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Full Length Research Paper

Shell disease of *Neoepisesarma mederi* crabs and its associated secondary infections

Sharmila Joseph F.R*, S. Ravichandran and K. Kumaravel

Centre of Advanced Study in Marine Biology, Faculty of Marine Sciences, Annamalai University, Parangipettai – 608502, Tamil Nadu, India.

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Shells of crabs are infected with a high prevalence of diseases than any other crustacean. Bacteria, viruses, fungi and several other pathogens influence higher percentage of shell disease in crabs. Infections in crab affected growth and metabolism and it may also lead to death. This work deals with the microscopic examination of shell diseased crabs with reference to its variation in hemocytes count, histopathology and biochemical estimation. The total heterotrophic bacterial (THB) population in normal shell was found to be 5.15 x 10^4 ± 0.09 Colony forming unit (CFU) ml^{-1} and THB load was noted to be slightly increased than in normal shell and the count was 5.95 x 10^4 ± 0.12 CFU ml^{-1} in the infected shell. The current findings suggest a positive correlation between bacterial shell disease and the secondary infection affecting hemolymph (total hemocyte count), proximate composition (protein, carbohydrate and lipid) and histopathology of the animal (gills, hepatopancreas and heart). The total hemocyte count and differential count were experimented which showed lower number of total hemocytes in infected crabs while no highly accountable difference was found in differential count. Hematology studies reported total hemocyte as 9.93 x10^6 ± 0.07 cells ml^{-1} in normal and 6.525 x 10^6 ± 0.12 cells ml^{-1} in shell diseased hemolymph. The level of protein, carbohydrate and lipid contents suffered a mild alteration in crabs with bacterial shell disease. Histopathology of gills had shown variations such as dilated lamellae and hemocyte accumulation (nodules), hepatopancreas showed mild difference with increased necrosis, appearance of hemocytic nodules in the hemal spaces and heart with very mere infection while no reportable changes were accomplished in muscle tissue.

Key words: Shell disease, bacteria, hematology, histopathology, biochemical composition.

INTRODUCTION

Brachyurans, support an important sport and commercial fishery along the Indian waters. They possess immense scientific, economic, and ecological value with anti-microbial (White et al., 1985), anti-leukemic (Alain et al., 2011), anti-coagulant (Preyanat et al., 2003) and cardio active properties (Fort et al., 2007). They are also used as tools in drug action mechanisms. Shell disease is a common syndrome in decapod crustaceans that cause various types of erosive lesions on the shell (Johnson, 1983; Sindermann and Lightner, 1988). High prevalence of shell disease has been associated with stressful environments, such as intensive aquaculture (Sindermann, 1990), impounded populations (Taylor, 1948), or polluted natural environments (Gopalan and Young, 1975; Young and Pearce, 1975). These shell diseases can also be considered as a biomarker for

*Corresponding author. E-mail: sharmilajoseph88@gmail.com. Tel: 9843525530.

environmental degradation (Sindermann, 1990). Shell disease, the degradation of a crustacean's integument, is actually an external infection where a variety of microorganisms may attack the chitin of exoskeleton and has been known to affect crustaceans since 1900 (Rosen, 1970). The term shell disease was coined by Hess (1937) to describe exoskeleton lesions on the American lobster, *Homarus americanus*.

Histological studies have shown that gills, hepatopancreas and heart of diseased crabs were also affected significantly (Vogan et al., 2001). Although the disease is not believed to be fatal in its initial stages, death is known to result from adhesion of successive moult shells at lesion sites leading to incomplete withdrawal from exuviate at moult (Smolowitz et al., 1992). Alterations in immune reactivity within invertebrates have been shown in response to various external and artificial stimuli, including changes in temperature and salinity, pollutants (Le Moullac and Haffner, 2000), and natural or artificially induced infections (Ford et al., 1993). Vogan et al. (2001) have recently explained that severely shell diseased edible crab, *Cancer pagurus* display systemic haemocoelic bacterial infections in combination with damage to the gills and hepatopancreas. No specific studies in *Neoepisesarma mederi* crab have yet been reported which deals with shell diseases and their secondary infection in ecologically important mangrove crabs. Hence this study focuses on comparative search of secondary infection in hemocytes, biochemical composition and internal organs of bacterial shell diseased as well as disease free crabs.

