Physiological Studies on Stress and Morbidity during Post-harvest Handling and Storage of Western Rock Lobster *Panulirus cygnus*. II. Standard Autopsy Techniques and Immune System Competency

Professor Louis H. Evans

Project No. 1996/344

Physiological Studies on Stress and Morbidity During Post-harvest Handling and Storage of Western Rock Lobster *Panulirus cygnus*. II. Standard Autopsy Techniques and Immune System Competency

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OBJECTIVES:

1. To identify suitable immune system parameters which can be used to evaluate stress responses and health status in captive lobsters and to apply those parameters in a study of stress induced by post harvest handling procedures.
2. To investigate the causes of mortality in captive lobsters held in processing factories. This study will focus on bacteriological and histopathological examinations and result in the development of a standard protocol for autopsy of lobsters.
3. To evaluate the influence of air exposure with and without physical handling on immune parameters.
4. To evaluate the efficacy of immune parameters in predicting survival of lobsters held under a range of simulated truck transport and live shipment conditions.

NON TECHNICAL SUMMARY:

OUTCOMES ACHIEVED:

A suite of immune system laboratory assays for use in health and stress assessment in spiny lobsters was developed and standardized. The majority of these tests are simple to perform and could be carried out by trained factory staff. Normal values for individual parameters in postharvest *Panulirus cygnus* and deviations from normal in moribund lobsters were determined. A standard protocol for the autospy of lobsters was also developed. These assays and the autopsy protocol will be of use in the investigations of disease conditions in *P. cygnus* and other spiny lobster species and in optimising postharvest practices in lobster fisheries.

Investigations of causes of mortality in postharvest lobsters showed that lobsters that became moribund during storage and were removed from factory tanks (reject lobsters) often had bacterial infections. The source of the bacterial infections is unknown but was not related to a pre-existing condition in the lobsters prior to capture. The infections most likely resulted from physical handling, wounding and exposure to environmental extremes during or after capture. Characteristic changes in the levels of immune parameters were shown to occur in reject lobsters, suggesting that these assays can be used to provide a quantitative measure of health status in lobsters.

Lobsters were shown to exhibit significant changes in immune parameter levels following exposure to behavioural stressors such as visual and/or physical disturbance as well as to environmental stressors such as air exposure with or without water sprays. The observations provided a physiological basis to the well recognized importance of minimizing handling stress and air exposure in lobster postharvest practices. The relative impact on lobster health and survival of different types of capture and postharvest stressors remains to be determined.

Need for the research

The western rock lobster industry of Western Australia is the largest single species fishery in Australia. Lobsters are marketed as live product, whole cooked lobsters, whole frozen lobsters or frozen tails. Lobster processors aim to maximize the proportion of the catch that is 'fit for live' so as to have flexibility in their marketing operations. Whether or not a postharvest lobster is fit for live export is dependent on their physiological condition. The physiological condition of a posthavest lobster is primarily determined by acute stress responses to environmental and behavioural stressors during capture and postharvest processing. Excessive stress leads to poor health and poor survival during storage and live export. The overall aim of this project was to develop a suite of laboratory tests that could evaluate the health and acute stress status of lobsters and to use these tests to determine the influence of different handling procedures on lobster health. The project received strong support from processing companies and fishers wishing to improve their handling and holding methods so as to reduce postharvest stress.

Project aims

The project had two major aims – to develop and use the suite of laboratory tests in studies of different postharvest stressors (behavioural stressors such as physical handling or visual disturbance and environmental stressors such as air exposure) and to develop a standard autopsy method and apply this technique to a study of the causes of mortality in postharvest lobsters.

Development and evaluation of immune parameter tests

The immune system indicators investigated included total hemocyte (blood cell) counts (THC), differential hemocyte counts (measures of the different types of blood cells; DHC), clotting time (the time taken for hemolymph to clot), antibacterial activity (antibacterial activity in lobster hemolymph), phagocytic capacity (the ability of hemocytes to sequester and destroy bacteria and other foreign agents), bacterial colony count (the number of live bacteria in the hemolymph), %prevalence bacteremia (the proportion of lobsters with bacteria in their hemolymph) and quantitative histopathological measurements (quantification of occurrence of specific types of histological features seen in fixed sections of lobster tissues). Standardised procedures were developed and normal ranges established for six of the parameters. All but one parameter (phagocytic capacity) were analysed in both 'accepted' (healthy lobsters that were graded fit for live export) and 'reject' lobsters (unhealthy lobsters; not fit for live export) and the variation from normal values which occurs in reject lobsters evaluated. Significant findings of the study were:

- Characteristic changes in the level of some immune parameters (THC, %granular cells, clotting time, prevalence of bacteremia) occurred when lobsters became weakened through poor postharvest handling practices. This finding means that measurement of an appropriate suite of immune parameters in a batch of lobsters could provide a quantitative measure of the health of the lobsters.
- Behavioural stressors such as physical handling, visual disturbance and, possibly, noise and light exposure, caused reproducible changes in the levels of two parameters (THC and clotting time) while environmental stressors such as air exposure or wounding also resulted in changes in several parameters. These findings suggest that measurement of an appropriate suite of immune parameters can provide a measure of acute stress status of a batch of lobsters.
- Simulated truck and live shipment trials showed that a selected suite of immune parameters could accurately differentiate between holding conditions that resulted in high lobster survival (submerged storage) and those that resulted in high mortality (held in air with or without spray treatments at ambient temperatures). This finding means that these tests can be used to evaluate the efficacy of different postharvest practices.
- In the same factory trials, the immune parameters successfully predicted the outcome (classification of lobsters into those that would survive the simulated truck transport and live shipment and those that would not) in 66% of lobsters (trial 1) and 79% of lobsters (trial 2). Addition of the physiological parameters (measured by an affiliated research team) to the analysis improved the discrimination to 77% (trial 1) and 81% (trial 2).
- Variations occurred in some of the parameters with the moult stage. Seasonal and/or diurnal variations may also occur but these were not studied. No differences in the parameters have been observed with the sex of the lobster but sex differences in each of the parameters, if any, have still to be fully evaluated. This means that normal values with respect to moult stage, time of year, time of day and lobster sex will have to be determined if these parameters are to be used by processing factories.

Development of autopsy procedure and studies on causes of postharvest lobster mortality

A standard protocol for autopsy of lobsters was developed and documented. Autopsies were conducted on 135 lobsters, 49 reject lobsters and 86 accepted lobsters. Only a small proportion of lobsters exhibited pre-existing disease conditions (mainly parasite infections) thus eliminating pre-existing disease conditions as a main cause of postharvest mortality. Wounding as a cause of mortality was not investigated apart from recording the occurrence of missing appendages and exoskeleton lesions in accepted and reject lobsters at autopsy.

The contribution of bacterial infection as a cause of mortality was investigated by both bacteriological and histopathological investigations. No evidence was obtained to suggest that the postharvest lobsters were dying of an infection by a highly pathogenic bacterial species or strain. Seventeen to ninety percent of the reject lobsters had bacteria in their hemolymph and a high proportion of these lobsters had inflammatory reactions in their tissues. The level of bacteremia and inflammatory lesions in accepted lobsters was low. These observations suggest that opportunistic

bacterial infections could play a significant role in postharvest mortality in those lobsters that have been weakened due to prior stressor exposure. Further studies on the relationship between bacteremia and tissue injury in postharvest lobsters should be conducted.

Lobsters dying in the simulated truck transport and live shipment trials were found to have a marked inflammatory reaction in the bladder and antennal glands. The cause of the inflammation was not determined. Similar histopathological lesions were observed in postharvest *Jasus edwardsii* following live transport and storage in air (see Final Report, FRDC 98/304), suggesting that this inflammatory reaction may be a contributing factor to mortality during live export. Further studies should be conducted on the prevalence and cause of this condition in postharvest lobsters.

Approximately half of the reject lobsters had no histopathological evidence of tissue injury so the precise cause of mortality in this group was not identified. It is possible that these lobsters died from acute organ failure. A full analysis of biochemical as well as immunological parameters should resolve the question of the precise cause of mortality in this group of lobsters.

Acknowledgements

The advice, assistance and funding support from the Fisheries Research and Development Corporation (FRDC) and the critiques and review by colleagues from the FRDC Rock Lobster Post Harvest Sub-program is gratefully acknowledged. We also wish to thank the staff of lobster processing factories in Western Australia, in particular Geraldton Fishermen's Cooperative Pty Ltd, Kailis Bros Pty Ltd and Lobster Australia Limited, for their advice and support.

CHAPTER 2 GENERAL INTRODUCTION

1. Background

1.1 Description of rock lobster post harvest handling procedures

The western rock lobster industry of Western Australia is the largest single species fishery in Australia, with landings valued at $209 million in 1997/98 (McKoy and Sen 1999). Lobsters are caught in baited pots set at depths of approx 10–150 m. The pots are hauled onto a bench on the deck of the fishing boat using a mechanical winch. All lobsters in the pot, comprising legal and undersized animals, are removed from the pot immediately after it is winched onboard and placed in a box for sizing. Legal sized lobsters (carapace length exceeding 73-75mm) are placed into above or below deck holding tanks and undersized animals are returned to the water.

The holding tanks onboard fishing boats are fitted with some form of flow-through water system. However, the sizes and shapes of the tanks and the location of the inlet and outlet pipes vary from boat to boat as does the size of the water pump. These differences result in a variation between boats in the water flow rates and patterns within the holding tanks which in turn affects water quality parameters such as dissolved oxygen. Variations in environmental conditions in the onboard holding tanks, combined with differences in individual handling procedures, results in lobsters being delivered to the wharf in a range of physiological conditions. Since the physiological condition of the lobsters upon arrival at the factory is the major determinant of whether the lobsters will be fit for live export, considerable effort is being exerted by processing factories to encourage fishers to improve their handling and holding methods so as to reduce post harvest stress.

Upon arrival at the wharf the crates of lobsters are either immediately delivered to the processing factory or transported to the factory in trucks. The period of time in which they are stored in the trucks varies from about 30 mins to many hours, depending of the relative locations of the wharf and the processing factory. Processing companies have developed a range of methods of storing the lobsters in trucks during transport including storage in air, under water or in some form of spray system. Temperatures are generally lowered during transport to minimise deterioration in health status associated with this procedure. Truck transport, or transport onboard carrier boats, a post harvest procedure in the Abrolhos lobster fishery, is generally recognised as a detrimental practice and lobsters arriving at the factory by these means tend to be weaker and of lower health status than those delivered directly off the fishing boats.

Immediately upon arrival at the factory the lobsters are graded. This procedure comprises the lobsters being placed on a conveyor belt from which they are removed by factory grading staff and either placed in live holding tanks (*accepted (healthy) lobsters*) or forwarded to the freezing or cooking sections of the factory. The grading procedure is based on visual observation of the number of missing legs or other wounds along with visual and tactile observations of muscle tone and general vigour.

Lobsters destined for live export are held in the factory tanks for periods from several days to two weeks before being shipped to market. During this time any lobsters showing signs of weakness or poor health are removed (*reject (unhealthy) lobsters*).

Live shipment involves immobilising the animals through a cold-stunning procedure and placement in a foam box between layers of a packaging material, usually pine wood chips. The foam containers are then delivered to the airport in a truck and air freighted to overseas markets.

During the 1997/98 Western Australian rock lobster season approximately 40% of harvested lobsters were marketed as live product, the remainder, being sold as whole cooked lobsters (45%), whole frozen lobsters or frozen tails (15%) (McKoy and Sen, 1999). Processing factories report high survival of live exports, with mortality rates of 1% to 4% generally being experienced (Phillips, 1994). On occasions, however, higher mortalities, often of unexplained origin, are observed.

1.2 Likely causes of morbidity and mortality in post harvest lobsters

Lobsters are exposed to a range of harmful and stressful events during capture and post harvest handling and storage (Table 1). Assuming that the animals are relatively healthy when entering the trap, the development of weakness or poor health (morbidity) and/or death (mortality) must result from physical damage occurring during the processes of capture and post-capture processing or from physiological responses to post harvest stressors. These adverse physiological reactions occur through exposure of lobsters to environmental stressors that either alarm the lobsters, initiating an acute stress response, or cause a marked alteration in a physiological process such as oxygen uptake or ion regulation.

Table 1 Stress factors in lobster post harvest handling

Factor	Examples
Handling Stress	Winching up in pot Removal from pot Transfer to factory Packaging operations
Hypoxia Stress	On boat handling Transport to factory and to markets Exposure to low oxygen levels in tanks
Temperature Stress	Exposure to variations in environmental temperature on boat and during transport Dip treatment prior to packaging
Behavioural Stress	Limb autotomy from a variety of stimuli Crowding and aggression
Toxicity Stress	Exposure to high environmental ammonia Exposure to other dissolved toxins (e.g. copper, excreta)
Salinity Stress	Exposure to high and low salinity environments

Four likely causes of morbidity and mortality in post harvest lobsters can be hypothesised:

- Cell injury and organ failure due to physiological disturbances - air exposure, rough handling and other stressors
- Opportunistic bacterial infections resulting from impaired immunity induced by above stressors
- Wounding - increased likelihood of bacterial infections
- Pre-existing disease conditions - weakens ability to resist stress

Stress reactions are likely to be a contributing factor to each of the above disease mechanisms.

Exposure to air, temperature extremes and physical processes leading to wounding or appendage loss are likely to be the three main predisposing causes of mortality in post harvest lobsters. These can lead to morbidity and mortality in lobsters by a number of different pathways (Fig. 1). The actual cause of death is likely to be failure of vital organ function through cell injury caused by irreversible physiological dysfunction, intracellular acidosis or opportunistic bacterial infections arising from either physical injury or from an impairment in the lobster's host defense responses (La Via and Hill, 1975; Wood et al., 1983; Wedemeyer (1997); McDonald and Milligan, 1997). Stress responses induced by exposure to environmental stressors during capture and handling can lead to death through either of these pathways and are undoubtedly an important factor in the development of weakness or poor health in post harvest lobsters.

Figure 1 **Pathogenesis of rock lobster post harvest mortality**

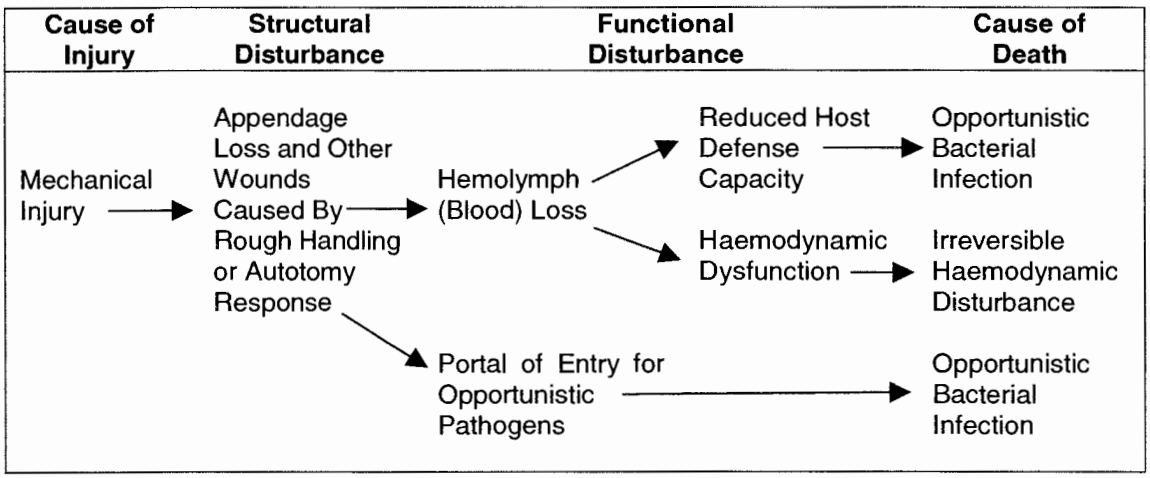


Figure 1(a) Mechanical injury

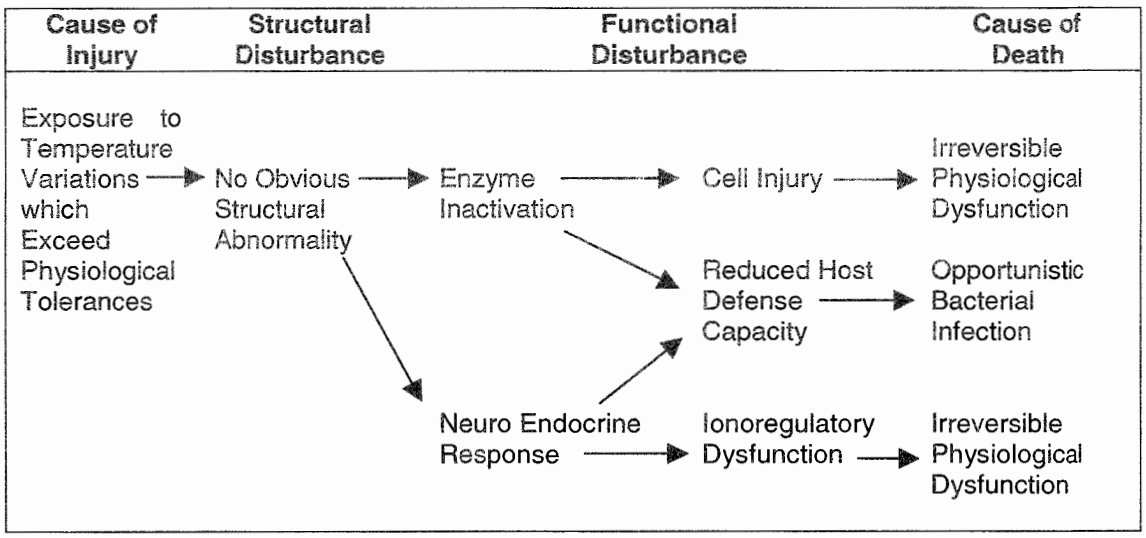


Figure 1(b) Exposure to temperature variation which exceed physiological tolerances.

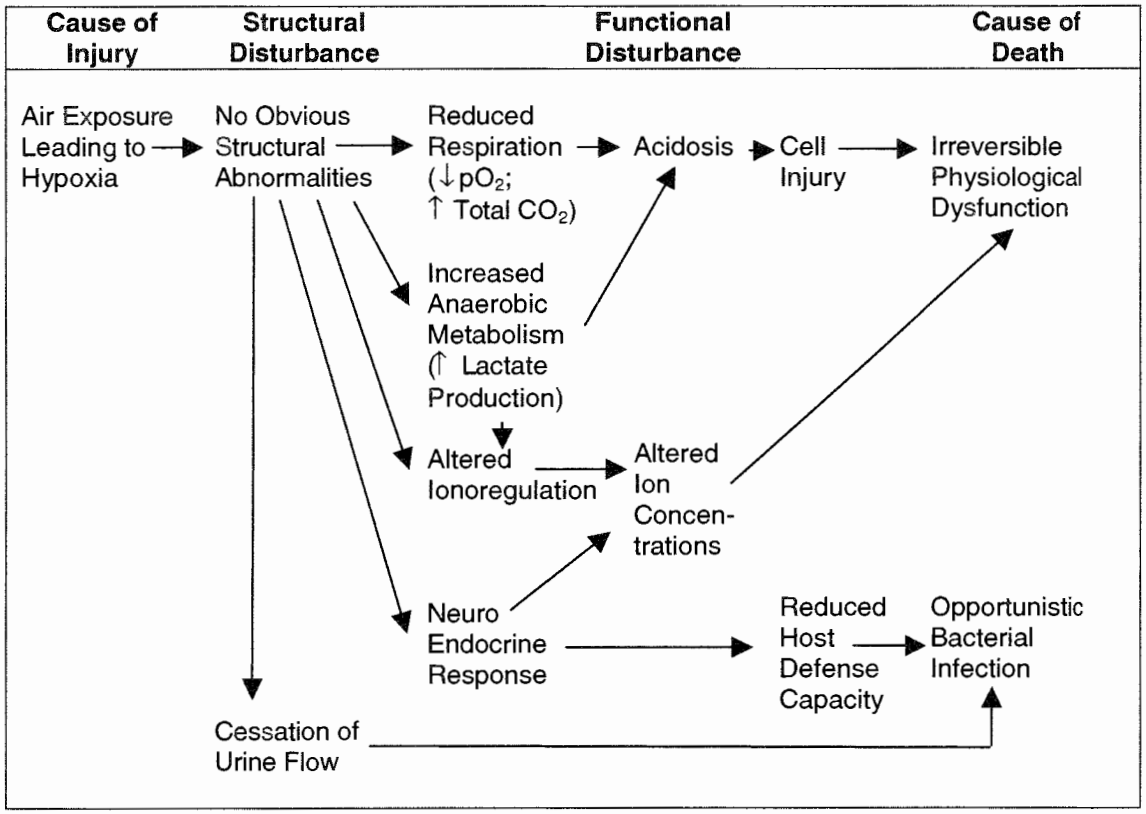


Figure 1(c) Air exposure leading to hypoxia

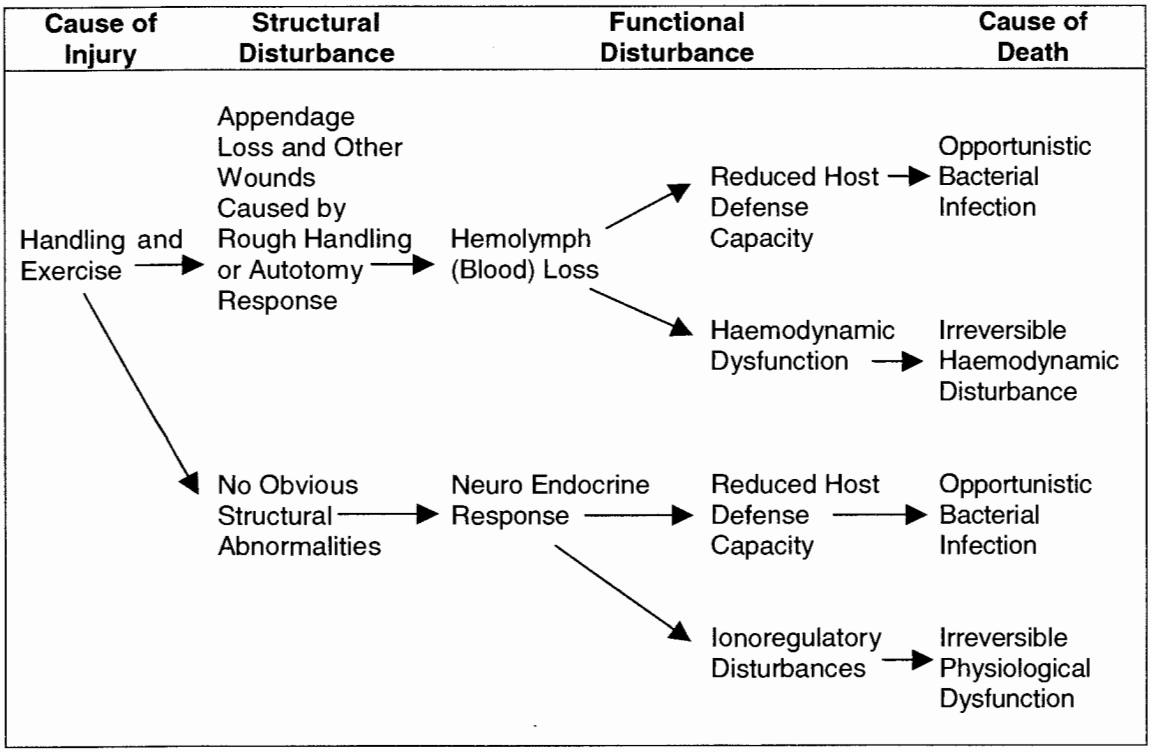


Figure 1(d) Handling and exercise

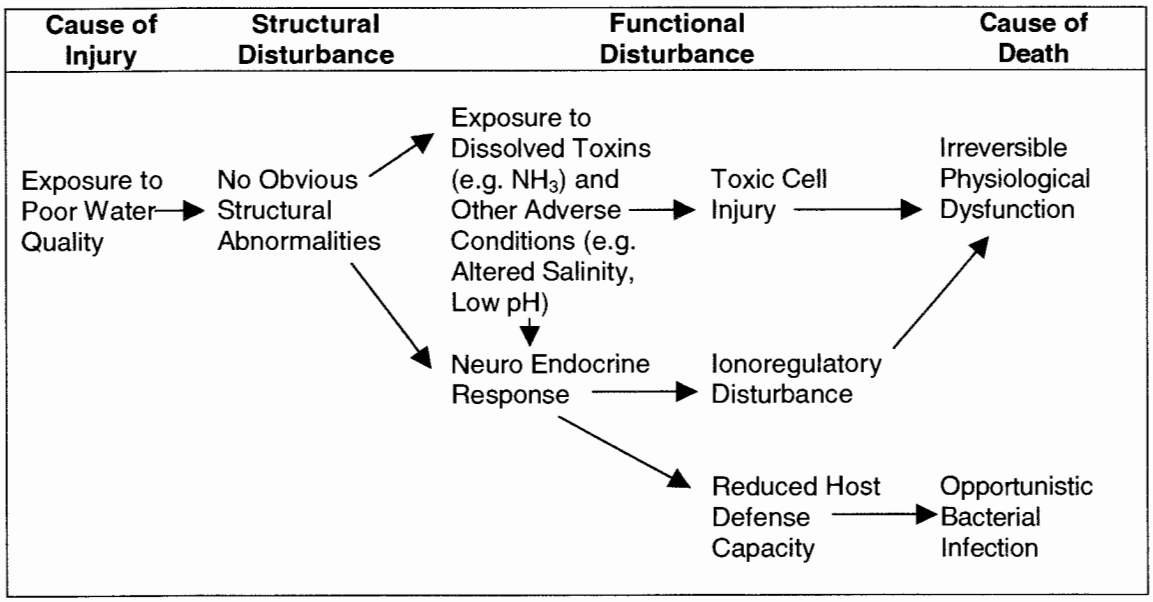


Figure 1(e) Exposure to poor water quality

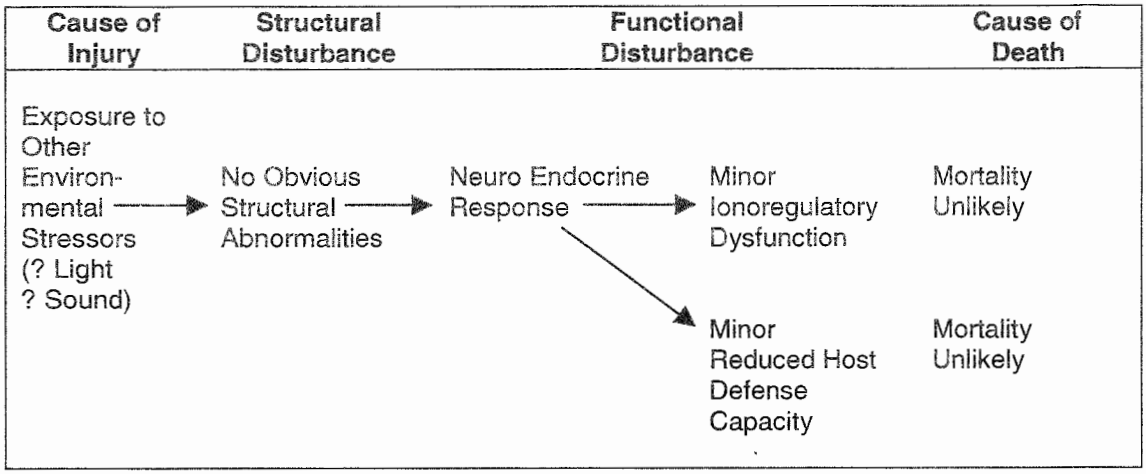


Figure 1(f) Exposure to other environmental stressors

1.3 Current state of knowledge of stress and immunity in rock lobsters

A major part of this study has concentrated on enumeration of circulating hemocytes in lobster hemolymph. Alteration in blood cell numbers in response to exposure to environmental stressors is a well described stress reaction in vertebrate animals (Wedermere 1996). Erythrocytes increase rapidly in an acute stress response, causing an increase in the hematocrit. The increase in the number of red cells is due to contraction of the spleen which expels blood cells into the circulation. Swelling of red cells induced by ionic and osmoregulatory changes which accompany acute stress responses also contributes to the increased hematocrit. Leucocrit, on the other hand, tends to decrease after exposure to stressors, largely as a consequence of a reduction in circulating lymphocytes. The decrease in lymphocytes has been attributed to an effect of the stress hormone, cortisol, on lymphocyte production.

Invertebrate animals do not possess the equivalent of erythrocytes, their blood cells comprising different forms of leucocyte-type cells, called hemocytes (or a similar term).

Three morphologically different hemocyte types can be identified in most crustacean species (Bauchau, 1981; Söderhäll and Smith, 1983; Sequeira et al., 1995; van de Braak et al., 1996). The specific functional activity associated with each hemocyte type such as prophenoloxidase activation, phagocytosis and coagulation (Lanz et al., 1993; Cerenius and Söderhäll, 1995), occurs in response to environmental stimuli.

Stewart et al. (1967) noted that total hemocyte counts (THC) in the lobster *Homarus americanus* declined in the presence of food deprivation. These workers (see review Stewart, 1975) demonstrated a decline in THC in bacterial infections in lobsters. In an early review of hemocytes, hemolymph and defense reactions in crustaceans, Rabin (1970) listed THC as being affected by injection of sheep red blood cells, bacteria, diet, starvation, moult cycle and gaffekemia infections. Field and Appleton (1995) observed a decrease in THC in the presence of dinoflagellate parasites. While Smith et al. (1995) have shown a decline in THC in shrimp, *Crangon crangon* exposed to harbour dredge spoils. Truscott and White (1990) reported circatidal rhymicity in

THC in *Carcinus maenus*, highest THC levels occurring during the high water period. In another study, shrimp hemocyte differentiation was shown to proliferate up to sixfold compared with the number in non-infected non-stimulated individuals (Sequeira et al., 1996) in animals infected with *Fusarium* spp. pathogens.

Few studies have been conducted on the responses of differential hemocyte counts (DHC) to different stressors. Bauchau (1981) showed that DHC changed during the moult cycle, and Sequeira et al. (1995) showed that changes could also be related to sex in *Panulirus japonicus*. On the other hand, Tsing et al. (1989) did not observe significant differences in DHC in *P. japonicus* with moult stage or sex.

Hemocytes are intimately involved in host defence reactions in crustaceans (see reviews, Bauchal, 1981; Söderhäll and Cerenius, 1992; Bachére et al., 1995). One of the defense mechanisms possessed by lobsters is non-specific antibacterial activity of their hemolymph (Rabin, 1965; Cornick and Stewart, 1968; Evans et al., 1968, 1969a,b; Weinheimer et al., 1969; Paterson et al., 1976, Mori and Stewart, 1978a,b; Chisholm and Smith, 1995; Ueda et al., 1990, 1994, 1999) sometimes termed antibacterial activity. It has been suggested that naturally occurring antibacterial activity may reflect the status of the immune system and can be used as an indicator of the lobster health conditions (Ueda et al. 1990, 1994). Other defense mechanisms include phagocytosis, nodule encapsulation and cytotoxicity.

Lobster health is influenced by a range of factors, one of the most important of which is stress. Exposure of aquatic animals to environmental stressors is known to result in physiological responses leading to short or long term changes in cardiovascular and respiratory function, energy metabolism, fluid and ionic balance, acid base balance and immunity (Selye, 1973; Barton and Iwama, 1991; Thompson et al., 1993; McDonald and Milligan, 1997; Iwama et al., 1997; 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, detrimental long term effects can occur. Adverse effects resulting from stress exposure include reduced resistance to disease, reduced growth, impaired reproduction and reduced survival (Pickering and Pottinger, 1989; Lee and Wickins, 1992; Iwama et al., 1997). In post harvest handling of lobsters both mild and extreme stressor exposure is likely to occur. In the latter case the physiology of the lobster may be so disturbed as to result in mortality.

Stress responses in fish, and, presumably lobsters can be divided into three stages, primary, secondary and tertiary, the reactions in the previous stage inducing those in the subsequent stage (Mazeaud et al., 1977; Barton et al., 1986; Barton and Iwana, 1991). Stress can also be defined as either 'simple' or 'compound' (McDonald and Milligan, 1997). Simple responses are limited to those physiological disturbances directly provoked by the initial nervous response and stress hormone secretion. Simple stress responses normally result in little direct harm to the animal, under conditions of good water quality. Some examples of simple stressors are routine post harvest practices of handling, confinement and transport. Compound responses to stress result from additional physiological disturbances evoked from the compounding effects of metabolic responses and stress hormone action as may occur following exposure to an extreme stressor such as prolonged, vigorous exercise (e.g. tail flapping) or air exposure. These stressors provoke anaerobiosis and lead to

extracellular acid-base disturbances through the release of lactic acid from muscle tissues.

Rapid, short-lived stress responses in scaphognathite activity and heart rate of crustaceans to a variety of tactile, chemical and visual stimuli have been the subject of numerous studies (Larimer, 1964; Ashby and Larimer, 1965; Wilkens and McMahon, 1972; Wilkens and Young, 1975), and likened to a startle reaction (Wilkens, 1976). Cardiac and ventilatory responses of longer duration (hours to more than a day), as occur following the trauma of handling and surgery, have also been investigated (McDonald et al., 1977, 1980; McMahon and Wilkens, 1977). Metabolic responses to environmental stressors are another area of investigation and have been studied in a number of crustacean species including crabs (Smith et al., 1995; Zou et al., 1996), prawns (Hall and van Ham, 1998) and lobsters (Taylor and Whiteley, 1989; Spicer et al., 1990; Whiteley and Taylor, 1992; Paterson and Spanoghe, 1997; Schmitt and Uglow, 1997).

In contrast to physiological and biochemical responses to environmental stressors, few studies have been conducted in crustacean species on the stress responses of immune parameters such as circulating hemocytes, coagulation factors or phagocytic capacity. Prior to commencement of this project there appeared to have been only one report on the influence of physical handling on circulating hemocytes in a decapod crustacean (Hamann, 1975) and no reports of other stressor effects on hemocytes in lobsters other than changes observed in starvation and bacterial infection (Stewart et al., 1967; Stewart, 1975). Information on the likely influences of post harvest handling stressors on the differential distribution of hemocytes, clotting rate, hemolymph antibacterial activity and phagocytic capacity was also lacking.

2. Need

Prior to the commencement of the research described in this report a study was conducted on the need for investigations of stress physiology in rock lobsters aimed at improving post-harvest handling procedures (Phillips, 1994). The objective of the review was to clearly establish the scope of the proposed physiological studies. The review was restricted to assessment of rock lobsters taken under current legislation and held and sold as live lobsters.

Three main research questions on western rock lobster post harvest handling physiology research arose from this study:

1. Is lobster mortality in the processing factory and during shipment due to handling and transport causing irreversible physiological failure, or is it due to impaired host defenses (breakdown of the immune system) leading to opportunistic bacterial infections?
2. If mortality is due to physiological failure, what are the key physiological systems affected by post harvest handling, transport and what are their tolerance limits?
3. If mortality is due to impaired host defenses, what are the key host defense parameters that can be used to assess host defense status.

Based on these research questions it was recommended that a research program be initiated to determine stress indicators for the western rock lobster and that the program include research into methods of assessing immunocompetence in lobsters. The development of a standard protocol for the autopsy of moribund lobsters was also identified as an urgent priority and was recommended for funding. The study described in this report was one of two projects implemented in response to the recommendations of this review. The other project was aimed at investigating physiological indicators of lobster stress status. At the commencement of the project it was anticipated that the final stress or health status indices would combine both immune and physiological indicators.

The perceived outcome of the project, assuming that one or more of the immune system components proved to be suitable as a stress and/or health status indicator, was the availability of a tool for fishers or processors to measure stress and/or health status in a batch of lobsters. This capability was seen to be of value to fishers and processors in the improvement of post harvest handling procedures and in maximising the proportion of the catch that are 'fit for live'. The investigation of reject lobsters would provide insight into the likely causes of lobsters becoming weak in holding tanks.

3. Objectives

The main aim of this project was to evaluate the application of immune system indicators in the assessment of the stress or health status of post harvest lobsters. In this context, the term 'health status' refers to the likelihood of the lobster dying within one or two weeks. The immune system indicators investigated included total hemocyte (blood cell) counts, differential hemocyte counts (measures of the different types of blood cells), clotting time (the time taken for the hemolymph to clot), antibacterial activity (antibacterial activity in lobster hemolymph), phagocytic capacity (the ability of hemocytes to sequester and destroy bacteria and other foreign agents), bacterial colony count (the number of live bacteria in the hemolymph) and quantitative histopathological measurements (quantification of occurrence of specific types of histological features seen in fixed sections of lobster tissues).

A secondary aim was to determine common causes of mortality of lobsters in live holding tanks. This aim was achieved by conducting autopsy investigations on lobsters from live holding tanks which showed behavioural and gross features indicating they were weak and unlikely to survive live export (reject lobsters).

The specific objectives of the project were as follows:

- *To identify suitable immune system parameters which can be used to evaluate stress responses and health status in captive lobsters and to apply those parameters in a study of stress induced by post harvest handling procedures.*

- *To investigate the causes of mortality in captive lobsters held in processing factories. This study will focus on bacteriological and histopathological examinations and will result in the development of a standard protocol for autopsy of lobsters.*

- *To evaluate the influence of temperature change on immunological and physiological stress responses*

(Following recommendations from the Steering Committee this objective was modified to a laboratory based study of the influence on immune stress parameters of handling with and without air exposure).

- *To study the influence of hormonal secretions on immunological and physiological stress responses.*

(Following recommendations from the Steering Committee this objective was changed to an investigation of the influence of holding conditions on immune stress parameters achieved through a series of simulated truck transport and live shipment trials conducted in a lobster processing factory (Geraldton Fishermen's Cooperative). Some studies on hormones produced by lobsters were commenced by the other project team in 1998 but the factory trials were given precedence over hormone investigations and this work was discontinued.)

- *To investigate innovative techniques which will boost immunocompetence but not adversely affect marketability of live product.*

(Following recommendations from the Steering Committee this objective was changed to an investigation of the influence of holding conditions on immune stress parameters achieved through a series of simulated truck transport and live shipment trials conducted in a lobster processing factory (Geraldton Fishermen's Cooperative.)

CHAPTER 3 GENERAL METHODS

1. Experimental approach

The research conducted in this project comprised five main areas of study – development of test methodology; determination of normal values for immune test procedures; acclimation and stress tests performed in the Muresk Marine Laboratory (MML), Fremantle, and at the premises of Geraldton Fishermen's Cooperative, Geraldton, measurement of stress parameters in lobsters collected from commercial fishing boats and factories and autopsy studies in accepted and reject lobsters obtained from factory tanks.

The methods developed for the immune tests were adapted from existing published procedures (total hemocyte counts, differential hemocyte counts and %granular cells, antibacterial activity, phagocytic capacity, clotting time) or new quantitative procedures were developed (bacteremia; quantitative histopathology). The emphasis in test development was to produce a test procedure which could be conducted in the factory by trained factory personnel. This aim was achieved with four of the tests (total hemocyte counts, %granular cells, clotting time and bacteremia), all of which were demonstrated to be of value in assessing lobster condition.

Normal values for the different parameters were determined by performing measurements on baseline lobsters collected by SCUBA and by measuring the different parameters in post harvest lobsters stored in factory tanks for 1-7 days. While immune parameters in the latter group were unlikely to be at baseline levels, the 'normal' values determined in these lobsters will be the most appropriate values for industry personnel to assess condition or stress status of post harvest lobsters tested using the tests developed in this project.

Information on the responses of western rock lobsters to a range of environmental stressors was obtained by exposing the lobsters to selected stressors under controlled laboratory conditions. The stress tests were performed by selecting the stressor to be tested (e.g. air exposure, wounding, physical handling) and comparing the immune parameter responses elicited by these stressors with the levels of the same immune parameters in control, unstressed lobsters. The time required for acclimation prior to these trials was also assessed by conducting a long term (64 day) acclimation trial in which both immune and physiological parameters were measured.

Autopsies were conducted following the dissection procedure developed in a previous FRDC project FRDC 94/134.07. Both reject lobsters, those judged by factory staff to be weak and unlikely to survive live transport (reject lobsters) and apparently healthy lobsters, those judged by factory staff to be 'fit for live' (accepted lobsters) were examined. Autopsies were also performed on some of the lobsters sampled during stress tests. Autopsies were conducted on 139 lobsters, 56 healthy (accepted), 49 unhealthy (reject) lobsters and 34 lobsters of uncertain health status.

2. Study sites

2.1 Muresk Marine Laboratory

The acclimation and stress trials were conducted in tanks and aquaria at the Muresk Marine Laboratory (MML), a laboratory facility developed in a leased building adjacent to the TAFE Fremantle Maritime Centre, Fremantle. The laboratory was established through funding provided by FRDC and Curtin University and comprises two temperature controlled rooms, an analytical laboratory, a recirculating tank storage system and staff offices. The recirculating seawater system consisted of two $8m^3$ tanks connected to a heat exchanger, biofilter and protein skimmer. The FRDC laboratory, one of the temperature controlled rooms, contained three banks of 8 x 250L glass aquaria. Each bank of aquaria was fitted with a temperature controlled recirculating water system including a sump tank, biofilter, protein skimmer and heat exchanger while the room within which the aquaria are housed was insulated and fitted with an air conditioner. A black plastic cover was placed around each battery of 8 tanks to reduce visual disturbance of lobsters. Most experiments conducted in MML were carried out in the FRDC laboratory.

2.2 Processing factories or depots

In addition to the laboratory trials experiments were also conducted in depots and processing factories owned by processing companies in Western Australia – Geraldton Fishermen's Cooperative Pty Ltd, Lobster Australia Pty Ltd and Kailis Bros. Pty Ltd. Lobsters were collected either immediately after arrival at the factory or depot or after storage for 1-7 days in the factory tanks. In one experiment lobsters transported to the depot and subsequently to the processing factory were sampled. This study was conducted in Jurian Bay and in Geraldton. Other studies on lobsters transported in trucks were carried out in Fremantle.

Tank systems used by the companies supporting the project were either recirculating or flow-through systems with lobsters being held in either raceways or fibreglass or concrete holding tanks. Typical holding tanks or raceways used in the study are shown in Figs. 2 and 3.

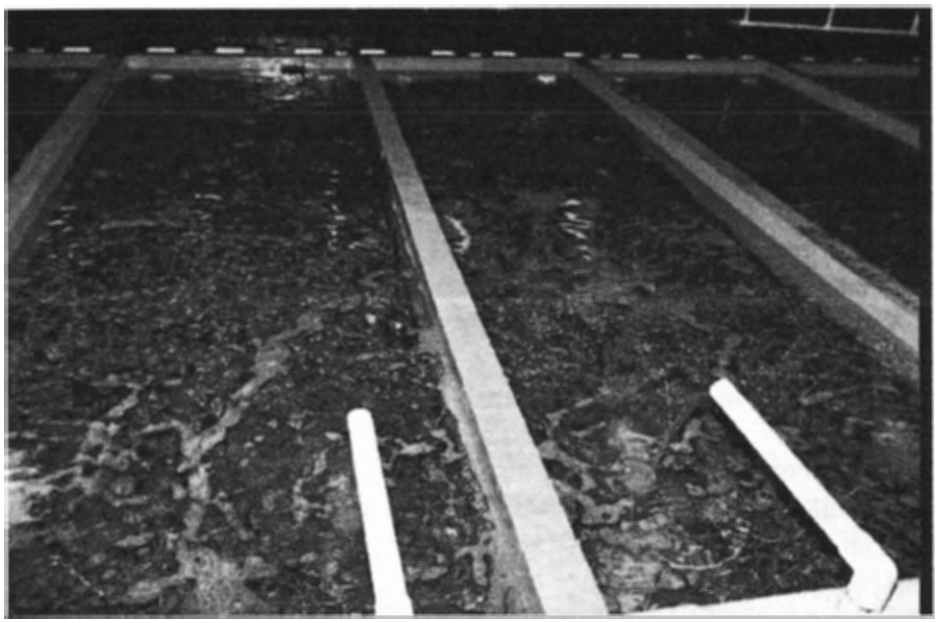


Figure 2 Raceway holding system at Lobster Australia Limited

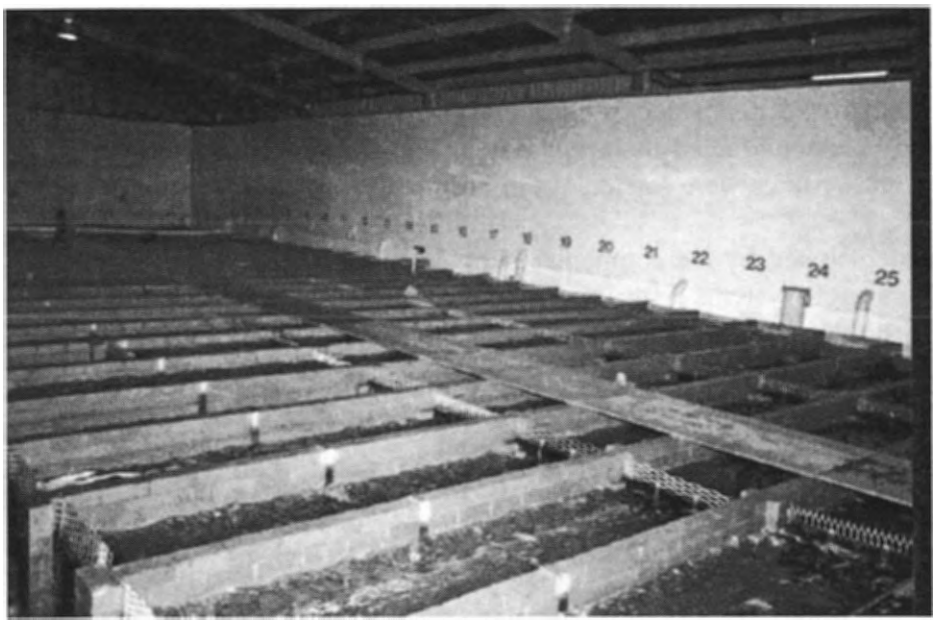


Figure 3 Lobsters in holding tank at Lobster Australia Limited

In some studies lobsters were collected while on board fishing boats (Figs. 4, 5 and 6) or underwater by SCUBA collection.

Figure 4 WA rock lobster fishing vessel

Figure 5 Lobster capture operation

Figure 6 Collection of hemolymph on board lobster fishing vessel

2.3 Experimental design and data analysis

The basic experimental design used in all laboratory experiments was to expose a number of treatment groups of lobsters to a given stressor, examine the changes with time in the selected immune parameters and compare these changes, if any, with levels of the same parameter in control, unstressed lobsters. The number of lobsters in each treatment group varied from 8 to 20 depending of the design of the experiment. In some preliminary experiments a smaller number of lobsters was studied. However, in most experiments at least 8 lobsters were examined per treatment group. In numerous instances the sample size number was restricted by the availability of only 24 aquaria in the FRDC laboratory at MML.

Data was analysed using both SSPS and Minitab statistical packages. A range of statistical analyses were performed including analysis of variance, multivariate analysis, Chi square and t-tests. Turkey's, Duncan's and Least Square Tests were used to demonstrate statistically significant differences between treatment groups with a 95% level of significance ($P<0.05$).

2.4 Laboratory and field trials performed

A summary of the laboratory and field trials performed during the course of the project is as follows:

1. Red and white lobster experiments December 1995 - June 1996 (Kailis Bros Pty Ltd)
2. Anticoagulant for THC and DHC, (MML) - April - December 1996
3. Antibacterial activity assay experiments (MML) - September 1996 - September 1998

4. Wounding experiment - August 1996 (MML)
5. Truck trial and boat trial (1 boat) (Jurian Bay & Geraldton) - December 1996
6. Geraldton Fishermen's Cooperative boat trial (2 boats) - December 1996
7. Acclimation trial (MML) - November 1996 - January 1997
8. SCUBA collections (Geraldton) - June & July 1997, April and November 1998
9. THC Methodology experiments (MML) - February - December 1997
10. Geraldton Fishermen's Cooperative boat trial (3 boats) - April 1997
11. Geraldton Fishermen's Cooperative first factory trial & SCUBA collection - April 1998
12. Air exposure and handling stress trial (MML) - May 1998
13. Emersion trial (MML) - June 1998
14. Geraldton Fishermen's Cooperative second factory trial - November 1998
15. Minor stressors trial (MML) - January 1999
16. Geraldton Fishermen's Cooperative third factory trial - March 1999
17. Factory studies of immune parameters (Lobster Australia Limited) - April & May 1999

CHAPTER 4 DEVELOPMENT OF TEST METHODOLOGY

1. Hemolymph sampling techniques

1.1 Hemolymph sampling site

1.1.1 Influence of sampling site on THC and clotting time values

A preliminary study was conducted to determine whether the site of hemolymph sampling affected the THC value. Lobsters were obtained from commercial catch from Geraldton and held in recirculating holding tanks at MML for several weeks, during which period lobsters were fed with mulies and mussels three times a week. In the experiment, lobsters (N=8) were bled from four anatomical bleeding sites: left (l) and right (r) ventral sinuses, pericardial sinus and abdominal artery. The sites were bled in a circulating order in order to prevent a possible effect of time lag on the THC result. Lobsters were bled one at a time, with the maximum time between removal of the first and fourth hemolymph sample being 2 min. In each lobster the first sample was taken either from pericardial sinus or ventral sinus on the base of the fifth walking leg or from the abdominal artery. All hemolymph samples (200 µL) were collected using a 23G needle and 1 mL calibrated syringe containing 200 µL of precooled Na-cacodylate based anticoagulant (Appendix 1). Samples were stored up to 48 h in ice until analysed. THCs were analysed by the standard procedure described below.

The anatomical sampling site had a significant effect on THC value with the abdominal artery sample giving the lowest counts (t-test, $P<0.05$) compared to ventral sinuses and pericardial cavity (Fig. 7). It was concluded that lobsters could be bled either from the pericardial sinus or from either of the ventral sinuses. In most of the early experiments the latter sites were used. Following the demonstration of heart injury through pericardial sampling (see below) the ventral sinus bleeding sites were used exclusively for hemolymph sampling.

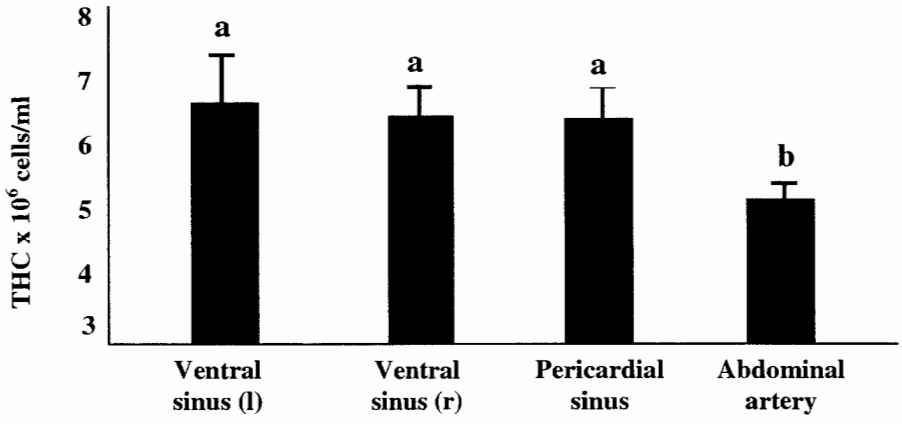


Figure 7 Total hemocyte counts in samples taken from different anatomical locations (Mean±SE). Mean values with different letters are significantly different ($P<0.05$).

The influence of sampling site on clotting time was also studied. Hemolymph samples were removed from the venous sinuses at the base of the right and left fifth walking legs and clotting time determined as described in Section 4.1. The mean values for clotting times determined on 8 lobsters (61.0±6.1 secs and 52.3±5.0 secs left leg and right leg respectively; mean±SE) were not significantly different ($P<0.05$).

1.1.2 Heart histology following pericardial puncture

Hemolymph samples obtained in the first factory trial at Geraldton Fishermen's Co-operative were all withdrawn from the pericardial cavity rather than the ventral sinus. Examination of histological sections of heart sections from lobsters sampled within several days of hemolymph sampling revealed an extensive hemocytic aggregation in the heart tissues in some of the lobsters (Fig. 8). The appearance of the lesion was suggestive of a needle prick wound in the heart. As a result of this observation, and the demonstration of a lower THC in hemolymph samples taken from the abdominal sinus (Fig. 7) it was decided that all hemolymph samples should be taken from the ventral sinuses.

