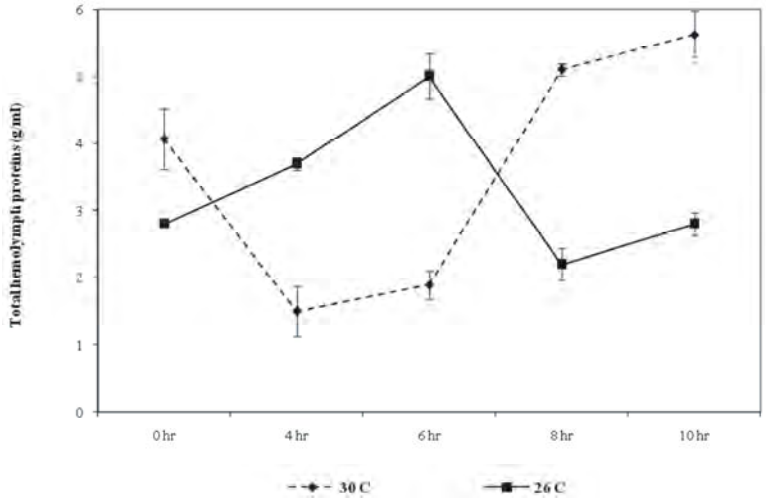

Fig. 2: Time-course changes in total protein on transfer from experimental (26°C and 30°C) to control (28°C)

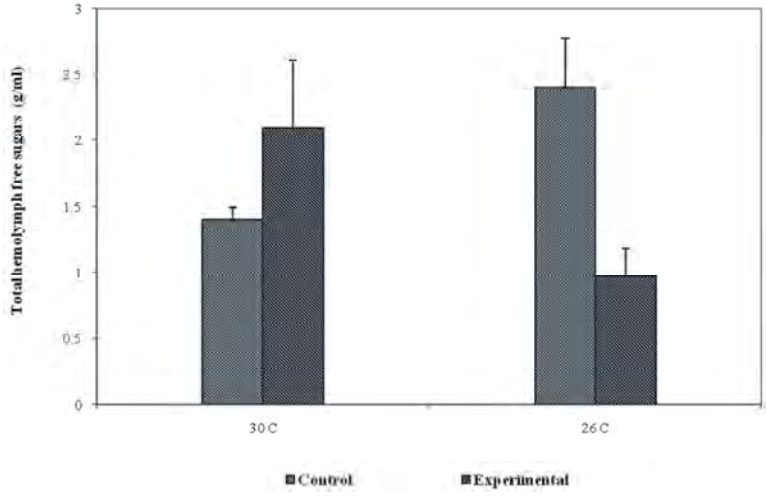

Fig. 3: Variations in the level of total free sugars of *P. pelagicus* on exposure to varying temperatures

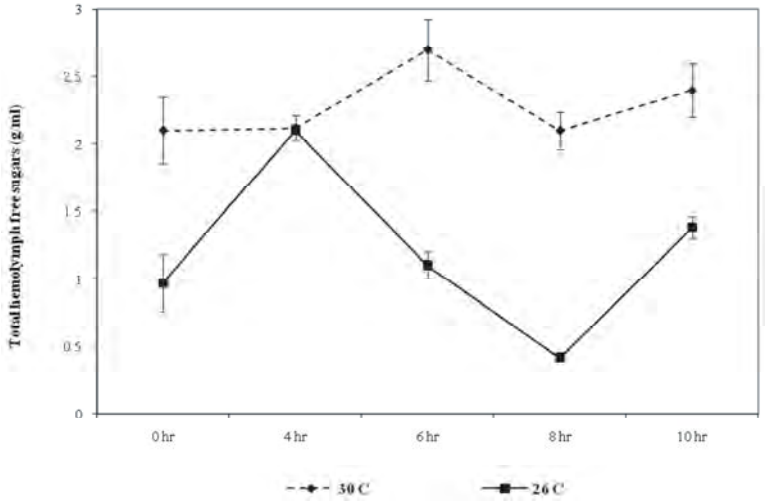

Fig. 4: Time-course changes in total free sugars on transfer from experimental (26°C and 30°C) to control (28°C)

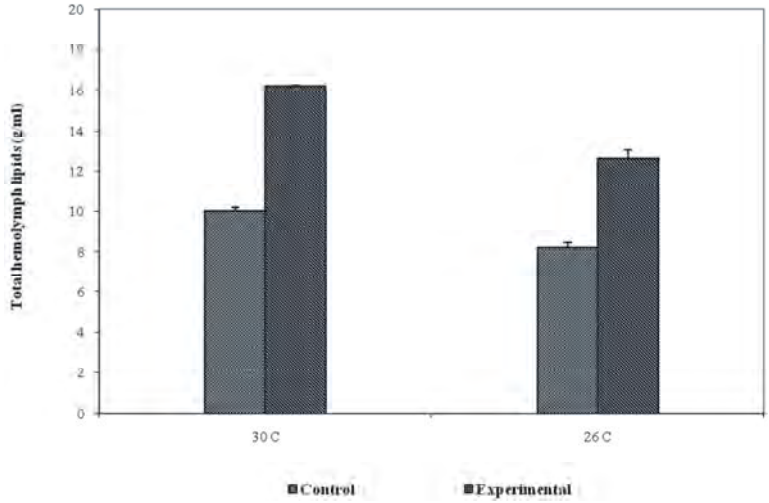

Fig. 5: Variations in the level of total lipids of *P. pelagicus* on exposure to varying temperatures

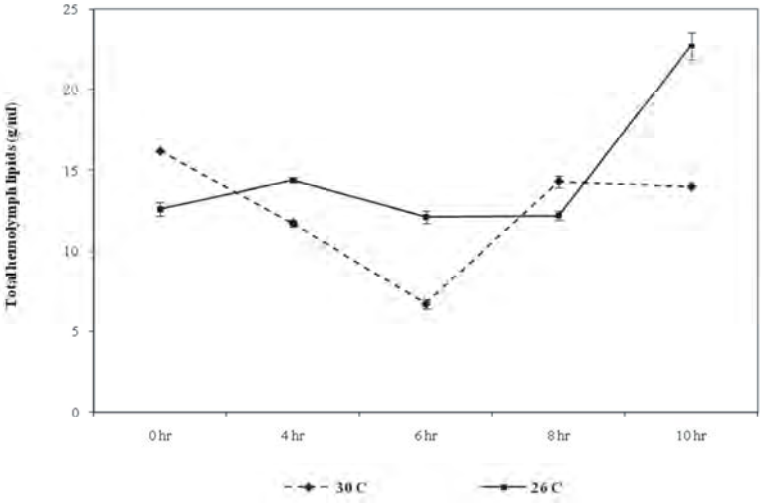

Fig. 6: Time-course changes in total lipids on transfer from experimental (26°C and 30°C) to control (28°C)

2.8±0.17 g/ml after 10 hrs (Fig.2). Significant variations are witnessed in the level of total protein during the hours of recovery and varying water temperature studied ($F<0.05$).

Total Free Sugars: Increase in the level of total free sugars was observed at 30°C (2.1±0.5 g/ml) with a decrease at 26°C (0.97±0.21 g/ml) when compared to the control ($t<0.1$, $t<0.05$) (Fig.3). After treatment at 30°C, the total free sugar level steady increased from 2.12±0.09 g/ml to 2.4±0.42 g/ml after 10 hrs of incubation. Similar trend was observed with the experimental group II crabs which were exposed to 26°C water temperature. After 4 hrs of recovery, the total free sugar level increased to 2.1±0.03 g/ml which gradually dropped to 0.41±0.08 g/ml after 8 hrs. Significant increase to 1.38±0.08 g/ml was observed after 10 hrs of recovery (Fig.4). Significant variations are witnessed in the level of total free sugars during the hours of recovery ($F<0.05$) while insignificant variations are observed with respect to water temperature studied ($F>0.05$).