MATERIALS AND METHODS

Animal collection

Crabs were collected during low tide (pre-monsoon) from Vellar estuary (Lat.11º29″ N and Long. 79º46″ E), South-East coast of India in sterile polythene bags using sterile forceps and subjected to experiments. After capture, both infected (n=20) and un-infected (n=20) crabs (females) were acclimatized to laboratory conditions (Salinity 10-34 ppt, Temperature 22-30°C, pH 8.0-8.5) in separate tubs.

Enumeration of total heterotrophic bacterial (THB) population

The shells of both shell diseased and disease free *N. mederi* crabs were dissected using sterile scissors and forceps. The carapace were crushed in homogenizer and serially diluted. Bacteria were isolated by pour plate method in Zobell marine agar (2216). Bacterial colonies were obtained after incubation of 24 h at 37°C. The plates were examined and counted for the number of colonies per plate. The microbial load in the given sample was calculated and is expressed as colony forming units (CFU) per ml of the sample. Pure cultures screened from randomly isolated colonies were transferred to nutrient agar slants and stored at 4°C for future use.

Hemocyte counts

Hemolymph was collected from both disease free and shell diseased *N. mederi* crabs from walking limp (pereiopods 2 to 5) using sterile gauge needle in eppendorf tubes. The hemolymph was diluted with sodium citrate buffer (pH – 4.6) in the ratio of 1:5. Hemolymph stain was prepared using Giemsa stain (5:1 dilution) and observed under microscope for hemocyte count (Vogan and Rowley, 2002).

Total hemocyte count

Total hemocyte counts were established from hemolymph of both normal and infected *N. mederi* by an improved Neubauer hemocytometer using the formula; Cell count = N x D/A x 10 x 10^3 cells/ml where, N is the total number of cells counted; D is the dilution of hemolymph; A is the total area counted and Factor 10 to convert area into volume (in µl).

Differential count

Differential counts were conducted on slides prepared by 100 µl of diluted cell suspension (1x10^4 cells ml^{-1}). The percentage of different cells was determined by the formula; Number of a particular hemocyte type / Total number of all types of hemocytes x 100.

Biochemical composition of crab tissue

Tissues were collected from each crab separately after dissection using sterile scissors and subjected to proximate analysis of protein, carbohydrate and lipid using standard methods of Lowry et al. (1951), Dubois et al. (1956) and Folch et al. (1956) respectively.

Histological examination of crab tissue

To determine whether shell disease intrudes structural changes in internal tissues like gills, hepatopancreas, heart and muscle tissue, and both un-infected and infected crabs' histological studies were conducted. The animals were anesthetized by ketamine vapours and the anesthetized animals were immediately dissected. Gills, heart, hepatopancreas and muscle tissue were kept in separate sterile slides and subjected to section cutting into 2 to 3 regions per block. The sections were washed with sterile distilled water and stained with eosin. The stained sections were examined histologically using 100X microscope.

Data analysis

Data were treated statistically by one way analysis of variance (ANOVA) and t-test (Two-Sample Assuming Equal Variances) to test the significance. Results were considered significant if P≤0.05.

RESULTS

Enumeration of total heterotrophic bacterial (THB) population

The total number of bacteria in shells of infected and un-

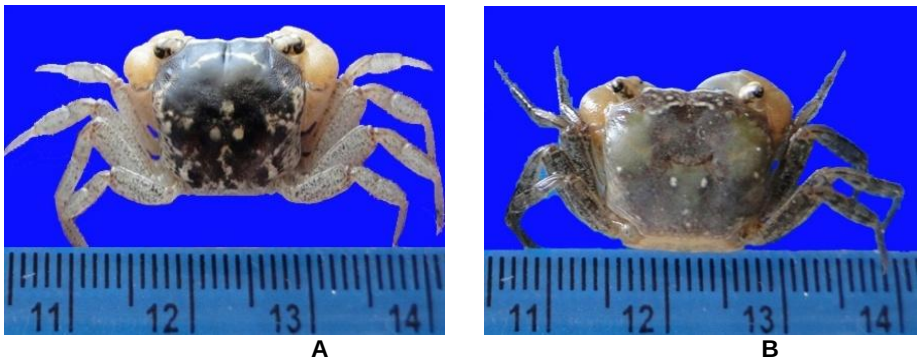

Figure 1. Study animal. A - Normal crab; B - Infected crab.