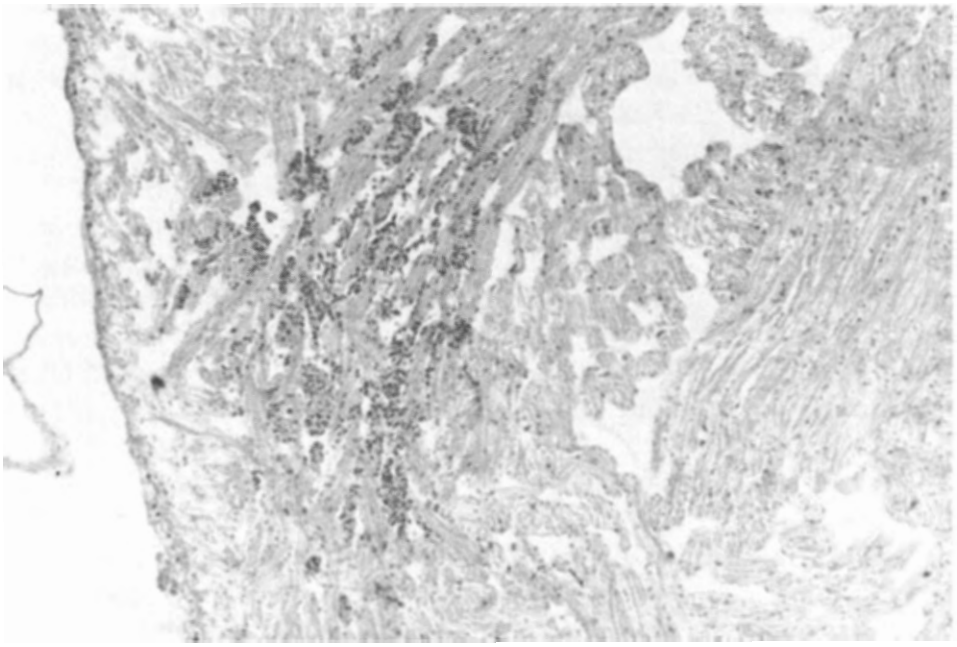


Figure 8 Histological section of lobster heart with needle prick wound (H&E x100)

1.2 Standard hemolymph collection and storage procedure

Samples for THC, DHC and phagocytic capacity were collected from the ventral sinus by puncture of the arthrodial membrane at the base of the fifth walking leg using a pre-cooled syringe containing anticoagulant. Samples were immediately transferred into 2mL Eppendorf tubes (Treff AG, CH-9113, Degersheim, Switzerland) held in ice and mixed well. The anticoagulant used in THC and DHC assays was 0.1% glutaraldehyde in 0.2M sodium-cacodylate buffer pH 7.0 (Appendix 1) and a modified PanBSS based anticoagulant (Aono et al., 1994) preparation (Cht 4, Section

7.4.1.1) was used for assay of phagocytic capacity. Samples were analysed following procedures described in subsequent sections. THC samples were either counted immediately or held at +4°C in a refrigerator for up to 24h and then counted. It was found that samples could be held up to 48h without any decrease in THC values but most assays were performed within 24h.

Samples for antibacterial activity assay, clotting time and bacterial colony count were collected without anticoagulant using a 2 mL calibrated syringe. Samples were then either stored on ice or frozen prior to analysis (antibacterial activity) or analysed immediately (clotting time and bacterial colony count).

2. Total Hemocyte Counts

2.1 Introduction

At the commencement of this study there had been few reports in the literature on acute stress responses in invertebrate species and no reports of these types of studies in lobsters. Accordingly, in order to validate some of the experimental observations on circulating hemocyte responses to stressors in lobsters, and to overcome occasional problems of availability of test animals for laboratory experiments, some experiments on THC responses were conducted in another decapod crustacean species, the freshwater crayfish *Cherax tenuimanus* (marron). Where appropriate, the results of these studies are included in this report.

Total hemocyte counts (THC) were estimated using both manual and automated techniques. THC values were found to be affected by technical factors including the anticoagulant preparation (Cht 4, Section 2.3), hemolymph sampling site (Cht 4, Section 1.1) and time interval between disturbance and hemolymph removal (Cht 4, Section 2.5). The effect of these factors on THC values was determined and a standard counting technique developed.

2.2 Basic counting procedures

The method used to count hemocytes in lobster hemolymph was based on the standard blood cell counting method using the Spencer Bright-Line Improved Neubauer counting chamber (hemocytometer). 200 µL of hemolymph was drawn into the syringe preloaded with 200 µL of Na-cacodylate anticoagulant (Appendix 1) avoiding air bubbles and coagulation. The hemolymph and anticoagulant solution (a total of 400 µL) was then placed in a 2 mL Eppendorf tubes held in ice. 100 µL of the mixture was transferred to another Eppendorf tube containing 400 µL of precooled 3% saline solution (in some studies the preparation was diluted 1:1). The resulting preparation was throughly mixed by inverting the tube 3 times. The samples were kept in ice or held at +4°C in a refrigerator until analysed.

THCs were estimated using a hemocytometer and microscope (x 100; manual method) or Coulter Counter (automatic method; Cht 4, Section 2.8). The hemocytometer was carefully filled with sample solution, enough to fill the platform but not the surrounding ditch. The sample was left to settle for 5 min before counting, so that the cells sedimented onto the platform surface. The distribution of the cells

was checked before counting by moving the chamber to ensure that there was an even distribution throughout the counting chamber. If the cells were not evenly distributed, the chamber was thoroughly cleaned and reloaded.

THC results were expressed as cells/mL using the following formulae:

$$THC = (C \times K \times 1000) / 0.4$$

where C is cell count from the hemocytometer and K is sample dilution factor.

For measurements using the Coulter Counter the hemolymph-anticoagulant solution (1:1) was diluted to 1:200 using particle free isotonic solution. For each individual sampling set a quality control check was performed by comparing the THC values obtained from 10-20 samples with those obtained on the Coulter Counter. The Coulter Counter settings suitable for a 500 µL sample of diluted lobster hemolymph were found to be: sensitivity 0.707; controls 64; threshold 4 and 85. Coincidence correction tables were checked if Coulter Counter readings exceeded 20000 units.

2.3 Influence of anticoagulant and outlier values on THC result

2.3.1 Preliminary studies on marron THC values

The influence of anticoagulant preparation used in the THC measurements was studied in both marron and in lobsters. In the marron study THC measurements were performed on 30 marron collected from a marron farm and delivered to the laboratory. Three different observers collected hemolymph from 10 marron each (observer A, B and C), with observers A and B using the sodium-cacodylate anticoagulant preparation (Appendix 1) and observer C using an anticoagulant containing citrate and EDTA Durliat & Vranckx (1981).

The mean THC value obtained with the sodium-cacodylate anticoagulant by Observer A was significantly different from than that obtained by Observer C using the citrate based preparation (Table 2). There was no significant difference between the mean THC value obtained by Observer B and the other two observers.

Table 2 Influence of anticoagulant preparation on THC obtained in farmed marron (Mean±SE)

Observer/Anticoagulant*	THC x 10^6 Cells/ml
A/1	3.50 ±0.45 (10)[a]
B/1	3.05 ±0.24 (10)[ab]
C/2	2.51 ±0.34 (10)[b]

* A,B,C = Observer A,B&C respectively
1 = Sodium-cacodylate anticoagulant
2 = Citrate-EDTA based anticoagulant
Mean values in the same column with different superscripts are significantly different ($P < 0.05$).
(n) = number of marron studied.

The results suggest that THC values in the sodium-cacodate buffer were significantly higher than those obtained using the citrate based buffer.

2.3.2 Lobster anticoagulant studies

An extensive study of the influence of anticoagulant preparation on lobster THC values was conducted as part of the development of the phagocytic capacity assay Cht 4, Section 7). It was found that with some preparations the THC did not change significantly over the 60min observation time while with others there was a marked decrease in THC with time. This investigation, along other incidental observations of a fall in THC values with time in lobster hemolymph samples prepared using the citrate based anticoagulant preparation, confirmed the necessity of choosing an anticoagulant preparation which would provide adequate preservation of hemocytes and prevention of coagulation. Based on the results of the study of the influence of anticoagulant preparation on the differentiation of the three cell types found in lobster hemolymph (Cht 4, Section 3.2) and the above findings it was decided to use the sodium-cacodylate anticoagulant in both THC and DHC assays.

2.4 Accuracy and reproducibility of THC manual method

The accuracy of the THC measurement is dependent on a number of factors. One factor is the number of cells or fields counted when using the manual THC method (Fig. 9). During the course of the project this number varied depending on the accuracy desired and the time available for counting. As a standard practice at least 150 cells were counted.

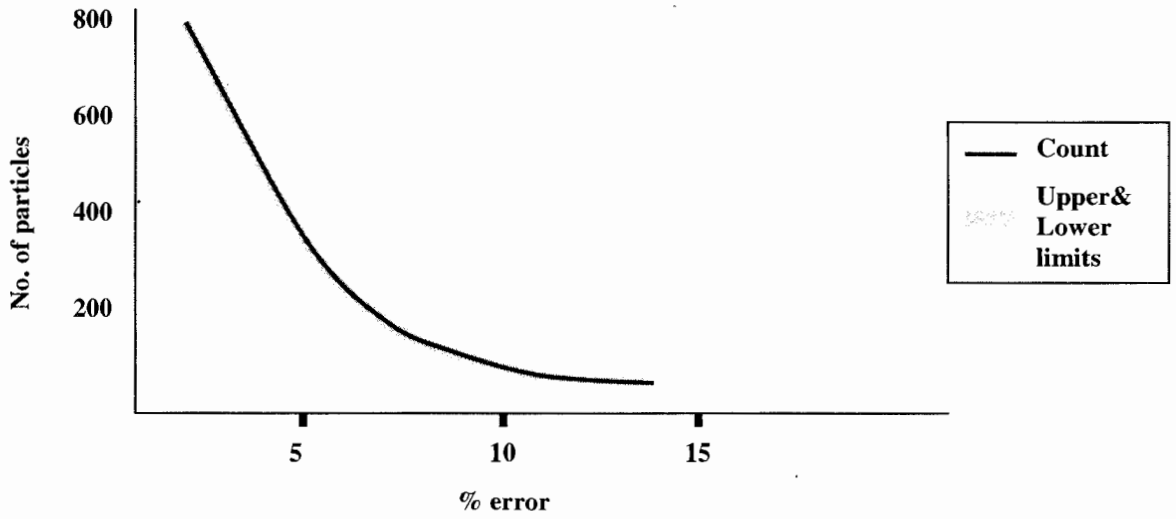


Figure 9 Counting error in hemocyte counting chamber in relation to number of cells counted (adapted from Dacie and Lewis, 1991).

The reproducibility of the THC manual assay method was tested by two observers conducting 10 THC analyses on hemolymph samples from three lobsters (8 estimations on one lobster and 2 on the other lobster), each sample being counted by each observer. Only one hemolymph sample was collected from each lobster so as

to avoid errors introduced by elevations in THC due to stress responses to handling (see below). The lobsters had been held for approximately six months in a tank at MML at the time of hemolymph collection. The mean THC value for the ten estimations conducted by Observer A was within 4% of that obtained by the other observer (Table 3). The coefficient of variation (CV%) calculated by averaging the CV%s for each duplicate measurement, was 4.10% which indicated good reproducibility for repeated observations by different observers.

Table 3 Reproducibility of THC assay

Lobster No.	Assay No.	Observer A	Observer B
1	1	3.48	3.59
	2	3.41	3.37
	3	3.33	3.50
	4	3.54	3.53
	5	3.27	3.30
	6	3.64	3.24
	7	3.15	3.33
	8	3.16	3.77
2	9	7.26	8.10
	10	7.82	7.96
Mean±SE		4.20±0.56	4.37±0.61
CV%		4.10	

2.5 Time interval between disturbance and hemolymph collection

The influence of the time interval between disturbance and hemolymph collection was studied. Lobsters were obtained from the commercial catch from Geraldton and held in recirculating holding tanks for several weeks, during which period lobsters were fed with mulies (*Sardinops neopilchardus*) and mussels (*Mytilus edulis*) three times a week. In the experiment, lobsters (N=6-8) were bled from pericardial sinus 0, 30, 60, 120 and 180 sec after a physical handling event (grasping the lobster around the carapace and removing from the water). Handling procedures were similar to those followed by lobster graders in commercial factories. Hemolymph samples were stored as described above (Cht 4, Section 1.2) and THC analyses performed by the manual method.

Total hemocyte counts increased significantly ($P<0.05$) in handled lobsters after 60 sec of the handling event (Fig. 10). It was concluded that in all future studies the hemolymph sample should be taken in less than 60 secs from initial contact with the lobster.

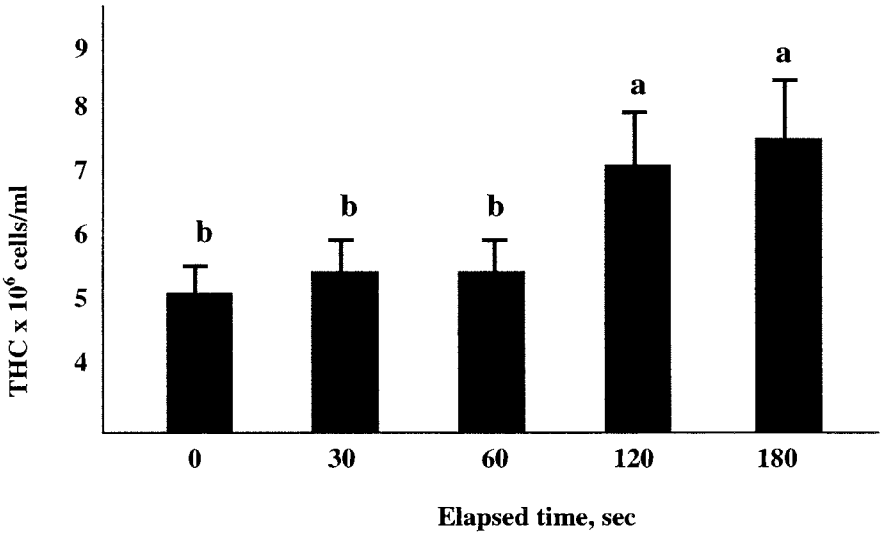


Figure 10 Effect of sampling time lag on THC (Mean±SE). Mean values with different letters are significant different (P<0.05).

2.6 Influence of moult stage on total hemocyte counts

A preliminary study of the influence of moult stage on THC values was conducted. Moult stage and THC were determined in lobsters sampled as part of an acclimation study carried out MML during December 1997 and January 1998. Lobsters were held in the recirculating tank system (Cht 3, Section 2.1) and fed using mussels or mulies three times a week. The water temperature was kept constant at 21°C during the experiment and other water quality parameters (DO, pH, ammonia, nitrate) were closely monitored and partial water exchanges performed when required to maintain values within acceptable limits. Pleopod strips were cut off from sampled lobsters and stored in 3% saline solution for later analysis of moult stage (Lyle and MacDonald, 1983). Prior to the analyses, samples were stored at +4°C in a refrigerator. THC analyses were performed and the results compared to the moult stage at the time of hemolymph collection (Fig. 11). Substages of D_1 were pooled prior to statistical analysis.

The mean THC values increased up to molt stage D_2, and then declined. The mean THC values in stage D_{3-4} and stage C were significantly lower than that of stage D_2. The SE was significantly higher in D_2 and D_{3-4} lobsters compared to the earlier moult stages (P<0.05).

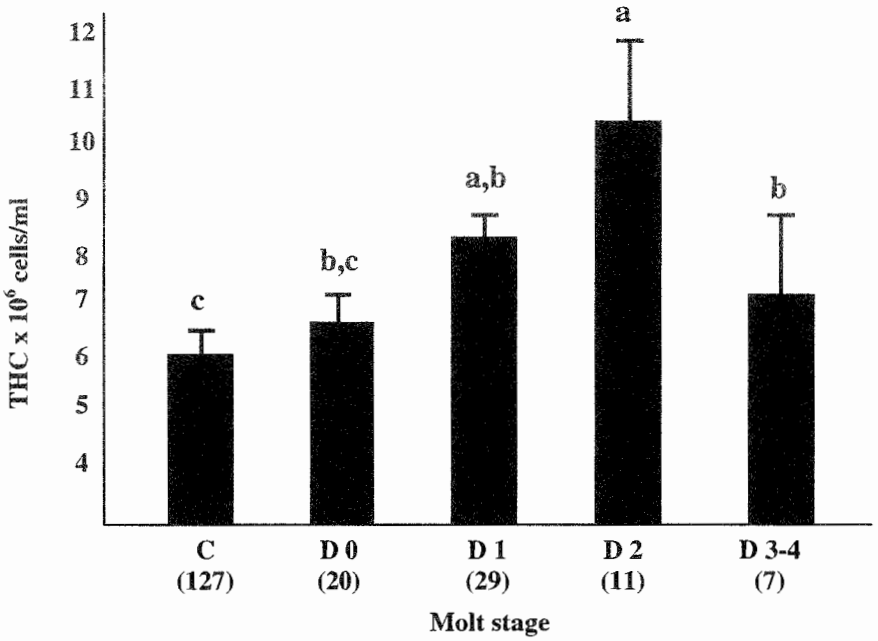


Figure 11 Influence of moult stage on THC (Mean±SE). Mean values with different letters are significant different (P<0.05).
(n) = Number of lobsters studied

The conclusion from this study was that moult stage had a significant influence on THC with lower values being seen in C, D0 and D3-4 stage lobsters compared to lobsters in moult stages D1 and D2. However, this conclusion is preliminary and needs to be confirmed through a controlled study with larger sample sizes. A second conclusion was that only C stage lobsters should be used in experiments where the choice of test animals was an option in the experimental design.

2.7 Influence of lobster sex on THC

Four data sets were examined for sex differences in THC values. The first data set comprised 67 white and red lobsters (43 males and 24 females) studied in Fremantle in 1995/96 (Cht 7, Section 1). No differences in THC between sexes were found (P>0.05). The second data set comprised the 174 lobsters sampled at MML in December, 1996 and January, 1997 during the acclimation trial (see Section 2.6 above) (147 males and 27 females) while the third set comprised 80 lobsters (40 males and 40 females) sampled at a Fremantle processing factory in April, 1998 (Cht 7, Section 4). Only lobsters in moult stage C or D0 from treatment groups with mean values lower than the upper limit for normal, relatively unstressed lobsters (8.79 x 10^6 cells/ml; see Cht 5, Section 3.1) were included in the analyses. No difference in THC between sexes was found (P>0.05).

2.8 Coulter Counter study

2.8.1 Comparison of manual and automatic THC methods

While providing useful information about stress and health status of lobsters, THC measurements are time consuming and labor intensive. In order to improve the

efficiency of the THC assay, the application of an automated hemocyte counting technique was investigated. Two automatic cell counters, a Coulter Counter (Coulter Electronics, Model ZB1) and an H1 Analyser (Bayer Technicon) were tested both under laboratory conditions and during practical sampling in the factory trials conducted in Geraldton. THC values were determined for the same hemolymph sample using both manual and automatic techniques. A high correlation was observed (r^2=0.95) between THC determined by manual counting and the corresponding value obtained with the Coulter Counter (Fig. 12), based on both field and laboratory tests. Less consistent results were obtained with the H1 Analyser. The reasons for differences in performance between the two counters were not investigated. It was concluded that the Coulter Counter was the automatic counter of the choice, being simple to operate, reliable and field compatible.

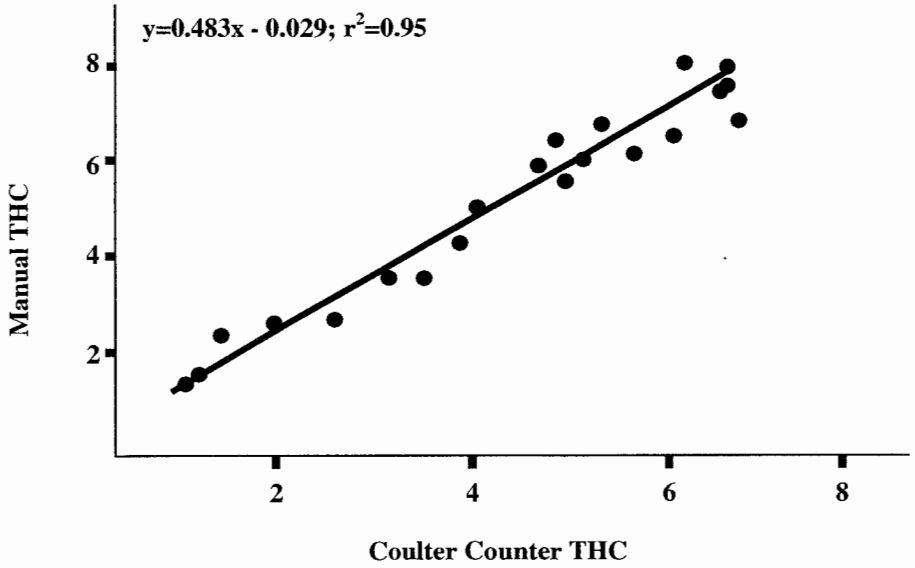


Figure 12 THC values obtained by manual and Coulter Counter methods

Automatic counting was used in all experiments involving large numbers of THC estimations (the factory studies in Geraldton). It was also found that samples could be stored up to 48h without any significant effect on the THC result obtained with the Coulter Counter.

2.8.2 Reproducibility of the automatic THC method

The reproducibility of the Coulter counter method on THC determination was tested in two separate experiments. In the first trial 20 repeated estimations were performed on a single hemolymph sample. The coefficient of variation (SD x mean THC^{-1} x 100%) was 0.62%. In the second experiment, 30 different samples were counted three times each. The mean CV% obtained by averaging the individual CV% from the 30 sets of triplicate counts was 1.17%. When the mean CV% of the three sets of duplicate counts were compared (rep pairs 1 & 2, 1 & 3 and 2 & 3) values of 0.94 to 1.13% were obtained. This result showed that increasing the repeat estimates for 2 to 3 did not improve the accuracy of the final THC measurement. Accordingly, duplicate assays were performed for all automatic THC determinations. The

reproducibility of the automatic THC method (approx. 1%) was considerably higher than that of the manual method (approx. 4%; Cht 4, Section 2.4).

3. Differential Hemocyte Count (DHC) and %Granular Cell Assays

3.1 Sample preparation and staining technique

Hemolymph samples (200μL) were collected from the ventral sinus in the base of the fifth walking leg (unless otherwise stated) using a 23G needle and 1 mL calibrated syringe containing 200μL of precooled sodium-cacodylate based anticoagulant (Appendix 1). The hemolymph preparation was then placed in an Eppendorf tube, the tube was capped and inverted several times to achieve thorough mixing. One drop (30μL) was placed on a glass slide with an automatic pipette and a glass spreader was used to produce a uniform smear. The prepared smears were air dried and fixed in absolute methanol. Staining was performed by the May-Grünwald-Geimsa technique (a Romanowski stain) and counting performed following standard procedures (Dacie and Lewis, 1991). A minimum of 150 cells were analysed in each smear.

3.2 Anticoagulant preparation

Microscopic examination of hemolymph, collected during early field trials into a caffeine based anticoagulant, had shown evidence of gross cellular deterioration together with marked cellular clumping. An investigation was therefore conducted into developing an anticoagulant that would have the ability to:

- Arrest primary hemostasis (cellular aggregation)
- Arrest secondary hemostasis (hemolymph gelation)
- Preserve hemocyte nuclear and cytoplasmic proteins (morphological features)
- Maintain a stable preparation for a minimum of 24hr at room temperature

Two mechanisms for inhibiting the clotting process were the basis of anticoagulant development:

- Fixation of protein systems
- Chemical capture of Mg^{++} and Ca^{++}

Published reports of anticoagulants found to be suitable for use with crustacean hemolymph preparations were examined (Durliat and Vranckx, 1981, 1983; Söderhäll and Smith, 1983; Aono et al., 1993, 1994; Lanz et al., 1993; Vargas-Albores et al., 1993; Guzman et al., 1993; Sequeira et al., 1995) and six preparations were selected for study (Table 4).

Table 4 Anticoagulant preparation examined using lobster hemolymph

		Composition	Mode of Action
1	Söderhäll & Smith, 1983	pH/EDTA-citrate	Chemical capture
2	Aono et al., 1994	pH/L-cysteine	Chemical capture
3	Sequeira et al., 1995	pH/glutaraldehyde	Protein fixation
4	Lanz et al., 1993	pH/glutaraldehyde	Protein fixation
5	Durliat & Vranckx 1981	pH/Citrate	Chemical capture
6	Guzman et al., 1993	pH/EDTA	Chemical capture

Hemolymph was collected using a pre-cooled 1ml syringe loaded with an equal volume of chilled anticoagulant as described in Cht 4, Section 1.2. The resulting preparation was examined at x100 – x400 using a binocular microscope. Fixed hemolymph smears were prepared as described in Cht 4, Section 3.1.

The results obtained were as follows:

Three Hours Post Collection
All anticoagulants (1-6) successfully arrested hemolymph clotting and cellular clumping.

Twelve Hours Post Collection
Anticoagulants 1, 2, 5 and 6 continued to arrest hemolymph clotting. However, strong cellular clumping and lysis were evident together with salt precipitation. Anticoagulants 3 and 4 maintained good cellular integrity.

Twenty Four Hours Post Collection
No cellular adhesion or clumping were seen in anticoagulants 3 and 4 but cellular features stained with increasing intensity, indicative of overfixation with glutaraldehyde. Poor cell preservation and extensive cell clumping seen in all other preparations.

Anticoagulants 3 and 4 (Table 4) were selected for further study and further adjustments to anticoagulant buffer pH, molarity and glutaraldehyde concentration were trialled. Optimum cell preservation and minimal cell clumping was achieved with a 0.1% glutaraldehyde in 0.2M sodium cacodylate buffer pH 7.0 and a collection ratio of 1 ml hemolymph to 1 ml anticoagulant. The preparation prevented cellular clumping and maintained cellular morphological features for 24hr when collected at room temperature. Good preservation was also achieved after 48-96hr storage when hemolymph specimens were placed on ice immediately after collection and maintained at 4°C.

3.3 Description of hemocyte types

Three different hemocyte types were identified in the smears (Fig. 13).

1. Hyalin cells (HCs); mean size 8-10 µm; round shape, sometimes irregular (raisin-like); dark blue or violet nucleus; little or no cytoplasm.
2. Granular cells (GCs); mean size 35-40 µm; round shape; clearly visible dark or pale blue nucleus; cytoplasm with either pink, blue or colourless granules;

possibly three subgroups; eosinophilic, basophilic and neutrophilic granulocytes.

3. Semi-granulocytes (SGCs); mean size 25-40 µm; round or irregular shape; typically pink or sometimes light violet nucleus; small amounts of cytoplasm.

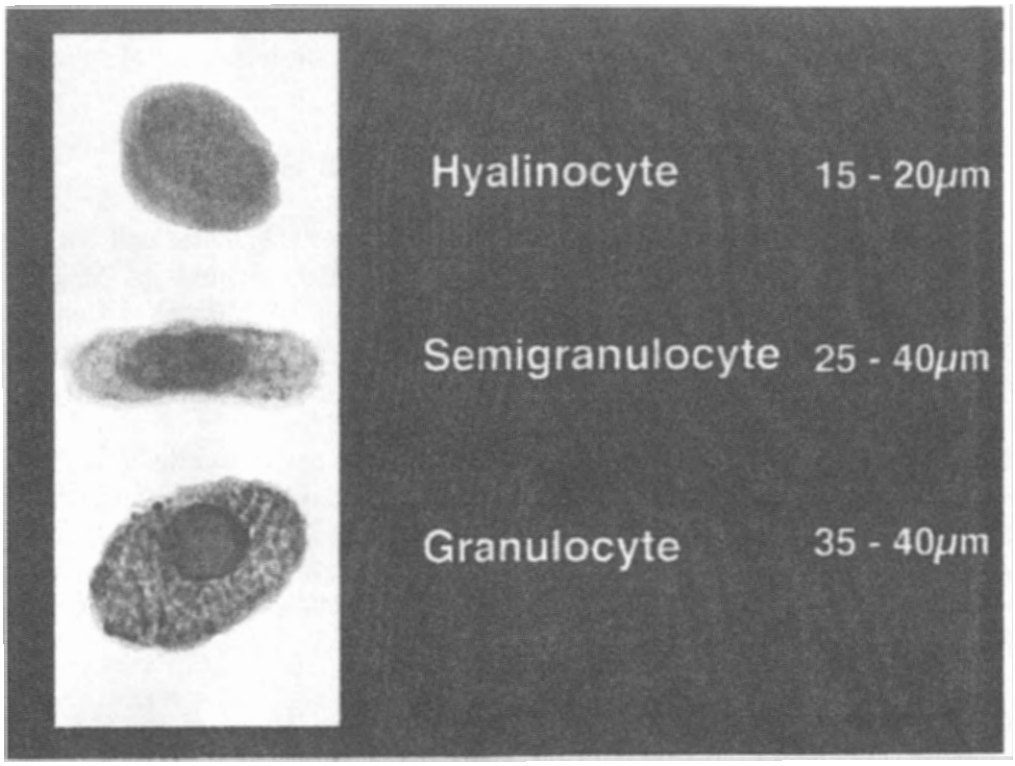


Figure 13 Morphological features of the three main hemocyte types found in *Panulirus cygnus*. Average diameter of long axis also shown

3.4 Influence of moult stage on differential hemocyte counts

A preliminary study of the influence of moult stage on DHC was conducted. Moult stage and DHC were determined in lobsters sampled as part of an acclimation study carried out MML during December 1997 and January 1998. Details of the experimental procedures are given in Cht 4, Section 2.6. A total of 47 DHC analyses were performed and the results compared to the moult stage at the time of hemolymph collection (Table 5).

Table 5 Influence of moult stage on DHC (Mean±SE)

	Combined	D_0 (13)	D_1^{I} (11)	D_1^{II} (6)	D_1^{III} (5)	D_2 (7)	$D_{3\text{-}4}$ (5)
Hyaline cells	21.2±1.4	16.7±3.1^{a}	23.1±3.0^{a,b}	21.1±3.6^{a,b}	30.3±1.9^{b}	23.8±1.9^{a,b}	15.1±4.5^{a}
Granular cells	7.0±0.6	7.8±0.9	7.5±1.7	6.8±1.6	6.0±1.3	6.1±1.2	6.3±1.9
Semi-granular cells	71.9±1.7	75.5±3.7^{a,b}	69.4±4.0^{a,b}	72.1±4.8^{a,b}	63.7±1.7^{b}	70.1±2.6^{a,b}	71.9±1.7^{a}

Mean values in the same row with different superscripts are significantly different ($P<0.05$)
(n) = number of lobsters studied

Unfortunately all lobsters studied were in the D moult stage so information on possible variations in DHC in other stages of moult was not obtained. No significant changes occurred in the proportion of granular cells throughout the D stage of the moult (Table 5). The proportion of hyaline cells was significantly higher in D_1^{111} stage compared to D_{3-4} and D_0 stages and a similar, inverse trend was seen in semi-granular cells. However, in view of the small sample sizes and the absence of data on C stage lobsters, a firm conclusion with respect to the influence of moult stage on DHC results cannot be made at this stage.

3.5 Reproducibility of DHC and %granular cell assays

The reproducibility of the DHC assay (including the % granular cell assay method) was tested by an observer conducting duplicate DHC estimations on hemolymph smears prepared from 10 lobsters. No statistically significant differences were observed between the mean values obtained for the two sets of measurements (Table 6).

The coefficients of variation for determination of the proportional distribution of hyaline cells, semi-granular cells and granular cells, calculated by averaging the CV% for each duplicate measurement, were 31.4%, 4.5% and 9.0% respectively. The reproducibility of the semi-granular and granular cell assays was therefore acceptable but that of the hyaline cell assay exceeded the CV% value commonly regarded as indicating acceptable reproducibility (<20%).

Table 6 Reproducibility of DHC assay

	Cell type (%)					
	Hyaline		Semi-Granular		Granular	
Lobster No	1	2	1	2	1	2
1	19.5	12.0	75.0	81.5	5.5	6.5
2	12.0	4.5	80.5	89.0	7.5	6.5
3	30.0	14.0	69.0	85.0	1.0	1.0
4	13.0	11.0	76.0	78.0	11.0	11.0
5	12.0	12.5	80.0	80.0	8.0	7.5
6	7.5	8.0	90.5	89.5	2.0	2.5
7	15.0	23.0	75.0	69.0	10.0	8.0
8	5.0	11.0	78.5	77.0	16.5	12.0
9	4.0	5.0	95.0	94.0	1.0	1.0
10	6.5	13.0	87.0	79.5	6.5	7.5
Mean±SE	12.5±2.5	11.4±1.7	80.7±2.5	82.3±2.3	6.9±1.6	6.4±1.2
CV%	31.4		4.5		9.0	

A further reproducibility test was performed for the %granular cell assay. In this test two different observers performed the duplicate assays. No significant difference was observed between the mean value for %granular cells (Table 7). The CV (16.4%) was higher than that obtained with one observer performing the assay (9.0%) but was still within acceptable units.

Table 7 Reproducibility of %granular cell assay

	%Granular cells Observer	
Lobster No	A	B
1	6.5	7.0
2	4.8	10.0
3	1.3	1.5
4	11.8	11.0
5	11.8	7.8
6	2.5	2.5
7	8.5	7.0
8	13.5	16.0
9	2.0	3.0
10	8.8	7.8
Mean±SE	7.12±1.40	7.35±1.38
CV%	16.4	

The lack of reproducibility in the hyaline cell assay arose primarily from inconsistencies in the differentation of this cell type from the semi-granular cell type. This inconsistency arose from a lack of clearly identifiable features in the stained preparations of the two cell types and differences in personal judgements of identification features. Attempts to improve the differentiation of the cell types by applying different staining techniques (modifications to the May-Grünwald Giemsa and Sudan Black staining methods) were not successful.

A lack of precision in identification was not encountered in the %granular cell assay. Granular cells are relatively easy to identify and count. However, because of the low numbers of granular cells present in the smear (5-10% of the total number of hemocytes) the coefficient of variation tended to be higher than seen in other immune stress tests.

It was concluded that since granular cells are more easily recognised in stained hemolymph smears than hyaline or semi-granular cells, only these cells would be quantified in future studies. This decision was supported by the fact that the estimation of %granular cells rather than DHC was considerably less time consuming and therefore less expensive to perform. However, in studies of reject lobsters it was of value to consider the differential distribution of all three cell types and full DHC analyses were therefore performed with reject lobsters.

4. Clotting time

4.1 Method development

The method for the clotting time assay was based on a simple procedure presently in use in the Aquatic Science Research Unit, Curtin University (Fewtrell, 1998) which is a modification of published methods (Battelle and Kravitz, 1978; Kopácek et al., 1993). The clotting time was evaluated by examining the flow of 25 µL of hemolymph inside a plain soda lime glass capillary tube (inner diameter 1.1-1.2 mm, length 75 mm, Chase®). The tube was maintained in vertical position and turned 180°

when the lower part of the hemolymph column reached a distance of exactly 3mm from the lower open end of the capillary tube. The clotting time was defined as the point when the flow of hemolymph ceased.

It was found that in order to achieve reproducible results the above procedure must be carefully followed. In addition, it was important that the assay was commenced immediately following hemolymph removal. As a routine procedure the same hemolymph sample was used for both bacteremia and clotting time assays. The hemolymph was removed as described in Section 5.1, 2-5 drops immediately placed on the surface of the agar plate and the remaining hemolymph sample placed in an Effendorf tube. Using an automatic pipette, 50μL of hemolymph was removed and placed in a second tube which was then aspirated into the capillary tube by placing the tip of the tube in contact with the surface of the hemolymph. Care was taken to ensure that no air bubbles entered the capillary tube. The tube was then repetitively inverted as described above and the time taken for movement of hemolymph within the tube to cease was recorded (clotting time).

4.2 'No clot reaction'

A surprising finding was that the hemolymph of a proportion of lobsters (approx 10%) did not clot despite the rocking motion being continued for more than two minutes. Following this finding it was decided that observations would be terminated after 90 secs and any sample which had not ceased to move by this time was designated 'impaired clotting' or no clot reaction. The proportion of lobsters with hemolymph exhibiting this characteristic was expressed as %prevalence for the group of lobsters under study. In three experiments on post harvest factory stored and laboratory stored lobsters in which clotting time was measured, the %prevalence in the total group of lobsters sampled during the course of the experiments ranged from 6.3 to 18.8% (Table 8). Higher %prevalence results were observed in laboratory stored lobsters compared to factory stored lobsters.

Table 8 Percentage prevalence of 'no clot' reaction in factory and laboratory stored lobsters

Source of Lobsters	Number	% 'No Clot'
Fremantle Factory (April '99)	80	6.3
Fremantle Factory (May '99)	16	12.5
MML (Jan '99)	48	18.8
MML (May '99)	16	18.8

4.3 Preliminary study of effect of stressor exposure on clotting time

A preliminary study of the influence of stressor exposure on clotting time was carried out as a part of the long term immersion trial in MML (Cht 6, Section 5). Hemolymph was collected from eight groups of lobsters (n=4) either exposed to air (treatment) or held submerged (controls).

This preliminary trial showed that there was a tendency for clotting time to be initially reduced in lobsters exposed to air (Table 9). The general conclusion was that there were significant trends between treatment and control groups, with treatment groups showing either shorter or similar clotting rates to those of the controls. An ANOVA analysis showed a significant difference between the treatment and control groups ($P<0.05$), which was not as evident in the breakdown of the group comparisons (Table 8). However, due to small sample size (n=4) these findings were not conclusive and further tests were conducted. The results of this study are provided in Jussila et al. (2001) and briefly described below.

Table 9 Preliminary study on influence of air exposure on clotting time. Means with different superscripts are significantly different ($P<0.05$).

Sampling and air exposure time (h)	Clotting time (sec)	
	Treatment	Control
0	35 ± 8^{b}	$68 \pm 10^{a,b}$
1	34 ± 6^{b}	$51 \pm 4^{a,b}$
3	$66 \pm 14^{a,b}$	$62 \pm 6^{a,b}$
24	$64 \pm 7^{a,b}$	78 ± 6^{a}

4.4 Influence of physical handling and exercise on clotting time

Based on the results of the preliminary trial, an experiment on the effect of a stressor (2 min exercise) on the hemolymph clotting time was performed. In this experiment lobsters (n=8) were sampled after 2 min of physical handling and exercise (physical removal from tank, physical disturbance including tail flapping) and then returned to the aquaria. A post exercise sample was taken from the same lobsters 60 min post exercise (repeated sampling). Initial and final controls (n=8) were also sampled to control for possible diurnal variations and sampling disturbance in the studied parameters.

The clotting time experiment showed that a mild stressor, 2 min of exercise, slightly decreased the hemolymph clotting time in the immediate post-exercise samples (Fig. 14), while there was a significant decrease in clotting time 60 min post-exercise. Both of the control groups had similar clotting times. The results provided a strong indication that physical disturbance and and/or exercise caused a decrease in clotting time in the western rock lobster.

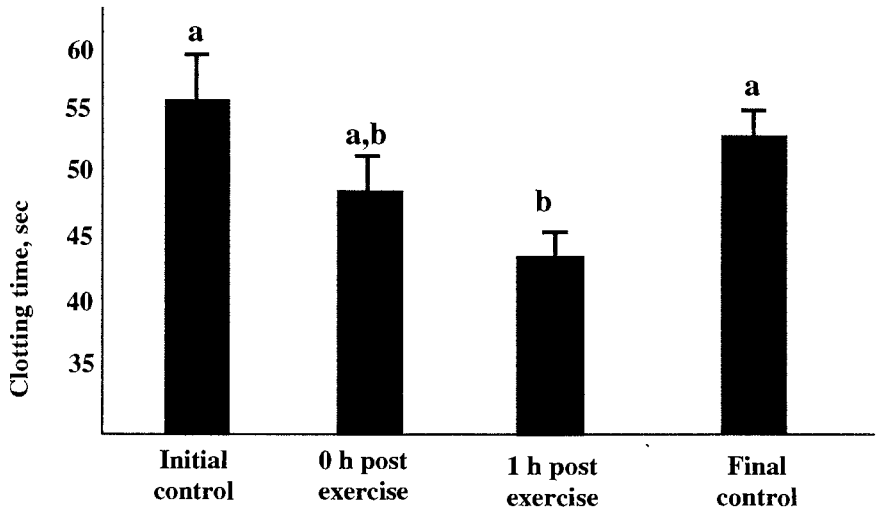


Figure 14 Hemolymph clotting time after 2 min physical disturbance and exercise. Means with different superscripts are significantly different ($P<0.05$).

4.5 Reproducibility of clotting time assay

The reproducibility of the clotting time assay was studied using two approaches. In the first study (Table 10) two observers determined the clotting time in multiple hemolymph samples taken from three different lobsters. The lobsters had been held for approximately six months in a tank at MML at the time of hemolymph collection. The hemolymph sample was taken from the lobster and placed into two Eppendorf tubes which were then taken by the individual observers and the assay performed as described in Section 4.1. A high level of precision was obtained, the mean clotting time of Observer B being only 0.3% lower that that of Observer A (Table 12). The coefficient of variation of the duplicate values was 3.7%.

Table 10 Reproducibility of clotting time assay

Lobster No.	Clotting Time (sec)	
	Observer A	Observer B
1	38	42
	34	34
	30	30
	30	29
2	34	31
	30	30
	35	34
	26	29
3	20	17
	25	25
Mean±SE	30.2 ± 1.7	30.1 ± 2.0
CV %	3.7	

In the second study the clotting time was determined by two observers during a stress trial conducted at MML using the same procedure described above. The lobsters had been held for approximately four months in a tank at MML at the time of hemolymph collection. The mean clotting time for the first observer (52.8±21.4 secs; Mean±SD) for the 13 pairs of estimations was 9.5% lower than that obtained by the second observer (58.0±29.7 secs) indicating a lower precision than was seen in the other reproducibility test.

4.6 Influence of lobster sex on clotting time

The influence of lobster sex on clotting time was studied by measuring clotting time in 80 lobsters (40 males and 40 females) sampled at a Fremantle processing factory in April, 1999. There was no significant difference (t-test; $P>0.05$) between the clotting time for male (39.9±1.6 secs) and female (40.8±1.3 secs) lobsters.

5. Bacteremia Assay

5.1 Assay procedure

The presence of bacteria in the hemolymph of lobsters was determined by two different procedures – a qualitative method (presence or absence of bacteria in a hemolymph sample; expressed as %prevalence of bacteremia in a group of lobsters) and a quantitative approach in which the number of colony forming units per ml of hemolymph (CFU/ml) was estimated. In the latter approach it was occasionally impossible to count the number of colonies due to the presence of large numbers of bacteria in the hemolymph. To overcome this difficulty, and to retain the ability to provide a quantitative measure of bacteria numbers, a ranking scheme was developed (Table 11). Results obtained in the latter part of the study were reported as either CFU/ml or as a bacterial colony count rank. Prior to this time %prevalence was the measure used to assess bacterial numbers in lobster hemolymph.

Table 11 Bacterial colony ranking scheme

CFU/ml	Colony Rank
<10 or 25*	0
10 or 25 – 250	1
251 – 500	2
501 – 750	3
751 – 1000	4
1001 – 1250	5
1251 – 1500	6
1501 – 1750	7
1751 – 2000	8
2001 – 2250	9
2251 – 2500	10
2501 – 2750	11
2751 – 3000	12
>3000	13

* Different lower unit depending on whether 2 drops (25 CFU/ml) or 5 drops (10 CFU/ml) placed on agar plate

The prevalence of bacteremia in a group of lobsters was assessed by the following procedure. A hemolymph sample was collected in a 2ml sterile syringe from the ventral sinus at the base of the fifth walking leg following sterilisation of the sample site by swabbing with 70% alcohol. Three drops of hemolymph were placed on a marine blood agar plate and the sample was spread over the surface of the plate by gently agitation. The plates were carefully inverted and left at room temperature for up to 5 days. If any colonies were observed on the agar surface after the incubation period the lobster was designated as exhibiting bacteremia.

For the quantitative measure of bacteremia, two drops of hemolymph were carefully placed on the surface of the agar plate. Care was taken to ensure that the surface of the drop did not contact the agar during application as this would introduce inaccuracies in volume delivery. The volume of a drop was found to be approximately 20µl and this value was used to calculate CFU/ml. Towards the end of the project this step was modified to apply 5 drops to the plates in order to improve accuracy and simplify the calculation of CFU/ml. Following application of the hemolymph to the agar surface the plates were carefully inverted and left to incubate at room temperature for 3 days. The number of colony forming units in the drops was summed and the results were expressed as CFU/ml.

When results were expressed as colony rank rather than CFU/ml, data were analysed using non parametric statistics.

5.2 Reproducibility of bacteremia assay

The reproducibility of the bacteremia assay was studied by performing duplicate assays on 8 lobsters, one hemolymph sample being withdrawn from the right ventral sinus (RVS) and the other from the left ventral sinus (LVS). Duplicate results from other immune parameters studied in the same experiment (clotting time and THC) are also added for comparison (Table 12).

Table 12 Reproducibility of bacteremia assay

Lobster No.	Bacteremia Mean CFU/ml		Bacteremia Mean Colony Rank		THC ($x10^6$ cells/ml)		Clotting * Time (sec)	
	LVS	RVS	LVS	RVS	LVS	RVS	LVS	RVS
1	0	0	0	0	2.06	1.96	46	50
2	0	0	0	0	4.63	4.05	54	47
3	0	0	0	0	8.64	8.42	-	-
4	0	0	0	0	8.20	5.92	45	55
5	2.5	0	1	0	15.02	10.01	-	-
6	0	2.5	0	1	7.20	4.52	61	59
7	2.5	5.0	1	2	14.38	14.26	37	36
8	0	0	0	0	5.84	6.30	71	72
Mean	0.63	0.94	0.25	0.38	8.25	6.93	52.33	53.17
±SE	±0.41	±0.66	±0.16	±0.26	±1.59	±1.37	±5.01	±4.95

LVS = Left ventral sinus
RVS = Right ventral sinus
*- = No clotting within 90 secs.

The same or similar results for CFU/ml and bacteremia were obtained in all eight lobsters. However, the overall levels of bacteremia were low. Thus, while these results provide a preliminary assessment of the reproducibility of the bacteremia assay in lobsters with low bacteremia the test should be repeated in lobsters with a high level of bacteria. The reproducibility of the other parameters was similar to that observed in other studies of reproducibility (Cht 4, Sections 2.4 and 4.5).

5.3 Frequency distribution of bacterial colony rankings

The frequency distribution of mean colony ranks observed in well acclimated lobsters in a presumably low stress status (Cht 5, Table 29(a), Fremantle (2) April '99, pooled results for lobster groups (1) and (2) (20 lobsters)) and a presumably high stress status (Cht 5, Table 29(b) MML Jan '99, pooled results for lobster groups (1) and (2) (16 lobsters)) showed different distribution patterns (Fig. 15). In the lobsters with presumably low stress status (K&F) the colony ranking was either 0 (no bacteremia) or 1 (25-250 CFU/ml). In the presumably high stress group the majority of lobsters had either a low colony ranking or a high ranking with few intermediate values. A frequency distribution similar to that seen in the presumably high stress group was also seen in lobsters sampled during the November and March GFC trials (Fig. 16; Cht 7, Section 5).

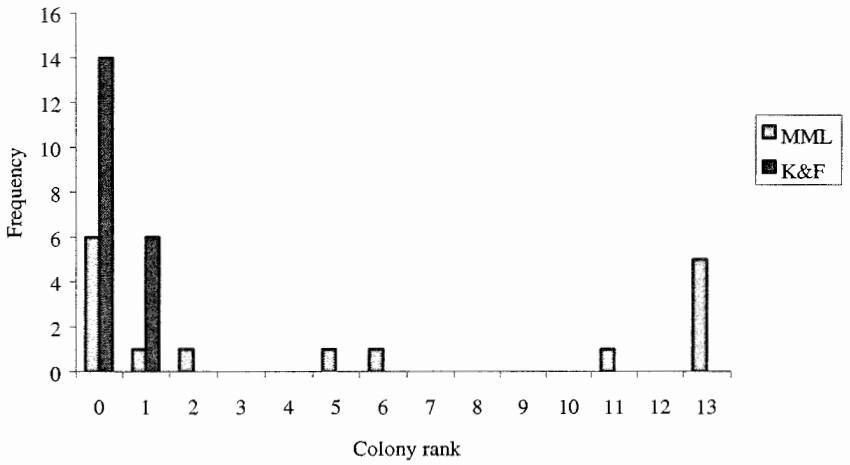


Figure 15 Frequency distribution of colony ranks of lobsters with high stress status (MML, laboratory stored lobsters, n=16) compared to frequency distribution of colony ranks of lobsters with low stress status (K&F, factory stored lobsters, n=20)

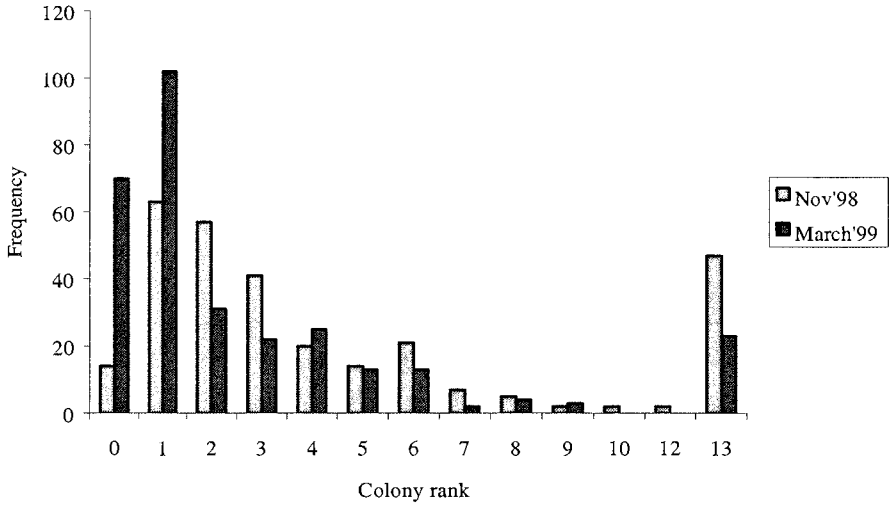


Figure 16 Frequency distribution of colony ranks of lobsters from the November and March factory trials (n=294 (November trial); 313 (March trial))

The results suggest that bacteremia will be occasionally seen in individual members of a group of relatively unstressed lobsters but the number of bacteria in the hemolymph will be low. When a group of lobsters are in a high stress state those lobsters with bacteremia are likely to have higher levels of bacteria in their hemolymph. The transition from no bacteria to large numbers of bacteria would appear to be rapid, resulting in few observations of lobsters in the transition state (colony ranks 4-12).

5.4 Influence of physical handling and air exposure on bacteremia

The influence of physical handling and air exposure on bacteremia was studied. Ten lobsters were removed from a factory tank, placed in a foam box and shaken for 2 mins. They were then left in air for two hours and hemolymph was removed for bacteremia assay (Fig 17; test; no repetitive bleeding). A second group of ten lobsters from the same tank was sampled at the commencement of the test and a third group was sampled after 2h. (Fig 17; control; no repetitive bleeding).

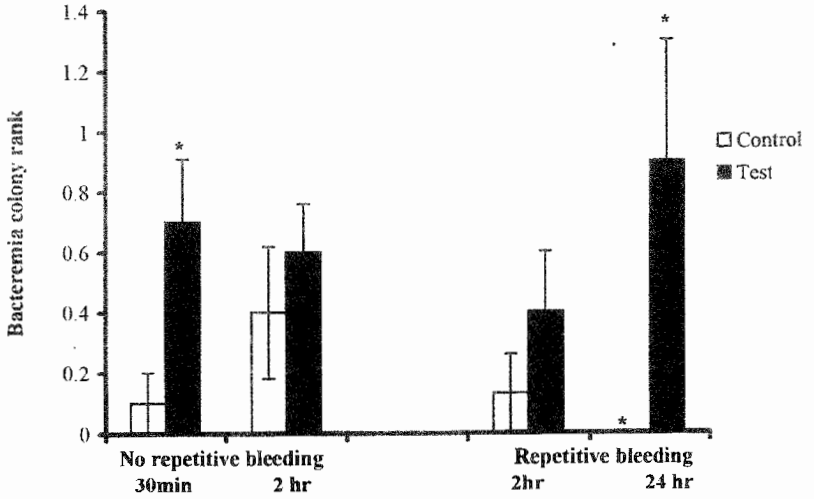


Figure 17 Bacteremia levels following handling and air exposure with or without repetitive bleeding (mean colony rank)
* Bacteremia level in test lobsters significantly higher than bacteremia level in matching control ($P < 0.05$)
n = 10 lobsters: times = length of air exposure

In a further trial, laboratory stored lobsters were removed from a tank at MML, sampled for bacteremia (2h and 24h controls, repetitive bleeding). Matching groups of test lobsters were stored in air at ambient temperatures and transported to another laboratory at Curtin University and then sampled again (2h and 24h test; repetitive bleeding).

In both studies the test lobsters showed higher levels of bacteremia compared to control and these differences were significant in the ½h no repetitive bleeding and the 24h repetitive bleeding groups. The results suggest that bacteremia develops following exposure to a handling and air exposure stressor.

5.5 Bacterial species found in well acclimated lobster hemolymph

Speciation of bacteria present in well acclimated lobster hemolymph was performed on stored lobsters exhibiting high levels of bacteremia (Cht 6, Section 7). The hemolymph was sampled for the bacteremia assay and cultured as described above. Most bacterial colonies cultured from these lobsters had a similar colony morphology. Three samples of the bacterial species producing these colonies were submitted to Agriculture WA for bacteriology and identified as *Rhodococcus maris*.

Bacteriology was also performed on 25 well acclimated factory stored lobsters examined in the first year of the study. This study was performed by the UWA Department of Microbiology under the supervision of Assoc. Prof. Thomas Riley. None of the well acclimated lobsters had bacteria in their hemolymph. However, *Vibrio alginolyticus* and other *Vibrio* species were present in the hemolymph of one of the 25 lobsters sampled before placement in factory tanks (fresh arrivals) and gram negative bacilli were present in the hemolymph of another of the 25 fresh arrivals examined for bacteriology.

6. Antibacterial Activity Assay

6.1 Development of methodology

6.1.1 Initial method

The initial method used for assessing antibacterial activity in western rock lobster in this project was based on the original method described by Ueda et al. (1990) for Japanese spiny lobster *Panulirus japonicus*. Some modifications were introduced to the method. According to Ueda et al. (1990), the methodology provided an accurate means of assessing antibacterial activity in the serum of lobsters.

6.1.1.1 *Serum preparation*

After swabbing the sampling surface of lobster with 70% ethanol, the hemolymph was collected from the pericardial sinus with a sterile 2ml syringe and 24-gauge needle and transferred into a sterile Eppendorf test tube. The hemolymph was allowed to form a firm clot at 4°C overnight. The clot was then broken up and centrifuged at 2,000 x g for 15 min at 4°C. The supernatant, which constituted a small fraction of serum, was transferred to another sterile Eppendorf tube. The remaining clot was dragged to the top of the tube with a sterile glass rod and left there overnight at 4°C to allow further retraction, which resulted in more serum. This serum was combined with the previous portion and used for bactericidal assays.

