

HISTOMORPHOLOGICAL CHANGES IN *PENAEUS INDICUS*
DUE TO THE EFFECT OF LOW SALINITY
AND LOW OXYGEN IN WATER

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CERTIFICATE

This is to certify that this Dissertation is a bonafide record of the work done by Smt. Sharmila Aziz under my supervision and that no part thereof has been presented before for any other degree.



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CONTENTS

Page No.

PREFACE	1
INTRODUCTION	4
MATERIALS & METHODS	9
RESULTS	14
DISCUSSION	31
SUMMARY	40
REFERENCES	43

PREFACE

The hydrological parameters like dissolved oxygen (DO) concentration, salinity, temperature and other physical characteristics of water have a great impact on the survival, growth and other physiological activities of the culture animals. Adequate knowledge on the effects of these parameters on the organisms is inevitable for successfully culturing the economically important aquatic animals on a large scale.

Water quality management is an integral part of mariculture. Nearly every problem that arises in an aquaculture system is the result of, or leads to degradation of water quality, which in turn may lead to oxygen depletion. This often triggers the sudden outbreak of diseases. Hence, DO is a critical hydrological characteristic which should be carefully monitored in the culture system.

Salinity of the culture medium is as important as the DO factor for successful culture activities. The influx of fresh water, especially during the monsoon in the brackish water system, where most of the culture activities are carried out at present, brings the salinity level to such an extent that it brings about a lot of undesirable changes in the culture animals leading to varying degrees of morbidity and mortality.

Though innumerable diseases of economically important crustaceans have been described, most of them are of undetermined etiology. This reveals the fact that besides the instinct pathogenicity of certain disease causing agents, the effect of environmental stressors like DO, salinity and temperature fluctuations also play vital roles.

The objectives of the present study were:

- (1) To determine the effect of low salinity and low DO levels of water on the animals in respect of clinical symptoms.
- (2) To note different clinicopathological findings viz. total haemocyte count (T.H.C.) and glucose content of the haemolymph in the affected animals.
- (3) To bring to light the gross changes in various tissues and organs of the animals exposed to the above conditions.
- (4) To study the various pathomorphological alterations in different tissues and cells of the exposed animals.

The effects of environmental stressors are often difficult to assess and frequently become apparent only after considerable resource and effort have been expended. This causes immense economic losses due to the large-scale mortality of culture animals. It should be possible to observe structural-functional alterations in individual cells or groups of cells at an early stage of a stress response. Some of these cellular responses may be generalised, whereas others are likely to be specific to a particular type of stressor. Histological changes serves as excellent early warning indicators of environmental stress. This provides indications about the degree of stress and of the adaptive capability of the organism. Hence in mariculture activities the histomorphological studies of the stressed animals may prove to be of immense worth and can help in reducing the large-scale mortalities by timely implementing the control measures.

Although many classical studies are made dealing with human and domestic animals, very few studies are made on the histopathology of aquatic animals and by comparison a negligible proportion is being dealt with aquatic invertebrates. Since histomorphological studies in the stressed prawns are still very meagre, the present study was undertaken to elucidate the alterations at cellular level.

Histopathological changes in the 5 organs examined in the stressed animals indicated that they may serve as early warning indicators of stress. The muscle appears to be one of the most important organs for this purpose, as frank lesions were abundant in the muscle tissue of the exposed animals. Tissue responses in the hepatopancreas, heart and gill also proved as useful indicators of stress. The degenerative changes in the gills and exoskeleton are helpful for early detection of stress.

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INTRODUCTION

Water quality may be direct or accessory etiologic agent of prawn diseases. Dissolved oxygen, salinity, pH, temperature, ammonia, Nitrate and Nitrite contents are the various parameters which affect the normal behaviour in aquatic animals. Among these, dissolved oxygen and salinity in optimum quantity can be said to be the most important parameters for proper growth and normal physiology of the aquatic animals. The crustaceans are not exceptions. The dissolved oxygen (DO) and salinity requirements varies from species to species and also for different stages in the life history.

Irrational exploitation of the natural populations of many of the economically important crustacean species have lead to the complete depletion of these species. As a result, the mariculture facilities have been extended leading to an overcrowding of these species in the culture systems. This has made them more prone to diseases. An optimum level of these essential parameters are inevitable for the healthy existence of these animals. A decrease or increase in these parameters brings about a lot of stress in the animals. The signs of stress become evident morphologically as well as histologically. The present study is intended to bring to light these changes which are of great biological interest.

Valuable informations on the normal haematological and histological studies on prawns and other decapods have been recorded by workers like Travis (1955), Bunt (1968), Welinder (1974), Williams and Lutz (1975), Foster and Howse (1978), Pequeux et al. (1979), Al-Mohanna et al. (1985), Martin and Graves (1985) and many others.

Though a lot of work have been done on the physiological and biochemical changes in prawns brought about by the environmental stressors, studies on the histomorphological changes are remarkably meagre except for the works of Rigdon and Baxter (1970), Bielawski (1971), Johnson (1974), Aquacop (1977), Lakshmi et al. (1978), Sis et al. (1980), Rajamani (1982), Pequeux et al. (1984), Lee et al. (1985). Baticados et al. (1986), Momoyama and Matsuzato (1987) and Nash et al. (1987, 1988). These workers have shown that several histomorphological disorders are brought about by the synergistic effect of stressors like variations in DO level, salinity, pH, temperature etc.

Idiopathic muscle necrosis (IMN) is considered to result from predisposing environmental stressors including extremes and sudden fluctuations in salinity, temperature, hyperactivity, high concentration of quinaldine, overcrowding, exposure to direct sunlight, physical handling and exposure to air. (Venkataramiah 1971 a,b; Lightner 1977, 1983; Brock 1983 etc.).

Localised increase in blood cells or haemocytes can be induced by a variety of factors like parasitism, injury leading to shell damage etc. (Des Voigne & Sparks, 1968).