Total Lipids: A similar trend was observed in the both the experimental groups. The total lipid level significantly increased to 16.2±0.03 g/ml on 30°C temperature treatment while it increased to 12.6±0.44 g/ml on 26°C temperature treatment, when compared to the control ($t<0.1$, $t<0.05$) (Fig. 5). The crabs of the experimental group I (30°C), experienced a decrease in total lipid level till 6 hr of recovery period (6.7±0.3 g/ml) and an increase thereafter to 14.3±0.39 g/ml after 8 hrs. The experimental group II crabs (26°C), on a recovery period of 4 hr had a higher concentration of 14.4±0.17 g/ml, which declined upto 8 hrs and then increased steadily to 22.7±0.89 g/ml after 10 hrs (Fig. 6). Significant variations are witnessed in the level of total lipids during the hours of recovery and at varying temperatures ($F<0.05$).

DISCUSSION

Many intertidal animals live under conditions where temperatures regularly approach the upper lethal limit and, in such cases, it is likely that mechanisms will be required both to repair thermal damage and to protect the organism from further thermal damage as the temperature increases. Thus the temperature effects on an animal may be reflected in its physiology. Past investigations of temperature effects on populations have concentrated upon comparison of rate functions such as oxygen consumption, ciliary activity, heart beat and thermal limits of tissues and / or whole organisms [6]. It is expected that the ability to exist at an environmental temperature is expressed in the physiological and biochemical responses of the animal. The nature of these responses to temperature may vary with species or stage of the life cycle. Thus, not only is there a variation in the rate function of metabolic change with temperature adaptation, but the nature of the metabolic change with temperature adaptation, but the nature of the metabolic reaction or pathway may be altered [25].

Heat shock (HS) involves the sudden exposure of cells, tissues and organisms to a temperature well above normal, but below lethal. The response usually induces the synthesis of one or more heat shock proteins and commonly results in induced thermotolerance (ITT); that is, survival under temperature conditions that otherwise would be lethal [26,27,28]. The hsp70 proteins of the brine shrimp *Artemia* showed a variable pattern of stage-specific and heat activation [29]. Frenkel *et al.* [30] have observed an increase in brain Hsp of 70 kDa (Hsp70) expression after a heat-shock treatment. Endogenous Hsp70 levels were higher than control levels when the barnacle, *Balanus glandula* was exposed to 34°C for 8.5 h [31]. Relative to controls animals held at a constant temperature, there was a slight elevation of Hsp70 only among heat shocked trilobite larvae in the 6 h recovery treatment [32]. Zhang *et al.* [33] have identified two hsp90 cDNAs post heat shock in *Portunus trituberculatus.* Amalia *et al.* [34] have identified 8 heat shock proteins in the copepod, *Calanus finmarchicus* in response to freezing, dessication and diapause.

Rahman *et al.* [35] demonstrated induced thermotolerance in larvae of the prawn, *Macrobrachium rosenbergii* at 37°C for 30 min lowered the rate of metamorphosis. The upper thermal tolerance and expression of heat shock protein 70 (Hsp70) were examined in the gill, heart tissues, hepatopancreas and skeletal muscles of the juveniles of the freshwater prawn *Macrobrachium malcolmsonii* that had been acclimated at two different temperatures (20°C and 30°C) [36]. Expression of Hsp70 mRNA has been reported to peak at 1 h of heat stress and decrease at 5.5 h [37].

Interspecific differences in the heat-shock response, especially among congeneric species, often correlate positively with thermal extremes in the environment [38-45]. Some of these studies have shown how Hsp levels vary over hours in response to thermal stress under natural [46-48] as well as laboratory conditions [42]. Several variations in chemical constitution of tissues have been associated with differences in environmental temperatures. Diwan and Nagabhushanam [49] have studied the effect of temperature and salinity on the heat

tolerance of the freshwater crab, *Barytelphusa cunicularis* in terms of total water content, total protein, fat, glycogen and blood glucose. The biochemical composition of the embryos of *Callinectes sapidus* at 12-16°C and greater than 24°C was studied [50]. Embryos spawned at 16°C had initially greater lipid levels, an equivalent amount of protein and a lower carbohydrate and caloric content than embryos cultured at 26°C. In the present study, the crabs (*P. pelagicus)* were exposed to varying water temperatures of 30°C and 26°C for 2 hrs, which resulted in changes in the biochemical constituents. The total protein tends to increase with increase in temperature (30°C) and decrease with decrease in temperature (26°C) when compared to the control (28°C). Recovery periods showed a prominent increase in the total protein levels.

According to Zhou *et al.* [15] the concentrations of 14 FAA in serum decreased directly with decreasing temperature, with a magnitude of between 12.15% and 400.89% in the white shrimp *Litopenaeus vannamei.* Concentrations of 5 FAA in hepatopancreas increased directly with decreasing temperature, while that of 8 FAA, remained unchanged or declined at the last stage of the cold stress when water temperature decreased from 18°C to 13°C. Exceptionally, concentration of taurine in hepatopancreas continually decreased during the entire procedure. The lowest and the highest temperature significantly decreased the total hemocyte count (THC), whereas haemocyte volume and haemolymph glucose concentration did not differ significantly in a series of experiments with *Carcinus aestuarii* [18].

Dean and Vernberg [51] studied the effects of temperature acclimation on carbohydrate metabolism in decapod Crustacea. Serum glucose content gradually increased with decreasing temperature, while hepatopancreas glycogen content just sharply declined at the early stage of the cold stress (28 to 23°C) but slightly changed then (23 to 13°C) in the white shrimp *Litopenaeus vannamei.* Though there was significant increase in the total free sugars after 30°C treatment in the present study, its level during the recovery period was stable and did not return to normal even after 10 hrs of recovery.

Stoner *et al.* [19] have undertaken experiments to explore how temperature mediates growth and energy allocation in newly metamorphosed juveniles of the red king crab, Paralithodes camtschaticus. Lipid class analysis revealed a trend towards higher proportions of storage lipids in larger crabs cultured at 12°C than in crabs cultured at low temperatures. High proportions of essential fatty acids in all crab groups coupled with elevated levels of triacylglycerols in 12°C animals, indicate that rapid growth does not negatively affect condition in juvenile crabs.

One reason for carrying out this research was the idea that the use of the stress response and induced thermotolerance might be a way to enhance the performance of these animals during transport and upon release under field conditions. In fact, this rationale can be applied to many species of aquacultural importance [52]. The first step toward testing this idea requires a careful characterization of the stress response in the laboratory, using the species in question and that has been accomplished here for *P. pelagicus.*

Local fishermen of Palk Bay are solely dependent on the fishery of the blue swimmer crab, Portunus pelagicus while other edible crustaceans like shrimps, fishes are caught only in comparatively lesser amounts, thus crab fishery being the major source of their food and living. The present proposed study would be a preliminary step towards the identification of the ability of the crab to adapt itself to varying temperature regimes in laboratory conditions. This would provide an idea on the effect of intertidal temperature on the physiology of the crab, thereby enumerating the causes of their mortality. Further studies on tracing the genes of heat shock response in the crab and altering them to suit the changing environment, would be a boon for the fishermen on socio-economic grounds relying on crab fishery.