6.1.1.2 *Preparation of bacterial suspension*

Several *Vibrio* spp. were initially trialed as target cells for assessing the antibacterial activity of lobster hemolymph. Bacterial cultures were obtained from School of Biomedical Sciences, Curtin University of Technology and from Department of Microbiology, UWA and maintained on blood agar plates throughout the experiment. A fresh bacterial suspension was prepared in advance each time the assay was to be performed by inoculating peptone water with the colonies from agar plates and incubating the inoculum at overnight. The bacterial cells were harvested by centrifugation, washed two times with physiological buffered saline (PBS) and then prepared at the concentration of 1-2 x 10^4 cells/ml in 2%NaCl-PBS.

6.1.1.3 *Assay for antibacterial activity*

An aliquot of 0.1 ml of suspension of live bacteria was added to 0.7ml of 2% NaCl-PBS and 0.2 ml of serum, and this test mixture (T) was incubated at room temperature for 2 hours. Control mixture (C), which contained heated serum (60°C, 1 hour) instead of the fresh serum, and contamination control mixture (CC), which did not contain bacterial suspension, were incubated similarly. After incubation, 0.1ml from each mixture was spread onto agar plates and bacterial cells were allowed to grow for 48 hours at 27°C before colony counting. The antibacterial activity (ABF) was calculated using numbers of colony forming units (CFU):

$$ABF = 1 - (CFU \text{ from } T - CFU \text{ from } CC)/CFU \text{ from } C$$

The value of 1 represented the highest activity, the value of 0 the lowest activity.

Replacement of fresh serum with heated serum rather than with buffer solution in control mixture was the major modification of the original method. This modification was introduced as the buffer solution in the control mixture did not provide for an adequate bacterial growth which resulted in very low colony numbers from control mixtures. Heating was shown to inactivate serum bactericidal activity (Cornick and Stewart, 1968; Evans et al., 1968; Stewart and Zwicker, 1972), therefore heated serum was added to control incubation tubes to provide the similar nutrients as in experimental tubes.

6.1.1.4 *Outcomes of the initial assays*

Antibacterial activity was initially tested against four *Vibrio* spp., *V. parahaemolyticus*, *V. alginolyticus*, *V. fluvialis*, and *V. vulnificus*. "Red" lobsters kept in the hatchery tanks in the Aquatic Science Field Trial area after the end of the 1996/97 season were used as test animals for developing the assay. Antibacterial activity was demonstrated consistently only against *V. parahaemolyticus* in the range of 0.397-0.608 (Table 13).

Table 13 Antibacterial activity (ABF) of lobsters' sera against *Vibrio parahaemolyticus*

Lobster No	CFU from T	CFU from C	CFU from CC	ABF
1	322	524	6	0.397
2	262	486	0	0.461
3	259	550	0	0.529
4	TNTC	TNTC	0	-
5	259	660	0	0.608
6	62	121	0	0.488
7	68	171	0	0.602
8	322	549	0	0.413

TNTC = too numerous to count

6.1.2 Selection of a target bacterial species for the assay

Antibacterial activity of lobster serum against *V. parahaemolyticus* was compared with antibacterial activity against bacterial species from other genera to select the species which would elicit the strongest response from lobster serum. *Pseudomonas fluorescens*, *Ps. aeruginosa*, *Staphylococcus aureus*, *Bacillus cereus*, *Escherichia coli* W578 and *E. coli* A128 along with *Vibrio parahaemolyticus* were tested. Hemolymph from five animals was pooled to obtain a serum sample to be tested with seven bacterial species in a side by side assay. Four pooled samples were tested and the highest activity of 0.852 was demonstrated against *E. coli* A128 (Table 14).

Table 14 Antibacterial activity (ABF) of lobsters' sera against different bacterial species

Pooled sera N	Ps.fluor	Ps.aeru	S.aureus	B.cereus	V.para	E.coli A128	E.coli W578
1	0.000	0.167	0.782	0.000	0.225	0.865	0.390
2	0.010	0.454	0.535	0.123	0.000	0.900	0.495
3	0.231	0.394	0.485	0.000	0.425	0.826	0.528
4	0.476	0.174	0.558	0.000	0.029	0.816	0.412
Mean	0.179	0.297	0.590	0.031	0.170	0.852	0.456
±SE	±0.112	±0.074	±0.066	±0.031	±0.099	±0.019	0.033

Escherichia coli strain A128 was selected as target species for antibacterial activity assays as western rock lobster serum reacted more strongly with this species than with other species. The high activity allows for more reliable and sensitive assessment of antibacterial activity to be performed.

6.1.3 Reproducibility of ABF assay using initial assay technique

Hemolymph from five animals was pooled to obtain a serum sample to be tested for reproducibility of results. Three pooled sera were tested against *E.coli* A128 in nine separate assays for each preparation. The results of these assays are shown in Table 15.

Table 15 Antibacterial activity (ABF) in successive assays

Assay #	Pooled serum 1	Pooled serum 2	Pooled serum 3
1	0.383	0.684	0.776
2	0.411	0.691	0.718
3	0.410	0.737	0.684
4	0.438	0.698	0.760
5	0.397	0.698	0.712
6	0.398	0.771	0.676
7	0.369	0.766	0.771
8	0.411	0.737	0.725
9	0.383	0.755	0.743
Mean	0.400	0.726	0.729
SD	±0.020	± 0.034	± 0.036
C.V. %	5.0	4.7	4.9

Coefficient of variation ranged between 4.7 and 5.0%, which demonstrated an acceptable reproducibility of the assay.

6.2 Localization of antibacterial activity

6.2.1 Freezing-thawing experiment

Most early reports have assumed that antibacterial activity is located in lobster serum. However, in the recent research of Chisholm and Smith (1995), antibacterial activity was revealed in circulating hemocytes of squat lobster and Norwegian lobster. These authors suggested that in the earlier studies hemocyte lysis was not prevented which resulted in antibacterial activity release from hemolymph cells into plasma.

To establish the actual source of antibacterial activity in the western rock lobster we conducted the following test. Hemolymph mixed with anticoagulant was subjected to freezing and thawing several times. This treatment is supposed to cause hemocyte lysis which would result in release of antibacterial activity from hemocytes into plasma if such activity is present in hemocytes. Lysed hemocytes were then removed by centrifugation and antibacterial activity was determined in remaining plasma. This activity was compared with antibacterial activity of the serum of the same lobster obtained at the same time and analysed side by side. The results of this experiment are shown in Table 16.

Table 16 Comparison between serum antibacterial activity and antibacterial activity of plasma following its release from hemocytes after freezing-thawing

Lobster No	Serum ABF	Plasma plus hemocyte ABF
1	0.450	0.045
2	0.354	0.543
3	0.369	0.000
4	0.206	0.369
5	0.645	0.324
6	0.438	0.383
7	0.637	0.276
8	0.369	0.259
9	0.684	0.000
10	0.369	0.292
Mean	0.452^{a}	0.249^{b}
SE	± 0.049	± 0.057

Mean values in the same row having different superscripts are significantly different ($P < 0.05$)

The results of this experiment did not demonstrate any release of activity from lysed hemocytes. On the contrary, antibacterial activity in plasma which has undergone freezing and thawing was lower than in untreated serum. This suggested that freezing itself might have inhibited antibacterial activity. To confirm this hypothesis, the influence of freezing and thawing on antibacterial activity was tested.

6.2.2 Freezing in liquid nitrogen

Hemolymph of 2-3 lobsters was pooled and distributed into two test tubes. One tube was immediately immersed into liquid nitrogen ("nitrogen"). Another pair was left on ice ("normal"). Six such pooled hemolymph samples were obtained and distributed into subsamples in the same way. On arrival back to the laboratory, "nitrogen" subsamples were placed into a freezer while "normal" subsamples were placed in a fridge and processed as described in Cht 4, Section 6.1.1.1 to obtain sera. Prior to performing antibacterial activity assays, "nitrogen" subsamples were defrosted.

Antibacterial activity in hemolymph subjected to deep freezing and thawing was significantly lower than in hemolymph stored on ice (Table 17).

Table 17 Effect of freezing on antibacterial activity

Sample No	Subsamples*	
	"Normal"	"Nitrogen"
1	0.459	0.000
2	0.485	0.404
3	0.429	0.000
4	0.496	0.109
5	0.648	0.305
6	0.322	0.440
Mean	0.473^{a}	0.210^{b}
SE	± 0.043	± 0.081

* See text for explanation of "normal" and "nitrogen".
Mean values in the same row having different superscripts are significantly different ($P < 0.05$)

6.2.3 Lysis of hemocytes by sonication

As freezing-thawing method appeared to be unsuitable for hemocyte lysis, in the next trial sonication was used to destroy the hemocyte membrane and release hemocyte content into plasma. Hemolymph was collected into anticoagulant (20 mM N-ethyl-maleimide, 10% sodium citrate and 3% sodium chloride, NEM) at a ratio 1:1, sonicated for five minutes in iced water in a Branson 1200 sonicator and centrifuged. The supernatant containing plasma and a hemocyte lysate was analysed for antibacterial activity. The results were compared with the activity of sera obtained from the same lobsters. Antibacterial activity in plasma with hemocyte lysate was significantly higher than that in serum (Table 18).

Table 18 Effect of sonication on antibacterial activity in hemolymph

	ABF	
Sample No	Plasma + Hemocyte Lysate	Sera
1	0.778	0.231
2	0.683	0.167
3	0.784	0.130
4	0.722	0.237
5	0.742	0.435
Mean	0.742^a	0.240^b
SE	± 0.019	± 0.053

Mean values in the same row having different superscripts are significantly different (P <0.05)

It was concluded that antibacterial activity is present in hemocytes and therefore sonicated and centrifuged hemolymph should be used as a source of antibacterial activity in future experiments.

The hemocyte lysis by sonication was confirmed directly by comparing hemocyte numbers in hemolymph before and after sonication. Total hemocyte count decreased by 40% following sonication.

However, it was observed that numbers of *E. coli* colonies from both control and test incubation mixtures were lower with hemocyte lysate than with sera. The possible explanation for this phenomenon could be the inhibitory effect of anticoagulant into which hemolymph was collected. This issue was addressed in subsequent experiments (Cht 4, Section 6.4).

6.3 Development of rapid ABF Assay

An attempt was made to replace the present labour intensive and time consuming antibacterial activity assay by a rapid assay involving the use of lawn bacterial cultures on Petri dishes and 6-mm diameter assay discs impregnated with test hemolymph.

Hemolymph was collected into one of three anticoagulants. An assay disc with an aliquot of sonicated and centrifuged hemolymph was placed on a Petri dish freshly inoculated with *E. coli*. An assay disc with a corresponding anticoagulant only was used as a control.

The following anticoagulants were tested:

1. Tiger Shrimp Anticoagulant (TSAC), 10 mM tris-hydrochloride, 0.25 M sucrose and 0.1 M sodium citrate, adjusted to pH 7.6, the ratio of hemolymph to anticoagulant – 3:1 (Song and Hsieh, 1994).
2. PJBSS, 2.7 g NaCl, 0.95 g KCl, 2.8 g $MgSO_4$, 1.94 g $MgCl_2$, 0.044 g NaH_2PO_4 and 0.3 g glucose in 100 mL of an aqueous solution, adjusted to pH 7.8 using sodium bicarbonate, the ratio of hemolymph to anticoagulant – 1:1 (Aono and Mori, 1996).

3. NEM, 20 mM N-ethyl-maleimide, 10% sodium citrate and 3% sodium chloride, the ratio of hemolymph to anticoagulant – 1:1 (Durliat and Vranckx, 1981).

After 24 h, inhibition zones were observed around the discs containing hemolymph collected into NEM, as well as around NEM control discs. No inhibition zones were observed around other assay discs. This test suggested that antibacterial factors in hemolymph, which are most probably proteins of various molecular weights, are unable to diffuse from a disc into an agar, and rapid assay on an agar plate is unsuitable for testing antibacterial activity.

6.4 Selection of anticoagulant for ABF assay

From the results of the rapid assay study (Section 6.3) it was found that NEM had an inhibitory effect on bacterial growth and therefore could not be used as anticoagulant when hemolymph is collected for antibacterial activity assay.

Similarly, PJBSS did not meet the requirements of the assay as some coagulation was observed upon sonication of the hemolymph samples collected into this anticoagulant.

TSAC did not exhibit an inhibitory effect on bacterial growth and provided a good anticoagulation effect during the whole assay procedure. Therefore this anticoagulant was selected for future use.

The inhibitory effect of NEM observed in the agar plate assay is in agreement with lower numbers of *E. coli* colonies from both control and test incubation mixtures of plasma with hemocyte lysate compared to that of sera (see Section 6.2).

To confirm that ABF activity is released by sonication, the test described in Section 6.2.3 was repeated with only one modification – hemolymph was collected not into NEM anticoagulant but into TSAC. The results of this test are shown in Table 19.

Table 19 Effect of sonication on antibacterial activity in hemolymph collected in TSAC

Sample No	ABF	
	Plasma + Hemocyte Lysate	Sera
1	0.720	0.371
2	0.642	0.213
3	0.735	0.315
4	0.772	0.459
5	0.751	0.383
Mean	0.724^{a}	0.348^{b}
SE	± 0.022	± 0.041

Mean values in the same row having different superscripts are significantly different ($P < 0.05$)

Antibacterial activity in plasma with hemocyte lysate was significantly higher than that in serum (Table 19).

Based on the above described findings, in all experiments conducted after November 1998, the following procedure was used:

- Hemolymph was collected into TSAC at a ratio 3:1 and kept on ice.
- Hemolymph was sonicated for 5 min in iced water.
- Hemolymph was centrifuged at 3000g for 5 min.
- The supernatant was used as a source of antibacterial activity in the antibacterial activity assay with *E. coli* as a target species.

All samples were analysed for ABF within several hours after sampling.

7. Phagocytic Capacity

7.1 Introduction

One of the most important functions of hemocytes in host defence of crustaceans is the recognition and removal of foreign particles which enter the hemocoel. These cell-mediated defence mechanisms involve phagocytosis, nodule formation and encapsulation reactions, of which phagocytosis is the most common, representing the first line of defence after the foreign agent invades the hemolymph (Söderhäll and Smith, 1986; Battistella et al., 1996).

The capacity of hemocytes to phagocytose can be used as an indicator of the immune system potential to defend the host organism from invading bacteria. Phagocytosis is associated with a dramatic increase in the activity of the hexose monophosphate shunt and a sharp increase in oxygen uptake. Oxygen is reduced to superoxide anions (O_2^-) by NADPH oxidase using NADPH as the electron donor. The subsequent reactions involving superoxide anion lead to the production of potent antimicrobial agents, hydrogen peroxide, singlet oxygen and hydroxyl radicals, which are collectively called reactive oxygen intermediates (ROIs). Production of ROIs during phagocytosis is referred to as the respiratory burst.

Phagocytosis has been demonstrated in American lobster *Homarus americanus* both *in vivo* (Cornick and Stewart, 1968) and *in vitro* (Paterson and Stewart, 1974). The level of phagocytosis observed in lobster hemocytes (2%) was low in comparison with some other invertebrates such as molluscs (29-44%) and freshwater crayfish (10%) (Paterson and Stewart, 1974). However, considering the concentration of hemocytes and hemolymph volume in lobster, the authors concluded that the numbers of phagocytic hemocytes in lobsters provide for efficient protective mechanisms (Paterson and Stewart, 1974). Phagocytosis has not been studied in western rock lobster *Panulirus cygnus*.

7.2 Quantification of phagocytosis *in vitro*

Two principle approaches can be used in the quantification of phagocytosis *in vitro*. One approach involves counting the number of hemocytes showing phagocytosis as well as the number of particles contained in the hemocytes using phase contrast or electron microscopy. The other approach quantifies phagocytosis by evaluating ROI production. In the present study the latter approach was followed.

7.2.1 Luminol-dependent chemiluminescence assay

A flash of light is associated with the release of reactive oxygen intermediates during phagocytosis. This "native" chemiluminescence is very weak and cannot be easily detected. However, it can be amplified by adding luminol (5-amino-2,3-dihydro-1,4-phtalazinedione) to phagocytosing cells. Luminol interacts with the ROIs to produce larger and more measurable amounts of light (Klesius et al., 1985). This light production is called luminol-dependent chemiluminescence (LDCL). The intensity of light produced depends on the concentration of ROIs which in turn depends on the rate of phagocytosis taking place in hemocytes.

LDCL has been widely used in research on fish (Scott and Klesius, 1981; Hetrick et al., 1984; Klesius et al., 1985) and molluscs (Bachére et al., 1991; Anderson et al., 1992a, 1992b). Among crustacean species, LDCL was demonstrated in tiger shrimp *Penaeus monodon* where this assay was used to study shrimp response to immunostimulants (Song and Hsieh, 1994). No data on chemiluminescence response of lobster hemocytes are available.

7.2.2 Nitroblue tetrazolium reduction assay

Reactive oxygen intermediates, especially superoxide anion produced during phagocytosis respiratory burst, are efficient reducing agents. When soluble nitroblue tetrazolium (NBT), which readily accepts electrons from various reducing agents, is added during the initial stages of phagocytosis, it is reduced to an insoluble blue formasan. The formasan formed intracellularly can be extracted in an appropriate solution and the amount measured by spectrophotometry. The amount of formasan formed depends on the concentration of ROIs which in turn depends on the rate of phagocytosis taking place in hemocytes.

NBT reduction assay has been applied in molluscs (Anderson et al., 1992b; Lopez et al., 1994; Greger et al., 1995) and fish (Collazos et al., 1994). NBT reduction by phagocytosing hemocytes has been demonstrated in only one crustacean species, *Penaeus monodon* (Song and Hsieh, 1994).

7.3 Assay procedures

7.3.1 Luminol-dependent chemiluminesence assay (LDCL)

Hemolymph was withdrawn into the syringe filled with recommended volume of anticoagulant as described in Cht 4, Section 1.2. Hemocytes were separated from whole hemolymph by centrifugation at 2000 rpm for 10 minutes and then resuspended in anticoagulant to the original volume. The hemocyte concentration (cells/ml) was then determined by the standard counting procedure (Cht 4, Section 2.2).

Test mixtures contained 300µL of 10^{-4} M luminol, 300 µL of 12.5 mg/mL zymosan, 300µL of hemocyte suspension and 100 µL of medium. Phagocytosis was initiated by the addition of 300µL of 12.5 mg/mL zymosan. Immediately after all the reagents were added, the samples were placed in the LKB Wallace 1251 Luminometer and the chemiluminescence response (CL) was measured. Integration mode was used whereby CL responses were accumulated for 15 minutes and then for further 15 mins.

In the corresponding unstimulated mixture, zymosan suspension was replaced with an equal volume of medium and background LDCL was recorded. Chemiluminescence response was calculated by subtracting background LDCL values produced by unstimulated mixtures from LDCL values produced by stimulated test mixtures. The chemiluminescence responses were expressed as millivolts per second per hemocyte.

7.3.2 Nitroblue tetrazolium reduction assay (NBT)

The hemocyte suspension for NBT reduction assay was prepared as described above (Cht 4, Section 7.3.1). The number of hemocytes/ml in the test solution was determined as described above. The test mixture contained 1000µL of hemocyte suspension and 1000µL of 0.2% NBT in the selected medium. Phagocytosis was initiated by the addition of 200µL of 12.5 mg/mL zymosan. Unstimulated samples had 200 µL of medium added to it instead of zymosan. All mixtures were incubated for 60 minutes at room temperature with agitation at 15 minute intervals. The mixture was centrifuged (2000 rpm, 10 minutes), the supernatant discarded, and pellet resuspended in 2 mL of 70% methanol. The suspension was centrifuged as before, the supernatant discarded, and the pellet vigorously resuspended in 1.25 mL extraction solution (6 mL 2M KCl + 7 mL dimethyl-sulphoxide). The samples were centrifuged as before and the absorbance of the supernatant read at 625 nm. The absorbance was used as a measure of formazan produced on reduction of NBT. Phagocytic activity was expressed as the difference between formazan amounts in stimulated and unstimulated incubation mixtures divided by the number of hemocytes in the mixtures.

7.4 Selection of anticoagulant

7.4.1 Hemocyte viability study

7.4.1.1 *Anticoagulants tested*

The viability (stability) of hemocytes in seven different anticoagulant preparations was tested using the trypan blue dye exclusion method. Hemolymph was withdrawn into the syringe filled with recommended volume of ice-cold anticoagulant. 20µL of hemolymph-anticoagulant mixture was mixed thoroughly with 20µL of trypan blue solution. This mixture was loaded onto a hemocytometer with improved Neubauer ruling. Hemocytes were observed at 200X magnification and blue stained hemocytes (dead cells) were counted. All hemocytes within the central, triple-ruled area of the hemocytometer (25 squares) were counted and percentage of viable cells calculated. Hemocyte counts were carried out at time 0, 30 and 60 minutes after hemolymph was withdrawn from the lobster.

Seven anticoagulants were individually tested to determine the most suitable anticoagulant which would prevent clotting of hemolymph and maintain the viability of hemocytes. The following anticoagulants were used:

Anticoagulant 1 (AC1): NEM/Na citrate anticoagulant (Durliat and Vranckx, 1981). 20 mM N-ethyl-maleimide, 10% sodium citrate and 3% sodium chloride. : Hemolymph : Anticoagulant = 1:1

Anticoagulant 2 (AC2): Trypsin inhibitor anticoagulant (Durliat and Vranckx, 1981). 5% soya bean trypsin inhibitor and 3% sodium chloride. Hemolymph : Anticoagulant = 1:1.

Anticoagulant 3 (AC3): Alpha-C buffer (Adema et al., 1994). 20 mM glucose, 6 mM trisodium citrate, 5 mM citric acid, 2 mM EDTA, 3% sodium chloride, adjusted to pH 7.8. Hemolymph : Anticoagulant = 1:1.

Anticoagulant 4 (AC4): Tiger shrimp anticoagulant (Song and Hsieh, 1994). 10 mM tris-hydrochloride, 0.25 M sucrose and 0.1 M sodium citrate, adjusted to pH 7.6. Hemolymph : Anticoagulant = 3:1.

Anticoagulant 5 (AC5): Marine crustacean anticoagulant (Söderhäll and Smith, 1983). 0.45 M sodium chloride, 0.1 M glucose, 30 mM trisodium citrate, 26 mM citric acid and 10 mM EDTA. Hemolymph : Anticoagulant = 1:1.

Anticoagulant 6 (AC6): PanBSS (Aono et al., 1993). 2.81 g sodium chloride, 0.95 g potassium chloride, 2.8 g magnesium sulphate, 1.94 g magnesium chloride, 0.044 NaH_2PO_4, 0.3 g glucose and distilled water to a final volume of 100 mL. Solution adjusted to pH 7.8 using sodium bicarbonate. Hemolymph : Anticoagulant = 1:1.

Anticoagulant 7 (AC7): Modified PanBSS (Aono et al., 1994). 2.81 g sodium chloride, 0.95 g potassium chloride, 2.8 g magnesium sulphate, 1.94 g magnesium chloride, 0.044 NaH_2PO_4, 0.3 g glucose, 0.25 g N-ethyl-maleimide and distilled water to a final volume of 100 mL. Solution adjusted to pH 7.8 using sodium bicarbonate. Hemolymph : Anticoagulant = 1:1.

7.4.1.2 *Viability of hemocytes*

Five minutes after hemolymph withdrawal, the proportion of viable hemocytes was 89-100% in all anticoagulants (Fig. 18). As time progressed, percentage of viable hemocytes decreased steadily. At 60 minutes, trypsin inhibitor anticoagulant (AC2) had the highest proportion of viable cells, followed by AC4, AC1, AC3, AC7, AC5 and AC6 (Table 20). The percent of viable hemocytes in AC5 and AC6 was significantly lower than that in other anticoagulants ($P < 0.05$).

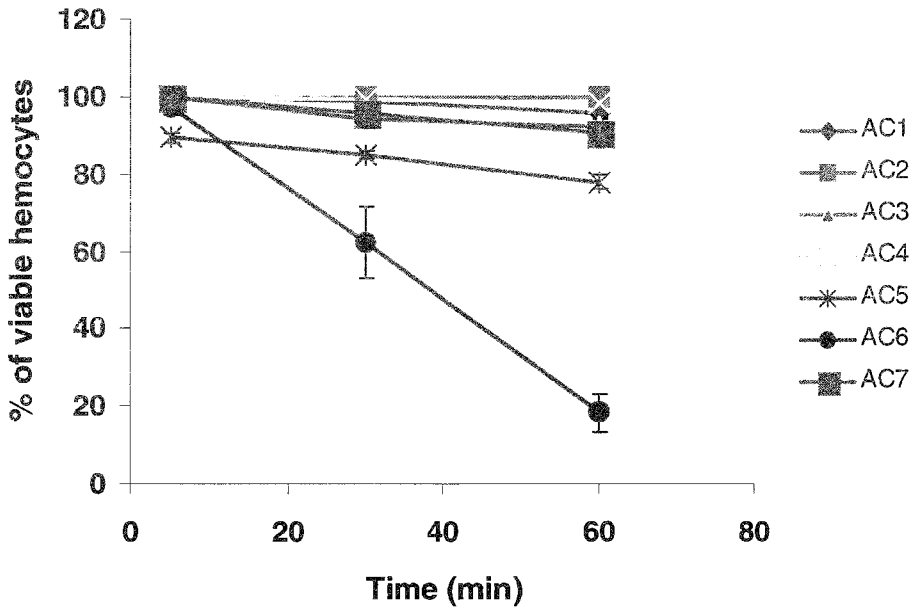


Figure 18 Decrease in percentage of viable hemocytes in different anticoagulants over time (% of viable hemocytes in each anticoagulant preparation after 30 and 60 min incubation)

Table 20 Viable hemocytes in different anticoagulants over time (Mean±SE, %). Mean values in the same column with different superscripts are significantly different ($P<0.05$)

Anticoagulant	5 min	30 min	60 min
AC1	99.7±0.2^{a}	98.8±0.5^{a}	95.8±1.6^{a}
AC2	100.0±0.1^{a}	100.0±0.0^{a}	100.0±0.0^{b}
AC3	99.8±0.1^{a}	94.4±2.3^{a}	92.1±2.7^{a}
AC4	99.7±0.2^{a}	99.3±0.2^{a}	98.4±0.5^{a}
AC5	89.4±1.4^{a}	85.0±1.0^{b}	77.9±1.8^{c}
AC6	97.6±2.2^{a}	62.3±9.2^{b}	18.5±4.9^{c}
AC7	99.7±0.1^{a}	95.9±2.3^{a}	90.6±2.8^{a}

7.4.2 Hemocyte loss

It was noticed that total number of intact or whole hemocytes decreased over time in most anticoagulants (Fig. 19).

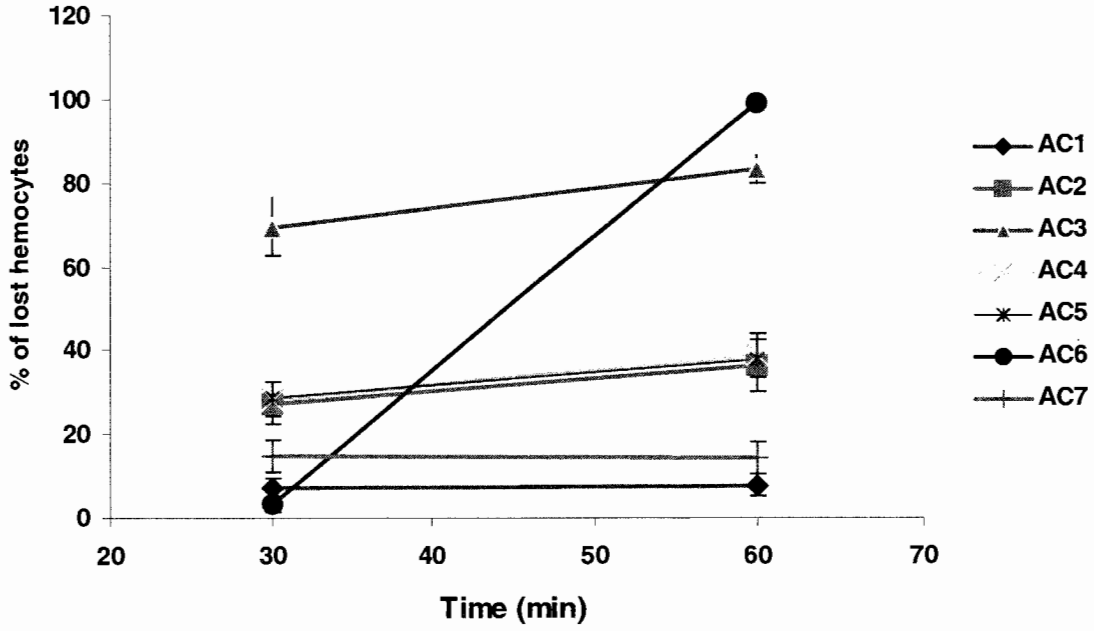


Figure 19 Hemocyte loss over time in different anticoagulants (% of viable hemocytes present in each anticoagulant preparation after 30 and 60 min incubation)

Loss of hemocytes may be due to adhesion, clumping and complete lysis of the hemocytes. The loss of hemocytes was calculated as follows:

$$\text{Hemocyte loss} = \frac{THC_5 - THC_{30 \text{ or } 60}}{THC_5} \times 100\%$$

When THC_5 = Total number of hemocytes at 5 min

$THC_{30 \text{ OR } 60}$ = Total number of hemocytes at 30 or 60 min

Hemolymph in AC1 and AC7 had the least hemocyte loss, 7% and 14% respectively, while hemolymph in AC6 and AC3 had significantly higher hemocyte loss of 99% and 83%, respectively after 60 minutes (Table 21; $P<0.05$).

Table 21 Hemocyte loss in different anticoagulants over time (Mean±SE, %). Mean values in the same column with different superscripts are significantly different ($P<0.05$)

Anticoagulant	30 min	60 min
AC1	7.2±2.67^a	7.7±2.7^a
AC2	27.3±5.0^b	36.2±6.2^b
AC3	69.6±6.9^c	83.0±3.4^c
AC4	28.4±4.2^b	38.8±5.2^b
AC5	28.5±4.3^b	37.9±5.4^b
AC6	3.2±1.9^a	98.8±0.5^c
AC7	14.7±3.8^a	14.4±3.8^a

7.4.3 Other effects of anticoagulants on hemocytes

Hemolymph samples remained fluid within 60 minutes after withdrawal for all seven anticoagulants tested. However, some changes such as hemocyte clumping and lysis were observed, varying with the type of anticoagulant used. These changes are presented in Table 22. It can be seen that AC1, AC2 and AC7 caused the least changes in the hemocytes. This corresponds with the results from the viability test where AC1, AC2 and AC7 were among anticoagulants most efficient in maintaining viability of hemocytes.

Table 22 Effects of anticoagulants on the hemocytes after 60 minutes from hemolymph withdrawal

Anticoagulant	Hemocyte clumping	Hemocyte lysis
AC1	-	+
AC2	-	-
AC3	+++	-
AC4	+	++
AC5	++	±
AC6	+++	-
AC7	+	±

(-) no visible reaction; (±) doubtful reaction; (+) weak visible reaction; (++) moderate reaction; (+++) very strong reaction.

7.4.4 Selection approach for anticoagulant of choice

Anticoagulants that resulted in highest hemocyte viability were AC1, AC2, AC3, AC4 and AC7. Of these five anticoagulants, two, AC3 and AC4, caused significant changes in hemocytes and were therefore rejected. Of three anticoagulants, AC1, AC2 and AC7 that caused insignificant changes in hemocytes while resulting in highest hemocyte viability, AC2 caused 36% hemocyte loss, therefore it was not considered as an efficient anticoagulant for subsequent research in phagocytosis. Between the remaining two, AC7 was selected as the better medium since it showed doubtful hemocyte lysis, while AC1 showed a visible although weak reaction.

7.5 Preliminary laboratory stress trial

7.5.1 Experimental design

A study was conducted to investigate the effects of handling with and without short-term air exposure on phagocytic activity in the western rock lobster. The experiment was conducted in the three independent recirculating systems located in the cool room at MML (Cht 3, Section 2.1). Each system comprised one row of eight tanks. The three rows of tanks were divided into four blocks so that a randomised block design could be applied. Each block contained one of the six treatments (tanks). For the LDCL assay each tank contained one lobster. This experiment was repeated once to give a final sample size of 8 lobsters per treatment group. For the NBT assay each

tank held two lobsters. All lobsters were held in oyster mesh cages within the tanks and a handling disturbance was induced by mild shaking of the cage. One group were left immersed during the handling event and the other group was emersed by siphoning the water out of the tank prior to shaking the cage. Lobsters were acclimatised to the tanks for seven days prior to the experiment.

The following six treatments were used:

- Control 0 (T1) – control lobsters sampled at time 0 (undisturbed and immersed).
- Stressed Immersed 5 (T2) – immersed lobsters exposed to 1 minute of handling and sampled after 5 minutes.
- Stressed Emersed 5 (T3) – emersed lobsters exposed to 1 minute of handling and sampled after 5 minutes.
- Stressed Immersed 120 (T4) – immersed lobsters exposed to 1 minute of handling every half an hour and sampled after 120 minutes since first handling.
- Stressed Emersed 120 (T5) – emersed lobsters exposed to 1 minute of handling every half an hour and sampled after 120 minutes since first handling.
- Control 120 (T6) – control lobsters sampled after 120 minutes (undisturbed and immersed).

Phagocytic activity was estimated by measuring luminol-dependent chemiluminescence and nitroblue tetrazolium reduction by lobster hemocytes (Sections 7.3.1 and 7.3.2).

7.5.2 Results

Luminol-dependent chemiluminescence responses as well as a reduction of NBT to formasan were observed in western rock lobster hemocytes. This clearly indicates that the lobster hemocytes produce a respiratory burst as a response to the phagocytic stimulation by zymosan. The number of hemocytes in hemocyte suspensions used for the both LDCL and NBT reduction assay was counted in order to calculate response produced by one hemocyte. This approach eliminated any errors introduced through variables causing fluctuations in total hemocyte counts.

Data on phagocytic activity of hemocytes in lobsters exposed to various stressors showed that nitroblue tetrazolium reduction assay was more sensitive in detecting changes in phagocytic activity compared to luminol dependent chemiluminescence assay (Table 23).

Table 23 Phagocytic activity in lobsters exposed to different stress stimuli. Mean values in the same column with different superscripts are significantly different (P<0.05)

Treatment Groups	NBT responses (arbitrary units x 10^{-8}) (mean±SE)	LDCL responses (mV/sec/cell x 10^{-9}) (mean±SE)	
		15 mins	30 mins
T1	11.8±3.4^{a}	20.1±9.2^{a}	21.8±9.7^{a}
T2	2.7±1.4^{b}	8.4±3.3^{a}	9.5±4.4^{a}
T3	3.6±2.6^{b}	16.8±3.9^{a}	20.0±8.4^{a}
T4	2.4±1.5^{b}	25.6±8.1^{a}	45.2±14.8^{a}
T5	5.1±2.5^{b}	24.6±6.3^{a}	46.5±18.0^{a}
T6	4.3±1.4^{b}	29.8±6.1^{a}	51.3±21.7^{a}

T1-T6 - See text for explanation of treatment groups

As can be seen from Table 23, stress exposure (T2-T5) reduced phagocytic activity of lobster hemocytes as measured by the NBT method. However, a reduction in NBT response was also seen in the final control group (T6) These lobsters could have been affected by diurnal fluctuations or by visual and aural disturbances resulting from researchers moving past the tanks to sample other lobsters. Since all eight tanks had a common water supply it is also possible that chemical stimuli from test lobsters affected the stress status of control lobsters. The possible influence of visual and aural disturbances on phagocytic response was studied in a subsequent experiment (Cht 6, Section 7) and will be further discussed in that section of the report.

7.6 Reproducibility of phagocytic capacity assay

As the development of this assay procedure was not completed until the latter part of the project no reproducibility studies were conducted.

8. Quantitative histopathology

8.1 Introduction

Quantitative histopathology is finding increasing application in the assessment of physiological responses to adverse environmental conditions. Common approaches to quantification include counting the number of selected lesions in a given area of tissue, semi-quantitative grading of lesions (0, +, ++ etc.) or simply assigning a presence or absence result to each tissue section/test animal and expressing the results as a % prevalence for the group of tissues or test animals. The approach has most often been used to measure the level of parasitic infections in test animals or to quantify a given pathological reaction such as gill hyperplasia or inflammatory lesions in liver or other tissues. There appear to be no reports in the literature where quantitative histopathology has been specifically applied to studies of stress reactions in decapod crustaceans.

One of the few histological responses to stressors which has been described in decapods is a reaction in muscle tissue, generally referred to as idiopathic muscle necrosis (Akiyama et al., 1982). This reaction occurs as a result of exposure of the organism to adverse environmental conditions, hyperactivity, overcrowding, physical

disturbance combined with emersion and exposure to quinaldine (Rigdon and Baxter, 1970; Lightner, 1977; Lakshmi et al., 1978; Akiyama et al., 1982; Nash et al., 1987; Evans et al., 1999a). The etiology of the lesion is poorly understood.

In this study of stress responses in the western rock lobster, quantitative histopathology was investigated as one approach to detecting and measuring stress reactions. The results of this study are given below. Some quantitative histopathology was also applied in the study of causes of mortality in reject lobsters (Cht 8) and in marron exposed to a handling stress (Section 8.5).

8.2 Investigation of histological parameters as stress indicators

Following a preliminary survey conducted on the Fremantle white and red lobsters it appeared that two specific histopathological features – hemolymph clots in the heart and idiopathic muscle necrosis (muscle myopathy), may occur as stress reactions in western rock lobsters.

The identifying characteristics of these two histopathological reactions were as follows:

- Hemolympth clots in heart – large aggregates of hemocytes exhibiting cell adhesion and usually incorporating coagulated hemolymph (Fig. 20). The clots were located in spaces between the muscle bundles of the heart and were distinguished from loose cellular aggregates which occur as a post-morteum artifact on the basis of the cell adhesion and coagulated hemolymph features.

- Muscle myopathy - the lesion was usually focal with some fibres within the muscle fascicle showing myofibril necrosis and degeneration but neighbouring fibres being unaffected (Fig. 21). Pyknotic nuclei were present in most lesions and karyorrhexis was also occasionally observed. Myofibrils in affected fibres were condensed and showed increased eosinophilia. In most lesions these was a clear zone surrounding the centrally condensed myofibrils, but this clear zone was not always present. In the latter type of lesion there was clear evidence of myofibril disorganisation and necrosis but the condensation reaction was absent and there was no clear zone around the affected myofibrils.

Figure 20 Heart section from *Panulirus cygnus* showing hemolymph clot in space between muscle fibres (H&E x 100)

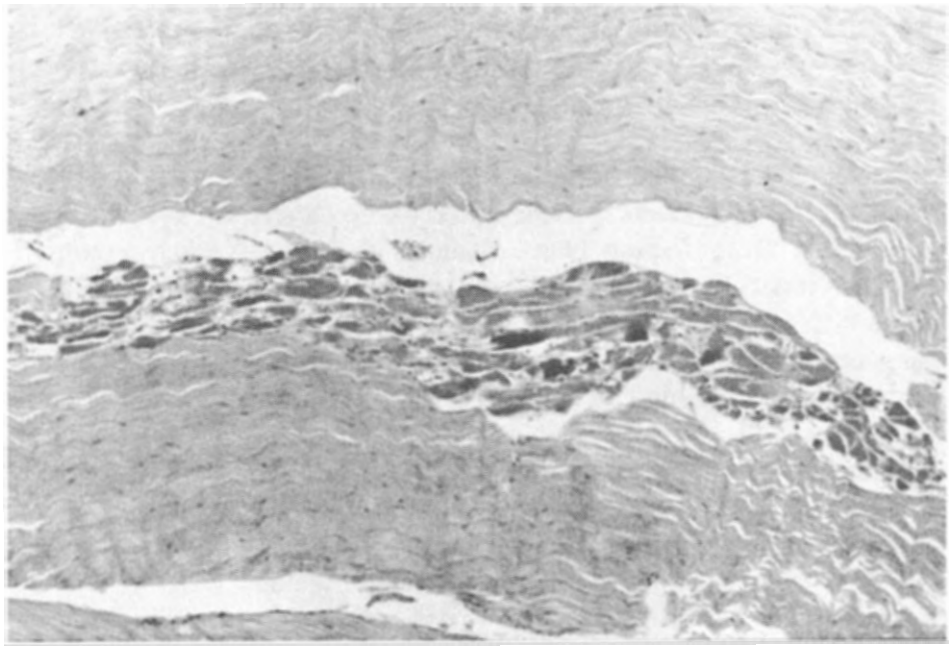


Figure 21 Muscle section from *Panulirus cygnus* showing muscle myopathy (H&E x 100)

Investigations aimed at evaluating the application of these two parameters as stress indicators were conducted in both marron and in lobsters.

8.2.1 Sampling methodology

Lobsters were sacrificed by cutting off the proximal area of the cephalothorax just behind the antennae, from dorsal to ventral surface, with a sharp knife. The walking legs were removed and a cut made at the junction of the cephalothorax and abdomen to separate the tail. Two longitudinal cuts were made on the carapace, one on either side close to the gill chamber. The carapace was separated from the underlying tissues to expose the main organs. Tissue samples (1-2g) were collected from the following organs: hepatopancreas (two pieces), antennal gland (two pieces), heart (two pieces), gills (two, one podobranch from each branchial cavity) and abdominal muscle (two samples taken from the abdominal muscle immediately adjacent to the carapace). These were removed as rapidly as possible and placed in seawater formalin to fix the tissues. The samples were processed, embedded in wax, sectioned and stained in haematoxylin and eosin (Bancroft and Stevens, 1977).

8.2.2 Test animals

Histopathological investigations were carried out on western rock lobsters collected at Fremantle factory (1995/96; Fremantle whites). All lobsters were delivered to the factory by trucks. Three treatment groups were studied: Factory arrivals (truck) (N=8) - sampled soon after arrival at the factory, Accepted, 16 h (N=9) – lobsters which showed no signs of morbidity on arrival nor after 16 h storage in factory tanks and Rejects 16 h (N=9) those which showed no signs of morbidity upon arrival but were moribund following 16 h storage in factory tanks.

Specimens were also sampled from the commercial harvest at a Geraldton factory in December 1996. Some lobsters were delivered to the factory by truck and others were collected directly from fishing boats. Samples from boat transportation included factory arrivals (boat) (N=10) - sampled immediately after boat arrival at the jetty and accepted 48 h (N= 7) - sampled after storage in factory tanks for 48 h. Samples from truck transportation included factory arrivals (truck 2) (N=6) - sampled at arrival to factory by trucks and accepted 16 h (N=10) - sampled after 16 h in factory tanks.

8.3 Quantitative measures for heart clots and muscle myopathy reactions

The clots in the heart and muscle myopathy reactions were assessed quantitatively. With the former, the clots seen in each heart section were counted and the approximate surface area of the section was determined by multiplying the average length by the average width (both determined by visual estimation and microscopic and micrometer measurements). The average number of clots/mm^2 surface area was then calculated.

Muscle myopathy was scored as present or absent and expressed as % prevalence for each treatment group of lobsters or marron.

8.4 Assessment of heart clots and muscle myopathy as stress indicators

The suitability of heart clots and muscle myopathy as stress indicators was assessed using two approaches:

- an experiment was conducted in which marron were subjected to a stressor (physical handling and air exposure) and the hemocyte and histopathological reactions to stress exposure examined. The reactions were quantified in marron exposed to the stressor and values compared to those obtained from control, undisturbed animals.

- a survey was conducted on the prevalence of these reactions in lobsters of presumably differing stress status - fresh arrivals (stressed lobsters), factory stored accepted lobsters (acclimated lobsters) and factory stored reject lobsters (weak lobsters). The reactions were quantified in the three groups of lobsters and mean values compared.

8.5 Marron stress study

8.5.1 Experimental design

The occurrence of idiopathic muscle necrosis and the changes in THC following exposure to physical handling and air exposure was studied. Sixty marron were purchased from a commercial marron farmer in the South West of Western Australia, transported to the laboratory in foam containers and acclimated communally in 6 aquaria for 14 days. During this period approximately half of the marron died and/or were cannibalised by other marron. Immediately prior to the trial the numbers of marron in each of 5 aquaria was adjusted to 6 per aquarium.

At the commencement of the trial, 18 marron from three aquaria were removed from the water and placed in two foam containers where they were left at ambient temperature for 2 h. Nine marron were returned to the aquaria and left overnight before being sacrificed and dissected (24 h test). The remaining nine marron were removed from the box and a hemolymph sample was taken by puncture from the base of the fifth walking leg with a calibrated 1 ml plastic syringe containing an equal volume of cooled citrate/EDTA anticoagulant (Durliat & Vranckx, 1981). The marron were then weighed, sexed, a uropod sample taken for moult stage assessment, sacrificed, dissected and heart and muscle samples collected for histology (2 h test). The muscle samples were taken from the tail immediately adjacent to the carapace. Hemolymph and heart and muscle samples were also collected from six control animals at the commencement of the experiment (three each from two aquaria) (0 h control) and from the remaining 6 animals in these two aquaria which were left undisturbed for the same time period as the 24 h test animals (24 h control). Total hemocyte counts were performed as described in Cht 4, Section 2.2. The heart and muscle tissues were processed into paraffin wax, sectioned, stained with H&E and the heart clot and muscle myopathy reactions determined as described above.

8.5.2 Results

The mean THC for test animals was significantly elevated ($P<0.05$) following physical handling and short periods of air exposure (Table 24). The THC value tended to fall to control levels following 24 h re-immersion. In contrast, muscle myopathy increased following removal from water and levels were still elevated in the 24 h test animals. The heart clot results showed no obvious trend due to stressor exposure.

Table 24 THC, heart clot and muscle myopathy responses to handling and air exposure in the freshwater crayfish *Cherax tenuimanus*

Treatment Group	THC (No. cells/mlx10^{-6})	Heart Clots (No/mm^2)	Muscle Myopathy (% prevalence)
0 h control	2.02±0.11(6)^{a}	0.13±0.06^{b}	16.4
2 h test	3.50±0.39(9)^{b}	0.07±0.03^{a}	55.6
24 h control	2.25±0.40(6)^{ab}	0.03±0.01^{a}	16.7
24 h test	2.64±0.52(9)^{ab}	0.07±0.01^{a}	44.4

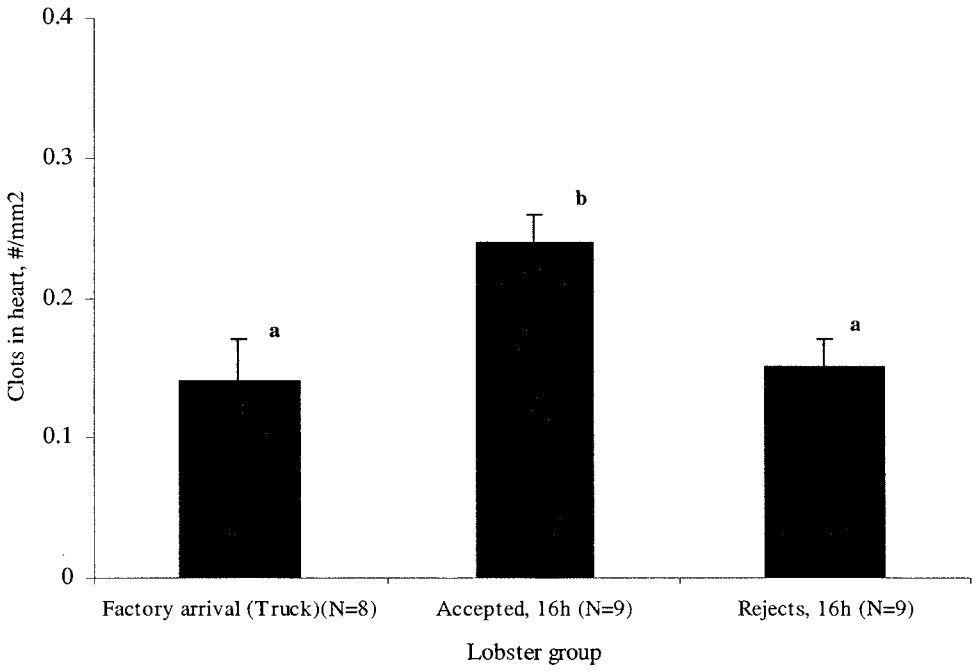
Mean values in the same column with different superscripts are significantly different (P<0.05)
(n) = number of animals studied

8.6 Lobster study

8.6.1 Clots in the heart

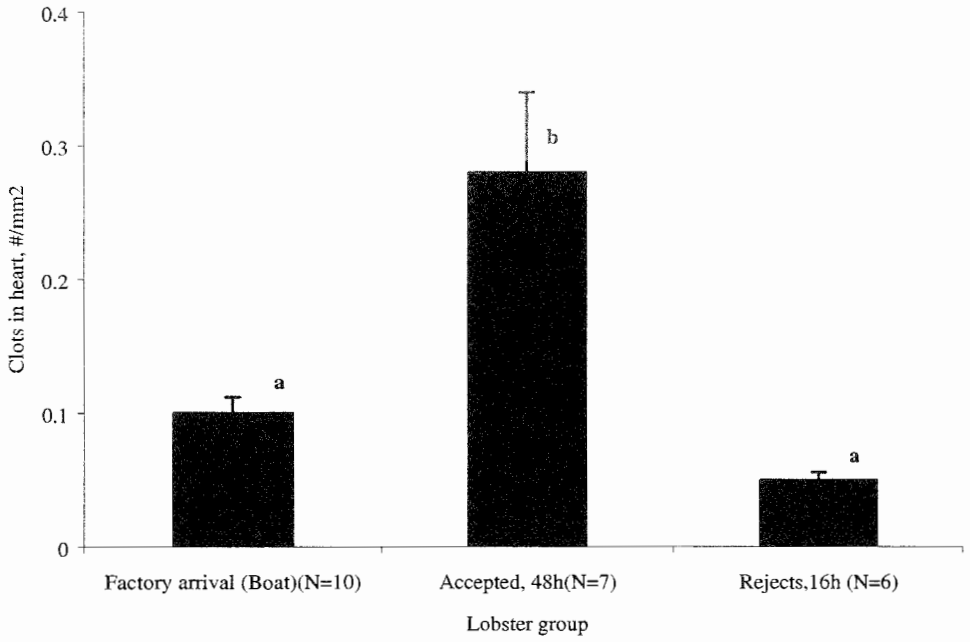
The number of clots in the heart was significantly elevated (P<0.05) following storage in factory tanks (Fig. 22a&b). Reject lobsters had similar levels of clots in the heart compared to the factory arrival lobsters.

Figure 22 Effect of transportation and holding on clots in the heart



(a) Fremantle whites (Dec '95)

Mean values with different letters are significantly different (P<0.05)



(b) Geraldton lobsters (Dec. '96)

Mean values with different letters are significantly different (P<0.05)

8.6.2 Muscle myopathy

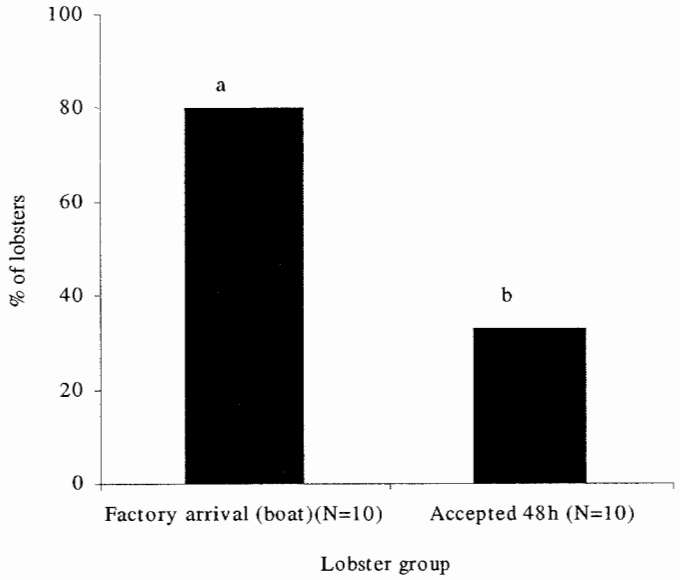
8.6.2.1 *Fremantle whites*

The percentage prevalence of muscle myopathy was 15.4% in accepted lobsters (N=13) and 40.0% in reject lobsters (N=10). This difference was not statistically significant (χ^2; P=0.180).

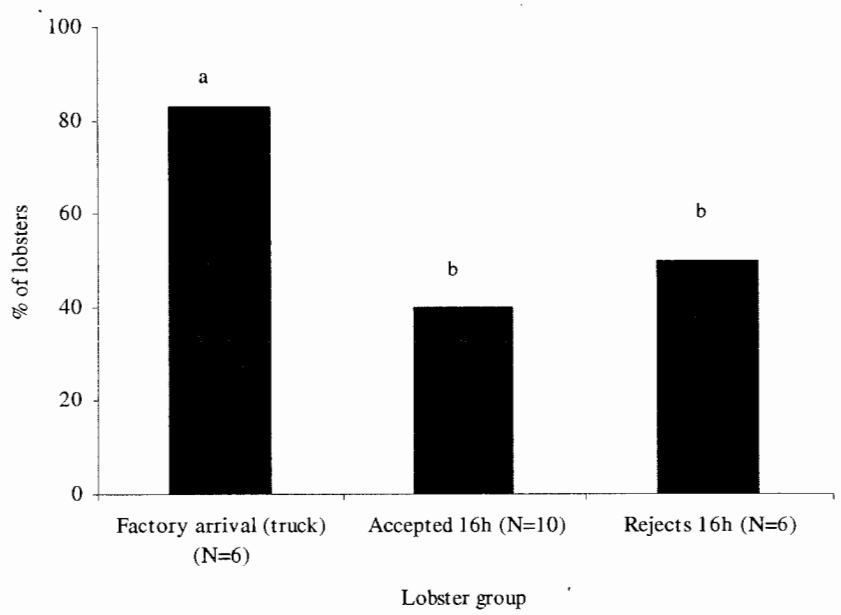
8.6.2.2 *Geraldton lobsters*

Muscle myopathy was significantly higher in stressed lobsters delivered to factory in a boat (factory arrivals) and declined sharply following acclimation in factory tanks (accepted lobsters) (Fig. 23a; χ^2, P-0.025). The levels of muscle myopathy in reject lobsters was similar to that in accepted lobsters stored in factory tanks for the same time period (Fig. 23b χ^2; P>0.05).