Rigdon and Baxter (1970) studied the histopathological aspects of the necrotic muscles in brown shrimp, Penaeus aztecus taken from the Galveston Bay, Texas. They could not determine the causes of necrosis nor the reasons for the variations in the magnitude of necrosis from one bait pen to the other.

Johnson (1974) observed necrosis at room temperature in P. aztecus immediately after a hyperactive state. The affliction of necrosis following or

during high motor activity suggested that this condition might be due to muscle fatigue, presumably under inadequate oxygen level in water.

AQUACOP (1977) found that under certain stresses, white opacity of the tail of shrimp is a common feature. In Penaeus merguensis, an unusual opacity of the tail muscles has been noticed by them.

Taylor et al. (1977) studied the responses of the shore crab, Carcinus maenas (L) to progressive hypoxia at different acclimation temperature and salinities.

Sis et al. (1980) reported changes in the gut of penaeid shrimp as a result of environmental stressors.

Wilkes and McMahon (1982) studied the effect of maintained hypoxic exposure on the Crayfish, Orconectes rusticus.

In crabs acclimated to dilute media such as low salinity, the posterior gills are essentially characterized by a complex and well developed network of large apical evaginated and digitated folds. These membranous folds produced a large and very characteristic extracellular compartment under the cuticle. (Pequeux et al., 1984). Although details may vary from one animal to another, a structure similar to that described in the posterior gills epithelium has been demonstrated in gills of many other osmoregulator crustaceans (Bulger, 1963; Komnick, 1963; Philpott and Copeland, 1963; Tandler, 1963; Copeland, 1964, 1968; Copeland and Fitzjarrell, 1968; Bielawski, 1971; Foster and Howse, 1978).

Clark (1986) showed the inhibition of moulting in penaeus semisulcatus by long term hypoxia. Shrimps kept for 17 days at an oxygen level of 2 parts per million did not moult and steady high mortality was observed. When the oxygen level was subsequently increased to 5 parts per million, the mortalities ceased and many moults were seen.

Dallavia (1986) studied the salinity responses of the juvenile penaeid shrimp penaeus japonicus.

Momoyama and Matsuzato (1987) observed muscle necrosis in cultured P. japonicus. Environmental or physiological stress due to high water temperature, low DO and overcrowding has been suspected to produce muscle necrosis in shrimp. But no relation was found between the occurrence of this disease and the water quality of the shrimp culture ponds in their study.

Nash et al. (1987) observed idiopathic muscle necrosis in 28 - day old Machrobrachium rosenbergii post larvae. Avoidance of overstocking and increased DO in tank water have proved effective in preventing subsequent recurrences of the condition.

Nash et al. (1988) have also reported abnormal brown discolouration of the gills, soft shells and decreased survival in adult penaeus monodon, cultured in grow-out ponds rich in acid sulphate mangrove soils in Johor, Malaysia.

Venkataramiah (1971 a & b) could induce muscle necrosis in healthy looking P. aztecus by overcrowding, lowering DO levels and changing other physico-chemical conditions of the holding media under laboratory condition.

Venkataramiah et al. (1973) studied the effect of salinity and feeding levels on the growth rate and food conversion efficiency of P. aztecus.

Lakshmi et al. (1978) studied spontaneous muscle necrosis in P. aztecus in relation to salinity and temperature changes. The incidence of necrosis and subsequent mortality of necrotic shrimp appeared to be directly related to the magnitude of the changes. Necrosis occurred mainly in the distal 4 to 6 segments.

Histopathological studies in soft prawns were conducted by Remesh (1988). The histopathological changes were observed in the exoskeleton, muscle, gills, hepatopancreas, heart and gut. It has been observed that the condition was most prevalent when the salinity and oxygen levels of water were very low whereas temperature was very high.

It is hoped that the present study may prove useful in elucidating various histomorphological changes that occur in Penaeus indicus subjected to low DO and low salinity in various degrees.

MATERIALS AND METHODS

Collection of specimens

The specimens were collected from the Narakkal prawn culture farm of CIBA by cast net operation. Prawns (Penaeus indicus) of both sexes and of the size range of 70-120 mm in length (from tip of rostrum to tip of telson) were collected and transported to the laboratory in 50 litre capacity plastic bins at a density of 1 number/2 litres, with occasional agitation of water.

Experimental set-up

Acclimation tanks

Four rectangular tanks of 200 litre capacity were used as the acclimation tanks. In each tank, 25 animals of both sexes were maintained in 20 ppt salinity sea water prepared by proportionately diluting 34 ppt seawater with tap water. The water was aerated adequately. Temperature was maintained at $27 \pm 2^{\circ}\text{C}$. The prawns were fed ad libitum with artificially prepared pellet feed and were maintained under three sets of conditions during the course of the experiment. They were:

- (i) Sea water with low oxygen and optimum salinity.
- (ii) Sea water with low salinity and optimum oxygen.
- (iii) Sea water with low oxygen and low salinity.

- (i) Sea water with low oxygen and optimum salinity

Eight, 10 litre capacity circular tanks were used where three animals were introduced in each tank. They were allowed to remain in the water

without providing any aeration. The dissolved oxygen content was checked using Winkler's method (Strickland and Parsons, 1968). When the dissolved oxygen content reached 2-3 ml/litre mild aeration was provided to maintain this low oxygen concentration constantly. The animals were maintained for a period of five days under such condition. Salinity was maintained at 20 parts per thousand. The dissolved oxygen content was checked every six hourly to ensure maintenance of dissolved oxygen level within 2-3 ml/litre using Winkler's method. The tanks were covered with wire gauze netting to prevent the animals from escaping from the tank. Eight animals, one from each tank was sacrificed on the 3rd, 4th and 5th day respectively. A control tank with three animals was maintained with salinity of 20 parts per thousand and DO of 6 ml/litre. One animal each from the control group was also sacrificed on these days for comparative study.