infected animals was estimated after isolation and growth on nutrient agar plates incubated at 37°C. The total heterotrophic bacterial (THB) population in normal shell was found to be 5.15 x 10^4 ± 0.09 CFU ml^{-1} and THB load was noted to be slightly increased than in normal shell (Figure 1A) and the count was 5.95 x 10^4 ± 0.12 CFU ml^{-1} in the infected shell (Figure 1B). The bacteria identified in infected shell were *Aeromonas*, *Pseudomonas*, *Flavobacterium* and *Bacillus*, three (*Aeromonas*, *Pseudomonas* and *Bacillus*) of which were found to be absent in the normal shells. *Alcaligens*, *Flavobacterium, Alteromonas* and *Photobacterium* were identified in normal shells.

Hemocyte classification

In *Neoepisesarma mederi*, four different hemocyte types were recognized based on their nuclear morphology, refractile nature of granules and staining characteristics with Giemsa stain (Figure 2).

Hemocyte counts

The circulating hemocytes for all un-infected (n=20) were found to be 9.93x10^6 ± 0.07 cells ml^{-1} whereas for all infected crabs (n=20), the total circulating hemocyte count was 6.525x10^6 ± 0.12 cells ml^{-1}. Using Giemsa staining method, hemogramme was observed to consist of almost similar number of differential hemocytes both in un-infected and infected *N. mederi* crabs which were recorded as 41.38 ± 1.19% (normal) and 41.3 ± 0.02% (infected) hyaline cells (H); 26.61 ± 1.98% (normal) and 26.58 ± 1.2% (infected) basophilic granular (BG) cells; 22.06 ± 2.92% (normal) and 21.87 ± 1.98% (infected) basophilic/eosinophilic granular cells (BEG) and 10.54 ± 2.4% (normal) and 10.02 ± 1.9% (infected) eosinophilic granular (EG) cells. One way analysis of variance done showed that total hemocytes count among un-infected and infected crabs differed significantly (P<0.05) while differential count differed insignificantly (P>0.05).

Proximate composition

Protein

The content of total protein in wet tissue of experimental crabs was estimated by spectrometry at 540 nm and calibration curve. The protein content was found to be 8.494 ± 0.876% in un-infected healthy crabs and 6.438 ± 0.764% in infected crabs. One way analysis of variance showed that protein content among un-infected and infected crabs differed significantly (P<0.05).

Carbohydrate

The level of total carbohydrate in wet tissue of experimental crabs was estimated by spectrometry at 490 nm and calibration curve. The total carbohydrate level was found to be 0.8008 ± 0.029% in un-infected crabs and 0.654 ± 0.025% in infected crabs. One way analysis of variance showed that carbohydrate content among un-infected and infected crabs differed significantly (P<0.05).

Lipid

The percentage of total lipid in wet tissue of experimental crabs was estimated and found to be 2.8 ± 0.01% in un-infected crabs and 1.926 ± 0.07% in infected crabs. One way analysis of variance showed that lipid content among un-infected and infected crabs differed significantly (P<0.05).

Histopathology

Gills

A pair of gills was present on either side of the branchial chamber in *Neoepisesarma mederi*. The structure consisted of pairs of flattened lamellae branching from