Figure 23 Effect of transportation and holding on prevalence of muscle myopathy



(a) Geraldton (Dec '96), boat lobsters



(b) Geraldton (Dec '96), truck lobsters

Mean values with different letters are significantly different ($P<0.05$)

8.7 Conclusions

The statistically significant increase in the level of hemocyte aggregations in heart tissues in accepted lobsters compared to factory arrivals suggests that this reaction is a secondary stress response, occurring after the exposure to the initial stressor. The aggregation of hemocytes following stress exposure may be a method of resolution of stress related changes in the hemolymph. The failure of reject lobsters to exhibit an increased heart clot response could have arisen from the low hemocyte counts which are typically seen in weak lobsters (Cht 8) or some other impairment to the immune response.

Marron heart sections also exhibited hemocyte aggregates similar to those seen in the lobster heart sections, but exposure to a stressor did not cause a statistically significant change in the number of heart clots (Table 24). Other workers have observed intravascular clotting in living freshwater crayfish exposed to environmental stressors (Söderhäll pers. observ.). While it is apparent that the marron used in this experiment experienced a stress response, as demonstrated by the statistically significant increase in THC (Table 24), it is possible that the stressor used in this experiment (air exposure and physical handling) was insufficient to elicit a hemocyte aggregation response in this species.

Muscle myopathy levels were very high (approx 80% prevalence) in lobsters dissected immediately upon arrival at the factory. The decrease in the prevalence of this lesion in factory stored lobsters (accepted lobsters) suggests that the condition, or the process(es) leading to the appearance of this lesion in fixed muscle tissue sections, resolves following tank storage for 16-48h. Muscle myopathy also increased following stressor exposure in marron (Table 24) but did not resolve within 24h. The results provide evidence, as has been found in other species, that muscle myopathy is a stress response in both lobsters and marron. However, there may be species differences in the time taken for the condition to resolve.

While both of the histological reactions investigated showed promise as stress indicators, the methodology required to quantify heart clots or muscle myopathy is laborious and expensive and requires a highly skilled operator for reliable and repeatable results. For these reasons further investigations of these parameters in post harvest lobsters were discontinued following the initial studies conducted in the first 18 months of the project.

CHAPTER 5 NORMAL RANGES FOR IMMUNE PARAMETERS

1. Introduction

If the immune tests developed in this project are to be applied to industry situations it is necessary to establish baseline values for each parameter and 'normal ranges' for apparently healthy post harvest lobsters stored in factory tanks. Two approaches to establishing these ranges were investigated – measurement of immune parameters in undisturbed lobsters collected by SCUBA diving and measurement of parameters in well acclimated, apparently healthy lobsters from factory tanks. The former approach was only utilised for THC assays since technical difficulties precluded conducting other assays on hemolymph samples collected by SCUBA (apart from one study on DHC). However, establishment of normal ranges from results obtained with factory lobsters was achieved for all immune tests except the phagocytic capacity, the last test to be developed.

The minimum acclimation time period used for derivation of normal values for apparently healthy post harvest lobsters held in factory tanks was 16h. Some results obtained in lobsters held in tanks at MML have also been considered in this analysis. However, since these lobsters may have had a compromised health status due to water quality variation and other factors their results were not included in the final determination of normal ranges.

Due to time and funding limitations seasonal and/or diurnal variations in immune parameters were not studied. Other factors which might influence an immune parameter test result, such as sex and moult stage, were not studied in detail for all parameters. This means that while the test procedures reported here can be used in industry to improve post harvest practices, some applications will be hampered by the lack of information on variations in relation to moult stage, time of year, time of day and lobster sex.

2. Source of baseline and acclimated lobsters

2.1 SCUBA collection procedure

Lobsters (N=10-20) were sampled by SCUBA divers working in pairs (Paterson et al., 2001) with one diver using a wire loop snare to catch a lobster (baseline lobsters). Following capture, one diver held the lobster with the abdomen bent under the cephalothorax exposing the arthroidial membrane between the posterior margin of the carapace and the tergum of the first abdominal segment. The other diver took a 200µL hemolymph sample from the pericardial sinus into a 1 mL syringe preloaded with 200µL of sodium-cacodylate anticoagulant. Another 1 mL sample was then taken for assays other than THC. Immediately, the lobster and the sample were taken to the surface and delivered to the fishing boat for further analyses. Samples were taken from both free-ranging lobsters and from animals in pots. In the latter case, lobster pots were set according to standard industry practices and, at the time the pot would normally be raised, divers removed animals from the pots using the wire loop.

Lobsters were sampled in June 1997, July 1997, April 1998, and November 1998 mainly during 11am-3pm from depths ranging from 4 to 12 m. During the lobster season (15 November – 30 June) lobsters were caught from commercial fishing grounds close to lobster pots. Hemolymph samples were stored in ice and THCs later analysed by the manual method.

2.2 Acclimated lobsters

Acclimated post harvest lobsters were obtained from two sources, (1) lobsters delivered to a processing factory and stored in holding tanks for 16 h - 5 days (factory lobsters; 15 groups of 10-20 lobsters) (2) lobsters which were obtained either from the commercial catch or from a WA Department of Fisheries fishing vessel. These lobsters were delivered to MML and held for varying time intervals, up to 6 months prior to sampling (laboratory lobsters).

Factory lobsters were delivered to the factory directly from the lobster fishing boats or by truck from a factory depot. Lobsters were sorted and graded following the standard procedure and only those fit for live were sampled. Lobsters were bled following standard sampling procedure and hemocyte samples were analysed by the standard method for each immune parameter (Chapter 4).

3. Determination of normal ranges

3.1 Total hemocyte counts

The mean values for baseline (SCUBA) and acclimated factory lobsters are shown in Fig. 24 and Tables 25 & 26. Mean values for baseline THCs for free ranging lobsters (wild) ranged between 4.70±0.50 and 5.47±0.77 x 10^6 cells/mL. Significantly higher THCs were obtained in one group of three lobsters sampled in pots 7.91±0.50; (Fig. 24) but the mean THC of the other group of ten 'pot' lobsters was similar to that of the free range lobsters. Mean±SE from the pooled data from the three free range samples (C & D0 stage lobsters only) was 5.02±0.69 x 10^6 cells/mL.

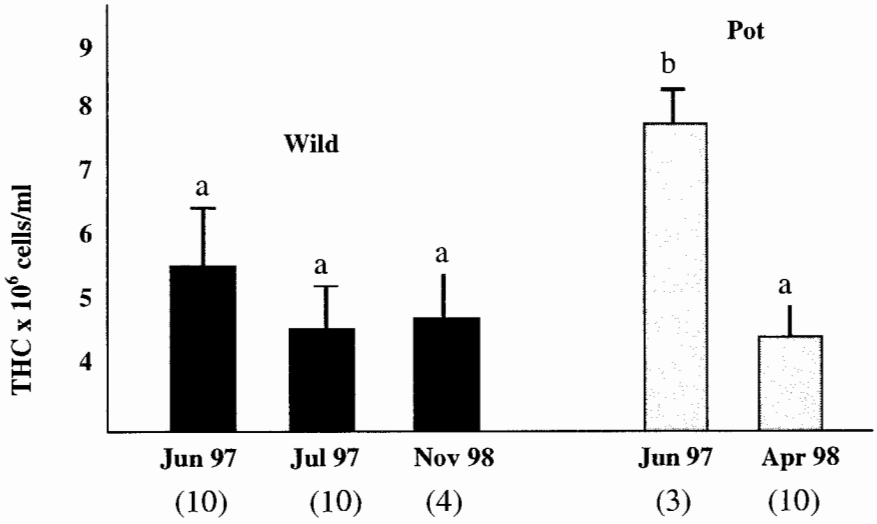


Figure 24 Total hemocyte counts (THC) in western rock lobster (*Panulirus cygnus*) sampled underwater by SCUBA collection. Different letters above the bars indicate statistically significant differences ($P<0.05$). See text for explanation of 'wild' and 'pot'.
(n) = No. lobsters

The significant difference seen between THC in one group of lobsters obtained from pots (Fig. 24; Pot, Jun '97) and the THC values obtained with all other groups of lobsters collected by SCUBA suggests that lobsters from the 'Pot, Jun '97' group may have experienced a stress response at the time of sampling, resulting in an elevation of circulating hemocytes. However, since there was only 3 lobsters sampled in this group, the low sample number may have been the reason for the high mean value.

The mean THC values for 18 groups of 10-23 lobsters delivered to the factory by either boat or truck and stored for 16 h to 5 days ranged from 3.92 to 8.49 (Table 25).

Table 25 THC in post harvest lobsters held in factory tanks (No cells/ml x 10^{-6}; mean±SE)

Delivery Mode	Location and Date	Storage Time 16-24 h	Storage Time 30-120 h
Truck	Fremantle (1) Dec '95	5.63±0.70 (13)	-
Truck	Fremantle (1) Feb-Jun '96	5.35±0.70 (12)	-
Truck	Geraldton Dec '96	6.32±0.60 (10)	6.29±0.61 (10)
Truck	Geraldton Dec '96	8.25±0.35 (10)	7.65±0.57 (10)
Boat	Geraldton Dec '96	-	7.27±0.51 (20)
Boat (1)	Geraldton Apr '97	6.04±0.61 (10)	4.93±0.44 (10)
Boat (2)	Geraldton Apr '97	8.02±0.62 (10)	8.49±0.77 (10)
Boat (1)	Geraldton Jun '97	7.39±0.77 (10)	4.23±1.12 (10)
Boat (2)	Geraldton Jun '97	7.11±0.53 (10)	7.30±0.68 (10)
Boat (1)	Geraldton Dec '97	7.57±0.64 (15)	7.27±0.48 (23)
Boat (2)	Geraldton Dec '97	6.45±0.57 (10)	7.18±0.48 (20)
Truck or Boat	Geraldton Apr '98		3.92±0.10 (10)*
Truck or Boat	Geraldton Nov '98		4.27±0.47 (10)*
Truck	Fremantle (2) Apr '99		6.14±1.08 (10)
Truck	Fremantle (2) Apr '99		7.42±1.42 (10)
Truck	Fremantle (2) Apr '99		7.00±0.50 (10)
Truck	Fremantle (2) May '99		6.46±1.01 (16)
Truck	Fremantle (2) May '99		5.42± 0.50 (16)

* Some lobsters sampled after 16-24 h
Mean ± SD = 6.51 ± 1.25

In those studies in which lobsters from the same delivery were tested after 16h and 40-48h, there was a significant decrease in THC after the longer acclimation time in only two of the eight groups of lobsters studied (Boat (1) Apr '97; Boat (1) Jun '97), suggesting that in most cases 16hr acclimation was sufficient time for THC values to stabilise. The normal range for factory stored lobsters calculated from the 26 mean values shown in Table 25 (mean ± 2SD) was 4.01 - 9.01. This range was slightly higher than that obtained for the baseline lobsters, suggesting that the factory lobsters were still exhibiting elevations in THC resulting from post harvest stressors. There was no significant difference between the THC values obtained in acclimated lobsters sourced from three different factories, one in Geraldton and two in Fremantle (Table 25; Fremantle (1) and Fremantle (2)) or in lobsters which had been initially delivered to the factories in trucks or direct from boats.

THC assays were performed on 13 groups of 10-14 C-stage laboratory lobsters during the acclimation trial conducted in MML from November, 1996 – January, 1997 (MML acclimation lobsters; Cht 6, Section 2; Table 26).

Table 26 THC in laboratory lobsters held in MML tanks (mean±SE)

(a) Acclimation trial

Acclimation Time (Days)	No. Studied	THC x 10^6 cells/ml (Mean±SE)
0	13	8.43±1.22
1	14	5.49±0.72
2	13	5.42±0.68
4	14	4.27±0.51
8	10	5.30±0.88
15	13	7.97±0.76
22	13	5.72±0.61
29	14	7.29±0.85
39	14	10.05±0.94
43	14	7.04±0.82
50	14	9.78±0.59
57	14	6.79±0.84
64	14	7.09±0.95

Mean±SD (excluding Days 0, 39 & 50) = 6.24±1.16

The THC values for two of these 13 groups (Days 39 and 50) were outside the normal range calculated from the factory lobster data, suggesting that these lobsters were stressed at the time of sampling. Possible explanations for these elevated values are given in Cht 6, Section 2.3. The THC value obtained on Day 0, immediately prior to placement in the tanks, was also higher than any other value with the exception of those obtained on Days 39 and 50. If the results for Days 0, 39 and 50 are excluded from determination of mean and standard deviation a normal range (mean±2SD) of 3.92 - 8.56 is obtained. This range is similar to that obtained from the factory lobster data.

In conclusion, it would appear that the THC in post harvest lobsters stored in factory tanks is likely to be slightly higher than that of baseline, undisturbed lobsters (free range lobsters) but similar to that seen in laboratory lobsters held in re-circulating tank systems for extended periods. The recommended normal range for post harvest lobsters stored in factory or laboratory tanks, obtained by averaging the two normal ranges, is therefore 3.97 - 8.79 x 10^6 cells/ml.

3.2 Differential hemocyte counts; % granular cells

Difficulties were experienced in performing hemolymph smears on board the fishing vessels following collection of samples by SCUBA divers. As a consequence DHC assays were only performed during one of the SCUBA sampling trips (June 1997). The mean ±SE DHC values obtained from lobsters were as follows: hyaline cells 20.1±3.2%, granular cells 5.4±1.2% and semi-granular cells 73.6±3.1%. These values were similar to the mean DHC levels obtained in 47 laboratory lobsters sampled during the acclimation trial (Cht 4, Section 3.4): hyaline cells 21.2±1.4%, granular cells 7.0±0.6% and semi-granular cells 71.9±1.7% (Table 5).

A summary of DHC results obtained in factory lobsters is shown in Table 27.

Table 27 DHC values in acclimated factory lobsters (mean±SE)

Acclimation Time	Location & Date	No. Studied	DHC % Hyaline Cells	Semi-granular Cells	Granular Cells
16 h	Fremantle Dec '95	13	29.1±2.9	62.9±2.9	8.0±1.9
	Fremantle Feb-June '96	12	34.4±3.7	54.9±3.8	10.7±1.4
	Geraldton Dec '96 (1)	10	22.6±2.0	71.3±1.9	6.1±0.8
	Geraldton Dec '96 (2)	10	20.2±1.6	73.7±1.5	6.0±1.1
48 h	Geraldton Dec '96 (1)	9	16.1±1.9	74.4±3.4	9.5±2.6
	Geraldton Dec '96 (2)	10	15.8±1.50	74.7±2.1	9.5±1.6
	Geraldton Dec '96 (3)	20	22.6±1.50	69.2±1.7	8.2±0.8
	Fremantle Apr '99 (2)	10	11.9±2.4	81.5±2.2	6.6±0.5
16 h-120 h	Geraldton Mar '98	10	22.5±2.5	71.2±2.0	6.3±0.9
Mean±SD			21.7±6.9	70.4±7.6	7.9±1.7

The proportion of hyaline cells obtained in different lobster groups ranged from 11.9 to 34.4% and the proportion of semi-granular cells from 54.9 to 81.5%. The normal range (mean±2SD) for these two cell types is 7.9 to 35.5 for hyaline cells and 55.2 to 85.6 for semi-granular cells.

The proportional distribution of granular cells in factory stored lobsters was similar to that seen in the MML acclimated lobsters, ranging from 5.9 – 10.7% but higher than that seen in the baseline lobsters (5.4%). However, since only one study was conducted on baseline lobsters, more data are required before a definitive conclusion can be reached that post harvest lobsters have elevated %granular cells compared to baseline lobsters. The normal range (mean±2SD), obtained from data obtained with factory stored lobsters, is 4.5 to 11.3.

3.3 Clotting time

The data set for determination of normal range for clotting time is smaller than for THC or DHC as this assay was developed later in the project. In addition, this test could not be performed on lobsters collected by SCUBA as this was not technically feasible. Lobsters with hemolymph which failed to clot were treated separately in the data analysis.

Clotting time results obtained with acclimated factory stored and laboratory stored lobsters are shown in Table 28.

Table 28 Clotting time values in factory and laboratory stored lobsters

(a) Factory stored lobsters

Location and Date	Acclimation Time	Clotting Time (sec) (mean±SE)	% Prevalence 'no clot'
Geraldton Nov '98	16h – 5d	28.6±1.5 (10)	NA
Fremantle (2) Apr '99 (1)*	48h	39.3±1.8 (9)	10
(2)	48h	36.0±1.5 (8)	20
(3)	48h	42.3±4.2 (10)	0

(b) Laboratory stored lobsters

Location and Date	Acclimation Time	Clotting Time (Sec) (mean±SE)	% Prevalence 'no clot'
Aug '98	2 mon	56.0±4.0 (8)	NA
Jan '99	3 mon	56.5±8.0 (8)	37.8
May '99 (1) **	7 mon	51.3±8.7 (6)	25.0
May '99 (2)	7 mon	47.4±2.9 (7)	12.5
May '99 (3)	7 mon	61.0±6.1 (7)	12.5

* Batch number of three groups of lobsters held in the same (1&2) or different (3) tanks and sampled within 120 mins of each other
** One group of lobsters sampled on three different occasions
NA = Not Available

Mean clotting times in factory lobsters ranged from 28.6 to 42.3secs while the %prevalence 'no clot' ranged from 0–20%. Mean clotting times for lobsters held for extended periods (2–7 months) in the recirculating systems at MML were longer than those seen in the laboratory lobsters and ranged from 47.4–61.0 secs. The reason for the longer clotting times seen in laboratory stored lobsters is unkown but may be related to poor nutrition or sub-optimal water quality conditions leading to a deterioration in health status in the laboratory stored lobsters.

The normal range, determined by estimation of mean±2SD of the four mean values obtained with the factory stored lobsters was 37±12 (25-49) secs.

3.4 Bacteremia assays

As was the case with the clotting time assay it was not technically feasible to determine bacteremia in lobsters collected by SCUBA. Accordingly, normal values for bacteremia were assessed from studies on factory stored lobsters. In addition, some results obtained in laboratory stored lobsters were included in the analysis.

Factory stored lobsters were sampled as part of the field studies of post harvest handling practices. The laboratory stored lobsters were held in the tanks at Muresk Marine Laboratory for 4–6 months and sampled during the 1999 stress experiment and other experiments conducted in May 1999. The %prevalence of bacteremia, CFU/ml or colony rank values were determined as described in Cht 4, Section 5.1.

The levels of bacteremia in factory stored lobsters ranged from 0-50% prevalence in groups of 8 to 13 lobsters stored in factory tanks in Fremantle and in Geraldton (Table 29). In those groups in which a quantitative measure of bacteremia was

obtained, the levels ranges from 2.5-12.5 CFU/ml and 0.10-0.50 mean colony rank. Similar results were obtained with laboratory stored lobsters with one exception (Jan '99) on which occasion high levels of bacteremia were observed. High levels of bacteremia were also occasionally observed in the control serum sample of laboratory stored lobsters assayed for antibacterial activity.

Table 29 Bacteremia in factory and laboratory stored lobsters

(a) Factory lobsters

Location & Date	Acclimation Time	No. Studied	Bacteremia		
			Prevalence %	CFU/ml	Colony Rank
Fremantle (1) Dec '95	16h	13	0	-	-
Fremantle (1) Feb-June '96	16h	12	0	-	-
Geraldton Dec '97	16h	10	0	-	-
Geraldton Apr '98	16 - 120h	10	0	-	-
Geraldton Nov '98	16 - 120h	10	20	-	-
Fremantle (2)(1)* Apr '99	48h	10	50	12.5	0.50
(2)	48h	10	10	2.5	0.10
(3)	48h	10	30	10.0	0.30
Fremantle (2) May '99		8	38	9.4	0.38

(b) Laboratory lobsters

Location & Date		Acclimation Time	No. Studied	Bacteremia		
				Prevalence %	CFU/ml	Colony Rank
Jan '99	(1)**	3 mon	8	50	-	3.15
	(2)		8	87.5	-	4.25
May '99	(1)***	3 mon	8	13	12.5	0.13
	(2)		8	0	0.0	0
	(3)	7 mon	8	25	31.0	0.25

* Batch number of three groups of lobsters held in the same (1&2) or different (3) tanks and sampled within 120 mins of each other

** Batch number of two groups of lobsters held in individual aquaria and sampled within 120 mins of each other

*** One group of lobsters sampled on three different occasions

In order to provide a meaningful assessment of bacteremia levels in post harvest lobsters it is important to consider both %prevalence and mean colony rank (or mean CFU/ml if this statistic can be determined). The data shown in Table 29 provides an example of the reasoning behind this conclusion. Two groups of lobsters (factory lobsters: Fremantle April '99 (1) and laboratory lobsters: Jan '99 (1)) had the same %prevalence (50%). However, the number of bacteria in the hemolymph of lobsters

in the latter group (colony rank 3.15±1.26 (Mean±SE)) was considerably higher than the hemolymph bacteria numbers in the former group (colony rank 0.50±0.20 (Mean±SE)). These two groups of lobsters were clearly experiencing very different bacterial contamination of hemolymph despite the %prevalence values being the same for each group. The biological significance of these observations has still to be determined. One explanation relates to the stress status of lobsters at the time of sampling. A lobster group with low %prevalence and low colony rank is likely to be relatively unstressed, one with a high %prevalence and low colony rank is probably in a moderately stressed state and lobsters with a high %prevalence and high colony rank are likely to be in a high stress status. Alternatively, the differing colony rank values may be a reflection of differences in bacterial load in the culture tanks. Further studies are required to determine the causes of bacteremia in lobsters and the biological significance of differences in colony rank.

Healthy, unstressed lobsters should not contain bacteria in their hemolymph. However, since post harvest lobsters are likely to be in a state of mild stress, it is suggested that a group of post harvest lobsters with a %prevalence bacteremia of up to 50% and a mean bacterial colony rank of up to 0.50 should be considered to be 'normal'. As more information becomes available on the factors contributing to bacteremia in post harvest lobsters these values may be lowered.

3.5 Antibacterial activity assay

The level of ABF found in 7 groups of acclimated factory lobsters is shown in Table 30.

Table 30 Antibacterial factor in acclimated factory lobsters

Delivery Mode	Location and Date	ABF (mean±SE)	
		16-24h acclimation	40-48h acclimation
Boat	Geraldton Dec '96	-	0.386±0.058 (16)
	Geraldton Apr '97	0.087±0.051 (8)	0.281±0.093 (6)
Truck	Geraldton Dec '96 (1)*	0.641±0.086 (9)	0.164±0.058 (5)
	Geraldton Dec '96 (2)*	0.409±0.108 (7)	0.365±0.103 (6)

* Two different truck deliveries
(n) = No. of lobsters studied

The mean ABF values varied widely, ranging from 0.087 to 0.641 ABF units, with two high values (0.641 and 0.409) being observed in lobsters transported to the factory in trucks and stored for 16h. The ABF levels of lobsters from the same truck deliveries decreased with further storage (0.164 and 0.365 respectively after 48h storage), suggesting that the values observed after only 16h acclimation were elevated as a result of the truck transport conditions. The normal range calculated from the mean±2SD of the 7 mean values reported in Table 30 was 0.333±0.362 (0-0.695) ABF units. If the two mean values obtained from the lobsters transported in trucks and acclimated for only 16h are removed, the normal range of ABF in post harvest lobsters would be 0.257±0.258 (0-0.515) ABF units.

It should be noted that the antibacterial activity test development was not completed until towards the end of the study period with numerous modifications being introduced during the course of method development. The effect of these modifications was to increase the magnitude of the ABF units obtained by the assay. Since the results from factory stored lobsters reported in Table 30 were all obtained with earlier methodology it is likely that the normal range quoted above will be an underestimate of values likely to be obtained with the final methodology in post harvest lobsters.

3.6 Phagocytic capacity

No studies were performed on phagocytic capacity in post harvest lobsters so a normal range for this parameter in post harvest lobsters cannot be specified. However, of the two studies performed with laboratory lobsters in MML the levels of phagocytic capacity observed in the control lobsters during two stress trials were 11.8±3.4 and 13.4±3.2 phagocytic capacity units.

3.7 Summary of normal ranges for immune stress tests

Based on the results presented in this section the recommended normal ranges for the mean values of immune parameters derived from estimations on 8 or more factory stored lobsters acclimated for 16 h or more are as follows.

THC	:	$3.97 - 8.79 \times 10^6$ cells/ml		
DHC	:	Hyaline cells	:	7.9 - 35.5%
		Semi-granular cells	:	55.2 - 85.6%
		Granular cells	:	4.5 - 11.3%
Clotting time	:	25 - 49 secs		
Bacteremia	:	Mean colony rank ≤0.50		
		Bacteremia ≤50%		
ABF	:	0 - 0.515 ABF units		

As more data is accumulated the upper limits (bacteremia and ABF) and lower units (%granular cells) of the normal ranges may decrease and increase respectively.

CHAPTER 6 LABORATORY STUDIES OF RESPONSES TO POST HARVEST STRESSORS

1. Stressors examined

Five major studies of the influence of stressor exposure on immune parameter levels in lobsters were conducted. The stressors used in these studies were physical handling and disturbance with and without air exposure, wounding, emersion and visual and/or aural disturbances. An acclimation study was also conducted in which immune and other hemolymph parameters were assessed over a 64 day period.

2. Acclimation experiment

2.1 Aim

The main aim of this experiment was to determine the length of time required for an immune system parameter (THC) to stabilise in post harvest lobsters.

2.2 Experimental procedures

Lobsters were obtained from a Fisheries WA research vessel after being caught near the Abrolhos Islands off Geraldton and transported to Fremantle Fishing Boat Harbour. Upon arrival at the harbour the lobsters were immediately transported to MML and placed in the recirculating holding system (Cht 3, Section 2.1). A total of 14 lobsters were sampled upon arrival at MML for immunological and physiological assays (Day 0) and hemolymph samples were collected from successive groups of 14-15 lobsters on Days 1, 2, 4, 8, 15, 22, 29, 39, 43, 50, 57 and 64. Following hemolymph collection the lobsters were transferred to another holding tank so as to avoid any lobsters being sampled twice during the acclimation period. Lobsters were fed with West Australian pilchards (mulies, *Sardinops neopilchardus*) and mussels (*Mytilus edulis*) three times a week for the period of observations. Water quality parameters (ammonia, nitrite, pH, temperature and salinity) were measured every two days for the first week and 3-5 times/wk thereafter. Dissolved oxygen levels were checked before and after placement of lobsters in the tanks and at the completion of the trial. Partial water exchanges were performed on 8 occasions during the trial to maintain water quality parameters within acceptable ranges.

Lobsters were moult staged and THC and refractive index (RI, an indirect measure of hemolymph protein concentration) assays were performed on all lobsters following procedures described in this report (THC; Cht 4, Sections 1.2 and 2.2) or previously described (RI; Tsvetnenko et al., 1995; Paterson et al., 2001). DHC analyses were also performed on selected lobsters using previously described methods (Cht 4, Section 3.1). Vigour index was determined using the criteria developed by Spanoghe (1996) (Appendix 2). A range of other physiological parameters including hemolymph pH were also measured by the other research team participating in the study.

2.3 Results

2.3.1 Water quality

Water pH (7.8±0.0), salinity (37±0.4 ppt) and temperature (19.6±0.1 °C) (mean±SE) remained relatively constant throughout the period of the trial. Ammonia concentrations rose from 0.2 mg/l on Day 2 to 6 mg/l on Day 5, necessitating several partial water exchanges to be conducted. An increase in nitrite concentrations occurred soon after the increase in ammonia concentrations, rising from 3 mg/l on Day 5 to 14 mg/l on Day 12, the general pattern of change in these two water quality parameters being typical of that seen in a marine biofilter subjected to a shock loading. The ammonia levels fell after the first week and were maintained at between 0.2-0.8 mg/l thereafter. Nitrite levels remained between 8-12 mg/l until Day 27 when they fell to 4 mg/l and then steadily declined to 1.0 mg/l on Day 64.

2.3.2 Variation in THC values over 64 days

Only lobsters in moult stages C or D0 were included in the analysis. The mean THC values declined for the first five days and then fluctuated thereafter (Fig. 25). The THC values obtained on Day 50 (9.78±0.59 x 10^{-6} cells/ml; mean±SE) was significantly higher that the mean THC obtained on days 1, 2, 4, and 22 ($P<0.05$). There was no significant difference between any other of the mean values.

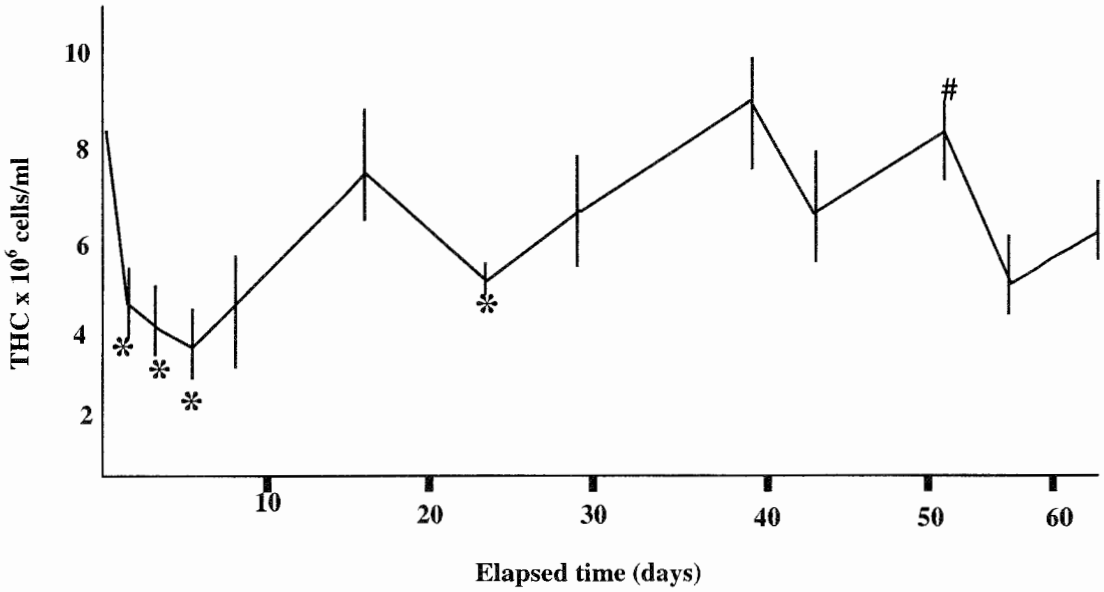


Figure 25 Total hemocyte counts in western rock lobsters *(Panulirus cygnus)* held in a recirculating water system for an extended time period (Mean±SE).
* Mean THC value significantly lower than mean THC marked # ($P<0.05$)

2.3.3 Correlation between THC and RI or hemolymph pH

The THC results were compared with two other hemolymph parameters measured in the same lobsters - hemolymph pH and refractive index (RI). These results were obtained in association with the other group of researchers collaborating in the study (Paterson et al., 2001). Regression analyses of THC with hemolymph pH and total protein in hemolymph samples taken from the same lobsters are shown in Figs. 26 and 27 respectively. A highly significant inverse correlation was obtained between the THC and hemolymph pH (Fig. 26; $R^2 = 0.624$; $P = 0.001$) while THC and total protein showed a weak significant positive correlation (Fig. 27; $R^2 = 0.306$; $P = 0.05$). These relationships are better displayed by graphing individual THC values against pH or RI obtained on the same sample and smoothing the data by averaging the results obtained for each 5 successive THC results (moving average; Figs. 28 & 29). The relationship between pH and THC is also better displayed by comparing pH with THC^{-1} on each measurement day (Fig. 30).

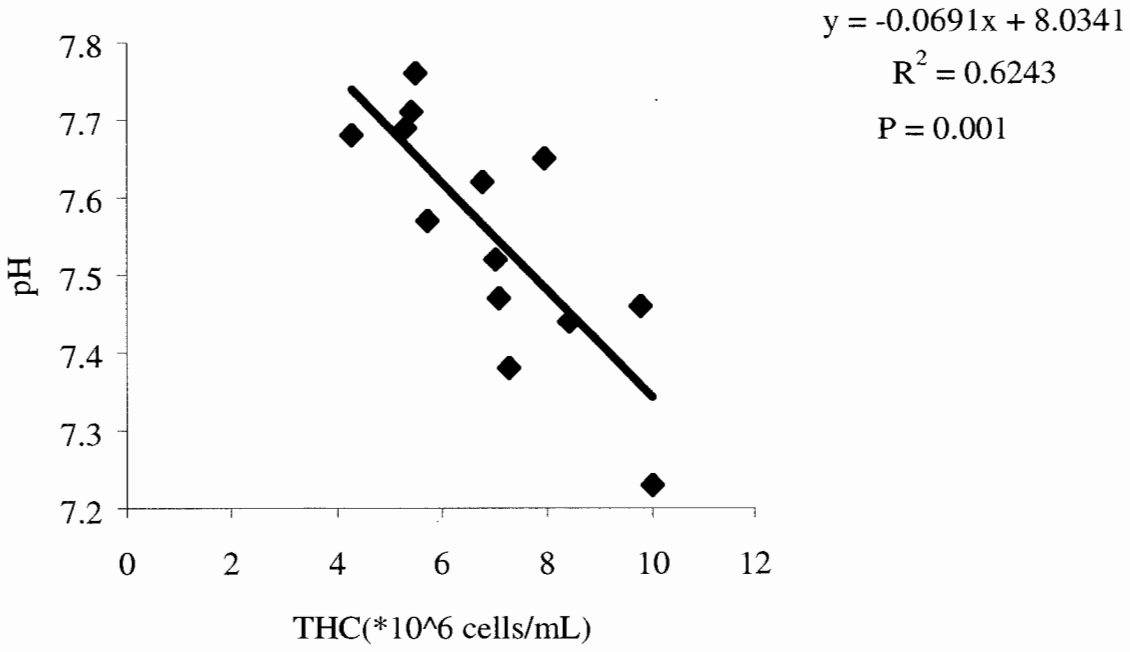


Figure 26 Mean hemolymph pH and THC variations over time

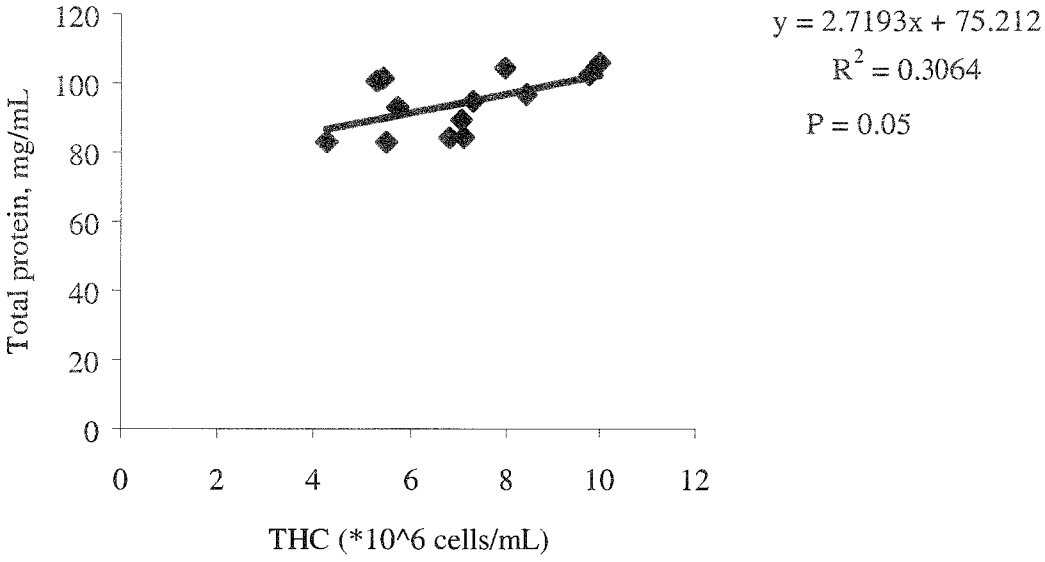


Figure 27 Mean total protein and THC variations over time

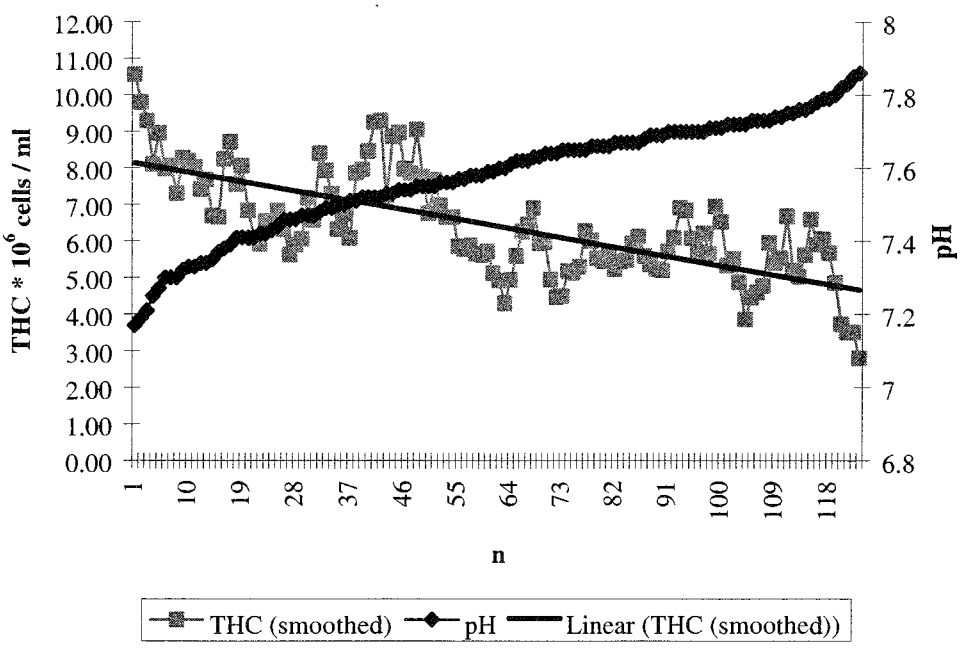


Figure 28 Inverse correlation between moving average THC and pH of successive groups of 5 lobsters

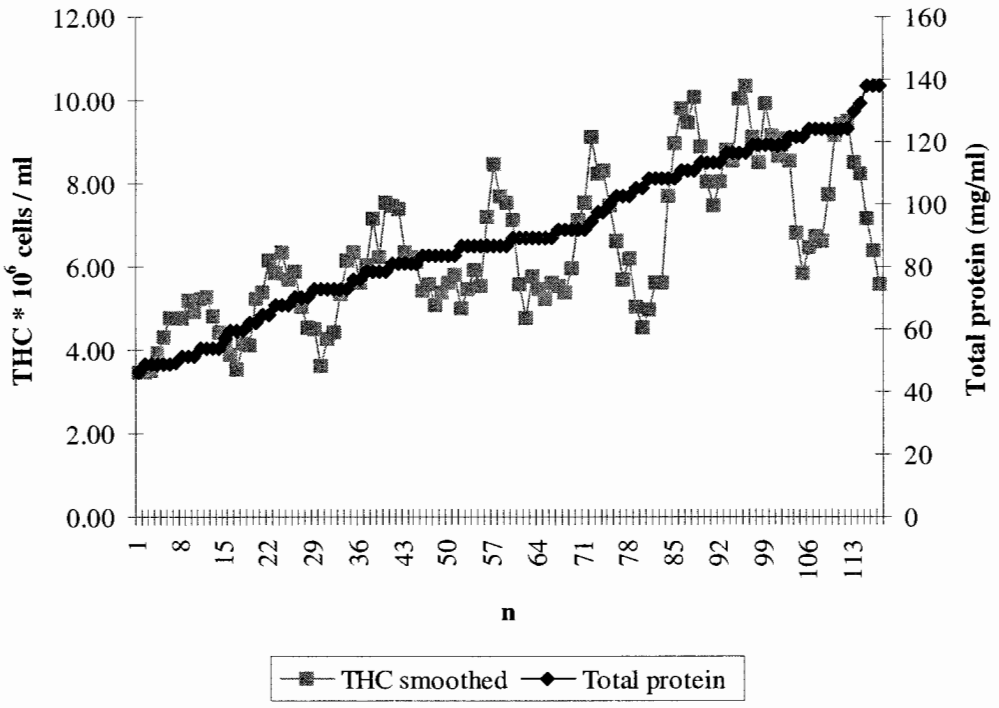


Figure 29 Correlation between moving average THC and RI for successive groups of 5 lobsters

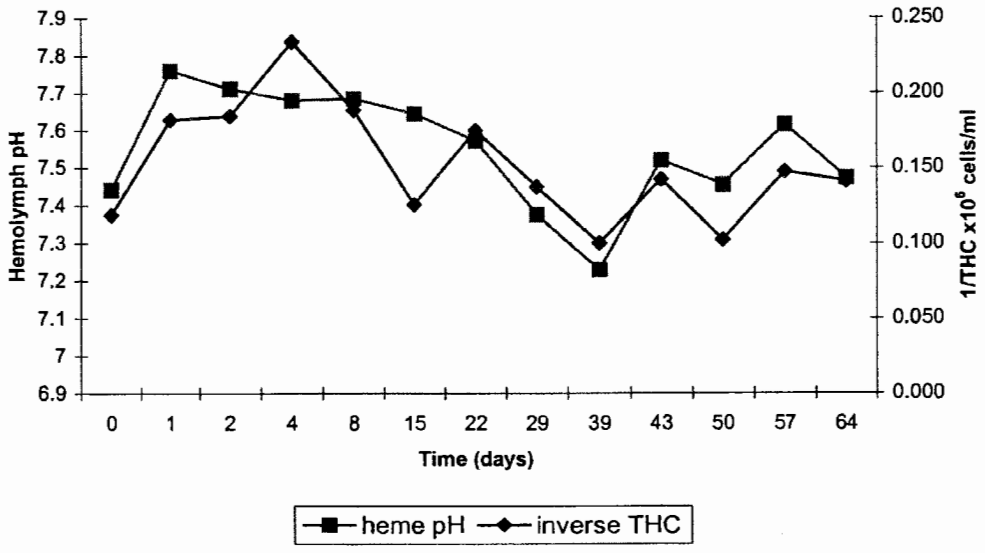


Figure 30 Correlation between hemolymph pH and THC^{-1}

2.3.4 Comparison of THC and daily barometric pressure measurements

As antedotal reports suggest that lobster stress levels may be affected by climatic conditions (large swell; easterly winds) it was decided to examine the relationship, if any, between barometric pressure and THC levels. The results are shown in Fig 31. There was no significant correlation between the two parameters (linear regression; R^2 = -0.259; P=0.393). However, there was a significant correlation between barometric pressure values and THC values over the period day 8 – day 64 (R^2 = 0.701; P = 0.03). The relationship between barometric pressure and THC is best displayed by comparing barometric pressure with THC^{-1} (Fig. 32).

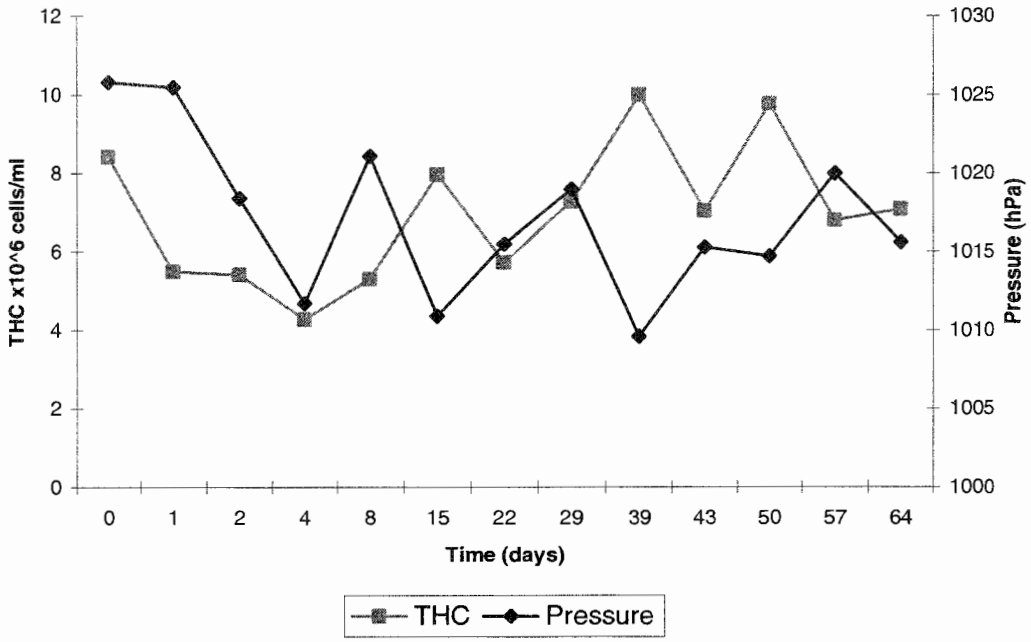


Figure 31 Comparison between THC and barometric pressure readings

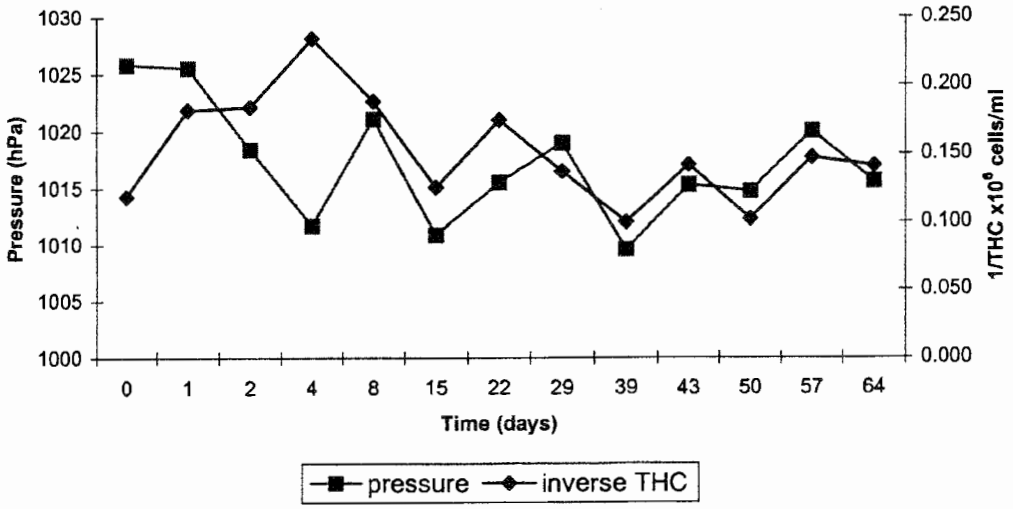


Figure 32 Correlation between barometric pressure and THC^{-1}

2.3.5 Vigour index

A highly significant positive correlation (linear regression; $R^2 = 0.665$; $P = 0.001$) was observed between mean vigour index and mean THC (Fig. 33) for each group of lobsters. The lowest vigour indices (2.57, 3.28 and 3.36) were observed on days 0, 50 and 39 respectively, the three days on which the highest THC were obtained.

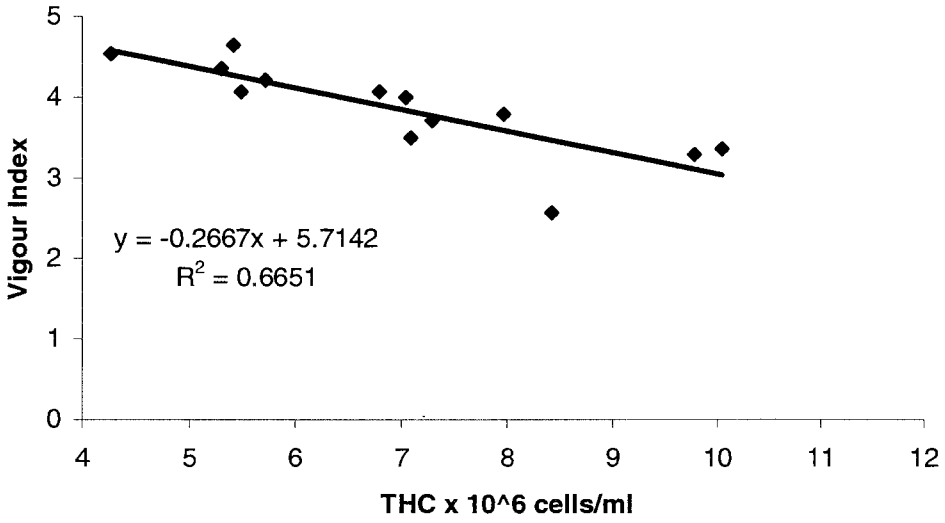


Figure 33 Comparison between mean vigour index and mean THC for each group of lobsters

2.4 Discussion

The initial fall in THC following 24h acclimation in the recirculating holding system was similar to that seen in other studies where lobsters were transported to factories in trucks and placed in holding systems for 24-48h (Cht 7, Section 1 & 2). This fall has been interpreted as a return to baseline THC levels following an elevation in circulating hemocytes caused by stress responses to post-harvest handling procedures (Cht. 7).

The mean THC levels seen in lobsters held in the system for the first 8 days after arrival at MML (4.27 – 5.49 x 10^6 cells/ml; Table 26, Fig. 26) were similar to those seen in baseline lobsters collected by SCUBA. This suggests that the lobsters quickly acclimated to the new environment and that a period of 16-24h is sufficient for THC to return to baseline levels. THC levels rose after Day 8 and fluctuated between 5.72 and 10.05 x 10^6 cells/ml. Two of these mean values, 9.78 and 10.05 x 10^6 cells/ml (Days 50 and 39 respectively), were outside the normal range calculated for baseline or well acclimated factory lobsters (Cht 5, Section 3.1). One of these mean values (9.78 x 10^6 cells/ml; Day 50) was significantly elevated compared to four other mean THC values obtained during the trial. The results suggest that the two groups of lobsters tested on Days 39 and 50 were experiencing a stress response and consequent elevation of circulating hemocyte number at the time of hemolymph collection.

The cause to the apparent stress response is unknown. Although transient high levels of ambient ammonia and nitrite were observed on occasions during the trial there was no obvious relationship between levels of ammonia and nitrite levels in the holding system water and mean THC. Other workers have also observed a lack of response in stress reaction indicators (heart rate and scaphognathite rate) in the prawn, *Nephrops norvegicus* exposed to high levels of ambient ammonia (Schmitt and Uglow, 1997). Similarly, there was no obvious relationship between atmospheric pressure and mean THC although the two parameters did show a significant correlation after 8 days of acclimation (Fig. 32).

A significant correlation was observed between mean THC and RI (Fig. 27). As the hemolymph protein concentration as measured by RI increased so did the THC. Dall (1974) has drawn attention to the importance of taking into consideration hemolymph water volumes when interpreting apparent variations in hemolymph parameters such as protein concentrations and total circulating hemocytes. He pointed out that a decrease or increase in the parameter, measured in terms of volume of hemolymph, could be due to either a change in the total mass of the hemolymph component or in the volume of hemolymph water. Thus, the correlation between THC and RI observed in this study could be interpreted as indicating that the number of circulating hemocytes and the total amount of hemocyanin (the major protein component of lobster hemolymph; (Durliat and Vranckx, 1989) were similarly affected by the processes leading to changes in their circulating levels. Alternatively, it is possible that the changes in these parameters in acclimated lobsters were primarily a reflection of alterations in water content of lobster tissues.

A correlation was also observed between THC and hemolymph pH, increasing THC being accompanied by decreasing hemolymph pH (Fig. 30). Furthermore, the mean value for hemolymph pH on Day 39, the day on which the highest THC was recorded, was the lowest mean pH observed throughout the acclimation trial. The hemolymph pH in lobsters sampled on Day 50 was also low compared to most other observations. A fall in hemolymph pH has been observed as a stress response in crustaceans (McMahon et al., 1978; Taylor and Whiteley, 1989). The observation of low pH values in the two groups of lobsters with excessively high levels of circulating hemocytes adds further weight to the conclusion that these lobsters were stressed at the time of sampling.

Vigour index results also point to a stressor event influencing lobster health during the experiment as evidenced by the low vigour results being observed on the same days as high THC and low pH. While transport stress was the likely cause of deviations in these parameters on day 0 the stressor causing changes in stress parameters in days 39 and 50 is uncertain.

In conclusion, the results suggest that elevations in THC values caused by capture and transport to the laboratory resolved within 24h of the lobsters being placed in the holding system. The levels of THC seen in the acclimated lobsters were similar to those seen in baseline (SCUBA collector) lobsters for the first 8 days of the trial but unexplained elevations in THC, presumably due to stress responses to an unknown stressor, occurred thereafter. Despite the apparent exposure of lobsters to a stressor(s) on at least two occasions during the trial, the majority of the groups of lobsters

exhibited THC values similar to those seen in baseline or well acclimated factory lobsters (Fig. 25, Table 25).

2.5 Relevance to industry practices and recommendations

The results suggest that a minimum acclimation period of 24h is required to allow elevations in THC caused by transport and handling disturbances to resolve. Thus, assuming that stress responses in THC contribute to a decline in post harvest lobster condition (strength), processors should allow at least 24h acclimation before subsequent handling disturbances.