In the present study, a temperature of 28±2°C for the stress exposure experiments was chosen as it marks an extreme situation that occasionally arises in the native environments of the test species, *P. pelagicus* and thus can be expected to produce significant physiological results and ensure the survival of the test individuals. Results obtained demonstrated that the highest and lowest temperature tested influenced crab biological responses and indicated that *P. pelagicus* modulated its cellular and biochemical parameters in order to cope with temperature.

Hence, by the present study, variation of total proteins and lipids in hemolymph reflects a balance of biochemical degradation and synthesis under the combined stress of cold and heat stress and also gives information that proteins and lipids may play important roles such as functional substrates or energy utilization for the animal to sustain prolonged and intensified heat or cold stress. So, dietary protein and lipid quality and quantity during stress should be paid more attention during feed formulation.

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ORIGINAL ARTICLE

Molt cycle related changes and effect of short term starvation on the biochemical constituents of the blue swimmer crab *Portunus pelagicus*

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Abstract Synthesis and hardening of a new exoskeleton are essential to the arthropod molting process. The present study emphasizes the variations in the levels of hemolymph total free sugars, hepatopancreas glycogen and cuticular proteins during the molting stages of *Portunus pelagicus*. It also reports the effect of short-term starvation conditions on the biochemical constituents of the hemolymph. Intermolt crabs were subjected to 6 days of starvation and hemolymph samples were taken. Standard biochemical procedures were followed toward the quantification of total proteins, total free sugars and total lipids. The total free sugar level in the hemolymph of *P. pelagicus* was observed to increase during early premolt D_0 (3.108 ± 0.032 g/ml) and a gradual decrease till late postmolt B stage (0.552 ± 0.124 g/ml), suggesting the need for total free sugars to provide energy for the apolysis process. Increase in the levels of hepatopancreas glycogen was observed from 1225 ± 0.04 μg/mg in early premolt D_0 to 1700 ± 0.3 μg/mg in late premolt D_{2-3}. This is in correlation with the decreased levels of free sugars during premolt stages, suggesting an increase in the storage of glycogen reserves in the hepatopancreas. Cuticular proteins increased during stage B (2.702 ± 0.093 g/ml) and stage C (3.065 ± 0.012 g/ml), indicating exoskeleton hardening and mineralization. Results of the starvation studies clearly showed a steady decline in the level of total free sugars till day 6 (0.099 ± 0.00 g/ml) when compared to the control (8.646 ± 0.08 g/ml). Gradual decrease of total lipids was also observed from the first day of the experiment (6.088 ± 2.44 g/ml) to the last day of the study (0.401 ± 0.20 g/ml) which was 85% lesser than the control (8.450 ± 0.49 g/ml)suggesting the efficient usage of total sugars to consolidate the loss of energy reserves during starvation. The knowledge of Molt-cycle events can be used as a tool for the evaluation of the developmental state providing a morphological reference system for physiological and biochemical studies related to crab aquaculture. Starvation studies enlightens that increasing carbohydrate levels in crab feed together with good protein content

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could alleviate the natural effects of starvation, improve farm productivity and reduce the deleterious impact of nitrogen pollution generated by rich-protein feeds used in crab farming.

1. Introduction

Comparative biochemistry in crustaceans has developed in recent years due to the interest in aquaculture, but has taken many initial hypotheses from the insect biochemistry field. Insects are and will remain as a very useful model for biochemical research and precisely due to its evolutionary closeness, crustacean biochemistry can take advantage of the available insect knowledge (Law and Wells, 1989). The biochemical changes occurring in crustaceans during molting, feeding and starvation are indicators of their nutritional requirements and are an important basis for determining suitable diets.

Crustaceans differ from insects in that they combine to molt and grow even after the attainment of sexual maturity. In large decapod crustaceans such as crabs and lobsters, the female reproductive cycle is completed within the protracted intermolt period and molting is initiated only after reproductive arrest (quiescent period). Conversely, in many soft shelled shrimps and prawns, molting is permitted to occur during the course of oogenic cycle, thus revealing a close synchronization between molting and the reproductive process (Subramoniam, 2000). The pattern of changes, an increase in blood glucose and protein levels during premolt, a decrease following the molt and more or less rapid return to the intermolt level, have been reported (Telford, 1974). Renaud (1949) described the cycling of carbohydrates and lipids through the molt cycle of *Cancer pagurus* and an apparent increase in blood reducing substances during premolt. In a study of spiny lobster, *Panulirus argus*, Travis (1955, 1957) has shown a detailed pattern of the use of glycogen during the molt cycle, from which the elevated level of blood glucose in premolt and postmolt decreases could be predicted. Telford (1968) has cited several sugars including mannose, galactose, fructose, maltose and trehalose in marine decapods. Presumably, they play a less prominent role in the metabolism of freshwater crayfishes. McWhinnie and Saller (1960) found glucose to makeup 25% of the total reducing substances in the blood of *Orconectes virilis*, which is in close agreement with the observation of Telford (1974). There have also been studies which sought to determine relatedness among crustacean and insect exoskeletal proteins. Dennell (1947) showed similarities between the crustacean exoskeleton and the insect exoskeleton with respect to composition, deposition and hardening. Many insect and crustacean exoskeletal proteins have also been shown to share characteristics such as acidic isoelectric points and molecular weights of 31,000 daltons or smaller (O'Brien et al., 1991). More recently, immunological crossreactivity between crustacean exoskeletal proteins and insect storage hexamerins has been demonstrated (Stringfellow and Skinner, 1988; Kumari and Skinner, 1993).

Soluble glycoproteins from the organic matrix of the cuticle of brachyurans have been studied at all stages of the molt cycle. Electrophoretic patterns of extracts of lectin-binding proteins from the calcified exoskeleton layers of the Bermuda land crab *Gecarcinus lateralis* change dramatically during and after apolysis (Kumari and Skinner, 1995). These pre-molt changes in the existing cuticle relate to mineral dissolution and resorption rather than mineral deposition. Various mild aqueous solvents were used to extract glycoproteins from the anecdysial (intermolt) cuticle of the Atlantic shore crab *Carcinus maenas*, and the complex array of electrophoretic bands described from these extracts contained both *O*-linked and *N*-linked glycans (Compere et al., 2002).

Studies on the starvation of crustaceans in biochemical composition are intended to yield information that remains useful in understanding the ecophysiology of a population (Lehtonen, 1996). Artificially induced fasting and starvation may enlighten the metabolic routes used in hierarchical order and may describe novel biochemical and physiological adaptation mechanisms (Barclay et al., 1983). The ability of an organism to survive and recover from long periods of starvation is vital. Starvation can lead to a severe deficiency of nutrients. Therefore, starvation studies may be useful predictors to determine energetic and metabolic requirements (Guderley et al., 2003). Furthermore, the knowledge derived from the understanding of their biochemical processes may be the basis to optimize crustacean pond rearing efforts.

