केन्द्रीय समुद्री मात्स्यिकी अनुसंधान संस्थान
CENTRAL MARINE FISHERIES RESEARCH INSTITUTE
(भारतीय कृषि अनुसंधान परिषद)
(Indian Council of Agricultural Research)
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SOME IMMUNOBIOLOGICAL ASPECTS OF THE SPINY LOBSTER *PANULIRUS HOMARUS* (LINNAEUS, 1758)

THESIS SUBMITTED IN PARTIAL FULFILLMENT
OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

Fish and Fisheries Science (Mariculture)

OF THE
CENTRAL INSTITUTE OF FISHERIES EDUCATION
(DEEMED UNIVERSITY)
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BY

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JULY 2003

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CERTIFICATE

Certified that the thesis entitled **"SOME IMMUNOBIOLOGICAL ASPECTS OF SPINY LOBSTER *PANULIRUS HOMARUS* (LINNAEUS, 1758)"** is a record of independent bonafide research work carried out by **MISS. BINDHU VERGHESE** during the period of study from September 1998 to February 2003 under our supervision for the degree of **Doctor of Philosophy** in Fish and Fisheries Science **(Mariculture)** and the thesis has not previously formed the basis for award of any degree, diploma, associateship, fellowship or any other similar title.

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I hereby declare that the thesis entitled **"SOME IMMUNOBIOLOGICAL ASPECTS OF THE SPINY LOBSTER *PANULIRUS HOMARUS* (LINNAEUS, 1758)"** is an authentic record of the work done by me and no part thereof has been presented for the award of any degree, diploma, associateship, fellowship or any other similar title.

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सारांश

पालूलिरस होमारस एक वाणिजा प्रधान महाचिंगट है जिसको अर्न्तरष्ट्रीय बाजारों में बडी माँग है. सजीव अवस्था में इसका निर्यात होता है, पर माँग में स्थास्थ्य अवस्था का बडा अमर है. इसके प्रतिरोधी अभिलक्षण इस अध्ययन का विषय था. *पी. होमारस* के रक्तलसिकाओं (haemolymph) में तीन प्रकार के कोश हैं, ये हैं हाइलिन, सेमिग्रानुलर और लार्ज ग्रानुलर कोश. रक्ताणु (haemocyte) का उद्‌भव रक्त जनन ऊतकों से होता है जो कि जीव के एपिगास्ट्रिक भाग में स्थित है. इसका आकार 30-875 micro gm है. एक स्वस्थ महाचिंगट का रोग प्रतिरोध स्थिति में रक्ताणु का काऊंट (count) 8.7 + 3.8X10^6 कोश मिली $^{-1}$ और फीनोलोक्सिडेस सक्रियता (phenoloxidase activity) 30.61+5.6 1U मिग्रा प्रोटीन $^{-1}$ मानी गई है. शारीरिक दंड जैसे छिल्का उतारना (moulting) , नेत्रवृंत अपरदप (eyestelk ablation) देने पर प्रतिरक्षी प्राचलों (immune parameter) में व्यतियान दिखाया पडा (*पी* < 0.05%). भूखी स्थिति में रक्ताणु का कुल काऊंट और फीनैल ऑक्सिडेस सक्रियता कम होते हुए दिखाई पडी. खाद्य पर किये प्रतिरक्षी परीक्षणों से व्यक्त हुआ कि मछलियों की तुलाना में शंबुओं से खिलाना प्रतिरोध की दृष्टि से अच्छा है. पर्यावरणीय घटक जैसे लवणीयता, pH , विलीन आक्सिजन और अमोनिया ने *पी.होमारस* के प्रतिरक्षी तंत्र में सुव्यक्त प्रभाव दिखाया. सूखी अवस्था में परिवहन करने पर फीनोल आक्सिडेस सक्रियता को दबाते हुए देखा जबकि रक्ताणुओं की काऊंट में व्यतियान नहीं था. बाक्टीरिया टीकाकरण में, पहले 30 मिनिटों में रक्ताणुओं का निम्नतम काऊंट और फीनोल आक्सिडेस की उच्चतम सक्रियता दिखायी पडी. दोनों 24 घंटों के बाद पूर्वस्थिति में पहूँच गए. लामितारिन जो समद्री शैवाल से बनाया निचोड है, से टीकाकरण करने पर भी समान परिणाम देख गया. चिटोसान जोडके बनाए गए देशी खाद्य प्रतिरक्षी प्रणाली की क्रियाविधियों को बढाते हुए देखा. वाणीज्यक प्रतिरोद उत्तेजकों के प्रयोग करने पर प्रतिरक्षा में देखनेलायक गुण नहीं दिखाया था. कवच प्रणियों के स्वास्थ्य प्रबंधन में सूचकों के रूप में, फीनोल आक्सिडेस विश्लेषण और रक्ताणुओं के काऊंट स्वीकार जा सकता है. यह खेतों के मोनिटरन केलिए भी अपनाया जा सकता है. जलकृषि के सफल विकास केलिए जन्तुओं की स्वास्थ्य स्थिति का नियमित मॉनिटरन अत्यंत महत्वपूर्ण मुद्दा है.

ABSTRACT

Panulirus homarus (Linnaeus, 1758) being a species of great commercial importance fetches high demand in the international market. The demand of live lobsters depends on the health status of the animals. Study on the defence mechanisms in *P. homarus* showed three cell types in the haemolymph: hyaline cells, semi granular cells and large granular cells. The haemocytes are produced from the haematopoetic tissue located in the epigastric region, they are lobular with a size range of 30-875 µm. The immune status of healthy lobsters showed that the total haemocyte count was $8.7 \pm 3.8 \times 10^6$ cell ml^{-1} and the phenoloxidase activity 30.61 ± 5.6 IU mg $protein^{-1}$. Studies on physiological stress like moulting, eyestalk ablation showed variation in the immune parameters ($P < 0.05$). Upon starvation the THC and PO activity was found to decrease drastically. Feed trials showed that mussels, the preferred diet of lobsters was found to show better immune response over those fed with fish diet. Environmental stress factors like salinity, pH, dissolved oxygen and ammonia showed significant effect on the immune system of *P. homarus*. Live transportation of lobsters by dry method was found to suppress the PO activity but no change was seen in THC. Injection studies with bacteria showed the lowest peak during 30 minutes incubation for THC whereas it showed highest peak for PO activity for the same incubation time. Both THC and PO levels came back to normal after 24 hours. On injection with laminarin an immunostimulant similar result was obtained. Farm made artificial feed supplemented with chitosan was found to enhance the activity of the immune system. Satisfactory result for immune enhancement was not observed with commercial immunostimulant supplement. Analysis of serum PO activity and total haemocyte count can be used as indicators of the health in crustaceans, which can be monitored, even in farm conditions. Routine monitoring of health status is of great importance with regard to successful aquaculture practices.

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INTRODUCTION

1. INTRODUCTION

Farming of crustaceans like shrimps, prawns and crabs are carried out on a large scale globally. Commercial spiny lobster culture is yet to become a reality, as the hatchery technology for seed production has not so far been perfected, though larval cycle of a few species have been completed. The potential of lobster farming is enormous as live lobsters fetch the highest unit price in the international market. The local merchants hold live lobsters purchased from the fishermen in small tanks until sold to the exporters. The poor holding conditions result in stress and poor health leading to mortality during transportation. Crowding also results in accumulation of metabolic wastes, which attracts pathogenic bacteria and thereby causes infections. Some of the exporters hold smaller grade, low priced lobsters for short-periods until they moult and grow to the next higher price grade. Diseases may set in by poor management of the holding and fattening systems. Faster growth and higher survival can be expected only when the animals are healthy. An animal is said to be healthy when the physiological processes controlling growth, defense and reproduction are normal. On the other hand, a stressed or diseased animal is one, which experiences some form of a physiological imbalance and threatens its survival. Stress results in damage to body tissues resulting in abnormal physiological function. The physiological damage may be the consequence of a sudden environmental change: either prolonged exposure to air (outside the water) or to a chemical toxin or the invasion of body tissues by infectious organisms. Exposure to these agents stimulates a whole state of physiological mechanisms, the host defense and immune processes, in order to counteract the threat and repair of the damaged tissue.

Different types of physical, chemical and biological agents cause disease in aquatic crustaceans, which is mostly due to exposure to pathogenic organisms such as viruses or bacteria, inadequate nutrition or due to changes in culture conditions. Diseases may be mild with little adverse effects on the animal or severe that threatens the survival of the organism. Whether or not a lobster will succumb to a disease is dependent on the physiological state of the host, the

environment and the invading organism. Decapod crustaceans exhibit a wide range of host defense reactions, which are aimed at preventing tissue injury or infections (Smith and Chisolm 1992; Bachere *et al.* 1995; Evans *et al.*, 2000). The success of these defense reactions in preventing or overcoming the disease is strongly influenced by the existing health status of the host at the time of exposure. Health status in turn is influenced by prior exposure to environmental stressors. Excessive stress in response to the environmental stressors weakens lobsters and pre-disposes them to the disease.

In order to avoid disease outbreaks, it is essential that stock health is optimised within the economic constraints of the production or holding system. However, while optimal stock health shall be the aim of any processor or aquaculturist, this is difficult to achieve in practice. Under most rearing and holding conditions, lobsters are exposed to various forms of environmental stressors, e.g. crowding and confinement, adverse water quality, handling and grading procedures, and all these have the potential to affect health. In well designed and properly maintained holding systems, the level of stress experienced by the lobsters does not significantly compromise performance or product quality. The successful manager is able to balance the trade-off between operational costs and stock health in such a way as to maximise financial returns from the operation. On the other hand, in poorly designed or maintained systems, stock health is compromised resulting in reduced production, poor product quality and lesser profits. Disease prevention through effective health management shall therefore be the goal of any commercial operation based on either lobster wild-stock harvest or aquaculture.

Lobsters are stressed when a factor called stressor causes their internal physiology to deviate from the normal. These responses are normal physiological reactions to changes in environmental conditions. These conditions include a wide range of factors such as water quality parameters (oxygen levels, pH, salinity, temperature, presence of metabolic wastes), physical factors (handling, injury, air exposure), behavioural interactions and nutrient availability. Exposure to these stressors leads to short and long term changes in cardiovascular and respiratory functions, energy metabolism, fluid and ionic

balance, acid-base balance and immunity (Barton and Iwama, 1991; Thompson *et al.*, 1993; Hall and van Ham, 1998). If the stressor is mild and of short duration, the physiological disturbances are temporary. However, if the stressor is extreme, or if there is prolonged exposure even to a mild stressor, long-term detrimental effects can occur. These include reduced resistance to disease, poor growth, impaired reproduction and low survival (Pickering and Pottinger, 1989; Lee and Wickins, 1992). During post-harvest handling of lobsters, both mild and extreme stresses are likely to occur. In the latter case, the physiology of the lobster may be so disturbed as to result in mortality.

Physiological stress is a likely cause of mortality in cultured and post harvest lobsters. Moulting, though a physiological activity is a high energy demanding physiological process, which cause immense stress that consequently lead to mortality, under unfavourable conditions. Environmental stresses like hypoxia stress can be caused by reduced oxygen in culture tanks and this condition is also encountered during post-harvest handling and transportation. Low dissolved oxygen is a critical environmental factor especially during moulting and after feeding when higher levels of oxygen are required to meet the energy demands. Variations in water temperature in culture systems can cause temperature stress. However, this is not a serious problem in tropical conditions as there is not much variation in the temperature of culture water. Similarly dip treatments for live packing is yet another factor that may cause temperature stress. Sudden exposure to high and low salinity in culture environments can cause salinity stress. Fluctuation in pH in the culture tanks is yet another important stress factor. High light intensity, crowding, isolation and aggression can also lead to stress. High levels of ammonia, especially un-ionized ammonia from excretion or from the decomposition of uneaten feed remains is found to be lethal to the animal. Exposure to heavy metals and other dissolved metals like copper, which leaches from the cooling tubes during temperature conditioning at the time of packing is also likely to cause stress and mortality.

Inadequate and poor nutrition may result in stress, which make the animal weak and susceptible to infection. Pathogenic organisms cause disease out-breaks, which affect the normal physiological process and lead to mortality.

A healthy animal can withstand the challenge of a disease agent better than one in poor health, unless the agent has the capacity to cause disease regardless of the health status. Some viruses and highly virulent bacteria and some form of nutrient deficiencies or chemical exposures fall in the latter category. Crustaceans respond to these stress factors by their innate immune system, consisting of both cellular and humoral factors. The cellular immune system includes phagocytosis, encapsulation, nodule formation, phenoloxidase activity etc; and the humoral system includes agglutination, bactericidal activity and killing factors. Haemocytes play a crucial role in the immune response because of their participation in phagocytosis, encapsulation, nodule formation and cytotoxic mediation. Examination of haemolymph is one method to study whether the animal is under stress, as it is known that infectious diseases reflect back on the blood of crustaceans (Bang, 1971). It has been reported that infections reflect on the quality of haemolymph in crustaceans. Many of the parameters that can be measured in crustacean blood are stress indicators of some kind (Paterson and Spanoghe, 1997). Ofcourse, simple information that the lobsters are stressed after harvest may not be of much practical use. Of increasing concern particularly in relation to the success of aquaculture ventures is the effect of environment (season, temperature, salinity, diet, reproduction, pollution etc.) on immunity and hence, disease resistance by the host. In the case of spiny lobsters occurring in Indian waters, nothing is known about the impact of environmental parameters on the defense mechanism. The objective of the present study is therefore, to understand the immune status of a stressed animal from that of a normal animal. In order to assess the physiological state of an animal under stress compared to a normal lobster, the studies were conducted on variations in haemocytes number and defence reaction in response to factors like culture conditions, diet, different types of stress and starvation. The effect of immunostimulants in improving the functional properties of haemolymph, and thereby enhancement of immunity was also investigated.

Immune system parameters such as haemocyte counts (Stewart *et al.*, 1967; Persson *et al.*, 1987; Evans *et al.*, 1992; Sequiera *et al.*, 1996; Jussila *et al.*, 1997; 1999), bactericidal activity (Söderhäll and Cerenius, 1992, Ueda *et al.*, 1994, 1999), haemolymph clotting (Jussila *et al.*, 2001), phenol oxidase activity

(Le Moullac *et al.,* 1998; Cheng and Chen, 2000) and superoxide anion production (Sung *et al.*, 1998) have been used as rough stress or disturbance indicators in crustaceans. However, in Indian spiny lobsters, no detailed study was conducted in relation to the stress factors. The present study was undertaken to study these responses for improvement in lobster holding and farming operations so that appropriate management strategies could be formulated to overcome disease situations and thereby prevent economic losses. Lobsters being a high value seafood, product quality is important and therefore the study is of utmost commercial significance.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

2.1. Immune response mechanism

Studies on the internal defense mechanism in crustaceans began since the work of Elie Metchnikoff in the 1890's, the pioneering work on phagocytosis and inflammatory process. Like wise, Cantacuzene started his investigation on humoral defense responses and came out with a series of papers from 1912 to 1934. But unfortunately, the work was languished until 1960's when Sinderman (1971) renewed interest on research on the major crustacean diseases of economic significance, particularly 'Gaffkaemia' in lobsters and fungal infections in crayfish. It is commonly agreed that arthropods and invertebrates in general, don't possess immunoglobulin (Ratcliffe *et al.*, 1985), or memory following the first encounter with a pathogen. In other words, they do not possess an acquired immunity like the vertebrates (Schapiro, 1975). Recently, invertebrates were found to possess an innate, non–adaptive immune system employing a large variety of circulating molecules. Arthropods with their open circulatory system have immediate, non-inducible defense and coagulation mechanisms to entrap parasites and prevent blood loss. These reactions are carried primarily by the circulating haemocytes (Lackie, 1980). Circulating cells also mount phagocytic, cytolytic or inflammatory responses which broaden the number of immune response the invertebrates can employ in response to an invasion (Roch, 1999). Absence of immunoglobulins invertebrates are potentially useful models for analysis of non-specific processes such as Ig-independent phagocytosis and cytotoxicity, despite the numerous observations of the various humoral and cellular defense reactions in different species (Ratcliffe and Rowley, 1979).

The primary immune response in crustaceans is non specific cellular immunity (McCumber, 1983; Anderson, 1992). Arthropods possess very effective defense mechanisms for sequestration and clearence of foreign bodies from the haemocoel. The defense mechanisms are usually detected as cellular and humoral responses to bacterial infection and parasites (Soderhall and Unestam,

1979), the main role of which are to maintain the exoskeletal integrity, foreign agent recognition, inactivation and elimination from the internal organs, and repair of the wounds. All these activities are not mutually exclusive as they depend on circulatory haemocytes, fixed phagocytes and fibrocytes.

The chitinous exoskeleton of spiny lobsters is an effective barrier, which prevents the entry of infectious agents, provides muscle anchorage and also protects the underlying soft tissue. The first barrier presented by exoskeleton against invasion is a very thin proteolipid epicuticular membrane (Unestam, 1973; Malloy, 1978; Fisher, 1988). Underlying this is the exocuticle, which is an impermeable layer for even the pathogens which are capable of producing extra cellular chitinases. In contrast, the soft endocuticle is easily penetrated by such agents (Unestam, 1973). Maintenance of the epicuticle depends on the diet (Fisher *et al.*, 1976) and therefore it is evident that the rate of infection also depends on the nutritional status of the host. Rapid sealing of wounds in exoskeleton is very essential to prevent loss of haemolymph and to minimize the opportunistic invasion. Once the pathogen gains entry into the body, the innate immune system starts its action.

2.2. Cellular factors of innate immunity

The circulating haemocytes of crustaceans *in vivo* and other invertebrates play a crucial role in the defense mechanisms in crustaceans (Rabin, 1970). Studies on crayfish *Parribacus leniusculus* and the shore crab *Carcinus maenas* show that crustaceans possess different functions in immunity such as phagocytosis, encapsulation, cytolysis, haemolysis, cytotoxic mediation, cell adhesion and degranulation (Smith and Soderhall, 1983 ; Johansson and Soderhall, 1985; Martin and Graves, 1985; Ratcliffe *et al.*, 1985; Soderhall and Smith, 1986; Persson *et al.*, 1987a; Kobayashi *et al.*, 1990; Soderhall and Cerenius, 1992; Bachere *et al.*, 1995). Crustaceans have three morphologically different haemocytes: hyaline, semi-granular and granular cells (Hearing and Vernick, 1967; Bauchau, 1981; Michael *et al.*, 1980; Sternshein and Burton, 1980; Blaxhall, 1981; Soderhall and Smith, 1983; Benjamin and James, 1987; Hose *et al.*, 1987; Tsing *et al.*, 1989; Hose *et al.*, 1990; Manjula *et al.*, 1997; Jussila *et al.*, 1998). It has been accepted but not proved that the three types of cells represent

the developmental stages of a single cell line, with granulocytes being the terminal stage (Bodammer, 1978; Mix and Sparks, 1980; Jussila *et al.*, 1998). However, there is lot of confusion regarding the classification of haemocytes. Cornick and Stewart (1978) described four types of cells in *Homarus americanus* based on cell histochemisrty and morphology. They described two hyaline types, two granular types, one eosinophilic and one chromophobic. Williams and Lurtz (1975) presented evidence of five types in *Carcinus maenas* based on histochemistry. They classified granulocytes based on the glycogen staining of granules. Baracco and Amirante (1992) found two groups of semi-granulocytes in *Squilla mantis* which were ultrastructurally distinct. Johnston *et al.* (1973) reported two types of haemocytes cell lines in *Carcinus maenas*, the alpha cells and the beta cells.

Hose and Martin (1989) found that hyalinocytes initiate coagulation and they lyse in the presence of bacterial toxins and seawater, while granulocytes and semi-granulocytes are involved in phagocytosis and encapsulation. Hyaline cells are typical phagocytic cells (Soderhall *et al.*, 1986; Bauchau, 1981). The semi-granular cells also have phagocytic capacity, but they encapsulate non-specifically to a variety of particles (Persson *et al.*, 1987a) and are labile. They degranulate spontaneously to some extent on glass cover slips *in vitro* (Soderhall and Smith, 1986; Johansson and Soderhall, 1985) or when encapsulating particles. They have small granules in their cytoplasm. Granular cells are almost fully packed with large granules (Soderhall and Smith, 1986; Bauchau, 1981). Semigranulocytes and granulocytes adhere readily to glass and plastic and releases long filipodia in the haemocytes as in *Cancer irroradiatus* (Newman and Feng, 1982), *Homarus americanus* (Goldberg *et al.*, 1984, 1986) and *Squilla mantis* (Barraco and Amirante, 1992). It has been reported that during fungal infection a specific β-1,3 – glucan binding protein (β GBP) in the plasma recognises and binds the fungal cell wall (Duvic and Soderhall, 1990). Then semi-granular and granular haemocytes respond to β GBP complexed with glucans by degranulation and release of the proPO activating system (Soderhall and Cerenius, 1998), which includes cell adhesive and opsonic protein (Tyson and Jenkin, 1973) and peroxinectin from storage granule (Johansson *et al.*, 1995). Finally, released peroxinectin stimulate phagocytosis by hyaline cells (Thornqvist *et al.*, 1994) or encapsulation by semi-granular cells (Kobuyashi *et al.*, 1990).

The number of free haemocytes can vary and can, decrease dramatically during an infection (Persson *et al.*, 1987b; Smith and Soderhall, 1983a; Smith *et al.*, 1984; Lorenzon *et al.*, 1999). Thus, new haemocytes need to be compensated and proportionately produced and it is commonly believed that haemocytes are released continuously, although at varying rates from the haematopoietic tissue. The location and function of the haematopoietic tissue is not well understood. In most crustaceans, the sheet like haematopoietic tissue is situated on and covers the dorsal and dorsolateral sides of the stomach and is surrounded by the connective tissue (Johansson *et a*l., 2000). In *Nephrops norvegicus,* it is believed to be a thin sheet of tissue on the dorsal and lateral surfaces of the cardiac stomach, and probably on the floor of the cephalic cavity as it shows a marked seasonal cycle of activity (Field and Appleton, 1995). The location in prawns and shrimps is better known (Bell and Lightner, 1988) and haematopoietic tissue has been found to occur in the same locations in spiny lobsters. Haematopoietic cells of different morphology are organized and densely packed in small lobules, and some of these morphological cell types are seen in the interlobular spaces. The situation is found in the crab *C. maenas* (Ghirett-Magaldi *et al.*, 1977), the lobster, *Homarus americanus* (Martin *et al.*, 1993a) and the crayfish *P. leniusculus* (Chaga *et al.*, 1995). The arrangement is found to be different in shrimps such as *Sicyonia ingentis* (Hose *et al.*, 1992), where the haematopoiesis occur in paired epigastric haematopoietic nodules.

Among the cellular response in crustaceans, phagocytosis has received the most attention and there is considerable evidence from *in vitro* studies that this process plays an important part in the removal of foreign particles from blood (Reade, 1968; McKay and Jenkin, 1970; Anderson *et al.*, 1973; Paterson and Stewart, 1974; Tyson and Jenkin, 1974; Paterson *et al.*, 1976; Smith and Ratcliff, 1978; Amstrong, 1979; Brehelin and Arcier, 1985; Bayne, 1990; Sagarista and Durfort, 1990; Bell, 1993). Cell co-operation and communication are necessary for most of the defense mechanisms, which occurs when a parasite is recognized and when an immune response is initiated (Tyson and Jenkin, 1974; Segal, 1984; Soderhall and Smith, 1986; Johansson and Soderhall, 1989; Soderhall and Cerenius, 1992). Phagocytosis and encapsulation is a universal phenomenon amongst the invertebrates. The mechanism of

phagocytosis has been studied in insects and molluscs extensively. Phagocytosis is a defense employed mechanism when the foreign agent is smaller than the haemocyte (Hed, 1986).

When micro organisms are engulfed by these haemocytes, a series of microbicidal substances including reactive oxygen species are produced during phagocytosis, in a process called respiratory burst (Leslie and Allen, 1987; Leslie, 1987; Song and Hsieh, 1994). The generation of $O_2^{\cdot}$ (superoxide anion) has been reported in haemocytes of shore crab *Carcinus maenas* (Bell and Smith, 1993) the tiger shrimp *P. monodon* (Song and Hsieh, 1994) and the blue shrimp *P. stylirostris* (Le Moullac *et al.*, 1998). If foreign particles are larger than 10 μm in diameter, encapsulation rather than phagocytosis occurs or they are immobilized by nodular aggregation (Ratner *et al.*, 1983; Soderhall *et al.*, 1984a; Ratclifffe, 1985) following clotting. Encapsulation reactions by haemocyte preparations obtained from *Panulirus interruptus* were studied by Hose *et al.* (1990) who showed that the reaction involved the semi-granular haemocytes and fibrocytes. Haemocytes (granulocytes and semi-granulocytes) cluster around the foreign body forming encapsulations that are many cell layers thick. The outer cells retain a more normal shape while inner cells become flattened. Diffuse melanisation occurs in the compact core and in the intracellular matrix forming a thick brown leathery capsule. Such capsules are not resorbed. Encapsulation response is merely the clumping of basophilic amoebocytes around damaged tissue as in earthworms or a more structured capsule may be produced. Encapsulation in insects may take several forms or involves several types of haemocytes, depending on the species of the insect investigated. The adhering cells may form the three distinct layers or may be less organized capsule with two distinct regions (Smith and Ratcliffe, 1978).

Inflammation has been studied in detail in penaeid shrimps and the process appears to be identical in spiny lobsters (Martin *et al.*, 2000). Injection of shrimp with carmine is followed by accumulation of carmine in the dorsal abdominal vein, heart and gills. By 30 hr post injection, carmine was observed only in the gills, heart and injection site (Fontaine and Lightner, 1974). Histologically the carmine forms tightly packed extra cellular masses, at the

injection site, which are infiltered and phagocytosed by haemocytes. Circulating carmine particles are then trapped by fixed phagocytes lining the blood vessels and in sinusoids of the gill filaments. These particles finally accumulate in the distal gill filaments and heart. Brown melanised nodules consisting of necrotic haemocytes containing phagositised carmine develop in the periopods and as cysts in the connective tissues of the gill cover by a process of filtration rather than through the action of fixed phagocytes and are subsequently shed at moulting (Martin *et al.*, 2000). Carmine containing haemocytes also migrate through the midgut epithelium and into the lumen of the antennal gland. Smith and Ratcliff (1980) studied clearance of foreign agents from gills of the crab *Carcinus maenas*. They found that there were two mechanisms in operation: aggregation of haemocytes into 12-25 µm diameter clumps of 5 to 50 haemocytes containing trapped bacteria; and the formation of elongate, diffuse networks of phagocytic haemocytes in the gill blood sinuses. Aggregation of semi-granulocytes and granulocytes is accomplished by a combination of binding by pseudopodia and humoral factors. This occurs in response to foreign agents such as *Vibrio* sp. (Johnson, 1976; Newman and Feng, 1982) and is the precursor to encapsulation for foreign agents too large to phagocytise. Aggregation is often accompanied by extensive pre-mortem clotting of plasma and, in severe cases, the aggregation and plasma clotting can obstruct haemolymph leading to massive focal necrosis (Johnson, 1976). Haemocytes also cluster around clots which are presumably resolved in time.

Degranulation is also a neglected area of study in spiny lobsters. Observation of histological material shows that mature granulocytes appear to aggregate near foreign agents and degranulate in the same way as molluscan haemocytes. This process was studied in quahog *(Mercenaria mercenaria*) by Mohandas *et al.* (1985) who showed that bacteria stimulate haemocytes to extrude intact lysosomes into the haemolymph, a process referred to as degranulation. The resulting release of lysosomal hydrolases is assumed to be responsible for associated host and non-host tissue damage (Feng, 1988; Hose and Martin 1989, Watanabe, 1999) and may be one mechanism by which bactericidal activity is seen to rise in lobster haemolymph after inoculation of formalin-killed bacteria (Sinderman, 1971). Smith and Soderhall (1983b) studied

the induction of degranulation and lysis of haemocytes in the fresh water Cray fish. HLS in shrimp was found to be capable of cell adhesion and degranulation of penaeid haemocytes (Perazzolo *et al.*, 1997). Recent work on degranulation of human eosinophils has suggested that the eosinophils do not discharge granules to the cell surface (exocytosis) but by lysis (Watanabe, 1999), and the process in lobster haemocytes may be the same.

Reactions leading to wound repair in spiny lobsters consist of rapid haemocyte accumulation and aggregation at the wound site followed by intravascular clotting. Cells circulating in the haemolymph of crustaceans are thought to play important role in wound repair (Fontaine and Lightner, 1973). Clotting is initiated by contact of hyalinocytes with seawater (Hose and Martin, 1989). The clot results from direct conversion of a soluble fibrinogen (coagulogen) into cross linked fibrin through the action of a coagulin released by haemocyte rupture (Fuller and Doolittle, 1971; Durliat and Vranckx, 1981; Ghidalia *et al.*, 1981; Hose *et al.*, 1990; Clare and Lumb, 1994; Aono and Mori, 1996). This is followed by melanisation of the wound area to form a dense black membrane beneath which the new epidermis forms. Melanin is produced by the action of the enzyme polyphenoloxidase on melanin precursors (Unestam and Nylund, 1972; Bauchau, 1981) and has antimicrobial properties (Nyhlen and Unestam, 1980; Söderhäll and Ajaxon, 1982). The epidermis involutes into the wound utilizing the haemocyte network as basal support. New cuticle is formed by this epidermal layer and lies beneath the melanin membrane (Fontaine, 1975). In association with the haemocyte response a dense network of collagen-like fibres forms. This fibrous tissue is not resorbed but remains as a scar (Fontaine and Lightner 1975). The phenomenon has been extensively studied in chelicerates, mainly *Limulus polyphemus* and its close relatives. Several excellent accounts of clotting in arthropods have already been published (Durliat, 1985; Levin, 1985). The haemolymph coagulation mechanism of the spiny lobster *Panulirus japonicus* was investigated *in vivo*. Aono and Mori (1996) found that plasma induces cytolysis in hyaline and semi granular cells; the clotting enzyme transglutaminase is released by lysis of these haemocytes as the enzyme causing gelation of plasma. Haemolymph clotting time was used as an indicator of stress in *Panulirus cygnus* (Jussila *et al.*, 2001). Changes in the number of circulatory haemocytes and

clotting time has been claimed to be an indicator of stress in crustaceans (Durliat and Vranckx, 1983; Persson *et al.*, 1987; Smith *et al.*, 1995; Jussila *et al.*, 1997, 1999).

In addition to phagocytosis and encapsulation, arthropods have further defense mechanisms to ward off of foreign objects by a process called melanisation, deposited either by cells in the capsule, formed during encapsulation(Chakrabarthi, 1993) or by precipitation from haemolymph (Poinar and Leutennegar, 1971; Fisher and Brady, 1983). Many crustacean researchers have documented that the proPO activating system functions in non-self recognition and host defense (Soderhall , 1982; Ratcliffe *et al.*, 1985; Smith and Soderhall, 1991; Soderhall *et al.*, 1994). Foreign agent recognition, inactivation and elimination is effected through both cellular and humoral host defense responses. Immuno-recognition is thought to be mediated through the prophenoloxidase system, a cascade of serum proteases and prophenoloxidase present in the haemocytes, which is activated by the presence of non-self molecules initiating melanization (Soderhall *et al.*, 1983; Soderhall and Smith, 1986; Soderhall *et al.*, 1990; Cereinius and Soderhall, 1995; Soderhall *et al.*, 1996; Sung *et al.*, 1998; Gollas-Galvan *et al.*, 1999). Melanization is induced by the complex enzymatic cascade, which involves the activation of enzyme prophenoloxidase (proPO) to its active form phenol oxidase (PO). Phenoloxidase is present in the inactive form in many arthropods and is activated by proteases (Ashida *et al.*, 1974; Soderhall and Unestam, 1979; Soderhall and Cereneus, 1992; Aspan *et al.*, 1990, 1995) organic solvents or detergents (Inabe *et al.*, 1963). In addition to the denaturing agents and physical conditions like temperature, pH, dissolved oxygen can also affect proPO activity (Ashida and Soderhall, 1984; Dunphy, 1990; Brivio *et al.*, 1996; Le Moullac *et al.*, 1998). In crayfish *Astacus astacus*, pro-phenoloxidase, the precursor of phenoloxidase is located with in the haemocytes (Soderhall *et al.*, 1979) and specifically activated by non-self molecules, such as β- 1,3 glucans, a glucan common to most fungal groups (Unestam and Soderhall, 1977; Soderhall and Unestam, 1979). The proPO activating system may operate as a recognition system in crayfish, and it is suggested that this system functions as a complement-like system in arthropods (Ashida and Soderhall, 1984). The link between phenoloxidase and coagulation was studied by Nagi *et al.* (2000) in *Tachypleus tridentatus*. It had been concluded

that the two systems have a common ancestral protease cascade. Phenoloxidase can oxidise phenols into quinones that will then polymerize non-enzymatically to melanin (Nappi and Vass, 1993). Serine proteases can inturn be specifically activated by polysaccharides from microbial surface such as β- 1,3 glucans from fungi (Soderhall, 1982) and peptidoglycans or Lipopolysaccharides (LPS) from gram negative bacteria and gram positive bacteria, respectively(Soderhall *et al.*, 1990), suggesting the involvement of proPO activating system in crustacean non-self recognition (Preston and Taylor, 1970; Soderhall and Smith., 1986; Johansson and Soderhall, 1989; Soderhall and Cerenus, 1992; Soderhall *et al.*, 1982). Phenoloxidase on activation is normally involved in several processes, including cuticle tanning and hardening and humoral melanisation of foreign bodies (Soderhall and Smith, 1986; Soderhall *et al.*, 1990).

2.3. Humoral factors of innate immunity

Subsequent host defense responses comprise cellular mechanisms together with humoral responses involving the actions of circulating antibacterial factors, lectins and other immunologically active molecules (Smith and Chisholm, 1992). Invertebrates do not exhibit acquired immunity (Roch, 1999) although proteins, with domains belonging to the immunoglobulin superfamily, have been demonstrated (Lanz Mendoza and Faye, 1996). The relative importance of cellular and humoral host defense mechanisms in spiny lobsters has yet to be determined. It would seem, however, that circulating haemocytes play a central role in both through their involvement in immuno-recognition and in the processes of inactivation and elimination. Factors that bind to and cause aggregation or agglutination of foreign particles have been reported for many invertebrate species from sponges to urochordates (Schapiro, 1975; Cooper and Lemni, 1981; Cooper, 1985; Ratcliffe *et al.*, 1985; Amirante, 1986). The humoral factors upon which most attention is focused are the haemagglutinin so called because of their ability to agglutinate vertebrate erythrocytes *in vitro* (Cushing, 1967; Cohen, 1968; McKay *et al.*, 1969; Cornick and Stewart, 1973). In addition, the agglutinins may agglutinate bacteria (Tyson and Jenkin, 1973; Scott, 1971b; Miller *et al.*, 1972; Huang *et al.*, 1981), the specificity and titre of which varies between the invertebrate species. These factors are not found in all crustacean species,

compared with other invertebrates and the titre is found to be quite low (Brown *et al.*, 1968).

Agglutinins occur naturally in plasma or serum, and it is now recognised that these agglutinins are lectin–like, i.e. molecules which have receptors for certain specific carbohydrate determinants (Liener *et al.*, 1986). The function of haemagglutinin is less obvious, as they are inhibited by sugars and this affinity for polysaccharides has encouraged several workers to classify them as lectins. Lectins serve a variety of biological functions in molluscs (Ratcliffe *et al.*, 1985; Renwrantz, 1983; 1986) but information about a similar function in crustaceans is lacking. Cornick and Stewart (1978) has noted that agglutinin activity is associated with haemolymph in *Homarus americanus*, and Amirtante and Basso (1984) have demonstrated it with monoclonal antibodies that haemagglutinins in *S. necrulis* is present on the membranes of granular haemocytes as well as free in the plasma. Ratanapo and Chulavatnatol (1990) reported that agglutinin in *P. monodon* occurs in the ovary, testis, hepatopancreas, muscle as well as the haemolymph. Agglutinins can be distinguished as two types in crustaceans that which are in blood and those associated with reproductive system. Agglutinins which react with N-acetyl neuramic acid have been found in the blood of lobsters (Cornick and Stewart, 1973; Hall and Rowlands, 1974) and crayfishes (Imai *et al.*, 1994; McKay *et al.*, 1964; Miller *et al.*, 1972). Recently lectins have been purified and characterised in many crustaceans such as lobster (Hartmen *et al.*, 1978), the shrimps, *P. indicus* (Maheswari *et al.*, 1997; Jayasree, 2001), *P. californiensis* (Vargas-Albores et al., 1993) and *P. monodon* (Ratanapo and Chulavatnatol, 1990).

In crustaceans, killing factors have been reported to be less compared to the agglutinins. They include factors which are effective against viruses (Mc Cumber *et al.*, 1979), bacteria (Stewart and Zwicker, 1972; Soderhall and Smith 1986; Chisholm and Smith, 1991) or Fungi (Nyhlen and Unestam, 1980). Bactericidins have attracted more attention especially in crabs and lobsters due to the high market value of these animals. Bactericidal activities in lobsters appear to be effective against many bacteria except the pathogen *G. homari* (Cronick and Stewart, 1968, 1975; Stewart and Zwicker, 1972). In crabs too

bactericidins against *G. homari* were found to be absent (Cornick and Stewart, 1968, 1975). Killing activity has been found to be induced by pre-treatment of the host with live or heat killed bacteria (Stewart and Zwicker, 1972; Mori and Stewart, 1978; Adams, 1991; Chisholm and Smith, 1995). However, it was found that these responses appear to have limited specificity and the time taken to reach maximum effect is highly variable from 36 – 48 hr (Adams, 1991) to 7 days (Evans *et al.*, 1969). It is well established that injection of foreign agents into the haemocoel of crustaceans produces a marked haemocytopenia with in a few hours, and recovery of cell number can take place with in two days (Smith and Ratcliffe, 1980b; Smith *et al.*, 1984). In lobsters bactericidal activity has been found to recede predominantly in hepatopancreas and small amount present in haemolymph is not associated with haemocytes (Mori and Stewart, 1978). In contrast, Smith and Ratcliffe (1978) and White *et al.* (1985) failed to detect bactericidal activity or bacteriostatic factors in the plasma or serum of *C. maenas*. Franchin and Ottavian (1990) have shown that phagocytes of crayfish, *Procambarus clarki* produce lysosomal enzymes which effectively degrade and remove foreign material. Haemocyte phagocytosis in a number of animal species has only been proved indirectly by detection of phagocytosis related lysosomal enzymes such as α-naphthyl acetate esterase, β-glucuronidase and acid phosphatase (Hearing, 1969; McHenery *et al.*, 1979; Birmingham and Jeska, 1981; Lentzen *et al.*, 1984; Moore and Gelder, 1983). Sung and Song (1999) studied the intra haemocytic activity of lysosomal enzymes in *P. monodon* and *M. rosenbergii*.

Studies in *C. maenas* on the role of peptides in bacterial killing did not provide any clue on lysis and reduction in bacterial turbidity following incubation with bacterial suspension with haemocyte extracts. It was found that the killing factors reside predominantly in granular cells (Hall and Soderhall, 1982), which are associated with phenoloxidase activity (Soderhall and Smith, 1986). In penaeids, Bachere *et al.* (2000), Unestam (1973) and Nyhlen and Unestam (1980) have observed deposition of melanin around fungal hyphae in the tissues of crayfish. Soderhall and Ajaxon (1982) found that quinones and melanin are fungi toxic (Soderhall *et al.*, 1979). In addition to these, in crustacean plasma there is another factor which is a glucan binding molecule (Duvic and Soderhall,

1990). It is a monomeric glycoprotein with molecular mass of 100 kDa and bind to laminarin (Duvic and Soderhall, 1990), a specific elicitor of the prophenoloxidase activating system (Soderhall and Unestam, 1979).