3. Wounding experiment

3.1 Aim

The aim of this experiment was to investigate the influence of handling and wounding on THC, DHC and vigour index measurements in laboratory stored lobsters.

3.2 Experimental procedures

Lobsters used in this experiment were obtained either from the commercial catch or from a Fisheries WA research vessel and were mature commercial size A animals or slightly smaller. Test animals were acclimated in the experimental system for one week before the experiment and were fed West Australian pilchards (mulies, *Sardinops neopilchardus* or mussels, *Myticus edulis*) three times a week until 48h before the commencement of the experiment when feeding ceased.

The experimental system comprised eight 180L seawater aquaria fitted with a temperature controlled recirculating water system (Cht 3, Section 2.1). The water temperature was maintained at 21±1°C and photoperiod at 14h light and 10h dark. Water quality parameters (dissolved oxygen, ammonia, nitrite, pH and temperature) were monitored during the acclimation and experimental periods and excess food and debris were siphoned from the tanks twice a week.

One group of lobsters (35 animals; injured lobsters) were removed from the tanks and injured using a sterile scalpel by inserting the scalpel through the ventral tail membrane and forming a 10 x 25 mm deep cut in the tail muscle. The wound was made in the third articular membrane approx 2cm laterally from the abdominal artery and ventral nerve cord. After wounding the lobsters were returned to the tanks and sampled after 10, 30 and 60 min and 1 and 5 days (7 lobsters/time point). A second group of lobsters (35 animals; handled only lobsters) were handled similarly to the test lobsters but were not wounded. A third group of lobsters (14 lobsters; control lobsters) were sampled immediately prior to the commencement of the experiment and immediately following completion of sampling on Day 5, 7 lobsters being sampled at each time point. Physical and biological parameters (wt (g), OCL (mm), sex, presence of exoskeleton lesions and moult stage were recorded for all test and control lobsters, vigour index was assessed following the criteria introduced by

Spanoghe (1996) (Appendix 2) and the hemolymph samples were removed from the ventral sinus at the base of the fifth walking leg.

Hemolymph samples (200 µL) were removed with a 23G needle and a 1mL syringe containing 200 µL precooled Na-cacodylate anticoagulant and analysed for THC, DHC and antibacterial activity as previously described (Cht 4). Hemolymph protein was analysed indirectly using a refractometer (Shibuya S-1) and converted to protein content in mg/dL using the formula established by Paterson et al. (1999). Histopathology was performed as described in Cht 4, Section 8.

3.3 Results

Water quality parameters remained within acceptable levels throughout the acclimation and experimental periods with oxygen saturation exceeding 95%, pH ranging from 7.6 – 8.1, water temperature 21±1 °C and ammonia and nitrite levels not exceeding 1 mg/L.

THC levels in the injured lobsters increased following removal from the tank and wounding and peaked within 30min of commencement of the experiment (Fig 34). The levels then steadily declined over the next 5 days. THC levels in the handled only lobsters also rose following removal from the tank but did not reach maximum levels until 60 min later.

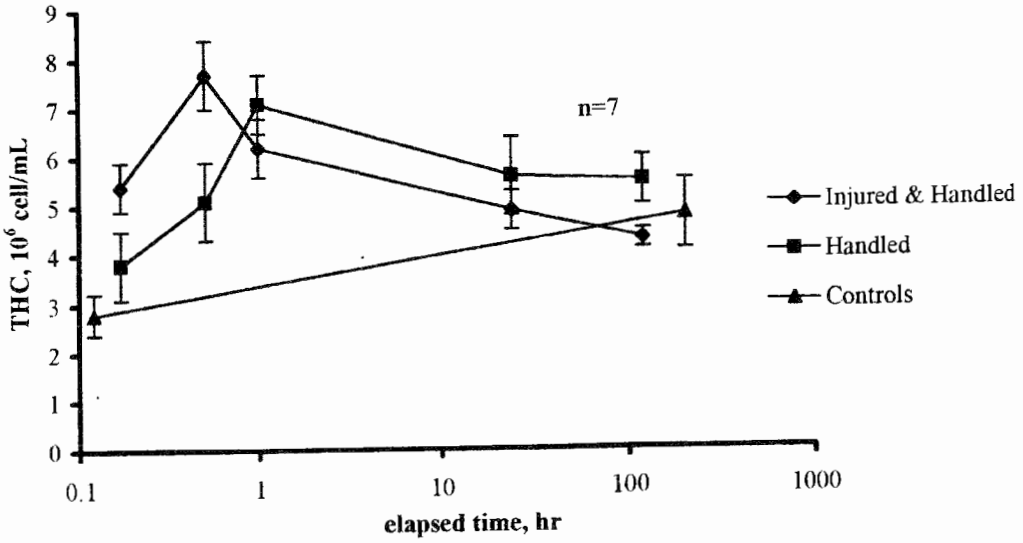


Figure 34 Influence of handling and wounding on THC levels in laboratory stored lobsters (mean±SE). Logarithmic scale on x axis

The THC level in the handled only lobsters was significantly lower that that of the injured lobsters 30 min after removal from the tank ($P<0.05$) but levels were similar to those of the injured group at all time points thereafter. The mean THC value for the initial control lobsters was significantly lower ($P<0.05$) than the mean THC for most of the other groups of lobsters including that of the final control group.

There was no significant difference in the DHC values for the three hemocyte types between the injured and the handled only lobsters at any of the time points (Table 31; P>0.05). However, there was a tendency for the proportion of semi-granular cells to increase and the proportion of hyaline cells and granular cells to decrease in the injured lobsters five days after wounding. This apparent change was not seen in the handled only lobsters.

Table 31 Differential hemocyte counts (DHC) in control, injured and handled only lobsters (Mean±SE)

Cell type	Lobster group	Initial undisturbed control	10 min	30 min	60 min	1 d	5 d
HC %	Injured		9.3±1.7	12.4±3.8	9.7±0.9	13.5±2.4	7.5±2.0
	handled only	12.8±2.1	9.3±2.8	13.4±1.3	11.3±1.8	11.7±1.1	10.3±1.6
GC %	Injured		13.1±1.0	9.4±1.0	12.7±2.1	11.0±1.9	6.6±1.5
	handled only	8.6±1.3	9.1±0.8	7.4±2.5	13.1±2.0	7.8±1.5	9.5±2.1
SGC %	Injured		77.5±2.3	78.2±4.3	77.6±2.1	75.5±2.6	85.7±1.9
	handled only	78.6±2.2	81.5±2.3	78.2±4.3	79.1±3.2	80.4±1.4	80.1±2.9

HC % = percentage of hyaline cells; GC % = percentage of granular cells; SGC % = percentage of semi-granular cells

The DHC results for the initial control lobsters were similar to those of the injured and the handled only lobsters with the exception of the Day 5 values for the injured group. Unfortunately, due to technical difficulties, a DHC was not obtained on the final control lobsters so a comparison of the results from these lobsters with those of the other two groups was not possible.

ABF values were highly variable within each lobster group. No significant differences were observed in the ABF levels in injured and handled only lobsters (Table 32; P>0.05).

Table 32 Antibacterial activity (ABF±SE) in control, injured and handled only

Time after wounding	Treatment	
	Handled only	Injured
Initial control (time 0)	0.196±0.104	
60 min	0.396±0.230	0.480±0.139
1 d	0.455±0.132	0.223±0.144
5 d	0.226±0.147	0.268±0.131

Variations in vigour index were observed in both injured and the handled only lobsters during the course of the experiment (Table 33). However, there was no obvious trends in the data over time or between different treatment groups apart from a low vigour index at the commencement of the trial (3.38±0.18) and a low value in

the 60 min injured group (3.29±0.18). The latter value was significantly lower than the value obtained for the 5 day injured group.

Table 33 Mean vigour index and rating proportions in control, injured and handled only lobsters (%)

		Vigour Index Rating proportion, %			Mean±SE
		3	4	5	
0	Initial control	63	37	0	3.38 ± 0.18^{ab}
10 min	Injured	29	71	0	3.71 ± 0.18^{ab}
	handled only	29	71	0	3.71 ± 0.18^{ab}
30 min	Injured	44	28	28	3.86 ± 0.34^{ab}
	handled only	29	29	42	4.14 ± 0.34^{ab}
60 min	Injured	71	29	0	3.29 ± 0.18^{a}
	handled only	14	43	43	4.29 ± 0.29^{ab}
1 d	Injured	29	57	14	3.86 ± 0.26^{ab}
	handled only	14	86	0	3.86 ± 0.14^{ab}
5 d	Injured	0	57	43	4.43 ± 0.20^{b}
	handled only	14	86	0	3.86 ± 0.14^{ab}
	Final control	43	43	14	

See Appendix 2 for descriptions of vigour ratings 3, 4 & 5

Hemolymph protein concentrations in the injured and the handled only lobsters were similar 10 min and 30 min after commencement of the study but deviated thereafter (Fig. 35).

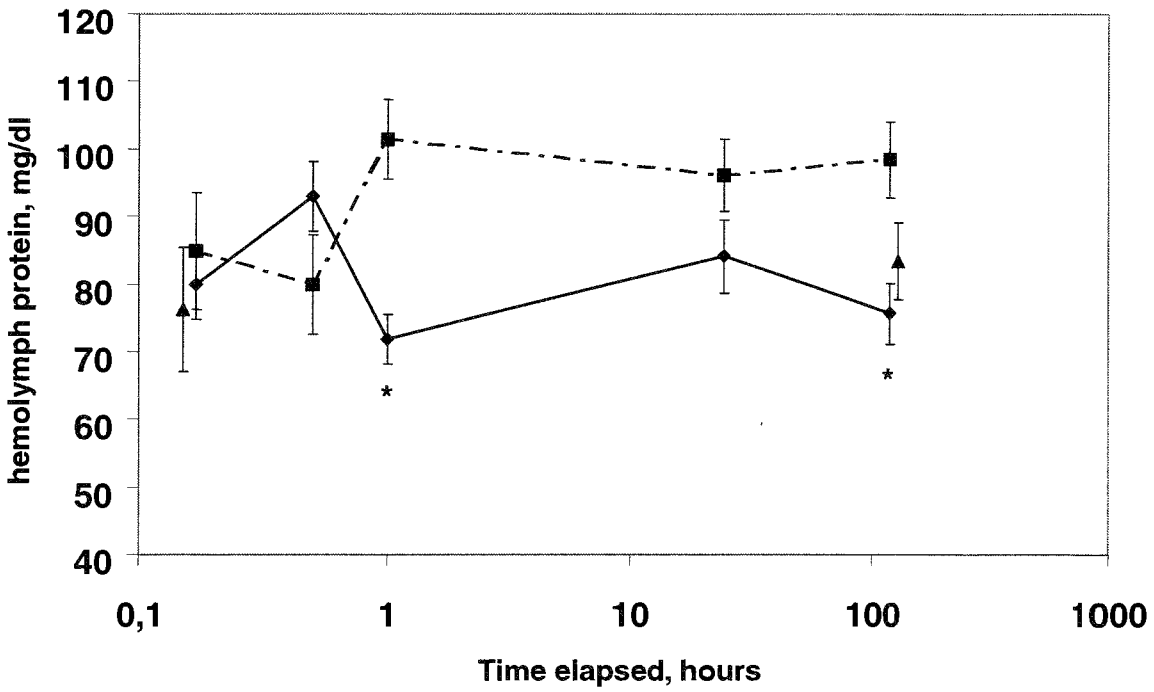


Figure 35 Influence of handling and wounding on hemolymph protein concentrations in laboratory stored lobsters (mean±SE). Logarithmic scale on x axis
■ = handled once (dashed line); ◆ = handled and wounded (solid line); ▲ = undisturbed control; ✶ = significant difference between mean THC ($P < 0.05$)

The hemolymph protein concentration in the handled only lobsters was higher than that of the injured lobsters in all measurements after 30 min and this difference was significant at the 1h and 5d time points ($P<0.05$). There was a weak but significant positive correlation between protein concentration and THC level for the same lobster ($R^2 = 0.51$; $P<0.05$). The hemolymph protein concentrations in the initial and final control groups were similar.

A correlation analysis showed there was no significant correlations between the presence of tail blisters and THC, ($P = 0.217$; $R^2 = -0.141$) nor between vigour index and THC ($P = 0.867$; $R^2 = 0.019$) and vigour index and % granular cells ($P = 0.964$; $R^2 = 0.005$)

Histopathology of muscle tissues adjacent to the wound site revealed focal aggregations of hemocytes in the wound area.

3.4 Discussion

The major finding of this study was that handling lobsters with or without inflicting an injury caused a rapid increase in the levels of circulating hemocytes. Observations of a rapid rise in THC levels following handling disturbances were subsequently made on numerous occasions during the course of this project. Similar findings have been reported for freshwater crayfish (Hamann, 1975). Handling disturbances are known to have a marked and rapid effect on respiratory and circulatory performance (McMahon et al., 1978) and the neuro-hormonal processes leading to these changes may be similar to those causing an increase in circulating hemocytes. The rapid increase in total numbers of hemocytes in the circulation suggests that they are released from a storage site rather than produced *de novo* in response to stressor exposure. It has been suggested that hematopoietic tissue can store large numbers of mature hemocytes (Söderhäll, pers. comm.) and may function as the storage site for rapid mobilisation of hemocytes.

The delay in the rise in THC in handled only lobsters (Fig. 34) compared to the injured lobsters suggests that the superimposition of two stressors resulted in a more rapid response that one stressor alone. Alternatively, wounding may be a specific stressor which illicits a very rapid increase in circulating THC levels. Further studies on the causes and mechanisms of release of hemocytes in response to environmental stressors are warrented.

Hemocytes are a major participant in wound healing in decapod crustaceans (Bauchau, 1981; Durliat, 1985; Söderhäll and Cerenius, 1992), the processes of hemolymph gelation and hemocyte aggregation ensuring that wounds are sealed and healing reactions commenced. Although hemocyte aggregates were observed in tissue sections of the wound site these aggregation reactions did not appear to lead to a decrease in hemocyte levels in the hemolymph. It would appear that the number of hemocytes removed from circulation through aggregation at the wound site was relatively small and a consequent decrease in hemocyte count was masked by the increase in circulating hemocytes caused by the handling disturbance.

This study revealed a delayed but persistent decrease in hemolymph protein concentration in wounded lobsters compared to lobsters which were not wounded.

One explanation for this observation is that hemolymph gelation reactions caused by tissue damage resulted in a decrease in the concentration of coagulogen, the clotting protein in decapod crustacean hemolymph, and a consequent decrease in hemolymph protein concentration. Plasma coagulogen may account for up to 20% of the total blood protein in lobsters (Durliat, 1985) so part, at least, the observed decrease in protein concentration in the wounded lobsters could have been due to a loss of coagulogen from the hemolymph. Chen et al., (1993) have reported changes in clottable protein concentrations during and after injury, the proteins presumably taking part in the healing process. However, the magnitude of the difference between the protein levels after 1h in wounded compared to non-wounded animals (30%) suggests that decreased protein concentration in the wounded lobsters may have also been partly due to an increase in hemolymph volume resulting from stress related volume disturbances. Hemodilution has been described as a rapid response to handling stressors in freshwater salmonids (McDonald and Milligan, 1997) but whether a similar response occurs in crustaceans is unclear.

The lack of a consistent response in vigour index to a handling event indicates that this parameter does not provide an accurate measure of alarm type acute stress responses in spiny lobsters. Spanogue (1996) has presented evidence for a decline in vigour index in post harvest lobsters which have been weakened though the rigours of capture, handling and transport. The conclusion is in accordance with the low vigour index observed in the 60 min injured group in this experiment. It would appear that this parameter has application in assessing a weakened state in spiny lobsters, as is likely to occur following wounding or prolonged air exposure, but has limited value in detecting and assessing responses which occur during the alarm phase of the stress response. In addition, as discussed in Tod and Spanoghe (1997), an induced stupor resulting from exposure of lobsters to low temperature environments is another major cause of reductions in vigour ratings in post harvest lobsters.

Antibacterial activities also showed no consistent response to wounding or handling and were highly variable within each treatment group. Whether the stressors studied in this experiment (physical disturbance and wounding) were of insufficient magnitude to affect circulating levels of antibacterial activity or whether these stressors do not initiate changes in ABF levels is unknown.

Of particular interest was the significant difference between the THC values in the initial compared to the final control lobsters. An increase in THC levels in apparently undisturbed lobsters was seen on a number of occasions in this project and was studied in detail in a later experiment (see Section 6). However, these later studies mainly focussed on short term THC responses in control lobsters (hrs). The long time period between the first and second measurement on the control lobsters in this experiment (4 days) should have been more than sufficient for THC levels to return to baseline levels, based on the results obtained in the acclimation trial (Section 2) and in post harvest lobsters stored in factory tanks (Cht 5, Section 3.1). Furthermore, the mean THC in the initial control lobsters, 2.7 x 10^6 cells/ml, was lower than the lower limit of the normal range for post harvest lobsters (Cht 5, Section 3) while that of the final control, 4.7 x 10^6 cells/ml, fell within the normal range. Thus, it would appear that the significant difference in THC between these two groups of lobsters may have in part been due to an abnormally low value being observed prior to commencement of the study rather than to an elevation in the final control THC value.

Low THC levels are typically seen in freshwater crayfish reared in sub-optimal environments (Evans et al., 1999b) and in lobsters and freshwater crayfish with inadequate nutrition (Stewart et al., 1967; Jussila and Evans, 1998). The reason for the difference in THC levels in initial control lobsters seen in this study is unknown but may have been due to poor nutrition or sub-optimal holding conditions. It is possible, therefore, that the responses to wounding and/or handling in the number and differential distribution of circulating hemocytes, lobster vigour and hemolymph protein concentrations seen in the study may have influenced by a lack of health or nutritional status of the test animals.

3.5 Relevance to industry practices and recommendations

The results suggest that lobsters exhibit an acute immune system stress response to handling procedures and this response may be exacerbated by simultaneous exposure to handling and wounding. Wounding appears to lead to a weakened state and a compromised immune system as evidenced by the fall in vigour index, the shift in the proportional distribution in hemocyte type and a possible decrease in levels of circulating coagulogen. It can also be concluded that significant wounding, as was inflicted in this experiment, may require a long time period for immune capacity (circulating levels of coagulagen, hyaline and granular cells) to return to normal. However, these conclusions are preliminary since the findings were not confirmed in a repeat experiment and since the low levels of circulating hemocytes in the initial control lobsters suggests that test animals had a compromised immune status prior to the commencement of the study. It is recommended that further studies be conducted on the influence of wounding on immune status in the western rock lobster and the likely effects of wounding (including autotomy) inflicted during capture and transport, on the survival of lobsters during storage and live export.

4. Handling and air exposure trial 1

4.1 Aim

The aim of this experiment was to compare the effects of emersion with and without handling on three stress/health indicators – THC, hemolymph glucose and vigour index.

4.2 Experimental procedures

The experimental system, acclimation conditions, source of test animals and monitoring of water quality was the same as described for the wounding experiment (Cht 6, Section 3.1).

Lobsters were held in individual oyster mesh cages, two lobsters per cage and exposed to air with (handling and emersion lobsters) and without (emersion lobsters) a handling stressor. The stressor comprised 1 min of gentle shaking of the oyster mesh cage and preventing lobsters gaining a hold on the mesh. The lobsters were exposed to air either by physically removing the cages holding the lobsters from the aquaria (handling and emersion) or by gently siphoning the water from the tank without

excessive disturbance of the lobsters (emersion lobsters). Lobsters were sampled after 5 min and 120 min, the latter group being subjected to the handling disturbance every 30 min after commencement of the trial. Control groups of lobsters were sampled immediately before commencement of the trial and immediately after the sampling of the last treatment group (initial and final control lobsters respectively).

THC and vigour index were determined as described in Cht 6, Section 3.1 and hemolymph glucose was analysed by the glucose oxidase method using Sigma kit reagents. Before the assay hemolymph samples were deproteinised in an equal volume of $0.6molL^{-1}$ perchloric acid. Each lobster was also analysed for sex and moult stage and the extent and location of exoskeleton lesions was recorded.

4.3 Results

Water quality measurements showed acceptable water quality was maintained during the experiments with oxygen saturation above 95%, pH between 7.6-8.1, ammonia and nitrite levels low and water temperature 21±1°C.

The THC in the handled and emersed lobsters was significantly elevated 5min after the handling event and fell slightly over the next 120 min (Fig. 36; $P<0.05$). The THC in lobsters emersed without handling showed a initial tendency to fall, the mean THC in the 5 min emersed lobsters being lower than that of initial control lobsters, and then increase. The THC level in the final control was higher than that of the initial control but this difference was not significant ($P>0.05$).

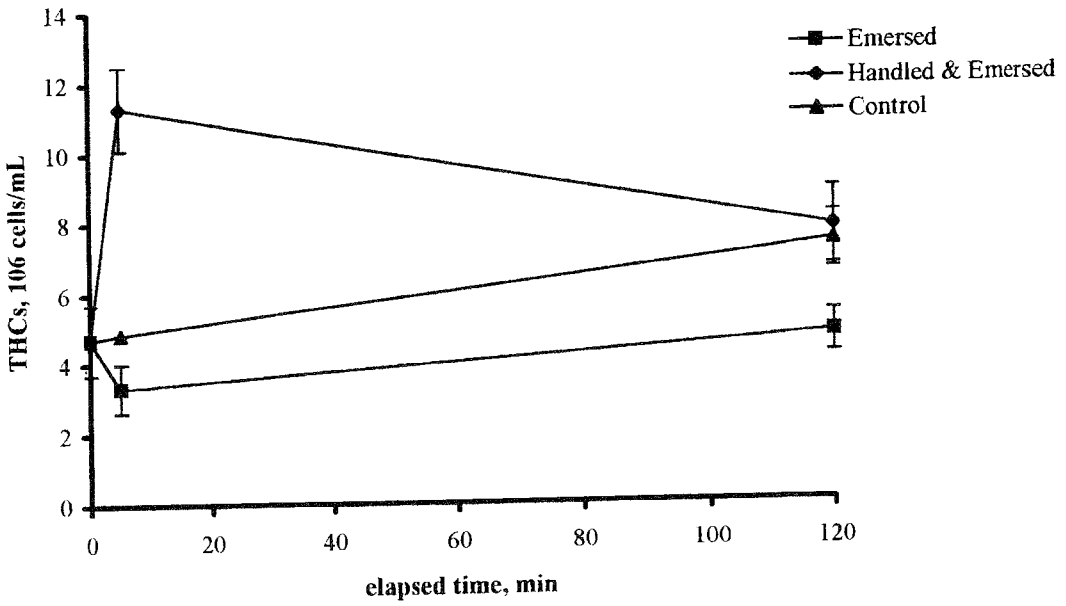


Figure 36 Influence of air exposure with and without physical disturbance on THC levels in laboratory stored lobsters (mean±SE)

Hemolymph glucose was significantly elevated ($P<0.05$) after 120 mins in both groups of lobsters, regardless of whether air exposure was accompanied by a handling disturbance or not (Fig. 37).

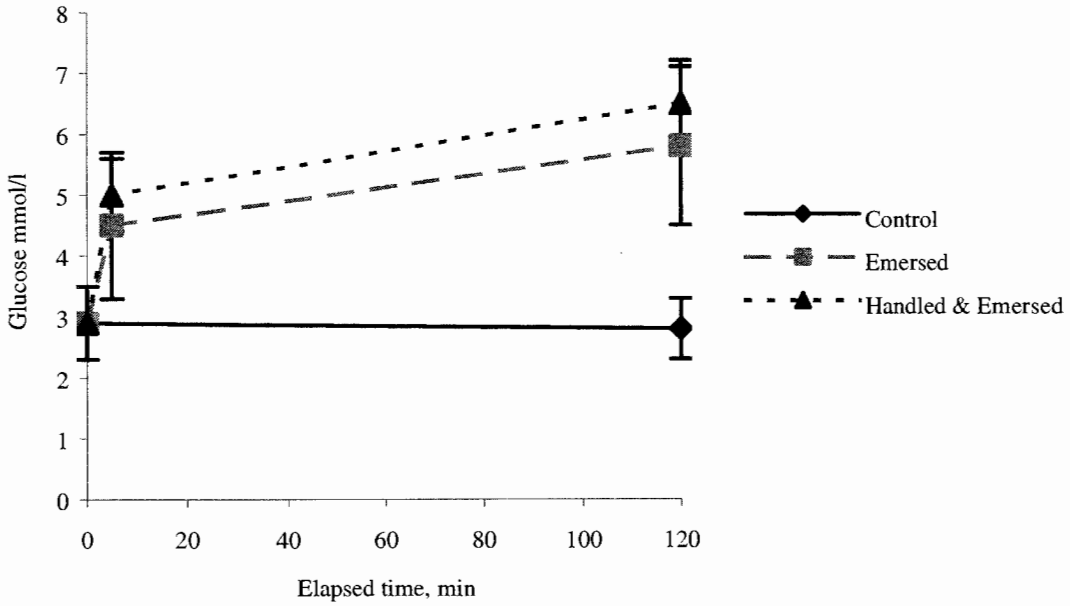


Figure 37 Influence of air exposure with and without physical disturbance on hemolymph glucose levels in laboratory stored lobsters (mean±SE)
◆ = emersion (solid line); ■ = handled and emersion (dashed line);
▲ = undisturbed control

Vigour index tended to be lower in lobsters which were handled and emersed compared to lobsters whch were emersed without handling on to control lobsters (Table 34).

Table 34 Vigour index rating following air exposure with and without handling

		Vigour index rating proportion, %		
		3	4	5
Initial	control	13	50	37
5 min	Emersion	13	50	37
120 min	Emersion	13	25	62
5 min	Handling and emersion	25	75	0
120 min	Handling and emersion	50	38	12
Final	control	13	62	25

4.4 Discussion

The major finding of this study was that air exposure with or without handling caused a marked elevation in hemolymph glucose while elevations in THC levels were only

seen in the lobsters which were handled during emersion. The pattern of a rapid increase in THC levels following handling was similar to that seen in the wounding experiment (Cht 6, Section 3.2) and confirmed these and other observations of a rapid increase in circulating hemocyte levels in response to physical disturbance. The absence of a rise in THC in the lobsters which were emersed without handling was a surprising result. The results of a later study (Cht 6, Section 6) confirmed this finding, THC levels in that study showing a significant decrease 3h after emersion. The pattern of change is suggestive of withdrawal of circulating hemocytes into a storage site such as the hematopoeitic tissue. These observations suggest that air exposure alone may not have a markedly adverse effect on immune functions affected by circulating hemocytes providing the period of exposure is relatively short.

The increase in plasma glucose following air exposure is in agreement with findings by other workers. Increases in plasma glucose following exposure to air has been demonstrated in lobsters (Telford, 1968; Spicer et al., 1990; Schmitt and Uglow, 1997), shrimp (Hall and Van Ham, 1998), crabs (Lynch and Webb, 1973; Johnson and Uglow, 1985; Santos and Colares, 1990; Schmitt and Santos, 1993; Santos and Keller, 1993) and in freshwater crayfish (Telford, 1974; Santos and Keller, 1993; Jussila et al., 1999). This elevation is considered to be the result of asphyxia and/or a combination of different factors such as handling and placement in an alien environment (Telford 1974; Santos and Colares, 1990).

The rise in plasma glucose was rapid, a significant rise being detected in the handled and emersed group within 5 min of emersion and a similar, though not significant, increase being observed in the lobsters emersed without handling. The effects of emersion on hemolymph glucose and hyperglycaemic hormone (CHH) levels in the crab *Cancer pagurus*, also showed that the increase in hemolymph glucose was a rapid response, being observable after about 30 min of air exposure and reaching significantly higher levels than control immersed animals after 45 min. A significant increase in CHH levels was detected during the first 15 min of emersion, confirming the findings of other workers (Keller and Andrew, 1973) that increases in CHH levels lead to a measurable hyperglycaemia within 15-30 min. Relative contributions of these stress responses to handling or to lack of oxygen through emersion has not been investigated in detail by other workers but has been recently addressed in Paterson et al. (2001). The handling stressor utilised in this study evoked a fall in vigour index when combined with air exposure. The results suggest that a combination of repetitive physical disturbance and prolonged (2 h) air exposure leads to a weakened state in *Panulirus cygnus*. Similar findings by Spanoghe (1996) support this conclusion.

4.5 Relevance to industry practices and recommendations

The THC results suggest that immune system functions in *Panulirus cygnus* affected by the number of circulating hemocytes may not be adversely influenced by a short term (less than 2h) air exposure providing handling disturbances are minimised. However, other immune functions, not studied in this experiment may be affected. Changes in energy metabolism caused by repeated physical disturbance and air exposure, are clearly induced by those simulated post harvest procedures. The significance of these changes on lobster health and survival are unclear and should be further evaluated.

5. Handling and air exposure trial 2

5.1 Aim

The aim of this experiment was to compare the effects of handling with and without emersion on four stress/health indicators – THC, % granular cells, vigour index and phagocytic capacity.

5.2 Experimental procedures

The experiment was conducted in the FRDC laboratory at MML using the three recirculating systems described in Cht 3, Section 2.1. The three rows of eight tanks were divided into four blocks so that a randomised block design could be applied. Six treatment groups were used as described below.

The experiment was repeated three times, twice using 4 lobsters per treatment (Experiment 1; 1 lobster per tank) and once using 8 lobsters for each treatment (Experiment 2; 2 lobsters per tank). In the latter experiment phagocytic capacity was measured by the NBT assay method (Cht 4, Section 7.3.2) whilst in the former set of experiments the LDCL method (Cht 4, Section 7.3.1) was used. The results of the former two experiments were pooled and the combined data were used in the data analysis.

Lobsters used in the first experiment were mature commercial size A animals obtained from a Fisheries WA research vessel and held in the recirculating holding system at MML for approximately 9 months before testing. A significant proportion (approximately 25%) of the lobsters used in Experiment 1 were in moult stages D1-3. In addition, these animals had been used in other stress tests prior to the pre-acclimation period and had experienced multiple samplings. Lobsters used in Experiment 2 were freshly arrived lobsters obtained from a processing factory in Geraldton two weeks prior to commencement of the experiment and were mostly unsampled before the experiment. All animals from both experiments were acclimated in the experimental system for two weeks before the experiments and were fed West Australian pilchards (*Sardinops neopilchardus*) and mussels (*Mytilus edulis*) three times a week until 48h before the commencement of the experiment when feeding ceased.

Lobsters were held in individual oyster mesh cages, one (LDCL assay) or two (NBT assay) lobsters per cage and handled while immersed in seawater (immersed lobsters) or after being removed from the water (emersed lobsters). The handling stressor comprised 1 min of gentle shaking of the oyster mesh cage so as to prevent lobsters gaining a hold on the mesh. Lobsters were sampled after 5 min and 120 min, the latter group being subjected to the handling disturbance every 30 min after commencement of the trial. Control groups of lobsters were sampled immediately before commencement of the trial and immediately after the sampling of the last treatment group (initial and final control lobsters respectively). Treatment groups were designated as follows:

Control 0 - control lobsters sampled at time 0 (undisturbed and immersed)

Immersed 5 - immersed and handled lobsters sampled after 5 mins

Emersed 5 - emersed and handled lobsters sampled after 5 mins

Immersed 120 - immersed and handled lobsters sampled after 120 mins

Emersed 120 - emersed and handled lobsters sampled after 120 mins

Control 120 - control lobsters sampled after 120 mins (undisturbed and immersed)

THC (lobsters in moult stages C and D0 only) and %granular cells were determined as described in Cht 4, Sections 2.1 and 3.1 respectively, vigour index using the criteria described by Spanoghe (1996) (Appendix 2) and phagocytic capacity as described Cht 4, Section 7.3. Each lobster was also analysed for sex and moult stage and the extent and location of exoskeleton lesions were recorded. Water quality parameters were monitored as described in Section 3.

5.3 Results

Water quality measurements gave similar results to those obtained in previous experiments and showed that acceptable water quality was maintained during the experiments.

Mean values for THC, %granular cells, phagocytic capacity and vigour index obtained in the two experiments are shown in Table 35.

Table 35 Stress parameters in immersed and emersed lobsters

(a) LDCL assay

Treatment group	Stress Parameter (Mean±SE)			
	THC ($x\text{-}10^6$ cells/ml)	% granular cells (%)	Phagocytic capacity (arbitary units $x\ 10^{-9}$)	Vigour index
Control 0	8.20±0.90 (7)[a]	4.6±0.6[a]	20.1±9.2[a]	3.4±0.2
Immersed 5	7.83±1.10 (5)[a]	6.0±1.0[a]	8.4±3.3[a]	3.5±0.2
Emersed 5	6.93±0.90 (5)[a]	6.9±1.5[a]	16.8±3.9[a]	3.8±0.2
Immersed 120	8.03±0.39 (7)[a]	6.1±1.4[a]	25.6±8.1[a]	3.1±0.1
Emersed 120	8.21±1.07 (6)[a]	8.1±1.4[a]	24.6±6.3[a]	3.4±0.2
Control 120	10.31±2.37 (5)[a]	3.8±0.9[a]	29.8±6.1[a]	3.4±0.2

(n) = No. of animals
Means in columns with different superscripts are significantly different (P<0.05)

(b) NBT assay

Treatment group	Stress Parameter (Mean±SE)			
	THC (x-10^6 cells/ml)	% granular cells (%)	Phagocytic capacity (arbitary units x 10^{-9})	Vigour index
Control 0	5.86±1.25 (7)[a]	5.0±0.3[a]	11.8±3.4[a]	4.9±0.1[a]
Immersed 5	4.80±0.22 (8)[a]	6.4±1.3[a]	2.7±1.4[b]	4.8±0.2[a]
Emersed 5	5.25±0.86 (6)[a]	3.3±0.4[a]	3.6±2.6[b]	4.5±0.3[a]
Immersed 120	7.44±1.32 (8)[a]	6.4±1.1[a]	2.4±1.5[b]	4.9±0.1[a]
Emersed 120	7.86±1.03 (8)[a]	4.7±0.9[a]	5.1±2.5[b]	2.4±0.2[b]
Control 120	5.52±0.86 (7)[a]	7.1±1.0[a]	4.3±1.4[b]	5.0±0.0[a]

(n) = No. of animals
Means in columns with different superscripts are significantly different (P<0.05)

THC levels showed no significant differences with handling or emersion in either experiment. However, in second experiment the THC levels tended to increase in both emersed and immersed lobsters 120 min after the handling event. Duncan's multiple comparison test showed that the THC values obtained in the first experiment were significantly higher than those obtained in the second experiment.

No significant alteration in phagocytic capacity was observed in the first experiment when the LDCL technique was used as the assay procedure (Table 35a). However, significant changes were observed in the second experiment. A decrease in phagocytic capacity occurred within 5 min of the handling event regardless of whether the test animals were immersed or emersed and levels were still low when stressed lobsters were sampled 120 min after initial stressor exposure (Table 35b; P<0.05). A significant decrease in phagocytic capacity with time was also seen in the control lobsters.

Handling with and without emersion had no effect on %granular cells (Table 35a&b; P>0.05). Vigour index was not affected by handling and/or emersion in the first experiment but significant differences between treatment groups were observed in the second experiment with vigour index being significantly reduced after 120 min emersion and handling (Table 35b; P<0.05).

The levels of tail blisters, tail erosion and missing legs in the different treatment groups in the two experiments are shown in Table 37. The overall prevalence of tail blisters and missing legs was considerably higher in the lobsters used in Experiment 1 compared to that in lobsters used in Experiment 2. Both groups showed similar levels of tail erosions. There were no significant differences between the levels of the three forms of exoskeleton lesions within the different treatment groups in either experiments (Chi-square; P>0.05).

A comparative assessment of interactions between LDCL response, NBT response and THC and variables such as the number of tail blisters (0 – 5), extent of tail erosion (scale of 0 – 5) and number of missing legs (0 – 6) was performed using one-way and two-way ANOVAs (Table 37). Neither tail blisters, tail erosion or missing legs showed any significant differences when tested individually by one-way ANOVA against THC, LDCL or NBT response except for THC and tail blisters in Experiment

2. The latter analysis showed that there was a significant increase in THC with increasing blisters ($P<0.05$). However, this result was based on the observation of high incidence of blisters (3 or 5) in just 3 lobsters, the majority of the test animals (81%) having no blisters.

Table 36 Presence of exoskeleton lesions in control and test lobsters

(a) LDCL assay

Treatment Group	Exoskeleton Lesions (Mean±SE) Tail Blisters (No./lobsters)	Tail Erosion (Scale 0-5)	No. Missing Legs
Control 0	1.4±0.5	5.0±0.0	2.0±0.3
Immersed 5	0.4±0.2	4.5±0.4	2.1±0.5
Emersed 5	0.8±0.4	3.6±0.8	1.5±0.8
Immersed 120	1.9±0.6	4.6±0.3	2.9±0.4
Emersed 120	1.1±0.5	4.8±0.2	1.5±0.5
Control 120	2.0±0.6	5.0±0.0	2.0±0.6

(b) NBT assay

Treatment Group	Exoskeleton Lesions (Mean±SE) Tail Blisters (No./lobsters)	Tail Erosion (Scale 0-5)	No. Missing Legs
Control 0	0.25±0.2	3.2±0.7	0.62±0.3
Immersed 5	0.00±0.0	4.5±0.1	0.12±0.1
Emersed 5	0.28±0.3	4.3±0.5	0.28±0.2
Immersed 120	0.38±6.4	4.2±0.5	0.00±0.0
Emersed 120	0.12±0.1	3.9±0.7	0.12±0.1
Control 120	1.25±0.6	4.0±0.4	0.25±0.2

Significant interactions were demonstrated between some of the exoskeleton variables when treatments were included in the analysis (Table 37). The two-way ANOVA demonstrated significant interactions between treatments and tail blisters for THC (both experiments) and phagocytic capacity (NBT assay), tail erosion and THC (Experiment 1) and phagocytic capacity (NBT assay) and missing legs and THC (Experiment 1). Overall, the most prevalent interaction between an exoskeleton lesion and the stress parameters studied occurred with tail blisters.

Table 37 ANOVA analysis of interactions between stress parameters and exoskeleton lesions

Exoskeleton Lesion	THC [b]		Phagocytic Capacity (LDCL assay)	Phagocytic Capacity (NBT assay)
	Exp 1	Exp 2	(Exp 1)	(Exp 2)
Tail Blister	**	*	N.S.	*
Tail Erosion	**	N.S.	N.S.	*
Missing Leg(s)	**	N.S.	N.S.	N.S.

a N.S. = Not Significant; * = P<0.05; ** = P<0.01
b Exp 1 = Experiment 1 (using LDCL assay)
Exp 2 = Experiment 2 (using NBT assay)

5.4 Discussion

The major finding of this study was that handling with or without air exposure caused phagocytic activity, as measured by the NBT method, to decrease. A tendency for THC to rise following physical disturbance was seen in one study (Experiment 2) but not in the other (Experiment 1). The absence of a rise in THC 5 min after handling in the lobsters exposed to air confirms other observations from this project (Cht 6, Section 4.3 & Section 6) of a tendency for THC to remain the same or to fall following emersion.

Generation of reactive oxygen intermediates (ROIs) during phagocytosis has been studied by assessment of luminol-dependent chemiluminescence (LDCL) in fish (Scott and Klesius, 1981; Hetrick et al., 1984; Klesius et al., 1985) and molluscs (Larson et al., 1989; Le Gall et al., 1991; Bachère et al., 1991, Anderson et al., 1992a, 1992b; Noel et al., 1993; Volety and Chu, 1995; Bramble and Anderson, 1997) but there have been few studies of this reaction in crustaceans. LDCL has been demonstrated in the shrimp, *Penaeus monodon* where the assay was used to investigate response to immunostimulants (Song and Hsieh, 1994). No data on the generation of ROIs by lobster hemocytes are available and this appears to be the first report of assessment of phagocytosis in lobsters using this approach.

The results obtained in this stress experiment showed that phagocytic capacity, as measured by the NBT method, was markedly reduced in animals sampled 120 mins after commencement of the experiment. Since the phagocytic capacity was reduced in both test and control animals it would appear that the stressor causing the reduction in generation of ROIs affected both groups similarly. The nature of this stressor is unknown. A further investigation of the likely cause of the decreased phagocytic capacity in lobsters used in stress experiments was carried out towards the end of the project and is described in Cht 6, Section 7.

The lack of a response in phagocytic activity as measured by the LDCL assay method could have resulted from a lack of sensitivity of this assay procedure compared to the NBT approach. Alternatively, the difference in experimental design used in the two experiments may have contributed to the differing results. In the first experiment only one lobster was placed in the mesh cage in each tank while the cages in the second experiment contained two lobsters. If the stimulation of a change in phagocytic

capacity is mediated by some form of visual or aural communication between stressed lobsters there would have been a greater opportunity for behavioural interactions in the cages containing two lobsters. The tendency for THC to increase after 120 mins in the NBT experiment but not in the LDCL experiment could also have resulted from this difference in experimental design. Further studies are required to determine the cause of the lack of consistency between the two sets of results and for the decrease in phagocytic capacity in the presumably unstressed, immersed control animals in the second experiment.

A negative correlation between THC and vigour index, previously observed in the acclimation experiment (Cht 6, Section 2), was again apparent in these trials. Vigour indices were higher in Experiment 2 than in Experiment 1 and a reverse trend was seen in THC. Furthermore, the only treatment group showing a significant decrease in vigour index compared to other groups in the same experiment (Experiment 2; emersed 120) also recorded the highest mean THC for the experiment. This negative correlation contrasted with results obtained with rejected lobsters in which a reversal in vigour was accompanied by a decreased THC (see Chapter 8). It would appear that the changes in THC seen in these laboratory stored lobsters differ from those affecting circulating hemocyte levels in reject factory lobsters.

It is possible that variation in immunocompetence or general health status influenced the findings of this study. The lobsters used in Experiment 1 had been held in the laboratory for a considerable length of time during which they had been repeatedly sampled and subjected to stress tests. The higher levels of exoskeleton lesions and the lower vigour index in this group of animals is evidence of their reduced health status. This may have influenced their response to the handling and emersion stressors, in particular the lack of response of phagocytic capacity as measured by the LDCL assay.

5.5 Relevance to industry practices and recommendations

The THC results confirm the previous conclusion (Cht 6, Section 4.5) that immune system functions in *Panulirus cygnus* affected by the number of circulating hemocytes may not be adversely influenced by a short term (less than 2h) air exposure providing handling disturbances are minimised. However, the reduction in phagocytic capacity in both control and test lobsters showed that an environmental stressor affecting lobsters was present during this experiment and further studies should be conducted into the stressor(s) and the effect of this exposure on lobster condition during post harvest handling.

6. Emersion experiment

6.1 Aim

The aim of this experiment was to evaluate the effects of 24h air exposure and subsequent 48h reimmersion on selected immunological parameters in western rock lobsters.

6.2 Experimental procedures

Commercial size lobsters for the experiment were obtained from Geraldton Fishermen's Co-operative and were acclimated in the MML communal storage tanks for 2 weeks prior to the experiment. After initial acclimation, lobsters were transferred into 180L glass tanks (four lobsters each tank) in individual oyster mesh cages for an additional 1 week acclimation. Lobsters were fed West Australian pilchards (mulies, *Sardinops neopilchardus*) during the 2 week acclimation period in the communal storage tanks and during part of the acclimation period in glass tanks.

Feeding was ceased 36 h prior to initial control sampling (-24h sample). Afterwards, all treatment group lobsters were exposed to air for 24h, and those to be sampled during recovery were then reimmersed. Control group lobsters remained immersed during the whole experiment.

Lobsters were sampled 24h before air exposure (-24h), during 24 h air exposure (0, 1, 3, 12, 24h), during 24 h recovery (0, 3, 8, 12, 24h) and 48h after being reimmersed. Each lobster was sampled only once during the experiment. The experiment was replicated three times and four lobsters were sampled in each treatment and control group in each replicate run.

During the experiment sampling related disturbances were minimised by planning the sampling carefully. Lobsters were held in individual oyster mesh cages (25 x 40 x 40 cm; W x L x H) during the experiment. They were sampled individually by removing one cage at a time from the tank and sampling the lobster outside of the experimental cool room. Before sampling, the water inlet to the glass tank was closed and water was siphoned from the tank to prevent lobsters from communicating via overflow water with lobsters in other tanks in the system.

Water quality parameters (dissolved oxygen, pH, temperature, ammonia and nitrite) were monitored during the experiment and relative humidity measurements were performed within drained tanks and in the experimental room.

A gross examination was carried out on each lobster and hemolymph samples were taken for total hemocyte counts (THC), refractive index (RI), antibacterial activity (ABF), bacteremia and histopathology. Assay methods used are described in Chapter 4. Each lobster was also analysed for sex and moult stage and the extent and location of exoskeleton lesions were recorded.

6.3 Results

Water DO saturation remained between 94-100% and nitrogen compounds below any reported harmful levels. Relative humidity within drained tanks and in experimental laboratory was between 90 and 100% and air temperature between 20 and 22°C.

THC levels declined sharply after lobsters were exposed to air, with a significant difference between treatment and control groups after 3 h of air exposure (Fig. 38; $P<0.05$). Later during the air exposure, the THC levels in treatment groups increased, with a slight overcompensation towards the end of 24 h air exposure. The THC levels

were similar in both treatment and control groups after lobsters were returned to water.

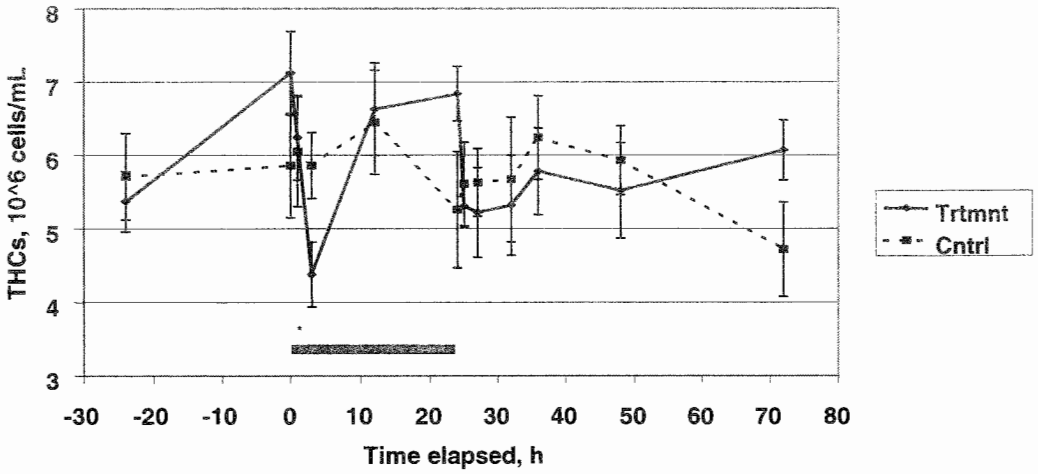


Figure 38 THC during long term air exposure and recovery (mean±SE). Black rectangle indicates air exposure time
* Treatment THC significantly lower than control (P<0.05)

A breakdown of THC results by intratank sampling order showed that lobsters sampled first had a significantly lower THC than the other three lobsters (Fig. 39; P<0.05).

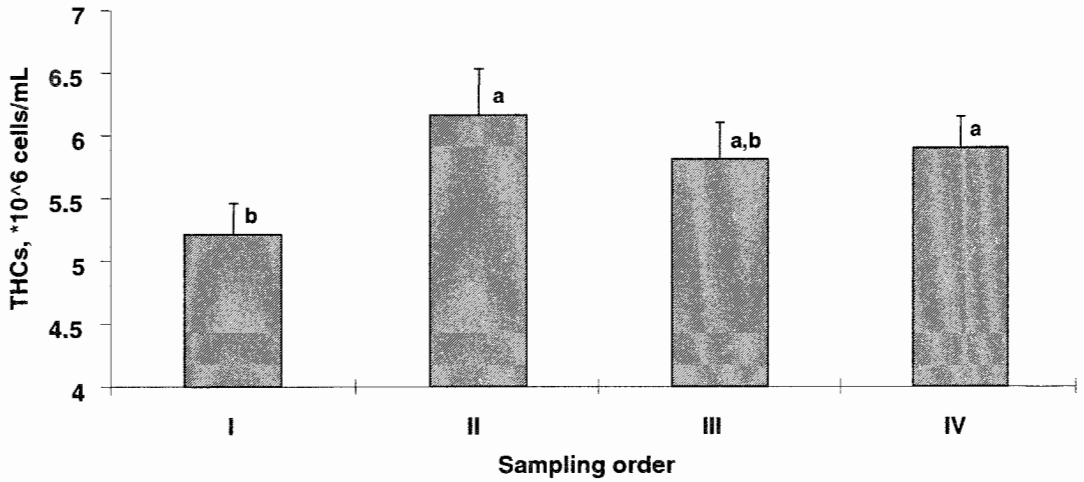


Figure 39 Influence of sampling order on THC result (mean±SE). Mean values with different letters are significantly different (P<0.05)

There were no significant differences between treatment and control groups in the refractive index values (Fig. 40) and no significant effect of sampling order on RI result (P>0.05).

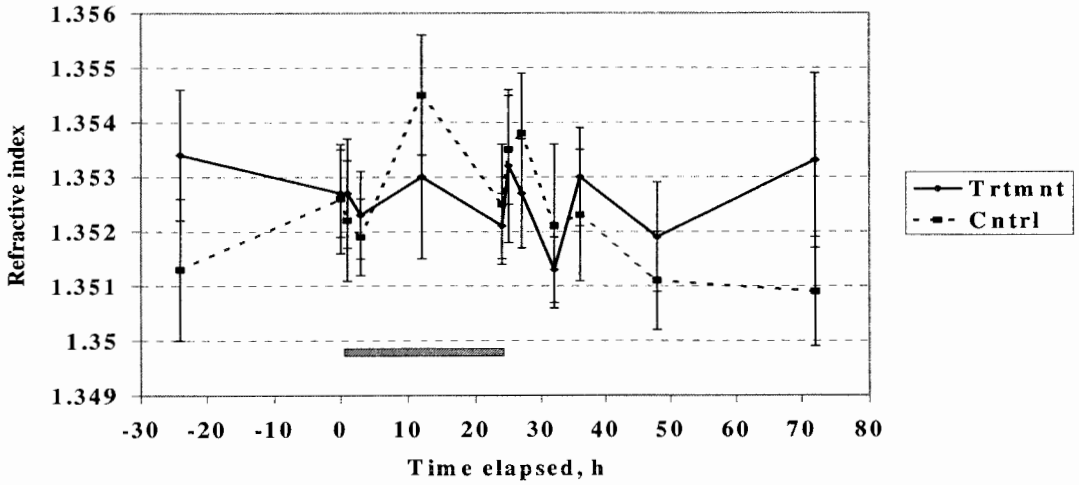


Figure 40 Hemolymph refractive index (RI) during long term air exposure and recovery (mean±SE). Dashed rectangle indicates the air exposure time

The levels of ABF observed in control and treatment lobsters are shown in Table 38.

Table 38 Antibacterial activity (ABF) in lobsters exposed to air (Mean±SE)

Treatment	ABF	
	Control	Treatment
24 h prior to aerial exposure	0.524 ± 0.065^{a} (7)	N/A
0 h prior to aerial exposure	0.192 ± 0.051^{bc} (12)	N/A
1 h aerial exposure	0.209 ± 0.109^{bc} (7)	0.342 ± 0.085^{abc} (10)
3 h aerial exposure	0.232 ± 0.070^{bc} (7)	0.266 ± 0.065^{bc} (8)
12 h aerial exposure	0.110 ± 0.083^{b} (5)	0.284 ± 0.122^{abc} (4)
24 h aerial exposure	0.088 ± 0.065^{b} (7)	0.210 ± 0.096^{bc} (7)
1 h reimmersion	0.330 ± 0.137^{abc} (4)	0.250 ± 0.126^{abc} (3)
3 h reimmersion	0.280 ± 0.096^{abc} (6)	0.192 ± 0.027^{bc} (6)
8 h reimmersion	0.301 ± 0.103^{abc} (4)	0.226 ± 0.082^{abc} (4)
12 h reimmersion	0.139 ± 0.089^{b} (7)	0.407 ± 0.078^{ac} (10)
24 h reimmersion	0.037 ± 0.037^{bc} (2)	0.246 ± 0.105^{abc} (4)

Mean values having different superscripts are significantly different
(n) = number of lobsters studied

Widely varying levels of ABF were observed between individual lobsters within most of the treatment groups with some lobsters in the group showing no ABF activity and others from the same group showing relatively high amounts. The mean values for control and treatment groups at each time point also showed a large variation. While statistically significant differences were observed between some mean values a statistically significant difference between a treatment and a corresponding control was only observed at the 12h reimmersion time point. Bacteremia was observed in 8.8% of lobsters studied, mostly at low levels, (Mean Colony Rank <0.50).

6.4 Discussion

The major finding of this study was that neither of the two immune parameters studied, THC and ABF, showed marked responses to long term emersion. THC showed a significant decrease during the first 3h of emersion and then increased for the remaining period of emersion. All THC values in control and treatment lobsters fell well within the normal range with the exception of the 3h emersion and the 72h control values which approached the lower limit of normal for post harvest lobsters. The results indicate that long term emersion did not have a marked effect on total circulating hemocytes. Whether longer periods of emersion alter THC cannot be predicted from these results.

Unfortunately, due to technical difficulties, no DHC values were obtained in this experiment. It is possible that long term emersion may have had an influence on the differential distribution of hemocytes and a consequent effect on the immune functions controlled by different hemocyte types. Other immune processes not assessed in this experiment may also be affected by long term air exposure.

The observation of a significant effect of sampling order on THC values indicates that rapid rises in THC can occur as a result of minor sampling disturbances such as noise, vibration, visual disturbance and/or chemical communication within a tank. The influence of such minor stressors on immune parameters was studied in a later experiment (Section 7) and shown to significantly affect some immune parameters.