Proteins are critical for artificially reared crustaceans and are an expensive component of feeds for decapod crustaceans (Kureshy and Davis, 2000). Therefore, the dietary protein quantity and composition should be optimized to grant maximal growth (Shiau, 1998). Feed protein contents between 30% and 57% (w/w) are recommended for suitable growth of different species of the penaeid shrimp (Cordova-Murueta and Garcia-Carreno, 2002; Kureshy and Davis, 2000; Shiau, 1998). During a 28-day starvation study, in the hepatopancreas of the shrimp *Penaeus japonicus,* the glycogen stores were rapidly depleted, presumably being converted to glucose and are used as an energy source (Cuzon et al., 1980). Tail muscle lipids diminished progressively and proteins were next mobilized, but more slowly, eventually accompanied by muscular atrophy. Similar results were obtained for the shrimp *Penaeus duorarum* (Schafer, 1968) and for the *Crangon crangon* (Cuzon and Ceccaldi, 1973). However, the purple shore crab *Hemigrapsus nudus* during a 23-day starvation period used preferentially proteins (Neiland and Scheer, 1953) as reported for other decapods more recently (Anger, 2001). Moreover, crustacean responses to starvation appear to be influenced by the developmental stage. Spiny lobster *Jasus edwardsii,* phyllosoma larvae during a 6–11-day starvation catabolized more lipids than carbohydrates and proteins in stages II, IV and VI. These larvae were 14–40% lighter than their fed counterparts (Ritar et al., 2003). The main lipid storage organ in crustaceans is hepatopancreas. Lipids are mobilized to and from this organ through lipoproteins that bind and carry these hydrophobic molecules in the aqueous hemolymph environment. High density lipoproteins (HDL) and very high density lipoproteins (VHDL) are the main lipoproteins found in crustacean species (Lee and Puppione, 1978; Yepiz-Plascencia et al., 2000, 2002).

Reports about the metabolic requirements of protein and lipids under starvation in crustaceans are very contrasting. As mentioned before, several authors report protein as the

main source of energy for starved crustaceans. During the starvation of crustaceans, there are three distinct phases of biomass degradation (Anger, 2001). Initially, energy-rich lipid reserves are preferentially mobilized, reflected in decreasing lipid:protein ratios, which is typical of short term food deprivation. When much of the accessible lipid pool has been depleted, proteins are increasingly utilized. A significant part of the lipid pool is bound in crucial cell structures such as membranes and hence is normally unavailable for energy metabolism. In the final phase of starvation prior to death, structural lipids may also be degraded so that the lipid:protein ratio decreases again (Mikami et al., 1995; Abrunhosa and Kittaka, 1997).

P. pelagicus (Linnaeus, 1758), the blue swimmer crab found in the intertidal estuaries of the Indian and Pacific Oceans, forms the important source of commercial fishery in the Thondi Coast. They are exported to South East Asian countries under live conditions. Because of their delicacy and larger size, the live mud crabs are always in greater demand and fetch a higher price in both national and international markets (Kathirvel, 1993). Swimming crabs, both *P. pelagicus* and *P. sanguinolentus* are being exported mostly in frozen and canned forms. The males are bright blue in color with white spots and long chelipeds. And the females are duller green or brown in color. Male and female *P. pelagicus* generally reach sexual maturity at a size of 70–90 mm in carapace width, when they are approximately one year old.

Besides seasonal changes in food availability, a common denominator in crustaceans and *P. pelagicus* in particular, is their constant feeding activity. Furthermore, they alternate episodes of feeding and fasting during development, which occurs through Molting and results in growing by sequential steps. Increase in body size at each ecdysis is non-linear; this is a hormonally controlled process which might last days or weeks, is continuous and accompanied by morphological, physiological and behavioral alterations occurring almost daily (Dall et al., 1990). This process requires a high amount of energy. Molting involves a series of stages with different feeding behavior. During intermolt, they feed actively, prior to molting, feeding declines until it stops completely during ecdysis. Finally feeding begins again in postmolt (Phlippen et al., 2000). Starvation can lead to a severe deficiency of nutrients. Starvation induction of crustaceans in the intermolt stage has been suggested to be a good model to try to understand the molecular and enzymatic changes that occur naturally during their growth process, although the effect of hormones must not be forgotten (Sanchez-Paz et al., 2003). Therefore, starvation studies may be useful predictors to determine energetic and metabolic requirements (Guderley et al., 2003).

The effects of starvation on the blue swimmer crab, *P. pelagicus* have not been examined in terms of accumulation and loss of the major body components viz., protein, lipid and carbohydrate. The findings of starvation studies can be used to determine the nutrients most critical as energy reserves and those catabolized or conserved in the face of increasing food deprivation. In view of the afore mentioned information, the present study was aimed primarily toward the examination of carbohydrate metabolism and cuticular proteins in *P. pelagicus* with reference to the molting cycle and secondly to study the effect of short-term starvation on biochemical constituents in adult males of *P. pelagicus* in the intermolt stage to gain insights on the connection between episodes of food shortage, metabolic preferences and sequence of the use of energy reserves.

2. Materials and methods

2.1. Collection & maintenance

Adult blue swimmer crabs, *Portunus pelagicus*, were caught from the Thondi Coast, Thondi (9°45'N 79°04'E). The crabs were transported to the laboratory in aerated plastic troughs. They were weighed and acclimatized for a week in tanks containing 10–15 cm of sand at the bottom at about 34 ± 2 ppt salinity and at room temperature (30 ± 2 °C). During the period, the crabs were fed with oyster (*Crassostrea madrasensus*) 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. Analysis of Molt stages

Setal development of *P. pelagicus* was observed on the basis of the epidermal retraction observed at the posterior median part of the swimmeret. Molt stages were determined using morphological changes of the seta as described by Drach and Tchernigovtzeff (1967) using a light microscope (Optika B-350, Italy).

2.3. Extraction of cuticular proteins

Cuticular proteins were extracted from the exoskeleton following the method of Otoshi (1994). Briefly, the dorsal carapace portion of the exoskeleton of *P. pelagicus* was always used so that contaminants such as hair bristles could be excluded. The exoskeleton was brushed clean and rinsed of any visible cellular material with deionized water. It was then dried at approximately 60 °C until constant weight was achieved. The dried exoskeleton was then ground with mortar and pestle at room temperature until it was a fine powder. The proteins from the ground exoskeleton were then extracted with a 1% KCl solution, pH 7.5, using 12 ml solution per gram of ground exoskeleton. The extraction mixture was incubated overnight at 4 °C. The mixture was then centrifuged, for 5 min at 13,000 rpm (Remi C-24 BL Cooling Centrifuge, India). The supernatant was stored at 4 °C until further protein analysis.

2.4. Starvation experiments

Only intermolt crabs were chosen for starvation experiments. After acclimation, the crabs were measured (carapace length, carapace width) and weighed (wet weight). Subgroups of 6 crabs each, were maintained for 0 (control group), 1, 2, 3, 4, 5 and 6 starvation days. The samples for biochemical analysis were obtained on every starvation day from the member of the subgroup.

2.5. Hemolymph sampling

Hemolymph samples were drawn from the crabs of various molting stages as well as from control and starved crabs through the arthrodial membrane of the pereiopods by using disposable syringes. Approximately 10 ml was obtained from each crab. The collected hemolymph was stored in separate vials under −20°C until further use.

2.6. Quantification of biochemical constituents

The biochemical composition of cuticular proteins, total free sugars in the hemolymph and hepatopancreas glycogen during the molt cycle and the level of total proteins, total free sugars and total lipids in the hemolymph of control and starved crabs was estimated following standard procedures. Estimation of total proteins and cuticular proteins was done as per the methodology of Bradford (1976). Estimation of total free sugars was done according to the methodology of Roe (1955). Estimation of glycogen in the hepatopancreas was done according to the methodology of Carroll et al. (1956). Estimation of total lipids was done according to the methodology of Barnes and Blockstock (1973). Extraction of lipids from sample was done following the procedure of Folch et al. (1957).