2.4. Effect of extrinsic and intrinsic factors

Stress makes animal more susceptible to microbial infections and diseases, which reduce its capacity to resist bacteria normally present in sea water (Lee and Wickins, 1992). Stress responses are normal physiological reactions to the environmental changes. This condition includes wide range of factors such as water quality parameters like dissolved oxygen levels, pH, salinity, temperature (Ravindranath, 1975; Steenbergen *et al.*, 1978), presence of toxins, pathogens, physical factors (Spices *et al.*, 1990), behavioural interaction and nutrient availability (Stewart *et al.*, 1967; Tsvetnenko *et al.*, 1999; Lee and Shiau, 2002). Exposure to these stressors leads to long term changes in cardiovascular and respiratory function, energy metabolism, fluid and ionic balance, acid-base balance and immunity (Thompson *et al.*, 1993; Hall and VanHan, 1998). If the stressors are mild and of short duration, the physiological disturbances are temporary. On the other hand if it is long and extreme, this may cause detrimental long term effects. These include poor growth, reduced resistance to diseases, impaired reproduction and lower survival (Lee and Wickin, 1992; Norton *et al.*, 1999).

The haemocyte count can vary greatly in response to infection, environmental stress and endocrine activity such as moulting (Smith and Ratcliffe, 1980; Persson *et al.*, 1987b; Smith and Johanston, 1992). Experimental injection of a fungal cell wall preparation or of a β-1,3 –glucan caused a rapid decrease in number of haemocytes followed by slow recovery (Persson *et al.*, 1987b) but in contrast, injection with crayfish parasite *Psorospermium haeckelii* gave dramatic increase in the number of free haemocytes (Presson *et al.*, 1987b; Soderhall and Cerenius, 1992). Smith and Ratcliffe (1980) observed that the total cell count reduced by 90% with in 30 min of injection with bacteria and it returned back to normal level after 24 hr. Where as Sung *et al.* (1991) observed that when *P. monodon* was immersed in a viable cell suspension, the cells were eliminated

within 12 hrs following invasion and completely undetectable after 24 hr. Decapod crustaceans have the capacity of rapidly clearing the invading bacteria from their haemolymph (Cornick and Stewart, 1968; McKay and Jenkin, 1970). It has been reported that lobsters, shore crabs and penaeid shrimps remove 75% of bacterial cells with in 10 min to 1hr after injection (Cornick and Stewart, 1968; White and Ratcliffe, 1982; Adams, 1991; Martin *et al.*, 1993b). *M. rosenbergii* on challenging with *Aeromonas* strains caused decrease in THC between 4 and 24 hr after injection Sung *et al.* (2000). The percentage of granulocytes also varied significantly. The clearence of injected materials and the reduction in total haemocyte count have been reported in the American lobster *H. americanus* (Stewart *et al.*, 1969) and in shore crab, *Carcinus maenas* (Smith and Ratcliffe, 1980). A significant reduction of THC was observed in the crayfish *P. leniusculus,* infected with the fungus *Aphanomyces astaci* (Presson *et al.*, 1987; Soderhall *et al.*, 1988) and in the Kuruma shrimp, *P. japonicus* infected with rod-shaped DNA virus (Henning *et al.*, 1998). The fate of these disappearing haemocytes is unclear. Factor and Beekman (1990) injected latex beads into lobsters and suggested that some phagocytic haemocytes may leave circulation and enter the connective tissue. Several studies described this aggregation of haemocytes in gills of animals injected with foreign material (Cornick and Stewart, 1968; Smith and Ratcliff, 1980; Martin *et al.*, 1993b).

In decapod crustaceans, the THC and DHC, which are associated with the cellular defense vary with species and in relation to intrinsic factors (Hose *et al.*, 1992; Giulianini *et al.*, 1998). It was reported that THC of blue shrimp, *P. stylirostris* in the intermoult is significantly lower than that in the post-moult and pre-moult. (Chen *et al.*, 1988; Le Moullac *et al.*, 1997). Sequiera *et al.* (1996) while studying the moult cycle in *P. japonicus* found that there was variation in different haemocyte count. It was observed that the hyaline cells were dominant just before and after the moult, where as they decreased during the intermoult. Later Cheng and Chen (2002) studied the effect of moulting on haemocytes and found lowest THC in pre-moult and highest in inter moult in *M. rosenbergi.*

Bacterial infection following stress can occur rapidly during capture and transport (Johnson, 1976; Paterson and Spanoghe, 1997) making it extremely

difficult to ensure that unstressed crustaceans have been sampled. Patersson *et al.*, (1999) studied the effect of stress during post harvest handling with respect to blood lactic acid in *P. cygnus*. The influence of environmental changes during transportation and the effects that live marketing stressors may have on the immune capability of high value crustaceans such as spiny lobsters are virtually unascertained. Recently, Gomez-Jimenez *et al.* (2000) studied the effect of cooling in relation to immunological parameters like THC and pro-phenoloxidase activity. Several authors have suggested that haematological parameters such as THC and phenoloxidase activity might represent sensitive indicators of environmentally induced immunosuppression in marine crustaceans (Smith and Johanston, 1992; Hauton *et al.*, 1995; Jussila *et al.*, 1997). Jussila *et al.* (1999) showed that air exposure increase THCs and in injured lobsters, the THC increased quickly compared to the controls.

The effect of exposure to contaminated sediments was studied by Smith *et al.* (1995) in the common shrimp *Crangon crangon*. The exposed animals showed an elevation in recoverable haemolymph volume and reduction in THC and blood cell PO activity. The effect of mercury has been studied in the freshwater prawn *Macrobrachium idea*. Victor *et al.* (1990) found hyperplastic gill lamellae engorged with haemocytes whereas in *C. maenas*, THC showed no changes, but found that the metal affected haematopoiesis (Truscott and White, 1990). Evans *et al.* (1999) reported no change in THC number of the crayfish *Cherax tenuimanus* after 2 hr exposure to 0.1 or 0.5mg L^{-1} of dissolved copper and observed progressive decreases in haemocytes titre with 0.2 0r 0.8 mgL^{-1} copper after 2 weeks. In *Paleomon elegans* Lorenzon *et al.* (2001) found that upon immersion in artificial seawater containing Hg, Cd, Cu, Cr, Zn or Pb caused decrease in haemocyte count during first 8 hr though it returned back to initial level after 16 hr immersion. The greatest decrease in count was seen with lead.

In the crab *C. maenas*, a slow rise in temperature from 10^0C to 20^0C for 5 days increased blood cell number, although rapid change initiated no response (Truscott and White, 1990; Le Moullac *et al.*, 1998). Dean and Vernberg (1966) found that temperature affects clotting time, THC and levels of plasma protein in hermit crab, *Uca pungilator*. Chislom and Smith (1994) showed in *C.*

maenas that seasonal changes of temperature affect antibacterial activity of haemocytes. Temperature affects phagocytosis in *H. americanus* (Paterson and Stewart 1974). In *M. rosenbergii,* the phenol oxidase activity and THC was minimum at 33 - 34^{0}C (Cheng and Chen, 2000). High temperature is known to activate the proPO system (Sung *et al.*, 1998). A relation between salinity and IHHNV has been observed in the white shrimp *P. vannamei* (Bray *et al.*, 1994).

Low oxygen tension hampers the metabolic performances in shrimp and can reduce growth and moulting frequency and cause mortality (Madenjian *et al.*, 1987). The response of shrimp *P. stylirostris* exposed to severe hypoxia (1mg/l O_2 for 2hr) was measured in terms of THC, DHC, PO activity and respiratory burst by NBT reduction (Le Moullac *et al.*, 1998). In *P. monodon*, the phagocytic activity of haemocytes was less effective in oxygen–depleted shrimp (Direkbusarakom and Danayadol, 1998).

The stress provided by high salinity further augments retardation of growth due to infection (Bray *et al.*, 1994). The effects of salinity on plasma concentration and total haemocytic proPO have been studied by Vargas-Albores *et al.*, (1998) in *P. californiensis.* Cheng and Chen (2000) demonstrated that the phenoloxidase activity and THC was lower at lower salinity, and also at higher pH of 9 - 9.5. Ammonia, the end product of catabolism of protein, accounts for more than half the nitrogenous waste released by decapods crustaceans (Regnault, 1987). Ammonia is very toxic to aquatic animals which are commonest toxicant in aquatic environment in addition to excretion. Ammonification of unconsumed feed also adds to ammonia load. Elevated amount of environmental ammonia have been reported to affect growth and moulting (Chen and Kou, 1992), oxygen consumption and ammonia excretion (Chen and Lin, 1992) haemocyanin and protein level in haemolymph (Chen and Cheng, 1993). Lethal and sub lethal effects of ammonia in shrimp was studied with respect to metabolism and osmoregulation of internal medium (Chen *et al.*, 1990a, b). Cheng and Chen (2002) suggested that exposure to ammonia – N decreased the total production of superoxide.

2.5. Immune enhancers

Immunostimulants are substances, which enhances protection against invading pathogen by eliciting the non-specific immune system. The cell wall preparation of bacteria, fungi, mushroom and yeast are reported to be good source of immunostimulants (Raa *et al.*, 1992). The non-specific immune system in Atlantic salmon was found to be enhanced by glucan preparation from *Saccharomyces cerevisiae.* Laboratory studies have proven that immuno stimulants like Yeast glucan, *Vibrio* bacterin, LPS, and peptidoglycans are effective to reduce diseases in crustaceans (Sung *et* al., 1994; Song and Hseih, 1994; Itami *et al.*, 1994; Lorenzon *et al.,* 1999; Newman, 1996).

In crustaceans, glucans exert their effect by specifically activating prophenol oxidase, through a complex enzyme cascade (Ashida, 1981; Soderhall, 1981; Soderhall, 1983; Varges-Albores and Yepiz-placenta, 2000). *In vivo* injection of β -1, 3-glucan into the haemocoel of *A. astacus* caused rapid reduction in the number of circulating haemocytes, indicating a cellular defense reaction (Smith and Soderhall, 1983a). In *C. maenas* glucan injection specifically activated pro-phenol activating system (Smith *et al.*, 1984). Melanisation was triggered by glucans by activating proPO with in the haemocytes (Unestam and Soderhall, 1977; Soderhall and Unestam, 1979; Soderhall, 1981).

Song and Sung (1993) reported that M-glucan (a mixture of β- 1, 3 and β- 1, 6 glucan) can be used as a short term immunostimulants in *P. monodon.* Immersion treatment with yeast *β*- glucan enhanced growth and resistance to vibriosis in the tiger shrimp *P. monodon* (Sung *et al.*, 1994), which is enhanced with increased PO activity and superoxide anion production and an increased bacterial clearence (Sung *et al.*, 1996). Resistance to White Spot Syndrome Virus (WSSV) and vibriosis was reported by dietary incorporation of glucan (Rao *et al.*, 1996; Chang *et al.*, 1999). PO activity was enhanced in *P. monodon* and *M. rosenbergii* by glucan treatment (Sung *et al.*, 1998). Immunity was also found to be enhanced by the probiont bacterium *Bacillus* sp. in *P. monodon* (Sung *et al.*, 1991; Dunphy, 1991; Phianphak *et al.*, 1999; Rengipat *et al.*, 2000). Chemicals

like levamisole were found to increase immunostimulation in *M. rosenbergii* (Baruah and Prasad, 2001).

It is important therefore to have a healthy environment for a successful aquaculture practice. The environmental and physiological variations induce changes in the immune system in lobsters which are often found to be stressful resulting in reduction of immune vigour, which results in higher susceptibility to infectious diseases.

MATERIALS AND METHODS

3. MATERIAL AND METHODS

3.1. Collection and maintenance of specimens

Healthy *Panulirus homarus* (Plate I) specimens were obtained from the lobster merchants at Vizhinjam (Thiruvananthapuram) and Thikkodi (Kozhikode). The lobsters were transported in thermocol boxes with or with out wood shavings. Before transportation the metabolic rate was reduced bringing down the temperature to 13-16^{0}C and covering with wet news paper. The temperature inside the box was brought down by placing ice and then properly sealed. The lobsters were transported to the marine hatchery at Calicut with in 15 hours. The animals were then brought to normal temperature slowly to avoid stress and released into holding tanks with continuous aeration after which they were acclimatized to the desired condition, for about two weeks.

3.2. Experimental setup

The experiments were carried out at the Marine Hatchery Complex at Calicut Research Centre of Central Marine Fisheries Research Institute. All the experiments were carried out with three replicates in FRP tanks of 100 L capacity (Plate II & III). The arrangement of the tanks was in such a way that both the control and the experimental tank receive similar environmental conditions. Continuous aeration was given in all the tanks. Shelters made of tile or pipes were used as hideouts. The temperature during the experiment ranged from 26 ± 1^{0}C. All conditions were the same unless otherwise stated.

The water quality parameters like salinity, temperature, pH and DO were routinely checked. Necessary adjustments were made to maintain these parameters at optimum level for the experiments. The wastes accumulated in the bottom were siphoned out, fifty percent of water exchange was carried out every morning and evening before feeding the animals. The water exchange of near 100% was done once in a week.

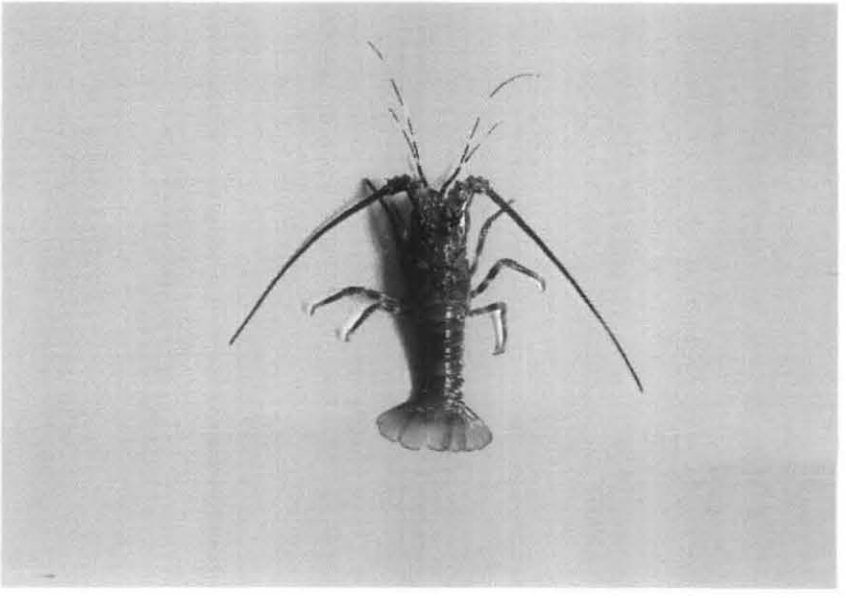


Plate I. *Panulirus homarus*

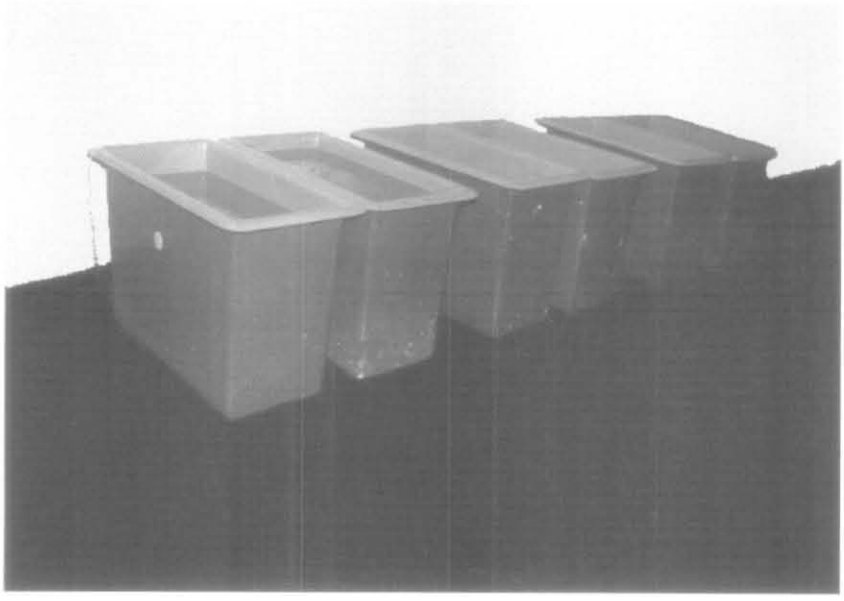


Plate II. Experimental setup

Plate III. Experimental setup

3.3. Feeding

The experimental animals were fed with green mussel (*Perna viridis*) and trash fish. The feed was given at the rate of 10% of the body weight. The feed was offered during evening hours as the animals exhibit nocturnal behaviour. Optimum feeding rate was maintained at optimum under the prevailing environmental conditions. Normally the animals were fed *ad libitum*. For specific rearing experiments artificial diets (farm made) were used.

3.4. Sampling and Observation

The animals were carefully handled during various experimental setups. Continuous aeration was given from a compressor of 10 Hp capacity through out the experimental period. The carapace length (mm) and wet weight (g), sex of animal and moult stages was determined following the standard procedure(Berry., 1971; Radhakrishnan.,1989) The animals were observed daily for activity and feed intake. Cannibalism and moulting were also noted. Cannibalism was rarely encountered after moulting.

3.5. Analysis

3.5.1. Characterisation of Haemocytes

Lobster haemolymph was collected from males and females weighing 100-120g using 1 ml syringe .The haemolymph was drawn by inserting the sterilised 1.5 in. 21G gauge hypodermic needle into the ventral sinus, located at the junction of carapace and abdomen of *P. homarus*. Haemolymph was drawn into cold formalin (10%) or into an anti coagulant .The syringe was rotated to insure thorough mixing and one or two drops were placed quickly on alcohol cleaned slides. A second clean slide was used to prepare a film by smoothly spreading the drop to the opposite end of the slide. Thick slides were used for light microscopic studies.

3.5.1.1. Light microscopic studies

The films were air dried and post fixed with absolute methanol for 5 min, and rinsed in distilled water. Stained for 5-10 min in Geimsa stain (Cornick and Stewart, 1978), rinsed in distilled water and allowed to dry and then covered with a cover slip. The haemocytes were observed under a phase contrast microscope. The entire slide was scanned randomly and a minimum of 100 haemocytes were examined. The dimensions of the haemocytes were measured using a micrometer. Cell length, width and diameter of the nucleus of 100 haemocytes were measured.

3.5.1.2. Electron microscopic studies

For studies on ultrastructure haemolymph was collected in a 1 ml syringe containing 0.4 ml of the fixative (2.5% gluteraldehyde in 0.1M cacodylate buffer containing 12% glucose, pH 7.8) and withdrawing 0.1ml of haemolymph into the syringe. The sample was transferred to a glass vial and mixed .The sample was then allowed to fix for 15 min at room temperature or overnight at 20^{0}C in a refrigerator. The fixed haemocytes were pelleted by centrifugation (1,000 g for 5 min) and washed in 0.1M Sodium cacodylate (pH 7.8 containing 24% sucrose), post fixed in 1% osmium tetroxide in 0.1M Sodium cacodylate for 1hr at room temperature are washed with the buffer. 2% Agarose made in cacodylate buffer was then added to the pellet and mixed thoroughly. Small pieces of pellets were then dehydrated with the acetone series of 30%, 50%, 70%, 80%, 90%, 95% and finally to absolute acetone giving two changes, 30 min each. The cells were filtered and embedded in Spurr's low viscosity resin. Thin sections were made on an ultra microtome and transferred to micro grid, stained for 1hr with Uranyl acetate and then with lead citrate for 5 min. The sections were observed and photographed using a Hitachi H 600 Transmission Electron Microscope located at the CMFRI Headquarters, Cochin..

3.5.2 Haematopoeitic Tissue

3.5.2.1. Light microscopic studies.

The lobsters were injected with Davidson's fixative of about 1% of their body weight using a 1ml syringe. The exoskeleton and epithelial layer of the dorsal cephalothorax were removed to expose the foregut. The HPT was then dissected out from the fore gut and cut into small pieces, and fixed overnight in Davidson's fixative. The tissues were then dehydrated in ascending grades of isopropyl alcohol. The tissue was first transferred in 70% alcohol and left overnight. From 70% the tissues were transferred to 80% and then to 90% for 30 min to one hour each. Two changes were given in 100% alcohol for 1hour each. The tissues were cleared by immersing in an alcohol-chloroform (1:1 v/v) mixture followed by two changes in pure chloroform.

The tissues were kept in a chloroform wax mixture for 2 hr and then transferred to paraffin wax for infiltration. Three changes were given in molten wax for duration of 1hr each. Paraffin moulded blocks was prepared and sections cut at 5-6 μ thickness in a rotary microtome. The sections were stained following Haematoxylin-Eosin method. The stained sections were observed and photographed.

3.5.3. Haemolymph Volume

Haemolymph volume was measured in lobsters of various size groups, which has been previously blotted dry and weighed. The lobsters were injected with 0.1ml of 1% amaranth solution in sterile saline and incubated for 15 min at 26 ± 2^{0}C(modification of amaranth dye method of Yeager and Munson , 1950). After incubation 0.5 ml of haemolymph was withdrawn and diluted with 4.5 ml of sterile saline containing the anticoagulant. The saline and the haemolymph were thoroughly mixed and centrifuged at 750g for 10 min. The supernatant was decanted into optically matched glass cuvettes and the absorbance read against a blank which contained 4.5 ml saline and 0.5 ml cell free haemolymph at a wave length of 530 nm using Genway 6300 spectrophotometer.

The concentration of dye in the haemolymph samples was derived directly from the calibration graph. This was constructed from the absorbance values of series of standard dye solutions by adding 0.5 ml of cell free

haemolymph to 4.5 ml of standard amaranth solution which was diluted to 1:100 (0.01%), 1:150 (0.0067%), 1:200 (0.005%) and 1:400 (0.00025%) concentrations. The concentration of dye (R) after dilution by haemolymph (x) is given by,

$$R = \frac{0.5}{10\,(X+0.1)}$$

And there fore the haemolymph volume (X)

$$X = \left\{\frac{0.5}{10R}\right\} - 0.1$$

3.5.4. Immune response parameter

3.5.4.1. Total Haemocyte Count (THC)

The THC was determined using a Neubauer chamber and the procedure similar to that used for human white blood cell counts was followed (Perazzolo *et al.*, 1997).

3.5.4.2. Differential Haemocyte Count (DHC)

The haemolyph collected for THC was used for DHC also: the haemolyph was made into a smear and allowed to dry, fixed with methanol for 5 min, and after which stained with Giemsa stain for 10 min and finally rinse in distilled water. The slides after drying were observed under a microscope and a minimum of 100 cells were counted in each slide.

3.5.4.3. Phagocytic assay (Smith and Ratcliffe, 1978)

Glass cover slips were placed at the bottom of sterile plastic Petri dishes and covered with 5 ml of saline (Carcarinus saline). Approximately 0.5 ml of haemolmph with the anti - coagulant was collected and added to the cover slips placed in the Petri dish, in an incubator at 20^{0}C .The haemocytes were allowed to settle and attach on to the glass substratum for 15 min, and yeast or bacterial

suspension at the rate of 1 x 10^8 cells ml^{-1} were added. After 1hour, the cover slips were removed and fixed with methanol for 1 min and stained with Giemsa in Phosphate buffered saline (1:1 v/v). The smear after 10 min was rinsed with distilled water and dried. The cover slip was then placed on a slide and mounted with DPX. The slides were then observed under the microscope for phagocytosis. The phagocytosis was calculated according to the formula by Blazer (1991).

$$\text{Phagocytic Index (PI)} = \frac{\text{No. of ingested Yeast/Bacterial cells}}{\text{Total no. of cells}}$$

A total of 100 cells were counted to calculate phagocytic index.

3.5.4.4. Phenoloxidase activity

3.5.4.4.1. Phenoloxidase activity of serum

Prophenoloxidase activity was measured spectrophotometrically by recording the formation of dopa chrome from L-dihydroxy phenylalanine (L-Dopa) (Perazzolo *et al.*, 1997). The haemolymph was collected and stored at 4^0C for 24 hrs and then repeatedly centrifuged at 2000 g to remove serum, which was used immediately. 20 µl of serum was pre-incubated with 20 µL of trypsin (1 mg ml^{-1}) for 30 min at 37^0C in 96 well micro titre plates. To each sample was added 20 µL of L-Dopa (3 mg ml^{-1}) and incubated for 5 min at 37^0C. 260 µL of distilled water was added to slow down the reaction. Activity of PO was detected spectrophotometrically using Bio-tek (EL 800) micro plate reader, after 5, 10, 20 and 60 min by the degree of absorbance at 490 nm by the formation of red pigment Dopa-chrome. For control, L-Dopa alone was incubated with 0.45 M Nacl monitored spontaneous oxidation. Phenoloxidase activity was expressed as change in absorbance min^{-1} mg $protein^{-1}$. Protein concentration of sample was determined by biuret method (Gornell *et.al.*, 1949).

3.5.4.4.2 Cytochemical analyses of phenoloxidase activity (Lanz *et al.*, 1993)

Haemocytes were fixed in neutral formalin solution (10% formaldehyde in 0.02 M phosphate buffer, pH 7), washed three times in PBS (0.01

M, pH 7) incubated in 1ml L-Dopa (mg ml^{-1}) for 10 hour at room temperature and observed under light microscope.

3.5.4.5. Super oxide anion production

Respiratory burst activity of haemocytes was quantified using the reduction of nitroblue tetrazolium (NBT) to formazon as a measure of superoxide anion production. This assay was conducted as described by (Song and Hsiech, 1994; Lee *et al.*, 2002). Approximately 0.1 ml of collected haemolymph was diluted with 0.4 ml of anticoagulant, and was centrifuged at 300 x g for 10 min at 4^0C. The resultant haemocyte pellet was resuspended to 10^8 cells/ml in a modified complete Hank's balanced salt solution (MCHBSS) containing 10 mM $CaCl_2$, 3mM $MgCl_2$, 5mM $MgSo_4$ and 24mg/ml HBSS. Haemocyte suspension (100 µL) was added to flat-bottomed 96 well microtitre plates (10^5 haemocytes/well) and cyto-centrifuged at 300 x g for 10 min at 4^0C using Remi C 30 refrigerated centrifuge. After removing the supernatant, 100µL of trypsin (2 mg ml^{-1}) was added and allowed to react for 30 min at 37^0 C. MCHBSS was added to remaining haemocyte suspension as a control. NBT (100 µL, 0.3% in MCHBSS) was added to haemolymph and incubated for 30 min at 37^0C.The staining reaction was terminated by removing the NBT solution and adding absolute methanol. After three washes with 70% methanol, the haemocytes were air dried and coated with a solution of 120 µl 2M KOH and 140 µL dimethyl sulfoxide was added to dissolve the cytoplasmic formazon. The optical density of the dissolved cytoplasmic formazon was measured at 630 nm with a Bio-tek (EL 800) micro plate reader. The ratio of OD 630 nm of trypsin elicited haemocytes to OD 630 nm of control haemocytes was used as an index for comparing the effect of different salinities on O_2^- generation.

3.5.4.6. Haemagglutination titre

The haemolymph was collected without the anticoagulant and stored at 4^0C overnight and the serum was isolated by breaking the clot and continuous centrifugation at 3000 rpm. 50 µl of PBS or normal saline was added in the wells of 96 well U shaped microtitre plates, in all the wells except the first well.100 µl of

serum was added in the first well and from that 50 µl was taken and serially diluted in the rest of the wells avoiding the last well. 50 µl of 1% chicken RBC was added in all the wells. The plate was then incubated for 24 hours at 37^0c, the slide was then observed for agglutination.

In the case of bacterial agglutination, the same procedure was used except that $1x10^8$ cells of bacterial suspension in PBS were used instead of chicken blood.

3.5.4.7. Antibacterial activity

3.5.4.7.1. Spread plate method (Smith *et al.*, 1995)

The bacteria (*Vibrio alginolyticus* obtained from National collection centre for industrial micro organism) were grown to log phase in peptone broth at 37^0 C. The cells were then harvested by centrifugation at 400 x g for 10 min and washed twice in sterile marine saline (MS) (0.5 M NaCl, 20mM $Cacl_2.6H_20$, 11mM KCl, 43mM Tris). The concentration was then adjusted to 2 x $10^8 ml^{-1}$.The suspension was further diluted 1: 10 with Marine Saline to make working solution of 2 x 10^7 cell ml^{-1}. Haemolymph was used for antibacterial assay; 800 µL of haemolymph was incubated with 100 µL of bacterial suspension and 100 µL of sterile marine broth in a sterile tube. The controls were incubated with 900 µl of sterile saline supplemented with marine broth and 100 µL of bacterial suspension. Incubate the tubes for up to 12 hrs the samples were taken at intervals of 0 hour, 3 hour, 6 hour and 12 hour to determine the number of viable bacteria.100 µL aliquots were taken aseptically after shaking and serially diluted with two tenfold dilutions in 3.2% NaCl and plated in triplicate on to pre-dried Nutrient agar(HiMedia) plates. All plates were incubated for 48 hrs at 37^0C.The number of colony forming units (cfu) were then estimated by direct counting.

$$\text{Survival Index} = \frac{\text{cfu at time } t_t}{\text{cfu at time } t_0} \times 100$$

3.5.4.7.2. Turbidometric assay

Bacterial suspension was washed in marine saline to obtain a final concentration of 2 x 10^6 ml^{-1} of *Vibrio alginolyticus*. 100 µL of serum was incubated with 100 µL of bacterial suspension in a 96 well flat bottomed titre plates, for controls 50 µLof marine saline and 50 µL of sterile marine broth and 100 µLof bacterial suspension was added. The absorbance was measured at 570 nm against a series of blank consisting 150 µL of marine saline and 50 µL of sterile marine broth, for a time intervals of 0, 1.30, 13.30, 15.30, 24 hour.

3.5.4.8. Acid phosphatase activity

Acid phosphatase activity was measured by the following method. 2ml of buffer substrate (containing 0.01M disodium phenyl phosphate and citric acid) was added into the test and control test tubes and kept aside at 37^{0}C for few minutes in a water bath; 0.2 ml of serum was then added to the test and incubated both the tubes for 1 hour at 37^{0}C. Removed from the water bath and added 1 ml of NaOH and 1 ml of $NaHCO_3$ to both the test tubes. Then 0.2ml of serum was added to the control. 1 ml each of 4-aminophenozone and potassium ferricyanide were then added and thoroughly mixed. Standards were prepared taking 1.2 ml buffer substrate and 1 ml of working standard phenol solution and blanked with 1.2 ml buffer and 1ml water. To both tubes NaOH, $NaHCO_3$, 4-aminophenozone and potassium ferricyanide were added as above and the absorbance were read at 520 nm.

Acid phosphatase activity (ACP)

$$ACP = \frac{\text{O.D of test-OD of control}}{\text{O.D of sample}} \times \frac{\text{Conc. of Standard}}{\text{Volume of test}} \times 100 \text{ KAU } L^{-1}$$

3.6. Experimental stress studies

Experimental stress was conducted for diffferent stress factors to understand the effect of these factors on the immune system.

The lobsters observed were all in the intermoult stage C with an average weight of 104 $\pm$ 43.88 g. Sex of the animal was not taken into account. Animals were maintained at salinity 35 ± 2 ppt, pH, 7.78 $\pm$ 0.2, dissolved oxygen, 4.17 $\pm$ 1.43 and temperature, 25 $\pm$ 0.3 ^{0}C. They were fed with mussel meat at the rate of 10% body weight.

3.6.1. Physiological stress

3.6.1.1. Moulting

For moult studies the animals were observed in three stages: the Pre- moult (stage D), the Inter moult (Stage C), and the Post–moult (Stages A and B).The moult studies usually ranged from 45 - 50 days. Care was taken to monitor the same individual through out the three stages. Ten animals of weight range 100-120 g, were monitored to study the immune changes during the moult cycle. Sex of the animal was not taken into consideration. All rearing conditions were maintained optimum through out the period.

3.6.1.2. Eye-stalk ablation

Animals in the inter moult stage was used for eye stalk ablation studies. Bilateral ablation was conducted. Five lobsters were ablated using heated scissors so that the wound was sealed off immediately, thereby prevent bleeding. The optimum rearing conditions were maintained at optimum. The experiments were carried out for two weeks and haemolymph was drawn with care from individual animals and analysed for the immunological parameters like THC, DHC and proPO activity.

3.6.2. Nutritional stress

3.6.2.1. Starvation

Lobsters were starved for about four weeks. Control animals were fed with mussel meat at the rate of 10% body weight. All other experimental conditions were maintained at optimum. Haemolymph samples were drawn at 7 days interval and analysed for changes in the immune parameters like THC, DHC, phagocytosis, proPO and acid phosphatase.

3.6.2.2. Effect of fish and mussel as feed

Experiments were conducted in triplicates for each food (fish and mussel). The green mussel *P. viridis* and the sole fish *Cyanoglossus* sp. were used. The experiments were carried out for a period of two weeks. The haemolymph was drawn to study changes in immunological parameters.

3.6.3. Environmental stress

3.6.3.1. Salinity

Lobsters were gently acclimatised to the salinities 20, 25, 30, 35, 40 and 45 ppt. Lower salinities were obtained by mixing seawater with fresh water and higher salinities by adding salt crystals. All trials were carried out in triplicates, in 100 L FRP tanks. The experiments were carried out for 2 weeks; the animals were gently caught with minimum stress. The following parameters were observed from the blood samples collected, THC, DHC, Phenoloxidase and superoxide anion production.

3.6.3.2. pH

Lobsters were acclimatized to different pH levels of 5.0, 7.5, and 9.0. The pH was adjusted with 1N HCl or 1N NaOH. All trials were done in triplicates in FRP tanks of 100 L capacity. Except the pH, all other conditions were maintained at optimum in all the tanks. The experiments were carried out for two weeks. The parameters analysed were THC, DHC, Phenoloxidase, superoxide anion production and acid phosphatase.

3.6.3.3. Dissolved Oxygen

For oxygen stress experiments, only two conditions were noted the hypoxic and normal levels of oxygen. Hypoxic condition was maintained by maintaining dissolved oxygen levels below 1 mgL^{-1}, for which no aeration was given and for normal condition, the dissolve oxygen level was maintained at 5 mgL^{-1}. All other parameters were maintained at optimum levels. Lobsters were not fed during the experimental period. The experiment was carried out for 24 hrs.

The haemolymph was measured individually for all the parameters analysed THC, DHC, Phenoloxidase and super oxide anion production.

3.6.3.4. Ammonia

The animals for ammonia stress condition were maintained for a week in different concentration of ammonia-N, 0.5 mg L^{-1}, 1.5 mg L^{-1}, 3 mg L^{-1} .Ammonia -N concentration was prepared by dissolving 38.2 g of NH_4Cl in 1L distilled water to make 10,000 mgL^{-1} as stock solution. Water was replaced daily to maintain the concentration. Stress tests were carried out in triplicate. The immunological parameters like THC, DHC, phenoloxidase and super oxide anion production was analysed.

3.6.4. Handling stress

3.6.4.1. Transportation

The effect of transportation stress was studied while bringing the lobsters to the laboratory for rearing experiments. The animals before dry packing were brought down to a temperature of 16^{o}C, so as to minimize the metabolic rate. To reduce the temperature, cold seawater was used; the animals were given a slow dip in this water and draped with damp newspaper made into many layers. Wood shavings were then laid over the thermocol box floor and bottles of seawater ice were used to reduce the temperature inside the box. The animals were laid in rows with minimum congestion so as to increase the survival rate. The box was then sealed tight to prevent loss of temperature. The boxes were then transported to the hatchery with in 15 hrs. As soon as the container reached the hatchery, haemolymph samples were collected for analysis and the animals were then released to fresh sea water for further studies. The samples were then analysed for THC and PO activity.

3.6.5. Pathogen stress

3.6.5.1. Bacterial challenge

Groups of 7-8 lobsters of similar size 100-140 g were injected with 0.1 ml of heat killed *Vibrio alginolyticus* obtained from National collection centre

for industrial micro organism ($1x10^9$ cells ml^{-1}) and incubated. The animals were maintained in sterile seawater. Samples of haemolymph were drawn at intervals of 30 min, 1, 3, 6, 12 and 24 h. Control lobsters were inoculated with 0.1 ml of sterile saline and similarly treated. The haemolymph samples were analysed for *immune* parameters like THC, DHC and proPO.

3.6.6. Immunostimulants

3.6.6.1. Effect of elicitors

The elicitors used in the experiment were heat killed *Vibrio anguillarum*, *Micrococcus lysidodyticus,* common yeast cells *Saccharomyces cereviceae*, (obtained from National collection centre for industrial micro organism) trypsin, 10% SDS and sea water were used. Prior to the experiment the cell concentration was adjusted to 5 $x10^8$ cell ml^{-1} in PBS at pH 7.4. The other elicitors used were different concentration of $MgCl_2$ and $CaCl_2$ at different concentration of 5 mM, 10 mM, 50 mM and 100 mM. For control, no elicitor was used. The serum was incubated with the elicitors for 30 min at room temperature. The samples then received 20 µL of L-Dopa (3mg ml^{-1}) and activity was measured similar to PO activity estimation of serum.

3.6.6.2. β-1, 3-Glucan (Laminarin) injection

Ten lobsters were maintained for *in vivo* studies using laminarin. An immunostimulant 0.1% of laminarin in saline were injected, using 1ml syringe, into the unsclerotinized membrane at the base of the fifth periopod. The laminarin was administered at the rate of 0.2 mg laminarin per millilitre of haemolymph. Control animals were inoculated with saline only. They were maintained in sterile filtered seawater under optimum rearing conditions. The haemolymph was drawn after 30min, 3h and 24h. The samples were then analysed for immune parameters.

3.6.6.3. Supplementation of farm made feed

Lobsters were fed with artificial diets containing yeast and chitosan which are believed to contain. β -1, 3-Glucan. The feed was prepared as moist diet. The constituents of the feed were 30 g agar, 60 g mussel meat, six ground

egg shell and 1 litre water boiled to make cake and air dried and used. This feed was used for control animals and for experimental animals the feed was either enriched with chitosan for feed I and yeast for feed II at the rate of 150 mg Kg^{-1}. The experiment was carried out for two weeks and examined for the changes in immune parameters.

3.6.6.4. Supplementation of commercial feed

Commercial immunostimulants "Allways" was used to study the immune enhancement. Eight lobsters each in triplicates were maintained for a period of one month. Control lobsters were fed with the same diet with out the immunostimulant. In this study farm made prepared diet was used. The immunostimulants was coated on to the feed and air dried just before feeding. The immunostimulant was fed at the rate of 0. 5 g for 25 g of feed.