(ii) Sea water with low salinity and optimum oxygen

Eight, 10 litre capacity tanks were maintained, two each with similar salinity. Three animals were introduced in each tank. The low salinities to which the animals were subjected were 1 ppt, 2 ppt, 3 ppt and 4 ppt. The required salinities were obtained by diluting the sea water with sufficient amount of fresh water. The volume of sea water required was found out using the following formula:

$$\text{Volume of sea water required} = \frac{\text{Desired salinity} \times 1000}{\text{Known higher salinity of sea water.}}$$

The salinity was checked using argentometric method (Strickland and Parsons, 1968). The water was well aerated. The tanks were covered with wire gauze

netting to prevent animals from escaping from the tank. The course of the experiment was 5 days. The salinity in each tank was checked daily and sufficient amount of fresh water was added to bring down the salinity, which increased due to evaporation. Eight animals, two from each set of tanks were sacrificed on the 3rd, 4th and 5th day respectively. A control tank with three animals was maintained with salinity of 20 parts per thousand and DO of 6 ml/litre. One animal each from the control group was also sacrificed on these days for comparative study.

(iii) Sea water with low oxygen and low salinity

Eight, 10 litre, capacity circular tanks were used. Three animals were introduced in each tank. They were allowed to remain in the water without providing any aeration. The dissolved oxygen was checked using Winkler's method. When the dissolved oxygen content reached 2-3 ml/litre mild aeration was provided to maintain this low oxygen concentration constantly. The salinities to which the animals were subjected were 1 ppt, 2 ppt, 3 ppt and 4 ppt. Two tanks had similar salinity and the animals were maintained for a period of five days. The dissolved oxygen content was checked every six hourly to ensure maintainance of dissolved oxygen level within 2-3 ml/litre using Winkler's method. The salinity in each tank was checked daily by argentometric method and sufficient amount of fresh water was added to bring down the salinity which increased due to evaporation. The tanks were covered with wire gauze netting. Eight animals, two from each set of tanks were sacrificed on the 3rd, 4th and 5th day respectively. A control tank with three animals was maintained with salinity of 20 parts per thousand and DO of 6 ml/litre. One animal each from the control group was also sacrificed on these days for comparative study.

SCHEMATIC DIAGRAM SHOWING THE EXPERIMENTAL SET-UP

(I) LOW OXYGEN AND OPTIMUM SALINITY

DO = 2-3 ml/litre

S = 20 ‰



CONTROL



(II) LOW SALINITY AND OPTIMUM OXYGEN

DO = 6 ml/litre

S = 1 ‰

DO = 6 ml/litre

S = 1 ‰



CONTROL

DO = 6 ml/litre

S = 3 ‰

DO = 6 ml/litre

S = 4 ‰



(III) LOW OXYGEN AND LOW SALINITY

DO = 2-3 ml/litre

S = 1 ‰

DO = 2-3 ml/litre

S = 2 ‰



CONTROL

DO = 2-3 ml/litre

S = 3 ‰

DO = 2-3 ml/litre

S = 4 ‰



* (3 Animals were maintained in each tank)

Haematological studies

(i) Total haemocyte count (THC)

26 gauge hypodermic needle attached to a 1 ml glass syringe was used for drawing haemolymph directly from the heart, which was pre-treated with an anticoagulant i.e. Tri-sodium citrate. Haemolymph was collected on the 3rd, 4th and 5th day prior to sacrificing them for histological studies. 0.05 ml of haemolymph from the collected sample was drawn into a WBC pipette. It was diluted with WBC diluting fluid (Analytical solution, SD's Lab-chem industry) and shaken well. A drop of this was used to charge the Neubauer counting chamber and the haemocytes were counted under low power objective in light microscope. The number of cells were expressed as cells per cubic millimeter.

(ii) Haemolymph glucose determination

A part of the haemolymph was utilized for determining the glucose content using a glucometer (model:Ames).

Morphological studies: Gross changes

The morphological and behavioural changes that occurred in the animals were noted from time to time. The external changes in the different organ tissues were also noted.

Histological studies

The animals were fixed at the end of each experiment after killing them by injecting the fixative into the abdomen and carapace. They were either fixed as a whole or dissected out and the tissues fixed separately. The

fixatives used were Neutral buffered formalin, Bouin's fluid and 10% formalin. After fixation, washing, dehydration, clearing and embedding in Paraffin wax (Merck 58-60 C), sections were cut in a manual rotary microtome (Weswox Optik MT-1090 A). The tissues selected for the study were hepatopancreas, heart, gills, muscle and gut. Staining was done using Haematoxylin and Eosin method. Occasionally Gram's stain and Giemsa stain were used wherever necessary. Stained sections were examined under a binocular research microscope (Olympus). Photomicrographs were taken wherever necessary.

RESULTS

The present study on Penaeus indicus was carried out for a period of 3 months from June 1989 to August 1989. The results obtained has been placed under 3 main headings viz., changes under low oxygen condition, changes under low salinity condition and changes under low oxygen and low salinity condition.

I. CHANGES UNDER LOW OXYGEN CONDITION

(i) Gross morphological changes:

The animals soon after introduction in the low oxygen condition showed vigorous struggling movements by beating rapidly the pleopods and hitting the sides of the container with the rostrum. They came to the surface of water showing signs of stress by paddling. These initial changes in the behaviour continued for about 5 to 8 minutes after which the animals settled to the bottom and became calm. By about the fourth day brownish areas of necrosis ranging from 4-8 mm in diameter were noticed in the posterior abdominal region. These areas of necrosis appeared initially as a brownish pink tinge (Fig. A) which turned opaque subsequently.

(ii) Haematology:

Total haemocyte count (T.H.C)

There was an increase in the number of haemocytes in the haemolymph. The average value was found to be 12,333.33 cells/mm³ with a range of 10,900-26,250 cells/mm³ (Table No. 1).

Haemolymph glucose content

There was decrease in the glucose content (hypoglycemia) with mean value of 9.50 mg/dl and with a range of 9-10 mg/dl (Table No. 2).