2.7. Data analysis

The results of the study were subjected to Two-way ANOVA to test whether the variations in the biochemical constituents among the various stages of molting and between the starvation periods are significant.

3. Results

3.1. Variations in the biochemical composition during the different molting stages

Results of the biochemical analysis when subjected to Two-way ANOVA clearly enumerated variations among the biochemical parameters during the different stages of molting ($F < 0.05$).

3.1.1. Total free sugars in hemolymph

Variations were observed in the levels of total free sugars in the hemolymph of *P. pelagicus* during the different stages of molting. Greater level of 3.108 ± 0.03 g/ml was observed during premolt D_0 stage, following which a steady decline was noticed thereafter till postmolt B stage (0.552 ± 0.12 g/ml). Intermolt C stage had a significant increase in the free sugar titer of 1.318 ± 0.15 g/ml) (Fig. 1).

3.1.2. Hepatopancreas glycogen

Depletion of glycogen in the hepatopancreas was observed after the late premolt stages D_{2-3} (1700 ± 0.30 μg/mg). Postmolt B stage had a minimal level of 425 ± 0.02 μg/mg followed by postmolt A (837 ± 0.23 μg/mg). An increase in the glycogen level was observed thereafter in intermolt C (1012 ± 0.43 μg/mg) till late premolt stage (Fig. 2).

3.1.3. Cuticular proteins

A steady decline in the level of cuticular proteins was observed right from the early premolt stage D_0 (1.469 ± 0.32 mg/ml) in correlation with the onset of setogenesis. The protein level dropped to 0.688 ± 0.476 mg/ml during late premolt stage D_{2-3} and a significant increase in glycogen was noticed in postmolt A (0.854 ± 0.08 mg/ml) and postmolt B (2.702 ± 0.09 mg/ml). Cuticular proteins were found to be highly concentrated in the exoskeleton in the intermolt stage C (3.065 ± 0.02 mg/ml) (Fig. 3).

3.2. Variation in the survival, weight and body measurements of *P. pelagicus* on starvation

During the experiment no mortalities were recorded. Relatively significant reduction in body weight on day 6 (53.04 ± 2.34 g) when compared to the control (77.05 ± 3.03 g). Insignificant variations were observed in carapace length and carapace width of the starved crabs when compared to the control (Fig. 4).

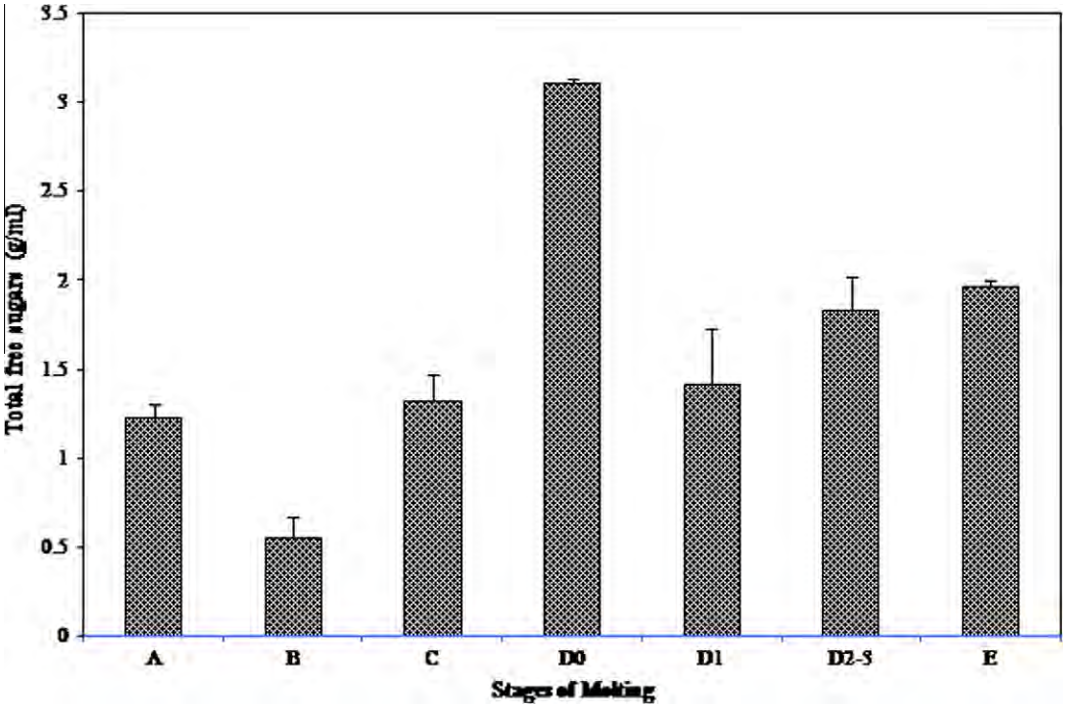

Figure 1 Variations in the total free sugars in the hemolymph during molting.

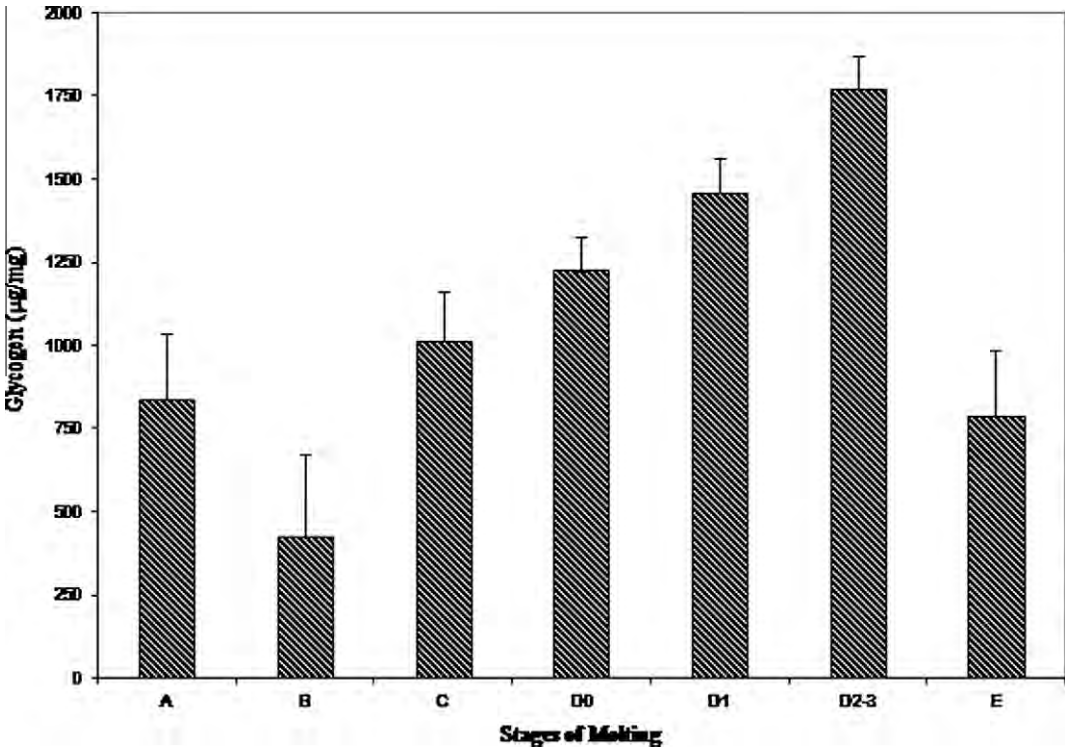

Figure 2 Variations in the hepatopancreas glycogen during molting.