RESULTS

4. RESULTS

4.1. Cellular characterisation

Haemocytes were classified into three broad classes according to the morphological criteria such as size and shape of cells, presence of refractile granules in cytoplasm, characteristic staining of the nucleus and intracytoplasmic content with Giemsa stain (Cronick and Stewart, 1978). Hyaline cells (HC) are the smallest among the haemocyte cell types. They are round to oval in shape with a mean cell length of 9.25 ± 1.52 µm and cell width of 5.85 ± 0.69 µm. Giemsa staining revealed a round large nucleus in the centre occupying almost the entire cell volume. The mean nuclear diameter is 5.38 ± 0.92 µm. The reduced cytoplasm is agranular. Semi-granular cells (SGC) are larger than the hyaline cells and are oval or spindle shaped. The mean cell length is 10.07 ± 1.79 µm. The cytoplasm contains granules that are sparsely dispersed. Giemsa staining showed the eosonophilic cytoplasmic content. The nucleus is central or eccentric and is oval in shape with a mean nuclear length of about 5.79 ± 1.52 µm. Large granular cells (LGC) are oval in shape with an average cell length of 13.2 ± 1.6 mm (Plate IV). The granular cytoplasmic content is abundant and highly refractile in the LGC. The giemsa staining reveals numerous highly eosonophilic granules in the cytoplasm. The nucleus is centrally located and round with a nuclear diameter of about 3.8 ± 0.6 µm (Table I). The TEM analysis also revealed three types of cells. The hyaline cells in the TEM analysis were found to show granules but few and less dense (Plate V). The large granular cells (Plate VII) and the semi-granular cells (Plate VI) were differentiated from the size of the granules the later have bigger granules as compared with the latter.

4.2. Hematopoietic tissue

In the light microscopic studies, the hematopoietic tissue of *P. homarus* was observed as a thin (80-900 µm thick) layer of tissue loosely bound to the dorsal surface of the foregut. The tissue is covered by an incomplete layer of loose connective tissue and contains muscle fibers, blood vessels and ovoid

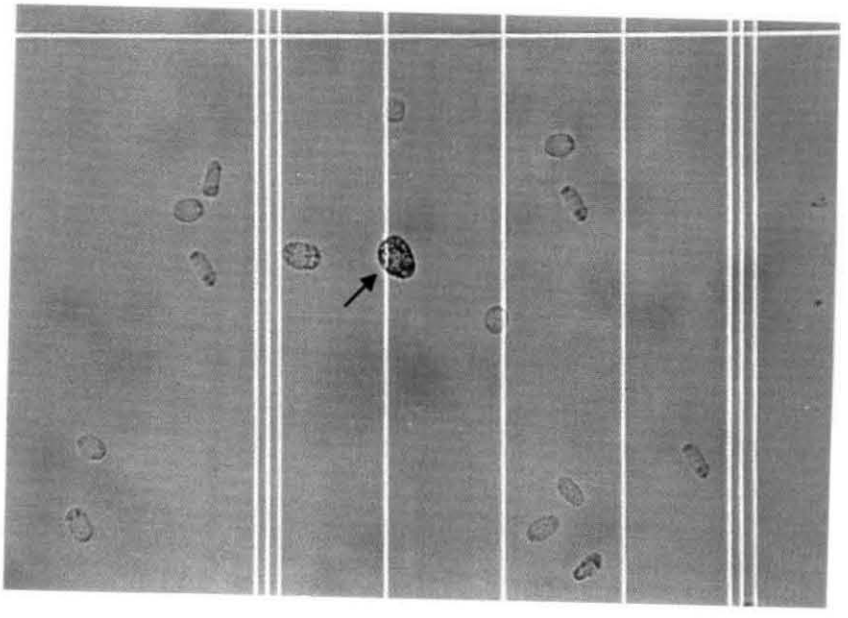


Plate IV. Formalin fixed haemocytes, arrow showing large granular cells

Table I. Cellular dimensions of the three types of haemocytes

	Hyaline	Semi-granular	Granular
Cell Length	9.25±1.52 µm	10.07±1.79 µm	13.2±1.6 µm
Cell Width	5.85±0.69 µm	5.64±1.2 µm	8.3±1.4 µm
Nuclear Length	5.38±0.92 µm	5.79±1.52 µm	3.8±0.6 µm
Nuclear Width	4.35±0.76 µm	3.85±0.638 µm	3.6±0.48 µm

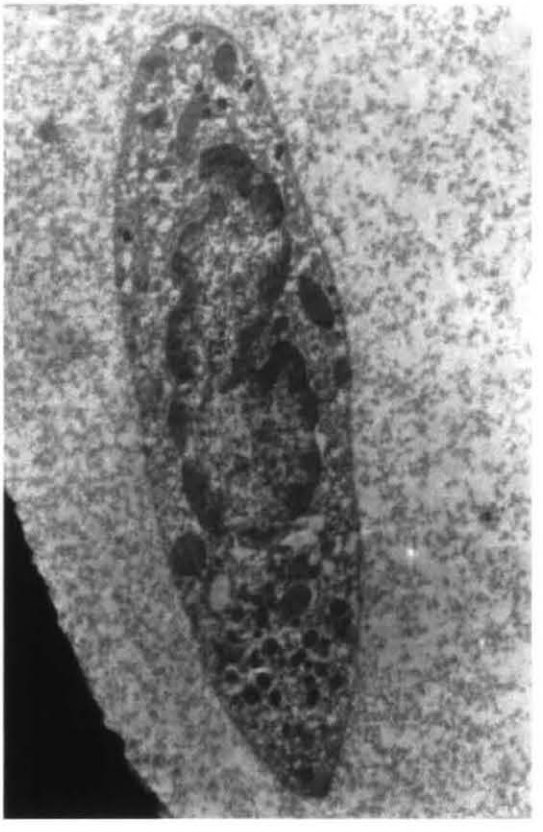


Plate V. TEM view of hyaline cell with few granules (8000X)

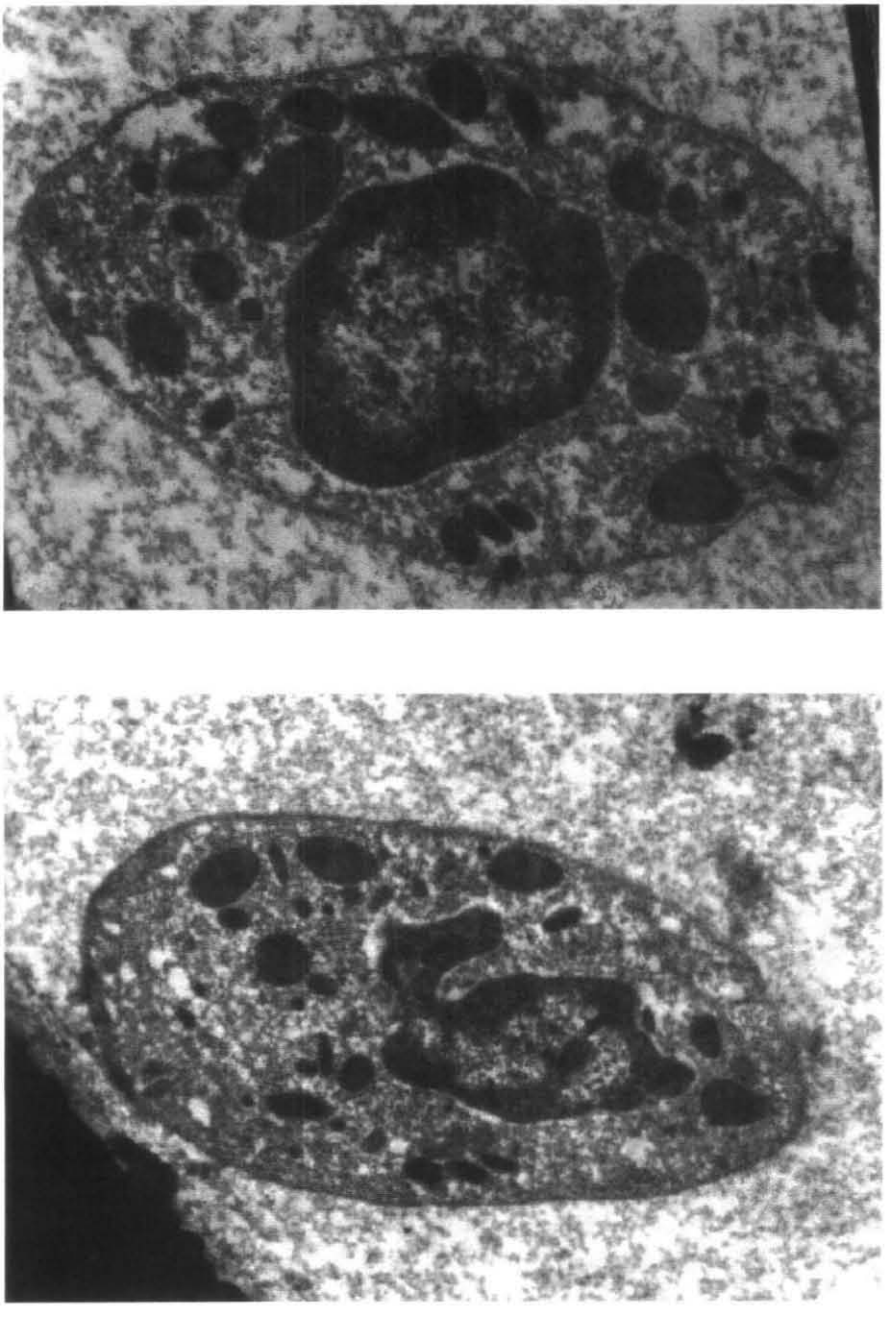


Plate VI. TEM view of semi-granular cells with less dense granules (8000X)

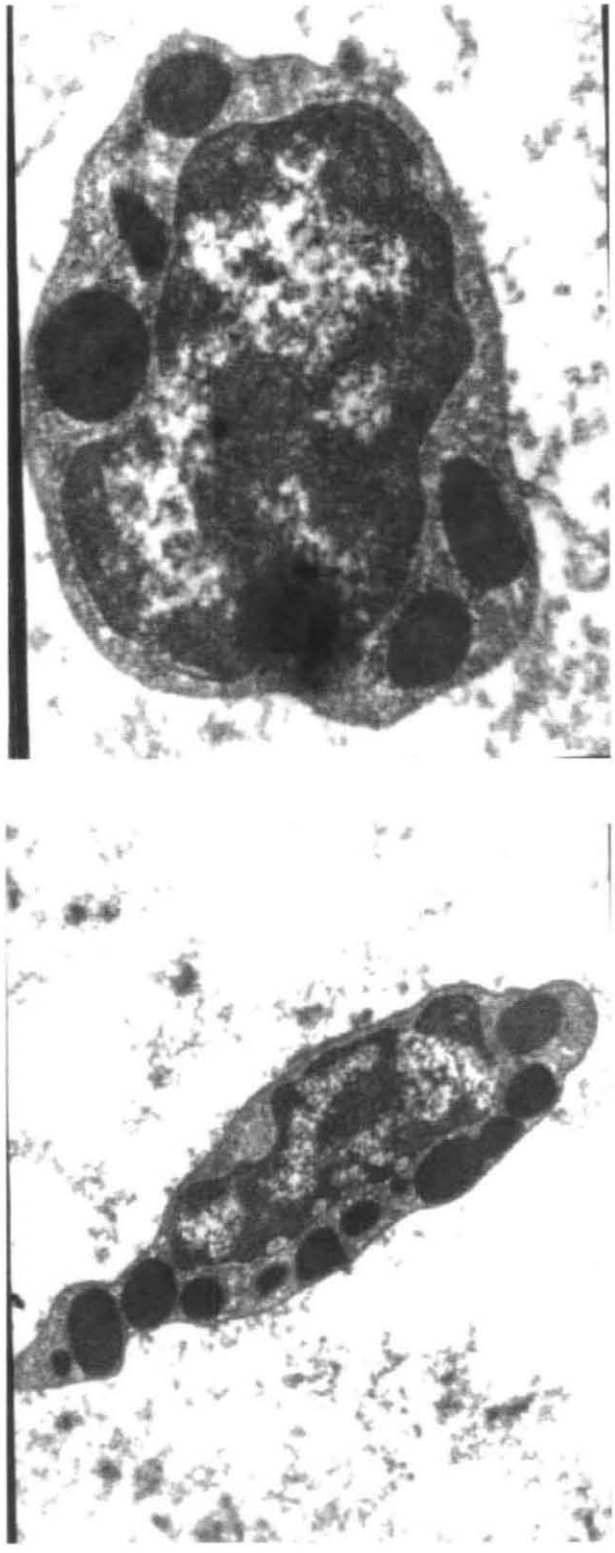


Plate VII. TEM view of large granular cells with large dense granules in the cytoplasm (8000X)

lobules including stem cells and maturing haemocytes. The lobules are more abundant in the foregut region.

The hematopoietic lobules are ovoid and the size ranges from 30-875 µm (Plate VIII). Most lobules are clearly separated from the adjacent lobules, although some appear as fused. Each lobule contains a cluster of densely packed stem cells and maturing haemocytes surrounded by connective tissue layers (Plate IX). This layer is composed of a fibrillar basement membrane (0.2 µm thick).

4.3. Immune parameters of a healthy lobster

The various immunological parameters of unstressed healthy lobsters maintained in the laboratory conditions were categorized. The animals observed were in the size range of 80 to 250 g and in stage C (intermoult) of the moult cycle. Though many functions of the lobster haemocytes are analogous to vertebrate immune cells, there is no consensus on the definition of haemocyte types. The mean Total Haemocyte Count (THC) was 8.7 ± 3.8 x 10^6 cells per ml of haemolymph (Plate XVI). The mean relative percentages of different haemocyte types were HC: 64.74 ± 8.75, SGC: 26.22 ± 6.57 and LGC: 8.4 ± 4.8. The blood volume and body weight showed positive correlation with each other (Fig. I). The mean phenoloxidase activity was observed to be 30.61 ± 5.6 IU mg $protein^{-1}$. The cytochemistry studies for phenoloxidase activity of haemocytes showed 34.8 ± 5.37% of positive reaction (Plate X & XI). The haemagglutination titer ranged from 1:32 to 1:128 in the haemolymph and from 1:64 to 1:256 in the case of serum (Plate XII). The mean phagocytic index was 5.85 ± 1.2 % when challenged with yeast cells (Plate XIV), and 9.2 ± 2.3% in the case of bacterial cells (Plate XV). The superoxide anion production showed a mean range of 1.3 ± 0.19. The serum acid phosphatase activity showed a mean value of 4.3 ± 0.69 KAU. The clotting time was found to be 0.48 ± 0.03 seconds for a healthy lobster. In bactericidal studies by plate count method, no growth was found in the plates after 3hr and 6 hr incubation of haemolymph with bacteria, *Vibrio alginolyticus* in contrast to the control with only bacteria (Plate XIII). Turbidometric studies for bactericidal activity also showed no significant increase in O.D in serum sample in comparison to the control sample (Fig. II).

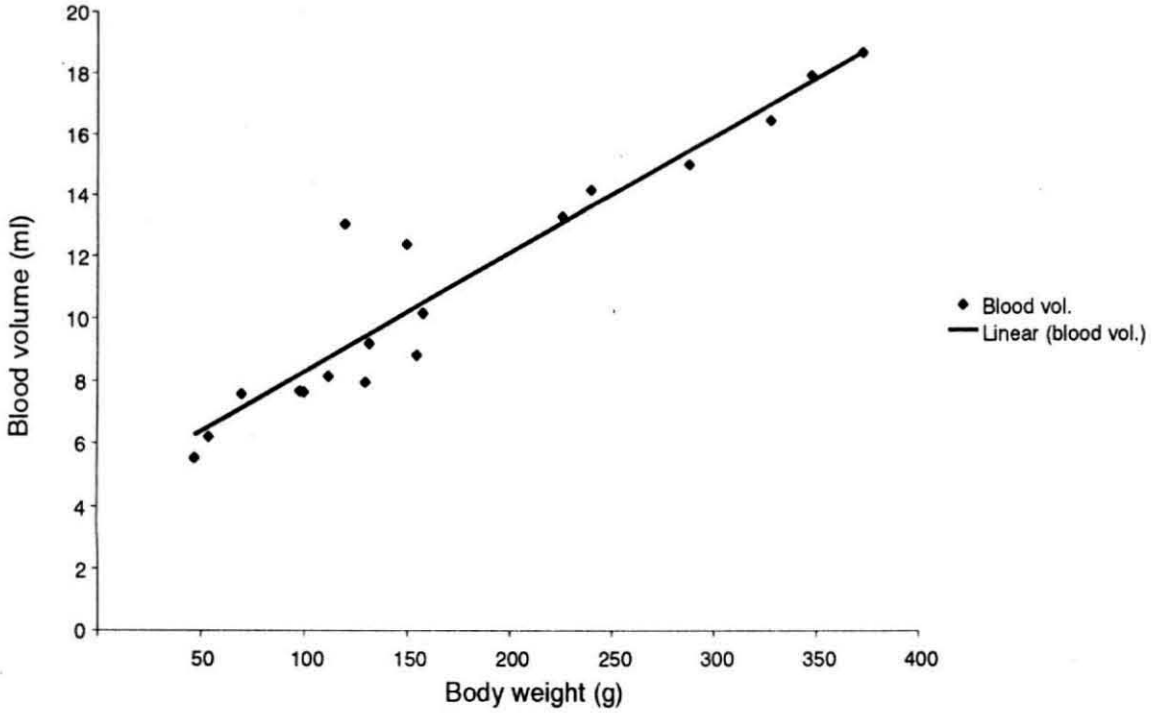


Fig. I. Relationship between body weight and blood volume in *P. homarus*

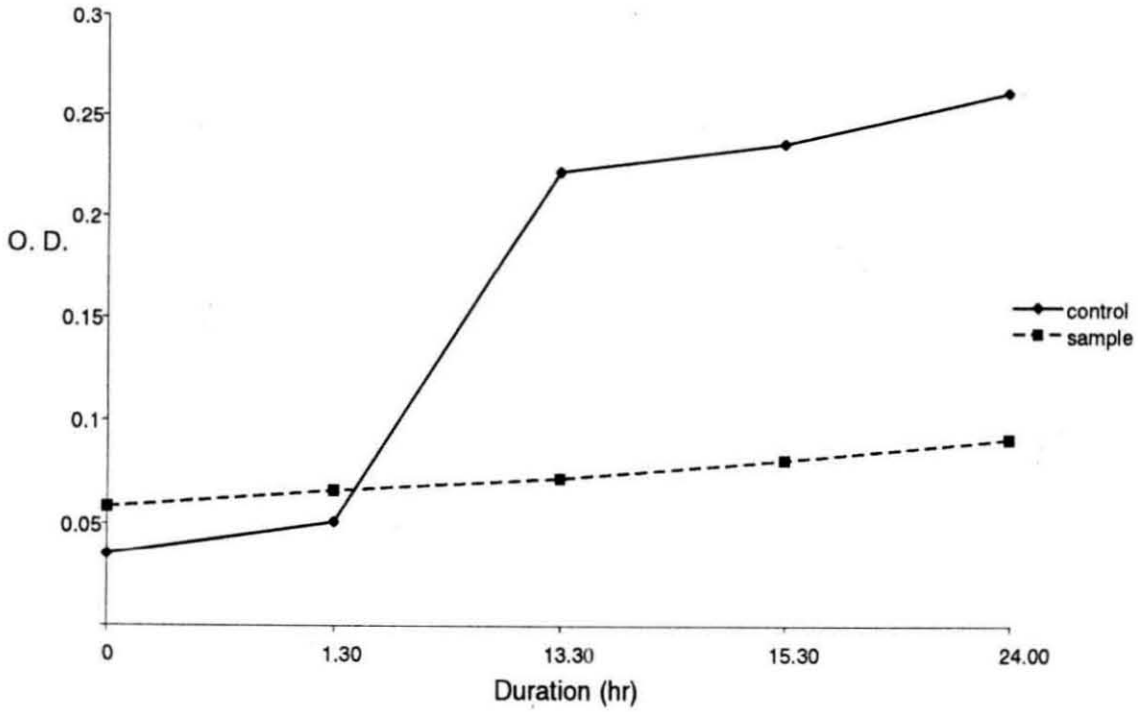


Fig. II. Turbidometric assay of bactericidal activity of *P. homarus* serum

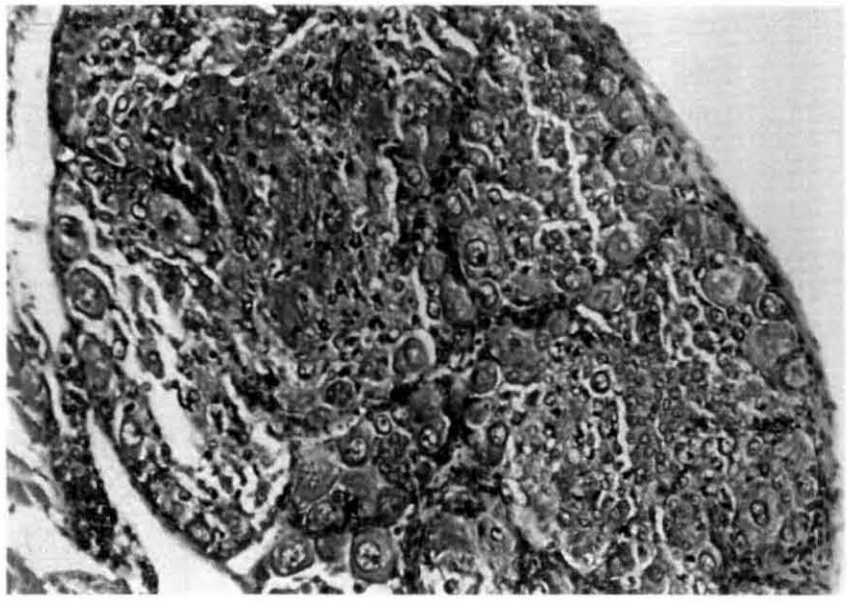


Plate VIII. Light microscopic view of lobster HPT lobule (1000X)

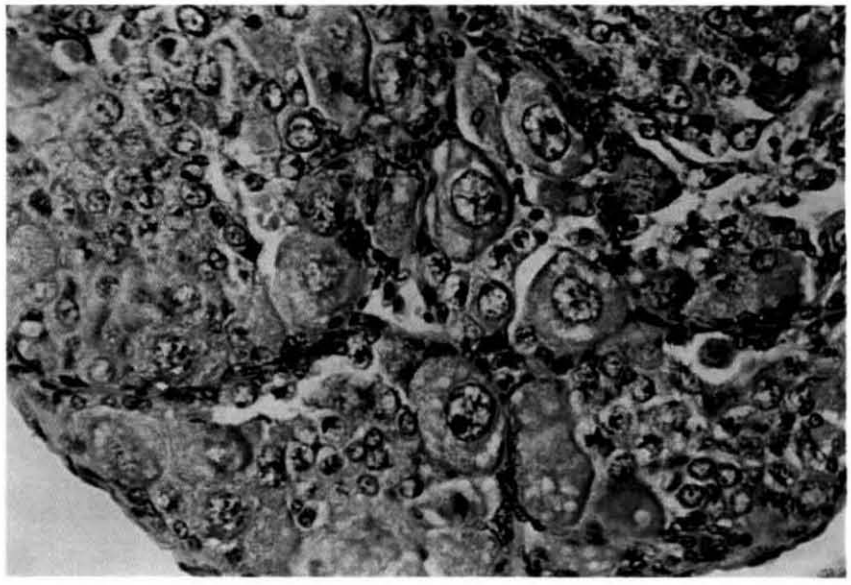


Plate IX. Lobules of HPT with stem cells, maturing and mature cells (400X)

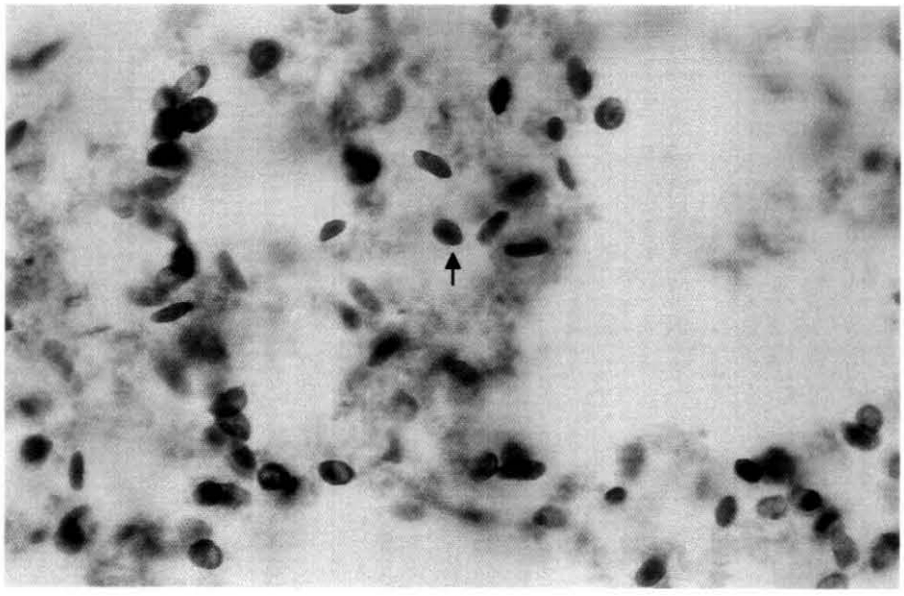


Plate X. Phenoloxidase activity of haemocytes (400X)

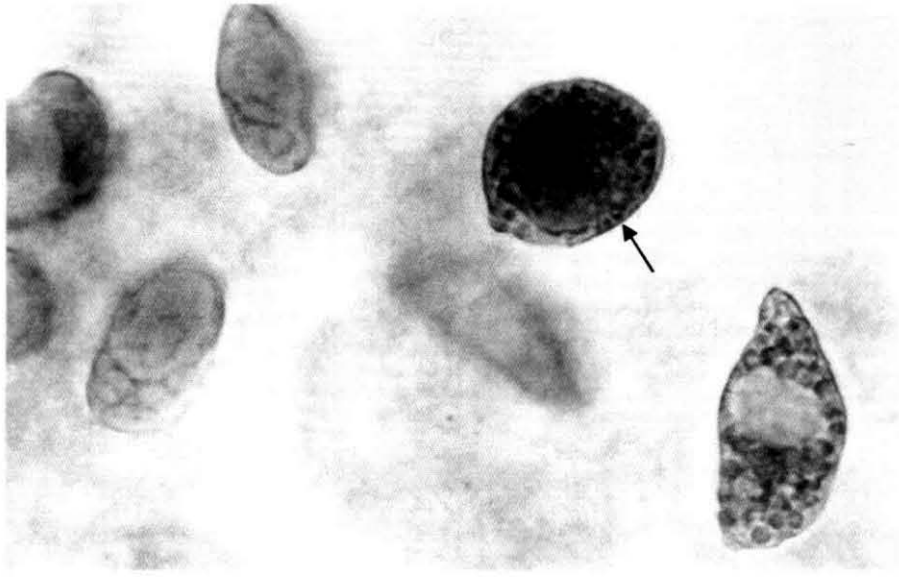


Plate XI. Phenoloxidase activity showing highly melanised large granular cells (1000X)

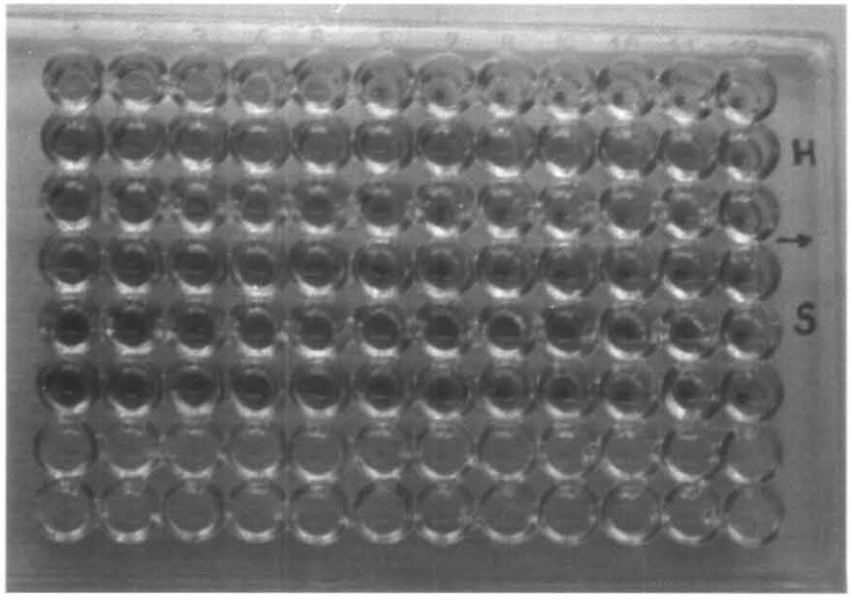


Plate XII. Haemagglutination of lobster serum (S) and haemolymph (H) against chicken RBC

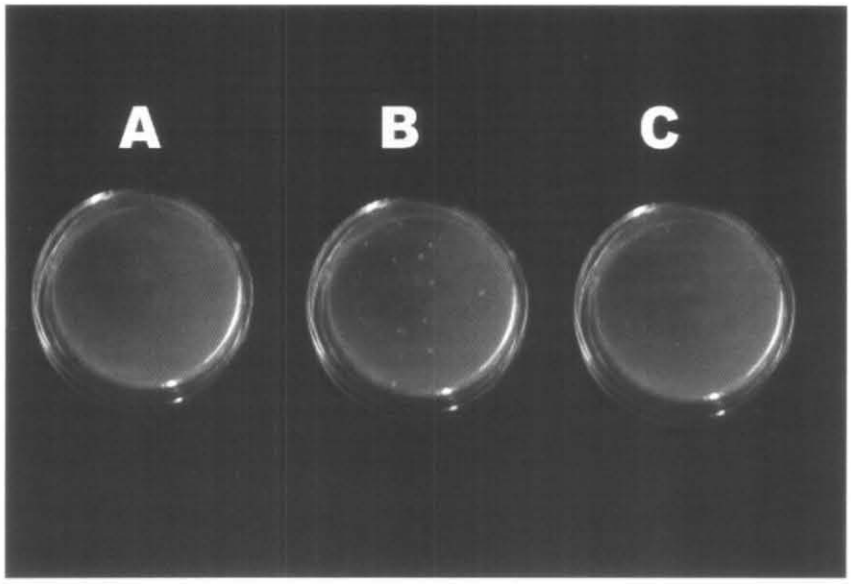


Plate XIII. Bactericidal activity of lobster serum at 3hrs (A) and 6hrs (C) against control (B)

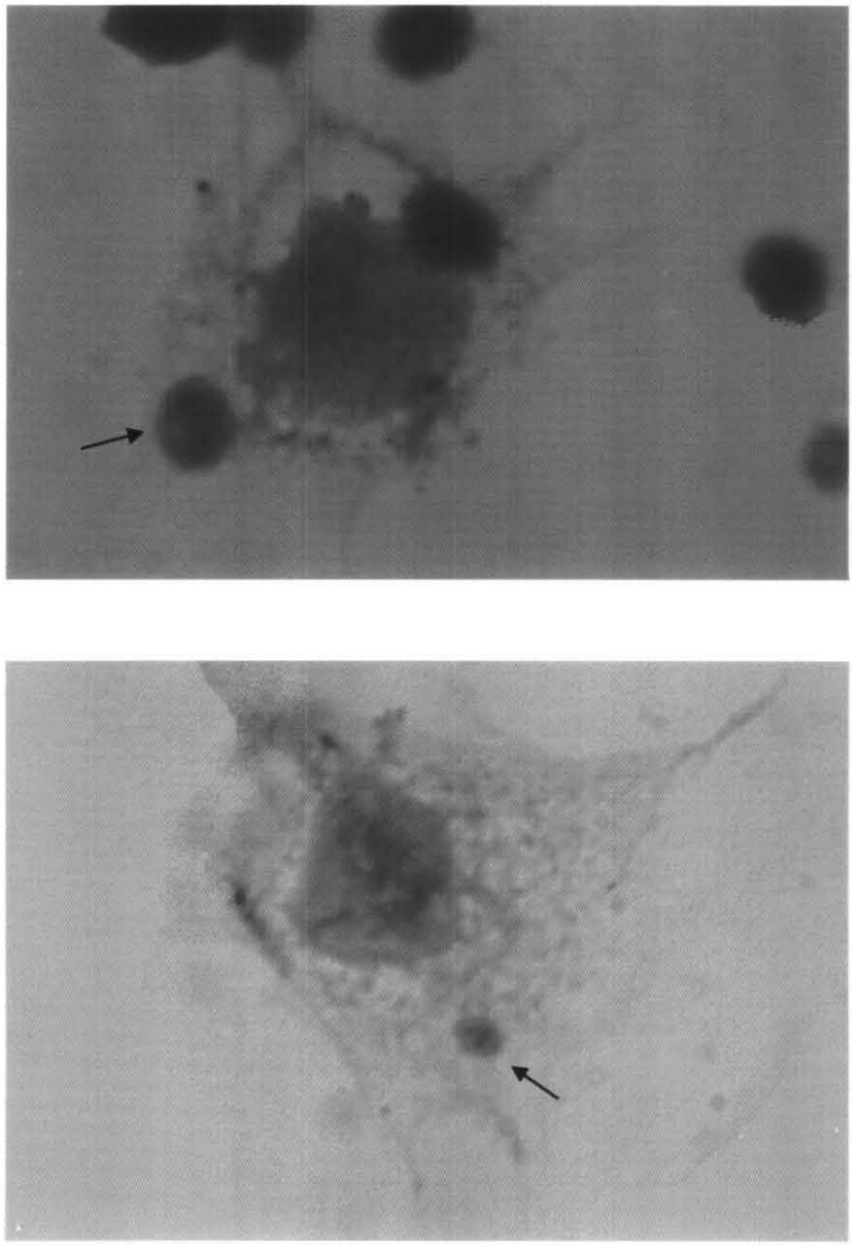


Plate XIV. Phagocytosis of yeast cells by lobster haemocyte (1000X)

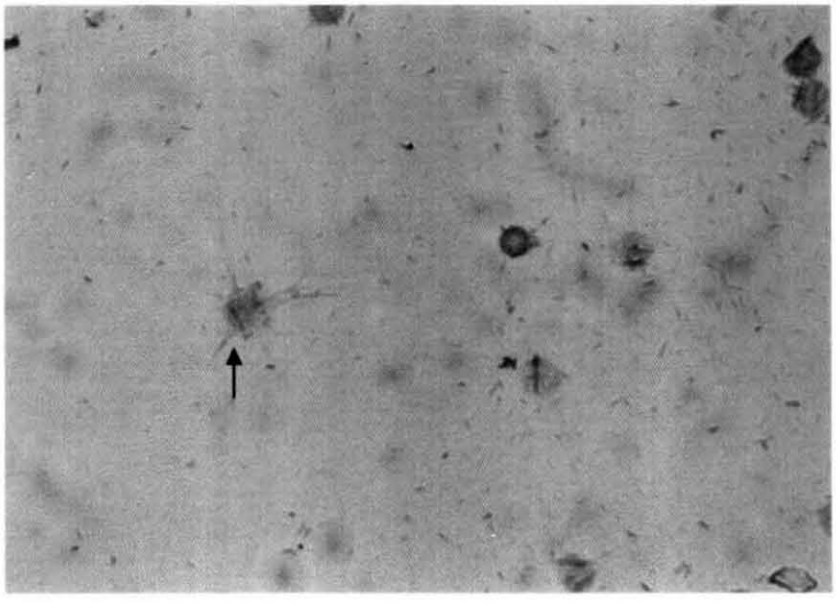


Plate XV. Phagocytosis of bacterial cell by lobster haemocytes (400X)

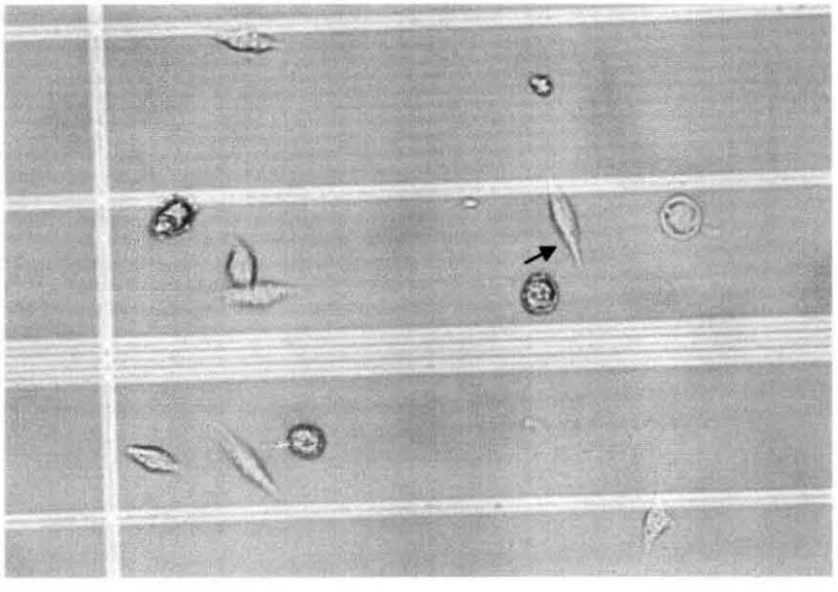


Plate XVI. Live haemocytes with extended filipodia in haemocytometer (400X)

4.4. Effect of stressors on immune parameters

4.4.1. Physiological stress

4.4.1.1. Effect of moulting

The THC in the intermoult stage showed a mean value of 8.75 ± 0.72 x10 ^{6} cells ml^{-1} and that in the postmoult was 13.06 ± 0.48 x 10 ^{6} cells ml^{-1}. In the premoult, the mean THC value was 11.98 ± 0.56 x 10 ^{6} cells ml^{-1} (Fig. III). The ANOVA analysis showed significantly low THC (Table II a & b) in the intermoult (stage C) compared to those in the postmoult (stage A and B) and in the premoult (stage D_0, D_1, D_2, D4). During postmoult, the relative percentages of different haemocytes were as follows. Mean HC: 72.57 ± 6.45, SGC: 24 ± 6.59 and the LGC: 4.57 ± 2.99. In the intermoult, the mean relative percentage of different haemocytes is HC: 68.82 ± 5.11, SGC: 20.31 ± 5.7 and LGC: 7.59 ± 2.92. In the premoult stage, the mean percentage for HC was 63.38 ± 7.82, SGC: 29.75 ± 6.63, and LGC: 6.56 ± 3.54.

During intermoult, the phenoloxidase activity measured in the serum of lobsters was found to be a mean of 31.91 ± 3.67 IU mg protein^{-1}, whereas in the postmoult the value was 18.07 ± 1.56 IU mg protein^{-1} and in premoult, 21.07 ± 2.85 IU mg protein^{-1}. The phenoloxidase activity was found to be significantly higher (P<0.05) in the intermoult than in the premoult and in the post moult.