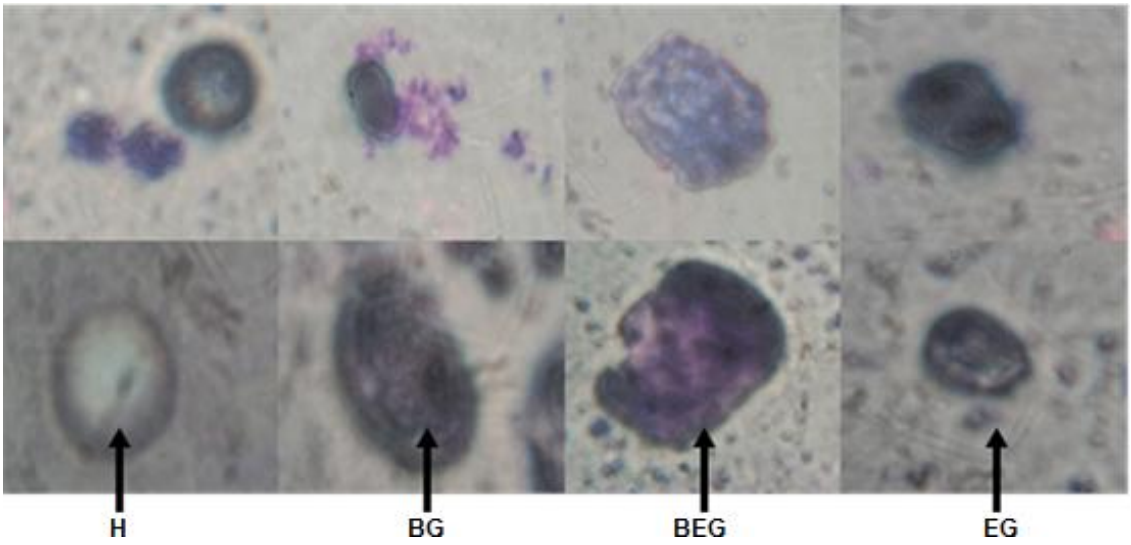

Figure 2. Hemocyte classification of *N. mederi* species. H – Hyaline cells; BG – Basophilic granular cells; BEG – Basophilic/Eosinophilic granular cells; EG – Eosinophilic granular cells.

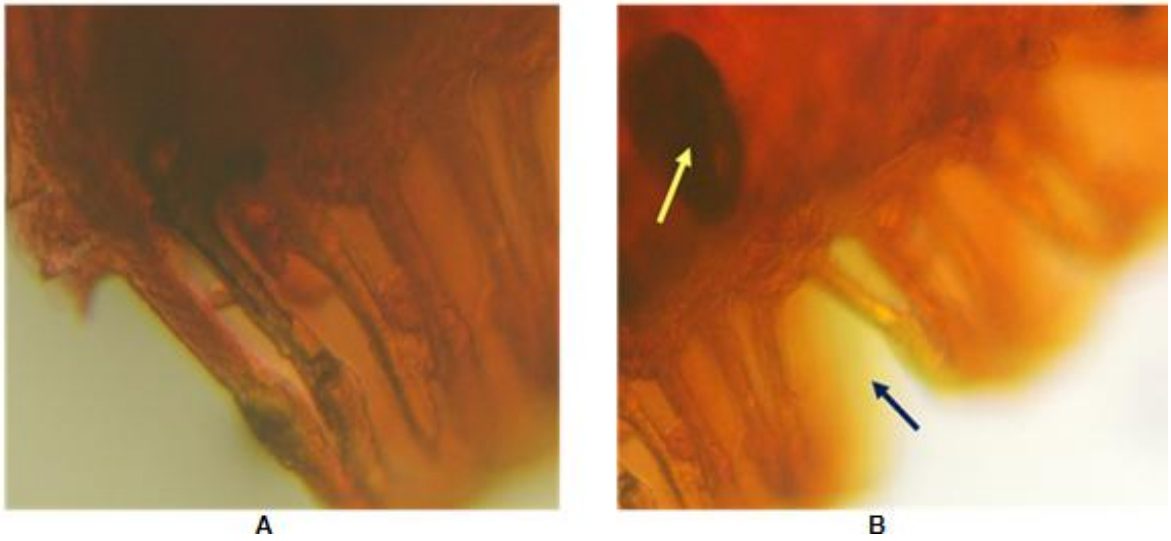

Figure 3. 100 X micrographs of gills of *N. mederi*. A) Section of a normal gill B) Section of infected gill showing hemal nodules and dilated lamella.

the central branchial stem, which had afferent and efferent hemal channels on each and each lamella was covered by a thin epithelium. Distinct histological changes in the gill were observed in crabs with shell disease. The lamellae were dilated and hemocyte accumulation (nodules) was also observed (Figure 3).

Hepatopancreas

As the apparent severity of shell necrosis increased, hemocytic nodules appeared in the hemal spaces (Figure 4). In the most severely affected animals, a massive destruction of epithelial cells was observed in some areas. A distinct lack of free hemocytes was also observed in the inter-tubular spaces.