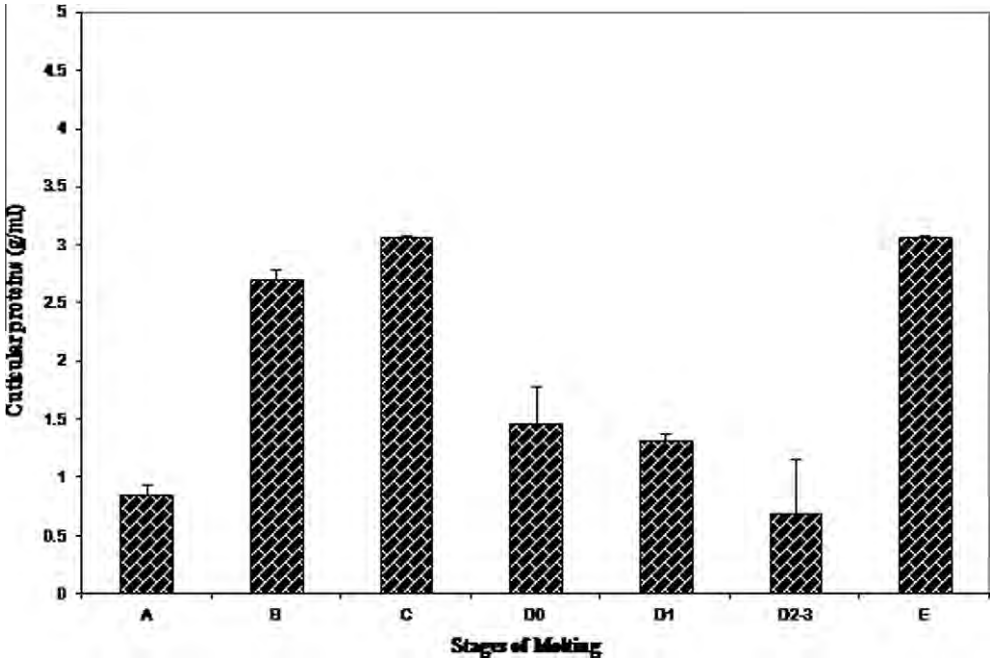

Figure 3 Variations in the cuticular proteins during molting.

3.3. Biochemical composition of hemolymph on starvation

Along the starving periods, significant differences were observed in the relative biochemical composition of the hemolymph of the experimental crabs. A drastic decrease was observed in the levels of the constituents from day 2 of the starvation period except the total protein level. Two way–Analysis of Variance showed significant variations within the days of starvation and between all the biochemical constituents studied ($F < 0.01$).

3.3.1. Total proteins

Significant decrease in the level of total protein was observed on day 1 (2.670 ± 1.11 g/ml) when compared to the control

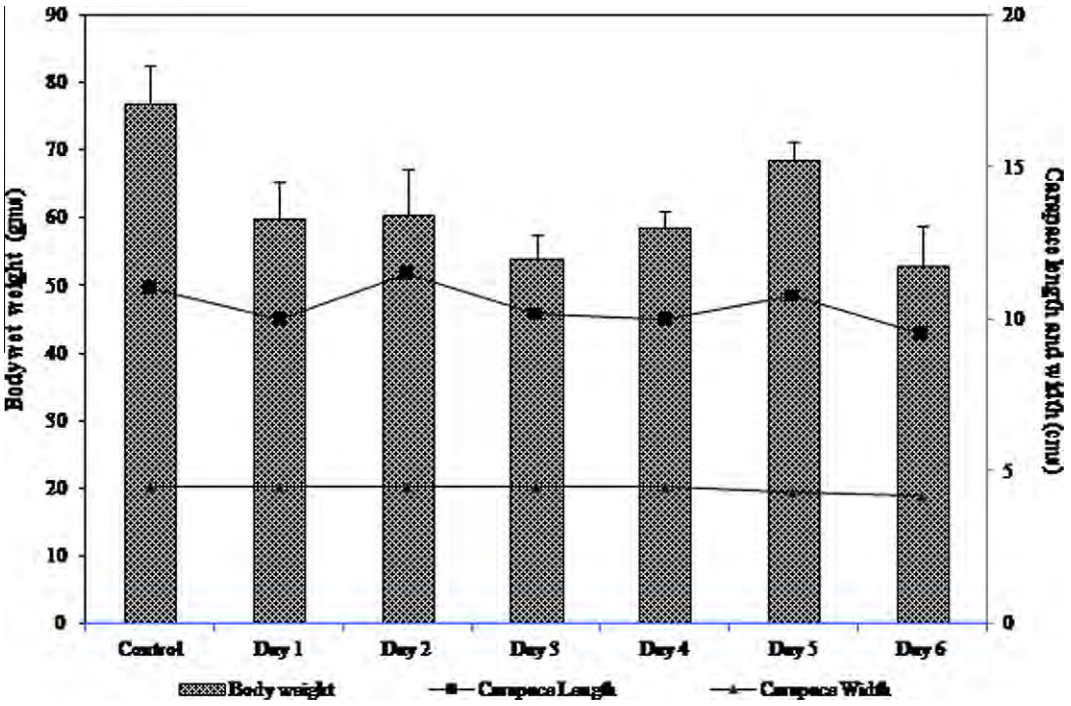

Figure 4 Weight and body measurements of *P. pelagicus* during starvation.

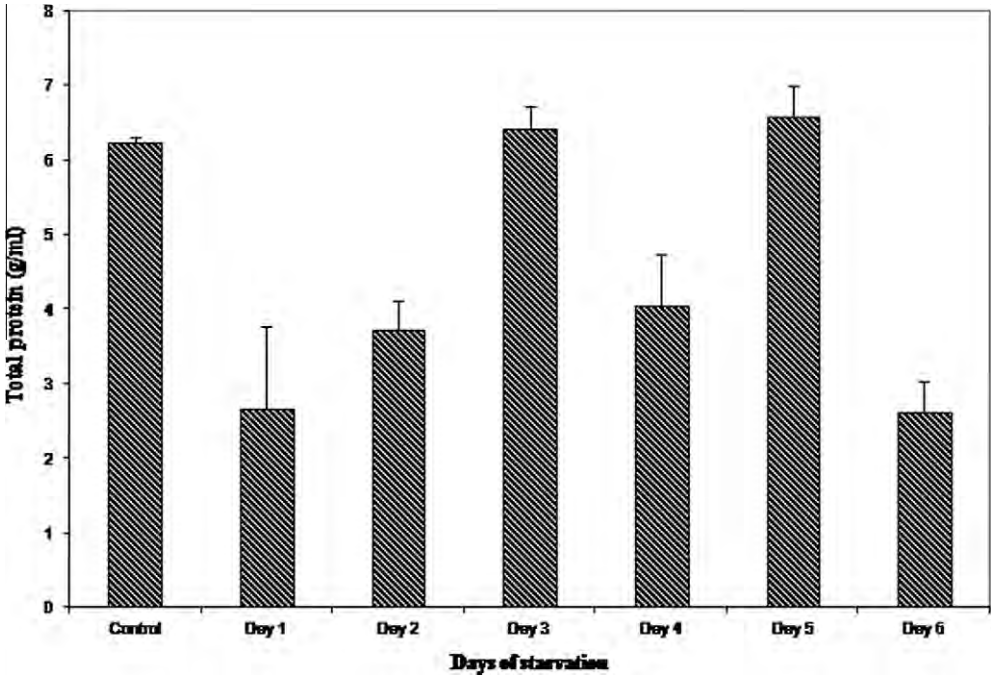

Figure 5 Variations in the hemolymph total protein on starvation.