No evidence was obtained to suggest that long term air exposure affects the circulating levels of antibacterial components in lobster hemolymph. ABF assays in this experiment were performed using a less sensitive method than was finally adopted in this study and, due to the large number of assays required, samples were stored at 4°C for several days before analysis. These factors may have contributed to the lack of demonstration of a significant effect of air exposure on antibacterial factor activity. The observation of the wide range of ABF values in lobsters within a particular treatment group suggests that either the assay method was imprecise or that environmental factors not controlled in the experimental design were the major determinants of ABF levels in the control and treatment lobsters. The low coefficient of variation obtained in a reproducibility test using the same procedure as was used in this assay (approx. 5%; Cht 4, Section 6.1.3) suggests that the latter interpretation of the ABF findings is more plausible than the former. The nature of these environmental factors is unknown but could relate to variations in prior exposure to bacteria causing induction of ABF or to genetic variations in natural levels in ABF.

Measurements of RI, an indirect measure of protein levels in lobster hemolymph, showed no significant influence of prolonged air exposure on circulating protein concentration. These findings are in contrast with those of the wounding experiment in which there was a significant decrease in protein concentration in the hemolymph of wounded lobsters tested 1 and 5 days after the wound was made. The results suggest that air exposure alone does not markedly affect hemolymph protein concentration and add weight to the previous conclusion that the decrease in protein levels seen in the handled and wounded lobsters was a direct consequence of wounding.

6.5 Relevance to industry practices and recommendations

The observation of an effect of sampling order on THC provides further evidence of the occurrence of an alarm phase stress reaction to physical or chemical environmental stimuli in *Panulirus cygnus*. While such reactions may be harmless, if superimposed on other stress reactions, or if repeated stimuli result in repeated alarm responses, a reduction in health status could occur. The results thus add further weight to the recommendation that post harvest stressors which cause alarm reactions (physical handling etc.) should be minimised.

Our results show that THC levels tended to fall when lobsters are initially exposed to air and return to normal levels after several hours of air exposure. This finding could be interpreted as indicating that air exposure does not have a detrimental effect on immune function. However, such a conclusion would be premature since circulating hemocyte numbers are only one measure of immune function. A detailed study of the influence of air exposure on a range of immune parameters such as phagocytic capacity, clotting time, antibacterial activity, bacteremia status and hemocyte differential distribution is required in order to fully evaluate the effect of emersion on immunity in lobsters. A preliminary investigation aimed at describing the different patterns of response of a range of immune parameters to short term emersion (2h) and handling was performed towards the end of the project (see Section 7). However, a detailed evaluation of the influence of long term air exposure on immunity and, hence, post harvest lobster survival, was not conducted. It is recommended that the emersion experiment described above be repeated and include estimations of all immune parameters investigated in this project.

7. Definitive laboratory stress trial

7.1 Aim

The aim of this experiment was to describe the pattern of response of all immune parameters investigated in this study (THC, DHC, clotting time, ABF, bacteremia and phagocytic capacity) to major (handling and emersion) and minor (noise, light, visual disturbances etc) post harvest stressors.

7.2 Experimental procedures

Lobsters used in this experiment were obtained from a Fisheries WA research vessel and were mature commercial size A animals or slightly smaller. Test animals were acclimated in the MML communal storage tanks for approximately 3 months prior to the experiment. Three days before commencing the experiment, lobsters were transferred into individual oyster mesh cages (two lobsters each cage) held in 180L glass tanks connected to a recirculating water system. Lobsters were fed West Australian pilchards (mulies, *Sardinops neopilchardus*) and mussels (*Mytilus edulis*) once a day during acclimation period in the communal storage tanks. Feeding was ceased following transfer to the glass tanks.

Two recirculating systems were used in the experiment, one located in the FRDC laboratory (Room 1) comprising two rows of eight 180L seawater aquaria fitted with

a temperature controlled recirculating water system (Cht 3, Section 2.1) and the other a similar system located in the adjoining laboratory (Room 2). The former system was used to house the test lobsters and one group of controls and the latter housed a second group of control lobsters. The water temperature in these systems was maintained at 21±1°C. Photoperiod in the FRDC laboratory was maintained at 14h light and 10h dark while dark conditions were maintained in the other laboratory except during the two sampling periods. Water quality parameters (dissolved oxygen, ammonia, nitrite, pH and temperature) were monitored during the acclimation period and kept within acceptable levels and excess food and debri was siphoned from the holding tanks twice a week.

Lobsters were exposed to a handling stressor for 1 min (physical removal from the tank of the mesh cage holding the lobsters and gentle shaking of the cage so as to prevent lobsters gaining a hold on the mesh). Hemolymph samples were taken from the base of the fifth walking leg of test lobsters after 5 min and 120 min, the latter group being subjected to the handling disturbance every 30 min after commencement of the trial (5 min and 120 min stressed lobsters). Lobsters from the 120 min sampling group were left in air in the mesh cages during the period of the trial. Control groups of lobsters held in tanks in both rooms were sampled immediately before commencement of the trial (initial disturbed control lobsters) and immediately after the sampling of the last treatment group (final disturbed control lobsters).

Researchers performing the sampling and handling of the lobsters were encouraged to talk and make noises while they were in Room 1. The black plastic covers normally surrounding the tanks in Room 1 to reduce visual disturbances were removed and researchers moved freely around the tanks in full view of control lobsters held in water as well as the emersed lobsters. Room 2, on the other hand, was only entered on two occasions during the trial – once to take hemolymh samples from control animals at the commencement of the trial (initial undisturbed control lobsters) and the other time to take samples at the completion of the experiment (final undisturbed control lobsters). The environmental conditions of the two rooms used in the experiment are shown in Fig. 41.

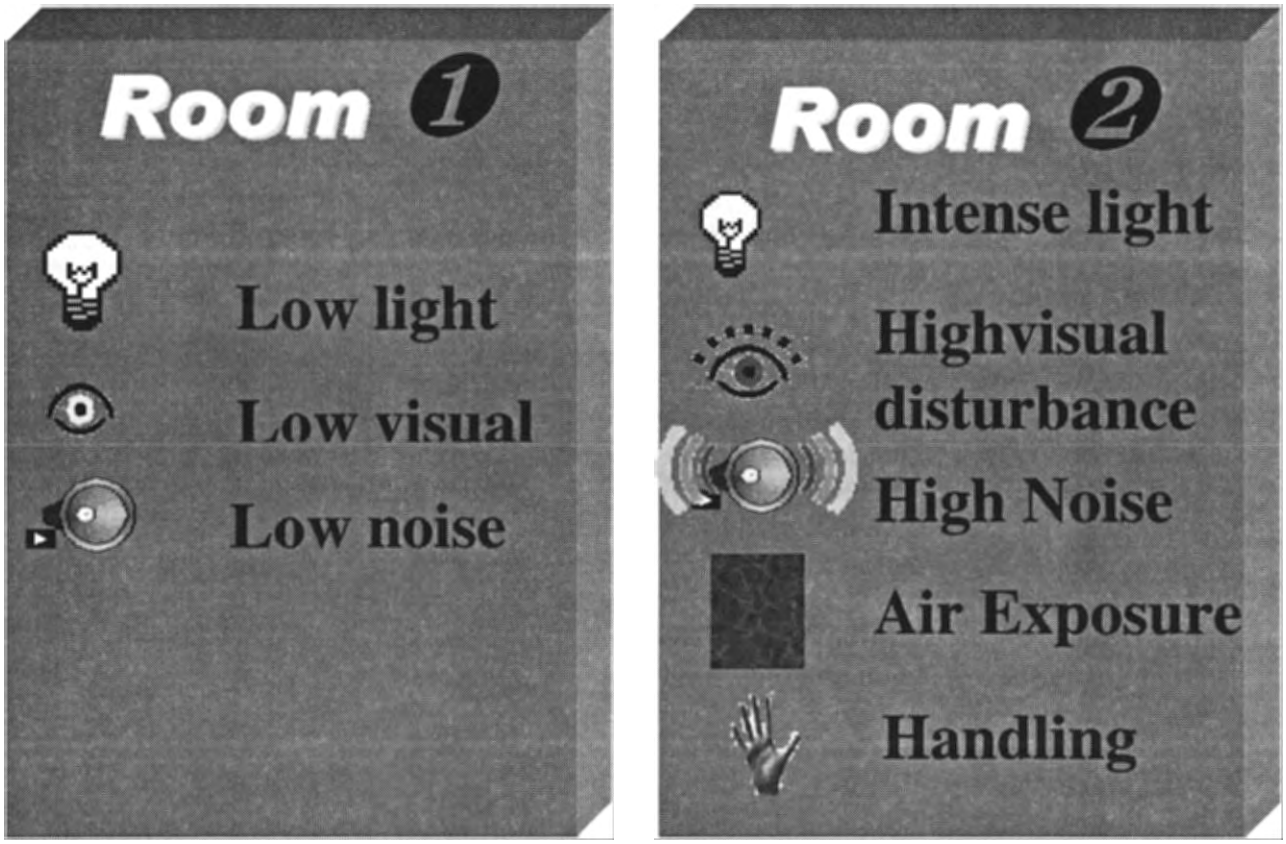


Figure 41 Environmental conditions in rooms 1 and 2 in definitive stress trial

Four hemolymph samples were removed from each lobster using the sampling procedure described in Cht 4, Section 1. One sample (200μL) was collected into a 1mL syringe containing 200μL precooled Na-cacodylate anticoagulant and analysed for THC and DHC (Cht 4, Section 2 & 3 respectively). A second sample was collected into a 1 mL syringe, three drops of hemolymph were placed on a blood agar plate for quantitative assessment of bacteremia (Cht 4, Section 5) and the remaining hemolymph was transferred to an Eppindorf tube for measurement of clotting time (Cht 4, Section 4). The third sample (200μL) was collected into a 1 mL syringe containing 200μL precooled modified PanBSS anticoagulant (Appendix 1) and analysed for phagocytic capacity (Cht 4, Section 7; NBT assay) and a fourth sample (200μL) was collected into a 1 mL syringe containing 200μL precooled TSAC anticoagulant (Appendix 1) and assayed for antibacterial activity (Cht 4, Section 6). Each lobster was also analysed for sex and moult stage and the extent and location of exoskeleton lesions was recorded.

7.3 Results

A summary of the analysis of variance of immune parameters obtained in stressed and control lobsters is given in Table 39. Statistically significant differences ($P<0.05$) were observed in the mean values of three of the immune parameters (THC, clotting time and phagocytic capacity; $P<0.05$) while the other three parameters (% granular

cells, bacteremia and ABF) showed no significant differences among treatments groups and controls (P>0.05).

Table 39 Immune parameters in stressed and control lobsters (mean±SE)

Treatments	THC (10^6 cells mL^{-1}) (8)	Clotting Time (sec) (4-8)	Bacteremia (mean colony rank) (8)	% GC (%) (8)	Phagocytic Capacity (NBT Units x 10^{-8}) (8)	ABF Units (8)
Undisturbed control 0	3.49±0.85^{c}	56.5±8.03^{a}	2.9±1.3^{a}	5.4±0.76^{a}	13.29±1.98^{a}	N/A
Undisturbed control 120	4.55±0.69^{bc}	55.5±2.62^{a}	3.4±1.3^{a}	8.9±2.03^{a}	13.84±3.27^{a}	N/A
Disturbed control 0	4.84±1.18^{bc}	46.5±9.07^{b}	4.6±1.1^{a}	7.0±1.59^{a}	13.35±3.23^{a}	0.498±0.053^{a}
Disturbed control 120	5.51±1.12^{abc}	43.0±3.15^{b}	3.9±1.4^{a}	7.6±1.52^{a}	6.38±1.36^{b}	0.333±0.036^{a}
5 min stressed	7.85±0.9^{a}	40.6±3.06^{b}	2.7±1.4^{a}	5.4±0.59^{a}	4.85±0.69^{b}	0.498±0.062^{a}
120 min stressed	6.55±0.92^{ab}	35.4±1.51^{b}	1.2±1.1^{a}	7.2±0.95^{a}	6.49±0.75^{b}	0.420±0.092^{a}

Mean values in the same column with different superscripts are significantly different (P<0.05)
(n) = number of animals studied

Handling and emersion caused a statistically significant elevation of THC after 5 min compared to the THC in disturbed control animals at the commencement of the experiment (Fig. 42; P<0.05). The circulating hemocyte numbers in lobsters sampled after 120 min emersion and repetitive handling were lower than those present in lobsters sampled after 5 min but the difference was not significant (P>0.05). THC levels in the disturbed control lobsters at the completion of the experiment were lower than those in the stressed lobsters but the differences were not significant (P>0.05).

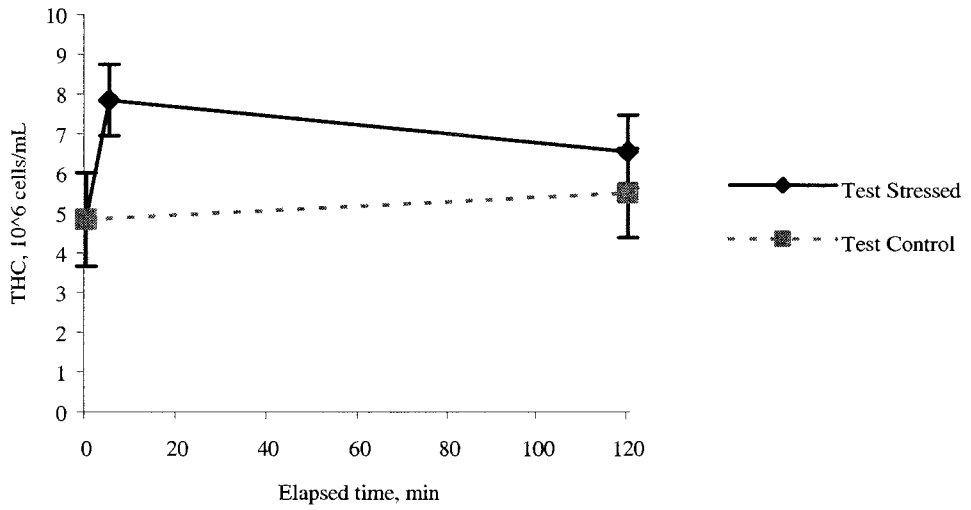


Figure 42 THC levels in stressed and disturbed control lobsters (Mean±SE) ◆ = stressed lobsters (solid line); ■ = disturbed control lobsters (dashed line); ✶ = mean value significantly different from initial control (P <0.05)

THC levels in the undisturbed controls were lower than those of the disturbed controls both at the commencement and at the completion of the experiment (Fig. 42) but these differences were not statistically significant (P>0.05). There was a tendency for the THC in both sets of control animals to rise during the experiment but the differences were also not significant (P>0.05).

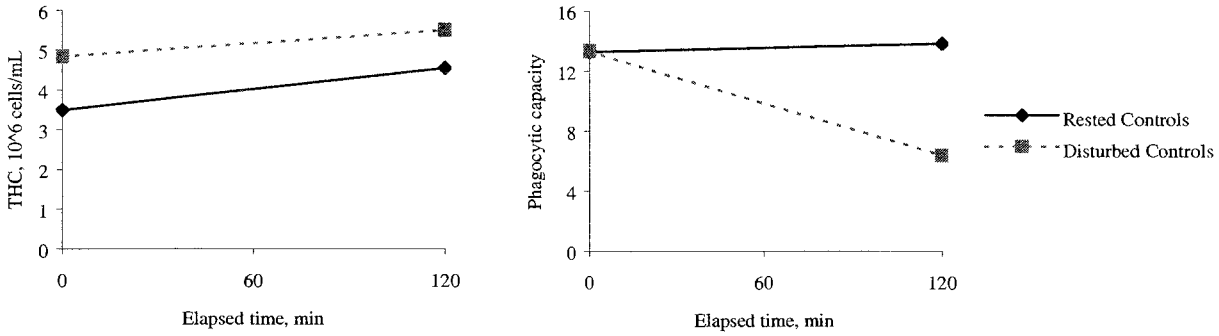


Figure 43 THC and phagocytic capacity in disturbed and undisturbed control lobsters (Mean±SE)

Handling and emersion caused a statistically significant decrease in phagocytic capacity after 5 mins and phagocytic capacity remained low after 120 min emersion and repetitive handling (Fig. 44; P<0.05). Phagocytic capacity in the disturbed controls also decreased significantly over the 120 time period, the mean value after 120 min being 56.2% of the initial value (P<0.05).

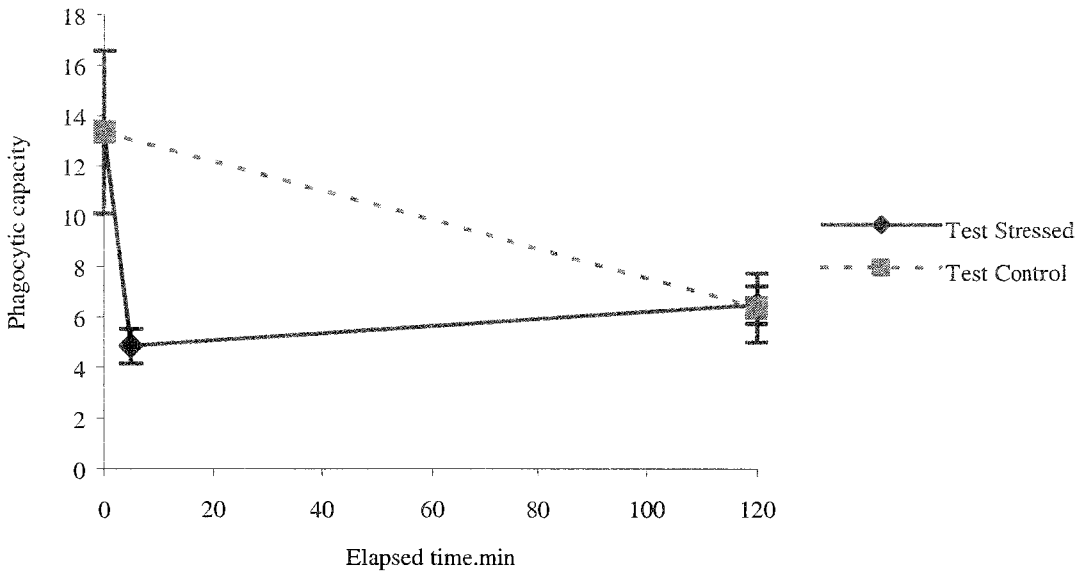


Figure 44 Phagocytic capacity levels in stressed and disturbed control lobsters (Mean±SE) ◆ = stressed lobsters (solid line); ■ = disturbed control lobsters (dashed line)

The phagocytic capacity in the undisturbed controls was similar to that of the disturbed controls at the commencement of the experiment (13.29 units compared to 13.35 units respectively) and remained high at the completion of the experiment (13.84 units) (Fig. 43). The phagocytic capacity in the disturbed controls, on the other hand, was significantly lower than that of the undisturbed controls 120 min after emersion and repetitive handling (6.38 units compared to 13.84 units respectively; $P<0.05$).

There was a tendency for clotting time to decrease compared to initial disturbed control levels 5 min after emersion and handling and to continue to decrease thereafter but these differences were not statistically significant (Fig. 45; $P>0.05$). The clotting time in the disturbed control lobsters at zero time and 120 min were similar.

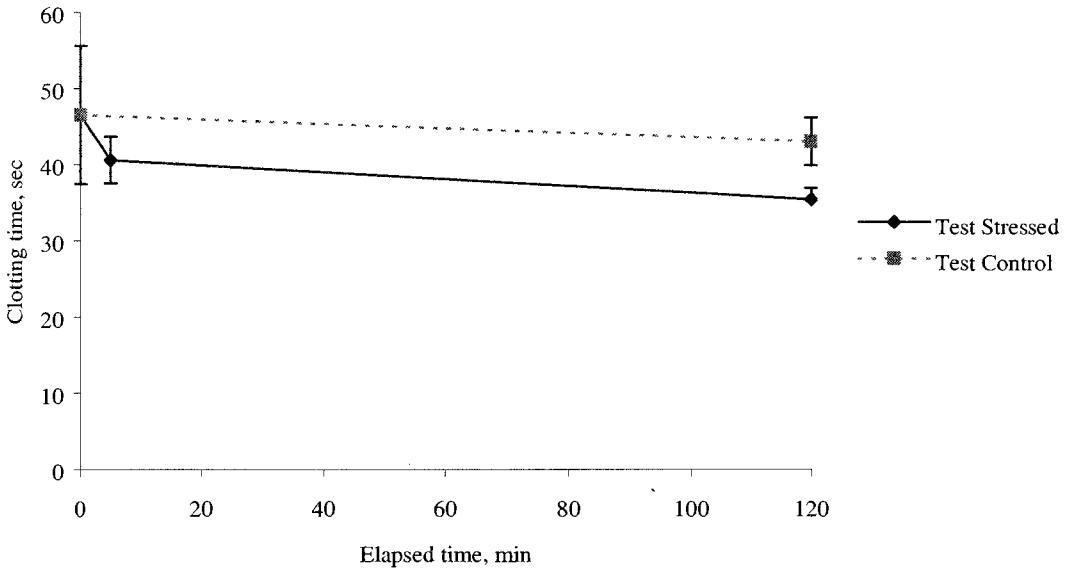


Figure 45 Clotting time in stressed and disturbed control lobsters (Mean±SE)
◆ = stressed lobsters (solid line); ■ = disturbed control lobsters (dashed line);

The clotting time in the undisturbed control lobsters was significantly higher than that of the disturbed control lobsters at both the commencement and the completion of the experiment (Table 39). %granular cells, in contrast, showed no significant difference between the initial or the final values for the disturbed and undisturbed controls (Table 39; $P>0.05$).

7.4 Discussion

A major finding of this experiment was that three of the immune parameters studied responded to a handling and an emersion event (THC, phagocytic capacity and clotting time) and the other three did not (% granular cells, bacteremia and ABF). The other major finding was that control lobsters held in the high disturbance room (Room 1) showed altered levels of immune parameters compared to controls held in the low disturbance room (Room 2). This difference was particularly evident in the phagocytic capacity results.

The patterns of change in THC and clotting time in response to emersion and handling stress observed in this study were similar to those seen in previous experiments (Cht 6, Sections 3 and 4; Cht 4, Section 4.4) – an increase in THC and a decrease in clotting time within 2 h of a handling event. Similarly, the pattern of change in phagocytic capacity following the handling and emersion event was similar to that observed in the previous stress trial (Section 5) including the decrease in phagocytic capacity in the disturbed control lobsters. A decrease in phagocytic capacity has been demonstrated as a stress response in molluscs (Anderson et al., 1992) but has not been previously described in lobsters. However, the contribution of the emersion and handling disturbance to the decrease in phagocytic capacity observed in this experiment is uncertain, given that the phagocytic capacity decreased to a similar degree in both control and stressed lobsters.

The failure of DHC values to change following an emersion and handling stressor confirmed earlier findings of a lack of response in the differential distribution of hemocytes to disturbance stressors (Section 3, Table 31). Similarly, no evidence was obtained in other studies of ABF to suggest that this parameter was affected by exposure to emersion (Section 6, Table 38) or handling and emersion (Section 3, Table 32). In contrast to the present findings, bacteremia was found to increase following handling and emersion in another experiment on post harvest lobsters (Cht 4, Section 5.4; Fig 18). However, in the latter experiment the control lobsters contained either no bacteria in their hemolymph or very low levels. The presence of high numbers of bacteria in the hemolymph of lobsters used in the present experiment may have masked an increase in bacteremia in response to emersion and handling.

It can be concluded that some immune parameters show an alarm phase type of stress response and others do not. This conclusion has an important consequence with respect to the parameters used to assess stress and health status in industry based experiments aimed at improving the quality of marketed product.

THC and clotting time levels in control lobsters held in the high disturbance room were significantly higher and lower respectively than those in control lobsters sampled at the same time point as control animals in the low disturbance room. This pattern of change is typical of that seen in stressed lobsters and suggests that the disturbed control lobsters were experiencing a mild stress reaction at the time of sampling.

The experimental design utilised in this study with *Panulirus cygnus* allowed lobsters to be exposed to different levels of several types of minor stressors including light intensity, noise and visual disturbance. Further studies are required to determine the contribution of these minor stressors to the mild stress reaction seen in disturbed control lobsters and whether such stressors have a detrimental effect on post harvest lobsters. Anecdotal reports from some lobsters processors suggest that maintenance of low light conditions may improve the health and condition of lobsters. If these reports are accurate a difference in light exposure may have caused the difference in the stress levels of lobsters held in the two rooms. Alternatively, differences in physical disturbances induced by researchers moving around the laboratory may have produced the differences in stress levels. Our previous demonstration of an effect of sampling order on lobster THC (Section 6) shows that this parameter is very sensitive to disturbance events.

It is of interest to note that THC and clotting time showed a different pattern of response to these minor stressors compared to phagocytic capacity. The THC and clotting time values in initial undisturbed control lobsters were significantly lower than corresponding values in the initial disturbed control lobsters, whilst there was no difference in the phagocytic capacity of these two groups of lobsters. However, there was a highly significant difference between the phagocytic capacity of these two groups of lobsters at the end of the experiment. These observations suggest that phagocytic capacity was affected by a stress inducing process or processes which differed from those affecting THC or clotting time.

A major difference between the environmental conditions in the two experimental rooms was that Room 1 contained lobsters which were being held in air and

physically disturbed while Room 2 did not. It is possible that the disturbed lobsters emitted a noise that was detected by the control lobsters in Room 1 but not by control lobsters in Room 2. This noise may have elicited an alarm reaction in all lobsters in Room I which manifested as a decrease in phagocytic capacity. The possibility of stressed lobsters generating noises which affect other lobsters should be investigated in *Panulirus cygnus* and, if demonstrated, their effect on health and survival during storage and transport determined.

The levels of bacteremia in the experimental animals were not only high compared to other studies (Cht 4, Section 5.4; Cht 5, Section 3.4) but there was also a wide variation within each treatment group, as evidenced by the high standard errors compared to other studies (Cht 7, Section 5 Table 53). The explanation for the wide individual variation, and for the overall high levels of bacteremia, is unknown. It is possible that there were large bacterial populations in the holding tanks and this resulted in increased bacterial numbers in lobster hemolymph. Variations between individuals with respect to host defense mechanisms which exclude bacteria from the hemocoel could have lead to variations in bacteremia levels. It is possible that the patterns of deviations in immune parameters observed in response to emersion, handling and minor disturbances may have been influenced by the abnormally high bacteremia.

7.5 Relevance to industry practices and recommendations

The observation of a significant elevation of THC and a reduction in clotting time in response to emersion and handling and to other minor stressors, provides further evidence of the occurrence of an alarm phase stress reaction to physical or chemical environmental stimuli in *Panulirus cygnus*. While such reactions may be harmless, if superimposed on other stress reactions, or if repeated stimuli result in repeated alarm responses, a reduction in health status could occur. The results thus add further weight to the recommendation that post harvest stressors which cause alarm stress responses in lobsters should be identified and changes in post harvest handling practices introduced to minimise such reactions. In particular, the influence of light and noise on stress levels of lobsters stored in factory tanks should be evaluated.

Further studies should be conducted on the factors inducing acute alarm stress responses in lobsters and of the influence of these, if any, on the health status of lobsters during storage and transport. Of particular importance would be the determination of whether exposure to multiple minor stressors has a detrimental effect on post harvest lobsters and, if so, how this effect could be minimised.

CHAPTER 7 FIELD STUDIES OF POST HARVEST HANDLING PRACTICES

1. Fremantle 1995/96 study – comparison of immune parameters in white and red lobsters

1.1 Aim

The aim of these trials were to describe the variation, if any, in THC and DHC in lobsters transported to a factory in a truck and acclimated in factory tanks.

1.2 Experimental procedure

Fifty lobsters were sampled after delivery to the Fremantle No. 1 factory by trucks. Lobsters were divided into two groups – fresh arrivals (whites: n = 13; reds: n = 12), accepted lobsters (whites: n = 13; reds; n = 12). A further 22 lobsters (whites: n = 10; reds: n = 12) were graded as rejects after 16 hr tank storage. The histopathology results obtained with these lobsters are presented in Chapter 8. Whites were collected in December 1995, reds in May 1996. Fresh arrivals were sampled immediately after delivery to the factory while accepted and reject lobsters were sampled after 16 hr acclimation at 18°C in the factory tanks. The lobsters were graded as accepted and rejects by factory staff. The number of lobsters sampled each day ranged from 3 to 24, the normal sample size being 6 lobsters. Only lobsters with a hard, rigid exoskeleton were sampled.

1.3 Results

Detailed results are described in detail in the accompanying paper (Appendix 4; Jussila et al., 1997) and in Chapter 8. The main findings with respect to fresh arrivals and accepted lobsters are given below.

THC values were significantly higher in fresh arrivals than in accepted lobsters in both white and red lobsters ($P<0.05$) (Table 40).

Table 40 THC in fresh arrivals and accepted lobsters (no. cells/mL x 10^6; mean±SE)

	Fresh arrivals	Accepted lobsters
White lobsters	8.5 ± 1.2^a	5.6 ± 0.7^b
Red lobsters	15.9 ± 1.1^a	5.3 ± 0.7^b

Means in the same row with different superscripts are significantly different ($P<0.05$)

The % granular cells was lower in the accepted lobsters in both the white and red groups and this difference was significant in the white lobsters (Table 41; $P<0.05$). The proportion of semi-granular cells was significantly higher, and that of the hyaline cells significantly lower, in the accepted lobsters compared to the fresh arrivals in the white group of lobsters ($P<0.05$). However the proportions of these cells types in the two different groups of red lobsters were similar ($P>0.05$).

Table 41 Differential distribution of hemocyte types (HC, hyaline cells; GC, granular cells; SGC, semi-granular cells) in fresh arrivals and accepted lobsters (%,mean ± SE)

	HC	GC	SGC
White lobsters			
Fresh arrivals	37.0±4.4[a]	11.9±2.1[a]	51.1±5.4[b]
Accepted lobsters	29.1±2.9[b]	8.0±1.9[b]	62.9±2.9[a]
Red lobsters			
Fresh arrivals	34.6±3.8[a]	13.1±1.7[a]	52.3±4.7[b]
Accepted lobsters	34.4±3.7[a]	10.7±1.4[a]	54.9±3.8[b]

Means in the same row with different superscripts are significantly different (P<0.05)

A comparison of the levels of different hemocyte types in individual lobsters revealed a strong negative correlation between the hyaline cells and the semi-granular cells in both white (r = -0.95; P<001) and red (r = 0.90; P<0.001) lobsters, a weaker correlation, but still highly significant, between the granular cells and the semi-granular cells in white (r = -0.57; P<0.001) and red (r = -0.46; P<0.001) lobsters but no correlation between hyaline cells and granular cells.

1.4 Discussion

The THC values observed in the fresh arrivals were at the upper limit (white lobsters) and above the upper limit (red lobsters) of the normal range for post harvest lobsters (Cht 5, Section 3.1) whilst the THC levels in the accepted lobsters, sampled after 16h acclimation in the factory tanks, were both within the normal range. These findings add further weight to the conclusion that 16-24h acclimation in factory tanks is sufficient for THC to return to baseline levels. The high THC values obtained in the fresh arrivals is similar to other findings (see below, Section 2), of an elevation in THC in lobsters delivered to factories in trucks. The publication describing the results of this study on THC and DHC in post harvest lobsters (Jussila et al., 1997) was the first published report on the influence of post harvest conditions on immune parameters in lobsters.

The change in the proportional distribution of hyaline cells in the accepted compared to fresh arrival lobsters from white lobsters was similar to that seen in the Day 5 hemolymph sample taken from wounded lobsters in the laboratory study of handling and wounding (Cht 6, Section 3). Whether the processes initiating this change in proportional distribution in cell types were the same in the two studies is unknown. It is possible, however, that a major stressor (truck transport or wounding) causes an alteration in hemocyte differentiation or production in the western rock lobster resulting in a change in the DHC.

1.5 Relevance to industry practices and recommendations

The elevated THC in fresh arrivals and the altered DHC in accepted lobsters provides evidence that truck transport causes an acute stress response in lobsters and this affects the hemocyte differentiation dynamics following storage in factory tanks. These two parameters could therefore be used to evaluate changes in truck transport

procedures aimed at reducing stress and improving the health of lobsters transported to factories in trucks.

2. Investigation of immune parameters in lobsters transported in trucks

2.1 Aim

The aim of this experiment was to investigate the influence on immune parameters of two different truck transport procedures – controlled temperature without a seawater spray and controlled temperature with a seawater spray.

2.2 Experimental procedures

Lobsters were commercially harvested by fishers and landed at the jetty at Jurian Bay. They were placed in a truck and transported a short distance to the Geraldton Fishermen's Cooperative Depot where they were stored in a cool room equipped with a seawater spray. Vigour indices were recorded on ten lobsters and hemolymph samples were taken for analysis of immune parameters. Lobsters were then transferred into one of two different trucks, one equipped with a refrigeration system (no spray) and the other with a spray system in addition to refrigeration (spray). Cooling was achieved by controlled temperature fans which circulated chilled air at approximately 15°C throughout the interior of the truck. The spray unit comprised a reservoir tank which was chilled and a pump and re-circulating spray system which delivered a fine spray over the lobsters. The lobsters were held in insulated containers which had channels in the floor to allow the sea water to be returned to the chilling unit.

Following placement in the trucks the lobsters were transported to the Geraldton Fishermen's Cooperative Factory in Geraldton where a second group of ten lobsters were tested and the remainder placed in the factory tanks. Two further groups of ten lobsters were sampled after 16 and 40 h holding in the factory tanks.

Physical and biological parameters (wt (g), OCL (mm), sex, presence of exoskeleton lesions and moult stage) were recorded for lobsters, vigour index was assessed following the criteria introduced by Spanoghe (1996) and hemolymph samples were removed from the ventral sinus at the base of the fifth walking leg.

Hemolymph samples (200 µL) were removed with a 23G needle and a 1mL syringe containing 200 µL precooled caffeine based anticoagulant (Appendix 1) and analysed for THC, DHC and antibacterial activity as previously described (Cht 4, Sections 2.2, 3.1 and 6.4). The target species used in the ABF assay was *E.coli* A128. Histopathology was performed as described in Cht 4, Section 8.

2.3 Results

The mean THC in the depot lobsters (12.15 x 10^6 cells/mL) was above the upper limit for the normal range for post harvest lobsters (8.79 x 10^6 cells/mL) and remained elevated following delivery to the factory in the trucks (Fig. 46). Lobsters delivered to

the factory in the truck without a spray system had a significantly lower mean THC (10.48±0.42 x 10^6 cells/mL) than the THC observed in lobsters transported in the truck with a spray system (11.98±0.45 x 10^6 cells/mL) but these values still exceeded the upper limit of the normal range.

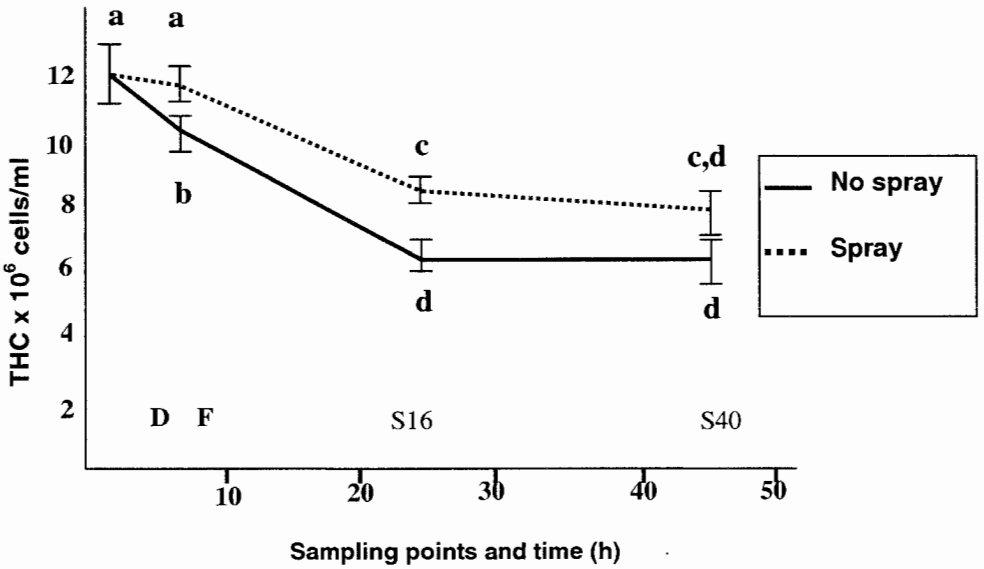


Figure 46 THC levels in lobsters transported to the factory in trucks with and without a spray system and acclimated in factory tanks (mean±SE). Letters refer to sampling points: D depot; F factory; S16 Storage tank, 16 h; S40; Storage tanks, 40 h. Means with different letters are significantly different (P<0.05).

After 16h acclimation the mean THC in both groups of lobsters fell. However, the mean THC in lobsters transported with the spray system was significantly higher than that of lobsters transported without spraying. After 40h acclimation there was no significant difference between the mean THC of the two groups of lobsters.

The proportional distribution of semi-granular cells and hyaline cells observed in the depot lobsters differed from values previously obtained in well acclimated post harvest lobsters, % semi-granular cells being lower and hyaline cells being higher than previously observed values. Following transport to the factory and subsequent acclimation in factory tanks there was a significant shift in the proportional distribution of hemocytes (Fig. 47). The pattern of this change in distribution differed slightly in lobsters transported by the two different methods. The overall change seen in all lobsters was a decrease in % hyaline cells and an increase in % semi-granular cells. This shift in distribution occurred more rapidly in the lobsters transported in trucks fitted with the spray system than in those transported without a spray system.

Figure 47 (a)

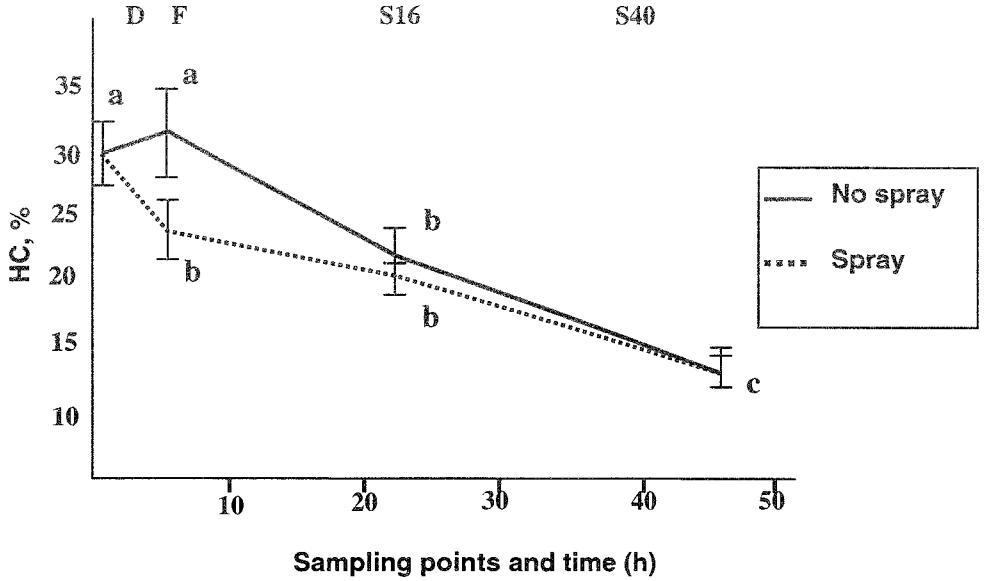


Figure 47 (b)

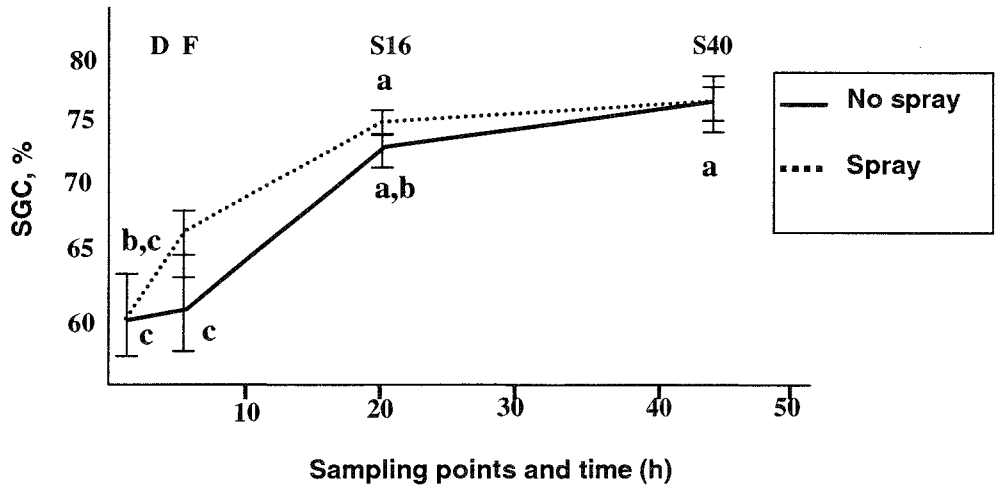


Figure 47 (c)

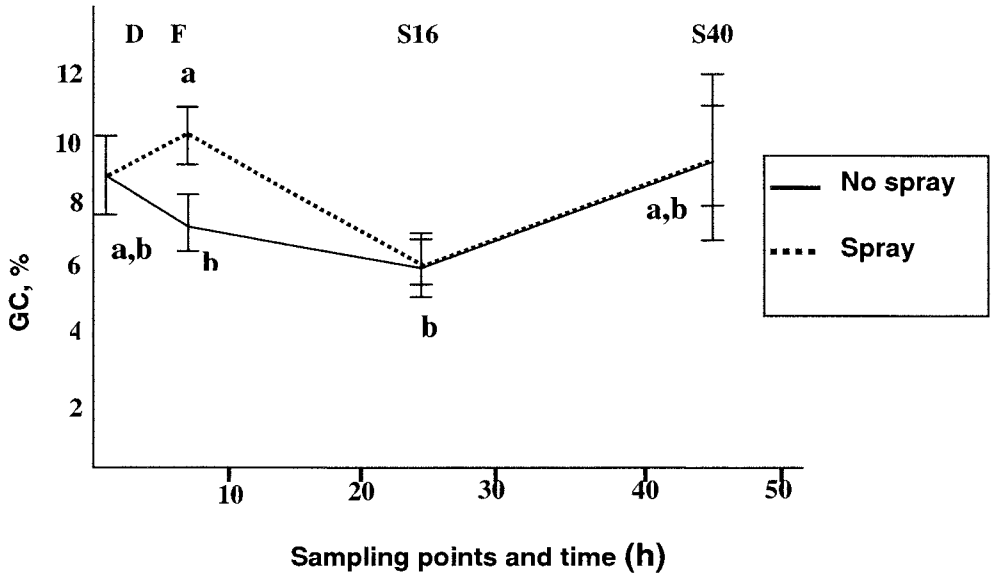


Figure 47 DHC levels in lobsters transported to the factory in trucks with and without a spray system and acclimated in factory tanks (mean±SE). Letters refer to sampling points: D depot; F factory; S16 Storage tank, 16h; S40; Storage tanks, 40h. Means with different letters are significantly different (P<0.05).

With the exception of one sampling point (factory, F) there was no significant change in the % granular cells in either group of lobsters. At sampling point F the % granular cells in lobsters transported with a spray system was significantly higher than that of lobsters transported without spraying.

The ABF levels in lobsters at the factory sampling point were similar in both groups of lobsters and little change was seen in the first 16 hr of acclimation (Table 42). After 40 hours in the factory tanks, antibacterial activity in lobsters transported in the truck without a spray system decreased significantly from the 16h level. The antibacterial activity at the 40h sampling point in lobsters transported in the truck with a spray system was also lower than the value observed after 16h acclimation but the difference was not significant. Due to technical reasons no mean ABF value was obtained for the depot lobsters.

Table 42 Influence of truck transfer conditions on ABF in post harvest lobsters (mean±SE)

Transport condition	Sampling point	ABF units (Mean±SE)
Spray	Factory	0.614 ± 0.097^{ab} (6)
	Storage tank 16h	0.409 ± 0.108^{ab} (7)
	Storage tank 40h	0.365 ± 0.103^{ab} (6)
No Spray	Factory	0.553 ± 0.115^{ab} (5)
	Storage tank 16h	0.641 ± 0.086^{a} (9)
	Storage tank 40h	0.164 ± 0.058^{b} (5)

Means with different subscripts are significantly different (P<0.05)
(n) = number of lobsters studied

The results obtained for the vigour index (VI) are shown in Table 43. Vigour indices in lobsters transported without spraying were consistently lower than those transported with a spray and this difference was significant immediately following arrival at the factory (sampling point F) and after 16h storage in factory tanks (T-test; P<0.01).

Table 43 Influence on truck transport conditions on vigour index (VI) distributions and means

Sampling point	Transport condition	VI, %				VI
		2	3	4	5	(Mean±SE)
Depot				100		4.0 ± 0.1^{a}
Factory	No spray	20	60	20		3.0 ± 0.2^{b}
	Spray		30	60	10	3.8 ± 0.2^{a}
Storage tank 16 h	No spray	30	40	30		3.0 ± 0.3^{b}
	Spray			80	20	4.2 ± 0.1^{a}
Storage tank 40 h	No spray	10	20	70		3.6 ± 0.2^{ab}
	Spray		10	90		3.9 ± 0.1^{a}

Means with different superscripts are significantly different (P<0.05)

2.4 Discussion

The major findings of this study was that lobsters sampled at the depot and the factory had significantly elevated THC, lower % semi-granular cells and higher %hyaline cells than previously observed in well acclimated post-harvest lobsters. These changes are typically observed in lobsters experiencing a marked, acute stress reaction. It would appear that either the lobsters delivered to the jetty from the fishing boat were highly stressed or that the short truck transport from the jetty to the depot caused a marked stress response in the lobsters which did not resolve, or was exacerbated, following the subsequent transport to the factory. While similar studies of changes in physiological parameters in lobsters under post-harvest transport and holding conditions have been reported (Whiteley and Taylor, 1992; Riley et al., 1997; Tod and Spanoghe, 1997) this and the previous study are the first reported investigations of the effect of truck transport per se on post harvest lobster immune parameters.

Elevated levels of circulating hemocytes were also observed in the study of white and red lobsters transported to the Fremantle No. 1 factory in trucks (Cht 7, Section 1). Furthermore, the pattern of change in the proportional distribution of hemocytes following 16h acclimation in the factory tanks in the white lobsters studied in Fremantle was the same as that seen in the white lobsters examined in this study an increase in %semi-granular cells and a decrease in % hyaline cells and %granular cells. The similarity of results in these two different studies adds weight to the previous conclusion that truck transport results in an increase in THC and a change in DHC. However, it is also possible that these changes were induced by environmental conditions experienced by lobsters in the pots or the fishing boats prior to truck transport. This issue was further examined in a series of later studies of changes in THC and DHC following pot capture and holding in fishing boat tanks (see below, Cht 7, Section 3).

Both vigour index and THC tended to be lower in lobsters transported in the truck without a spray system compared to the mean values seen in lobsters transported in the other truck. This observation is in contrast to previous findings (Chapter 6, Sections 2 and 4) of an inverse relationship between THC and vigour index but in keeping with other observations on reject lobsters of a positive relationship. One major difference between this and the previous studies was the reduction in temperature during transport. It is possible that slight differences in ambient temperatures between the two trucks may have occurred, leading to a effect on vigour index (Tod and Spanoghe, 1997) which was unrelated to other stressors affecting these two parameters.

No consistent change in antibacterial activity was seen in this experiment with the exception of the significantly lower antibacterial activity was observed in lobsters held in tanks for 40h following transport without spray. The biological significance of this change is unknown. Aono et al., (1990) have reported a decrease in ABF in the spiny lobster *Panulirus japonicus* exposed to transport and salinity stress. Noga et al. (1994) also reported a decrease in antibacterial activity in crabs collected from polluted compared to less polluted areas. Norton et al., (2001), on the other hand, found a very high antibacterial titre in a specimen of *Panulirus ornatus* with a tail lesion compared to levels observed in apparently healthy lobsters.

With the exception of the November factory trial (Cht 7, Section 5) the overall trend with ABF results for most of the experiments in which this parameter was measured was one of considerable variation between individuals in the same groups and a lack of consistency between mean values of different treatment groups. Thus, the apparent fall in ABF values seen after 40h acclimation in one group of lobsters but not in the other may have simply been a spurious result. Alternatively, it is possible that transport under spray leads to excessive exposure to bacteria which induce antibacterial factor activity. The experiment should be repeated to confirm this difference in response in ABF to truck transport conditions.

Significant differences were observed in some THC, DHC and vigour index values for lobsters transported by the two different methods. Lobsters transported in the truck with no spray had a lower vigour than those transported with a spray. Hyaline cells took longer to return to normal values in the lobsters transported without a spray system and THC values were lower in lobsters transported without a spray. However,

in this context a high THC is indicative of transport stress, suggesting that the spray truck provided less stressful conditions than the truck with no spray. Overall the differences were relatively minor and could not be used to make definitive statements about the efficacy of these two transport methods.

2.5 Relevance to industry practices and recommendations

The elevated levels of THC seen in lobsters transported in trucks are in agreement with the findings of the previous study (Cht 7, Section 1). It would appear that this parameter may have application in assessing the effect of truck transport conditions on stress/health status of lobsters. However, the wide variation between individual lobsters in a given treatment group and the sensitivity of this parameter to a whole range of stressors will probably mitigate against this application of THC measurements.

3. Boat studies

3.1 Aim

The aim of this study was to describe the variation, if any, in selected immune parameters at various points in the post harvest chain from point of capture to 48h acclimation in factory tanks.

3.2 Experimental procedure

Lobsters were sampled onboard commercial fishing vessels immediately after they were brought to the surface in pots (pot), just prior to being delivered to the factory (jetty), after a routine grading procedure (grade) and after they had been held in factory tanks for 24 or 48 h (tank). Hemolymph samples were taken from either the base of the fifth walking leg or from the pericardial sinus and THC was determined by the manual method described in Chapter 4. Assays were performed within 12 h of sampling.

A total of seven boat trips were studied - December 1996 (1 boat), April 1997 (two boats), June 1997 (two boats) and December 1997 (two boats). In the December 1996 trial DHC and antibacterial activity was also assayed using the procedures described in Chapter 4. ABF levels were also studied in April 1997 in one boat. Between 10 and 20 lobsters were sampled at each point in the post harvest chain.

3.3 Results

Statistically significant differences between THC values of lobsters from the same boat during the same trip were observed in three trips (April 1997, Boat 2; June 1997. Boat 1 and December 1997, Boat 1) (Table 44). However, there was no consistent pattern to these variations, the highest THC values in each trip being recorded at different stages of the handling chain.

Table 44 Total hemocyte counts (THC) at various points in the post harvest chain

	Pot	Jetty	Grade	Tank 24h	Tank 48h
December 1996	8.3±0.6[a]	7.9±0.3[a]	-	-	6.9±0.3[a]
April 1997					
Boat 1	4.8±0.6[a]	5.5±0.8[a]	6.7±0.6[a]	6.0±0.6[a]	4.9±0.4[a]
Boat 2	6.3±0.7[b]	8.5±1.0[ab]	6.3±1.0[ab]	8.0±0.6[ab]	8.5±0.8[a]
June 1997					
Boat 1	4.9±1.1[a]	10.3±1.0[c]	10.0±0.9[c]	7.4±0.8[b]	4.2±1.1[a]
Boat 2	7.7±0.6[a]	8.9±0.9[a]	8.8±0.7[a]	7.1±0.5[a]	7.3±0.7[a]
December 1997					
Boat 1	15.2±5.2[a]	-	7.6±1.0[b]	7.6±0.6[b]	7.7±0.6[b]
Boat 2	7.0±1.0[a]	7.9±2.0[a]	6.7±0.6[a]	6.5±0.6[a]	6.8±0.4[a]

Mean values within the same low different superscripts are significantly different ($P<0.05$)

Antibacterial activities were studied at various points in the post harvest chain in two of the seven boat trips. No statistically significant differences were observed at any post harvest point in either of the two studies (Table 45).

Table 45 Antibacterial activity levels at various points in the post harvest chain

Post harvest stage	December 1996	April 1997
Pot	0.342±0.059 (13)	0.216±0.197 (3)
Jetty	0.456±0.067 (16)	0.109±0.028 (10)
Grade	-	0.125±0.056 (7)
Tank, 24h	-	0.087±0.051 (8)
Tank, 48h	0.386±0.058 (16)	0.281±0.093 (6)

(n) = No. of lobsters studied

Minor but statistically significant differences were observed in the relative proportions of different hemocyte types in pot lobsters compared to jetty and tank 48h lobsters in the one study performed on this parameter (Fig 48; $P<0.05$). The proportion of both hyaline cells and granular cells increased during boat transport and that of semi-granular cells decreased. The proportion of granular cells decreased during tank storage but no significant change was seen in the proportions of hyaline cells and semi-granular cells during this post harvest stage.