(iii) Histopathology:

Histopathological changes were most evident in the muscle and heart tissues under this set of condition. Changes of moderate degrees were also seen associated with the exoskeleton, hepatopancreas and gills.

(a) Muscle

Muscle tissues exhibited a variety of remarkable changes. The primary changes were characterized by focal to multifocal areas of degeneration with loss of cross striations which was followed by fragmentation of myofibrils. Muscle showed areas of marked necrosis with mild to moderate infiltration of haemocytes (Fig. 1). Non-inflammatory degenerative changes with oedema were noticed in the muscle which showed opacity on exoskeleton (Fig. 2). Nuclei were either pyknotic or karyorrhectic. Zenker's necrosis with ground glass appearance or hyalinization was frequently observed under this set of conditions. Muscle bundles showed hyperchromatism in many areas.

(b) Heart

The myocardial fibres showed extensive fragmentation and marked infiltration of haemocytes in the necrotic areas (Fig. 3). Occasionally focal aggregation of round cells identical to macrophages were seen which exhibited phagocytic activities.

(c) Exoskeleton

Thinning of epicuticle was a regular finding which was minimum on the third day and maximum on the fifth day. The subepicuticular layer showed thinning and exhibited uneven thickness at some places. Tonofibrils traversing the epidermal layer showed focal detachment from the uncalcified layer of the endocuticle. There was separation of the exoskeleton from the underlying muscle. Mild to moderate infiltration of haemocytes was observed in the widened space.

(d) Hepatopancreas

The hepatopancreatic tubules exhibited severe tubular necrosis, with majority of tubules showing extensive degeneration (Fig. 4) by the fifth day. Thickening of the connective tissue capsule was more evident with loss of tubular cells. In many tubules only few secretory cells were left behind rendering distention of the lumen. Most of the cells were fragmented and disjointed with loss of architecture.

(e) Gills

Gill epithelium in the lamellae exhibited flattened appearance due to atrophy. Mild inflammatory cell reactions was observed in some areas. However, this was not a regular feature.

(f) Gut

No specific lesions could be observed.

II. CHANGES UNDER LOW SALINITY CONDITION

(i) Gross morphological changes:

The behavioural changes observed were almost same as noticed in the

low oxygen condition. Some of the animals developed cuticular lesions in the second, third, fourth and fifth abdominal segments, and tips of pleopods by about the fourth day. These appeared as brownish mustard-sized spots which increased in size and became very evident by the fifth day (Fig. B). Thinning and softening of the exoskeleton was observed, which started developing on the third day. The muscle tissues were bluish in colour, fragile and moderately soft to touch. This condition was most evident under 2 ppt and 4 ppt salinity. The feeling of softness was due to accumulation of some fluid in between the exoskeleton and muscle which produced cripitating sound on pressing.

(ii) Haematology:

Total haemocyte count (T.H.C)

A moderate increase in the haemocytes was observed in this group of experimental animals. Average value of T.H.C. was 10,150 cells/mm³ with a range of 8,500 - 22,000 cells/mm³ (Table No. 1).

Haemolymph glucose content

Hypoglycemia was observed in the haemolymph glucose content. The mean glucose content was 9.625 mg/dl with a range of 9-10 mg/dl. (Table No.2).

(iii) Histopathology:

Histopathological changes were most evident in the muscle, heart and hepatopancreas.

(a) Muscle

The muscle tissues exhibited loss of cross striations. The myofibres showed extensive muscular necrosis. These areas of myofibrillar necrosis were

frequently characterized by mild to moderate infiltration of haemocytes. There was marked separation of the exoskeleton from the underlying muscle tissue with accumulation of haemocytes in the emptied space (Figs. 5 & 6). The exoskeleton in these cases were extremely thin with loss of tonofibrils. Strands of connective tissue forming a net work was observed separating the muscle from the exoskeleton. The accumulated cells were round and darkly stained and were found in small aggregates. Areas of mineralization resembling calcification were frequently observed in the muscle tissue. These areas were intensely basophilic with pyknotic nuclei. The changes were almost common in third, fourth and fifth days with slight variations in the intensity.

(b) Heart

Focal aggregation of haemocytes was observed in the myocardium. (Fig. 7 & 8). These aggregates appeared as micronodules containing round to oval cells with pleomorphic nuclei. The myocardial fibres were separated and appeared fragmented (Fig. 9). Areas of vacuolation in the myocardium was marked with moderate haemocytic infiltration (Figs. 10 & 11). In some areas, accumulation of proteinous material was observed as intensely stained homogenous material in the vacuolated myocardium. These areas also revealed lightly stained net work resembling fibrin (Fig. 12). Mineralization in the heart muscles was also frequently observed under this set of conditions as intensely basophilic structures. Most of the cases showed varying degree of pericardial oedema with diffusely scattered haemocytes (Fig. 13).

(c) Exoskeleton

The changes were almost similar as under low oxygen condition. Aggregates of haemocytes were observed histologically where gross lesions were

noticed externally.

(d) Hepatopancreas

Hepatopancreatic tubules showed moderate to extensive degeneration with the loss of secretory cells. These areas exhibited net-like appearance having lost all the tubular cells excepting the pink staining peritubular connective tissues (Fig. 14). Majority of tubules exhibited marked necrosis by the 5th day. Compensatory hyperactivity of the adjoining tubular cells were discernable where focal degenerative changes of tubular cells were evident (Fig. 15). The shrinkage of tubules with the distention of connective tissue (Fig. 16) and flattening of lining epithelial cells (Fig. 17) was in marked contrast with the normal hepatopancreatic tubules (Fig. 18) of the animals sacrificed on 3rd, 4th and 5th day. Accumulation of homogenous proteinous materials was frequently seen in the intertubular space.

(e) Gills

Degeneration of the epidermal covering around the lamellar sinuses was a regular finding in the experimental animals starting from the 3rd day onwards. Telangiectasis of the gill lamellae was observed with emphysema or distention of the lamellae (Fig. 19). Fibrinous strands were seen inside the distended lamellae with flattening of the epithelial cells.