(6.259 ± 0.01 g/ml). Thereafter, protein concentration peaked more than the control on day 5 (6.597 ± 2.35 g/ml), about 0.5% increase. An abrupt decline in the level of total proteins was observed (2.636 ± 1.52 g/ml) on day 6 which was approximately 30% of the control group (Fig. 5).

3.3.2. Total free sugars

A gradual but a sharp decline was observed in the level of free sugars on starvation from control to day 6. The level of free sugars decreased to almost 50% that of the control group (8.646 ± 0.08 g/ml) on the very first day of starvation

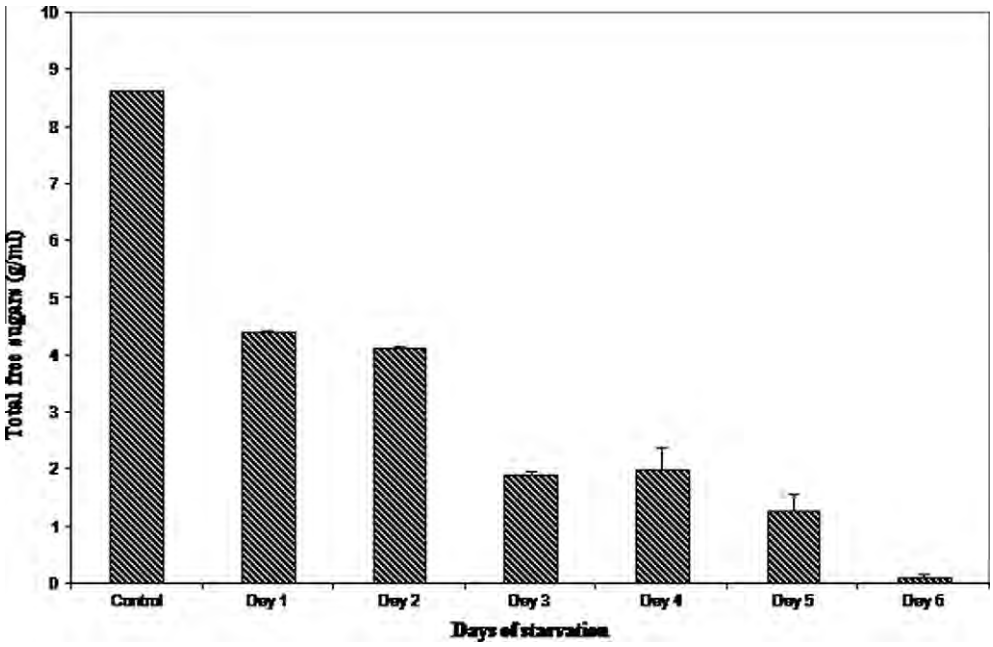

Figure 6 Variations in the hemolymph total free sugars on starvation.

(4.409 ± 0.00 g/ml). Reduction in the level of free sugars from 4.123 ± 0.02 g/ml on day 2–0.099 ± 0.00 g/ml on day 6 was recorded (Fig. 6).

3.3.3. Total lipids

The total lipid concentration of the hemolymph of the experimental crabs steadily decreased from day 1 till the termination of the experiment on day 2. Experimental crabs of day 3 showed a significant decrease in the level of total lipids (1.794 ± 0.088 g/ml) which was approximately 75% less than the control group (8.450 ± 0.49 g/ml). The final concentration on day 6 of starvation was 0.401 ± 0.20 g/ml, a 95% reduction in the total lipid level when compared to the control (Fig. 7).

4. Discussion

Arthropod growth patterns characterized by molt cycles present some trade-offs such as the need for variable muscle atrophy and restoration to accommodate the body inside the new slightly bigger exoskeleton. Various environmental conditions such as temperature, light (Bermudes and Ritar, 2008; Bermudes et al., 2008), salinity (Romano and Zeng, 2006), or

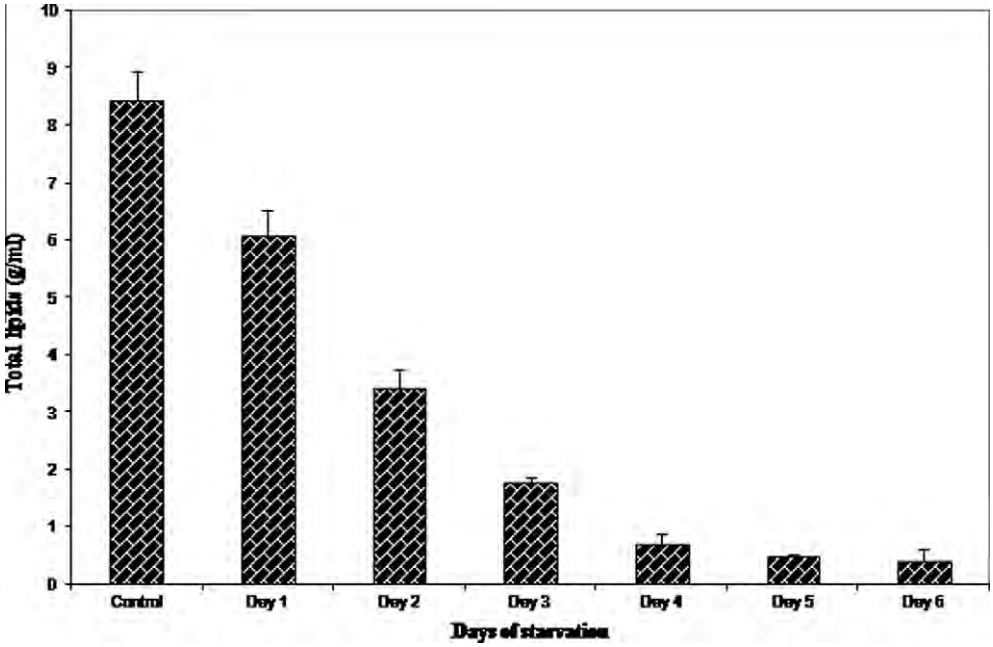

Figure 7 Variations in the hemolymph total lipids on starvation.

feeding treatments (Minagawa and Murano, 1993) can modify the molting cycle, which implies changes in numerous physiological, biochemical, and behavioral parameters (Chang, 1995; Anger, 2001).

Alvarez-Fernadez et al. (2005) have studied the role of lipid, protein, carbohydrate and nucleic acid in the molt cycle of Norway lobster, *Nephrops norvegicus*. In their study, the lipid content in hepatopancreas has been found to increase along the premolt period (stages D_0 and D_3) to cover the increase in energetic requirements in later stages. This increase in requirements results from starvation from stage D_3 until the end of the postmolt period, altogether with the formation of the new exoskeleton (Mayrand et al., 2000).