4.4.1.2. Effect of eyestalk ablation

The THC measured 2hrs after eyestalk ablation showed an increase in count from 5.5 ± 0.35 x 10^{6} cells ml^{-1} to 6.58 ± 0.13 x 10^{6} cells ml^{-1} (Fig. IV). The THC after one week was 6.8 ± 1.21 x 10^{6} cells ml^{-1}, which did not show any significant variation (P<0.05) from the THC obtained 2hrs after ablation. The relative percentage of haemocyte types was as follows: mean HC at 0hr was 66.25 ± 3.18, the SGC, 25.48 ± 4.7 and the LGC, 8.27 ± 1.5. After 2 hrs the HC was reduced to 61.3 ± 2.4, which was significantly lower from the 0 hr value. On the other hand, the mean SGC value was 20.6 ± 0.56, which was significantly lower than the 0 hr. The LGC showed significant increase in relative percentage with a mean value of 15.5 ± 0.07. The samples collected after a week did not show any significant variation from the 0 hr group. The mean relative

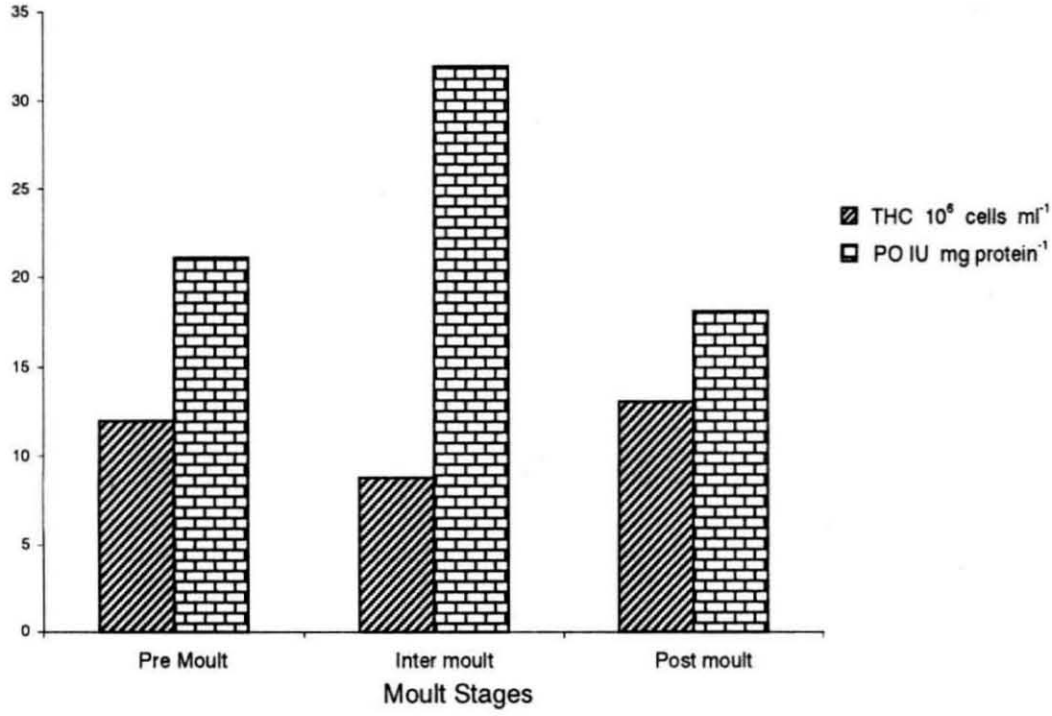


Fig. III. Effect of moulting on immune response of *P. homarus*

Table II a. ANOVA for influence of moult stages ($P<0.05$)

		Sum of Squares	df	Mean Square	F	Sig.
PO	Between Groups	317.722	2	158.861	19.812	.002*
	Within Groups	48.110	6	8.018		
	Total	365.832	8			
THC	Between Groups	30.147	2	15.073	42.977	.000*
	Within Groups	2.104	6	.351		
	Total	32.251	8			

* significant at $P<0.05$

Table II b. Multiple comparisons for different moult stages
LSD

			Mean Difference (I-J)	Sig.	95% Confidence Interval	
Dependent Variable	(I) MOULT	(J) MOULT			Lower Bound	Upper Bound
PO	1.00	2.00	-10.8333(*)	.003	-16.4907	-5.1760
		3.00	3.0000	.242	-2.6574	8.6574
	2.00	1.00	10.8333(*)	.003	5.1760	16.4907
		3.00	13.8333(*)	.001	8.1760	19.4907
	3.00	1.00	-3.0000	.242	-8.6574	2.6574
		2.00	-13.8333(*)	.001	-19.4907	-8.1760
THC	1.00	2.00	3.2233(*)	.001	2.0401	4.4065
		3.00	-1.0867	.066	-2.2699	9.654E-02
	2.00	1.00	-3.2233(*)	.001	-4.4065	-2.0401
		3.00	-4.3100(*)	.000	-5.4932	-3.1268
	3.00	1.00	1.0867	.066	-9.6542E-02	2.2699
		2.00	4.3100(*)	.000	3.1268	5.4932

1.00 - Pre-moult, 2.00 - Inter-moult, 3.00 - Post-moult, * significant at $P<0.05$

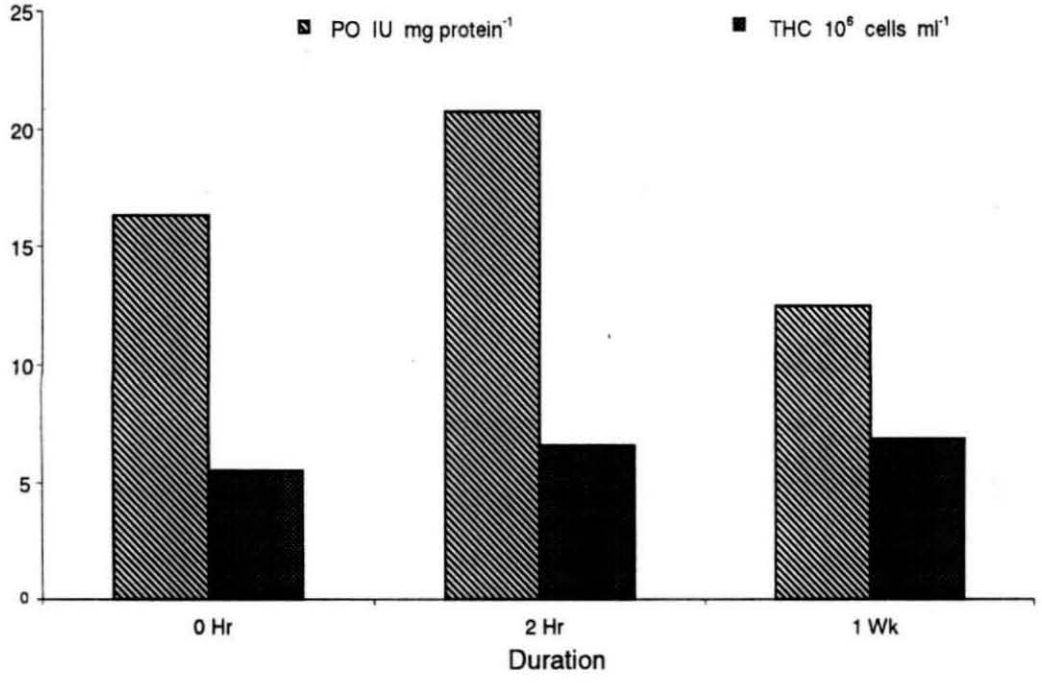


Fig. IV. Effect of eyestalk ablation on the immune parameters of *P. homarus*

Table III a. ANOVA table for the effect of ablation ($P<0.05$)

		Sum of Squares	df	Mean Square	F	Sig.
HC	Between Groups	24.755	2	12.377	1.997	.281
	Within Groups	18.596	3	6.199		
	Total	43.351	5			
SGC	Between Groups	43.299	2	21.649	2.115	.267
	Within Groups	30.714	3	10.238		
	Total	74.012	5			
LGC	Between Groups	65.091	2	32.546	25.377	.013*
	Within Groups	3.847	3	1.282		
	Total	68.939	5			
PO	Between Groups	136.642	2	68.321	26.729	.000*
	Within Groups	23.005	9	2.556		
	Total	159.647	11			
THC	Between Groups	2.101	2	1.051	1.964	.285
	Within Groups	1.605	3	.535		
	Total	3.706	5			

* significant at $P<0.05$

Table III b. Multiple comparisons for ablation studies

LSD

Dependent Variable	(I) EXP	(J) EXP	Mean Difference (I-J)	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
HC	no significant difference					
SGC	no significant difference					
LGC	0.00	1.00	-.6400	.611	-4.2440	2.9640
		2.00	-7.2850(*)	.008	-10.8890	-3.6810
	1.00	0.00	.6400	.611	-2.9640	4.2440
		2.00	-6.6450(*)	.010	-10.2490	-3.0410
	2.00	0.00	7.2850(*)	.008	3.6810	10.8890
		1.00	6.6450(*)	.010	3.0410	10.2490
PO	0.00	1.00	3.8650(*)	.008	1.3076	6.4224
		2.00	-4.3950(*)	.004	-6.9524	-1.8376
	1.00	0.00	-3.8650(*)	.008	-6.4224	-1.3076
		2.00	-8.2600(*)	.000	-10.8174	-5.7026
	2.00	0.00	4.3950(*)	.004	1.8376	6.9524
		1.00	8.2600(*)	.000	5.7026	10.8174
THC	no significant difference					

0.0 - Initial, 1.00 – 2hours, 2.00 – One week, * significant at $P<0.05$

percentage value for HC was 64.21 ± 1.64, the SGC: 26.87 ± 2.88 and the LGC: 8.91 ± 1.24.

Higher PO activity values were obtained 2 hrs after ablation compared to the lobsters before ablation. However, after one week, the activity showed a steady decrease. The mean value of PO activity of serum at 0 hr was 16.33 ± 0.87 IU mg protein^{-1} which was gradually increased to 20.73 ± 1.01 IU mg protein^{-1}. ANOVA analysis (Table III a & b) showed significant increase in the PO activity after ablation. Samples collected over a week showed a decrease in PO activity from the 0 hr with a mean value of 12.46 ± 2.42 IU mg protein^{-1}.

4.4.2. Nutritional stress

4.4.2.1. Effect of starvation

The total haemocyte count during the second and third week of starvation was significantly lower than the well-fed control lobsters ($P< 0.05$). The THC after a week's starvation did not vary significantly from the control group. The mean value of THC of the control group was 7.84 ± 1.11 x 10 ^{6} cells ml^{-1}, where as in the sample observed after one week, the value was 6.55 ± 1.03 x 10 ^{6} cells ml^{-1} (Fig. V). The THC observed 2 weeks after starvation was 4.53 ± 0.34 x 10 ^{6} cells ml^{-1} and after three weeks, the value was 2.37 ± 0.48 x 10 ^{6} cells ml^{-1}.

The PO activity measured in the serum after three weeks of starvation was significantly low ($P <0.05$) (Table IV a & b) with a mean value of 12.24 ± 3.41 IU mg protein^{-1}. The first and second week of starvation also showed a steady decrease in PO activity in comparison with the control, being 22.62 ± 3.57 IU mg protein^{-1}and 18.51 ± 4.36 IU mg protein^{-1} respectively.

The serum acid phosphatase activity in the first week of starvation did not show any significant difference from the control group but during second and third week of starvation, the activity was found to show significant reduction ($P<0.05$). The mean value for acid phosphatase activity of serum in the control group was 4.32 ± 0.7 KAU, 4.51 ± 1.45 KAU after one week of starvation, 2.18 ± 0.79 KAU after two weeks and 1.62 ± 1.4 KAU after three weeks of starvation.

The phagocytic index was found to decrease significantly in relation to the period of starvation. The phagocytic index during the first week of starvation

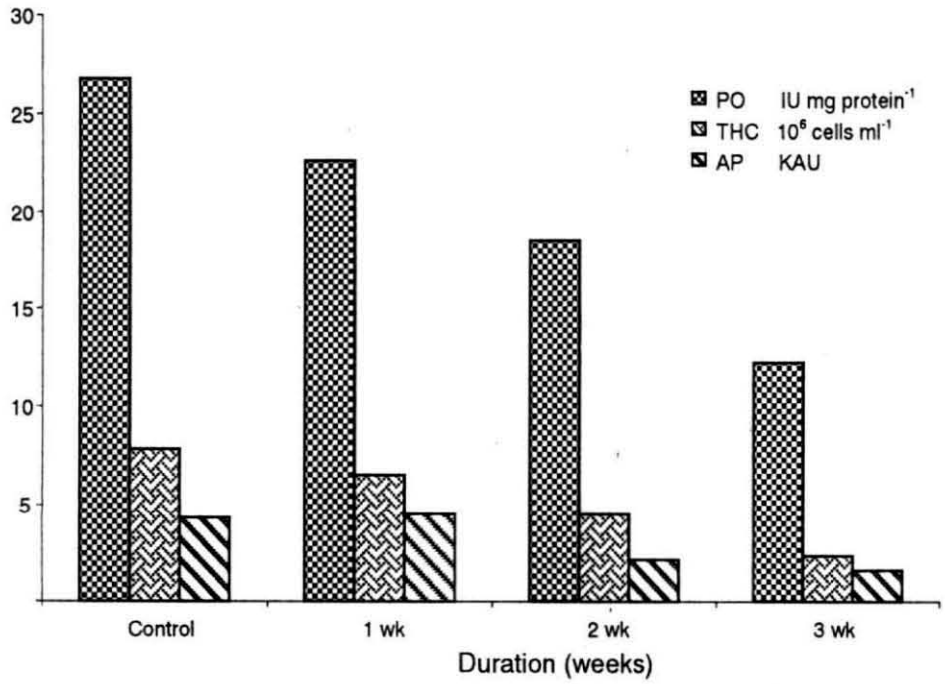


Fig. V. Effect of starvation on defense system of *P. homarus*

Table IV a. ANOVA table for the starvation experiment ($P<0.05$)

		Sum of Squares	df	Mean Square	F	Sig.
AP	Between Groups	17.092	3	5.697	14.287	.000*
	Within Groups	4.785	12	.399		
	Total	21.877	15			
PO	Between Groups	920.496	3	306.832	29.236	.000*
	Within Groups	293.860	28	10.495		
	Total	1214.355	31			
THC	Between Groups	102.707	3	34.236	51.339	.000*
	Within Groups	13.337	20	.667		
	Total	116.044	23			

* significant at $P<0.05$

Table IV b. Multiple comparisons for the starvation experiment

LSD

Dependent Variable	(I) EXP	(J) EXP	Mean Difference (I-J)	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
AP	1.00	2.00	.6250	.187	-.3479	1.5979
		3.00	1.9100(*)	.001	.9371	2.8829
		4.00	-.9350	.058	-1.9079	3.789E-02
	2.00	1.00	-.6250	.187	-1.5979	.3479
		3.00	1.2850(*)	.014	.3121	2.2579
		4.00	-1.5600(*)	.004	-2.5329	-.5871
	3.00	1.00	-1.9100(*)	.001	-2.8829	-.9371
		2.00	-1.2850(*)	.014	-2.2579	-.3121
		4.00	-2.8450(*)	.000	-3.8179	-1.8721
	4.00	1.00	.9350	.058	-3.7891E-02	1.9079
		2.00	1.5600(*)	.004	.5871	2.5329
		3.00	2.8450(*)	.000	1.8721	3.8179
PO	1.00	2.00	4.1100(*)	.017	.7920	7.4280
		3.00	10.3875(*)	.000	7.0695	13.7055
		4.00	-4.1363(*)	.016	-7.4543	-.8182
	2.00	1.00	-4.1100(*)	.017	-7.4280	-.7920
		3.00	6.2775(*)	.001	2.9595	9.5955
		4.00	-8.2463(*)	.000	-11.5643	-4.9282
	3.00	1.00	-10.3875(*)	.000	-13.7055	-7.0695
		2.00	-6.2775(*)	.001	-9.5955	-2.9595
		4.00	-14.5238(*)	.000	-17.8418	-11.2057
	4.00	1.00	4.1363(*)	.016	.8182	7.4543
		2.00	8.2463(*)	.000	4.9282	11.5643
		3.00	14.5238(*)	.000	11.2057	17.8418
THC	1.00	2.00	2.0183(*)	.000	1.0349	3.0018
		3.00	4.1725(*)	.000	3.1890	5.1560
		4.00	-1.2850(*)	.013	-2.2685	-.3015
	2.00	1.00	-2.0183(*)	.000	-3.0018	-1.0349
		3.00	2.1542(*)	.000	1.1707	3.1376
		4.00	-3.3033(*)	.000	-4.2868	-2.3199
	3.00	1.00	-4.1725(*)	.000	-5.1560	-3.1890
		2.00	-2.1542(*)	.000	-3.1376	-1.1707
		4.00	-5.4575(*)	.000	-6.4410	-4.4740
	4.00	1.00	1.2850(*)	.013	.3015	2.2685
		2.00	3.3033(*)	.000	2.3199	4.2868
		3.00	5.4575(*)	.000	4.4740	6.4410

1.00 –one week, 2.00 – two weeks, 3.00 – three weeks, 4.00 –control, * significant at $P<0.05$

was 4.49 ± 0.40 and 3.86 ± 0.38 during the second week and 2.58 ± 0.68 after three weeks as against the control value of 5.42 ± 0.9.

4.4.2.2. Influence of feed

In the experiments conducted to study the immune response in relation to the feed, the most preferred food (mussel) and the least preferred trash fish were studied. The results show that there was significant variation in the THC ($P<0.05$) over the two types of feed (Table V). The mean THC value for the mussel fed was 11.05 ± 1.74 x 10^6 cells ml^{-1} and that of fish fed, 8.19 ± 0.55 x 10^6 cells ml^{-1}. The relative percentage of haemocytes did not show any significant variation. In the mussel fed animals, the HC was 65.42 ± 3.25 where as in the fish fed group HC value was 66.13 ± 2.5. The SGC in the fish fed lobsters have a mean value of 27.55 ± 2.66 and the mussel fed 27.88 ± 2.09. Mean value for LGC was 6.3 ± 0.88 in the mussel fed compared to 6.66 ± 1.75 in the fish fed.

The PO activity of serum showed significant difference with the diet of mussel and fish with a mean value of 57.63 ± 20.46 IU mg $protein^{-1}$ and 39.81 ± 13.32 IU mg $protein^{-1}$, respectively (Fig. VI).

4.4.3. Environmental Stress

4.4.3.1. Effect of salinity

Total haemocyte count was more or less constant for salinities ranging from 30-40 ppt. The THC ranged from 7.36 ± 0.65 to 8.26 ± 0.3 x 10^6 cells ml^{-1}. In the lower salinities of 20 ppt and 25 ppt and higher salinity of 45ppt, a significant decrease in haemocyte number ($P < 0.05$) was observed. The values were 57.87 %, 28.93 % and 46.37 %, respectively compared to the control group at 35 ppt (100 %). It was observed that the spiny lobster *P. homarus* couldn't survive below 20 ppt.

The PO activity was observed to increase with increasing salinity. In lower salinities, the PO activity was less than that in normal salinities. The PO activity at 20 ppt and 25 ppt was significantly ($P<0.05$) (Table VI a & b) lower than that of 35 ppt, the values being 76 % and 50%, respectively (Fig. VII).

The intracellular superoxide anion (O_2^-) production ratio was found to increase with increasing salinity. In the highest salinity tested, the anion

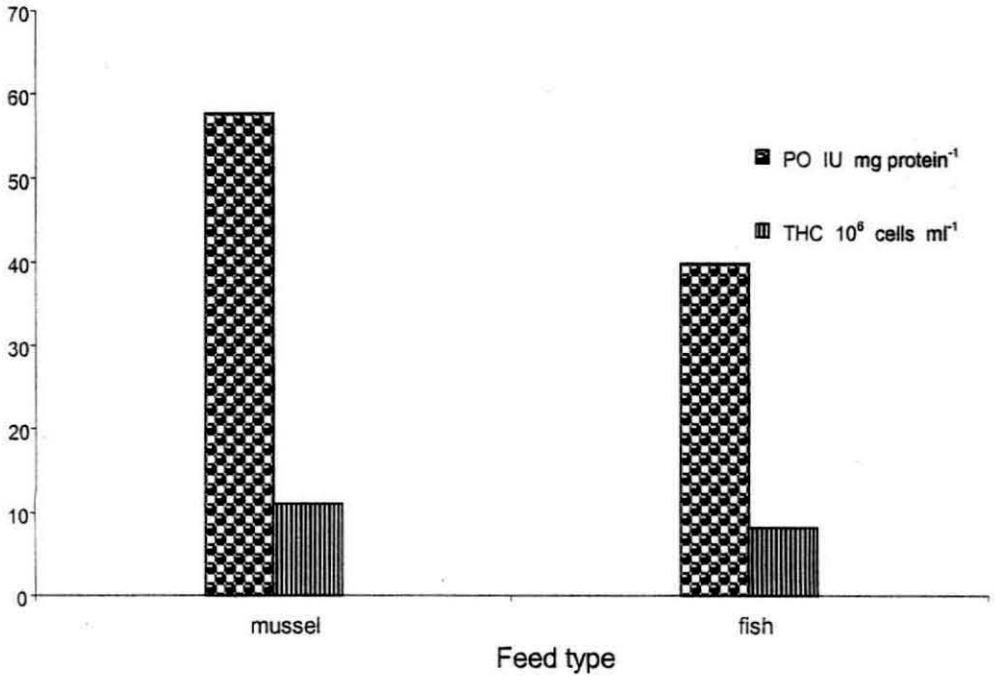


Fig. VI. Effect of different feeds on immune system of *P. homarus*

Table V. ANOVA table for the influence of feed ($P < 0.05$)

		Sum of Squares	df	Mean Square	F	Sig.
HC	Between Groups	1.141	1	1.141	.141	.719
	Within Groups	56.794	7	8.113		
	Total	57.935	8			
SGC	Between Groups	.241	1	.241	.041	.845
	Within Groups	41.301	7	5.900		
	Total	41.542	8			
LGC	Between Groups	.280	1	.280	.159	.702
	Within Groups	12.308	7	1.758		
	Total	12.588	8			
PO	Between Groups	1905.885	1	1905.885	5.441	.029*
	Within Groups	8056.885	23	350.299		
	Total	9962.770	24			
THC	Between Groups	16.359	1	16.359	7.376	.035*
	Within Groups	13.308	6	2.218		
	Total	29.667	7			

*significant at $P<0.05$

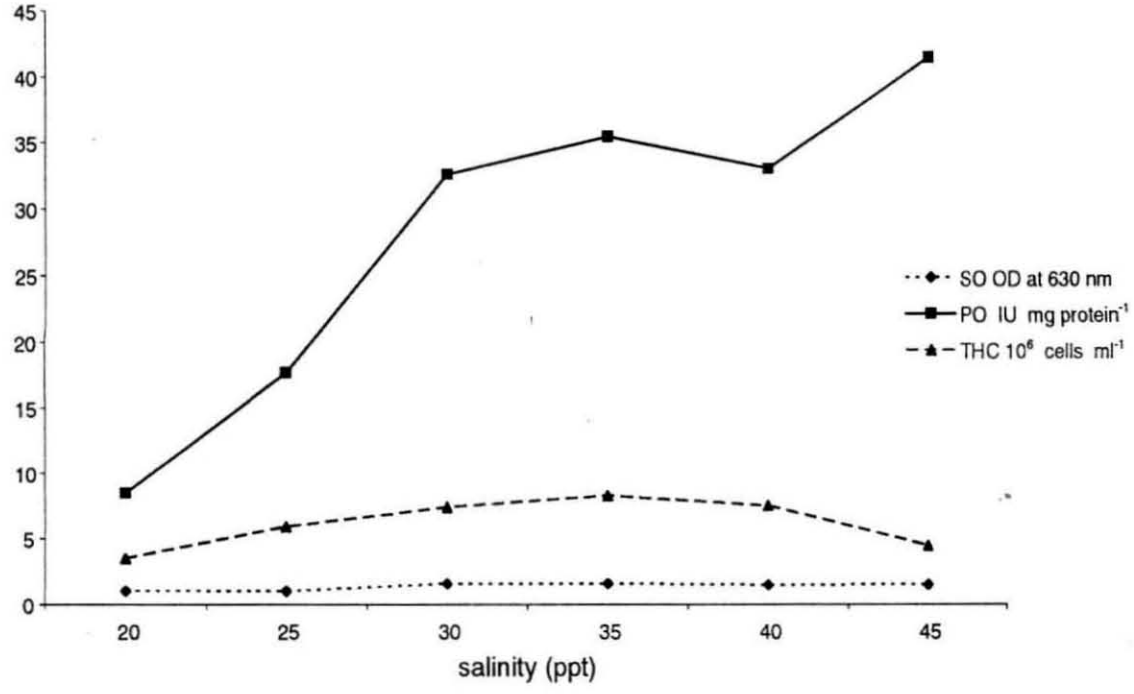


Fig. VII. Effect of salinity on immune system of *P. homarus*

Table VI a. ANOVA table for the effect of salinity ($P<0.05$)

		Sum of Squares	df	Mean Square	F	Sig.
PO	Between Groups	2302.463	5	460.493	71.849	.000*
	Within Groups	76.910	12	6.409		
	Total	2379.373	17			
SO	Between Groups	1.068	5	.214	312.639	.000*
	Within Groups	8.196E-03	12	6.830E-04		
	Total	1.076	17			
THC	Between Groups	53.571	5	10.714	30.585	.000*
	Within Groups	4.204	12	.350		
	Total	57.775	17			

* significant at $P<0.05$

Table VI b. Multiple comparisons for the effect of salinity

LSD

Dependent Variable	(I) SAL	(J) SAL	Mean Difference (I-J)		95% Confidence Interval	
					Lower Bound	Upper Bound
PO	20.00	25.00	-9.2000(*)	.001	-13.7038	-4.6962
		30.00	-24.1000(*)	.000	-28.6038	-19.5962
		35.00	-26.9567(*)	.000	-31.4604	-22.4529
		40.00	-24.5167(*)	.000	-29.0204	-20.0129
		45.00	-32.8900(*)	.000	-37.3938	-28.3862
	25.00	20.00	9.2000(*)	.001	4.6962	13.7038
		30.00	-14.9000(*)	.000	-19.4038	-10.3962
		35.00	-17.7567(*)	.000	-22.2604	-13.2529
		40.00	-15.3167(*)	.000	-19.8204	-10.8129
		45.00	-23.6900(*)	.000	-28.1938	-19.1862
	30.00	20.00	24.1000(*)	.000	19.5962	28.6038
		25.00	14.9000(*)	.000	10.3962	19.4038
		35.00	-2.8567	.192	-7.3604	1.6471
		40.00	-.4167	.844	-4.9204	4.0871
		45.00	-8.7900(*)	.001	-13.2938	-4.2862
	35.00	20.00	26.9567(*)	.000	22.4529	31.4604
		25.00	17.7567(*)	.000	13.2529	22.2604
		30.00	2.8567	.192	-1.6471	7.3604
		40.00	2.4400	.261	-2.0638	6.9438
		45.00	-5.9333(*)	.014	-10.4371	-1.4296
	40.00	20.00	24.5167(*)	.000	20.0129	29.0204
		25.00	15.3167(*)	.000	10.8129	19.8204
		30.00	.4167	.844	-4.0871	4.9204
		35.00	-2.4400	.261	-6.9438	2.0638
		45.00	-8.3733(*)	.002	-12.8771	-3.8696
	45.00	20.00	32.8900(*)	.000	28.3862	37.3938
		25.00	23.6900(*)	.000	19.1862	28.1938
		30.00	8.7900(*)	.001	4.2862	13.2938
		35.00	5.9333(*)	.014	1.4296	10.4371
		40.00	8.3733(*)	.002	3.8696	12.8771
SO	20.00	25.00	2.700E-02	.230	-1.9493E-02	7.349E-02
		30.00	-.5477(*)	.000	-.5942	-.5012
		35.00	-.5497(*)	.000	-.5962	-.5032
		40.00	-.4283(*)	.000	-.4748	-.3818
		45.00	-.4500(*)	.000	-.4965	-.4035
	25.00	20.00	-2.7000E-02	.230	-7.3493E-02	1.949E-02
		30.00	-.5747(*)	.000	-.6212	-.5282
		35.00	-.5767(*)	.000	-.6232	-.5302
		40.00	-.4553(*)	.000	-.5018	-.4088
		45.00	-.4770(*)	.000	-.5235	-.4305
	30.00	20.00	.5477(*)	.000	.5012	.5942
		25.00	.5747(*)	.000	.5282	.6212
		35.00	-2.0000E-03	.927	-4.8493E-02	4.449E-02
		40.00	.1193(*)	.000	7.284E-02	.1658
		45.00	9.767E-02(*)	.001	5.117E-02	.1442

	35.00	20.00	.5497(*)	.000	.5032	.5962
		25.00	.5767(*)	.000	.5302	.6232
		30.00	2.000E-03	.927	-4.4493E-02	4.849E-02
		40.00	.1213(*)	.000	7.484E-02	.1678
		45.00	9.967E-02(*)	.001	5.317E-02	.1462
	40.00	20.00	.4283(*)	.000	.3818	.4748
		25.00	.4553(*)	.000	.4088	.5018
		30.00	-.1193(*)	.000	-.1658	-7.2841E-02
		35.00	-.1213(*)	.000	-.1678	-7.4841E-02
		45.00	-2.1667E-02	.330	-6.8159E-02	2.483E-02
	45.00	20.00	.4500(*)	.000	.4035	.4965
		25.00	.4770(*)	.000	.4305	.5235
		30.00	-9.7667E-02(*)	.001	-.1442	-5.1174E-02
		35.00	-9.9667E-02(*)	.001	-.1462	-5.3174E-02
		40.00	2.167E-02	.330	-2.4826E-02	6.816E-02
THC	20.00	25.00	-2.3950(*)	.000	-3.4479	-1.3421
		30.00	-3.8933(*)	.000	-4.9463	-2.8404
		35.00	-4.7850(*)	.000	-5.8379	-3.7321
		40.00	-3.9983(*)	.000	-5.0513	-2.9454
		45.00	-.9617	.070	-2.0146	9.128E-02
	25.00	20.00	2.3950(*)	.000	1.3421	3.4479
		30.00	-1.4983(*)	.009	-2.5513	-.4454
		35.00	-2.3900(*)	.000	-3.4429	-1.3371
		40.00	-1.6033(*)	.006	-2.6563	-.5504
		45.00	1.4333(*)	.012	.3804	2.4863
	30.00	20.00	3.8933(*)	.000	2.8404	4.9463
		25.00	1.4983(*)	.009	.4454	2.5513
		35.00	-.8917	.090	-1.9446	.1613
		40.00	-.1050	.832	-1.1579	.9479
		45.00	2.9317(*)	.000	1.8787	3.9846
	35.00	20.00	4.7850(*)	.000	3.7321	5.8379
		25.00	2.3900(*)	.000	1.3371	3.4429
		30.00	.8917	.090	-.1613	1.9446
		40.00	.7867	.130	-.2663	1.8396
		45.00	3.8233(*)	.000	2.7704	4.8763
	40.00	20.00	3.9983(*)	.000	2.9454	5.0513
		25.00	1.6033(*)	.006	.5504	2.6563
		30.00	.1050	.832	-.9479	1.1579
		35.00	-.7867	.130	-1.8396	.2663
		45.00	3.0367(*)	.000	1.9837	4.0896
	45.00	20.00	.9617	.070	-9.1276E-02	2.0146
		25.00	-1.4333(*)	.012	-2.4863	-.3804
		30.00	-2.9317(*)	.000	-3.9846	-1.8787
		35.00	-3.8233(*)	.000	-4.8763	-2.7704
		40.00	-3.0367(*)	.000	-4.0896	-1.9837

* significant at $P<0.05$

production was lower than the control. At 20 ppt salinity, O_2^- production ratio was 35.6 % lower than at 35 ppt.

4.4.3.2. Effect of pH

The THC count was observed to be significantly lower at pH 5 and pH 9.5 compared to the control individuals at pH 8 (Fig. VIII). At pH 5, the mean range was 2.62 ± 0.43 x 10^6 cells ml^{-1} and at pH 9.5, it was 6.92 ± 1.03 x 10^6 cells ml^{-1}, compared to the control with mean value of 8.91 ± 1.59 X 10^6 cells ml^{-1}. The serum PO activity was also found to be significantly low at pH 5 and pH 9.5, compared to the control. The mean PO activity value was 14.26 ± 7.5 IU mg $protein^{-1}$and 15.69 ± 4.54 IU mg $protein^{-1}$, respectively for pH 5 and pH 9.5 compared to the control group (pH 8) with a PO activity of 25.87 ± 1.61 IU mg $protein^{-1}$.

The super oxide anion production did not show any significant variation between the three pH groups. The O_2^- production showed a mean value of 1.2 ± 0.25 and 1.04 ± 0.13 in pH 5 and 9.5, respectively as against the control, which showed a mean value of 1.34 ± 0.19.

There was significant reduction in serum acid phosphatase activity (*P<0.05*) at pH 9.5 compared to the control group (Table VII a & b). The value showed a mean of 1.43 ± 0.33 KAU and that of control was 4.85 ± 0.49 KAU. But the value at pH 5 did not show much variation from the control with a mean value of 3.2 ± 1.01 KAU.

4.4.3.3. Effect of dissolved oxygen (DO)

The THC count was found to be significantly low in the ANOVA analysis of animals kept at low dissolved oxygen of 1mg l^{-1}, which showed a mean value of 7.16 ± 1.51 x 10^6 cells ml^{-1}. In normal individuals, the mean value was 10.08 ± 1.83 x 10^6 cells ml^{-1}. The relative percentage haemocyte showed a significant increase in HC count, which showed a mean value of 68.1± 1.66 over the normal with a mean value 66.62 ± 2.43. The granular cells showed an increase in count in hypoxic condition, whereas the mean value for the semi granular cells was 22.15 ± 2.45 in contrast to the control the value was 24.78 ± 2.13 and that for LGC was 9.73±3.2 as against 8.59 ± 1.78 for control.

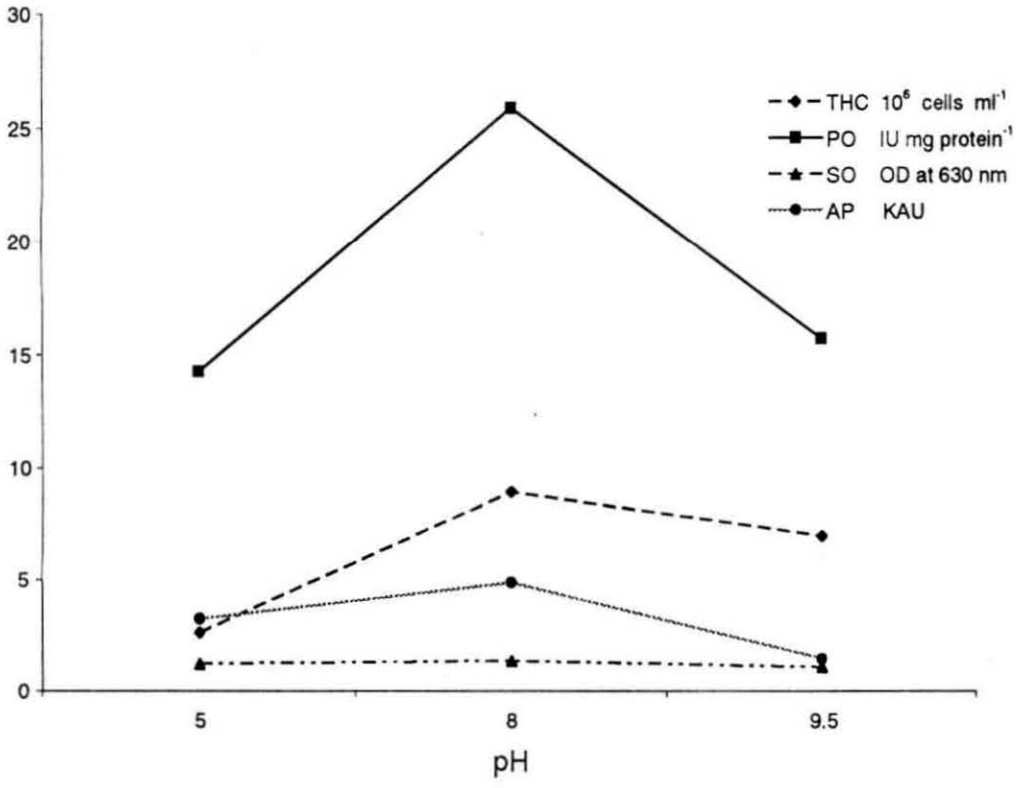


Fig. VIII. Effect of pH on the immune parameters of *P. homarus*

Table VII a. ANOVA table for the effect of pH ($P< 0.05$)

		Sum of Squares	df	Mean Square	F	Sig.
AP	Between Groups	11.706	2	5.853	12.786	.034*
	Within Groups	1.373	3	.458		
	Total	13.079	5			
HC	Between Groups	.495	2	.247	.067	.936
	Within Groups	29.518	8	3.690		
	Total	30.013	10			
LGC	Between Groups	70.754	2	35.377	26.274	.000*
	Within Groups	10.772	8	1.346		
	Total	81.526	10			
PO	Between Groups	480.967	2	240.484	8.922	.003*
	Within Groups	404.311	15	26.954		
	Total	885.279	17			
SGC	Between Groups	51.386	2	25.693	.453	.651
	Within Groups	454.111	8	56.764		
	Total	505.497	10			
SO	Between Groups	.369	2	.185	1.336	.292
	Within Groups	2.072	15	.138		
	Total	2.441	17			
THC	Between Groups	124.099	2	62.050	51.871	.000*
	Within Groups	17.943	15	1.196		
	Total	142.042	17			

* significant at $P<0.05$

Table VII b. Multiple comparisons for the influence of pH

LSD

Dependent Variable	(I) pH	(J) pH	Mean Difference (I-J)	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
AP	5.00	8.00	-1.6250	.096	-3.7782	.5282
		9.50	1.7950	.077	-.3582	3.9482
	8.00	5.00	1.6250	.096	-.5282	3.7782
		9.50	3.4200(*)	.015	1.2668	5.5732
	9.50	5.00	-1.7950	.077	-3.9482	.3582
		8.00	-3.4200(*)	.015	-5.5732	-1.2668
HC	no significant difference					
LGC	5.00	8.00	-8.3333E-02	.927	-2.1270	1.9604
		9.50	5.2242(*)	.000	3.1805	7.2679
	8.00	5.00	8.333E-02	.927	-1.9604	2.1270
		9.50	5.3075(*)	.000	3.4154	7.1996
	9.50	5.00	-5.2242(*)	.000	-7.2679	-3.1805
		8.00	-5.3075(*)	.000	-7.1996	-3.4154
PO	5.00	8.00	10.1783(*)	.004	3.7894	16.5672
		9.50	11.6117(*)	.001	5.2228	18.0006
	8.00	5.00	-10.1783(*)	.004	-16.5672	-3.7894
		9.50	1.4333	.639	-4.9556	7.8222
	9.50	5.00	-11.6117(*)	.001	-18.0006	-5.2228
		8.00	-1.4333	.639	-7.8222	4.9556
SGC	no significant difference					
SO	no significant difference					
THC	5.00	8.00	-6.2917(*)	.000	-7.6376	-4.9457
		9.50	-4.3017(*)	.000	-5.6476	-2.9557
	8.00	5.00	6.2917(*)	.000	4.9457	7.6376
		9.50	1.9900(*)	.007	.6441	3.3359
	9.50	5.00	4.3017(*)	.000	2.9557	5.6476
		8.00	-1.9900(*)	.007	-3.3359	-.6441

* significant at $P<0.05$

The PO activity was also found to decrease when the dissolved oxygen was reduced to 1mg ml^{-1} (Fig. IX). The PO activity during hypoxic stress was 14.51 ± 5.79 IU mg $protein^{-1}$ where as for normal it was 23.64 ± 4.21 IU mg protein $^{-1}$. Statistical analysis of ANOVA (Table VIII) also showed significant variation between the two experimental conditions.

The superoxide anion also showed significant decrease ($P<0.05$) in the hypoxic stressed animals compared to the normal. The superoxide anion was 0.93 ± 0.04 for animal under hypoxia and for normal it was 1.34 ± 0.19.