(f) Gut

No specific change was observed in the gut.

III. CHANGES UNDER LOW OXYGEN AND LOW SALINITY

(i) Gross morphological changes:

The behavioural changes were similar as observed in other set of

conditions. Brownish areas of necrosis ranging between 8 mm to 10 mm in diameter were seen in the posterior abdominal region (Fig. C) on the fourth day. They appeared as small pinkish brown areas which became opaque later by the fifth day. The animals were lethargic and moved very slowly.

(ii) Haematology:

Total haemocyte count (T.H.C)

Mild to moderate haemocytosis was observed in the haemolymph. The mean T.H. C. was 9, 125 cells/mm³ with a range of 6,450-18,150 cells/mm³ (Table No. 1).

Haemolymph glucose content

Hypoglycemia was observed in the haemolymph in these group of animals also with a mean value of 9.375 mg/dl and with a range of 9-10 mg/dl.

(iii) Histopathology:

Marked histological changes were observed in the muscle, gills, heart and hepatopancreas.

(a) Muscle

The myofibrils exhibited marked muscular necrosis. In advanced cases (fifth day) the degenerating myofibrils had a pale vacuolated moth-eaten appearance due to marked destruction of parenchyma (Fig. 20). Areas of mineralization resembling calcification were frequently observed (Figs. 21 & 22). Severe necrotic foci with mild to moderate infiltration of haemocytes were also observed.

(b) Heart

Heart exhibited myocardial degeneration with karyorrhectic nuclei in the muscle bands. Pericardial sac showed oedematous change with thickening and scattered haemocytes (Fig. 23). Hyalinization and vacuolation associated with moderate haemocytic infiltration was evident in majority of cases. Pericardium showed marked thickening due to hyperplastic changes and haemocytic infiltration.

(c) Exoskeleton

The changes were almost identical as in the other sets of condition.

(d) Hepatopancreas

The hepatopancreas exhibited marked tubular degeneration with complete loss of architecture in few cases (Fig. 24), where acinar structure was absent. In other cases empty hepatopancreatic acini were observed with complete denudation of the epithelial cells of the tubules. The thickened basal lamina was left alone in extreme cases (Fig. 25).

(e) Gills

Branchial cell hyperplasia occurred in the low salinity and low oxygen condition (Fig. 26). Marked to moderate haemocytic infiltration was observed in the gill lamellae and branchial septum.

(f) Gut

Increased number of granular eosinophilic cells were observed in the sub-serosal layer (Fig. 27) where marked oedema and extensive vacuolation was also noticeable.

TABLE No. 1

TABLE SHOWING THE TOTAL HAEMOCYTE COUNT (T.H.C) (Cells/mm³)

Condition	Number of animals examined	Range	Mean
I. Low oxygen	24	10,900-26,250	12,333.33
II. Low salinity	24	8,500-22,000	10,150.00
III. Low oxygen and low salinity	24	6,450-18,150	9,125.00
IV. Control	9	4,200-14,600	7,400.00

TABLE No. 2

TABLE SHOWING THE HAEMOLYMPH GLUCOSE CONTENT (mg/dl)

Condition	Number of animals examined	Range	Mean
I. Low oxygen	24	9-10	9.50
II. Low salinity	24	9-10	9.625
III. Low oxygen and low salinity	24	9-10	9.375
IV. Control	9	13-14	12.75

Fig. A. Initial lesions of necrosis seen as brownish pink coloured areas (arrow).

Fig. B. Brownish mustard-sized spots of necrosis (arrow).

Fig. C. Pinkish brown areas of necrosis (arrow).

Fig. 1. Muscle showing areas of necrosis and haemocytic infiltration. Note the loss of striation.

H & E x 400

Fig. 2. Photomicrograph showing non-inflammatory degenerative changes and oedema of the muscle.

H & E x 200

Fig. 3. Heart showing fragmentation of myocardial fibres with marked infiltration of haemocytes.

H & E x 400

Fig. 4. Hepatopancreas exhibiting severe necrosis. Note the loss of tubular cells.

H & E x 100

Fig. 5. Photomicrograph showing accumulation of haemocytes in the space between the exoskeleton (arrow) and muscle in a fibrinous network (F).

H & E x 100

Fig. 6. Higher magnification of Fig. 5 showing haemocytic infiltration.

H & E x 400

Fig. 7. Heart with degenerated myocardium showing formation of micro-nodules (arrow).

H & E x 400

Fig. 8. Higher magnification of Fig. 7.

H & E x 1000

Fig. 9. Heart showing extensive degeneration of myocardium with fragmentation. Note the hyperchromatic fibres.

H & E x 400

Fig. 10. Heart showing vacuolation in the myocardium (Fourth day post exposure).

H & E x 100

Fig. 11. Higher magnification of Fig. 10.

H & E x 400

Fig. 12. Heart exhibiting accumulation of proteinous materials in the myocardium (arrow). Note the lightly stained areas resembling fibrin (F).

H & E x 400

Fig. 13. Note the pericardial oedema with diffusely scattered haemocytes.

H & E x 100

Fig. 14. Degenerated hepatopancreatic tubules (DT) showing peritubular connective tissue with complete loss of tubular cells.

H & E x 100

Fig. 15. Compensatory hyperactivity of the tubules adjoining the degenerating tubules (arrow).

H & E x 100

Fig. 16. Hepatopancreas showing the shrinkage of tubules (arrow) and distention of connective tissue (CT).

H & E x 100

Fig. 17. Hepatopancreas showing the flattening of the lining epithelial cells (arrow).

H & E x 100

Fig. 18. Hepatopancreas showing the normal tubules (T) and the connective tissue (CT).

H & E x 100

Fig. 19. Gills exhibiting marked emphysema or distension of the gill lamellae with accumulation of fibrinous materials (F). Note normal looking lamellae at the bottom.