Heart

Though no structural changes were observed in the myocardium or epicardium between control and shell diseased crabs but changes were observed in the number of hemocytic nodules (Figure 5).

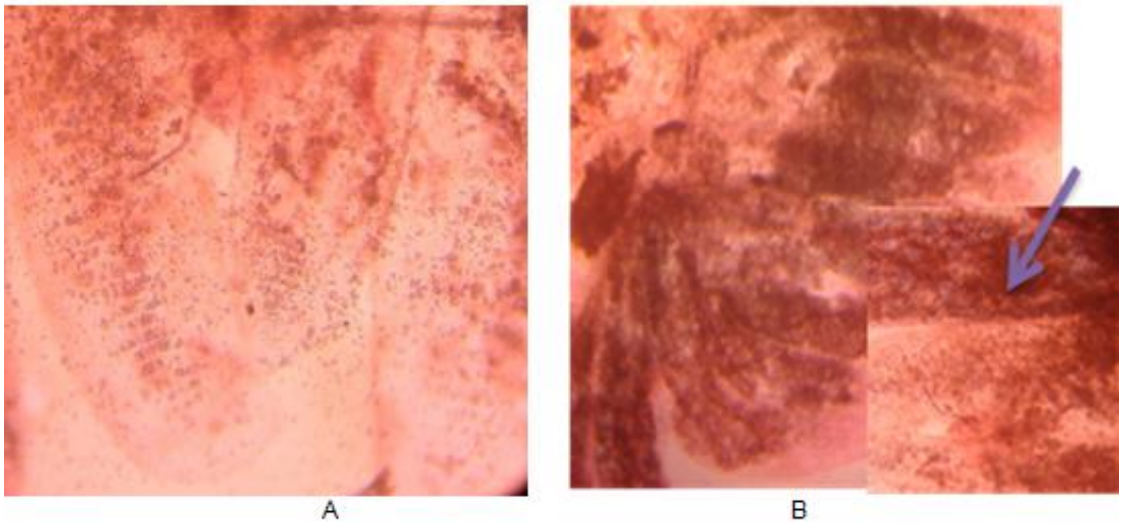

Figure 4. Section of hepatopancreas of *N. mederi* A) Normal hepatopancreas with free hemocytes B) Infected hepatopancreas showing hemocytic nodules in hemal spaces and lack of free hemocytes (inner image).

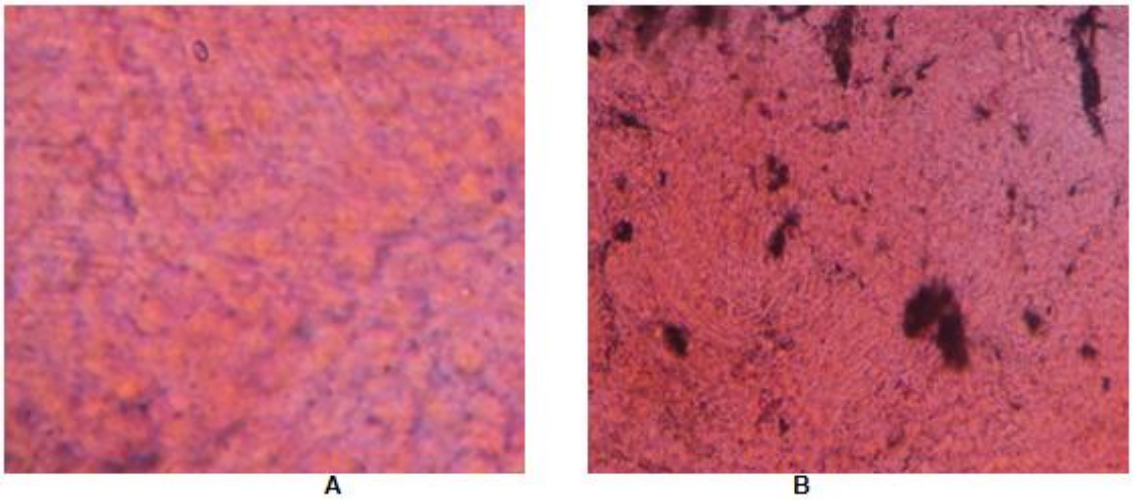

Figure 5. Section of heart A) Normal B) Infected showing hemal nodules (clumping of hemocytes).