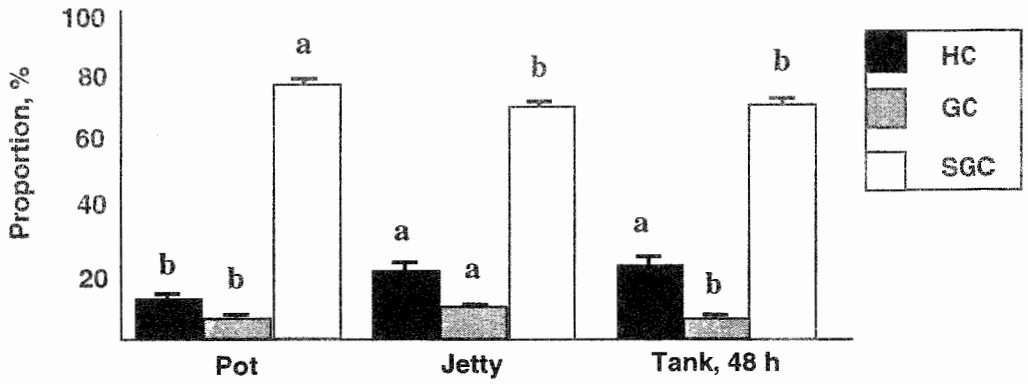


Figure 48 Percentage hyaline cells (HC), % granular cells (GC) and % semi-granular cells (SGC) at different points in the post harvest chain (mean±SE). Mean values for each cell type with a different letter are significantly different ($P<0.05$)

3.4 Discussion

The main finding of this study was that with one exception (June 1997, Boat 1) the mean THC of jetty or grade lobsters was not significantly different from that of lobsters stored in factory tanks for 24 – 48h. This observation contrasts sharply with those seen in lobsters transported in trucks in which a consistent decline in THC was seen following placement in factory tanks (Cht 7, Sections 1 and 2). Similarly, grading had no obvious effect on THC, the mean values of graded lobsters observed in the six studies performed being similar to those obtained at the completion of the boat journey (jetty lobsters). However, several THC values observed in the first three stages of the post harvest chain – pot, jetty and grade - exceeded or were close to the upper limit of the normal range. All values seen in the tank lobsters fell within the normal range. The demonstration of a significant elevation in THC prior to placement in factory tanks showed that some groups of lobsters studied were experiencing acute stress responses during boat transport and grading.

The THC values obtained in the pot lobsters varied from 4.9 – 15.2 x 10^6 cells/mL. This range of variation was higher than was seen at any other point in the post harvest chain. The wide range of values in the pot lobsters probably reflected different levels of alarm reactions in these lobsters at the time of sampling.

No significant differences were observed in antibacterial activity levels at different points in the post harvest chain. Similarly, while significant alterations were seen in some of the DHC values, these were minor compared to the changes in DHC seen in the truck study (Cht 7, Section 1). Overall the results suggest that boat transport did not have a major effect on any of the three parameters studied.

3.5 Relevance to industry practices and recommendations

Although the number of boat and truck trips studied was small, the results suggest that boat transport has less of an effect on immune stress parameters than truck transport. However, only three stress parameters were studied and only one of these, THC, in

detail. Further studies should be performed using all immune stress parameters developed in this project in order to validate the application of immune stress parameters in assessment of influences of boat and truck transport conditions on lobster stress and health status.

4. Factory trial No 1 – April 1998

4.1 Aim

The aim of this trial was to investigate changes in selected physiological and immunological parameters following exposure of lobsters to simulated truck transport conditions.

4.2 Experimental procedures

The experiment was carried out in March-April 1998 at Geraldton Fishermen's Co-operative factory, Geraldton. The lobsters, arriving on commercial fishing boats at the GFC live storage facility in Geraldton Fishermen Harbour, were graded and placed in crates (approximately 25 lobsters in each). They were exposed to air for 1h and placed in a truck container for 6h. The lobsters were sprayed with 14°C recirculating seawater (60 L/min/10 kg) while in the container.

After the treatments, lobsters were graded by GFC staff and sampled (10 fit-for-live and 10 rejects (if less than 10 rejects per treatment were available extra fit-for-lives were used). They were then tagged and held in live lobster tanks for 7d, packed according to the industry standards for live shipments, unpacked after 36h, and placed in the live lobster tanks for 24h. During this period lobsters were monitored for weakness and moribund features by the GFC factory staff and reject lobsters removed from the tanks were recorded.

All lobsters were sampled for immunological (THC, % granular cells, and ABF) and physiological variables (see FRDC 96/345 final report). The data was analyzed using a SSPS statistical package and also by discriminate statistical methods in order to establish a model predicting the fate of lobsters as observed in GFC live storage facility. Results of the discriminate analysis are presented in the FRDC 96/345 final report.

4.3 Results

A total of 350 lobsters were sampled for physiological and immunological parameters after they had been exposed to air for 1 h and then held in a truck container for 6h.

No statistically significant differences ($P>0.05$) in % granular cells were observed between the six observed categories (died in tanks, D; weak in tanks, W; lost legs in tanks, LL; fit-for-live, LA; weak at loadout, WL; no records, NR) (Fig. 49). However, despite there being no significant differences in % granular cells, the separation into different outcomes as predicted by the discriminate analysis model was improved when this parameter was included Paterson et al., (2001).

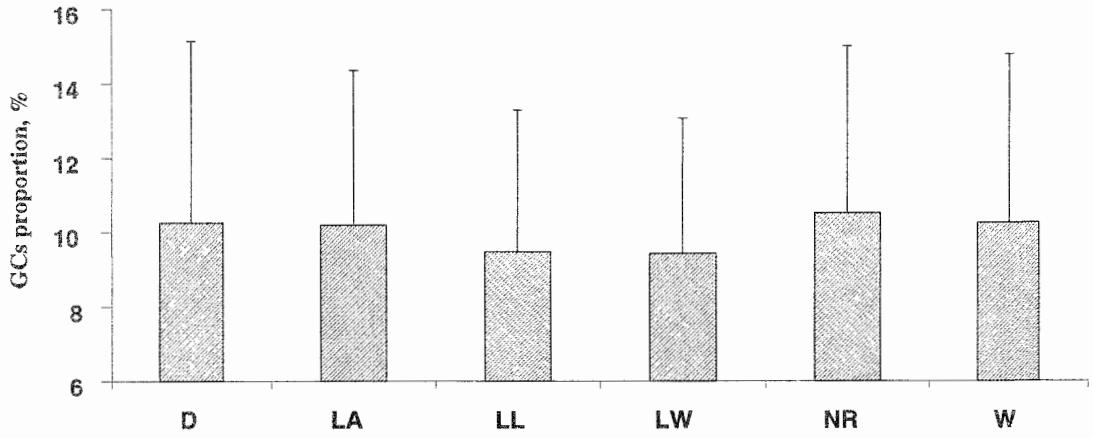


Figure 49 Percentage granular cells (GC) in different groups of lobsters held in live storage tanks after simulated truck transport (mean±SD), D: died in tank; LA: fit-for-live: LL: lost legs in tanks; LW: weak at loadout: NR: no records: W: weak in tanks.

The THCs showed significantly lower levels ($P<0.05$) in lobsters that had lost legs during live tank holding (Fig. 50). Unfortunately, the THCs could not be included in the discriminate model due to a technical problem in sample analyses during the experiment (resulted in roughly 30% of the results having to be removed from the analysis).

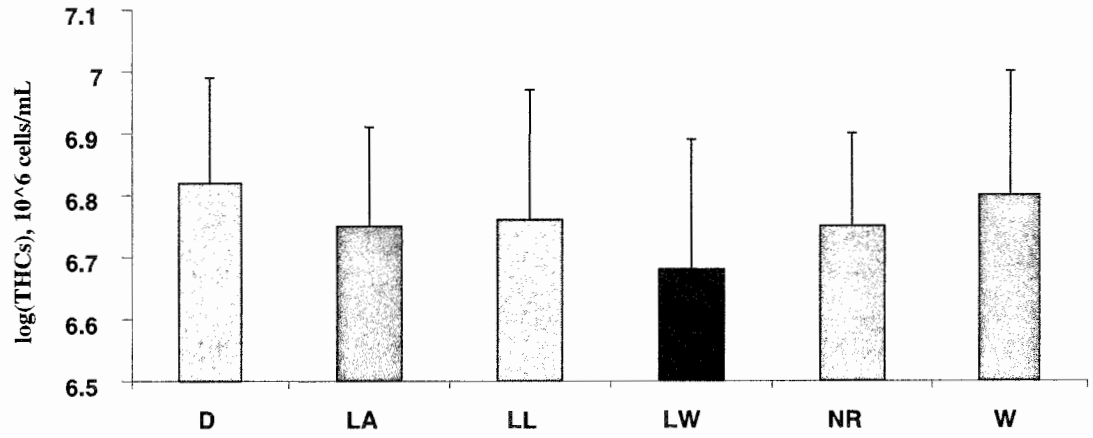


Figure 50 Total hemocyte counts (THC) in different groups held in live storage tanks after simulated truck transport (mean±SD). Black bar indicates significant difference ($P<0.05$) between mean THC and other mean values. D: died in tank; LA: fit for live: LL: lost legs in tank; LW: weak at loadout: NR: no records: W: weak in tank.

4.4 Discussion

This was the first of three similar trials conducted in 1998/99. This first trial achieved the goal of fine-tuning the experimental design and identifying problems which would preclude a successful completion of future trials. The identification of %granular cells as an immune parameter which is significantly affected by simulated truck transport confirmed earlier results in which it was found that this parameter was markedly reduced in reject lobsters (see Chapter 8).

4.5 Relevance to industry practices and recommendations

The experiment showed that % granular cells could be used as part of the model in predicting the fate of lobsters under post-harvest handling conditions. THC assays might also be useful, but their role as part of the model has to be studied further. The model was shown to be a promising tool with a prediction rate of 70% (when the most suitable physiological and immunological parameters were included) suggesting that it could be used as a tool to assess the disturbance experienced by lobsters under different conditions.

5. Factory trials No 2 and 3 – Nov/Dec 1998 and Mar 1999

5.1 Description of trials performed

5.1.1 Experimental approach

Two factory trials aimed at comparing responses to simulated live transport in selecting immune system stress indicators and at evaluating several different approaches to storage of lobsters during transport, were performed at Geraldton Fishermen's Cooperative (GFC) factory in November/December 1998 ('white' lobsters) and in March 1999 ('red' lobsters). Lobsters from the commercial catch were subjected to five different storage treatments (flow-through submerged, recirculating submerged, flow-through spray, recirculating spray and humid air) for 6hr, graded, sampled and placed in storage tanks for a period of 7 days. At the end of this period the surviving lobsters were taken through a simulated live shipment and then re-tanked for 24 hr. Weak or dead lobsters were recorded during 7 day storage period and following the simulated live shipment to give four different outcome groups (dead or weak in tanks during 7 days storage, DW; dead or weak following simulated live shipment, LD0, dead or weak following simulated live shipment and subsequent 24 hr recovery in tank, LD1; and fit-for-live following simulated live shipment and subsequent 24 hr recovery in tank, SS). The results obtained from four (March trial) or five (November/December trial) different immunological tests performed on the hemolymph samples collected from lobsters in the four different outcome groups and following the five different storage treatments were then compared in order to determine:

- which of the immunological parameters, when included in the discriminate analysis, enhanced the ability to discriminate between different outcomes

- which storage treatment(s) caused the most post-harvest stress (as judged by the degree of deviation of mean values of hemolymph immune parameters from normal).

A similar experimental approach was taken in the two factory trials. The same experiment was repeated on three successive days and the data for each day from each treatment group were pooled. The November/December trial on 'white lobsters' was conducted from 23/11/98 to 5/12/98 and the March trial on 'red' lobsters from 1/3/99 to 11/3/99. Approximately 300 lobsters were sampled in each trial.

Lobsters (pink 'A' category; 390-490g) from a number of different boats were placed in a tank at the GFC live holding facility where they were acclimated for 24 h. During the storage in the tank the lobsters were fed with chopped fish (3% wet weight) so as to simulate having been caught from a baited pot immediately prior to transport and storage. On the day following capture and placement in the tank a sample of 45-46 lobsters was taken from the holding tank, placed in two crates and weighed so as to give approximately 21kg/crate. The crates were placed into each of five treatment cubicles (two crates/treatment), the environmental conditions of which simulated the following transport/storage approaches:

- Flow through submerged
- Flow through spray
- Recirculating SW submerged (79 l water/system; approx. equivalent to 4:1 ratio of water volume:lobster wet weight)
- Recirculating SW spray (79 L water/system)
- Humid air (~ 100% relative humidity)

The lobsters were held in the cubicles for 6hr during which water quality parameters (ammonia, protein, oxygen and pH) were measured at 1hr intervals.

At the completion of the exposure interval lobsters were tagged and graded. Twenty lobsters from each treatment were sampled while the remaining 20-26 unsampled lobsters served as controls for the effects of blood sampling and tagging. The following immunological tests were performed on all hemolymph samples:

- Total hemocyte counts (THC)
- % granular cells
- Antibacterial factor (ABF) (November trial only)
- Bacteremia
- Clotting period

All lobsters were placed in factory tanks for 7 days during which moribund or dead lobsters were removed and recorded by research and factory staff. The sampled lobsters were then taken through a simulated load-out following established practices used by GFC staff. After 24hr in foam containers lobsters were removed and placed in factory tanks to recover for 24 hr. Animals dying during the 'in transit' or recovery period were recorded. In the March trial the lobsters were graded following the simulated live shipment. Post-shipment grading was not performed in the November/December trial.

Tag numbers of all lobsters were recorded for the following outcome groups:

- Died or weak in tanks (DW)
- Dead or weak following simulated live shipment (LD0)
- Dead or weak after simulated shipment and 24hr acclimation (LD1)
- Fit-for-live following simulated live shipment fit-for-live following simulated live shipment and subsequent 24 hr recovery in tank (SS)

The total number of lobsters in each of these outcome groups were then analysed using SPSS in order to determine significant differences between treatment and outcome groups.

In addition to the laboratory analyses the following gross observational data was recorded for each lobster:

- Date of collection
- Identifying no.
- Carapace length
- Sex
- Moult stage
- Appendage loss

5.1.2 Assay Procedures

(Note: Detailed descriptions of the methodology for each of the assays described below is provided in Chapter 4).

5.1.2.1 THC

THC was determined by both manual counting and automatic counting on a Coulter Counter. Results were expressed as No. cells/ml hemolymph.

5.1.2.2 Percentage granular cells

Percentage granular cells was determined by staining fixed hemolymph smears with May-Grunwald and Geimsa stain and counting hemocytes in the preparations using a binocular microscope.

5.1.2.3 Clotting period

Clotting period was determined using the method described in Chapter 4.

Data for samples which failed to clot (no clot reaction) were not included in the determination of mean values for treatment groups or health and survival outcomes.

5.1.2.4 Bacteremia

During the November/December trial the number of bacteria present in hemolymph samples (bacteremia) was estimated by placing three drops of hemolymph on an agar plate, shaking the plate to spread the sample over the agar surface and leaving the plates at room temperature for up to 5 days. Following incubation the number of colonies on the plates was counted. The confined space of the GFC room used for the trials resulted in unavoidable contamination of the plates, as evidenced by the presence of colonies on parts of the plate not covered by the hemolymph sample. Accordingly, in the March trial, the hemolymph drops were not spread on the plates and considerable care was taken to avoid contamination. This approach allowed hemolymph colonies and contaminant colonies to be readily distinquished.

5.1.2.5 Antibacterial activity

Antibacterial activity (ABF: previously called bactericidin) was measured in the November/December trial using the following approach.

Hemolymph was collected into anticoagulant and cell membrane was destroyed with ultrasound, which resulted in release of cell contents into plasma. Cell fragments were centrifuged and plasma was analysed for antibacterial activity as described before. Total antibacterial activity was expressed according to the following equation:

$$ABF = 1 - (T-AC)/C$$

Where T = colony numbers after incubating bacterial suspension with active plasma;
AC = colony numbers in plasma (contamination control);
C = colony numbers after incubating bacterial suspension with heat-treated plasma

ABF was not measured in the March trial as the pattern of results obtained with this assay in the November/December trial was similar to that obtained by simply measuring bacteremia. In addition, the expense of conducting ABF measurements in the March trial (approx $2500) was prohibitive.

5.1.3 Statistical analysis

Normality of data was tested using Kolmogorov-Smirnov test and the homogeneity of the variances of means was confirmed using Levene test. Data were log transformed if necessary prior to analyses. The data were analysed using either a Student T test or one - way ANOVA, and multiple comparisons were made using the Least Significant Differences multiple range test. Results were considered to be significantly different at $P \leq 0.05$.

5.2 Results

5.2.1 Water quality in holding systems

These results are fully documented in the final report for FRDC 96/345. The major feature of note were a marked increase in dissolved ammonia in the recirculating treatments and a higher temperature in March 1999 compared to November 1998.

5.2.2 Grading results

The initial grading of lobsters conducted immediately following removal of lobsters from the cubicles provides one method of assessing the effect of treatment on lobster condition. These results are shown in Figures 51, 52 and 53. The highest proportions of accepted lobsters occurred in the two treatment groups in which lobsters were submerged. The two spray treatments resulted in most lobsters being graded as weak. Recirculating spray treatment resulted also in high proportion of dead animals, while flowthrough spray system had high proportion of dead animals only in March, but not

in November trial. A high proportion of weak or dead lobsters was also observed in the group exposed to humid air, particularly in the March trial.

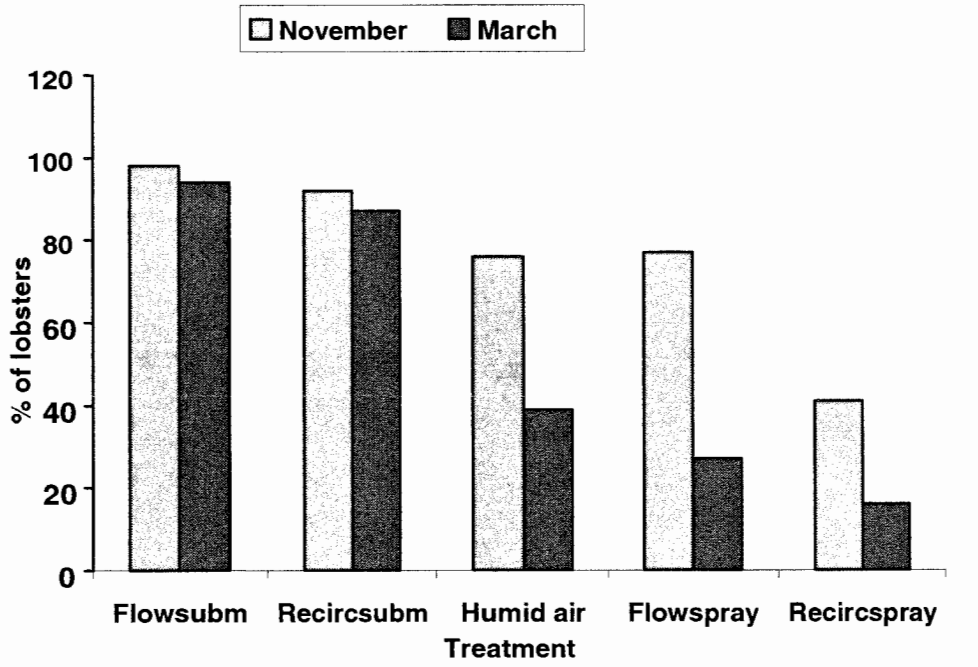


Figure 51 Percentage of lobsters graded as accepted

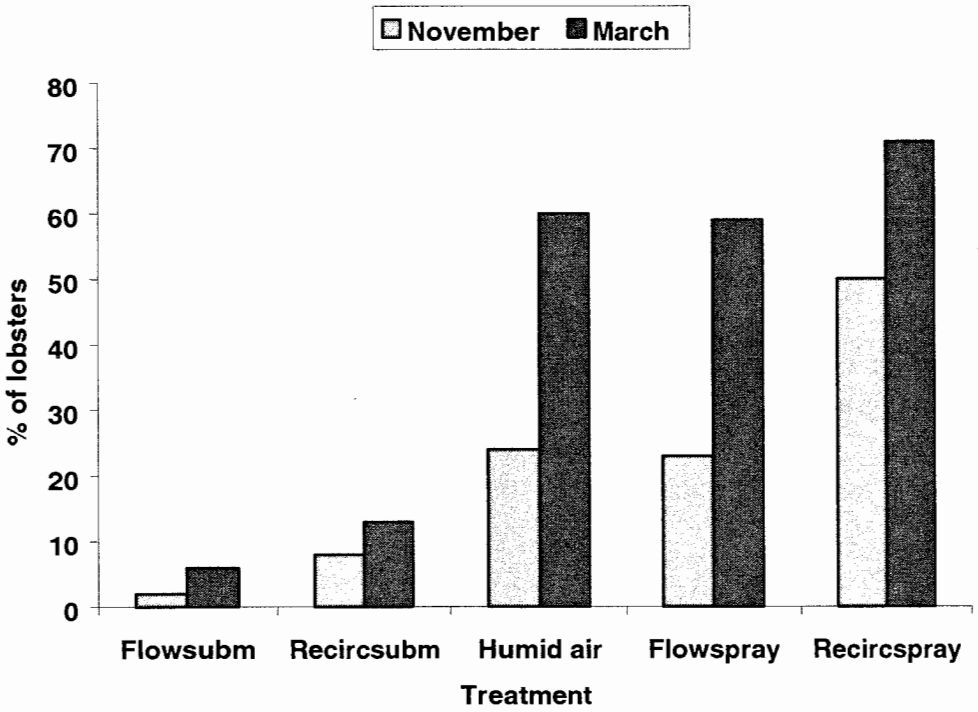


Figure 52 Percentage of lobsters graded as weak

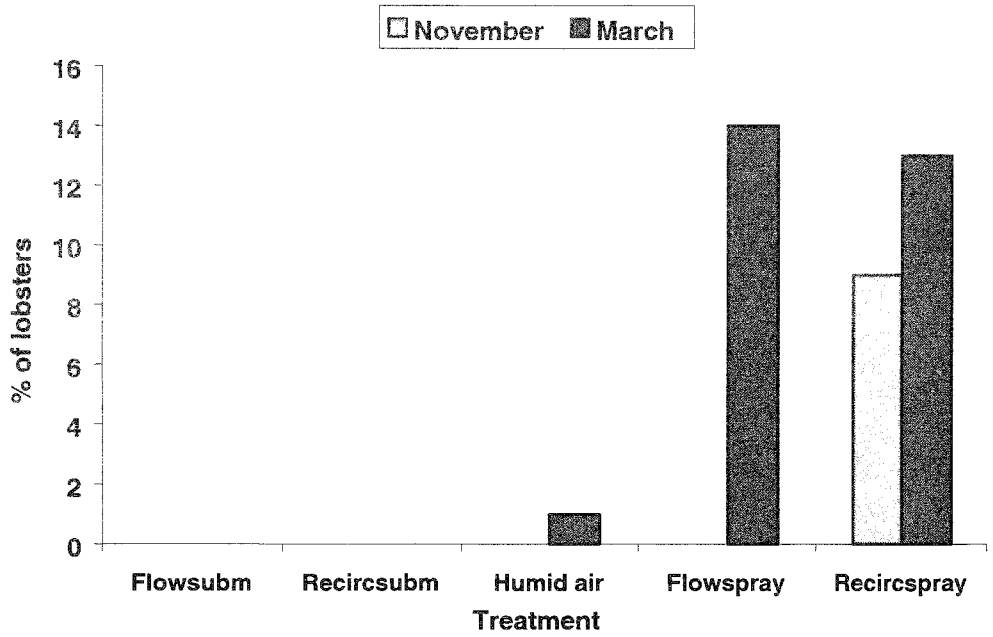


Figure 53 Percentage of dead lobsters following removal from treatment systems

The relative survival of lobsters after treatments followed by simulated shipment and recovery is shown in Figure 54. Only animals which were sampled after treatments, were included in this estimation. The mortality of untagged lobsters, which had not undergone sampling, was compared with that of tagged ones. There was no apparent additional mortality due to handling and sampling of the tagged lobsters.

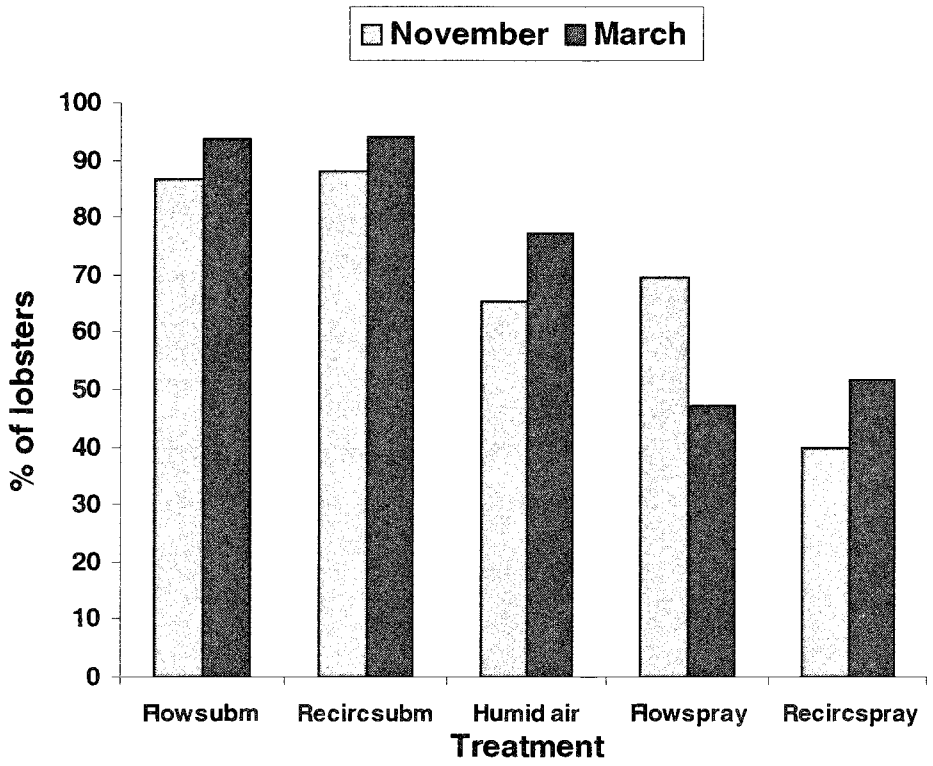


Figure 54 Percentage of lobsters survived simulated shipment procedure

5.2.3 Effect of moult stage on immune parameter results

The possible influence of moult stage on THC, DHC and clotting time was studied. Results from treatment groups in the March trial which had no significant difference in mean values for THC were pooled and mean values of THC, DHC and clotting time were calculated for each of five different moult stages (AB, C, D_0, D_1^1, D_1^{11} and D_1^{111}). These results are shown in Tables 46-48.

Table 46 Effect of moult stage on total hemocye count (THC) of pooled treatment groups of adult western rock lobster, *Panulirus cygnus*. Different superscripts within a column indicate significant differences ($P < 0.05$); combined treatments did not differ significantly in mean THC.

Combined treatments	moltstage	THC (x 10^6 cells mL^{-1}) mean ± SE	n
recircsubmerged	AB	7.61 ± 0.97 [b]	14
	C	7.50 ± 0.33 [b]	103
humid air	D_0	8.01 ± 0.52	2
	D_1^1	--	0
	D_1^{11}	16.14 ± 4.53 [a]	4
	D_1^{111}	4.22	1
recircspray	AB	6.42 ± 0.42 [a]	16
	C	6.79 ± 0.21 [a]	153
flowtsubmerged	D_0	8.26 ± 2.52 [a]	3
	D_1^1	8.93 ± 2.65	2
flowspray	D_1^{11}	9.44 ± 1.80 [a]	3
	D_1^{111}	--	0

Table 47 Effect of moult stage on percentage granulocytes of adult western rock lobster, *Panulirus cygnus*. Different superscripts within a column indicate significant differences ($P < 0.05$). The combined treatments did not differ significantly in mean THC.

Combined treatments	moltstage	% granular cells mean ± SE	n
recircsubmerged	AB	5.69 ± 0.66 [b]	13
	C	7.75 ± 0.38 [a]	88
humid air	D_0	9.50 ± 2.50	2
	D_1^1	-	0
	D_1^{11}	5.93 ± 1.09 [b]	3
	D_1^{111}	5.80	1
recircspray	AB	6.04 ± 1.01 [b]	13
	C	7.62 ± 0.34 [b]	121
flowsubmerged	D_0	7.55 ± 1.94 [b]	3
	D_1^1	1.0	1
flowspray	D_1^{11}	10.77 ± 2.79 [a]	3
	D_1^{111}	--	0

Table 48 Effect of moult stage on clotting time of adult western rock lobster, *Panulirus cygnus*. Different superscripts within a column indicate significant differences ($P < 0.05$). The combined treatments did not differ significantly in mean THC.

Combined treatments	moltstage	Clotting time (sec) mean ± SE	n
recircsubmerged	AB	46.45 ± 2.08 [a]	11
	C	44.18 ± 0.78 [a]	108
flowspray	D_0	47.00 ± 1.00	2
	D_1^1	-	0
	D_1^{11}	50.75 ± 5.95 [a]	4
	D_1^{111}	-	0
recircspray	AB	56.67 ± 3.41 [a]	12
	C	54.35 ± 1.12 [a]	113
humid air	D_0	63.00 ± 8.00	2
	D_1^1	68.00 ± 4.00	2
flowspray	D_1^{11}	62.67 ± 9.24 [a]	3
	D_1^{111}	48.00	1

Mean values for groups with more than three lobsters were compared. Although the sample numbers in some groups were very low, similar trends in the values were seen as those previously decreased (Cht 4, Section 2, Fig 11) with D_1^{11} values being higher than C stage values. This difference was significant for the 'recircsubmerged and humid air' group. Significant differences between C stage and D stage lobsters were also seen in the %granular cell results. It was therefore decided to only include data from C stage lobsters in the comparison of responses of THC, DHC and clotting time. All data were used for determination of mean values for bacteremia and antibacterial activity.

5.2.4 Effect of holding treatment on immune parameters

The results obtained for THC, %granular cells, clotting period, bacteremia and ABF for the five different treatments in the November 98 and the March 99 trial are shown in Tables 49-54.

Table 49 Effect of simulated transport method on total hemocyte count (THC) of adult Western rock lobster, *Panulirus cygnus*. Different superscripts within a column indicate significant differences ($P < 0.05$). Only C stage lobsters included in table.

	November 1998 THC (x 10^6 cells mL^{-1})		March 1999 THC (x 10^6 cells mL^{-1})	
Treatment	**n**	**mean ± SE**	**n**	**mean ± SE**
flowsubmerged	57	7.22 ± 0.78 [a]	57	7.07 ± 0.33 [b]
recircsubmerged	46	5.22 ± 0.44 [b]	55	7.12 ± 0.44 [b]
humid air	56	6.34 ± 0.46 [b]	48	7.83 ± 0.51 [a]
flowspray	52	6.62 ± 0.57 [b]	47	6.23 ± 0.33 [b]
recirspray	52	6.23 ± 0.52 [b]	49	7.02 ± 0.43 [b]

A consistent pattern of variation between the same treatment groups tested in November and March was not seen. In the November trial the mean THC for the flow-through submerged group was significantly higher than those of other groups but

this trend was not observed in March when the highest mean THC was seen in the humid air group.

Table 50 Effect of simulated transport method on %granular cells of adult western rock lobster, *Panulirus cygnus*. Different superscripts within a column indicate significant differences ($P < 0.05$). Only C stage lobsters included in table.

	November 1998		March 1999	
Treatment	**n**	**mean ± SE**	**n**	**mean ± SE**
flowsubmerged	49	10.21 ± 0.49 [b]	49	8.97 ± 0.48 [a]
recircsubmerged	48	10.53 ± 0.52 [a]	46	7.75 ± 0.51 [a]
humid air	48	10.04 ± 0.74 [b]	42	7.75 ± 0.56 [a]
flowspray	46	9.89 ± 0.64 [b]	39	7.65 ± 0.64 [a]
recircspray	49	8.75 ± 0.61 [b]	33	5.54 ± 0.48 [b]

The mean %granular cells obtained in the November trial for each treatment group was higher than that obtained for the corresponding treatment group in March for all simulated holding conditions. The results suggest that the November lobsters were stronger (less stressed) than the March lobsters. This was probably due to the fact that the March trial was conducted at a time when lobsters in the field were going through a synchronised moult, as evidenced by the relatively high percentage of lobsters in moult stages AB or D_1 (approx 15%; Table 46-48) and by anecdotal reports from factory staff.

The mean %granulocytes in the recirculating spray treatment was significantly lower than that of the recirculating submerged treatment in both trials. A similar trend was seen in the mean %granulocytes for the flow-through spray and the flow-through submerged treatments but the differences in mean values were not significant. Low values for %granulocytes are consistently seen in reject lobsters in a moribund state and are indicative of poor health status.

Table 51 Effect of simulated transport method on clotting time of adult western rock lobster, *Panulirus cygnus* in the November trial. Different superscripts within a column indicate significant differences ($P < 0.05$). Only C stage lobsters included in table.

Treatment	**n**	**clotting time (sec) mean ± SE**	**% no clot**
flowsubmerged	47	42.98 ± 1.49 [a]	9.62
recircsubmerged	48	44.88 ± 1.71 [a]	7.69
humid air	53	50.00 ± 1.96 [b]	3.64
flowspray	50	50.80 ± 2.33 [b]	3.85
recircspray	52	51.94 ± 1.99 [b]	7.14

Table 52 Effect of simulated transport method on clotting time of adult western rock lobster, *Panulirus cygnus* in the March trial. Different superscripts within a column indicate significant differences (P <0.05). Only C stage lobsters included in table.

Treatment	n	clotting time (sec)	% no clot
flowsubmerged	53	43.85 ± 1.06 [a]	7.02
recircsubmerged	51	43.90 ± 1.17 [a]	7.27
humid air	44	52.18 ± 1.83 [b]	10.20
flowspray	35	54.34 ± 1.90 [b]	25.53
recircspray	38	56.61 ± 1.89 [b]	20.83

The mean values for clotting times obtained in the November trial for each treatment group were similar to those obtained for the corresponding treatment group in March for all simulated holding conditions.

A consistent pattern of alteration in clotting time was seen in both trials with significantly higher times being observed in the two spray treatments and in the humid air treatment compared to the values obtained for the two submerged treatments. The pattern of alteration in clotting time in the different treatment groups (low levels in the two submerged treatments) was similar to that seen in the analysis of the outcome of the different simulated holding conditions with respect to survival (Figure 54) or to grading (Figures 51 & 52).

The percentage of lobsters in each treatment group whose hemolymph did not clot ranged from 3.64 – 9.62% in the November trial with no obvious correlation between failure to clot and treatment group. In the March trial, however, the percentage of lobsters whose hemolymph failed to clot was considerably higher in the two treatment groups with high levels of reject lobsters (flowthrough spray and recirculating spray). This finding is consistent with the conclusion drawn from the %granular cell analysis with respect to the health status of the March versus the November lobsters.

Table 53 Effect of simulated transport method on bacteremia in adult western rock lobster, *Panulirus cygnus*. Different superscripts within a column indicate significant differences (P < 0.05).

Treatment	November trial Colony Rank Mean ± SE	n	March trial Colony Rank Mean ± SE	n
Flow submerged	2.32 ± 0.30[a]	60	1.41 ± 0.26[a]	64
Recirc submerged	4.41 ± 0.52[b]	59	1.67 ± 0.26[a]	67
Humid air	4.93 ± 0.59[b]	58	4.11 ± 0.55[b]	66
Flow spray	4.84 ± 0.60[b]	57	3.57 ± 0.58[ab]	56
Recirc spray	6.23 ± 0.59[b]	60	3.13 ± 0.40[b]	60

The mean values for bacteremia (colony rank) obtained in the November trial for each treatment group were consistently higher than those obtained for the corresponding treatment group in March for all simulated holding conditions. However, as indicated in Section 5.1.2.4, this difference probably resulted from differences in methodology used in the two trials. Very low levels of bacteremia were seen in the two submerged treatments in the March trial and in the flowthrough treatment in the November trial.

The pattern of alteration in bacteremia in the different treatment groups (low levels in the submerged treatments) was similar to that seen in the analysis of the outcome of the different simulated holding conditions with respect to survival (Figure 54) or to grading (Figures 51 & 52).

95% of lobsters in November and 77% of lobsters in March were found to have bacteria in their hemolymph. However, control lobsters (graded as accepted by factory staff) taken from factory tanks during the week subsequent to the November trial, and in other trials at GFC, rarely exhibited bacteremia. This observation, combined with the finding of higher levels of bacteremia in the spray and humid air treatments compared to the submerged treatments, suggests that the development of bacteremia following exposure to adverse environmental conditions is a rapid process, occurring within hours of stressor exposure.

Table 54 Effect of simulated transport method on antibacterial factor in adult western rock lobster, *Panulirus cygnus*. Different superscripts within a column indicate significant differences

Treatment	n	ABF Mean ± SE
Flowsubmerged	52	0.232 ± 0.034^{a}
Recircsubmerged	57	0.237 ± 0.028^{a}
Humid air	53	0.336 ± 0.042^{ab}
Flowspray	50	0.353 ± 0.040^{b}
Recircspray	48	0.369 ± 0.043^{b}

The mean values for ABF in the two submerged treatments were significantly lower than those of the two spray treatments. The mean value for the humid air treatment tended to be higher than those for the submerged treatments but the differences were not statistically significant. These results are consistent with those obtained for bacteremia in the different treatment groups and suggest that induction of ABF in response to bacteremia is a rapid process, occurring within a few hours of the stimulus.

5.2.5 Conclusions from analysis of immune parameters in lobsters held in different simulated transport systems

Of the five parameters investigated, clotting time, ABF and bacteremia showed more consistent responses to the different simulated transport treatments and were more in agreement with the outcome of the trials with respect to the grading status or subsequent health and survival of lobsters than THC or %granulocytes. ABF is a laborious and costly test to perform and is not recommended for further study. The other three procedures, on the other hand, are simple tests which could be carried out in the factory by factory staff and should be further evaluated in post harvest lobster stress studies.

Similar mean THC, DHC, clotting time, bacteremia and ABF values were obtained for the recirculating treatments compared to the corresponding flow-through treatments, suggesting that exposure to high levels of ammonia did not have a

detrimental effect on the immune function of the test lobsters. This conclusion is supported by the observation of the similar results being obtained from grading and assessment of health and survival of lobsters after treatment exposure in spray or submerged treatments with low or high ammonia levels (Figures 51-54).

5.3 Analysis of health and survival outcomes

The results obtained for THC, %granular cells, clotting time, bacteremia and ABF for lobsters with different outcomes (Died or weak in tanks (DW); Fit-for-live following simulated live shipment (SS); Dead or weak following simulated live shipment (LD0); Dead or weak after simulated shipment and 24hr acclimation (LD1)) are shown in Tables 55–59.

Table 55 THC in lobsters with different outcomes following simulated transport and shipment. Different superscripts within a column indicate significant differences (P<0.05)

	November 1998		March 1999	
Outcome	**n**	**mean±SE**	**n**	**mean±SE**
Survivor (SS)	189	6.10 ± 0.29 [a]	186	7.42 ± 0.21 [a]
Tank reject (DW)	48	5.46 ± 0.31 [a]	60	6.01 ± 0.38 [b]
Retank reject(LD1)	22	6.81 ± 0.98 [a]	8	6.96 ± 0.73 [b]
Deadinbox (LD0)	5	4.76 ± 0.57 [a]	2	7.39 ± 3.31

Table 56 Percentage granular cells in lobsters with different outcomes following simulated transport and shipment. Different superscripts within a column indicate significant differences (P<0.05)

	November 1998		March 1999	
Outcome	**n**	**mean±SE**	**n**	**mean±SE**
Survivor (SS)	178	10.19 ± 0.30 [a]	150	8.52 ± 0.27 [a]
Tank reject (DW)	42	8.94 ± 0.70 [a]	48	5.24 ± 0.48 [b]
Retank reject(LD1)	19	9.00 ± 0.73 [a]	7	7.40 ± 1.24 [b]
Deadinbox (LD0)	4	7.08 ± 1.68 [a]	2	8.10 ± 0.70

Table 57 Clotting time in lobsters with different outcomes following simulated transport and shipment. Different superscripts within a column indicate significant differences (P<0.05)

	November 1998		March 1999	
Outcome	**n**	**mean±SE**	**n**	**mean±SE**
Survivor (SS)	179	46.42 ± 0.96 [b]	173	47.27 ± 0.80 [b]
Tank reject (DW)	44	56.66 ± 2.37 [a]	38	59.63 ± 1.67 [a]
Retank reject(LD1)	22	45.86 ± 2.88 [b]	8	46.13 ± 3.72 [b]
Deadinbox (LD0)	4	49.25 ± 3.84 [b]	2	50.00 ± 9.00

Table 58 Bacteremia in lobsters with different outcomes following simulated transport and shipment. Different superscripts within a column indicate significant differences ($P<0.05$)

	November trial		March trial	
Outcome	**Colony Rank**	**n**	**Colony Rank**	**n**
Survivors (SS)	4.2 ± 0.3^{a}	205	2.1 ± 0.2^{b}	226
Tankreject (DW)	6.1 ± 0.6^{a}	57	4.8 ± 0.6^{a}	64
Retankreject (LD1)	3.5 ± 0.7^{a}	26	2.5 ± 1.1^{ab}	10
Deadinbox (LD0)	5.2 ± 1.9^{a}	6	5.3 ± 3.8^{ab}	3

Table 59 ABF in lobsters with different outcomes following simulated transport and shipment. Different superscripts within a column indicate significant differences ($P<0.05$)

Outcome	**ABF**	
Survivors (SS)	0.268 ± 0.018^{b}	186
Tankreject (DW)	0.423 ± 0.045^{a}	44
Retankreject (LD1)	0.302 ± 0.064^{ab}	24
Deadinbox (LD0)	0.502 ± 0.157^{ab}	6

Tank rejects (DW) all showed significant differences in THC, %granular cells, clotting time, and bacteremia compared to survivors (SS) in the March trial. Similar trends were seen in the November trial but the differences were significant for only clotting time and ABF. A consistent pattern of results was not seen in LD1 and LD0 groups.

6. Statistical Analysis of the Physiological Studies on Stress and Morbidity during Post-Harvest Handling and Storage of Western Rock Lobsters (*Panulirus cygnus*)

6.1 Background

Statistical analyses were conducted on the variables gathered during the two factory trials to find out indicators to be included in a composite lobster stress index. Results of the analyses are discussed below.

6.2 White lobsters

Experiments for the white lobsters were conducted on November/December 1998. Parameters measured included total hemocyte count (THC), granular cells/hemocyte count (%GC), antibacterial activity, bacterial colony rank and clotting time. To examine which of the parameters are significantly different among the different storage treatments, a test of equality of group means was conducted. The results are shown in Table 60. Only those lobsters with complete data sets were included in the analysis.

Table 60 Tests of equality of group means

	Wilks' Lambda	F	df1	df2	Sig.
Result	.922	4.417	4	210	.002
sex	.987	.716	4	210	.582
legs	.972	1.486	4	210	.208
moult	.977	1.247	4	210	.292
bacteremia rank	.904	5.592	4	210	.000*
antibacterial activity	.970	1.600	4	210	.176
total hemocyte count	.954	2.504	4	210	.043*
clotting time	.934	3.723	4	210	.006*
%GC (granular cells/hemocyte count)	.985	.787	4	210	.535

* significantly different among groups

As shown in the table, bacteremia rank, total hemocyte count and clotting time were found to be significantly different among the various treatments. Among these indicators, bacteremia rank seems to be the most different among the treatments (lowest wilks' lambda). These parameters could therefore be used to examine the effects of the storage treatments on post harvest stress of lobsters.

To examine which storage treatments caused the most post-harvest stress, an analysis of group statistics were conducted as shown in Table 61.

Table 61 Group statistics of storage treatments

Storage treatments		Mean	Std. Deviation	Valid N (listwise)
Rsub	Result	1.25532	.67464	47
	sex	1.31915	.47119	47
	legs	.23404	.47607	47
	moult	1.06383	.24709	47
	bacteremia rank	4.48936	3.91692	47
	antibacterial activity	.24163	.20261	47
	total hemocyte count	4.77340	1.58289	47
	clotting time	44.04255	12.44459	47
	%GC (granular cells/hemocyte count)	10.66021	3.49851	47
Rspray	Result	1.79487	.89382	39
	sex	1.33333	.52981	39
	legs	.43590	.55226	39
	moult	1.12821	.65612	39
	bacteremia rank	6.23077	4.64226	39
	antibacterial activity	.34642	.27611	39
	total hemocyte count	6.60205	3.92996	39
	clotting time	49.56410	12.92243	39
	%GC (granular cells/hemocyte count)	9.35410	4.24250	39
Humid	Result	1.44186	.76539	43
	sex	1.18605	.39375	43
	legs	.32558	.47414	43
	moult	1.06977	.45750	43
	bacteremia rank	4.76744	4.40691	43
	antibacterial activity	.32799	.29719	43
	total hemocyte count	6.71860	3.69312	43
	clotting time	50.16279	13.50649	43
	%GC (granular cells/hemocyte count)	9.37093	5.19237	43
Fsub	Result	1.20930	.59993	43
	sex	1.30233	.46470	43
	legs	.30233	.55784	43
	moult	1.37209	1.32778	43
	bacteremia rank	2.20930	2.36601	43
	antibacterial activity	.24985	.24138	43
	total hemocyte count	7.34337	6.30920	43
	clotting time	43.37209	10.39236	43
	%GC (granular cells/hemocyte count)	10.19512	3.74495	43
Fspray	Result	1.34884	.57253	43
	sex	1.25581	.44148	43
	legs	.18605	.45018	43
	moult	1.11628	.62524	43
	bacteremia rank	5.23256	4.57653	43
	antibacterial activity	.33392	.28115	43
	total hemocyte count	6.86360	4.30538	43
	clotting time	52.18605	17.13567	43
	%GC (granular cells/hemocyte count)	9.86558	4.00285	43
Total	Result	1.40000	.72859	215
	sex	1.27907	.45986	215
	legs	.29302	.50483	215
	moult	1.14884	.75268	215
	bacteremia rank	4.55349	4.22671	215
	antibacterial activity	.29801	.26206	215
	total hemocyte count	6.42619	4.27066	215
	clotting time	47.76279	13.78996	215
	%GC (granular cells/hemocyte count)	9.91349	4.15578	215

The results suggested that flow-through submerged (FSUB) causes the least stress on white lobster, followed by recirculating submerged (RSUB). Lobsters under the flow-through submerged treatment recorded the lowest clotting time. Conversely, lobsters under recirculating spray (RSPRAY) did poorly, with the lowest THC and the highest bacteremia rank. The flow-through spray (FSPRAY) also did poorly as lobsters under this treatment had the highest clotting time and the second highest bacteremia rank.

In this experiment, lobsters were grouped into SURVIVORS (fit for live following simulated shipment); TANKRJCT (died or weak during 7 days storage); RETANRJCT (died or weak after simulated shipment and 24hr acclimation) and DEADINBOX (died or weak in tanks). To determine what factors best discriminate between survivors and non-survivors, a series of discriminant analyses were conducted. In discriminant analysis, a linear combination of the independent variable is formed which is then used as the basis for assigning cases to groups. Hence, the results can be used to classify new individuals into categories and predict whether lobsters will survive or not.

The first discriminant analysis used the four groupings described above. Three discriminant functions were produced as presented in Table 62.

Table 62 Standardized canonical discriminant function coefficients

	Function		
	1	2	3
storage treatments	-.049	.503	.371
sex	-.358	-.258	.118
legs	.183	.195	.256
moult	.082	-.505	.419
bacteremia rank	.276	-.023	.373
antibacterial activity	.449	-.116	.188
total hemocyte count	.067	-.069	-.893
clotting time	.463	.425	-.190
%GC (granular cells/hemocyte count)	-.568	.435	.189

The standardised discriminant function coefficients can be used as indicators of the relative importance of the parameters. Results show that ABF, clotting time and %GC appears to have the largest contribution to discriminant function 1 while storage treatments, moult, clotting time and %GC had the largest contribution to function 2. Function 1 accounted for 63.3% of the total between group variability. Further indicators are shown in the matrix structure (Table 63), which shows that %GC had the highest correlation with the discriminant function.

Table 63 Structure matrix

	Function		
	1	2	3
%GC (granular cells/hemocyte count)	-.549*	.423	.102
antibacterial activity	.474*	-.117	.194
sex	-.406*	-.281	.115
bacteremia rank	.344*	-.062	.294
clotting time	.446	.503	-.139
storage treatments	.044	.467	.179
moult	.066	-.459	.195
legs	.117	.225	.183
total hemocyte count	.061	-.062	-.672

Pooled within-groups correlations between discriminating variables and standardized canonical discriminant functions. Variables ordered by absolute size of correlation within function.

* Largest absolute correlation between each variable and any discriminant function

To assess the extent to which the discriminant function is able to correctly classify lobsters into the four groups (i.e., whether they would survive, die in the box, etc.), the classification results in Table 64 can be used. The table shows that the analysis was able to predict correct group membership for 47% of the cases. There was 46% correct prediction for SURVIVORS, 44% correct prediction for TANKRJCT, 47% prediction for RETANRJCT, and 100% correct prediction for DEADINBOX.

Table 64 Classification results[a]

			Predicted Group Membership[b]				Total
		Result	Survivor	Tankrjct	Retankrjct	Deadinbox	
Original	Count	Survivor	72	32	40	12	156
		Tankrjct	7	16	8	5	36
		Retankrjct	4	1	9	5	19
		Deadinbox	0	0	0	4	4
	%	Survivor	46.2	20.5	25.6	7.7	100.0
		Tankrjct	19.4	44.4	22.2	13.9	100.0
		Retankrjct	21.1	5.3	47.4	26.3	100.0
		Deadinbox	.0	.0	.0	100.0	100.0

[a] 47.0% of original grouped cases correctly classified
[b] Tankrjct = died or weak during 7 days storage
Retankrjct = died or weak after simulated shipment and 24h acclimation

The moderate predictability of the model could be due to violation of some assumptions, such as the homogeneity of variance assumption (box M is significant with p=.001) and sample size for twogroups is small (Tabachnich and Fidell 1996 suggest that sample size must be greater than 20 cases for each cell for the results to be robust).

To overcome the problem of sample size, a second discriminant analysis was conducted using two groupings: survivors and non-survivors. All lobsters which were weak/died regardless of storage treatments were classified under the latter category.

The results showed a 64.2% success rate of correct prediction overall, with 66% of the surviving lobsters correctly classified and 59.3% of the non-surviving lobsters correctly classified (Table 65).

Table 65 Classification results*

		Fresult	Predicted Group Membership Survivor	Non-survivor	Total
Original	Count	Survivor	103	53	156
		Non-survivor	24	35	59
	%	Survivor	66.0	34.0	100.0
		Non-survivor	40.7	59.3	100.0

* 64.2% of original grouped cases correctly classified.

The standardised discriminant function coefficient (Table 66) showed that %GC, clotting time and ABF are the best indicators of survival or non-survival for white lobsters with, %GC showing the highest pooled within-groups correlation between these discriminating variables and the standardised discriminant function.

Table 66 Standardized canonical discriminant function coefficients and matrix structure

	Discriminant function coefficients	Matrix structure*
storage treatments	-.196	-.595
sex	-.348	.422
legs	.083	.420
moult	.007	-.399
bacteremia rank	.159	.264
antibacterial activity	.384	.238
total hemocyte count	.318	.054
clotting time	.456	.047
%GC (granular cells/hemocyte count)	-.633	-.044

*Pooled within-groups correlations between discriminating variables and standardized canonical discriminant functions. Variables ordered by absolute size of correlation within function.

Analysis of group means also showed that ABF, clotting time and %GC are significantly different among survivors and non-survivors (Table 67). In general, lobsters that survived had low bacteremia rank, low ABF, and high %GC. In addition, clotting time was also slower than lobsters which did not survive (Table 68).

Table 67 Tests of equality of group means

	Wilks' Lambda	F	df1	df2	Sig.
storage treatments	1.000	.041	1	213	.840
sex	.985	3.335	1	213	.069
legs	1.000	.046	1	213	.830
moult	1.000	.061	1	213	.805
bacteremia rank	.993	1.457	1	213	.229
antibacterial activity	.983	3.702	1	213	.056
total hemocyte count	.994	1.190	1	213	.277
clotting time	.983	3.723	1	213	.055
%GC (granular cells/hemocyte count)	.966	7.421	1	213	.007

Table 68 Group statistics

Fresult		Mean	Std. Deviation	Valid N
Survivors	storage treatments	2.99359	1.47887	156
	sex	1.31410	.47931	156
	legs	.28846	.50812	156
	moult	1.14103	.77417	156
	bacteremia rank	4.33974	4.11684	156
	antibacterial activity	.27700	.24448	156
	total hemocyte count	6.23090	4.34306	156
	clotting time	46.65385	13.10240	156
	%GC (granular cells/hemocyte count)	10.38135	4.18370	156
Non-Survivors	storage treatments	2.94915	1.33163	59
	sex	1.18644	.39280	59
	legs	.30508	.50015	59
	moult	1.16949	.69858	59
	bacteremia rank	5.11864	4.49170	59
	antibacterial activity	.35358	.29889	59
	total hemocyte count	6.94254	4.06354	59
	clotting time	50.69492	15.19216	59
	%GC (granular cells/hemocyte count)	8.67644	3.84716	59
Total	storage treatments	2.98140	1.43704	215
	sex	1.27907	.45986	215
	legs	.29302	.50483	215
	moult	1.14884	.75268	215
	bacteremia rank	4.55349	4.22671	215
	antibacterial activity	.29801	.26206	215
	total hemocyte count	6.42619	4.27066	215
	clotting time	47.76279	13.78996	215
	%GC (granular cells/hemocyte count)	9.91349	4.15578	215

6.3 Red lobsters

The experiments on red lobsters were conducted during the March trials using 313 lobsters. The five storage treatments were also used. The results of the statistical analysis examining which treatment caused the least post-harvest stress is shown in Table 69.