H & E x 100

Fig. 20. Degenerating myofibrils exhibiting "moth-eaten" appearance due to marked destruction of parenchyma.

H & E x 100

Fig. 21. Muscular necrosis (N) with areas of mineralization.

H & E x 100

Fig. 22. Higher magnification of Fig. 21 showing mineralization.

H & E x 200

Fig. 23. Pericardial sac showing thickening and scattered haemocytes.

H & E x 100

Fig. 24. Extensive tubular degeneration of hepatopancreas showing complete loss of architecture.

H & E x 100

Fig. 25. Emptied hepatopancreatic acini (A) with thickening of the connective tissue.

H & E x 200

Fig. 26. Gills exhibiting hyperplasia of lamellar cells.

H & E x 100

Fig. 27. Sub-serosal layer of gut showing increased number of granular eosinophilic cells (arrow).

H & E x 100

DISCUSSION

The aquatic environment is under constant changes. Sometimes, the changes are so great and sudden that the animals inhabiting it are unable to cope up with the changes. These changes in the environment brings about a lot of stress in the animals. These signs of stress become evident in the behaviour and histological levels. Conditions like low dissolved oxygen and low salinity are two most important parameters that affect the normal physiological and metabolic activities in aquatic animals. It has been observed that a lot of histomorphological changes is brought about by the effect of these stressors. The present study was aimed to study the changes that occur at the tissue level due to the effect of environmental stressors like low dissolved oxygen and low salinity.

Kramer (1975) observed hyperactivity of juvenile and sub-adult Penaeus aztecus in low salinity and low oxygen concentration of water. The hyperactivity consisted of swimming, surfacing and strong abdominal contractions. In the present study too, similar behavioural changes were observed in Penaeus indicus. The animals after introduction in the low salinity and low oxygen condition showed vigorous struggling movements, by beating rapidly the pleopods, and coming to the surface of water showing signs of stress by paddling.

Venkataramiah et al. (1975), suggested that the muscular lesions which are probably caused by anoxia during or following a sustained hyperactivity in deficient conditions can lead to full blown necrosis. Brownish areas of necrosis was noticed in the posterior abdominal region, in the low salinity condition in the present study. These necrotic areas predominating the distal

segments are possibly the most metabolically active parts, more readily displaying the consequences of hypoxia in the hyperactive state. These areas which appeared initially as brownish mustard-sized spots increased in dimension and turned opaque later. This might be due to the anoxic conditions as reported above.

Lakshmi et al. (1978) observed irregular white foci in the abdominal region, especially in the distal four to six segments of P. aztecus subjected to salinity and temperature changes. They reported hyperactivity of the shrimps exposed to salinity and temperature changes. Hyperactivity as observed by them was characterized by swimming restlessly, or trying to jump out of the tank. They suggested that the prawns developed necrosis when they became immobile after the hyperactive state. In the present study also the animals developed opacity in the immobile state following hyperactivity in the low salinity or low oxygen condition. It may thus be explained that during the state of immobility the body metabolism gets disturbed which may lead to pathomorphological changes like muscular necrosis as a result of hypoxia.

Haematological findings in the present study revealed that there is considerable increase in the total haemocyte count (T.H.C.) under the stress of low salinity, low oxygen alone and in combination of these two factors when compared to the animals reared under optimum salinity and oxygen. Similar results were obtained by Sindermann (1971), Couch (1978) and Persson et al. (1987).

Persson et al. (1987) observed an increase in the total number of circulating haemocytes in the crayfish, Pacifastacus leniusculus affected with the parasitic fungus Aphanomyces astaci.

Decrease in the haemolymph glucose content from that of the normal was observed in the case of the prawns exposed to low salinity and low oxygen. Similar observations were made by Brock (1983) in terminal growth (TG) prawns and by Remesh (1988). Remesh (1988) studied the T.H.C. and glucose content of soft prawns (Penacus monodon and P. indicus) collected from the culture ponds. He observed an increase in the T.H.C. and a decrease in the glucose content and explained that haemocytosis may be as a result of acute inflammatory condition while hypoglycemia may be due to energy debt in the tissues owing to improper carbohydrate metabolism as an after effect of stress. Although tolerance, resistance, salinity and oxygen acclimation studies have not been taken up, the results of the present work indicated that dissolved oxygen level and salinity should not be allowed to fall below 4 ml litre and 4 ppt respectively in order to avoid any physical damage to the animals. The mariculturist must establish a time period at which the culture pond dissolved oxygen and salinity must be elevated to the minimum acceptable levels before these can cause any deleterious effect on the body tissues.

Venktaramiah et al. (1975) opined that advanced state of necrosis in the abdominal region usually leads to the death of the animal. These workers have reported that necrosis is a major problem in bait-shrimp holding tanks and in intensive culture operations. Extreme changes in salinity and temperature have been associated with necrosis in P. aztecus that are confined either in laboratory aquaria or in bait-shrimp holding tanks (Lakshmi et al., unpublished manuscript).

Necrosis is common among the shrimps stocked in bait pens in conditions of overcrowding, low oxygen pressures and sudden salinity-temperature fluctuations (Anon, 1971a). It is reported that the shrimps developed necrosis

on being transferred directly from their normal salinity of about 30 ppt to lower concentrations of 1.4 and 3.0 ppt on many occasions.

In another report (Anon, 1971b) it was shown that the shrimps affected with necrosis developed white patches along the abdomen, which resulted in the death of the animal in a couple of days. In the present study, areas of necrosis were evident as brownish mustard-sized spots which increased in size and became very evident on the fifth day.