Muscle tissue

No recordable alterations were observed in the muscle tissue of shell infected *N. mederi* (Figure 6).

DISCUSSION

Crabs are continuously affected by environmental fluctuations and management practices such as handling, crowding, transporting, fluctuating temperatures and poor water quality. All of these factors can impose candidate stress on the homeostatic mechanisms of crab rendering them susceptible to a wide variety of pathogens (Wang, 2011). Mu et al. (2009a, b), Meng et al. (2010) and more other researchers have reported aspects of crab immune system. Circulating hemocytes play an extremely important role in the defense reactions by phagocytosis, hemocyte clumping, the production of reactive oxygen metabolites and the release of microbicidal proteins (Smith and Chisholm, 1992, 2001; Smith et al., 2003). In addition, gills serve another important role in the immune response (Johnson, 1976a, b; White et al., 1985; Martin et al., 2000; Burnett et al., 2006). Johnson (1976a) provided histological evidence that nodule formation in response to stress-induced bacteremia in blue crabs might have adverse effects on gill function, including distention of gill lamellae and disruption of haemolymph flow.

Initially it was thought bacterial infection to be restricted

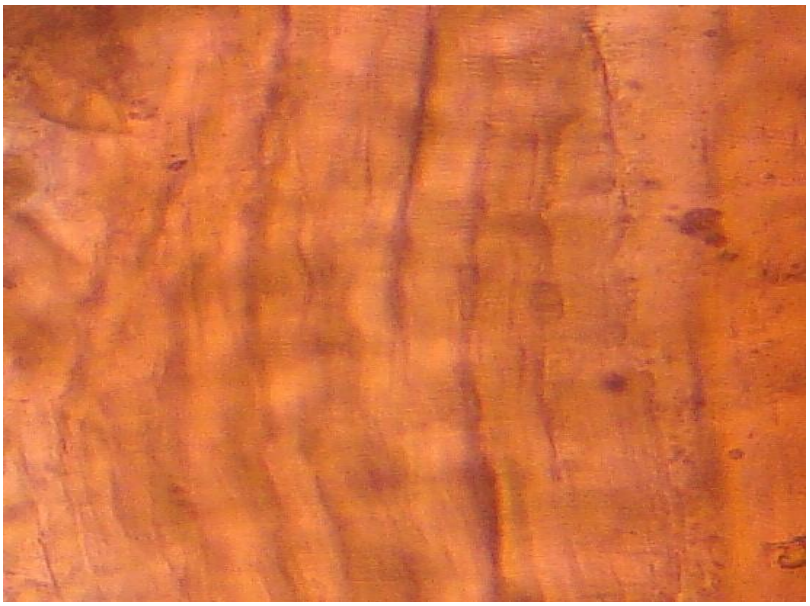

Figure 6. Section of muscle tissue of *N. mederi.*

to the exterior surfaces of the exoskeleton, recent studies showed that shell disease is not a disease caused by a single pathogen and solely restricted to the exoskeleton (Wang et al., 2011). Though the findings of Vogan and Rowley (2002) in the edible crab *Cancer pagurus* explains there is no significant correlation between bacterial shell infection and hemocyte count, in a previous study of Vogan et al. (2001), individuals exhibiting shell disease were shown to have internal infections. There was a linear relationship between the bacterial load and the severity of the disease. This study also revealed breakdown of the hepatopancreas and damage to gills associated with nodule formation (hemocyte clumping). Stage and gender of the animal also brings about considerable difference in their hemocyte count (Depledge and Bjerregaard, 1989; Horn and Kerr, 1963). The present investigation dealt with the hematology studies with female crabs within a range carapace width of 6 to 9 mm. Significant difference in the total hemocyte count and insignificant difference in differential count was observed between un-infected and infected *N. mederi* crabs.