4.4.3.4. Effect of ammonia

The THC of the sample before and after ammonia stress showed a decrease in all the three different concentrations of ammonia. The THC at 0.5 mgL^{-1} ammonia showed 25% decrease over the normal, with a mean value of 6.73 ± 1.41 x 10^{6} cells ml^{-1} over the 0 hr value of 9.08 ± 2.53 x 10^{6} cells ml^{-1}. For the ammonia concentrations at 1.5 mg L^{-1}, the value showed a decrease of 58.9% over the 0 hr group and those at 3 mg L^{-1} showed a decrease of 51.63% over the control (C). The relative haemocyte percentage did not show any significant variation from the control group and in animals exposed at 0.5 mg L^{-1} ammonia. Lobsters exposed to 1.5 mg L^{-1} of ammonia showed a decrease in the semi-granular cells from 28.45 ± 1.9 to 18.66 ± 1.9 and large granular cells from 7.86 ± 1.49 to 5.83 ± 0.87. The hyaline cells showed an increase from 63.68 ± 3.21 to 75.99 ± 2.44. In animals kept at 3 mg ml^{-1} of ammonia, the relative percentage of haemocyte showed increase in HC from 66.98 ± 0.04 to 78.27 ± 1.4, where as the semi granulocytes were reduced to 17.58 ± 0.69 from 24.99 ± 0.8 and the large granular cells from 8.02 ± 0.76 to 4.02 ± 0.76.

The PO activity also was found to decrease during ammonia stress. At 0.5 mg L^{-1} of ammonia, the mean value of PO activity was 28.81 ± 2.45 IU mg $protein^{-1}$ before the experiment and it reduced to 12.58 ± 2.08 IU mg $protein^{-1}$ after one week of ammonia exposure. At 1.5 mg L^{-1}, the PO activity was found to be 30.43 ± 5.81 IU mg $protein^{-1}$ for control and it was reduced to 11.17 ± 2.44 IU mg $protein^{-1}$ at the end of the experiment (Fig. X). Lobsters exposed to 3 mg L^{-1} also showed a decrease in PO activity from 27.22 ± 5.42 IU mg $protein^{-1}$ to 9.31 ± 1.78

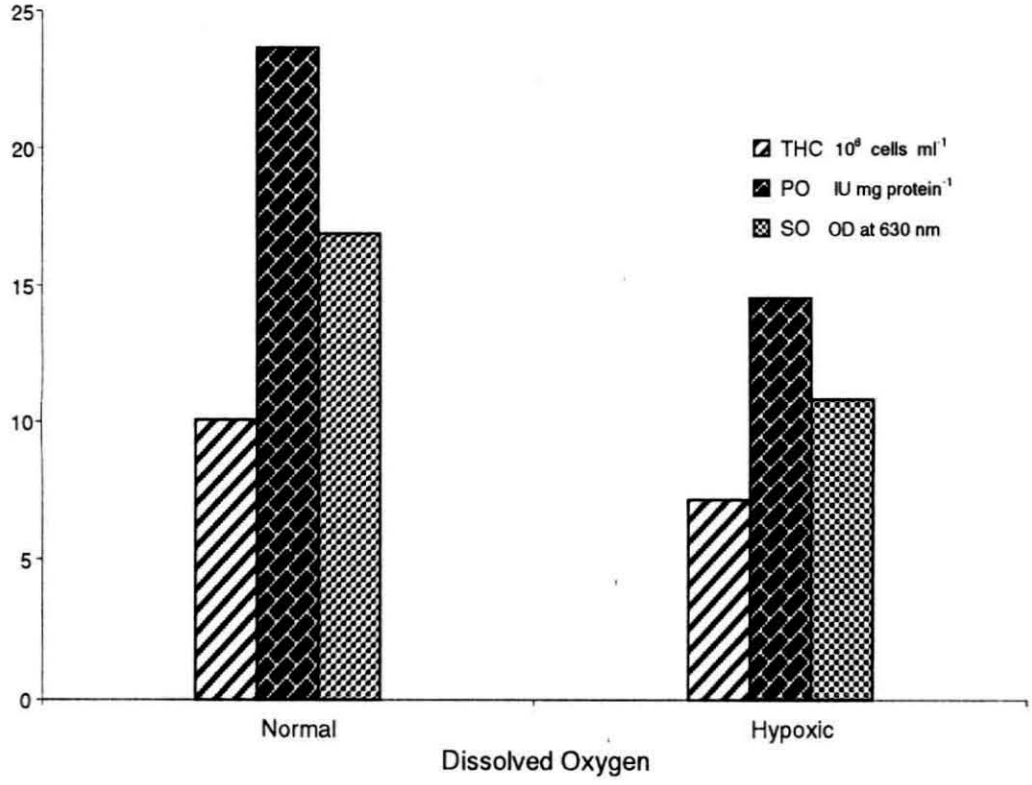


Fig. IX. Effect of dissolved oxygen concentration on immune response in *P. homarus*

Table VIII. ANOVA table for the effect of D.O. ($P<0.05$)

		Sum of Squares	df	Mean Square	F	Sig.
HC	Between Groups	4.396	1	4.396	1.015	.353
	Within Groups	25.982	6	4.330		
	Total	30.378	7			
LGC	Between Groups	2.611	1	2.611	.376	.562
	Within Groups	41.644	6	6.941		
	Total	44.255	7			
SGC	Between Groups	13.808	1	13.808	2.612	.157
	Within Groups	31.716	6	5.286		
	Total	45.523	7			
PO	Between Groups	457.915	1	457.915	17.865	.000*
	Within Groups	512.637	20	25.632		
	Total	970.552	21			
SO	Between Groups	.427	1	.427	21.575	.002*
	Within Groups	.158	8	1.978E-02		
	Total	.585	9			
THC	Between Groups	17.053	1	17.053	6.050	.049*
	Within Groups	16.913	6	2.819		
	Total	33.966	7			

* significant at $P<0.05$

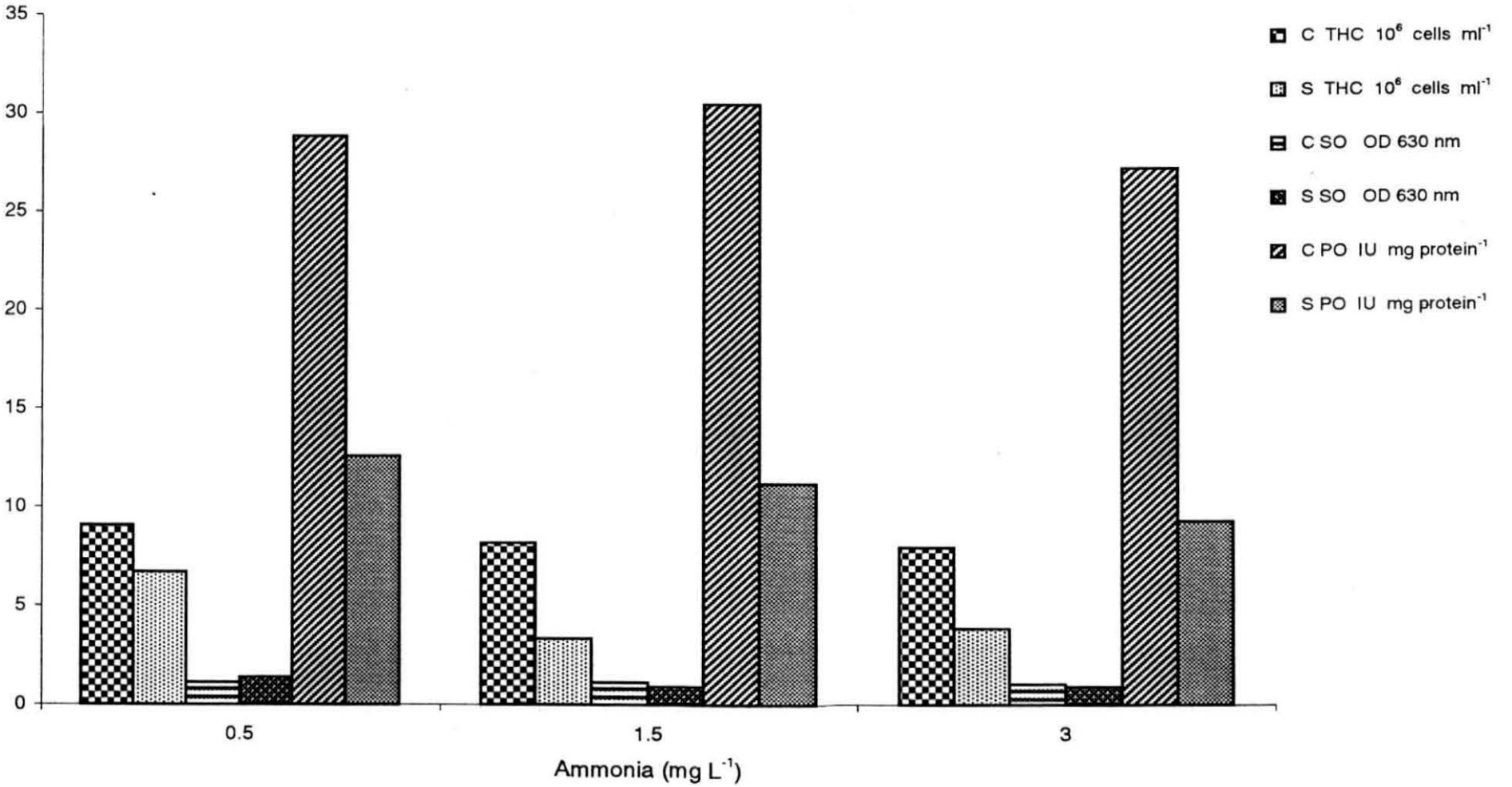


Fig. X. Effect of ammonia concentration on immune parameters in *P. homarus* (C - Control, S - sample)

IU mg protein^{-1}. The decrease was 56.33%, 63.3% and 65.8%, respectively for 0.5, 1.5 and 3.0 mg L^{-1} of ammonia.

The superoxide activity did not show any significant variation (Table IX) from the control and in the samples exposed to 0.5 mg L^{-1} ammonia, the mean was 1.11 ± 0.07 and 1.4 ± 0.12, respectively. The 1.5 and 3.0 mg L^{-1} ammonia showed significant reduction in superoxide anion production of 19.29% and 13.88%, respectively.

4.4.4. Handling stress

4.4.4.1. Effect of transportation

The THC did not show any significant variation from the control group during transportation (15 hours). This was 8.82 ± 2.23 x 10^6 cells ml-1 in the control group and 9.18 ± 5.87 x 10^6 cells ml^{-1} during transportation (Fig. XI).

The PO activity showed significant decrease (Table X) with transportation (P<0.05). The PO activity showed a mean value of 13.68 ± 11.59 IU mg protein^{-1} and 31.03 ± 11.47 IU mg protein^{-1} in the transported individuals and that of the control.

4.4.5. Pathogen stress

4.4.5.1. Effect of bacterial injection

The THC for the control and the sample was compared. In the control groups the THC was found to increase initially during the incubation and later it decreased and reached the normal level after 24 hrs. The sample THC decreased (77.9%) initially and after 30 minutes it increased and attained the initial level after 24 hrs of incubation (Fig. XII).

The PO activity also showed significant variation (Table XI) with highest PO activity at 30min of injection in sample groups (Fig. XIII), which slowly came down to the normal level after 24 hrs. However, there was no significant variation in the PO activity in the control groups with time.

Table IX. ANOVA table for the effect of ammonia ($P<0.05$)

		Sum of Squares	df	Mean Square	F	Sig.
HC	Between Groups	626.993	5	125.399	24.783	.000*
	Within Groups	70.838	14	5.060		
	Total	697.830	19			
SGC	Between Groups	365.453	5	73.091	25.228	.000*
	Within Groups	40.561	14	2.897		
	Total	406.013	19			
LGC	Between Groups	33.572	5	6.714	5.917	.004*
	Within Groups	15.886	14	1.135		
	Total	49.458	19			
PO	Between Groups	3530.414	5	706.083	53.907	.000*
	Within Groups	510.826	39	13.098		
	Total	4041.239	44			
SO	Between Groups	.512	5	.102	20.646	.000*
	Within Groups	5.950E-02	12	4.958E-03		
	Total	.571	17			
THC	Between Groups	95.617	5	19.123	6.571	.002*
	Within Groups	40.744	14	2.910		
	Total	136.361	19			

* significant at $P<0.05$

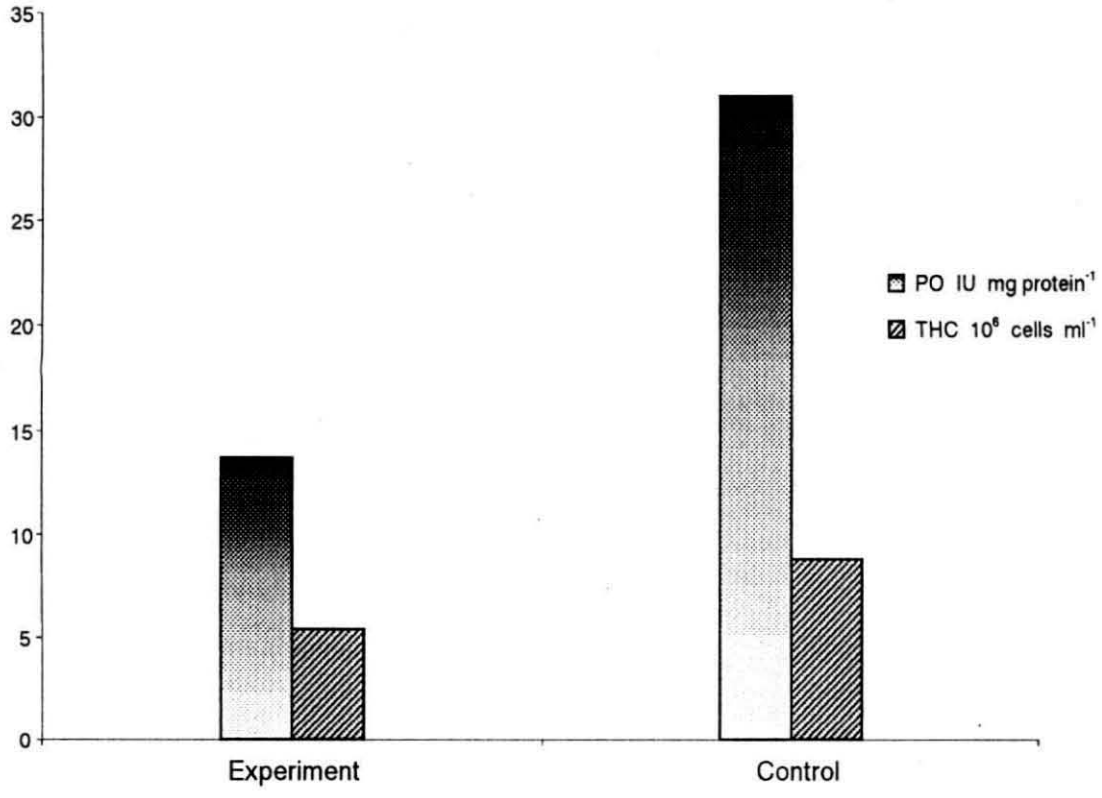


Fig. XI. Effects of transportation on immune system of *P. homarus*

Table X. ANOVA table for transportation experiment ($P<0.05$)

		Sum of Squares	df	Mean Square	F	Sig.
THC	Between Groups	28.866	1	28.866	8.771	.018*
	Within Groups	26.329	8	3.291		
	Total	55.195	9			
PO	Between Groups	2407.660	1	2407.660	18.115	.000*
	Within Groups	3987.376	30	132.913		
	Total	6395.036	31			

* significant at $P<0.05$

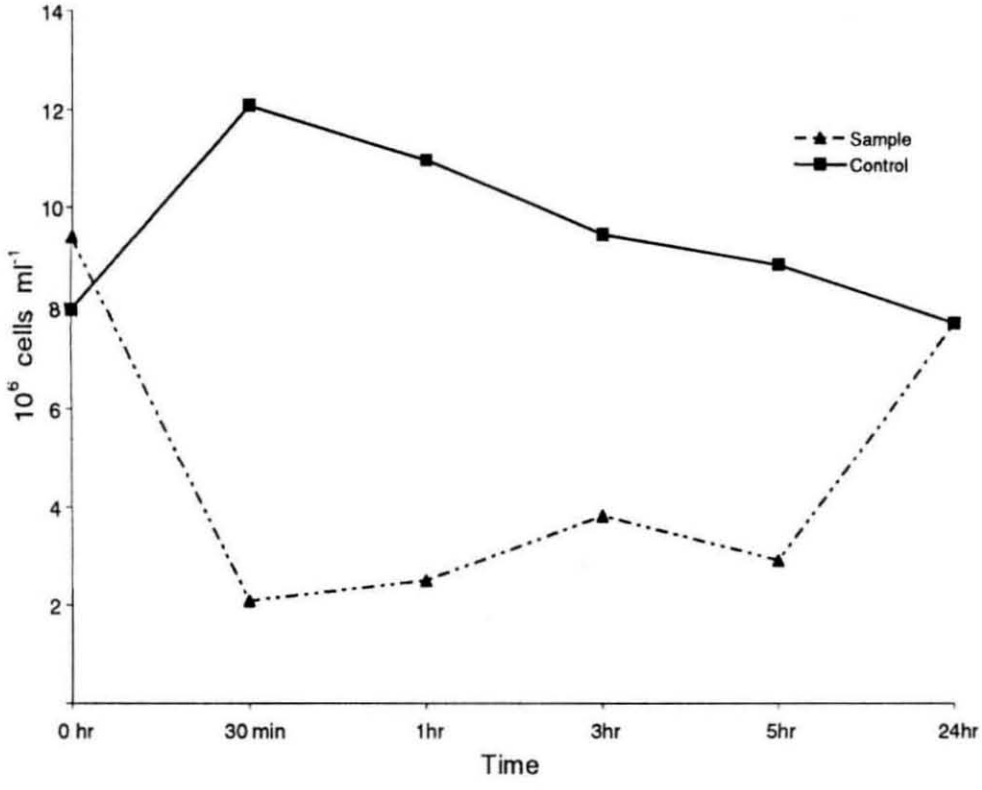


Fig. XII. Effect on THC due to *in vivo* bacterial challenge in *P. homarus*

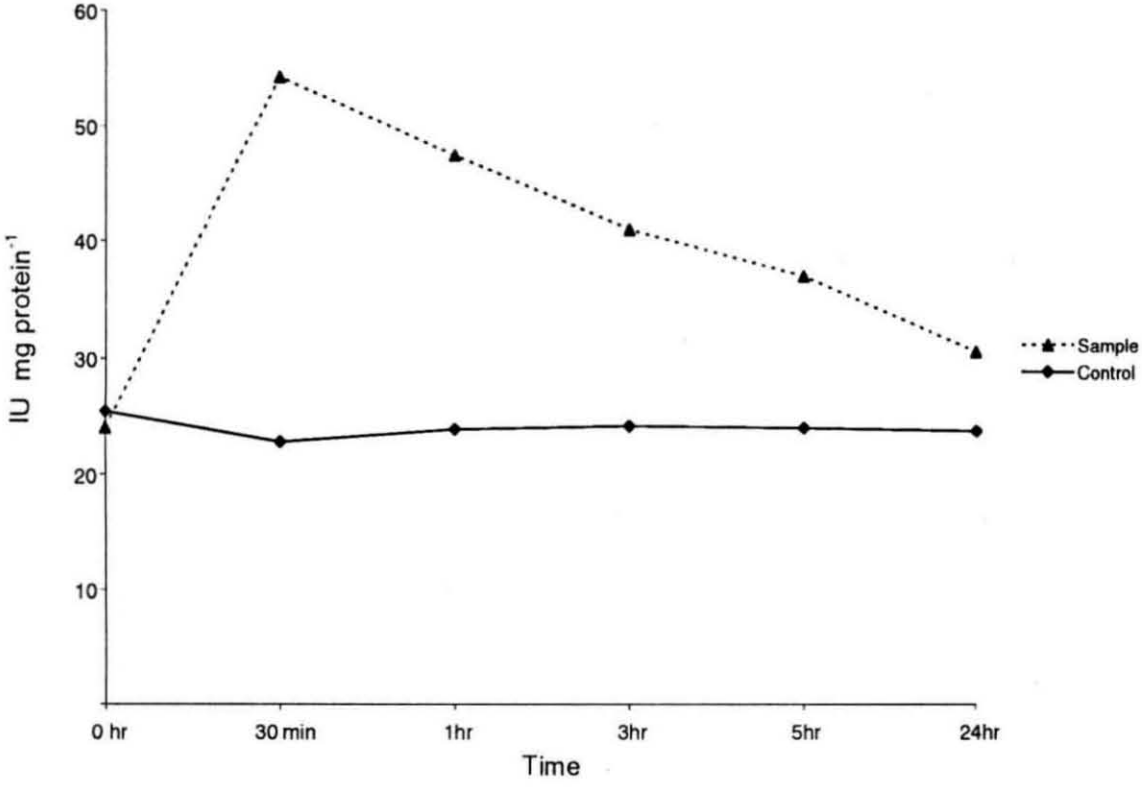


Fig. XIII. Effect on PO activity due to *in vivo* bacterial challenge in *P. homarus*

Table XI. ANOVA table for pathogen stress studies ($P< 0.05$)

THC		Sum of Squares	df	Mean Square	F	Sig.
Initial	Between Groups	1.170E-02	1	1.170E-02	.004	.949
	Within Groups	15.672	6	2.612		
	Total	15.684	7			
30 min	Between Groups	117.218	1	117.218	186.179	.000*
	Within Groups	3.778	6	.630		
	Total	120.996	7			
3 hr	Between Groups	15.472	1	15.472	18.208	.005*
	Within Groups	5.099	6	.850		
	Total	20.571	7			
6 hr	Between Groups	.173	1	.173	.253	.633
	Within Groups	4.108	6	.685		
	Total	4.281	7			
24 hr	Between Groups	.413	1	.413	.934	.371
	Within Groups	2.655	6	.442		
	Total	3.068	7			

PO		Sum of Squares	df	Mean Square	F	Sig.
Initial	Between Groups	.224	1	.224	.101	.767
	Within Groups	8.904	4	2.226		
	Total	9.128	5			
30 min	Between Groups	2282.280	1	2282.280	631.412	.000*
	Within Groups	14.458	4	3.615		
	Total	2296.738	5			
3 hr	Between Groups	1903.533	1	1903.533	394.055	.000*
	Within Groups	19.323	4	4.831		
	Total	1922.855	5			
6 hr	Between Groups	558.156	1	558.156	170.305	.000*
	Within Groups	13.110	4	3.277		
	Total	571.266	5			
24 hr	Between Groups	82.362	1	82.362	8.739	.042*
	Within Groups	37.700	4	9.425		
	Total	120.062	5			

*significant at $P<0.05$

4.4.6. Effect of immune enhancers

4.4.6.1. Effect of immune elicitors

The effect of immune elicitors on the phenoloxidase activating system of lobsters showed that of all the elicitors, seawater increased the phenoloxidase activity by 74.4% as compared to the control followed by yeast, 46.6%; heat killed *Vibrio anguillarum*, 32.18%; heat killed *Micrococcus lysidodyticus* 39.5% and Trypsin, 25.3%, respectively. On the other hand, 10% SDS showed a decrease at 60.98% over the control group (Fig. XIV).

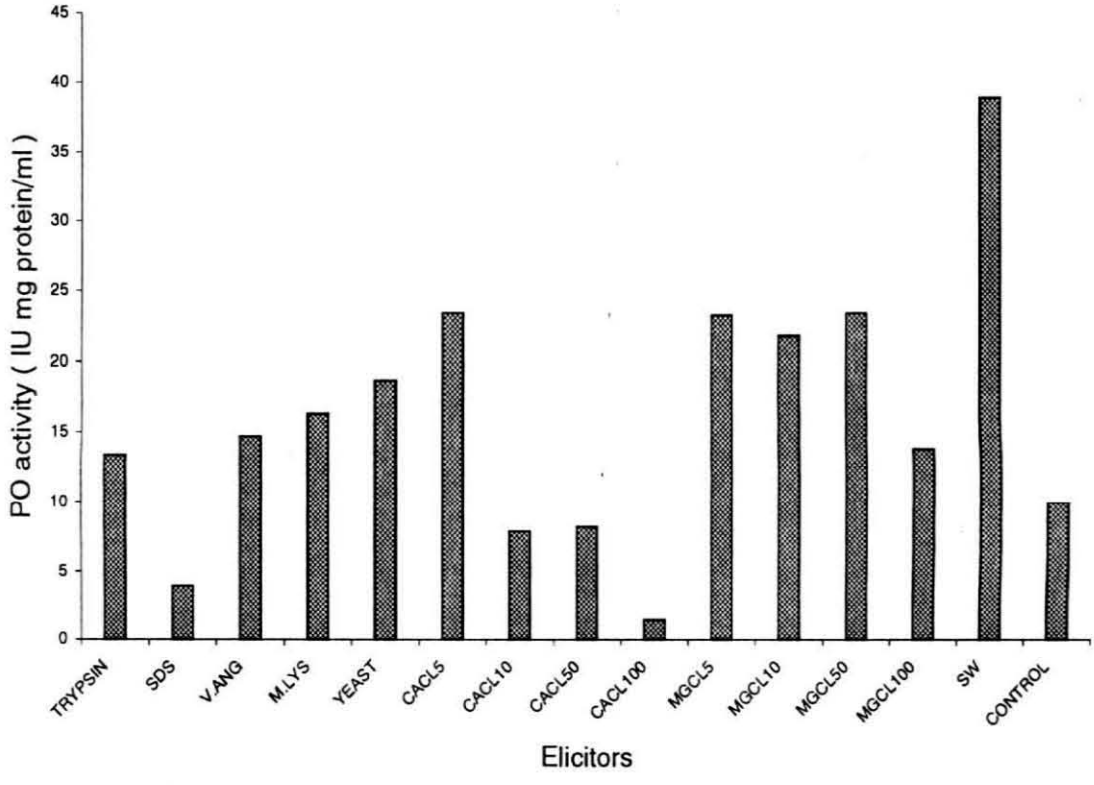
In a study with cations, $CaCl_2$ at 5 mM showed highest PO activity where as 100 mM $CaCl_2$ reduced the PO activity. In the case of $MgCl_2$ also the PO activity increased at 5 mM of $MgCl_2$ and reduced at 100 mM $MgCl_2$.

4.4.6.2. Effect of Laminarin

On injection with laminarin, the THC decreased in comparison with the control. The mean value before incubation was $7.7 \pm 1.74 \times 10^6$ cells ml^{-1}, which was reduced to $5.01 \pm 0.84 \times 10^6$ cells ml^{-1} at 30 min. The THC regained back to the normal levels after 24 hrs of incubation (Fig. XV). In control groups the initial mean value of THC was $7.62 \pm 0.7 \times 10^6$ cells ml^{-1}, where as the value increased to $13.85 \pm 0.49 \times 10^6$ cells ml^{-1} after 30 minutes of incubation and later the levels reached normal levels after 24 hrs of incubation. ANOVA for the effect of laminarin on immune parameters are given (Table XII).

The PO activity in contrast increased with laminarin injection during 30 min of incubation. The mean value increased from 12.26 ± 2.02 IU mg $protein^{-1}$ to 55.46 ± 2.65 IU mg $protein^{-1}$. After 24 hrs, it came to 19.97 ± 4.31 IU mg $protein^{-1}$. The PO activity of control groups (Table XII) did not vary much with saline incubation (Fig. XVI).

In comparison to the normal gill section (Plate XVII), the gill sections of lobsters incubated with 5 mg L^{-1} laminarin after 3 hours revealed the presence of small haemocyte clumps in blood sinuses. In the 6hr sample the lump was found to be large and compact (Plate XVIII).



V. ANG- *Vibrio anguillarum*, M.LYS- *Micrococcus lysidodyticus*, CACL- Calcium chloride (in mM), MGCL- Magnesium chloride, (in mM), SW- Sea water

Fig. XIV. Effect of different elicitors on the PO activity in *P. homarus*

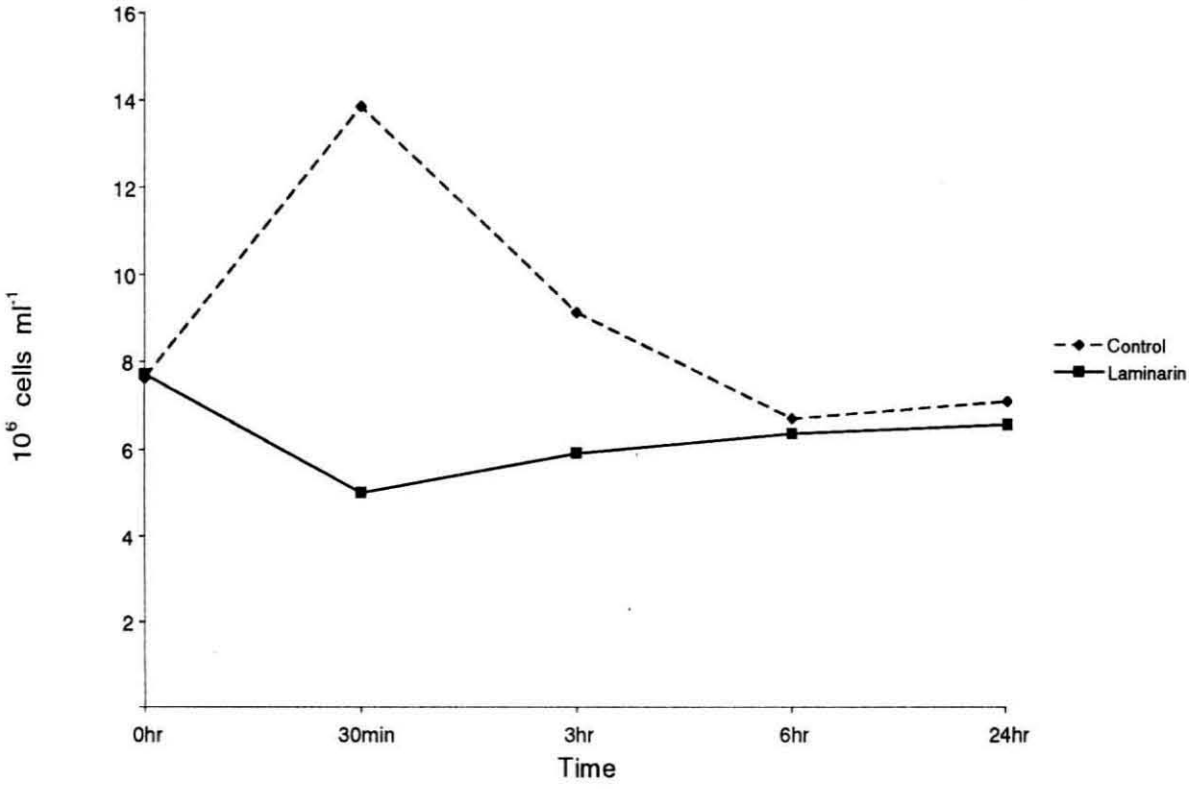


Fig. XV. Effect of *in vivo* challenge of laminarin on THC in *P. homarus*

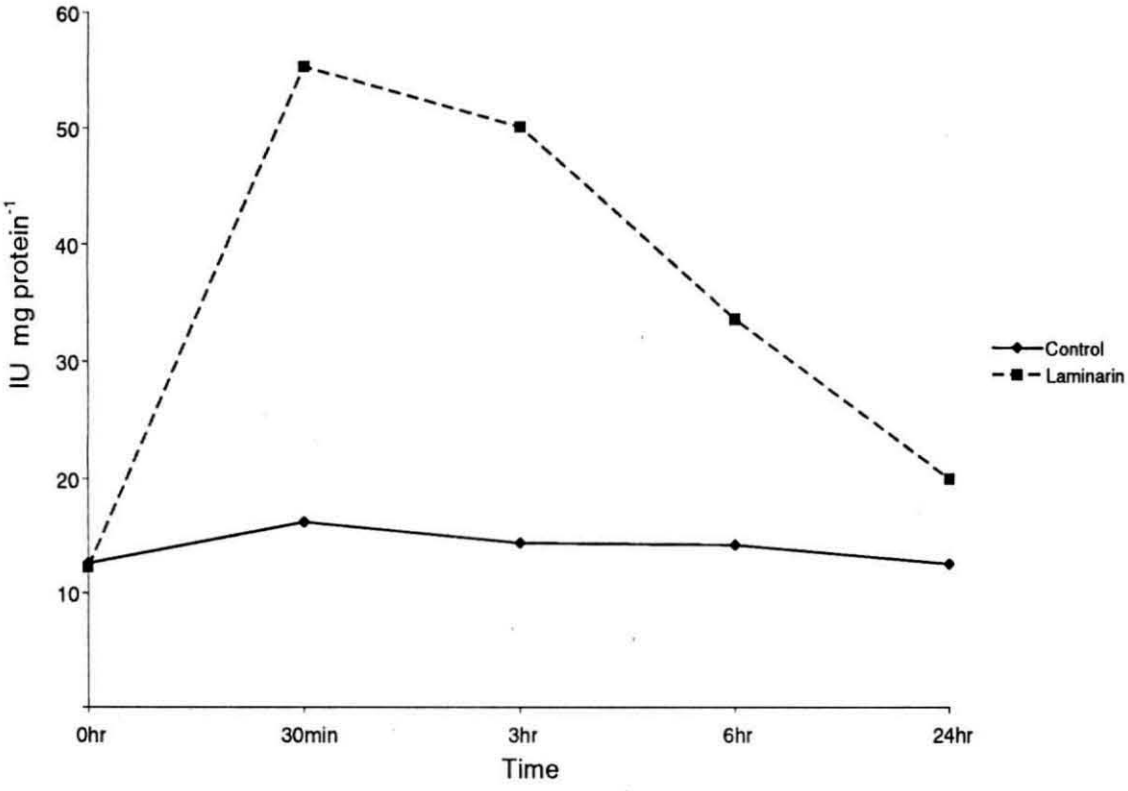


Fig. XVI. Effect of *in vivo* challenge of laminarin on PO activity in *P. homarus*

Table XII. ANOVA for the effect of laminarin as immune enhancer($P<0.05$)

THC		Sum of Squares	df	Mean Square	F	Sig.
Initial	Between Groups	4.220	1	4.220	3.222	.123
	Within Groups	7.858	6	1.310		
	Total	12.077	7			
30 min	Between Groups	199.800	1	199.800	158.796	.000*
	Within Groups	7.549	6	1.258		
	Total	207.349	7			
1 hr	Between Groups	143.736	1	143.736	84.032	.000*
	Within Groups	10.263	6	1.710		
	Total	153.999	7			
3 hr	Between Groups	63.563	1	63.563	461.673	.000*
	Within Groups	.826	6	.138		
	Total	64.389	7			
5 hr	Between Groups	70.746	1	70.746	252.711	.000*
	Within Groups	1.680	6	.280		
	Total	72.425	7			
24 hr	Between Groups	1.125E-04	1	1.125E-04	.000	.989
	Within Groups	3.378	6	.563		
	Total	3.378	7			

PO		Sum of Squares	df	Mean Square	F	Sig.
Initial	Between Groups	6.063	1	6.063	.264	.618
	Within Groups	229.593	10	22.959		
	Total	235.657	11			
30 min	Between Groups	2961.963	1	2961.963	53.988	.000*
	Within Groups	548.638	10	54.864		
	Total	3510.602	11			
1 hr	Between Groups	1651.115	1	1651.115	40.376	.000*
	Within Groups	408.938	10	40.894		
	Total	2060.053	11			
3 hr	Between Groups	839.679	1	839.679	19.884	.001*
	Within Groups	422.279	10	42.228		
	Total	1261.958	11			
5 hr	Between Groups	497.426	1	497.426	14.724	.003*
	Within Groups	337.839	10	33.784		
	Total	835.265	11			
24 hr	Between Groups	138.041	1	138.041	11.121	.008*
	Within Groups	124.128	10	12.413		
	Total	262.168	11			

*significant at $P<0.05$

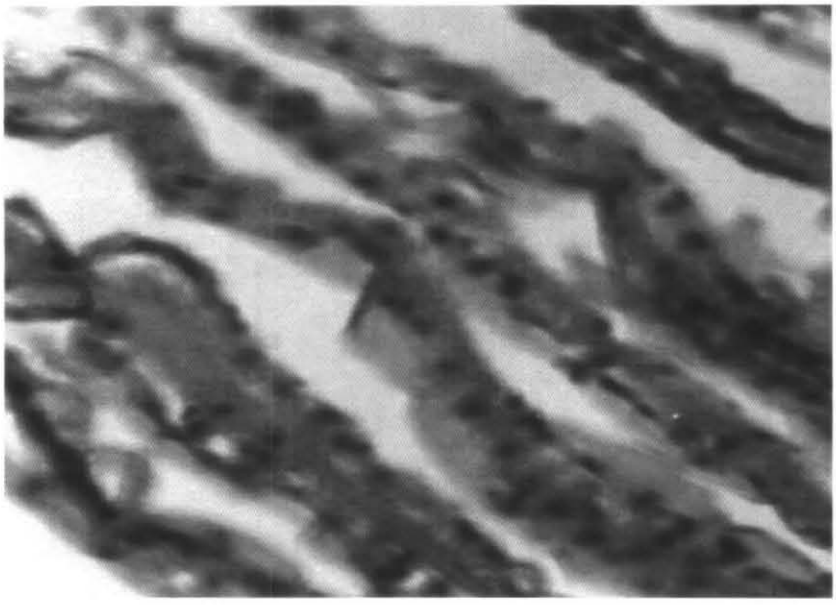


Plate XVIII. Normal gill section of lobster

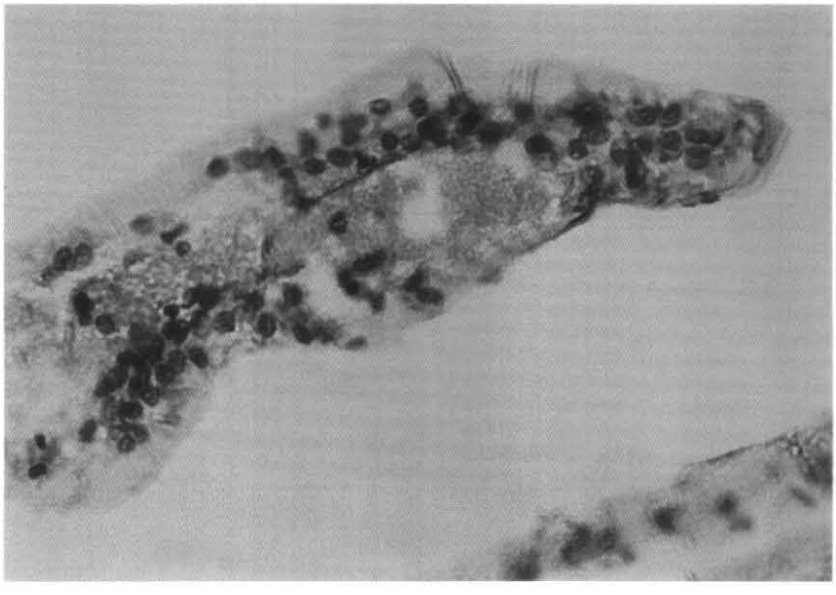


Plate XIX. Gill section of lobster after 6 hrs of incubation with laminarin, haemocyte clumping is visible

4.4.6.3. Effect of farm made feed incorporated with immuno-stimulants

The THC showed significant increase in animals fed with chitosan containing feed as against the control group. But there was no significant influence on the THC in the case of feed mixed with yeast cells. The mean value in control individuals was 7.33 ± 0.91 x 10^6 cells ml^{-1}, whereas for feeding with yeast and chitosan, the THC were 9.37 ± 1.38 x 10^6 cells ml^{-1} and 12.19 ± 0.32 x 10^6 cells ml^{-1}, respectively (Fig. XVII). The relative haemocyte percentage did not show any significant change in the number of HC, SGC and LGC cells in animals fed with yeast containing feed whereas in animals fed with chitosan containing diet the HC count were decreased to 58.6 ± 1.96 and that of SGC was 31.04 ± 2.03 and LGC, 10.34 ± 0.63.