Cook et al. (1984) have observed that potentially acid sulphate soils are common limiting factors on numerous brackish water prawn farms experiencing low productivity in southern peninsular Malaysia. These workers however could not suggest the actual mechanism which produces slow growth and high mortalities of prawns reared in the ponds. They also opined that associated alterations in water chemistry are more detrimental to the cultured animals than the effect of low pH alone. In addition, low levels of dissolved oxygen has been suggested as one of the major factors implicated with low productivity and high mortality (Cook et al., 1984, Simpson et al., 1984). The behavioural and the histomorphological changes observed in P. indicus in this work, supports the view that low dissolved oxygen is one of the major factors causing retardation in growth and large-scale mortality.

Nash et al. (1988) studied the pathological changes associated with adult P. monodon cultured in grow-out ponds developed from potentially acid sulphate mangrove soils. Histological and ultrastructural study showed ferric hydroxide accumulation in the gills and associated gill changes which led to hypoxic damage in other tissues. The gills exhibited oedema and separation of the opposing epithelial layers. The lamellae also exhibited hypertrophic, hyperplastic, necrotic and inflammatory changes. Epithelial hyperplasia led to multi-

focal lamellar thickening and focal fusion. Similar histological changes were noticed in the present study among the experimental animals exposed to water with low oxygen content. Besides these, Nash et al. (1988) also elaborated that the cuticular epithelium and striated body musculature exhibited several degrees of damage, which consisted of focal to multifocal areas of necrosis and haemocyte infiltration. The muscle fibres revealed fragmentation, hyalinization and vacuolation. The hepatopancreatic tissue showed slight focal necrosis with occasional basophilic cytoplasmic intravacuolar inclusions. Massive myocardial necrosis was also observed at places. The necrotic myocardial fragments displayed pyknotic or karyorrhectic nuclei.

Nash et al. (1988) considered extensive tissue necrosis to be directly related to hypoxia. They also opined that the effective surface area of the gills is limited which interferes with respiration and in turn brings about hypoxia and associated changes in the various tissues. Almost all these changes in various tissues were identical to histopathological lesions observed in the present study in the low oxygen condition of water. Therefore, it may be agreed that the tissue necrosis can be a sequelae to hypoxic condition arising from low dissolved oxygen in water.

Momoyama and Matsuzato (1987) examined Penaeus japonicus with whitish opaque patches in the abdominal muscles. Histological examination revealed that the skeletal muscles of the whitish areas were necrotic showing cross splitting of myofibrils, haemocytic infiltration at the necrotic foci and proliferation of fibrocytes. In extreme cases the muscle fibres had disappeared and replaced by connective tissue. Environmental or physiological stress due to high water temperature, low dissolved oxygen and overcrowding has been suspected for the cause of muscle necrosis in shrimp, by these workers. However, they could not find out any relation between the occurrence of this

disease and the water quality of the shrimp culture ponds in their study. But in the present study similar results were obtained in the low oxygen condition and to some extent in the low salinity condition, which shows that any of these factors or in combination may be responsible for producing these ill-effects.

Nash et al. (1987) also observed similar diffuse 'milky' white body opacity in 28-day-old Macrobrachium rosenbergii post larvae, cultured under intensive conditions in a hatchery. They observed muscular necrosis, eosinophilia, fragmentation, flocculation, vacuolation and mineralization of the muscle. They have also reported 'moth-eaten' appearance of the muscle fibres in advanced lesions as was recorded in the present study in the third group where the experimental animals were exposed to low oxygen (2-3 ml/litre) and low salinity (2 ppt and 4 ppt). Pronounced histological alterations could be noticed on the fifth day. It may thus be deduced that muscular necrosis can be seen in various species like P. japonicus, P. monodon and M. rosenbergii, and low dissolved oxygen and/or low salinity might be the possible factors responsible for it. It was not clearly understood why necrosis of the muscle occurred in the distal 4 to 6 segments rather than in the proximal ones. Similar observations have been recorded by Lakshmi et al. (1978) who too could not attribute any reason for this.

Lee et al. (1985) observed melanized necrosis of the shell, of the cuticular lining of the foregut, hindgut and gill and in the parenchymal tissues of the hepatopancreas, gill and hindgut in the crayfish, Procambarus clarkii maintained in aquaria containing various levels of dissolved calcium and ammonia. Although similar changes were noticed in the muscle, heart, hepatopancreas and gills of the animals subjected to low salinity and low oxygen stress, alterations in the gut was recorded as negligible. It is therefore presumed that other than low dissolved oxygen and low salinity there may be

some other factors which lead to histological changes in the gut.

Muscle necrosis has also been reported in crustaceans by workers like Rigdon and Baxter (1970), Lightner (1983) in penaeids, Delves - Broughton and Poupard (1976), Lightner (1977), Akiyama et al. (1982) in M. rosenbergii and by Delves - Broughton and Poupard (1976) in Palaemon serratus. The present study agreed to the finding of Rigdon and Baxter (1970) who opined that parasites or bacteria were not the etiological agents for spontaneous muscle necrosis. Akiyama et al. (1982) suggested that the muscle necrosis is of an idiopathic nature and not an infectious type. Lakshmi et al. (1978) in their study on P. aztecus subjected to salinity and temperature change observed that the extensive structural degradation of the muscle cells in the transitional and necrotic zones indicated that initial damage was of a physiological nature, induced by abrupt salinity and temperature changes. Nash et al. (1987) suggested that the absence of any microorganisms in association with the premortem pathological myofibre alterations in M. rosenbergii under intensive conditions, in the hatcheries, might be as a result of idiopathic nature of the lesions.

Hyperactivity in habitually sedentary prawns has been described as the common consequence of many potential stressors which may result in idiopathic muscle necrosis (IMN) (Lakshmi et al., 1978). The later workers opined that hyperactivity is usually followed by a period of reduced activity leading to complete exhaustion and immobility; this state has been correlated with lactic acid accumulation (Spotts and Lutz, 1981) and with the occurrence of IMN (Lakshmi et al., 1978) in prawns. Lactic acid is believed to be the major cause of post-activity acidosis in crustaceans (Phillips et al., 1977, McDonald et al., 1979, and McMahan et al., 1979). This and other haemolymph constituents may require 24-48 hours to return to pre-stress levels (McMahan et al., 1978). During this period the animals are more susceptible to the effects of further

stress such as low dissolved oxygen or infection (Spotts & Lutz, 1981). Nash et al. (1987) suggested that stress-induced hyperactivity leading to rapid development of muscle hypoxia and accumulation of lactic acid during anaerobic glycolysis are the most likely steps in the pathogenesis of IMN in 28-day-old M. rosenbergii. The present finding was observed to be in agreement with the opinion of the above workers.