Eric Floreto (2000) showed significant differences in the biochemical profiles of the various tissues between healthy and shell-disease affected lobsters. Biochemical constituents in crabs are known to vary with season, size of the animal, stage of maturity, temperature, availability of food etc. Nutrition has a direct relationship with biochemical composition of the animal (Manivannan et al., 2010). Ravichandran and Kannupandi (2004) reported biochemical composition of five mangrove crab *Sesarma. brockii, S. andersoni, S. plicatum, Metapograpsus messor* and *M. maculatus* among the species *M. messor* showed maximum proximate composition. All the crabs observed in the present study were females (brood and un-brood) and it is quite interesting that no infected male was identified. Results show a mild but reportable decrease in protein, carbohydrate and lipid level in shell diseased *N. mederi* than disease free controls. The results above could definitely show a slight consideration in biochemical variation and it's relation towards shell disease. It can be thought that the alteration in proximate composition of protein, carbohydrate and lipid of infected animals may be due to an unpredictable change in its feeding habit during the onset of bacterial attack.

The presence of melanised nodules in gills, heart and hepatopancreas suggest systematic bacterial infections in these animals (Johnson, 1976a, b; Smith and Ratcliffe, 1980). The breaches observed in lamellar epithelium combined with hemocytic infiltration and nodule formation around these foci identify a potential route of invasion by micro-organisms. In decapods, the epidermal cells of the gill lamellae, unlike the rest of the integument, possess only an ultra-thin covering of chitinous cuticle. Thus, although this thin covering is more vulnerable than the rest of the integument, its breach would still have to be through active chemical attack (Victor, 1993, 1994; Soegianto et al., 1999a), abrasive injuries or the extracellular enzymatic activities of micro-organisms (Lightner and Fontaine, 1975). Morado et al. (1988) showed erosion of the arthrodial membrane between the propodus and pereiopods of Dungness crabs *Cancer magister* that coincided with the appearance of nodules in the gills. Unexpectedly, the severity of shell disease in *Cancer pagurus* did not result dramatic changes to the

majority of immune parameters tested, including total hemocyte counts (Vogan and Rowley, 2002). Vogan et al. (2002) found that extracellular products (ECP) produced with shell disease could cause rapid death of the crab upon injection. The present study found distinct changes in histology of the gill displaying nodules which may be formed due to the lack of free running hemocytes. The disease can lead the animal to varying consequences like defective oxygen transport. Oxygen transport may be greatly affected as it is carried out by gills where hemocyte clumping was noticed in shell diseased animals. The lamellae were found to be dilated. Severe internal damage is associated with crabs showing highest amount of external damage in regions surrounding the branchial chambers. The carapace of the infected *N. mederi* was found to be too delicate when compared to normal ones.

Bowser et al. (1981) reported vacuole formation in hepatopancreas which resulted after the intra-hemocoelic injection of *Vibrio* spp. Pathogenecity of *Vibrio harveyi* was studied by Robertson et al. (1998) in *Penaeus vannamei* larvae and the results showed necrotic bundles in hepatopancreas of the infected animal. The present study also supports previous studies with necrotic bundles found scattered throughout the hepatopancreas of the shell-diseased *N. mederi*. Liberation of bacterial toxins may disturb the integration of hepatopancreas resulting in autolysis of tubules. Vogt (1997) support this through his findings in *Palaemon elegans* in which it was believed that exotoxin from Gram-negative bacteria caused destruction of hepatopancreas. Hemocytic nodules in hemal spaces and distinct lack of free hemocytes were also observed in hepatopancreas of shell-diseased *N. mederi* crabs.

Cardiac lumen of shell-diseased crabs showed number of hemocytic nodules while no structural alteration was observed. Though studies by Vogan et al. (2001) show similar type of result in the edible crab, *Cancer pagurus* it may not be inferred that the occurrence of nodules were found due to the impact of shell-disease. They may be formed due to the varied procedures followed in the histology studies. Further studies have to be done to confirm the results with heart. No previous results were found to suggest a muscle histological alteration coinciding with shell-disease of crustaceans. Muscles suffered no structural or observable changes also in the present histological investigation of bacteria infected *N. mederi* animals.

In conclusion, shell disease is well identified to cause secondary infection bringing alteration in the normal histology of the animal. Therefore, potential approaches should be designed such that a single compound or a strategy is sufficient for the development of immune activity in crab in order to control shell diseases as this induces loss of immunity as a result of its secondary infection. The influence of shell-disease and mortality of *N. mederi* has yet to be studied and it is suggested that lethal effects may depend on the type of infected microbe and its rate and degree of infection in the animal.

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