The PO activity was also found to increase in relation to the feed; in the control groups the mean value was 27.23 ± 5.21 IU mg $protein^{-1}$ and for the yeast fed, it was 46.49 ± 7.32 IU mg $protein^{-1}$ and for chitosan containing feed it was 84.98 ± 18.24 IU mg $protein^{-1}$. The ANOVA and multiple comparisons of means showed significant difference except in case of LGC (Table XIII a & b).

4.4.6.4. Effect of commercial immunostimulant

The study on the effect of commercial immunostimulants was limited to the THC and PO activity study. The THC analysis showed no significant difference ($P<0.05$) with the control groups (Table XIV). The mean THC value was 6.16 ± 1.52 x 10^6 cells ml^{-1} for the control groups where as it was 7.74 ± 1.64 x 10^6 cells ml^{-1} in the case of sample groups (Fig. XVIII). The percentage haemocyte count also did not show much variation from the control group. The HC for the experimental group was 63.1 ± 1.67 where as for the control, it was 65.3 ± 1.55; SGC for sample was 27.07 ± 3.19 and the control 26.32 ± 1.22; LGC for sample 9.82 ± 2.18 and the control 8.36 ± 0.51.

The PO activity also did not show any significant variation over the control groups with a mean value of the control being 23.99 ± 5.8 IU mg $protein^{-1}$ and that of sample being 26.89 ± 6.92 IU mg $protein^{-1}$.

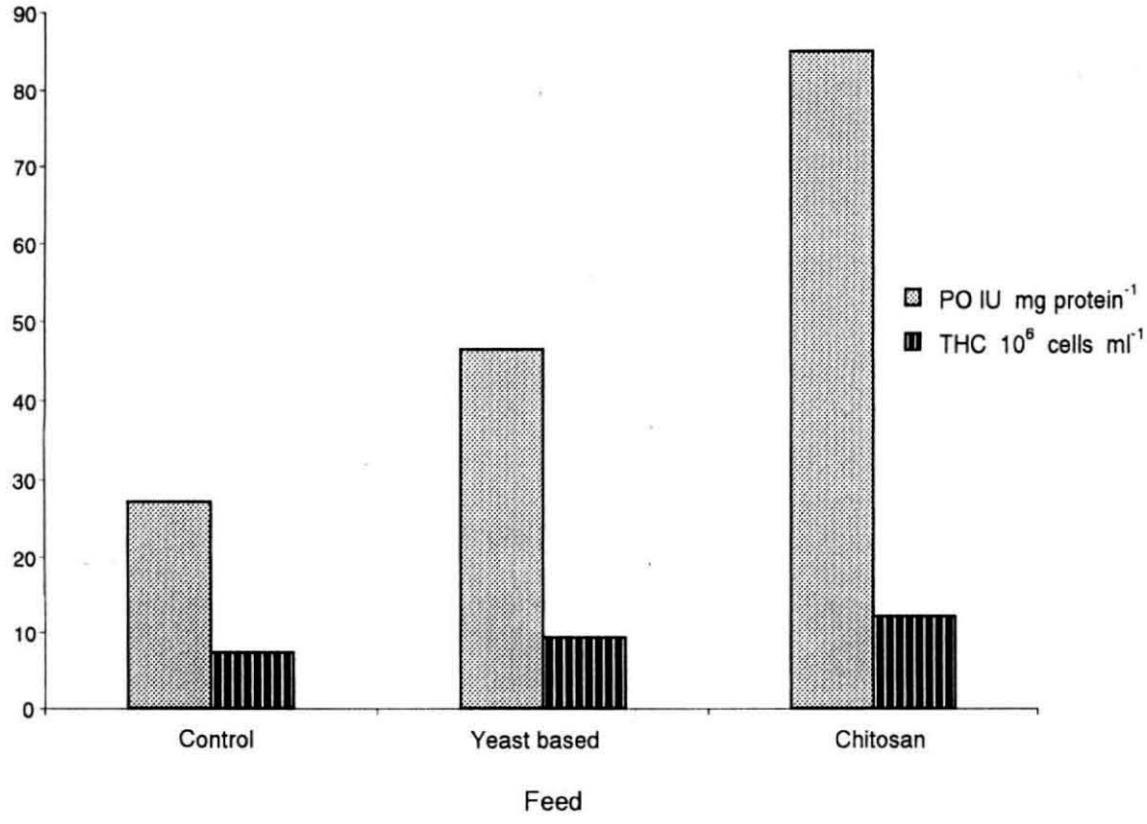


Fig. XVII. Effect of farm made feed on immune response in *P. homarus*

*significant at $P<0.05$

Table XIII a. ANOVA on the effect of immunostimulants through feed ($P<0.05$)

Farm-made feeds		Sum of Squares	df	Mean Square	F	Sig.
HC	Between Groups	71.527	2	35.763	7.332	.024*
	Within Groups	29.265	6	4.877		
	Total	100.791	8			
LGC	Between Groups	6.754	2	3.377	.514	.622
	Within Groups	39.388	6	6.565		
	Total	46.141	8			
PO	Between Groups	10375.878	2	5187.939	37.616	.000*
	Within Groups	2068.803	15	137.920		
	Total	12444.681	17			
SGC	Between Groups	74.767	2	37.383	4.802	.057
	Within Groups	46.709	6	7.785		
	Total	121.476	8			
THC	Between Groups	35.636	2	17.818	18.785	.003*
	Within Groups	5.691	6	.949		
	Total	41.328	8			

Table XIII b. Multiple comparisons for the immunostimulants through feed

LSD

Farm-made feeds			Mean Difference (I-J)	Sig.	95% Confidence Interval	
Dependent Variable	(I) FEED	(J) FEED			Lower Bound	Upper Bound
HC	1.00	2.00	1.4333	.457	-2.9790	5.8457
		3.00	6.5667(*)	.011	2.1543	10.9790
	2.00	1.00	-1.4333	.457	-5.8457	2.9790
		3.00	5.1333(*)	.029	.7210	9.5457
	3.00	1.00	-6.5667(*)	.011	-10.9790	-2.1543
		2.00	-5.1333(*)	.029	-9.5457	-.7210
LGC	no significant difference					
PO	1.00	2.00	-19.2567(*)	.012	-33.7087	-4.8047
		3.00	-57.7517(*)	.000	-72.2037	-43.2997
	2.00	1.00	19.2567(*)	.012	4.8047	33.7087
		3.00	-38.4950(*)	.000	-52.9470	-24.0430
	3.00	1.00	57.7517(*)	.000	43.2997	72.2037
		2.00	38.4950(*)	.000	24.0430	52.9470
SGC	1.00	2.00	-3.5567	.169	-9.1310	2.0177
		3.00	-7.0600(*)	.021	-12.6344	-1.4856
	2.00	1.00	3.5567	.169	-2.0177	9.1310
		3.00	-3.5033	.175	-9.0777	2.0710
	3.00	1.00	7.0600(*)	.021	1.4856	12.6344
		2.00	3.5033	.175	-2.0710	9.0777
THC	1.00	2.00	-2.0367(*)	.043	-3.9825	-9.0875E-02
		3.00	-4.8533(*)	.001	-6.7991	-2.9075
	2.00	1.00	2.0367(*)	.043	9.087E-02	3.9825
		3.00	-2.8167(*)	.012	-4.7625	-.8709
	3.00	1.00	4.8533(*)	.001	2.9075	6.7991
		2.00	2.8167(*)	.012	.8709	4.7625

*significant at $P<0.05$, 1.00 – control, 2.00- yeast, 3.00- chitosan

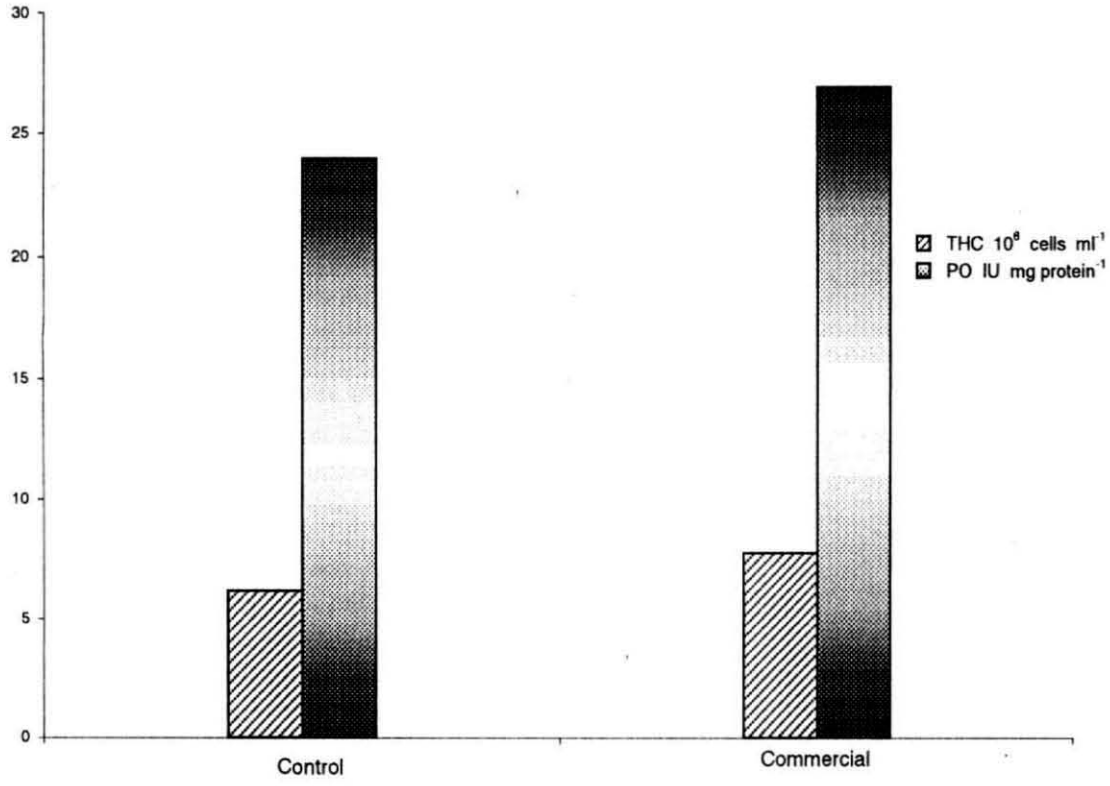


Fig. XVIII. Effect of commercial immunostimulant on lobster immune system

Table XIV. ANOVA on the effect of commercial immunostimulant ($P<0.05$)

Commercial (Allways)		Sum of Squares	df	Mean Square	F	Sig.
HC	Between Groups	12.396	1	12.396	4.646	.059
	Within Groups	24.011	9	2.668		
	Total	36.407	10			
LGC	Between Groups	5.381	1	5.381	1.643	.232
	Within Groups	29.468	9	3.274		
	Total	34.848	10			
SGC	Between Groups	1.437	1	1.437	.197	.668
	Within Groups	65.699	9	7.300		
	Total	67.136	10			
PO	Between Groups	33.640	1	33.640	.825	.379
	Within Groups	570:565	14	40.755		
	Total	604.205	15			
THC	Between Groups	5.516	1	5.516	2.182	.183
	Within Groups	17.696	7	2.528		
	Total	23.212	8			

*significant at $P<0.05$

DISCUSSION

5. DISCUSSION

5.1. Cellular characterisation

Based on the preliminary results the haemocytes in the haemolymph of *P. homarus* were classified into three groups: the hyaline cells designated as HC, which is characterised by small size and absence of granules in their cytoplasm; the semi granular cells (SGC) and the large granular cells (LGC), which can be distinguished from the size of their intra cytoplasmic granular content. SGC has small and less dense granules compared to LGC, which contains dense granular cells. There has been lots of confusion regarding classification of haemocytes in crustaceans. Johnson (1980) and Bauchau (1981) identified two types of granular haemocytes, those containing small granules and the other containing large granules. Many classification schemes have placed equal importance to the size, shape and staining characteristics of the cells. In the present study, in addition to the above characteristics the size of the cytoplasmic granules was also considered as the study based on cell size and shape alone was unreliable. Cell shape may not be a reliable basis of classification as the shape of the cell is highly variable *in vivo* and *in vitro* (Johnson, 1980). The haemocytes can be distinguished as hyalinocytes or granular cells even at the light microscopic level by observing the refractile or non-refractile granule. However, the difference is more pronounced under a phase contrast microscope in which the granular cells can be further distinguished as semi granular cells and large granular cells depending on the size of the granules.

Previous studies based on cell morphology and staining at the light microscopical level have divided haemocytes into three categories (Bauchau, 1981). The terminology refers to the number of granules in each cell type (Bauchau and Mengeot, 1978). Tsing *et al.* (1989) divided the haemocytes into three categories: undifferentiated haemocytes (UH), haemocytes with small granules (SGH) and those with large granules (LGH). The undifferentiated haemocytes of this study is identical to the hyalinocytes of the present work. The remaining cell types compare well with the present classification. The distinction of

LGH and SGH is in good agreement with the work of Martin and Graves (1985) in the shrimps, *S. ingestis* and *P. californiensis* and that of Tsing *et al.* (1989) in *P. japonicus* and *P. monodon.*

In most of the studies on crustacean haemocytes, the blood cells were separated into three categories. In the shrimp *Metapeneaus* sp; Dall (1964) used cell shape to distinguish between three types of cells, viz., lymphocytes, thigmocytes and large granular amoebocytes. These cells are comparable to the agranular, semi-granular and large granular cells of the present study. The study is comparable to the descriptions of crab and lobster haemocytes. Mix and Sparks (1980) identified three types of cells: the hyalinocytes, intermediate cells and esonophilic granulocytes in the crab *Cancer magister.* These studies used light microscopy and Wright's staining for haemocyte smear preparation. The classification favourably compares with the present scheme of classification. Hyalinocytes of crab lack granules that correspond to the hyaline cells of the lobsters. Intermediate and eosinophilic cells contain small and large granules which are comparable to semi granular and granular cells of lobsters, respectively. The TEM studies on the haemocytes of *C. sapidus* show three categories of cells: the hyaline, semi granular and granular cells (Bodammer, 1978), which also seems to agree with the present study in terms of terminology but differ on the fact that in Bodammer's classification, the hyalinocytes were found to contain small granules. However, the identities of other cells are in agreement with the present classification.

The scheme developed for spiny lobsters may also be comparable to the studies of Cornick and Stewart (1978) on the American lobster *H. americanus* and that of *P. homarus* (Manjula *et al.,* 1997). They identified four types of cells using Giemsa staining and light microscopy although the same procedure was used in the present study. In *P. homarus*, only three types of cells were identifiable. The prohyalocytes of the previous study was found to be comparable with the hyaline cells where as the hyalinocytes and esonophilic cells exhibited basophilic or eosinophilic staining to the granules, which was referred to as the semi granular cells of the present study. The chromophobic granulocytes were packed with large refractile granules as in the large granular cells of lobsters.

In another study based on shape and staining of cells, Hearing and Vernick (1967) identified three types of cells in *H. americanus*. However, the only difference was that they recorded granules in all the type of cells, which was not found to be in accordance with the present classification.

5.2. Hematopoietic tissue

Hematopoietic tissues of lobsters were in the form of ovoid lobules unlike the penaeids, where the bulk of HPT occurs as a pair of nodules on either side of the dorsoventral surface of foregut (Martin *et al.,* 1987; Hose *et al.*, 1992). In *P. homarus*, it was observed as lobules around the dorsal surface of the foregut, which agrees well with the earlier works of Martin *et al.* (1993 a) on *H. americanus*. They observed loosely attached lobules containing hematopoietic precursors and maturing haemocytes., which is similar to the HPT structure of present communication. Johnson (1980) described the lobular structure of epigastric hematopoietic tissue in *Callinectes sapidus*, which did not form nodules and was found to agree well with the present study. Studies conducted by Ghiretti-Magaldi *et al.* (1977) indicated that hematopoiesis in *C. maenas* takes place in specialized organ located in the gizzard wall. The differentiation of haemocytes takes place in the HPT, which was supported by the observations of Clare and Lumb (1994) in the blue crab *C. sapidus*. The functional and cytochemical criteria for differentiating the two categories were further supported by Martin *et al.* (1987). In the present study also it was found that the differentiation takes place with in the HPT. It can therefore be inferred that the two cell types arise from separate cell lineages.

5.3. Immune status of healthy lobsters

Haemolymph can be used as an effective tool to check the overall health as well as specific pathogenic process in crustaceans (Noga, 2000). The haemocyte count was reported to vary with infection suggesting its use as a health indicator (Soderhall and Smith, 1986; Persson *et al.*, 1987b). The haemocyte count was found to decrease considerably during viral and bacterial infections (Sequeira *et al.*, 1996), during starvation (Stewart *et al.*, 1967) and also

during stress (Le Moullac and Haffner, 2000). The THC and the relative percentage of haemocytes agree well with the earlier works done in healthy crustaceans (Jussila, 1997; Jussila *et al.*, 1999; Norton *et al.*, 1999). The blood volume studies show increase in body weight with corresponding increase in the volume of blood. But there was no significant difference in the THC count, which agrees well with the observations of Owens and O'Neil (1997) that THC and DHC are not influenced by age, weight and carapace length. The increase in blood volume with body weight was found to be in accordance with the studies carried out in *C. maenas* (Smith and Ratcliffe, 1980).

Phagocytosis has a pivotal role in crustacean defense system. *P. homarus* haemocytes was found to phagocyte both bacterial and yeast cells. This observation was supported by the results obtained in *C. maenas* in which the haemocytes were found to phagocyte the bacterial cells (Smith and Ratcliffe, 1978). It was found that stress reduced the phagocytosis in invertebrates (Dyryanda *et al.*, 1997). The work agrees well with the earlier works (Reade, 1968; McKay and Jenkin, 1970; Anderson *et al.*, 1973; Paterson and Stewart, 1974; Tyson and Jenkin 1974; Paterson *et al.*, 1976; Smith and Ratcliff, 1978; Amstrong, 1979; Brehelin and Arcier, 1985; Bayne, 1990; Sagarista and Durfort, 1990; Bell, 1993) .The health status of an individual can therefore be analysed for its capacity to phagocytose the pathogen and hence, rendering its use as an effective tool for bioanalysis.

There are very few studies on application of antibacterial activity as a crustacean health marker. The antibacterial activity by haemolymph of invertebrates has been concerned primarily with lysis of bacteria (McHenery *et al.*, 1979; Hultmark *et al.*, 1980; Kim, 1994). The spread plate technique for the study of antibacterial activity was used to analyse the blood of echinoids (Wardlaw and Unkles, 1978) and crustaceans (Chisholm and Smith, 1992; 1994). The tubidimetric assay was employed to demonstrate growth inhibition in crustaceans (Fenouil and Roch, 1991; Chisholm and Smith, 1994). In the present study, the serum was found to have bactericidal activity, which was demonstrated both by turbidometric and spread plate method. Antibacterial activity could be used as an effective tool to access the health status of crustaceans. In an earlier study,

decreasing antibacterial activity decreased in normal blue crabs from estuarine sites with high prevalence of disease was reported (Noga *et al.*, 1994). The effect of temperature on antibacterial activity in *C. maenas* showed that the activity was minimum at highest temperature. Yet in another study on the Japanese spiny lobster *P. japonicus*, Chilosam and Smith (1994) observed agglutination of the *Vibrio* sp. and *Pseudomonas* sp. strains by the serum. In spiny lobsters, which are exposed to reduced salinity and transportation, reduction in antibacterial activity was observed (Ueda *et al.*, 1990).

Lectin activity was identified in haemolymph of many crustaceans including prawns, crabs, crayfish, lobsters, and the penaeid shrimps (Fries, 1984 Ravindranath *et al.*, 1985; Cassels *et al.*, 1986; Ratanapo and Chulavatnatnol, 1990; Vargas-Albores *et al.*, 1992; 1993). In the spiny lobster *P. homarus*, the haemagglutination with chicken RBC in the serum as well as in the haemolymph was found to be in good agreement with the previous works. Norton *et al.* (1999), reported significant difference in haemagglutination titer with chicken RBC in the serum of sick and healthy *P. cygnus* and with fish RBC in *P. homarus* serum (Manjula *et al.* 1999). There is evidence of lectin level depression with inadequate nutrition in other invertebrates, which also varies with sex (Pauley, 1974) and the moult stages (Bang, 1967). These results suggest that agglutination and bactericidal activity can be used as health indicators in crustaceans.

The most well studied enzymatic system of crustaceans is the phenoloxidase cascade. Phenoloxidase (PO) is stored in the inactive form, 'proPO' in the crustacean haemocytes (Vargas-Albores *et al.*, 1993). In the present study, *P. homarus* serum was used for the study of PO activity and was found to give a good reproduction of PO activity which agreed with the observation of Perazzolo and Baracco (1997). They showed that fresh samples of HLS and serum of *P. paulensis* have considerable PO activity after induction with trypsin and LPS. The utilisation of serum to detect 'proPO' in the haemolymph of *P. paulensis* and eventually in other crustaceans of interest for aquaculture is thus possible. It appears as a possible alternative for obtaining the 'proPO' activating system for quick and preliminary analysis. This can also be used to study immune

status in growout system, as serum samples are easy to collect compared to the HLS.

The presence of 'proPO' enzyme in the semigranular and granular cells was studied by using L-DOPA (Smith and Soderhall, 1983; Johansson and Soderhall, 1985; Lanz *et al.*, 1993; Sung *et al.*, 1998). The 'proPO' system was present in the semigranular and granular cells with better representation in the granular cells. In the present work also, similar results were obtained during cytochemical analysis of haemocytes. The study was further supported by Soderhall and Smith (1983), who showed that by separating different haemocytes types using percoll gradient, they could isolate three types of cells in *C. maenas* haemolymph, *i.e.* the hyaline, the semigranular and the granular cells.

An important defense enzyme recently identified in crustaceans was the production of superoxide and hydrogen peroxide by haemocytes (Bell and Smith, 1993). This process is called respiratory burst and is an aerobic process, which releases oxygen radicals having potent microbicidal activity. In *P. homarus* the expression of superoxide radical was monitored as in the case of other crustaceans (Bachere *et al*, 1995; Cheng and Chen, 2000). Numerous other defense enzymes were also identified all within the cytoplasm of haemocytes, including acid phosphatase, non-specific esterases and peroxidase (Hose *et al.*, 1990; Tsing *et al.*, 1989). Many of these enzymes function intracellularly. Some may be released into the haemolymph during defense response (Chen *et al.*, 1988). The serum of *P. homarus* exhibited expression of acid phosphatase activity showing that serum is capable of defence using defensive enzymes.

Disease status in many crustaceans is associated with decreased clotting of haemolymph (Sequeira *et al.*, 1996). Little attention was given to practical use of these observations (Chen *et al.*, 1993). Haemolymph clotting time has only been measured in some earlier studies (Battelle and Kravitz, 1978; Gondko *et al.*, 1981; Smith *et al.*, 1995). The results were expressed in minutes while in the present work, it was found to clot within 60 sec, which agrees well with the observations of Jussila *et al.* (2001). Haemolymph can be used as an diagnostic tool as it has been observed that the presence of bacteria or environmental pollutants often changed the clotting time (Newman and Feng,

1982; Tsai *et al.*, 1991; Smith and Johnston, 1992). It has been observed that the THC was lowest in lobsters with longer clotting time and is similar to *P. cygnus* (Jussila *et al.*, 2001). The simple measurement of clotting time can provide valuable information of the health status and therefore can be used as a bioindicator in crustaceans.

5.4. Stress studies

5.4.1. Physiological stress

Moulting is an inevitable physiological process in all arthropods, which aid them in growth. But it is also true that this is the most vulnerable stage in the life cycle as the animal is more prone to infection during this period. In the present study, while analyzing the effect of moulting on the immune system variation in THC during different stages of the moult cycle was noticed. Similar variation in THC has been observed in *P. japonicus* in which the THC showed two periods of minimum and maximum levels; one in B_2 stage and other in late D_4 stage. The second minimum was preceded by an increase in haemocyte number in premoult. This observation agrees well with the results of the present study. In *P. homarus*, the THC during intermoult was at minimum, which was in concordance with the study in *P. stylirostris* (Le Moullac *et al.*, 1997). The THC showed highest peak at premoult and postmoult and was minimum during the intermoult. Similar variation was also observed in *P. japonicus* and *S. ingentis*. In *P. japonicus*, the THC showed a peak during the premoult and minimum during the intermoult (Tsing *et al.*, 1989). This increase in haemocyte numbers may provide an enhanced immune capability during periods of higher activity or when exposed to enhanced environmental bacterial loads. Change in haemocyte number may also be controlled by neuroendocrine hormones as it regulates moulting, colour variation and glycemia (Truscott and White, 1990). Monitoring THC as a measure of stress for freshwater crabs was suggested by Narain and Srivastava (1979).

In yet another study, Bauchau and Plaquet (1973) reported highest THC during intermoult stage in *E. sinensis*, which was not observed in *P. homarus* during the present study. But again this is contradictory to the report by Manjula *et*

al. (1997) in which the peak in THC was observed in the intermoult stage. In a similar study, Cheng and Chen (2001) observed high THC during the premoult stage in *M. rosenbergii* and this is comparable to the present study. The enhanced THC during the post moult and premoult can be attributed to compensation of immunodeficiency caused during moulting.

In contrast to the THC, the phenol oxidase activity was found to be highest during intermoult, compared to the premoult and post moult stages. This could be due to active participation of granulocytes by releasing enzymes needed for tanning of cuticle during the post and premoult. This could be correlated with the relative percentage of LGC. It is well known that prophenol oxidizing system is located in the LGC and SGC (Johansson and Soderhall, 1989; Hose *et al.*, 1990). The result is in concordance with the work done by Le Moullac *et al.* (1997) in *P. stylirostris*. The phenoloxidase activity per cell was maximum in the intermoult and less active in the premoult. The results from the present study show varying phenoloxidase activity in different stages of the moult cycle. The decrease in PO activity during premoult and postmoult may be due to the presence of increased amount of inhibitors of PO system, which has been detected in crayfish (Johansson and Soderhall, 1989). The low phenoloxidase activity in premoult and postmoult can be attributed to immunodeficiency during this stage. Le Moullac *et al.* (1997) observed high mortality rate during the pre moult than during the intermoult in shrimp injected with *Vibrio* AM23 suggesting that a high THC alone cannot give good protection during the premoult.

Eyestalk ablation studies have been restricted to marine prawns and lobsters and were undertaken with particular reference to moulting, vitellogenesis, ovulation and associated breeding activities. Bilateral eyestalk ablation accelerated moulting frequency and weight gain in juvenile, maturing and mature *P. homarus*, irrespective of their reproductive status (Radhakrishnan and Vijayakumaran, 1984). The viability of eyestalk ablation as a means to enhance the growth of juvenile spiny lobsters in commercial growout cultures showed that ablation could result in high gross yield (Juinio-Menez and Ruinata, 1996). But practically, no studies was carried out on the effect of ablation on the immune system of crustaceans .The present study is one of the first works to look into this

aspect in spiny lobsters. The THC counts showed significantly faster response in the bilaterally ablated lobsters compared to the controls within two hours of ablation. Rapid increase in THC on injury was observed by Jussila *et al.* (1999) in the rock lobster *P. cygnus*. On injury haemocytes are mobilized towards the point of injury, as haemocytes are the main defense against invasion and wound healing (Soderhall and Cerinius, 1992). The rapid rise in THC during ablation has been due to the release of haemocytes into circulation from the storage sites such as hematopoietic tissue, which is known to store large number of mature haemocytes. The percentage variation of hyalinocytes was significantly lower as compared to granulocytes, which showed a hike in their number during ablation.

In arthropods, melanin synthesis is involved in the process of sclerotisation, wound healing of cuticle and in defence reactions against invading micro organisms (Soderhall, 1982; Ratcliffe *et al*, 1985; Sugumaran, 1996). The rise in PO activity has also been found to increase due to ablation and this could be a defence reaction to repair the damage caused due to injury.

5.4.2. Nutritional stress

The relationship between feeding and increased THC may reflect the storage of carbohydrate in the haemocytes (Johnston *et al.*, 1973) and on the haemocyanin (Mosco *et al.*, 1989). In the present study, there was a sharp reduction in THC from $7.84X10^6$ cells ml^{-1} to $2.37X10^6$ cells ml^{-1} within three weeks of starvation indicating direct relationship between the haemocyte numbers and the nutritional status of the animal. Manjula *et al.* (1997) observed a reduction of 41.11% in THC during starvation in *P. homarus*, which is well in agreement with the present study. Stewart *et al.* (1967) demonstrated that starvation was found to decrease haemocyte count along with plasma proteins, which was treated as reserve material in *H. americanus*. In a recent study in *M. rosenbergii*, Cheng and Chen (2001) showed reduction in THC on feeding at 0.1% of the total body weight and increase in THC at 0.5% of the body weight. The serum PO activity of starved animal was also found to decrease during starvation, showing that poor nutrition may reduce the immune status and which in turn can make the animal more prone to infection.

There is little information on the serum acid phospahatase activity in decapods. The major studies available are on the cytochemical analysis, which are used for haemocyte classification. Acid phosphatase is considered to be an important enzyme helping in lysosomal activity. In a recent study on *P. monodon* and *M. rosenbergii*, intraheamocytic activity of lysosomal enzyme was shown to link with phagocytosis (Sung and Song, 1999). Tsing *et al.* (1989) reported the presence of acid phosphatase in the SGH of *P. japonicus*. However, Hose *et al.* (1987) did not detect any trace of acid phosphatase activity in either the hyalinocytes or in the granulocytes. There are no reports on the acid phosphatase activity in serum other than the preliminary study done by Roch and Latreille (1934), who identified a phosphatase in crab serum. However, no values were reported. In the present study, while monitoring changes in acid phosphatase activity during starvation, the activity was found to decrease during prolonged starvation. Dillon and Fisher (1983) demonstrated the relationship between acid phosphatase activities linked with stress related cell lysis. Decreased phagocytosis also agrees well with the acid phosphatase activity. Paterson and Stewart (1974) observed decreased phagocytic activity during stress, which is well in accordance with the present study.

Increased THC was observed in animals fed with mussel meat compared to fish meat. Though not much work was done on the significance of diet in relation to THC, Tsvetnenko *et al.* (1999) showed higher THC of $8.05\pm1.3X10^6$ cells ml^{-1} in mussel fed animals in comparison with a THC of $5.2\pm0.8X10^6$ cells ml^{-1} in those fed on artificial diets. Similar results were obtained in the present study. Reduced number of haemocytes in haemolymph concurs with previous findings on both freshwater crayfish and the western rock lobster *P. cygnus*. The THC in marron reared on nutritionally insufficient diets was lower than those reared in semi intensive culture (Jussila, 1997). The THC was suggested as stress or condition indicators in the western lobster. THC levels between 4 and 8×10^6 cells ml^{-1} were considered as normal whereas those below 4×10^6cells ml^{-1} were considered as weak (Jussila *et al.*, 1999).

In the present study, the haemocyte percentage did not show significant variation in contrast to the observations by Tsvetnenko *et al.* (1999),

who found an increase in the granular cells in lobsters fed with artificial diet as against those fed with mussel. This is contradictory to the present study.

Enhanced PO activity was observed in animals fed with mussel as against those fed with fish. This indicates that mussel is a better feed than fish for lobsters. Though no relevant literature was found on influence of feed on PO activity, certain immune enhancers were shown to increase the PO activity and have the ability to enhance the immune system (Rengipat *et al.*, 2000). Dietary incorporation of immunostimulants like glucan elevated the PO activity (Song and Sung, 1993), which agrees with the present result.

5.4.3. Environmental stress

The present study demonstrates that environmental parameters like pH, salinity, ammonia and dissolved oxygen affect THC, PO activity and superoxide anion production in *P. homarus*.

The fact that THC was significantly low at pH 5 and 9.5 indicates that lower or higher pH of seawater may affect the immune system in *P. homarus*. In a study conducted by Cheng and Chen (2000) in *M. rosenbergii*, high THC at pH 7.5 - 7.7 was observed. The differencial haemocyte count did not show significant variation in the study. The serum PO activity was high at pH 8, but was the highest at pH 7.5 - 7.7. There was a reduction in superoxide anion and acid phosphatase activity indicating a reduction in phagocytic activity. The NBT staining is used to evaluate the effect of pH stress (5.0) on phagocyte activity by quantification of O_2^- release, which is the first product of respiratory burst. The production of superoxide radicals by stimulated haemocytes did not reduce significantly in lobsters exposed to low and high pH, suggesting that the NADPH enzyme responsible for production of super oxide was not affected by pH variation (Bachere *et al.*, 1995).

The low oxygen tension hampers the metabolic performances in crustaceans and can reduce growth and moulting frequency. In the present study, hypoxia induced significant reduction in THC at 1 mg l^{-1} O_2 for 24 hrs. The result was found to agree with the observation in *P. stylirostris*, where the THC

decreased under hypoxic stress (Le Moullac *et al.*, 1998). The phenol oxidase activity was also found to decrease with hypoxia. However, the observation does not agree with the hypoxic stress studies made by Le Moullac *et al.* (1998).

Analysis of haemogram showed that there was no significant difference but the high PO activity can be attributed to the variation in LGC, as it is known that these cells are responsible for the production of phenol oxidase cascade. The superoxide anion production also showed significant reduction as against the observations in *P. stylirostris* in which no significant reduction due to hypoxia was reported. In another study with *P. monodon,* the phagocytic activity was found to be low (50% of control) during oxygen depletion (Direkbusarakom and Danayadol, 1998). This was found to be in agreement with the present study.

Ammonia is highly toxic to aquatic animals and can cause impairment in functioning of numerous organs (Colt and Armstrong, 1981). Lethal and sub- lethal effect of ammonia on shrimp have been largely reported with respect to metabolism and osmoregulation (Chen *et al.*, 1990a). Little is known about its effect on the immune system. In the present study, THC was found to decrease at 0.5, 1.5 and 3 mg L^{-1} of ammonia. In *P. stylirostris*, studies on dose-response effect of ammonia on immune system show significant reduction in THC at concentrations of 1.5 mg L^{-1} and 3 mg L^{-1} ammonia, which was found to be agreeable with the present study (Le Moullac and Hoffner, 2000). In contrast, Cheng and Chen (2002) in their studies with *M. rosenbergii* did not find any significant difference in THC at 0.55, 1.68 and 3.18 mg L^{-1} of ammonia-N concentration.

The amount of PO activity during ammonia stress was found to decrease by 56.33 %, 63.3 % at 65.8% for 0.5, 1.5 and 3 mg L^{-1} of ammonia, respectively. This was found to agree well with the studies conducted in *P. stylirostris* and *M. rosenbergii.* In *P. stylirostris,* the PO activity decreased to 60% and 50% in response to stress. On the other hand in *M. rosenbergii,* the reduction was about 36%, 37 % and 47%, which agrees well with the present study.

No significant differences in respiratory burst were observed in lobsters exposed to 0.5 mg L^{-1} ammonia but at concentration of 1.5 and 3 mg L^{-1},

there was significant reduction in O_2^- activity. In the experiment carried out on *M. rosenbergii*, in contradiction to the present study, an increase in O_2^- anion production was reported. In a study conducted in *P. stylirostris*, higher ammonia levels reduced phagocytosis potential, which is in total agreement with the present study.

It is reported that maximum growth of an organism occurs in isosmotic media, since the animal will be expending less energy for osmotic regulation. The studies on the impact of salinity on immune system showed increased THC with increasing salinity from 20 to 40 ppt but showed a decrease at 45 ppt indicating a salinity stress. Low haemocyte count is an indication of stress. In *P. paulensis*, 20% decrease in THC was reported when prawns were exposed to 34, 23 and 13 ppt salinity, which agrees well with the present result. In yet another study on *M. rosenbergii,* the THC was found to increase with increase in salinity, as was observed in the present study (Cheng and Chen, 2000).

PO activity was also observed to increase with salinity. Vargas-Albores *et al.* (1998) reported increase in total haemocytic 'ProPO' actvity in *P. californiensis* acclimated at 28, 32, 36, 40 and 44 ppt. In *M. rosenbergii* also the PO activity was found to increase with increase in salinity (Cheng and Chen 2000). Reduction in superoxide anion O_2^- at higher and lower salinity indicates a reduction in phagocytic activity to about 35.6 % at 20 ppt.

5.4.4. Handling stress

Palinurid lobsters like the homarids are relatively easy to be transported live by airfreight for long distances to export markets, with less mortality. Although losses are minor, cumulative revenue loss may be high. Very little physiological research has been conducted in post-harvest handling of lobsters. In the present study, by reducing the water temperature to 16^0 C, there was a significant decrease in THC but still higher reduction in PO activity was observed due to the temperature stress. Exposure to low temperature coupled with air exposure during transportation may have profound effect on the respiratory physiology of lobsters. The stress due to these factors may have profound effect on the immune system. Exposure to air along with handling was

found to be more exhausting (Jussila *et al.*, 1999). In *P. cygus*, changes in THC were reported to be quick during physical disturbances. Jussila *et al.* (1999) in their study on *P. cygnus* detected delayed and less marked peak in THC in handled lobsters. The result was found to agree with the study on the effect of cooling in *P. cygnus* by Gomez-Jimenez *et al.* (2000), in which the THC decreased with cooling at 4^0 C. A similar result was encountered in moribund lobsters (Jussila *et al.*, 1997). It is quite evident that 16^0 C cooling might evoke an impairment of cellular immune system in *P. homarus*. When an ectotherm experience extreme changes in temperature, the haemolymph can be expected to show certain physiological changes as soon as the haemolymph passes through the gills. The cells of the animal are not adapted to such rapid changes in temperature possibly resulting in considerable damage. This might have been manifested in the lower PO activity on exposure to low temperature. Consequently, the animal becomes highly vulnerable to physical damage and greater care is needed while handling and transporting the live lobsters.

5.4.5. Pathogen Stress

Rapid decrease in number of circulating haemocyte was observed in lobsters challenged with *Vibrio* sp. in comparison to the saline injected lobsters which showed an increase in THC. The result was found to agree well with the results of Smith and Ratcliffe (1980), where a fall in cell number in *C. maenas* was reported during the initial stage of injection. In a recent study by Sung *et al.* (2000) in *M. rosenbergii*, rapid fall in THC on incubation with *A. verornii* and *A. caviae* was reported. However, they did not observe an increase in THC in the saline treated prawns. Many other workers also have reported significant decline in circulating haemocytes during bacterial clearance (Cornick and Stewart, 1968; Tyson and Jenkins, 1973; Paterson *et al.*, 1976; Smith and Soderhall, 1983; Smith *et al.*, 1984; Martin *et al.*, 1993). The fate of these haemocytes still remains unclear. When body cavity of a crustacean is invaded by a large number of organisms, some are removed directly by phagocytosis, while others are confined to nodules or clumps of cells. In response to the presence of these infected tissues, it has been suggested that some haemocytes may migrate into the connective tissues (Factor and Beckman, 1990), the blood sinuses between the

hepatopancreatic tubules, and the gills (Fontaine and Lightner, 1974; Smith *et al.*, 1984; Sung and Song, 1996). Johnson (1987) has also suggested that in some tissues the fixed phagocytes may originate from circulating haemocytes.