In the present study, the level of total free sugars in the hemolymph was assayed during the molt stages, revealing increase in its level during the premolt stages. A gradual decline was observed thereafter on the onset of postmolt A and B, and a sharp increase in intermolt C was seen and a higher level during early premolt D_0. The results of the present study resemble those of Telford (1968), who has studied the changes in blood sugar composition during the molt cycle of the lobster *Homarus americanus*. Glucose levels were 35 percent higher in premolt than in intermolt and were 30 percent lower in postmolt. Besides its role in digestion, the digestive gland or hepatopancreas actively participates in the molt cycle, being the major site for storage glycogen, fats, and calcium during premolt and thus, in the mobilization of these reserves when needed in subsequent molt stages. When under stress (e.g., molting), the metabolic activities of the crabs increase rapidly, causing the hepatopancreas to release higher levels of metabolites into the hemolymph. Depletion of hepatopancreas glycogen in *P. pelagicus* was observed in the present study during postmolt A and B which arose to greater levels during intermolt C and early premolt D_0. This runs in line with the observed levels of total free sugars in the hemolymph observed in the present study, thus envisaging the mobilization of glycogen reserves from the hepatopancreas during the molt cycle to provide energy in the form of sugars.

Cuticle proteins are suggested to be involved in the calcification process (Andersen, 1999; Kragh et al., 1997) and in chitin binding. In the present study, the concentration of cuticular proteins was studied during the molting stages of *P. pelagicus* revealing its increase from postmolt B to intermolt C stage. This provides a positive correlation between the thickening of the epidermis and cuticle during postmolt B and intermolt C and the increase in the cuticular protein level. Hemolymph proteins have been found to increase during intermolt C because the internal tissue growth takes place during this stage (Passano, 1960a,b), and is considerably low in postmolt, premolt and molt. Because the growth starts to occur during the stages A–B (postmolt), and in the premolt stage D the rate of growth decreases and the rate of feeding also decreases (Freeman and Perry, 1985).

Starvation studies give indications of the energy resources utilized by crustaceans and provide clues to the biochemical pathways. The 100% survival of *P. pelagicus* in the present study showed the capacity of tolerance at starving conditions for 6 days which lies in coincidence with the observations of Comoglio et al. (2004, 2005, 2008) for *Litopenaeus vannamei* and the southern king crab *Lithodes santolla,* exposed to starvation for 12 days. *P. pelagicus* in the present study has suffered considerable weight loss during the period of starvation. Steffens (1989) has suggested that starvation affects metabolic activities and during this period essential processes are maintained at the expense of accumulated endogenous energy reserves, which sometimes result in the loss of weight. However, some authors have detected that some crustaceans such as shrimps and lobsters compensate the weight of organic matter that they use in starving conditions with water uptake so that no loss of weight is detected (Dall, 1974; Wilcox and Jeffries, 1976).

P. pelagicus presents a significant variation in its biochemical composition under starvation. In the present study, total protein in the hemolymph remained constant during the experiment with only a slight decrease in its content on day 1 and day 6 of the fasting period. These results disagree with many reports that maintain that protein is the main energy source for most crustaceans but may explain previous findings. Mayzaud and Conover (1988) have also reported an increase of ammonia excretion and low values of O:N during the starvation period. Muhlia-Almazan and Garcia-Carreno (2002) showed that in *L. vannamei,* hepatopancreatic trypsin activity was significantly affected by food shortage (differences of 35% between 2 and 120 h of starvation), while chymotrypsin activity decreased 40% at the same starvation level. Protein changes may occur in which the crustacean switches to the use of one energy reserve to another, depending on the developmental stage. In the copepod *Calanus finmarchicus*, the use of energy changed: during the first 10 days of starvation and the protein content showed a moderate decline, suggesting that this organism copes with starvation utilizing endogenous reserves different than protein; however, during the next 21 days, total protein content was drastically reduced (Helland et al., 2003). In subterranean aquatic crustaceans changes have also been found. After 28 days of starvation, the isopod *Asellus aquaticus* responded with an immediate, linear and large decrease of all the energy reserves, most of which were fully recovered after a 7-day refeeding period. In contrast, prolonged fasting (180 days) in the isopod *Stenasellus virei* was characterized by three successive phases: (1) an immediate, but low, depletion of both glycogen and arginine phosphate, followed by (2) the utilization of triacylglycerides associated with glycogen resynthesis and finally (3) a slow depletion of both proteins (demonstrated by a slight increase in ammonia excretion rate) and lipids, always associated with a glycogen resynthesis. As in *A. aquaticus, S. virei* energy reserves were fully recovered after a 15-day refeeding period (Hervant and Renault, 2002). Strategies of fuel reserves usage may change depending on the species and the larval stage (Le Vay et al., 2001).

Carbohydrates and lipids showed significant variations in the present study indicating both rapid accumulation and depletion. Azeiteiro et al. (2003) have reported that the carbohydrates and lipids were the most affected during the refeeding period, when the accumulation of these constituents did not reach the starting levels in *Mesopodopsis slabberi*. From the entire set of metabolites, studied, total free sugars was the most drastically affected by starvation, dropping constantly from the beginning of the study and stabilizing to approximately 5% of its initial value after 6 days of food deprivation. The results of the present study indicate that glucose is the first source used by the crab for dealing with the lack of food. While total free sugars were rapidly consumed, protein concentration decreased slightly. Although it cannot be ruled out that proteins are used as an energy source, the rapid decrease of glucose indicates that it is the first fuel utilized. This response

may be an adaptative strategy to avoid usage of high cost energy macromolecules, at the beginning of a food shortage episode. Prudent utilization of protein in very short starvation periods could represent energy protection in the case of prolonged food scarcity intervals.

One of the most important roles of lipids in crustaceans is related to reproduction, since they are associated with the maturation of oocytes and the survival of the initial larval stages. Total lipids decreased sensibly (90%) reaching the lowest values after 6 days of starvation. Both, sterols and acylglycerides may be the main cause. It has been proposed that in crustaceans, neutral lipids are preferentially catabolized during starvation, while polar lipids (phospholipids and cholesterol) are conserved due to their role as structural components of cell membranes (Heath and Barnes, 1970; Bourdier and Amblard, 1989; Stuck et al., 1996). A large reduction in total lipids (particularly a total depletion of triacylglycerides stores) as a response to starvation for the lake dwelling copepod *Acanthodiaptomus denticorni* was reported (Bourdier and Amblard, 1989). Similar results were found for larvae, adult and subadult lobsters (Stuck et al., 1996). Ritar et al. (2003) reported that lipid dry weight in lobster larval stages II, IV and VI, declined during starvation to 81%, 41%, and 73%, respectively, compared to fed larvae. Additionally, polar lipids were the only lipid class significantly reduced during starvation (45%, 38%, and 70%) in stages II, IV and VI, respectively. The next most abundant lipid class in phyllosoma was sterol, and was the only lipid class conserved during starvation at all stages.

Studies about the metabolism of crustaceans and their ability to adapt to environmental variations contribute to the understanding and elucidation of perhaps new mechanisms. More research is necessary to understand the biochemical and physiological aspects of crustacean nutritional requirements, especially considering the high degree of flexibility in the digestive physiology of crustaceans, as an essential part of their ability to grow, survive, and reproduce when the food supply changes or depletes.

5. Conclusion

The results of the present study emphasizes the role of biochemical constituents in the molting process and thereby throwing light on the molting stages that could be accounted for commercial procurement. Furthermore, the quantification of cuticular proteins of *P. pelagicus* through the molt stages in the present study is an initial step toward discovering answers for the following questions in the near future. In *P. pelagicus*, feeding takes place throughout the year, except during a few weeks of the Molting–mating period, when feeding ceases or is at a minimum. In this context, the results of the present study give new and relevant biological information about the physiological and biochemical responses during starving conditions about an important commercial species inhabiting the Palk bay.

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