Remesh (1988) while working on soft shell disease in prawns, observed marked thinning of endocuticle, marked muscular necrosis, flattening of branchial epithelial cells, degenerated hepatopancreatic tubules, myocardial degeneration and invagination of serosal layer of gut in spontaneous cases of soft prawns (P. indicus and P. monodon). During the period of the above study, the pond salinity ranged from 20.3 ppt to 2.2 ppt due to continuous dilution by monsoon rains and the dissolved oxygen content varied between 3.73 to 4.21 ml/litre. Although similar histological changes were observed in the present experimental study in the animals exposed to low salinity and low oxygen condition, typical cases of soft-prawns could not be seen to occur, excepting in few, probably because of the short duration of the course of the experiment. However it can be assumed that low dissolved oxygen and low salinity in water might be two of the major possible reasons for the soft-shell syndrome in prawns. Whether the soft-shelling or muscular necrosis in prawns is a reversible process or not remains a question. However, Lakshmi et al. (1978) pointed out that initial stages of muscular lesions was a reversible process under purely oxygenated water rather than normal aerated water. This was proved by transferring a number of necrotic shrimp into aquaria supplied with oxygen and leaving others under normal aeration. They observed that unless the affected shrimp were transferred to a well oxygenated medium the lesions developed into full necrosis.

These workers observed that muscular necrosis need not be a real threat to the wild shrimp in natural conditions because they can escape unfavourable conditions by burying into the substrate or by migrating into deeper water. Captive shrimp stocked in ponds are more likely to be effected by necrosis as they have no place to escape unfavourable environmental conditions.

Rao et al. (1982) observed that rapid ecological changes due to heavy rainfall, overcrowding in ponds or chemical pollutants may affect the dissolved oxygen content, temperature and pH, which subsequently lead to impairment of the metabolic pathway in prawn.

Taking all these views into consideration and basing on the findings of the present study, it is hoped that the gross lesions and histomorphological changes recorded as a result of low oxygen and low salinity, may serve as an eye opener for the prawn culturists. It can thus be advocated that the ponds consisting of sloping bottoms, soft substrates and capacity to exchange water volume quickly in order to maintain optimum oxygen and salinity level may perhaps reduce morbidity and mortality in prawn culture system.

SUMMARY

- (1) The present experimental study was carried out on Penaeus indicus maintained under low salinity and low oxygen conditions in the laboratory to find out the behavioural and histomorphological changes in these animals.
- (2) The experiment was carried out under 3 sets of conditions viz.,
 - (i) Sea water with low oxygen and optimum salinity.
 - (ii) Sea water with low salinity and optimum oxygen, and
 - (iii) Sea water with low oxygen and low salinity.
- (3) The low oxygen condition (2-3 ml/litre) was obtained by providing only mild aeration. The low salinity condition (S = 1 ppt, 2 ppt, 3 ppt and 4 ppt) was obtained by diluting the sea water with required amount of fresh water. The amount of sea water required was obtained using the following formula:
$$\text{Volume of sea water required} = \frac{\text{Desired salinity} \times 1000}{\text{known higher salinity of sea water.}}$$
- (4) The changes observed in the prawns were categorised under three main headings i.e. (i) Gross morphological changes (ii) Haematology and (iii) Histopathology.
- (5) Changes under low oxygen condition
 - (i) Gross morphological changes were characterized by pinkish to brownish necrotic areas of opacity ranging from 4-8 mm in diameter in the posterior abdominal region.

(ii) Haematological studies revealed an increase in the total haemocyte count (T.H.C) and a decrease in the haemolymph glucose content.

(iii) Histopathological studies revealed oedema, focal to multifocal areas of degeneration with mild to moderate infiltration of haemocytes in the muscle. Myocardial fibres showed extensive fragmentation and marked infiltration of haemocytes. Thinning of epicuticle was a regular finding. Hepatopancreatic tubules exhibited severe tubular necrosis. Gill epithelium in the lamella exhibited flattened appearance due to atrophy. No specific lesions in the gut could be observed.

Changes under low salinity condition

(i) Gross morphological changes were characterized by cuticular lesions in the third, fourth and fifth abdominal segments and tips of pleopods by about the fourth day. Thinning and softening of the exoskeleton was observed in 50% of cases.

(ii) Haematological studies revealed an increase in the T.H.C. and a decrease in the haemolymph glucose content.

(iii) Histopathological studies revealed extensive muscular necrosis. There was marked separation of the exoskeleton from the underlying muscle tissue with accumulation of haemocytes in the emptied space. Heart revealed areas of vacuolation with marked to moderate haemocytic infiltration. Shrinkage of hepatopancreatic tubules with flattened cells and distention of connective tissue was observed.

Distention of gill lamellae was a prominent feature in the gill. No specific lesions was observed in the gut.

(7) Changes under low oxygen and low salinity

(i) Gross morphological changes were characterized by small brownish areas of necrosis in the posterior abdominal muscles.

(ii) Haematological studies revealed an increase in the T.H.C. and a decrease in the haemolymph glucose content.

(iii) Histopathological changes were characterized by marked muscular necrosis, with "moth-eaten" appearance. Heart showed myocardial degeneration accompanied by oedema and haemocytosis. Hepatopancreatic tubules exhibited marked tubular degeneration with complete loss of architecture. Empty hepatopancreatic acini were also observed. Branchial cell hyperplasia was noticed in gills. Gut exhibited increased number of granular eosinophilic cells in the sub-serosal layer.

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