Phenol oxidase can be activated in crustaceans *in vitro* by LPS and is involved in host defense (Smith and Soderhall, 1991), which includes phagocytosis and bactericidal activity (Unestam and Soderhall, 1977). Sung *et al.* (1994) has reported the presence of PO in shrimp haemocytes. In the present study, the PO activity increased immediately after the challenges and returned back to control levels 24 hrs after the challenge. This increase may be due to the break down of granulocytes to initiate the activation of the 'proPO'. In *M. rosenbergii*, PO activity increase was reported on bacterial challenge and in immuno treated shrimp (Sung *et al.*, 2000; Sung *et al.*, 1996). These results confirm that haemocytes and PO activating system respond rapidly to external stimuli especially in the infection phase. This phenomenon was also observed in *S. ingentis* (Martin *et al.*, 1993).

The results from the present study and those of earlier work in decapods show that normal THC was restored after 72 and 96 hour challenge (Smith and Soderhall, 1983). Recovery of THC may be due to settled haemocytes returning to circulation and the production and release of fresh haemocytes from hematopoietic tissue.

5.5. Immune enhancers

It is well established that different molecules of microbicidal origin can specifically elicit the melanisation reaction commonly observed around foreign bodies intruding the body cavity of arthropods. Soderhall *et al.* (1994) had reported that the 'proPO' system of invertebrate haemocyte lysate is activated specifically by β -1-3 glucan from fungal cell wall and lipopolysaccharides and peptidoglycans from bacterial cells. Other researchers have found that external factors like temperature and calcium concentration also may affect 'proPO' system activation (Ashida *et al.*, 1983; Ashida and Soderall, 1984). The present study on the effect of heat killed *Vibrio anguillarum* and *Micrococcus lysidodyticus* was

found to enhance PO activity. The yeast cells were found to enhance the PO activity by 46%, which agrees with the earlier findings that phenoloxidase enzymes are toxic to fungi (Soderhall *et al.*, 1979). Previous studies have shown that treatment with β -1-3 and 1-6 glucan at 0.5 and 1 mg L^{-1} is sufficient to strengthen tiger shrimp resistance to Vibriosis *in vivo* (Sung *et al.*, 1994). When immersion is used to treat the shrimp, only one tenth of this dose reached haemolymph, which agrees well with the present result (Sung *et al.*, 1996).

The studies by Sung *et al.* (1998) showed that calcium and magnesium are required to enhance PO activity in the tiger shrimp and the giant freshwater prawn. Similar calcium mediated enhancement has been reported for several types of insects (Ashida *et al.*, 1983; Dunphy, 1991) and crustacean species (Soderhall, 1981; Ashida and Soderhall, 1984; Kondo *et al.*, 1992). Optimum levels of calcium ion varied with species: 50mM for *Lymanhia dispar*; 10-20 mM for *Galleria mellonella* (Dunphy, 1991), 10mM for *Astacus astacus* (Soderhall, 1981) and *Pacifastacus leniusculus* (Soderhall, 1981); 20 mM for Ca^{2+} and Mg^{2+} in *P. monodon* and *M. rosenbergii* (Sung *et al.*, 1998). In the present study with *P. homarus,* Ca^{2+} and Mg^{2+} at 5mM showed highest PO activity whereas 100mM of Ca^{2+} and Mg^{2+} inhibited the PO activity. Chen and Cheng (1993) has reported that ion concentration in haemolymph of Kuruma shrimp, *P. japonicus* vary with environmental conditions such as temperature and dissolved oxygen. These results suggest that the quality of pond water is critical to maintenance of proper concentration of Ca^{2+} or Mg^{2+} in order to increase PO activity and consequently enhance defense system.

In *P. homarus*, Ca^{2+} and Mg^{2+} ions enhance PO activity and high concentration strongly inhibits as in other crustaceans like crayfish (Soderhall, 1981; Smith and Soderhall, 1983; Ashida and Soderhall, 1984; Soderhall and Hall, 1984) and in *C. maenas* (Smith *et al.*, 1984). High concentration of calcium is known to prevent 'proPO' activation in arthropods (Ashida *et al.*, 1983; Soderhall, 1982). In the haemolymph of *Allogamus* sp. larvae, increase in Ca^{2+} concentration progressively decreased the PO activity *in vitro*. The results were supported by a marked enhancement in PO activity when haemolymph samples were incubated

in the presence of 20mM EDTA. Therefore calcium and magnesium ion level seems to play a key role in the modulation of 'proPO' system.

In vitro, LPS elicits a rapid and massive degranulation and depletion of haemocyte and *in vivo* the result was found to be fatal (Smith and Soderhall, 1983; Smith *et al.*, 1983). Handling stress, injury and slight blood loss associated with injury may affect the ability to withstand the toxicant challenge. Lorenzon *et al.* (1999) showed a decrease in circulating haemocyte after LPS injection in *P. elegans*, *C. crangon, S. mantis, M. vernalis* and *N. norvegicus*. The time to reach the negative peak was found to depend on the species. In *P. japonicus,* Sequeira *et al.* (1996) reported an increase of naturally proliferating circulating haemocytes after LPS administration and their presence reinforces observations concerning the involvement and importance of haemocyte proliferation in immune response. Thus, decrease of haemocyte during bacterial infections may be compensated by increased proliferation of circulating haemocytes (Sequeira *et al.*, 1996).

While probing the mechanism of defense enhanced by beta glucan, Sung *et al.* (1994) observed proPO activity in *P. monodon* fed with β -1-3 glucan. Meanwhile, Jorgenson *et al.* (1993) got similar results in rainbow trout and suggested that β -1-3 glucan administration enhances bactericidal activity, O^{2-} production of macrophages and serum lysosome activity. In addition, Itami *et al.* (1994) have reported that peptidoglycans can enhance the disease resistance of *P. japonicus* by increasing the phagocytic activity of the prawn haemocytes. Therefore, it can be concluded that β -1-3 glucan can act as a fundamental elicitor of host defense mechanism in the spiny lobster *P. homarus*.

Reduction in THC induced by β -1-3 glucan as has been observed in the present investigation shows that glucans can trigger a defense- like haemocytic response *in vivo*. Examination of gill sections taken from lobsters inoculated with 5mg L^{-1} laminarin after 3 hours revealed the presence of small haemocyte clumps in blood sinuses. In the 6hr sample the lump was found to be large and compact. This result hold good with the work of Smith *et al.* (1984) on *C. maenas*, where the cells were lost from the circulation.

The cell aggregation found in the gills of *P. homarus* on injection with bacteria was similar to that reported *C. maenas* injected with laminarin (Smith and Ratcliffe, 1980). β -1-3 glucan also can trigger activation of 'proPO' within the haemocytes (Soderhall and Unestam, 1979; Smith and Soderhall, 1983) and by specifically activating one or more serine proteases in enzymatic cascade (Soderhall, 1981; Soderhall, 1983). This activating pathway is therefore involved in haemocyte clumping. *In vitro*, β -1-3 glucan induce degranulation of the granular cells (Smith and Soderhall, 1983). Therefore, it can be suggested that glucans act initially on granular cells, inducing them to release phenoloxidase from granules, which then assist in activating the hyaline cells.

Studies by Sritunyalucksana *et al.* (1999) show that 0.002% LPS and 0.4% peptidoglycan activated PO activity. It has been recognized that defense mechanism in many invertebrates is often accompanied by melanisation. In arthropods, melanin synthesis is involved in the process of sclerotization and wound repair of cuticle as well as in defense reactions against invading microorganisms (Soderhall, 1982; Ratcliffe *et al.*, 1985; Sugumaran, 1996).

The activation of the PO cascade is achieved by extremely low quantities of microbial cell wall components (LPS), β -1-3 glucan and peptidogycan (Soderhall, 1982; Sugumaran and Kanost, 1993). PO activity in *P. californiensis* (Vargas-Albores *et al.*, 1996) and *P. paulensis* (Perazzolo and Barraco, 1997) was shown to increase with β -1-3 glucan treatment. Microbicial products such as LPS from *E. coli* strongly increase normal phenoloxidase activity in cell-free haemolymph of *Allogamus* sp. (Dunphy, 1990; Yoshida and Ashida, 1986). The endotoxin elicits the proPO system in *Locusta migratoria* (Brehelin *et al.*, 1989).

The advantage of using immune enhancers in growout is that they are natural substances and not antibiotic. Therefore, they may not produce residues and no chances of causing bacterial resistance or any environmental impact and human health hazard. A non-specific immunostimulant is more important in aquaculture than antibiotics or vaccines. Oral administration is a useful delivery system to a large mass of prawns in all stages. Feeding with SPGn or β -1-3 glucan enhanced resistance to artificial challenge of *Vibrio* sp. (Itami *et*

al., 1994). Yano *et al.* (1989) concluded that non-specific defense system can be activated by SPG injection by increasing phagocytic activity. Incorporating levamisole in the diet of *M. rosenbergii* (Baruah and Prasad, 2001) showed enhanced PO activity and NBT reduction. A similar effect was observed in the rainbow trout (Anderson *et al.*, 1989; Anderson and Jensy, 1992; Siwicki *et al.*, 1990) and in *Cyprinus carpio* (Siwicki, 1989) when stimulated with levamisole.

Chitosan was found to enhance immunity in brook trout against *A. salmonicida* (Anderson and Siwicki, 1994) and in *Labeo rohita* (Sahoo and Mukerjee, 1999). In fishes also it is seemed to enhance the non-specific immunity. Chitin and chitosan are acetyted and deacetylated form of muco-polysaccharide which are isolated from exoskeleton of crustaceans and fungal cell walls (Sakai *et al.*, 1992). No earlier works are available on the immunostimulant activity of chitosan in crustaceans. It was reported that chitosan at the rate of 150mg Kg^{-1} was found to increase immunity in *P. homarus* (Huxley *et al.*, 2000 personal communication) which supports the results of the present work.

SUMMARY

SUMMARY

- The objective of the present study was to understand the difference in the immune response of a stressed animal from a normal healthy animal by studying the effect of environmental as well as physiological parameters, so that an optimum culture condition can be maintained to maximize production.

- The study was conducted on the spiny lobster *Panulirus homarus* as they fetch high demand in the export market and recently small scale fattening practices have begun in certain parts of the country. The specimens for the study were obtained from local lobster merchants.

- The animals were kept for acclimatization under ideal conditions in the laboratory before starting the experiments.

- Experiments were conducted to study the effect of various stress factors such as environmental, physiological, nutritional, transportation and pathogen stress. Studies were also conducted with immunostimulants to understand their effect on immune parameters.

- The parameters observed for immune response studies were total haemocyte count, differential haemocyte count, phenoloxidase activity of serum, superoxide anion production and acid phosphatase activity.

- In invertebrates, haemocytes play a crucial role in defence mechanism, so a study was conducted to characterise the haemocytes in lobsters using the light as well as transmission electron microscopy. It was observed that *P. homarus* possess three types of cells: hyaline, semigranular and large granular cells.

- In *P. homarus* the haemocytes are produced from ovoid lobules found in a thin tissue around the foregut, called haemopoietic tissue. Light microscopic studies showed that these ovoid lobules contain stem cells and maturing haemocytes.

- Immune studies on healthy unstressed individuals were studied for the immunological parameters like phenoloxidase activity, THC, DHC, phagocytosis, haemagglutination so as to understand the general health parameter of a healthy lobster under ideal culture condition. The blood volume to weight ratio was also studied which was found to be positively correlated.

- Moulting even though was found to be an essential activity for growth was found to cause immense stress on the animal. It was observed that during post and pre moult the THC increased where as the PO activity was found to decrease making the animal more susceptible to infection.

- Eye-stalk ablation is usually done in lobster for increasing growth but the studies shows that it gives a rapid stress to the individual leading to a sudden increase in THC and an increase in PO activity, which is also a immediate response to injury.

- Nutritional stress like starvation decreases the immune response. It was found to considerably decrease the THC and PO activity of serum upon starvation.

- It was observed that the preferred diet of lobsters is mussels as against fish, which was also found to be giving better immune response as against the fish diet.

- Environmental stressor like low salinity was found to decrease the THC and PO activity of serum. Lower salinity was also found to decrease the superoxide anion production.

- High pH 9.5 and low pH 5.0 were found to decrease the THC and PO activity of serum. No significant variation was observed with regard to the superoxide anion production.

- Under the hypoxic condition, the THC and PO activity of serum decreased from normal. The superoxide anion production also showed significant reduction.

- Ammonia concentration even at 0.5 mg l^{-1} was found to suppress the immune response parameters.

- Transportation is yet another stress which showed a decrease in PO activity but surprisingly there was not much variation with regard to THC.

- *In vivo* studies with bacteria show increase in THC and increase in PO activity of serum just after 30 min of injection. However, lobsters attained normal state with in 24 hrs.

- Studies with immune enhancers show that seawater is the best elicitor for PO activity. The cations like calcium and magnesium have profound impact on PO activity enhancement. $CaCl_2$ and $MgCl_2$ at 5 mM concentration showed highest activity.

- *In vivo* studies using immunostimulant laminarin show increased THC and decrease PO activity immediately after 30 min of incubation followed by an increase in PO and decrease In THC levels. Lobsters regained normal state after 24 hrs.

- Immunostimulants were administered through farm-made feeds with Yeast and Chitosan as stimulants, which showed that chitosan was a good stimulant as compared to yeast. Commercial immunostimulants was also tried by did not come up with any positive results with respect to immune parameters.

REFERENCES

REFERENCES

Adams, A., 1991. Response of penaeids shrimp to exposure to *Vibrio* species. *Fish. Shellfish Immunol.*, 1:59-70.

Amirante, G. A. and Basso, V., 1984. Analytical study of lectins in *Squilla mantis L.*(Crustacea, Stomatopoda) using monoclonal antibodies. *Dev. Comp. Immunol.*, 8: 721–726.

Amirante G. A., 1986. Agglutinins and lectins of Crustaceans: their composition synthesis and functions. *In*: Haemocytic and Humoral Immunity in Arthropods, (ed. Gupta, A. P.). Wilney Interscience, New York, pp:359-380.

Amstrong, P. B., 1979. *In vitro* phagocytosis by *Limulus* blood cells. *J. Invert. Pathol.*, 34:145-151.

Anderson, R. S., Holmes, B. and Good, R. A., 1973. *In vitro* bactericidal capacity of *Blaberus cranifer* haemocytes. *J. Invert. Pathol.*, 22:127-135.

Anderson, D. P., Swicki, A. K., Dixon, O. W. and Lizzio, E. P., 1989. Immuno-stimulation by levamisole in raibowtrout (*Salmo gaidneri*) *in vitro*. *In*: Viruses of lower vertebrates. (ed. Anhe, W. and Kursta, K) Springer-Verlag Publication, New York, pp: 469-478.

Anderson, D. P., 1992. Immunostimulant, adjuvants and vaccine carriers in fish: Application to aquaculture. *Ann. Rev. Fish Dis.*, 2: 281–307.

Anderson, D. P. and Jeney, G., 1992. Immunostimulants added to injected *Aeromonas salmonicida* bacterin enhance the defense mechanism and protection in rainbow trout (*Onchorhynchus mykiss*). *Vet. Immunol Immunopath.*, 34:379-389.

Anderson, D. P. and Swicki, A.K., 1994.Duration of protection against *Aeromonas salmonicida* in brook trout immunostimulated with glucan or chitosan by injection or immersion. *Prog. Fish. Cult.*, 56: 258-261.

Aono, H., and Mori, K., 1996. Interaction between haemocytes and plasma is necessary for haemolymph coagulation in the spiny lobster, *Panulirus japonicus. Comp. Biochem. Physiol.*, 113A: 301-305.

Ashida, M., Dohke, K. and Ohnishi, E., 1974. Activation of prophenoloxidase III. Release of a peptide from prophenoloxidase by the activating enzyme. *Biochem. Biophys. Res. Comm.*, 57: 1089.

Ashida, M., 1981. A cane sugar factor suppressing activation of Phenoloxidase in haemolymph of the silkworm, *Bombyx mori. Insect Biochemistry.*, 11:57-63.

Ashida, M., Ishizaki, Y. and Ywahana, M., 1983. Activation of prophenoloxidase by bacterial cell walls or by β- 1, 3 glucans in plasma of the silkworm *Bombyx mori. Biochem. Biophys. Res. Commun.*, 113:562-568.

Ashida, M and Söderhäll, K., 1984. The Phenol oxidase activating system in crayfish. *Comp. Biochem. Physiol* ., 77B: 21-26.

Aspan, A., Sturtevant, J., Smith, V. J., and Söderhäll, K., 1990. Purification and characterization of a prophenoloxidase activating enzyme from crayfish blood cells. *Insect Biochem.*, 20:709–718.

Aspan, A., Huang, T.-S., Cerenius, L. and Söderhäll, K., 1995. cDNA cloning of prophenoloxidase from the freshwater crayfish *Pacifastacus leniusculus* and its activation. *Proc.Nat. Acad. Scie, USA* 92, 939–943.

Bachère, E., Miahle, E. and Rodriguez, J., 1995. Identification of defence effectors in the haemolymph of crustaceans with particular reference to the shrimp *Penaeus japonicus* (Bate): prospects and application. *Fish. Shellfish Immunol.*, 5B: 597–612.

Bachere, E., Mialhe, E., Noel, D., Buolo, V., Morvan, A. and Rodriguez, J., 1995. Knowledge and research prospects in marine mollusc and crustacean immunology. *Aquaculture.*, 132: 17-32.

Bachere, E., Destoumieux, D., Bullet, P., 2000. Peneaidins, antimicrbial peptides of shrimp: A comparison with other effectors of innate immunity. *Aquaculture.*,191:71-88

Bang, F.B., 1967. Serological responses among invertebrates other than insects. *Federation Proceedings*, 26: 1680-1684.

Bang, F.B., 1971. Transmissible diseases, probably viral in origin, affecting the amoebocytes of European shore crab *Carcinus maenas. Infect. Immunol.*, 3: 617-623.

Barracco, M.and Amirante, G. A., 1992. Morphological and cytochemical studies of the haemocytes of *Squilla mantis* (Stomatopoda). *J. Crust. Biol.*, 12: 372-382.

Barton, B. A. and Iwama, G. K., 1991. Physiological changes in fish from stress in aquaculture with emphasis on the response and effects of corticosteroids. *Annual Review of Fish Diseases,* 1: 649-59.

Baruah, N. D. and Prasad, K. P., 2001. Efficacy of levaisole as an immuno-stimulants in *Macrobrachium rosenbergii* (Deman). *J. Aqua. Trop.*, 16(2) : 149-158.

Battele, B. A. and Kravitz, E. A., 1978. Targets of octopamine action in the lobster: cyclic nucleotide changes and physiological effects in hemolymph, heart and exoskeletal muscle. *J. Pharmacol. Exp. Therapeutics*, 205: 438- 48.

Bauchau, A. G. and Plaquet, J. C., 1973. Variation in the number of haemocytes in crustaceans: Brachiura. *Crustaceana*, 24: 215-223.

Bauchau, A.G. and Mengeot, J.C., 1978. Structure et foncions des hemocytes chez les custaces. *Arch. Zool. Exp. Gen.*, 119: 227-248.

Bauchau, A. G., 1981. Crustaceans. *In*: Invertebrate blood cells (ed. Ratcliffe, N.A. and Rowley, A. F). Academic Press, London, pp. 385-420.

Bayne, C. J., 1990. Phagocytosis and non-self recognition in invertebrates. *Bioscience.,* 40(10) :723-731.

Bell, K.L., 1993. Studies on Phagocytosis of sore crab, Carcinus *maenas*. Ph.D thesis ,University of St.Andrews ,Fife,Scoltland

Bell, T. A., and Lightner, D. V., 1988. A handbook of normal penaeids shrimp histology. *World Aquacult. Soc.*, Louisiana.

Bell, K. L. and Smith, V. J., 1993. In vitro superoxide production by hyaline cells of the shore crab *Carcinus maenas* (L.). *Developmental and Comparative Immunology*, 17: 211-219.

Benjamin, L. R. and James, B.L., 1987. Light and Electron microscopy of haemocytes of *Ligia oceanica. J. Invert. Pathol.,* 49:19-25.

Berry,P.F.,1971.The biology of the spiny lobster *Panulirus homarus* (Linnaeus) off the east coast of Southern Africa.*S.Afr.Oceanogr.Res.Inst.Invert.Rep.*28:1-75

Birmingham, J. R. and Jeska, E. L., 1981. *In vitro* interaction of bovine mononuclear phagocytes and lipopolysaccharide from *Brucella abortus. AFR. Journal of Clinical Experimental Immunology.,* 2:233–241.

Blaxhall, P.C., 1981. A comparison of methods used for the separation of fish lymphocytes. *J. Fish biol.*, 18:177-181.

Blazer, V.S., 1991. Piscine macrophage function and nutritional influence: A review. *J. Aqua. Ani. Heal.* 3: 77-86.

Bodammer, J.E., 1978. Cytological observations on the blood and haematopoetic tissue in the crab, *Callinectes sapidus. Cell and Tissue Res.,* 187:79-96.

Bray, W. A., Lawrence, A. L. and Leung-Trujillo, J. R. ., 1994. The effect of salinity on growth and survival of *Penaeus vannamei*, with observation on interaction of IHHN virus and salinity. *Aquaculture.,* 122:133–146.

Brehelin, M and Arcier, J.M., 1985. Phagocytosis of inert particles: A comparative study in insects and marine crustaceans. *In*: Comparative Pathobiology (ed. Cheng T. C.). Plenum, New York. Vol. 8. pp.109-142.

Brehelin, M., Drif, L., Baud, L and Boemare, N., 1989. Insect haemolymph cooperation between humoral and cellular factors in *Locusta migratoria*. *Insec .Biochem.*, 19:301-307.

Brivio, M. F., Mazzi, C. and Scai. G., 1996. Pro PO system of *Allogamus auricollis* (Insecta): Effects of various compounds on Phenol oxidase activity. *Comp. Biochem. Physiol.*, 113B (2): 281-287.

Brown, R., Almodovar, L. R., Bhatia, H.M and Boyd, W.C., 1968. Blood group specific agglutinins in invertebrates. *J. Immunol.*, 100: 214-216.

Cassels, F. J., Marchalonis, J. J. and Vasta, G. R.,1986. Heterogeneous humoral and hemocyte-associated lectins with *N*-acylaminosugar specificities from the blue crab, *Callinectes sapidus* Rathbun. *Comp. Biochem. Physiol.* 85B, 23–30.

Catacuzene, J., 1912. Le probleme de l'immunite chez les invertebrates. *Compte rendu des sceances de la societe de biologie.*, 88:48-119.

Catacuzene, J., 1934. The cellular defence reactions of invertebrates. *Ann. Inst. Pasteur, Paris* 12, 273.

Cerenius, L. and Söderhäll, K., 1995. Crustacean Immunity and complement; a premature comparison? *Amer. Zool.*, 35:60-67.

Chackrabarti, R., 1993. Phenoloxidase activity in different tropical shrimps and the sensitivity to sulphiting agents. *Indian. J. Fish.*, 40(4):247-250.

Chaga, O., Lignell, M and Söderhäll, K., 1995. The haematopoietic cells of the fresh water crayfish, *Pacifastacus leniusculus. Ani. Biol.*, 4:59-70.

Chang, C. H., Su, M, S. and Chen, H. Y., 1999. A rapid method to quantify total haemocytes count of *Penaeus monodon*, using ATP analysis. *Fish. Pathol.*, 34(4):211-212.

Chen, J. C., Liu, P. C., Lei, S.C., 1990a. Toxicities of ammonia and nitrate to *Penaeus monodon* adolescents. *Aquaculture*, 89:127-137.

Chen, J. C., Ting, Y. Y., Lin, J. N. and Lin, M., 1990b. Lethal effects of ammonia and nitrate on *Penaeus chinensis* juveniles. *Mar. Biol.* 107: 427-431.

Chen, J. C. and Kou, Y. Z., 1992. Effects of ammonia on growth and molting of *Penaeus japonicus* juveniles. *Aquaculture*, 104:249-260.

Chen, J. C. and Lin, C.Y., 1992. Oxygen consumption and ammonia excretion of *Penaeus chinensis* juveniles exposed to ambient ammonia at different salinity levels. *Comp. Biochem. Physiol.*, 102 C: 287-291.

Chen, J. C. and Cheng, S. Y., 1993. Studies on haemocyanin and haemolymph proteins levels of *Penaeus japonicus* based on sex, size and moulting

cycle. *Comp. Biochem. Physiol.* Part B: *Biochem. Mol. Biol.*, 106 (2):293-296.

Chen, S. M., Rankin, S.M and Keeley, L. L., 1988. Characterisation of moult stages in *Penaeus vannnamei*: setogenesis and hemolymph levels of total proteins, ecdysteroids and glucose. *Biological Bulletin.*, 175:185-192.

Cheng, W. and Chen, J. C., 2000. Effects of pH, temperature and salinity on immune parameters of the freshwater prawn, *Macrobrachium rosenbergii. Fish Shellfish Immunol.,* 10: 387–391.

Cheng, W. and Chen, J. C., 2001. Effects of intrinsic and extrinsic factors on the haemocyte profile of the prawn, *Macrobrachium rosenbergii. Fish Shellfish Immunol.*, 11: 53-63.

Cheng, W. and Chen, J.C., 2002. The virulence of Enterococcus to freshwater prawn *Macrobrachium rosenbergii* and its immune resistance under ammonia stress. *Fish Shellfish Immunol.*, 12:97-109.

Chisholm, J. R. S and Smith, V. J., 1991. Antibacterial activity in *Carcinus maenas* haemocytes. *Dev. Comp .Immunol.*, 15:50-56.

Chisholm, J. R. S. and Smith, V. J., 1992. Antibacterial activity in the haemocytes of the shore crab, *Carcinus maenas. J. Mar. Biol. Ass. U. K.,* 72: 529–542.

Chisholm, J. R. S. and Smith, V. J., 1994. Variation of antibacterial activity in the haemocytes of the shore crab, *Carcinus maenas* with temperature. *J. Mar. Biol. Ass. U. K.,* 74: 979–982.

Chisholm, J. R. S and Smith, V. J., 1995. A comparison of anti bacterial activity in haemocytes of different crustacean species. .*Comp. Biochem. Physiol.*, 110A: 39-45.

Clare, A. and Lumb, G., 1994. Identification of haemocytes and their role in clotting in the blue crabs, *Callinectes sapidus. Mar. Biol.*, 118: 601-610.

Cohen, E., 1968. Immunologic observations of agglutinins of haemolymph of *Limulus polyphemus* and *Birgus latro. Trans. New York. Acad. Sci.*, 30: 427-443.

Colt, J. E. and Armstrong, D.A., 1981. Nitrogen toxicity to crustaceans, fish and molluscs. *In*: proceedings of Bio-engineering system for culture. (ed. Allen, L.J., Kinney, E.C.). Fish culture section of the American Fisheries society (FCS publ. I), Bethesda, pp. 34-47.

Cooper, E. L and Lemmi, C. A. E., 1981. Invertebrate humoral immunity. *Dev. Comp. Immunol.*, 5:3-21.

Cooper, E. L., 1985. Overview of humoral factors in invertebrates. *Dev. Comp. immunol.*, 9:577-583.

Cornick, J. W., and Stewart, J. E., 1968. Interaction of the pathogen *Gaffkya homari* with natural defense mechanisms of *Homarus americanus*. *J. Fish. Res. Bd. Can.*, 25: 697-709.

Cornick, J. W., and Stewart, J. E., 1973. Partial characterisation of natural agglutinin in haemolymph of lobster *Homarus americanus*. *J. invert. Pathol.*, 21: 255-262.

Cornick, J. W. and Stewart, J. E., 1975. Red crab (*Geryon quinquedens*) and snow crab (*Chionoecetes opilio*) resistance to infection by lobster pathogen *Aerococcus viridans* (var.) *homari*. *J. Fish. Res .Bd. Can.*, 32:702-706.

Cornick, J.W., and Stewart, J.E., 1978. Lobster (*Homarus Americans*) Haemocytes: classification differential counts and associated agglutinin activity. *J. invert. Pathol.*, 31:194-201.

Cushing, J.E., 1967. Invertebrates, immunology, and evolution. *Fed. Proc.*, 26:1666-1674.

Dall, W., 1964. Studies on the physiology of shrimp, *Metapenaeus mastersii* (Haswell) (Crustacea: Decapoda: Penaidae).I. Blood constituents. *Aust. J. Mar. Freshw. Res.*, 15: 145-161.

Dean, J. M. and Vernberg, F. J., 1966. Hypothermia and the blood of crabs. *Comp. Biochem. Physiol.* B., 17:19-22.

Dillon, T. M. and Fisher, D. T., 1983. Apparent water permeability and haemolymph acid phosphatase activity as indicator of stress in estuarine grass shrimp, *Paleomontes pugio*. *Comp. Biochem. Physiol.*, 74:377-382.

Direkbusarakom, S. and Danayadol, Y., 1998. Effect of oxygen depletion on some parameters of the immune system in black tiger shrimp (*Penaeus monodon*). *In*: Advances in shrimp biotechnology National center for genetic engineering and Biotechnology, (ed. Flegel, T. W.). Bangkok, pp 147-149.

Dunphy, G. B., 1991. Phenoloxidase activity in serum of two species of insects, the gypsy moth, *Lymantria dispar* (Lymanthriidae) and greater wax moth, *Galleria mellonella* (Pyralidae). *Comp. Biochem. Physiol.*, 98B: 535-538.

Dunphy, G. B., 1990. Phenoloxidase activity in the serum of two species of insects, the gypsy moth, *Lymantria dispar* (Lymantriidae) and the greater wax moth, *Galleria mellonella* (pyralidae). *Comp. Biochem. Physiol.* 95B: 321-325.

Durliat, M., and Vranckx, R., 1981. Action of various anticoagulants on haemolymph of lobsters and spiny lobsters, *Biol. Bull. Mar. Biol. Lab., Woods Hole,* 160: 55-68.

Durliat, M. and Vranckx, R., 1983. Analysis of clotting defects in diseased lobsters II. Immunochemical study of blood proteins. *Comp. Bio. Physiol.*, 76A (1): 103-108.

Durliat, M., 1985.Clotting process in Crustacea Decapoda. *Biol. Rev.*, 60:473-498.

Duvic, B. and Söderhäll, K., 1990. Purification and characterisation of a β -1,3-glucan binding protein from the plasma of the crayfish *Pacifastacus leniusculus. J. Biol. Chem.*, 265: 9332-9337.

Dyrynda, E. A., Pipe, R. K. and Ratcliffe, N. A., 1997. Sub-population of haemocytes in the adult and developing marine mussel, *Mytilus edulis*, identified by use of monoclonal antibodies. *Cell Tissue Res.* 289: 527-536.

Evans, E. E., Weinheimer, P. F., Painter, B., Acton, R.T. and Evans, M. L., 1969. Secondary and tertiary responses of induced bactericidin from the West Indian Spiny lobster, *Panulirus argus. J. Bact.,* 98: 943-946.

Evans, L. H., Fan, A., Finn, S. P., Dawson, S. A., Siva, C. J.and Lee, I. R., 1992. Nutritional status assessment studies in the freshwater crayfish, *Cherax tenuimanus. In*: Proceedings of the Aquaculture Nutrition Workshop, Salamander Bay, 15-17 April 1991 (Ed. by G. L. Allan and W. Dall). NSW Fisheries, Brackish Water Fish Research Station, Salamander Bay, Australia, pp. 87-91

Evans, L., Paganini, M., Fan, A., Fotedar, S., Fotedar, R. and Jussilla, J., 1999. Acute and chronic stress studies in freshwater crayfish. *In:* Book of Abstracts, World Aquaculture 99, and. Sidney, Australia 26 April–2 May 1999. pp. 249

Evans, L. H., Jones J. B. and Brock, J. A., 2000. Diseases of spiny lobsters. In: Spiny Lobsters. Fisheries and Culture 2nd edn. (ed. B. F. Phillips and T. Kittaka). Blackwell Scientific Publications, pp. 586-600.

Factor, J. R., and Beekman, J., 1990. The digestive system of the lobster, *Homarus americanus.* III. Removal of foreign particles from the blood by fixed phagocytes of the digestive gland. *J. Morphol.* 206: 293–302.

Feng, S. Y., 1988. Cellular defense mechanisms of oysters and mussels. *Amer. Fish. Soc. Spec. Pub.*, 18: 153-168.

Fenouil, E. and Roch, P., 1991. Evidence and characterisation of lysozyme in six species of freshwater crayfish from Astacidae and Cambridae families. *Comp. Biochem. Physiol.*, 99B: 43-49.

Field, R. H., and Appleton, P. L., 1995. A *Hematodinium*- like dinoflagellate infection of the Norway lobster *Nephrops norvegicus* observations on pathology and progress of infection. *Dis. Aquat. Org.*, 22: 115-28.

Fisher, W. S., Rosemark, T. R. and Nilson, E. H. ., 1976. The susceptibility of cultured American lobsters to a chitinolytic bacterium. *Proc. World Maricult. Soc.*, 7: 511-520.

Fisher C. W. and Brady U. E., 1983. Activation, properties and collection of haemolymph phenoloxidase of the American cockroach, *Periplanata americana*. *Comp. Biochem. Physiol.*, 75 C: 111-114.

Fisher, W. S. ., 1988. Shell disease of lobsters. *In:* Disease Diagnosis and Control in North American Marine Aquaculture, 2nd edn (ed. C.J. Sinderman and D.V. Lightener). Elsevier, Amsterdam, pp. 236-239.

Fontaine, C. T. and Lightner, D. V., 1973. Observations of the process of wound repair in penaeid shrimp. *J. Invert. Pathol* .,22:23-33

Fontaine, C. T., and Lightner, D.V., 1974. Observations on the phagocytosis and elimination of carmine particles injected into the musculature of the white shrimp *Penaeus setiferus*. *J. Invert. Pathol.*, 24:141-148.

Fontaine, C. T., 1975. Observations on the wound repair processes in the freshwater crayfish *Procambarus* sp. *J. invert. Pathol.*, 25: 391-393.

Fontaine, C. T. and Lightner, D.V., 1975. Cellular response to injury in penaeid shrimp. *Mar. Fish. Rev.*, 37: 4-10.

Franchin, A. and Ottaviani, E., 1990. Fine structure and acid phosphatase localization of haemocytes in the freshwater snail *Viviparus ater*, Gastropoda, Prosobranchia. *J. invert. Pathol* .,55:28–34.

Fries, C. R., 1984. Protein hemolymph factors and their roles in invertebrate defense mechanisms: A review. *In*: Comparative pathology (ed. Cheng, T. C.).Plenum, New York, Vol.6, pp: 49-109.

Fuller, G. M. and Doolittle R. F., 1971. Studies of invertebrate fibrinogen, II. Transformation of lobster fibrinogen into fibrin. *Biochemistry.*, 10B:1311-18.

Ghidalia, W., Vendrely, R., Montmory, C., Coirault, Y., and Brouard, M.O. ., 1981. Coagulation in decapod crustacea. *J. Comp. Physiol.*, 142: 473-8.

Ghiretti-Magaldi, A. Milanesi, C. and Tognon, G., 1977. Haemopoesis in crustacean decapoda: Origin and Evolution of haemocytes and Cyanocytes of *Carcinus maenas*. *Cell Differentiation.*, 6: 167-186.

Giulianini, P. G, Smullen, R. P., Bentky, M. G., Ferrreo, E. A., 1998. Cytological and immunocytochemical study of sinus gland in Norway lobster *Nephrops norvegicus* (L). *Inv. Rep. Dev.* 33(1): 57-58.

Goldenberg, P. Z., Huebner, E., and Greenberg, A.H., 1984. Activation of lobster haemocytes for phagocytosis. *J. invert. Pathol.*, 43: 77-88.

Goldenberg, P.Z., Greenberg, A.H., and Gerrard, J.M., 1986. Activation of lobster haemocytes: cytoarchitectural aspects. *J. invert. Pathol.*, 47: 143-154.

Gollas-Galván, T., Hernández-López, J and Vargas-Albores, F., 1999. Prophenol-oxidase from brown shrimp, *Penaeus californiensis*. haemocytes. *Comp. Biochem. Physiol.*, 122B (1):77.

Gomez-Jimenez, S., Uglow, R. F. and Gollas-Galvan, T., 2000. The effect of cooling and emersion on the total haemocytes count and Phenoloxidase activity of *Panulirus interruptus*. *Fish. Shellfish. Immunol.*, 10: 631-635.

Gondko, R., Michalak, W. and Swierezynski, B., 1981. Comparison of haemolymph content of protein and cation (Ca, Mg, Na, K), clotting time and pH in three crayfish species. *Comp. Biochem. Physiol.*, 69 A: 637-640.

Gornell, A. G., Baradawill, C. G. and David, M. M., 1949. Determination of total serum proteins by means of biuret reaction. *J.Biol.Chem.*,177:751-756.

Hall, J. L. and Rowlands, D. T. Jr., 1974. Heterogeneity of lobster agglutinins. II. Specificity of agglutinin–erythrocyte binding. *Biochemistry* 13: 828–832.

Hall, L and Söderhäll, K., 1982. Purification and properties of a proteinase inhibitor in crayfish haemocytes. *Jour. of invert. Pathol.*, 39:29-37.

Hall, M. R., and Van Han, E. H., 1998. The effects of different types of stress on blood glucose in the giant tiger prawn *Penaeus monodon*. *J. World Aquacult. Soc.*, 29: 290-300.

Hartman, A. L., Campbell, P. A. and Abel, C. A., 1978. An improved method for isolation of lobster lectin. *Dev. Comp. immunol.*,.2:617-625.

Hauton, C., Hawkins, L. E. and Williams, J. A., 1995. Circadial rhythmicity in the activity of phenoloxidase enzyme in the common shore crab, *Carcinus maenas*. *Comp.Biochem. Physiol.*, 111B:374–352.

Hearing, V. and Vernick, S.H., 1967. Fine structure of the blood cells of the lobster, *Homarus americanus*. *Ches.Sci.*, 8, 170-186.

Hearing, V. C., 1969. Demonstration of acid phosphatase in the granules of the lobster *Homarus americanus*. *Ches.Sci.*, 10: 24-28.

Hed, J., 1986. Methods for distinguishng injested from adhering. *Meth.Enzymol.*, 132:198-204.

Henning, O., Itami, T., Maeda, M and Kondo, M., 1998. Analyses of haemolymph immunoparameters in kuruma shrimp infected with penaeids rod-shaped DNA virus. *Fish Pathology*, 33: 389-393.

Hose, J. E., Martin, G. G., Nguyen, V., Lucas, J. and Rosenstein, T., 1987. Cytochemical features of shrimp haemocytes. *Biol. Bull.*, 173:178-187

Hose, J. E. and Martin, G. G., 1989. Defense functions of granulocytes in the ridgeback prawn *Sicyonia ingentis. J. Invert. Pathol.*, 53: 335-346.

Hose, J. E., Martin, G. G. and Gerard, A.S., 1990. A decapod hemocyte classification scheme integrating morphology, cytochemistry, and function. *Biol. Bull.*, 183:185-199.

Hose, J. E., Martin, G. C., Tiu, S. and McKrell, N., 1992. Pattern of hemocyte production and release throughout the moult cycle in the penaeid shrimp *Sicyonia ingentis. Biol. Bul.,* 183, 185–199.

Huang, M. T., Eble, A. F. and Hammen, C. S., 1981. Immune response of prawn *Macrobrachium rosenbergii*, to bacterial infection. *J .Invert. Pathol.*, 38:213-219.

Hultmark, D., Steiner, H., Rasmusson, T. and Boman, H. G., 1980. Insect immunity: Purification and properties of three inducible bactericidal proteins from haemolymph of immunized pupae of *Hyalophora cecropia. Eur .J. Biochem.*, 106:7-16.

Imai, T., Goto, R., Kittaka, J. and Kamiya, H., 1994. Lectins in the rock Lobster, Jasus *novaehollandiae* haemolymph. *Crustaceana.*, 67(10):121-130

Inabe, T., Suetake, Y. and Funatsu, M., 1963. Studies on tyrosinase in the housefly. II. Activation of protyrosinase by sodium dodecyl sulphate. *Agr.Biol.Chem.*, 27:332-337.

Itami, T., Takahashi, Y., Tsuchichitra, E., Igusa, H. and Kodo, M., 1994. Enhancement of disease resistance of Kuruma prawn *Penaeus japonicus* and increase in phagocytic activity of prawn haemocytes after oral administration of β-1, 3-Glucan (Schizophyllan) *In*: The third Asian fisheries forum. (ed. Chou L. M. Micro A. D, Lam T. J.). Asian fisheries society, Manila, Phillippines, pp. 375-378.

Jayasree, S., 2001. Purification and characterisation of a natural agglutinin in the haemolymph of prawn *Penaeus indicus* H. Milne Edwards. *J. Invert. Pathol.*, 77: 237-242.

Johansson, M. V and Söderhäll, K., 1985. Exocytosis of the phenoloxidase activating system from crayfish haemocytes. *J. Comp. Physiol.*, 156 B: 175-181.

Johansson, M. W. and Söderhäll, K., 1989. Cellular immunity in crustacean and the proPO system. *Parasitol. Today,* 5: 171–176.

Johansson, M. W., Lind, M. I., Holmblad, T., Thornqvist, P. O. and Söderhäll, K., 1995. Peroxinectin a novel cell adhesion protein from crayfish blood. *Biochem. Biophys. Res. Commun.*, 216:1079-1087.

Johansson, M. W., Keyser, P., Sritunyalucksana, K and Söderhäll, K., 2000. Crustacean haemocytes and haemopoeisis. *Aquaculture*, 191:45-52.

Johnston, M. A., Elder, H. Y. and Davies, P. S., 1973. Cytology of *Carcinus* haemocytes and their function in carbohydrate metabolism. *Comp. Biochem. Physiol.*, 46: 569-581.

Johnson, P. T., 1976. Bacterial infection in the blue crab, *Callinectes sapidus*: course of infection and histopathology. *J. Invert. Pathol.*, 28: 25-36.

Johnson, P. T., 1980. Histology of the Blue Crab, *Callinectes sapidus*.A Model for the Decapoda. Praeger, New York.

Johnson, P. T., 1987. A review of fixed phagocytotic cells of decapods crustaceans, with remark on haemocytes. *Dev. Comp. Immunol.*, 11:679-704.

Jorgensen, J. B., G. J. E. Sharp, C.J. Secombes and Robertsen, B., 1993. Effect of a yeast-cell-wall glucan on the bactericidal activity of rainbow trout macrophages. *Fish Shellfish Immunol.*, 3: 267-277.

Juinio-Menez, M. A., Ruinata, J., 1996. Survival, growth and food conversion efficiency of *Panulirus ornatus* following eyestalk ablation. *Aquaculture*, 146(3-4): 225-235.

Jussila, J., Jago, J., Tsvetnenko, E., Dunstan, B. and Evans, H., 1997. Total and Differencial haemocytes count in western rock lobsters (*Panulirus Cygnus* George) under post harvest stress. *Mar. Freshwat. Res.*, 48: 863-867.

Jussila, J., Jago, J., Tsvetnenko, E., Dunstan, R. and Evans, L.H., 1998. Total and differential haemocyte counts in western rock lobster (*Panulirus cygnus* George) under postharvest stress. *Mar. Freshwat,. Res.*, 48: 863-867.

Jussila, J., Paganini, M., Mansfield, S. and Evans, L. H., 1999. On physiological responses, haemolymph glucose, total hemocyte count and dehydration, of marron (*Cherax tenuimanus*) to handling and transportation under simulated conditions. *Freshwat. Crayf.*, 12: 154-167.

Jussila, J., Mc Bride, S., Jago, J. and Evans, L. H., 2001. Haemolymph clotting time as an indicator of stress in western lobsters (*Panulirus Cygnus* George). *Aquaculture*, 199:185-193.

Kim, K., 1994. Antimicrobial activity in gorgorian coral (Coelenterata, Octocoralla). *Coral Reefs*, 13: 75-80.

Kobayashi, M., Johansson, M. W. and Söderhäll, K., 1990. The 76 kD cell adhesion factor from Cray fish haemocytes promotes encapsulation *in vitro*. *Cell. Tiss. Res.*, 260:13-18.

Lackie, A.M., 1980. Invertebrate immunity. *Parasitology*, 80: 393-412.

Lanz, H., Tsutsumi, V. and Arechiga, H., 1993. Morphological and biochemical characterization of *Procambarus clarkii* blood cells. *Dev. Comp. Immunol.*, 17: 389-397.

Lanz Mendoza, H. and Faye, I., 1996. Immunoglobulin superfamily proteins in invertebrates. *In*: New Directions in Invertebrate Immunology. (ed. K. Söderhäll, S Iwanaga and G.R. Vasta). SOS Publications, Fair Haven, pp. 285-302.

Le Moullac, G., Le Groumellec, M., Ansquer, D., Froissard, S., Levy, P. and Aquacop., 1997. Haematological and phenoloxidase activity changes in the shrimp *Penaeus stylirostris* in relation with the moult cycle: protection against vibriosis. *Fish. Shellfish Immunol.*, 7: 227–234.

Le Moullac, G., Soyez, C., Saulnier, D., Ansquer, D., Avarre, J. C. and Levy, P., 1998. Effect of hypoxia stress on the immune response and the resistance to vibriosis of the shrimp *Penaeus stylirostris*. *Fish. Shellfish Immunol.*, 8: 621–629.

Le Moullac, G. and Haffner, P., 2000. Environmental factors affecting immune responses in crustacean. *Aquaculture*, 191:121-131.

Lee, D.O'C. and Wickins, J. C. 1992. Crustacean Farming. Blackwell Scientific Publications, Oxford.

Lee, M. H. and Shiau, S. Y., 2002. Dietary Vitamin C and its derivatives affect immune response in grass shrimp, *Penaeus monodon*. *Fish. Shellfish. Immunol.*, 12:119-129.

Lentzen, H., Agrawal, B., Noske, W. and Herken, H., 1984. Isolation and characterization of internalized glioma cell membrances. *Cell. Tiss. Res.*, 236:147–151.

Leslie, R. G. Q., 1987 .Evaluation and improvement of a rapid micro assay for measuring super oxide anion production by Phagocytes.1. spectrophotometric aspects. *J. Immunological Methods*, 103: 253-259

Leslie, R. G. Q. and Allen, R., 1987. Evaluation and improvement of a rapid micro assay for measuring super oxide anion production by Phagocytes. 2. Biochemical aspects. *J. Immunological Methods.*,103: 261-266

Levin, J., 1985. The role of amoebocytes in the blood coagulation mechanism of horse shoe crab, *Limulus polyphemus*. *In*: Blood Cells of Marine Invertebrates (ed. Cohen, W .D). AlanR. Liss, New York, pp:145-163

Liener, I.E., Sharon, N and Goldstein, I.J., 1986. The Lectins: Properties, Functions and Applications in Biology and Medicine. Academic press, New York, pp. 600.

Lorenzon, S., de Guarrini, S., Smith, V. J. and Ferrero, E. A. ., 1999. Effects of LPS injection on circulating haemocytes in crustaceans *in vivo*. *Fish. Shellfish Immunol.*, 9: 31–50.

Lorenzon, S., Francese, M., Smith, V. J. and Ferrero, E. A., 2001. Heavy metals affect the circulating haemocytes number in the shrimp *Paleomon elegans*. *Fish. Shellfish. Immunol.*, 11:459-472.

Madenjian, C. M., Rogers, G. L. and Fast, A.W., 1987. Predicting night-time dissolved oxygen loss in prawn pond of Hawaii: Part I. Evaluation of traditional methods. *Aquacult. Eng.*, 6:191-208.

Maheswari, R., Mullainadhan, P. and Arumugham, M., 1997. Characterisation of a natural agglutinin with affinity for acetylated aminosugars in the serum of marine prawn, *Penaeus indicus* (H. Milne Edwards).*Fish. Shellfish. Immunol.*, 7(1):17-28.

Malloy, S. C., 1978. Bacterial induced shell disease of lobsters (*Homarus americanus*). *J. wildl. Dis.*, 14: 2-10.

Manjula, P. L, Rahman, K. M. D. and Abraham, T.J., 1997. Haemocytes classification and differential counts in the Indian spiny lobster *Panulirus homarus* (Linnaeus). *J. Aquacult. Trop.*, 12:113-121.

Manjula, P. L, Abraham, T.J. and Rahman, K. M. D., 1999. Defense mechanisms and reactions in the Indian spiny lobster *Panulirus homarus* (Linnaeus): Responses to bacterial infection. *J. Aquacult. Trop.*, 14:173-180.

Martin, G. G. and Graves, B. L., 1985. Fine structure and classification of shrimp haemocytes .*J. Morph.*, 185: 339-348.

Martin, G. G., Hose, J. E. and Kim, J. J., 1987. Structure of hematopoietic nodules in the ridgeback prawn, *Sicyonia ingentis*: light and electron microscopic observations. *Journal of Morphology*, 192: 193-204.

Martin, G. G., Hose, J. E., Choi, M., Provost, R., Omari, G, McKrell, N. and Lam. G., 1993a. Organisation of haematopoitic tissue in the intermolt lobster, *Homarus americanus*. *J. Morph.*, 216:65-78.

Martin, G. G., Poole, D, Poole, C. Hose, J. E., Arias, M., Reynolds, L ., Mckrell , N and Whang, A., 1993b. Clearence of bacteria injected into the haemolymph of the penaid shrimp *Sicyonia ingentis*. *J. Invert. Pathol.*, 62:308-315.

Martin, G. G., Quintero, M., Quigley, M. and Khosrovian, H., 2000. Elimination of sequestered material from the gills of decapod crustaceans. *J. Crust. Biol.*, 20: 209-217.

McCumber, L.J., Hoffmann, E. M. and Clem, L. M., 1979. Recognition of viruses and xenogeneic proteins by the blue crab, *Callinectus sapidus*: a humoral receptor for T_2 bacteriophage. *J. Invert. Pathol.*, 33:1-9.

McCumber, L. J., Clem, L.W., 1983. Recognition of non–self in crustaceans. *Amer. Zoolt.*, 23: 173-183.

McHenery, J. G, Birkbeck, T. H and Allen, J. A., 1979. The occurrence of lysosome in marine bivalves .*Comp. Biochem. Physiol.*, 63B: 25-28.

McKay, D., Jenkin, C. R., and Rowley, D., 1969. Immunity in the invertebrates. 1. Studies on naturally occurring haemagglutinins in the fluid from invertebrates. *Aust. Jour. Exp. Biol. Med. Sci.*, 47: 125-134.

McKay, D. and Jenkin, C. R., 1970. Immunity in the invertebrates. The role of serum factors in phagocytosis of erythrocytes by haemocytes of the freshwater crayfish, *Parachaeraps bicarinatus. Aus. J. Exp. Biol. Med. Sci.*, 48: 139–150.

Michael, C., Mix, A., Albert, D. and Sparks, K., 1980. Hemocytes classification and differencial count in Dungeness crab, *cancer migister. J. Invert. Pathol.*, 35:134-143.

Miller, V. H., Ballaback, R. S., Pauley, G. B. and Kranssnis, S. M., 1972. A preliminary physiochemical characterisation of agglutinin found in the haemolymph of crayfish *Procambarus clarkii. J. Invert. Pathol.*, 19: 83-98.

Mix, M. C. and Sparks, A. K., 1980. Hemocyte classification and differential counts in the Dungeness crab *Cancer magister. J. Invert. Pathol.*, 35:134-143.

Mohandas, A., Cheng, T.C., and Cheng, J.B. 1985. Mechanism of lysosomal enzyme release from *Mercenaria mercenaria* granulocytes: a scanning electron microscope study. *J. Invert. Pathol.*, 46: 189-197.

Moore, C. A. and Gelder, S. R., 1983. The role of the "blunt" granules in hemocytes of *Mercenaria mercenaria* following phagocytosis. *J. Invert. Pathol.*, 41:369–377.

Mori, K. and Stewart, J. E., 1978. The haemolymph bactericidin of American lobster (*Homarus americanus*): Adsorption and activation. *J. Fish. Res. Bd. Can.*, 35:1504-1507.

Mosco, A., Riggo, D. and Savoini, A., 1989. Molecular characteristics, site of synthesis and tissue distribution of *Squilla mantis* haemocyanin. *In*: Biology of stomatopods (ed. Ferrero, E. A.).Selected symposia and monographs U.Z.I., 3. Mucchi, Modena, pp. 147-152.

Nagi, T and Kawabata, S. I., 2000. A link between blood coagulation and prophenol oxidase activity in arthropod defence. *J. Biol. Chem.*, 275 (38): 29264-29267.

Nappi, A. J. and Vass, E., 1993.Melanogenesis and generation of cytotoxic molecules during insect cellular immune reactions. *Pigm. Cell. Res.*, 6: 117-126.

Narain, A. S. and Srivastava, P. N., 1979. Pollution-related changes in blood cell counts and blood cell clumping of the freshwater crab, *Paratelphusa spinigera. Ind. J. Exp. Biol.,* 17: 971–973.

Newman, S. G., 1996. Non–specific immune stimulants to prevent shrimp disease. *Fisheries world*, April, p. 4-8.

Newman, M. C., and Feng, S. Y., 1982. Susceptibility and resistance of the rock crab *Cancer irroratus* to natural and experimental bacterial infection. *J. Invert. Pathol.,* 40: 75-88.

Noga, E. J., Arroll, T. A. and Fan, Z., 1994. Specificity and some physiochemical characteristics of antibacterial activity from blue crab *Callinectes sapidus. Fish. Shellfish. Immunol.*, 6: 403-412.

Noga, E J., 2000. Hemolymph biomarkers of crustacean health. In: Recent Advances in Marine Biotechnology, Vol. 5 Immunobiology and Pathology (eds. M. Fingerman and R. Nagabhushanam). Science Publishers Inc., New Hampshire, pp. 125-163.

Norton, J. H., Levy, N. and Field, K., 1999. A preliminary evaluation of 3 haemolymph tests to assess health status in tropical rock lobsters *Panulirus ornatus. In*: Proceedings International symposium on lobster health management, Aldelaide., pp. 116-120.

Nyhlen, L., and Unestam, T., 1980. Wound reactions and *Aphanomyces astaci* growth in crayfish cuticle. *J. Invert. Pathol.,* 36: 187-97.

Owens, L. and O'Neill, A., 1997. Use of clinical cell flow cytometer differential count of prawn *Penaeus monodon* haemocytes. *Diseases of Aquatic Organisms*, 31: 147-153.

Paterson, B. D. and Spanoghe, P. T., 1997. Stress indicators in marine decapod crustaceans, with particular reference to the grading of western lobsters (*Panulirus Cygnus*) during commercial handling. *Mar. freshwater .Res.*, 48: 829-834.

Paterson, B. D., Davidson, G. W. and Spanoghe, P. T., 1999. Identifying stress when western rock lobsters are stored out of water: the average and individual blood lactate concentration. *In*: Proceedings International symposium on lobster health management, Aldelaide., pp. 35-41.

Paterson, W. D. and Stewart, J. E., 1974. In *vitro* phagocytosis by haemocytes of American lobster (*Homarus americanus*). *J. Fish. Res .Bd. Can.*, 31:1051-1056.

Paterson, W. D. Stewart, J. E. and Zwicker, B. M., 1976. Phagocytosis as a cellular immune response mechanism in American lobster *Homarus americanus. J. Invert. Pathol.,* 27: 95-104.

Pauley, G. B., 1974. An attempt to immunize the blue crab, *Callinectus sapidus*, with vertebrate red blood cells. *Experientia.*, 29: 210-211.

Perazzolo, L. and Barracco, M., 1997. The phenoloxidase activating system of shrimp *Penaeus paulensis* and associated factors. *Dev. Comp. Immunol.*, 21: 385-395.

Persson, M., Vey, A. and Söderhäll, K. ., 1987a. Encapsulation of foreign particles in vitro by separated blood cells from crayfish, *Astacus leptodactylus. Cell. Tiss. Res.,* 247: 409–415.

Persson, M., Cerenius, L. and Söderhäll, K., 1987b. The influence of haemocyte number on the resistance of the freshwater crayfish, *Pacifastacus leniusculus*, to the parasite *Aphanomyces astaci. J. Fish Dis.,* 10: 471-477.

Phianphak, W., Rengpipat, S., Titivorakul, S. P., Menasveta, P. 1999. Probiotic use of lacto bacillus species for black tiger shrimp. *J. Sci .Res. Chula. Univ.* 24: 41-51.

Pickering, A. D. and Pottinger, T. G., 1989. Stress responses and disease resistance in salmonid fish: effects of chronic elevation of plasma cortisol. *Fish Physiol. Biochem.,* 7: 253-258.

Poinar, G.O. and Leutenegger, R., 1971. Ultrastructural investigations of the melanization process in *Culex pipiens* (Culicidae) in response to a nematode. *Journal of Ultrastructural Research*, 36:149-158.

Preston, J. W. and Taylor, R. L., 1970. Observations in Phenoloxidase system in the haemolymph of cockroach *Leucophaea maderae. J. Insect. Physiol.*, 16: 1929-1970.

Raa, J., Roerstad, G., Engstad, R. and Robertson, B., 1992. The use of immunostimulants to increase resistance of aquatic organisms to microbial infection. *In*: Disease in Asian aquaculture. (ed. Shariff, R., Subasinghe, P. and Arther, J. R.). Fish Health section, Asian Fisheries Society Manila, Philippines., pp. 39-50.

Rabin, H., 1970. Haemocytes, hemolymph and defense reactions in crustaceans. *J. Recticuloendothel. Soc.*, 7: 195-207.

Radhakrishman, E. V., 1989. Physiological and biochemical studies on the spiny lobster, *Panulirus homarus*. Ph.D Thesis, University of Madras.

Radhakrishnan, E. V. and Vijayakumaran, M., 1984. Effect of eyestalk ablation in spiny lobster *Panulirus homarus* (Linnaeus). 1. on moulting and growth. *Ind. J. Fish.,* 31, (1): 130-147.

Rao, A. V. P., Panchayuthapani, D., Murthy, A. and Ajithkumar, B. S., 1996. Resistance to diseases in tiger shrimp, *Penaeus monodon* through incorporation of glucan in feed. *Fish. Chimes*, 16(1):41-42.

Ratanapo, S. and Chulavatnatol, M., 1990. Monodin, a new sialic acid-specific lectin from black tiger prawn (*Penaeus monodon*). *Comp. Biochem. Physiol.*, 97B: 515–520.

Ratcliffe, N. A. and Rowley, A. R., 1979. A comparative synopsis of the structure and function of the blood cells of insects and other invertebrates. *Dev. Comp. Immunol.*, 3:189-194.

Ratcliffe, N. A., Rowley, A. F., Fitzgerald, S. W. and Rodes, C. P., 1985. Invertebrate immunity: basic concepts and recent advances. *Internat. Rev. Cytol.*, 97: 183– 350.

Ratner, S., and Vinson, S.B., 1983.Phagocytosis and encapsulation: Cellular immune responses in Arthropoda. *Am. Zool.* 23:95-104.

Ravindranath, M. H., 1975. Effects of temperature on the morphology of haemocytes and coagulation process in the mole crab Emerita.=*Hippa asiatica. Biol .Bull.*, 748:286-302.

Ravindranath, M.H., Higa, H. H., Cooper, E. L. and Paulson, J. C., 1985. Purification and characterization of an O-acetylsialic acid-specific lectin from a marine crab *Cancer antennarius. J. Biol. Chem.*, 260: 8850-8856.

Reade, P., 1968. Phagocytosis in invertebrates. *Aus. Jour. of Exp. Biol. Med. Sci.*, 46: 219-229.

Regnault, M., 1987. Nitrogen excretion in marine and fresh-water Crustacea. *Biol. Rev.*, 62: 1-24.

Rengipat, S., Rukpratanporn, S., Piyatiratitivorakul, S., Menasaveta, P., 2000. Immunity enhancement in black tiger shrimp (*Penaeus monodon*) by a probiont bacterium (Bacillus SII). *Aquaculture*, 191: 271-288.

Renwrantz, L. and Stahmer, A., 1983. Opsonizing properties of an isolated haemolymph agglutinin and demonstration of lectin-like recognition molecules at the surface of haemocytes from *Mytilus edulis. J. Comp. Physiol.*, 149B: 535–546.

Renwrantz, L., 1986. Lectins in molluscs and arthropods: their occurance, orgin and role in immunity. *In*: Immune mechanisms in invertebrate Vectors. (ed. Lackie, A.M.). Symposium Zoological Society, Oxford Clarendon Press, London, pp. 81-93.

Roch, P., 1999. Defense mechanisms and disease prevention in farmed marine invertebrates. *Aquaculture*, 172: 125-146.

Roche, J., and Latreille, M., 1934. Sur les phosphatases su sang et de l'hemolymphic. *Compt. Rend. Soc. Biol.*, 116: 1033-1034.

Sagrista, E. and Durfort., 1990. Ultrastructural study of haemocytes associated with haemolymph vessels of haepatopancreas of *Paleamonetes zariquieyi* (Crustacea, Decapoda). *J. Morph.,* 206: 173-180.

Sahoo, P. K. and Mukerjee, S. C., 1999. Influence of immunostimulants, chitosan on immuneresponse of healthy and cortisol-treated rohu (*Labeo rohita*).*J. Aqua. Trop.*, 14(3): 209-215.

Sakai, M., Kamiya, H., Ishii, S., Atsuto, S. and Kobayashi, M., 1992. The immuno-stimulanting effect of rainbow trout, *Onchorynchus mykiss. In:* Diseases in Asian Aquaculture (ed. Shariff, M., Sabarighe, R. P. and Arthur, J. R.). 1: 413-417.

Schapiro, H. C., 1975. Immunity in decapod crustaceans, *Amer. Zool.,* 15: 13-20.

Scott, M. T., 1971. A naturally occurring heamagglutinin in the haemolymph of the American cockroach. *Arch. Zool. Exp. Gen.*, 112: 73-80.

Segal, A., 1984. How do phagocytic cells kill bacteria? *Med. Biol.*, 62: 81-84.

Sequiera, T., Tavares, D. and Aralachaves, M., 1996. Evidence for circulation hemocyte proliferation in the shrimp *Penaeus japonicus. Dev. Comp. Immunol.,* 20:97-104.

Sindermann, C.J., 1971. Internal defenses of Crustacea: A review. *Fish. Bull.,* 69: 455-489.

Siwickii, A. K., 1989. Immunostimulanting influence of levamisole on non-specific immunity in carp (*Cyprinus carpio*). *Dev. Comp. Immunol.*, 13: 87-91.

Siwicki, A. K., Anderson, D. P. and Dixon, D. W., 1990. *In vitro* immunostimulation of rainbow trout (*Onchorhynchus mykis*) spleen with levamisole. *Dev. Comp. Immunol.*, 14: 231-237.

Smith V. J and. Ratcliffe, N. A., 1978. Host defense reactions of shore crab, *Carcinus maenas* (L), *in vitro. J. Mar. Biol. Asso. UK.*, 58: 367-379.

Smith, V. J. and Ratcliffe, N. A., 1980. Host defence reactions of the shore crab, *Carcinus maenas* (L.); clearance and distribution of injected test particles. *J. Mar. Biol. Ass. U. K.*, 60A: 89–102.

Smith, V. J. and Ratcliffe, N. A., 1980b. Cellular defense reactions of the shore crab, *Carcinus maenas*: in vivo hemocytic and histopathological response to injected bacteria. *Invert. Pathol.*, 35A: 65-74.

Smith, V. J. and Söderhäll, K., 1983a. β -1, 3-glucan activation of crustacean haemocytes *in vitro* and *in vivo. Biol. Bull.*, 164: 299-314.

Smith, V. J. and Söderhäll, K., 1983b. Induction of degranulation and lysis of haemocytes in the fresh water crayfish *Astacus astacus* by components of phenoloxidase activating system in vitro. *Cell and Tissue Research.*, 233: 295-303.

Smith, V. J., Söderhäll, K. and Hamilton, M., 1984. β-1, 3-glucan induced cellular defence reaction in the shore crab, *Carcinus maenas*. *Comp. Biochem. Physiol.*, 77A: 635–639.

Smith, V.J., Söderhäll, K., 1991. A comparison of phenol oxidase activity in the blood of marine invertebrates. *Dev. Comp. Immunol.*, 15(4): 251-261.

Smith, V. J. and Chisholm, J. R. S., 1992. Non – Cellular immunity in Crustaceans. *Fish. Shellfish Immunol.*, 2:1-31.

Smith, V. J. and Johnston, P. A., 1992. Differential haemotoxic effect of PCB congeners in the common shrimp *Crangon crangon*. *Comp. Biochem. Physiol.*, 101C: 641–649.

Smith, V. J., Swindlehurst, R. J., Johnston, P. A. and Vethaak, A. D., 1995. Disturbances of the host defense capability in the common shrimp *Crangon crangon*, by exposure to harbor dredge spoils. *Aquat. Toxiol.*, 32: 43-58.

Söderhäll, K. and Unestam, T., 1979. Activation of crayfish serum phenoloxidase: the specificity of cell wall glucan activation and activation by purified fungal glycoprotein. *Canad. J. Microbiol.*, 25: 404–416.

Söderhäll, K., Hall, L., Unestam, T. and Nyhlen, I., 1979. Attachment of phenol oxidase to fungal cell walls in arthropod immunity. *J. Invert. Pathol.*, 34: 285-294

Söderhall, K., 1981. Fungal cell wall β -1, 3 –glucans induce clotting and phenoloxidase attachment to foreign surfaces of crayfish hemolysate. *Dev. Comp. Immunol.*, 5: 565-573.

Söderhäll, K., 1982. Prophenoloxidase activating system and mealnisation- a recognition mechanism of arthropods? A review. *Dev. Comp. Immunol.*, 6: 601-611.

Söderhäll, K. and Ajaxon, R. ., 1982. Effects of quinones and melanin on mycelial growth of *Aphanomyces* spp. and extracellular protease of *Aphanomyces astaci,* a parasite on crayfish. *J. Invert. Pathol.*, 39: 105-9.

Söderhäll, K., 1983. β -1, 3-glucan enhancement of protease activity in crayfish haemocytes lysate. *Comp. Biochem. Physiol.* B 74:221-224.

Söderhäll, K. and Smith, V. J., 1983. Separation of the haemocyte populations of *Carcinus maenas* and other marine decapods, and prophenoloxidase distribution. *Dev. Comp. Immunol.*, 7: 229–239.

Söderhäll, K., Vey, A. and Ramstedt, M., 1984. Haemocyte lysate enhancement of fungal spore encapsulation by crayfish haemocytes. *Dev. Comp. Immunol.*, 8: 23-29.

Söderhäll, K. and Hall, L., 1984. Lipopolysaccharide induced activation of prophenoloxidase activating system in crayfish haemocyte lysate. *Biochem. Biophys. Acta.,* 797:99-104.

Söderhäll, K., and Smith, V.J., 1986. The prophenoloxidase activating system: the biochemistry of its activation and role in arthropod cellular immunity with special reference to crustaceans. *In:* Immunity in Invertebrates (ed. M. Brehelin). Springer-Verlag, Berlin, pp. 208-223.

Söderhäll, K., Johansson, M. W. and Smith, V. J., 1988. Internal defence mechanism. *In:* Freshwater crayfish: biology, management and exploitation (ed. D. M. Holdich and R. S. Lowery). Croom Helm Ltd., London, pp. 213–235

Söderhäll, K, Aspan, A. and Duvic, B., 1990. Propo system and associated proteins: role in cellular communication in arthropods. *Res. Immunol.*, 141: 896-901.

Söderhäll, K. and Cerenius, L., 1992. Crustacean immunity. *Ann. Rev. Fish Dis.,* 2: 3-23.

Söderhäll, K., Aspan, A. and Duvic, B., 1994. The Prophenoloxidase activating system and its role in invertebrate defense. *Ann. N. Y.Acad. Sci.*, 712: 155-161.

Söderhäll, K., Cerenius, L., and Johansson, M. W., 1996. The prophenoloxidase activating system in Invertebrate Immunology. *In*: Invertebrates in New Directions (ed. K. Söderhäll, S. Iwanaga, and G.R. Vasta). SOS Publications, Fair Haven, pp. 229-253.

Söderhäll, K. and Cerenius, L., 1998. Role of Phenoloxidase – activating system in invertebrate immunity. *Curr. Opin. Immunol.*, 10:23-28.

Song, Y. L and Sung, H. H., 1993. Vibriosis resistance in tiger shrimp (*Penaeus monodon*) induced by M-glucans treatment. *Oostende Belgium European Aquaculture society.*No.19 :97.

Song, Y.L. and Hsieh, Y.-T., 1994. Immunostimulation of tiger shrimp *Penaeus monodon* hemocytes for generation of microbicidal substances: analysis of reactive oxygen species. *Dev. Comp. Immunol.,* 18: 201–209.

Spices, J. I., Hill, A. D., Taylor, A. C. and Strang, R. H. C., 1990. Effect of aerial exposure on concentrations of selected metabolites in blood of Norwegian lobster *Nephrops norvegius* (Crustacea: nephropidae). *Mar. Biol.*, 105:129-135.

Sritunyalucksana, K., Sithisarn, P., Withayachumnarnkul, B. and Flegel, T.W., 1999. Activation of phenoloxidase, agglutinin and antibacterial activity in haemolymph of the black tiger prawn, *Penaeus monodon*, by immuno stimulants. *Fish. Shellfish. Immunol.*, 9: 21-30.

Steenbergen, F. J., Steenbergen, S. M. and Schapiro, H.C., 1978. Effects of temperature on phagocytosis of *Homarus americanus*. *Aquaculture*, 14: 23-40

Stewart, J. E., Cornick, J. W., and Dingle, J. R., 1967. An electronic method for counting lobster, *Homarus americanus* Milne Edwards. hemocytes and the influence of diet on hemocyte numbers and hemolymph proteins. *Can. J. Zool.*, 45: 291-304.

Stewat, J. E., Arie, B., Zwicker, B. M. and Dingle, J.D., 1969. Gaffkemia, a bacterial disease of the lobster, *Homarus americanus*: effects of pathogen, *Gaffkya homari,* on the physiology of the host. *Can. J. Microbiol.*, 15: 925-932.

Stewart, J. E. and Zwicker, B. M., 1972. Natural and induced bactericidal activities of the lobster *Homarus americanus*: products of haemocyte plasma interaction. *Can. J. Microbiol.*, 18: 1499-1509.

Strenshein, D. J. and Burton, P. R., 1980. Light and electron microscopic studies of crayfish hemocytes. *J. Morph.*, 165: 67-83.

Sugumaran, M., Kanost, M., 1993. Regulation of insect haemolymph Phenoloxidase. *In*: Parasites and pathogens of insects (ed. Beckage, N. H., Thompson, S. M. and Federici, B. A.). Academic press, San Diego, pp. 317-342.

Sugumaran, M., 1996. Roles of insect cuticle in host defense reactions. *In*: New directions in Invertebrate immunity (ed. Söderhäll, K., Iwanaga, S. and Vasta, G. R.). SOS Publications, Fair Haven, pp. 355-374.

Sung, H. H., Song Y.I. and Kou, G. H., 1991. Potential use of bacterin to prevent shrimp vibriosis. *Fish Shellfish Immunol.*, 1: 311-312.

Sung, H. H., Kou, G. H. and Song, Y. L., 1994. Vibriosis resistance induced by glucan treatment in tiger shrimp (*Penaeus monodon*). *Fish Pathol.*, 29: 11–17.

Sung, H. H. and Song, Y. L., 1996. Tissue location of *Vibrio* antigen delivered by immersion to tiger shrimp (*Penaeus monodon*). *Aquaculture*, 145: 41–54.

Sung, H. H., Yang, Y. L. and Song, Y. L., 1996. Enhancement of microbicidal activity in tiger shrimp plasma (*Penaeus monodon*) via immunostimulation. *J. Crust. Biol.*, 16: 279–285.

Sung, H. H., Chang, H. J., Her, C.H., Chang, J. C. and Song, Y. L., 1998. Phenoloxidase activity of haemocytes derived from *Penaeus monodon* and *Macrobrachium rosenbergii*. *J. Invert. Pathol.*, 71: 26-33.

Sung, H. H. and Song, R., 1999. Intrahaemocytic activity of lysosomal enzymes in *Penaeus monodon* and *Macrobrachiun rosenbergii*. *Fish. Shellfish. Immunol.*, 9: 505-508.

Sung, H. H., Hwang, S. F. and Tsai, F. M., 2000. Responses of Giant freshwater prawn (*Macrobrachium rosenbergii*) to challenge by two strains of *Aeromonas* spp. *J. Invert. Pathol.*, 76:278-284.

Thompson, I., White, A., Fletcher, T. C., Houlihan, D. F. and Secombe, C. J., 1993. The effect of stress on the immune response of Atlantic salmon (*Salmo salar L.*) fed different diets containing different amounts of vitamin C. *Aquaculture,* 114: 1-8.

Thronqvist, P. O., Johansson, M. W. and Söderhäll, K., 1994. Opsonic activity of cell adhesion proteins and β -1, 3-glucan binding proteins from two crustaceans. *Dev. Comp. Immunol.*, 18:3-12.

Truscott, R. and White, K, N., 1990. The influence of metal and temperature stress on the immune system of crabs. *Fuct. Ecol.,* 4: 455-461.

Tsai, I. H., Chen, Y. L. and Lu, P. J., 1991. Relationship between hemolymph-coagulation and disease in shrimp: (1) Coagulation rate *in vitro* and the hemolymph proteins of various shrimp species. *In*: COA Fisheries Series No. 29. Reports on Fish Disease Research XI (ed, G. H. Kou). Council of Agriculture ROC, Taipei, pp. 22–30.

Tsing, A., Arcier, J. M. and Bréhélin, M., 1989. Haemocytes of penaeids and palaemonid shrimps: morphology, cytochemistry and hemograms. *J. Invert. Pathol.,* 53: 64–77.

Tsvetnenko, E., Brown, J., Glencross, B. D. and Evans, L. H., 1999. Measures of condition in dietary studies on western rock lobster post-pueruli. *In:* Proceedings, International Symposium on Lobster Health Management, Adelaide, September, pp: 100-110.

Tyson, C. J. and Jenkin, C. R., 1973. The importance of opsonic factors in the removal of bacteria from the circulation of the cray fish. *Aus. J. Exp. Biol. Med. Sci.*, 51:609-650.

Tyson, C. J. and Jenkin, C. R., 1974. Phagocytosis of bacteria in vivo by haemocytes from the cray fish . *Aus. J. Exp. Biol. Med. Sci.*, 52: 341-348.

Ueda, R., Sugita, H. and Deguchi, Y., 1990. The serum bactericidal activity: a possible indicator as crustacean health condition. *In*: Proc. South. Pacific. Congr. Mar. Sci. Technol., Tokyo, pp. 333-340.

Ueda, R., Sugita, H. and Deguchi, Y., 1994. Bactericidal activities of the hemolymph of the Japanese spiny lobster, *Panulirus japonicus,* Decapoda, palinuridae. *Crustaceana.,* 67(2): 256-258.

Ueda, R., Sugita, H. and Deguchi, Y., 1999. Effect of transportation on the serum antibactericidal activity of *Penaeus japonicus* and *Ovalipes punctatus. Aquaculture,* 171: 221-225.

Unestam, T. and Nylund, J. E. ., 1972. Blood reactions in vitro in crayfish against a fungal parasite *Aphanomyces astaci. J. invert. Pathol.,* 19: 94-106.

Unestam, T., 1973. Fungal diseases of Crustacea. *Rev. Med. Vet. Mycology.,* 8: 1-20.

Unestam, T., and Söderhäll, K., 1977. Soluble fragments from fungal cell walls elicit defence reactions in crayfish. *Nature,* 267: 45–46.

Vargas-Albores, F., Guzma´n-Murillo, M.A. and Ochoa, J. L. 1992. Size dependent haemagglutinating activity in the haemolymph from sub-adult blue shrimp (*Penaeus stylirostris* Stimpson). *Comp. Biochem. Physiol.* 103A: 487–491.

Vargas-Albores, F., Guzmán, M. A. and Ochoa, J. L., 1993. A lipopolysaccharide-binding agglutinin isolated from brown shrimp., *Penaeus californiensis* Holmes, haemolymph. *Comp. Biochem. Physiol.,* 104B: 407–413.

Vargas-Albores, F., Baltazar, P. H., Clark, G. D and Barajas, F. M., 1998. Influence of temperature and salinity on the yellow legs shrimp, *Penaeus californiensis* Holmes, prophenoloxidase system. *Aquacult. Res.*, 29,549-553.

Varges-Albores, F., Yepiz_placencia, G., 2000. Beta glucan binding protein and its role in shrimp immune response. *Aquaculture*, 191:13-21.

Victor, B., Narayanan, M. and Jones Nelson, D., 1990. Gill pathology and haemocytes response in mercury exposed *Macrobrachium idae* (Heller). *J. Environ. Biol.*, 11: 61-65.

Wardlaw, A .G. and Unkles, S. E., 1978. Bactericidal activity of coelomic fluid from the sea urchin, *Echinus esculentis. J. Invert. Pathol.*, 32: 25-34.

Watanabe, K., 1999. The degranulation of eosinophils by cell lysis. *Asia/Pacific Microscopy and Analysis.*, 10: 9-10.

White, K. N. and Ratcliffe, N. A., 1982.The segregation and elimination of radio and flouresent-labelled marine bacteria from the haemolymph of shore crab, *Carcinus maenas. J. Mar. Biol. Assoc. U.K.*, 62: 819-833.

White, K. N., Ratcliffe, N. A. and Rossa, M., 1985. The antibacterial activity of haemocytes clumps in the gills of shore crab, *Carcinus maenas*. *J. Mar. Biol. Asso. U. K.*, 65: 857-870.

Williams, A. J. and Lutz, P. L., 1975. Blood cell types in *Carcinus maenas* and their physiological role. *J. Mar. Biol. Ass. U.K.*, 55: 671-674.

Yano, T., Magindaan, R. E. P. and Matsuyama, H., 1989. Enhancement of resistance to carp (*Cyprinus carpio*) to experimental *Edwardsiella tarda* infection by some β -1, 3-glucans. *Nipp. Sui. Gakk.*, 55: 1815-1819.

Yeager, J. F. and Munson, S. E., 1950. Blood volume of the roach, determined by several methods. *Arthropoda* .,1:255-265.

Yoshida, H. and Ashida, M., 1986. Microbial activation of two serine enzyme and prophenoloxidase in plasma fraction of haemolymph of silkworm, *Bombyx mori. Insect. Biochem.*, 16: 539-545.