Table 69 Group statistics for storage treatments

Treatment		Mean	Std. Deviation	Valid N
Rsub	Result	1.1321	.5560	53
	Legs	.2075	.4094	53
	Bactrank	1.7358	2.2884	53
	Thc	7.9554	4.4324	53
	Clottime	44.6038	7.1477	53
	%GC	7.7906	3.5316	53
Rspray	Result	1.4571	.5606	35
	Legs	.1429	.3550	35
	Bactrank	3.1429	2.9619	35
	Thc	7.2384	2.5792	35
	Clottime	57.1714	11.2759	35
	%GC	5.6743	2.9097	35
Humid	Result	1.2200	.5067	50
	Legs	.2800	.4965	50
	Bactrank	3.8800	4.4292	50
	Thc	8.2577	3.8815	50
	Clottime	51.4400	12.1608	50
	%GC	7.9460	3.2073	50
Fsub	Result	1.1633	.5533	49
	Legs	.2245	.4216	49
	Bactrank	1.4898	2.3015	49
	Thc	7.3921	2.5402	49
	Clottime	43.8776	8.1921	49
	%GC	8.5980	2.9042	49
Fspray	Result	1.4545	.6657	33
	Legs	.2121	.4151	33
	Bactrank	3.0606	3.6737	33
	Thc	7.1600	1.9844	33
	Clottime	55.1515	12.4226	33
	%GC	8.9333	3.7069	33
Total	Result	1.2591	.5743	220
	Legs	.2182	.4248	220
	Bactrank	2.5909	3.3223	220
	Thc	7.6653	3.3530	220
	Clottime	49.5773	11.4089	220
	%GC	7.8405	3.3905	220

As shown in the table, flow-through submerged (FSUB) caused the least post-harvest stress, while the recirculating spray (FSPRAY) caused the most stress in red lobsters. Results also suggest that clotting time, %GC and bacteremia rank are the parameters that are significantly different among lobsters in different storage treatments (Table 70). Lobsters under FSUB had the lowest bacteremia rank, the lowest clotting time and the second highest %GC. On the other hand, lobsters stored under RSPRAY recorded the highest clotting time, high levels of bacteremia (second highest among the groups) and had the lowest %GC (Table 69).

Table 70 Tests of equality of group means for storage treatment

	Wilks' Lambda	F	df1	df2	Sig.
Result	.944	3.163	4	215	.015
Legs	.990	.548	4	215	.701
Bactrank	.918	4.827	4	215	.001
Thc	.984	.898	4	215	.466
Clottime	.785	14.695	4	215	.000
%GC	.908	5.468	4	215	.000

Bacteremia, clotting time and the % GC were also found to be significant indicators of survival among the red lobsters (Table 71) but results for group statistics were not clear. Although surviving lobsters had low bacteremia rank and clotting time (2nd lowest for both parameters) and the highest %GC, lobsters under TANKRJCT had the lowest bacteremia rank, clotting time and the lowest %GC (Table 72).

Table 71 Group statistics for survival

Result		Mean	Std. Deviation	Valid N
Survivor	Treat	2.8011	1.3898	176
	Legs	.2159	.4262	176
	Bactrank	2.1250	2.8598	176
	THC	7.8050	3.3909	176
	Clottime	47.4773	10.2154	176
	%GC	8.2830	3.2472	176
Tankrjct	Treat	3.3030	1.3575	33
	Legs	.2424	.4352	33
	Bactrank	4.9091	4.4396	33
	THC	7.1810	3.4330	33
	Clottime	61.7576	10.4733	33
	%GC	5.9455	3.3953	33
Retankrjct	Treat	2.8889	1.2693	9
	Legs	.2222	.4410	9
	Bactrank	2.1111	1.9003	9
	THC	6.7702	2.0141	9
	Clottime	45.8889	9.8545	9
	%GC	6.0778	3.8925	9
Deadinbox	Treat	3.0000	2.8284	2
	Legs	.0000	.0000	2
	Bactrank	7.5000	7.7782	2
	THC	7.3895	4.6812	2
	Clottime	50.0000	12.7279	2
	%GC	8.1000	.9899	2
Total	Treat	2.8818	1.3929	220
	Legs	.2182	.4248	220
	Bactrank	2.5909	3.3223	220
	THC	7.6653	3.3530	220
	Clottime	49.5773	11.4089	220
	%GC	7.8405	3.3905	220

Table 72 Tests of equality of group means for survival

	Wilks' Lambda	F	df1	df2	Sig.
Treat	.983	1.211	3	216	.307
Legs	.997	.211	3	216	.889
Bactrank	.890	8.896	3	216	.000
Thc	.992	.546	3	216	.651
Clottime	.797	18.371	3	216	.000
%GC	.928	5.580	3	216	.001

The discriminant function, however, shows that for function 1, bacteremia rank, clotting time and %GC are the most important indicators among the variables included (Table 73).

Table 73 Standardized canonical discriminant function coefficients

	Function		
	1	2	3
Treat	.240	-.233	-.033
Legs	.006	-.075	.338
Bactrank	.500	.425	-.688
Thc	-.126	.248	.273
Clottime	.716	.229	.596
%GCc	-.406	.832	.206

The discriminant analysis correctly classified 52.3% of the cases (Table 74). While model assumptions of homogeneity of variances and no multicollinearity were met, the sample size for two groups (RETANRJCT and DEADINBOX) were too small. Consequently, it was decided to run a second discriminant analysis using 2 groups (surviving lobsters and non-surviving lobsters). The results of the analysis are shown in Table 75.

Table 74 Classification results*

			Predicted Group Membership				Total
		Result	Survivor	Tankrejct	Retankrjct	Deadinbox	
Original	Count	Survivor	87	18	55	16	176
		Tankrjct	1	22	4	6	33
		Retankrjct	2	1	5	1	9
		Deadinbox	0	0	1	1	2
	%	Survivor	49.4	10.2	31.3	9.1	100.0
		Tankrjct	3.0	66.7	12.1	18.2	100.0
		Retankrjct	22.2	11.1	55.6	11.1	100.0
		Deadinbox	.0	.0	50.0	50.0	100.0

* 52.3% of original grouped cases correctly classified

Table 75 Classification results*

			Predicted Group Membership		Total
		Final result	Survivor	Non-survivor	
Original	Count	Survivor	135	41	176
		Non-survivor	11	33	44
	%	Survivor	76.7	23.3	100.0
		Non-survivor	25.0	75.0	100.0

* 76.4% of original grouped cases correctly classified

Using two outcome groups, there was a high correct classification rate, with 76.4% of the red lobsters correctly classified into survivors and non-survivors. More than two-thirds of the surviving lobsters (76.7%) were correctly classified and 75% of non-surviving lobsters were correctly classified. The group statistics showed that lobsters that survived had lower bacteremia rank, higher %GC and had slower clotting time than lobsters which died in the box or after simulated storage treatments (Table 76). The three parameters were also found to be significantly different among surviving lobsters and those that did not survive (Table 77).

Table 76 Group statistics

Final result		Mean	Std. Deviation	Valid N
Survivor	Treat	2.8011	1.3898	176
	Legs	.2159	.4262	176
	Bactrank	2.1250	2.8598	176
	Thc	7.8050	3.3909	176
	Clottime	47.4773	10.2154	176
	%GC	8.2830	3.2472	176
Non-survivor	Treat	3.2045	1.3738	44
	Legs	.2273	.4239	44
	Bactrank	4.4545	4.2996	44
	Thc	7.1065	3.1728	44
	Clottime	57.9773	12.1645	44
	%GC	6.0705	3.4095	44
Total	Treat	2.8818	1.3929	220
	Legs	.2182	.4248	220
	Bactrank	2.5909	3.3223	220
	Thc	7.6653	3.3530	220
	Clottime	49.5773	11.4089	220
	%GC	7.8405	3.3905	220

Table 77 Tests of equality of group means

	Wilks' Lambda	F	df1	df2	Sig.
Treat	.987	2.979	1	218	.086
Legs	1.000	.025	1	218	.874
Bactrank	.921	18.706	1	218	.000
Thc	.993	1.531	1	218	.217
Clottime	.864	34.356	1	218	.000
%GC	.932	16.017	1	218	.000

The coefficients of the discriminant functions (Table 78) also indicate that clotting time, bacteremia rank and the percentage GC are the most important variables that discriminate among surviving lobsters.

Table 78 Standardized canonical discriminant function coefficients and matrix structure

	Discriminant Function Coefficients	Structure matrix*
Treat	-.260	.689
Legs	-.014	.508
Bactrank	.493	-.470
Thc	-.173	.203
Clottime	.629	-.145
%GC	-.507	.019

*Pooled within-groups correlations between discriminating variables and standardized canonical discriminant functions. Variables ordered by absolute size of correlation within function.

6.4 Model assumptions

In the latter discriminant analysis, model assumptions of samples size and homogeneity of variance were not violated (Box's M was not significant). Correlation between independent variables were also low (ranging from .007 to .224) indicating that multicollinearity was not a problem. Hence, the results of the discriminant analysis can be considered robust.

6.5 Conclusions

The results of the analysis showed that among the five treatments, flow-through submerged is the best storage treatment for transporting lobsters, while recirculating spray appears to cause the most post-harvest stress. Results of the experiment are consistent for white lobsters and for red lobsters. Lobsters stored under the former treatment had lower bacteremia rank, low clotting time, and high %GC, while lobsters in the latter storage had the reverse.

CHAPTER 8 HEALTH ASSESSMENT STUDIES USING IMMUNE PARAMETERS AND HISTOPATHOLOGY

1. Introduction

Measurement of immune parameters provides one approach to assessing health status of lobsters. In lobsters with poor health the immune system is compromised and variations in the levels of immune parameters can be expected. Another approach to assessing health status is to examine histological sections of selected lobster organs for the presence of disease causing organisms and/or pathological reactions. Little work has been published on either immunity analysis or histopathological investigations in lobsters. This investigation aimed to fill some of the knowledge gaps in this important area of lobster health assessment.

The aim of the study was threefold:

- Describe and quantify the pattern of variation in immune parameter levels in healthy compared to unhealthy lobsters
- Describe and quantify the occurrence of histopathological abnormalities in lobster tissues
- Determine the likely causes of mortality in captive lobsters
- Develop a reliable approach to assessing health status in captive lobsters.

2. Experimental procedures

2.1 Lobster autopsies

Autopsies were performed on lobsters used in the field studies of immune parameters (Chapter 7). Four main groups of lobsters were studied – Fremantle Whites (Dec 1996); Fremantle Reds (Mar/Apr 1997), Geraldton Whites (Factory trial no. 2 – Nov/Dec 1998) and Geraldton Reds (Factory trial no. 1 – April 1998). Two other groups of lobsters were also examined, lobsters used in the study of truck transport (Truck study, Dec 1997) and a group of 6 moribund lobsters that were used in an investigation of the effect of fixatives on lobster histology (Geraldton April 1998, Case study). A total of 139 lobsters were examined, 56 *healthy* lobsters (accepted - lobsters judged to be healthy by factory grading staff, stored in factory tanks for 24-48hr and judged to be healthy at the completion of storage), 49 *unhealthy* lobsters (rejects - lobsters judged to be healthy by factory grading staff, stored in factory tanks for 24-48hr and then judged to be unhealthy) and 34 lobsters of *uncertain health status* (fresh arrivals - lobsters sampled immediately upon arrival at factory prior to grading) (Table 79).

2.2 Immune parameters

Immune parameters were measured in all but one lobster group (Geraldton April 1998, Case study), early studies comprising measurements of THC, %granular cells and %prevalence bacteremia and later studies also including clotting time analysis. The methodology used for measuring bacteremia was different in the Fremantle

lobsters compared to the Geraldton lobsters, the former being a qualitative assessment of bacteremia and the latter being a quantitative assessment performed as described in Chapter 4. The techniques used to perform the immune parameter analyses are described in Chapter 4 and normal ranges are given in Chapter 5.

Table 79 Autopsies performed on healthy and unhealthy lobsters and on lobsters of uncertain health status[1]

Lobster group	Physiological condition	Number studied		
		Healthy	Unhealthy	Uncertain health
Fremantle	Whites	13	10	8
Fremantle	Reds	12	12	12
Truck Study, Dec '97	Whites	10	-	14
Geraldton April '98	Reds	11	11	-
Geraldton April '98 (Case study)	Reds	-	6	-
Geraldton Nov '98	Whites	10	10	-

[1] See text for explanation of lobster health status category and source of lobster study groups

3. Variation of immune parameters in unhealthy lobsters

3.1 Studies performed and results obtained

In all four studies reported, unhealthy lobsters were obtained from a factory tank and a matching healthy control lobster from the same factory tank was also examined. Hemolymph samples were taken immediately after removal from the tanks. THC, %granular cells and bacteremia was measured in all groups but clotting time was only recorded for the last group studied, the Geraldton Whites. Results obtained are shown in Table 80.

Table 80 Immune parameters in reject and accepted lobsters (mean ± s.e.)[1]

(a) THC (No cells/ml x 10^6)

Group	Fremantle		Geraldton	
	Whites	Reds	Whites	Reds
Healthy	5.6 ± 0.7^a	5.3 ± 0.7^a	4.3 ± 0.5^a	3.7 ± 1.2^a
Unhealthy	2.5 ± 0.5^b	3.3 ± 0.5^b	1.9 ± 0.4^b	0.8 ± 0.2^b

[1] See Section 2.1 for explanation of lobster groups
Means in columns with different superscripts are significantly different (t-test; $P<0.05$)

(b) Percentage granular cells (%)

Group	Fremantle		Geraldton	
	Whites	Reds	Whites	Reds
Healthy	8.0 ± 1.9^a	10.7 ± 1.4^a	5.9 ± 1.2^a	5.94 ± 1.0^a
Unhealthy	5.1 ± 1.5^a	5.1 ± 1.0^b	1.3 ± 0.7^b	0.4 ± 0.4^b

[1] See Section 2.1 for explanation of lobster groups
Means in columns with different superscripts are significantly different (t-test; $P<0.05$)

(c) Percentage prevalence bacteremia (%)

Group	Fremantle		Geraldton	
	Whites	Reds	Whites	Reds
Healthy	0^a	0^a	20^a	0^a
Unhealthy	50^b	17^a	90^b	82^b

[1] See Section 2.1 for explanation of lobster groups
Means in columns with different superscripts are significantly different (χ^2; $P<0.05$)

(d) Clotting rank

Group	Geraldton Whites
Healthy	2.9 ± 0.5^a
Unhealthy	5.6 ± 0.8^b

[1] See Section 2.1 for explanation of lobster group
Means in columns with different superscripts are significantly different (t-test; $P<0.05$)

With two exceptions (%granular cell and bacteremia results for Fremantle Reds, Tables 80b & c,) the value for the immune parameter in the reject group of lobsters was significantly reduced (THC, %granular cells) or elevated (bacteremia, clotting rank) compared to the corresponding value for the control group of lobsters.

Speciation was performed on bacterial cultures prepared from the Fremantle Whites and the Fremantle Red lobsters. Mixed cultures including *Vibrio alginolyticus* and other unidentified *Vibrio* species and gram negative bacilli were observed (Chapter 4, Section 5.5).

3.2 Discussion and conclusions

The results show that a loss of health status in post harvest lobsters, as demonstrated by a reduced muscle tone and lack of movement of body appendages, results in a reduction in circulating hemocyte numbers, a lower proportion of granular cells, increased levels of bacteremia in the hemolymph and a prolonged clotting time. The prolonged clotting time is not surprising, given that components from the hemocytes

participate in the clotting reaction and that the number of circulating hemocytes is reduced in the reject lobsters.

The presence of bacteria in the hemolymph combined with the evidence of a reduction in the proportion of circulating granular cells and a reduced number of hemocytes suggests that sequestration of hemocytes, in particular granular cells, into tissues has occurred as part of the host defence reaction to bacterial invasion of lobster tissues. The alternative hypothesis, that hemocytes, particularly granular cells, are lysed during the pathological reactions leading to weakness in lobsters, cannot be excluded as an explanation for the observed results. However, in view of the observation of an increased prevalence of inflammatory reactions in the tissues of unhealthy lobsters and the high proportion of granular cells seen in these reactions (see below), the major process leading to the changes in circulating hemocyte numbers and prolonged clotting time seen in unhealthy lobsters is likely be to movement of cells from the hemocoel into the tissue spaces.

4. Histopathological investigations

4.1 Studies performed

Tissues samples were obtained during autopsies and processed as previously described (Cht 4, Section 8.2.1). The tissues were fixed in one of three different types of fixatives, Bouins, Davidsons and seawater formalin (Bancroft and Stevens, 1977), depending on when the study was performed. All tissue sections were stained with haematoxylin and eosin and examined with an Olympus binocular microscope at 40 to 1000 x magnification. Tissue sections were examined for disease organisms and histopathological lesions.

4.2 Fixative study

A preliminary study was preformed in which tissues from the same lobster were fixed in two different fixatives, Bouins and Davidsons (Geraldton April 1998, Case Study). It was found that preservation of tissue architecture was superior in the Davidsons fixed sections, particularly in the sections of antennal gland and hepatopancreas. However, as previously reported for freshwater crayfish (Evans et al., 1988), granular cells were not strongly eosinophilic in tissues fixed with this fixative. Strong eosinophilic reactions of granular cells are desirable in histopathological studies of crustacean tissues since this cell type tends to predominate in early focal hemocytic aggregations and has been observed to be the first cell type to accumulate in hemocytic aggregation reactions to autotomy (Evans, unpublished observations). It is preferable, therefore, to use a fixative that highlights the granular cells, even if tissue architecture is not as well preserved. In order to achieve improved eosinophilia in granular cells a study was conducted on tissues fixed in seawater formalin. A strong eosinophilic reaction in the granular cells was demonstrated. This fixative was subsequently used for all histopathological studies (April, 1998 onwards).

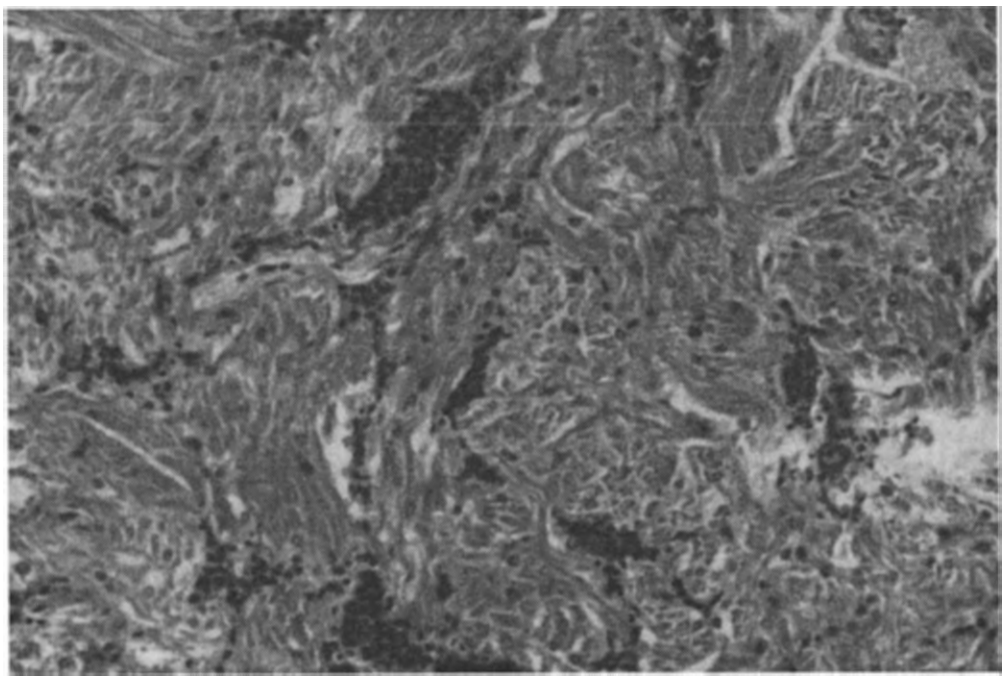
4.3 Disease organisms

Encysted life stages of two parasites were observed in lobster tissues, metacercariae of a microphallid parasite, *Thulakiotrema genitale* (Deblock et al., 1990) in the antennal gland of five lobsters, the abdominal muscle of one lobster and in the testis of another lobster and spores of an unidentified microsporidian parasite (Evans and Brock, 1994; Evans et al., 2000) in the heart of one lobster. Parasites were present in tissues of both healthy and unhealthy lobsters. Bacteria were also observed in sections of heart, antennal gland and abdominal muscle of a number of lobsters (see Section 4.4 below) and an unidentified yeast was seen infecting the antennal gland in two lobsters examined in the fixative study.

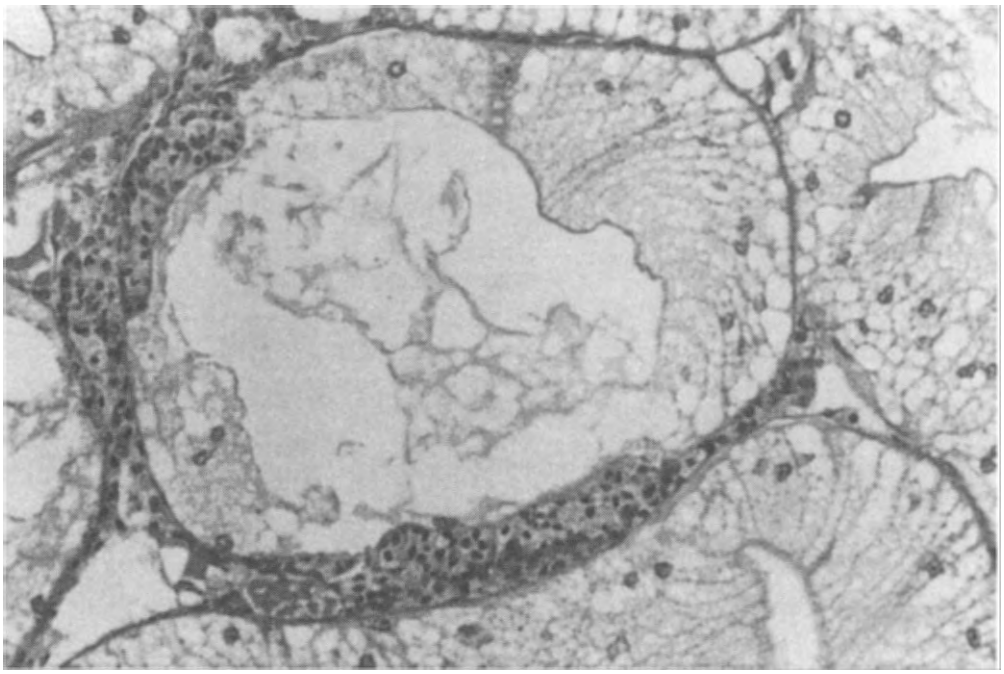
4.4 Pathological reactions in lobster tissues

The major type of pathological reaction observed in lobster tissues was a hemocytic aggregation, suggestive of an inflammatory response. The aggregate was often small, involving only a small number of hemocytes with granular hemocytes often predominating. This type of lesion was common in the heart and generally involved the encapsulation of a single heart muscle fibre by the aggregating hemocytes. However, in some tissue sections, mainly those of the heart but occasionally the hepatopancreas and antennal gland, massive inflammatory reactions were observed (Figure 55). Bacteria were occasionally seen in the area of the focal inflammation but this was not a common finding. Other inflammatory reactions observed included small nodules and encapsulations. Muscle myopathy was also a common finding and cardiac myopathy was seen in one lobster. Detailed descriptions of these pathological reactions will be provided in the final report for the project FRDC 99/202.

Figure 55 Examples of hemocytic aggregation lesions



(a) Lobster heart (H&E x 200)
(Note multiple foci of hemocytic aggregations lying between muscle bundles)



(b) Hepatopancreas (H&E x 200)
(Note aggregation of hemocytes in intertubular space)

4.4.1 Distribution of inflammatory reactions in lobster organs

A comparison was made of the prevalence of inflammatory lesions (hemocytic aggregations, small nodules and/or encapsulations) in various organs from healthy and unhealthy lobsters and from lobsters with uncertain health status. These results are shown in Table 81.

Table 81 Percentage prevalence of inflammatory lesions in lobster organs[1]

(a) Healthy lobsters

Lobster group[2]	Number Studied	%Prevalence				
		Heart	A.Gland	Hepat.	Musc.	Gill
Fremantle Whites	13	23.0	23.0	7.7	0.0	0.0
Fremantle Reds	12	16.7	8.3	0.0	0.0	16.7
Truck Study, Dec '97	10	40.0	10.0	0.0	0.0	10.0
Geraldton Reds	11	30.0	9.1	0.0	9.1	9.1
Geraldton Whites	10	10.0	30.0	0.0	0.0	10.0
Mean		23.9	16.1	7.7	9.1	11.5

[1] Abbreviations: A.Gland – antennal gland; Hepat. – hepatopancreas; Musc. – abdominal muscle
[2] See Section 2.1 for explanation of lobster groups

(b) Unhealthy lobsters

Lobster group[2]	Number Studied	%Prevalence Heart	A.Gland	Hepat.	Musc.	Gill
Fremantle Whites	10	40.0	10.0	20.0	0.0	20.0
Fremantle Reds	12	25.0	8.3	0.0	25.0	16.7
Geraldton April '98 (Case Study)	6	50.0	66.7	0.0	50.0	33.3
Geraldton Reds	11	54.5	54.5	9.1	9.1	27.3
Geraldton Whites	10	50.0	90.0	20.0	10.0	20.0
Mean		43.9	45.9	9.8	18.8	23.5

[1] Abbreviations: A.Gland – antennal gland; Hepat. – hepatopancreas; Musc. – abdominal muscle
[2] See Section 2.1 for explanation of lobster groups

(c) Lobsters with uncertain health status

Lobster group[2]	Transport to factory[3]	Number Studied	%Prevalence Heart	A.Gland	Hepat.	Musc.	Gill
Fremantle Whites	T	8	12.5	50.0	0.0	0.0	0.0
Fremantle Reds	T	12	16.7	66.7	8.3	0.0	58.3
Truck Study, Dec '97 – Jetty Lobsters[4]	B/T	10	30.0	30.0	0.0	0.0	20.0
Truck Study, Dec '97 – Truck Lobsters	T	6	66.7	33.3	0.0	0.0	33.3
Mean			31.5	45.0	4.0	0.0	27.9

[1] Abbreviations: A.Gland – antennal gland; Hepat. – hepatopancreas; Musc. – abdominal muscle
[2] See Section 2.1 for explanation of lobster groups
[3] T = delivered to factory in truck
B = delivered to factory by boat
[4] Jetty lobsters were taken from a boat to the Dongara factory by truck; truck lobsters were taken from the Dongara factory to Geraldton by truck.

Inflammatory lesions were observed in the tissues of healthy and unhealthy lobsters as well as in lobsters of uncertain health status. However, the prevalence of lesions tended to be lower in the healthy lobsters. A similar pattern of prevalence of inflammatory lesions in different organs was seen in all three lobster groups, the lesions being most often seen in the heart and antennal gland sections and least frequently seen in the hepatopancreas and the abdominal muscle.

4.4.2 Prevalence of systemic inflammatory responses in lobsters of differing health status

The occurrence of a single inflammatory lesion in one organ is unlikely to be of health significance, the lesions probably representing transitory reactions to minor tissue injury. Such minor reactions are not uncommon in histological sections of animal tissues and are generally ignored. However, if lesions are present in more than one organ, indicating systemic inflammation, it is possible that the lobster's host defence system is compromised and a deterioration in health status has occurred. This

hypothesis was tested by comparing the levels of systemic inflammation in lobsters of differing health status and examining the relationship, if any, of the occurrence of bacteremia and systemic inflammation. Systemic inflammation was defined as inflammatory lesions being present in more than one organ of the same lobster. The results are presented in Tables 82 and 83.

A statistical analysis of the prevalence of systemic inflammation in the two lobster groups of known health status is compared in Table 82. The mean prevalence of systemic lesions was higher in the unhealthy group (47.4%) than in the healthy group (5.9%). The difference between the prevalence of systemic inflammatory lesions in the matching healthy and unhealthy lobsters was statistically significant in two of the groups (Geraldton Reds and Geraldton Whites), but not in the other two groups.

Table 82 Prevalence (prev.) of systemic inflammatory lesions in healthy and unhealthy lobsters[1]

Lobster group[2]	Healthy		Unhealthy		P[2]
	Prev.	%prev.	Prev.	%prev.	
Fremantle Whites	2/13	15.4	4/10	40.0	0.097
Fremantle Reds	1/12	8.3	2/11	18.2	0.457
Geraldton Reds	0/11	0.0	5/11	45.5	0.007
Geraldton April '98 (Case study)	-	-	5/6	83.3	-
Geraldton Whites	0/10	0.0	5/10	50.0	0.007
Mean		5.9		47.4	

[1] See Section 2.1 for explanation of lobster groups
[2] Probability; χ^2 analysis

A comparison of the prevalence of systemic inflammation and the presence of bacteremia in post harvest lobsters in which bacteremia and histopathology results were available for the same lobster is shown in Table 83. Systemic inflammation was mainly seen in lobsters with bacteremia, only 2 lobsters exhibiting systemic inflammation in the absence of bacteremia. There was a strong correlation between the two health parameters (Pearson's correlation = 0.879) with the %prevalence of bacteremia exceeding that of systemic inflammation in 6 of the 7 groups of lobsters. A total of 15 out of the 27 lobsters with bacteremia showed no evidence of systemic inflammation.

Bacteria were observed in the foci of inflammation in histological sections of only 4 of the 16 lobsters exhibiting systemic inflammation.

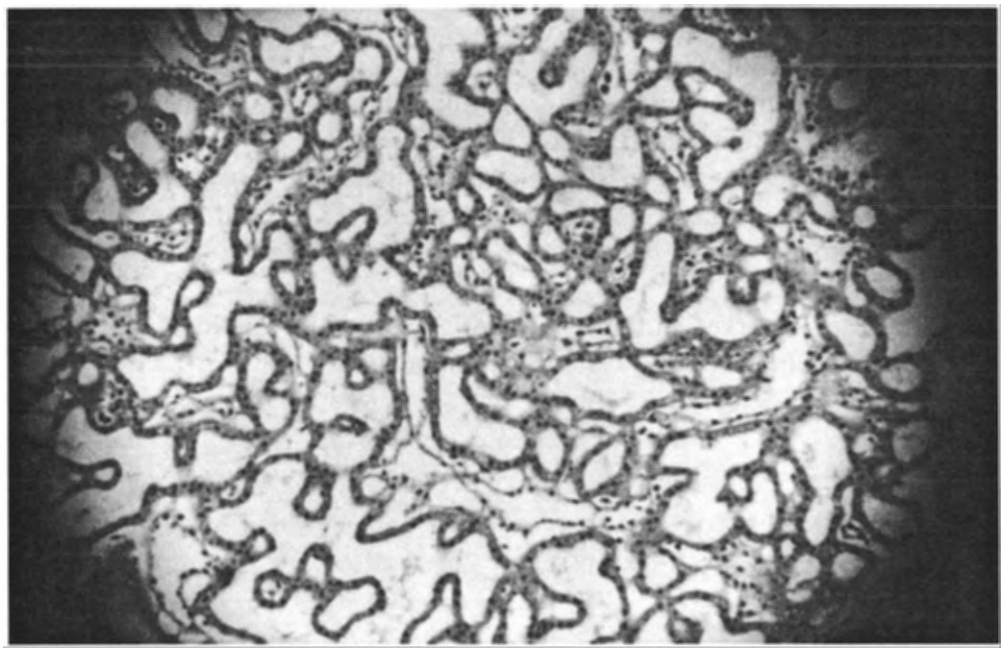
Table 83 Prevalence of systemic inflammation and bacteremia in post harvest lobsters

Health status	Lobster group	Number studied	%Prevalence				
			Bact.	S.I.	Bact. & S.I.	Bact. no S.I.	S.I. no Bact.
Healthy	Fremantle Whites	13	0.0	15.4	0.0	0.0	0.0
	Geraldton Reds	11	0.0	0.0	0.0	0.0	0.0
	Geraldton Whites	10	20.0	0.0	0.0	20.0	0.0
Unhealthy	Fremantle Whites	10	50.0	40.0	20.0	30.0	20.0
	Geraldton Reds	11	81.8	45.5	45.5	36.4	0.0
	Geraldton Whites	10	90.0	50.0	50.0	50.0	0.0
Uncertain health	Fremantle Whites	8	25.0	0.0	0.0	25.0	0.0

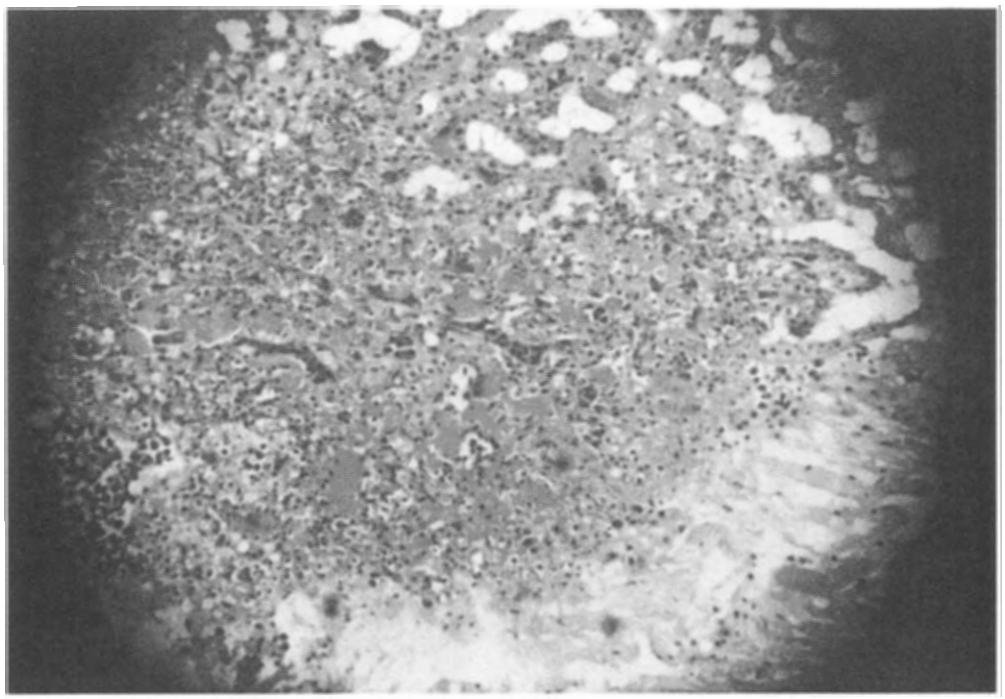
4.4.3 Pathological lesions suggestive of bladder infection

In two lobster groups, Geraldton Reds and Geraldton Whites, lesions suggestive of a bladder and, in some lobsters an associated antennal gland, infection were observed. Examples of these lesions are compared to the appearance of an unaffected organ in Figure 56. Similar lesions were seen in 90% of the Geraldton White lobsters and 50% of the Geraldton Reds. The same features were also seen in some antennal gland sections from *Jasus edwardsii* examined in another FRDC study (Evans et al., 2001). The dominant feature of the lesions was the high proportion of granular cells sequestered into the tissue spaces and the presence of an eosinophilic precipitate, suggesting of coagulated hemolymph.

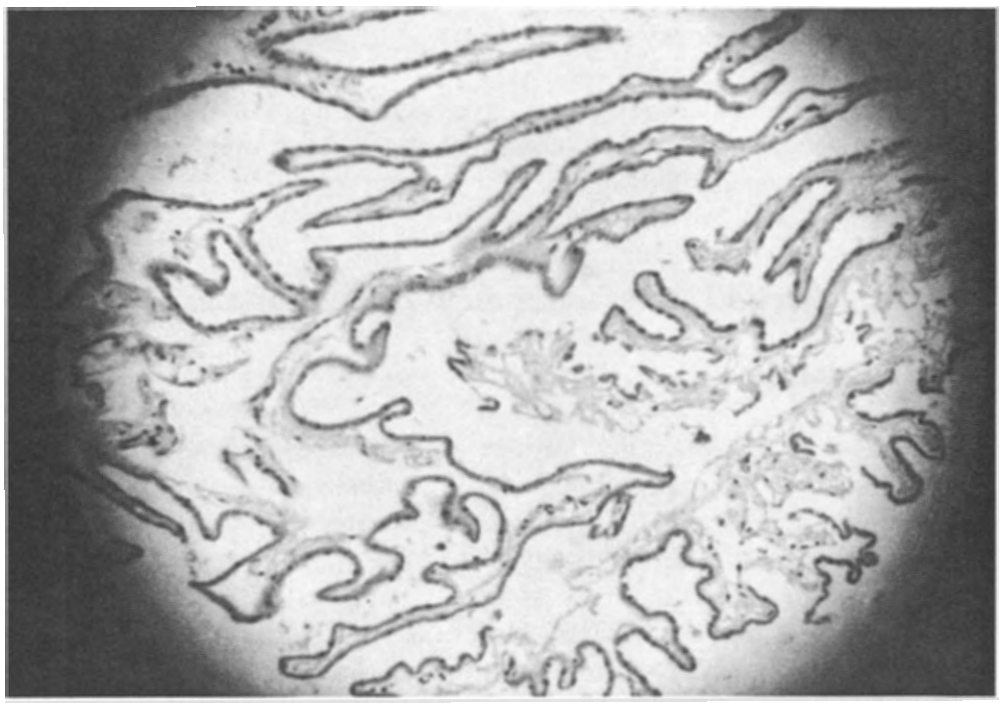
Figure 56 Histological evidence of marked inflammation in antennal glands and bladder



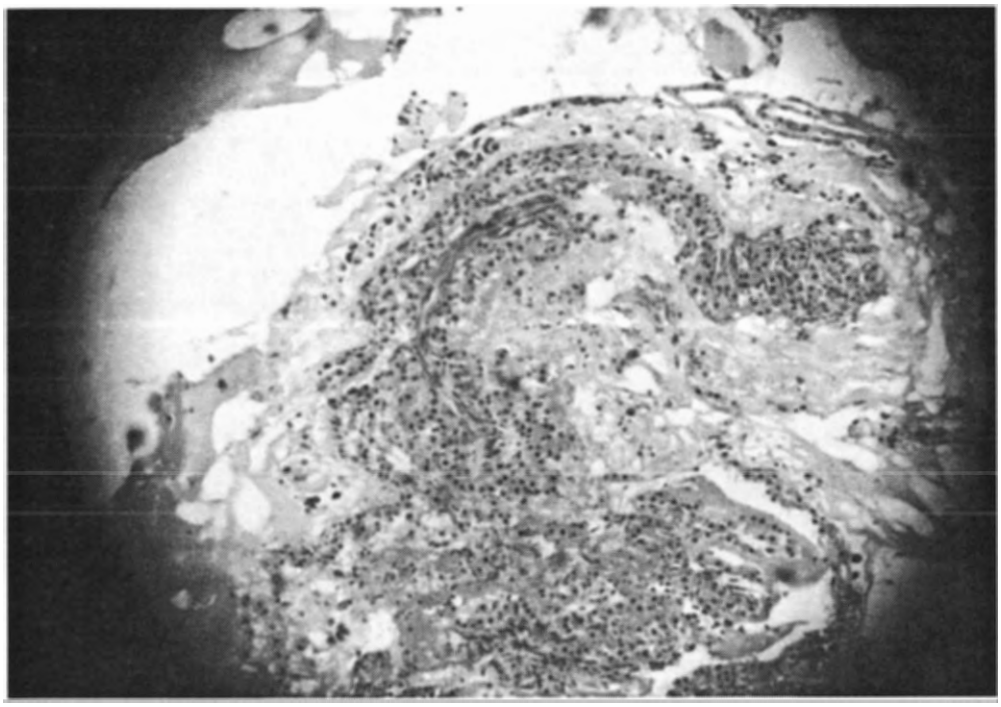
(a) Normal histological appearance of antennal glands (H&E x 100)



(b) Area of marked inflammation with hemocytic aggregation and coagulated hemolymph of antennal glands (H&E x 100)



(c) Normal histological appearance of bladder (H&E x 100)



(d) Area of marked inflammation in bladder with hemocytic aggregation of predominantly granular cells (H&E x 100)

4.5 Standard autopsy protocol

A standarised autopsy protocol was developed. The protocol is described in Appendix 3. Photographs showing the location of the various organs have been previously reported (Evans et al., 2001).

5. Health and stress assessment using immune parameters

5.1 Rationale

Health assessment of spiny lobsters will be an important procedure in lobster aquaculture and has application in the improvement of handling procedures for post harvest lobsters destined for live export. Assessment of stress status could also be of value in determining the contribution of a given post harvest handling procedure (eg. truck transport) to acute stress responses likely to be induced during capture and storage of post harvest lobsters. Health and stress status can both be assessed by measuring the status of the immune system. Immune status is decreased in lobster with poor health and is elevated in lobsters experiencing an acute stress response. In the former there is a reduction in THC and %granular cells and an elevation in clotting time and bacteremia (Table 80). In lobsters experiencing an acute stress response, on the other hand, there is an elevation of THC and a reduction in clotting time (Chapter 4, Sections 2.5 & 4.4). This response is the classical 'fight or flight' response first described by Selye (1973). In effect, immunocompetence is enhanced in anticipation of the need to counteract loss of hemolymph that could occur through wounding.

Since more than one immune parameter is altered in these different physiological states, it would be of value if the results from different assays could be combined in some way so as to provide an overall assessment of immunity. The aim of this part of the study was to derive a formula based on the four main immune parameters measured in this project (THC, %granular cells, bacteremia and clotting time) and use results obtained in various stress and health studies to evaluate the reliability of the formula in providing an assessment of immunity status.

5.2 Derivation of composite immunity parameter

5.2.1 Immunity Web calculation

The mean values for THC, %granular cells, bacteremia and clotting time were used to derive a single, quantitative measure of immunocompetence. Two quantitative measures were derived, one based on measurement of bacteremia using bacterial colony rank and the other using %prevalence bacteremia. The reasons for measuring bacteremia by these two different methods are discussed in Chapter 5, Section 3.

Various approaches to combining the mean values for the four parameters into one quantitative measure were evaluated. The approach that showed the most promise was to construct a polygon using the mean values for each of the four parameters and determine the area of the polygon using the formula: Area = 0.5 (AB + BC + CD + DA)

Where A = THC x 10^6 cells/ml
B = %granular cells
C = 10 - (0.1 x %prevalence bacteremia) or 10 - colony rank
D = 10 – clot rank or (clotting time x 0.1)

Mean values for THC and %granular cells were used directly while clotting time and bacteremia were expressed as 10 minus the mean values. This approach ensured that in the graphical presentation (and in the determination of enclosed areas), all immune parameter values showed a decreasing trend with a deterioration in health status while the two parameters affected by acute stress responses, THC and clotting time, were both displayed as increased levels in stressed lobsters.

An example of the polygon obtained using the average values for THC, %granular cells, %bacteremia and clot rank (see Chapter 5, Section 3.7), is shown in Figure 57. The area obtained using these values was 99.

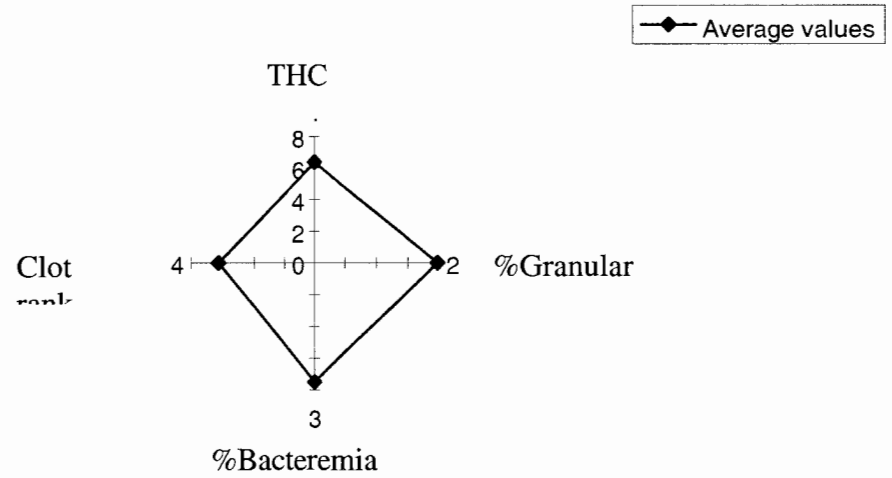


Figure 57 Immunity Web for average values for four immune parameters, THC (x 10^6 cells/ml), %granular cells, %bacteremia and clot rank. Average values obtained from normal range determinations (see Chapter 5)

The presentation of immunity results using a 'radar' plot diagram, termed an 'Immunity Web', clearly displayed the values obtained for each of the four immune parameters. Furthermore, the area enclosed by the polygon could be calculated and the areas for different treatment groups compared.

In analyses of IW areas shown below, those derived using %prevalence bacteremia are referred to as IW% and those obtained using bacterial colony ranks as IWR. A comparison of normal range values for these two parameters based on the normal ranges for THC, %granular cells, %bacteremia or bacterial colony rank and clot rank is given in Table 84.

Table 84 Normal ranges for Immunity Web areas

Parameter	Mean	Lower Limit	Upper Limit
Immunity Web using %prevalence bacteremia (IW%)	99	84	113
Immunity Web using bacterial colony rank (IWR)	114	84	150

5.3 Health assessment using Immunity Web areas

The results presented in Section 3 clearly demonstrate alterations in the immune parameters, THC, %granular cells, bacteremia and clotting time, in lobsters with poor health status compared to healthy lobsters. Immunocompetence in the unhealthy lobsters was significantly reduced compared to that in the healthy lobsters (Table 80). The results obtained with these groups of lobsters were used to derive Immunity Web areas (IW%). An example of the results obtained with this approach for healthy and unhealthy lobsters from the Geraldton Whites study is shown in Figure 58.

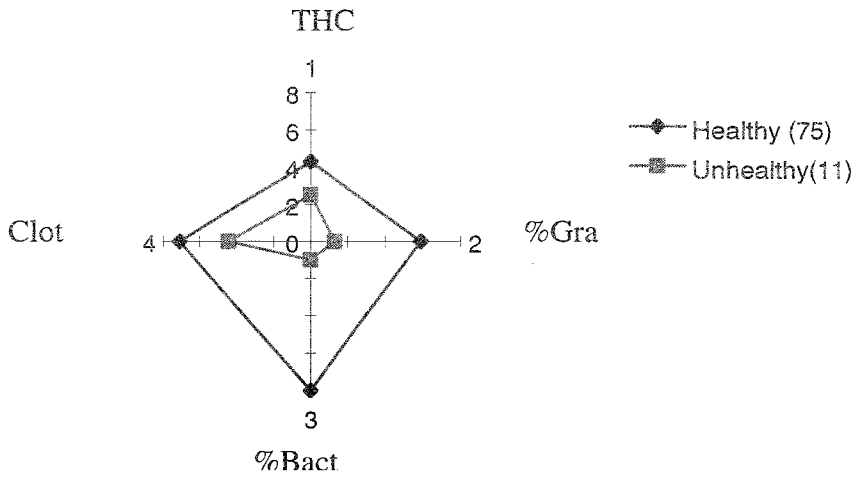


Figure 58 Immunity Web for Geraldton Whites Lobster Groups. Mean values for immune parameters in Geraldton Whites Healthy (n=10) and Unhealthy (n=10) lobster groups displayed using a 'radar' plot; (n) = Immunity web area; Abbreviations: THC (x 10^6 cells/ml); %Gran (%granular cells); %Bact (10 – (0.1 x %prevalence bacteremia); Clot rank (10 – clotting rank)

In the example shown in Figure 58 the Immune Web area (IW%) for the unhealthy lobsters, 11, was 85% lower than that of the healthy lobsters (75).

The Immunity Web areas (IW%) obtained for healthy and unhealthy lobsters in the Fremantle Whites, Fremantle Reds, Geraldton Whites and Geraldton Reds are compared in Figure 59. Since there were no clotting times measured in the Fremantle Whites, Fremantle Reds and Geraldton Reds studies, an estimated clot rank value of 5 was used in the calculations for both groups. The result depicted in Figure 59 is therefore the worst case scenario, since it would be anticipated that the clot rank for the unhealthy lobsters would be higher than that of the healthy lobsters.

The values obtained for IW% for the healthy lobsters were significantly higher than those for the unhealthy lobsters (paired t-test; $P<0.001$). The IW% for the Fremantle lobsters were higher than those of the Geraldton lobsters for both red and white lobsters in both treatment groups. The IW% values for healthy lobsters were slightly lower than the lower limit of the normal range (Table 84) for the Geraldton lobsters while the value for Fremantle Reds was slightly higher than the upper limit of the normal range. The value for the Fremantle Whites fell within the normal range. The IW% values for white lobsters were similar to those for red lobsters in both red and white groups of healthy lobsters.

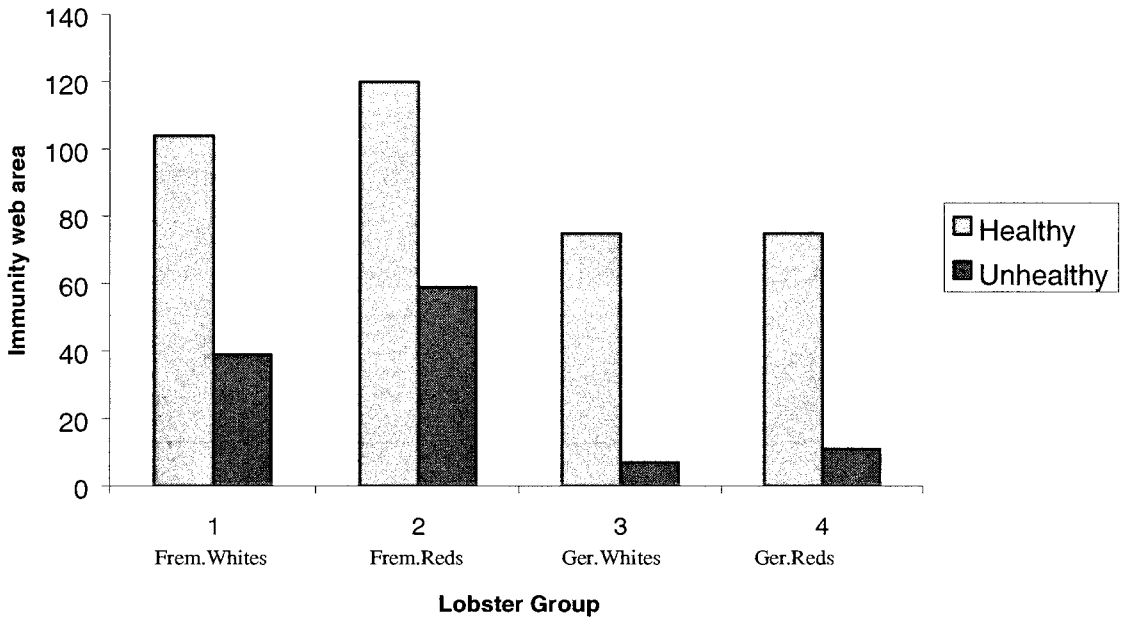


Figure 59 Comparison of Immunity Web areas for healthy and unhealthy lobsters. Abbreviations - Fremantle Whites (Frem Whites), Femantle Reds (Frem Reds), Geraldton Whites (Ger Whites) and Geraldton Reds (Ger Reds) studies.

In second test of the reliability of Immune Web areas as a measure of health status, a comparison was made of the mean %prevalence of systemic inflammatory reactions in the healthy and unhealthy lobster groups (Table 82) and the IW% values for the same treatment group. There was a strong correlation between these two parameters (Figure 60; Pearson's correlation coefficient = -0.824).

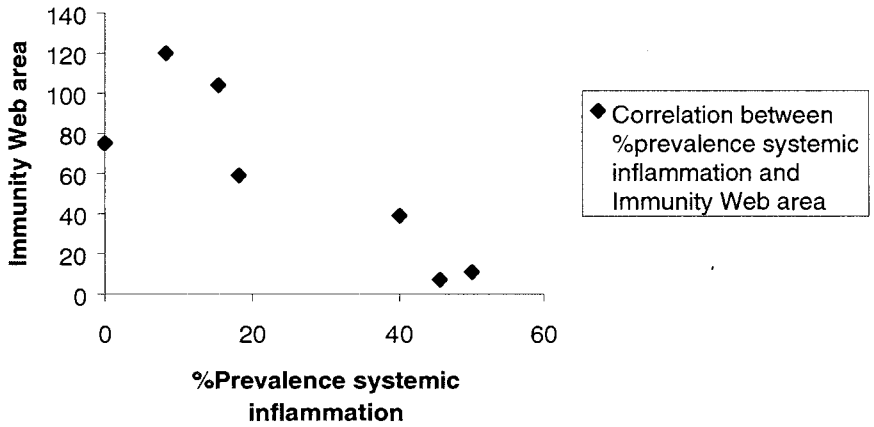


Figure 60 Correlation between mean %prevalence of systemic reactions and the Immunity Web area for healthy and unhealthy lobsters. Abbreviations - Fremantle Whites, Femantle Reds, Geraldton Whites and Geraldton Reds.

In another test of the reliability of Immune Web areas as a measure of health status, a comparison was made of areas obtained with the five different storage treatment groups used in the Factory 2 and 3 trials in Geraldton (Chapter 7, Section 5). In that study lobsters were stored under five different conditions (flow through submerged, recirculating submerged, humid air, flow through spray and recirculating spray treatments). The number of lobsters surviving storage under these different conditions and a subsequent 7 day storage and simulated live transport procedure was assessed and compared to the levels of immune parameters in hemolymph samples taken after 6hrs storage under the different conditions.

The Immune Web areas (IWR) obtained from the THC, %granular cells, bacterial colony rank and clotting times (Tables 49,50, 51, 52, 53) are shown in Table 85.

The pattern of change in IWR in the different treatment groups was similar in both trials, the highest values being obtained with the flow through submerged group and the lowest with the recirculating spray group. The results for the other three treatments were similar in the November trial but showed a decreasing trend in the March trial. All IWR values were within the normal range (Table 84) except for the flow through spray treatment in the March trial and the recirculating spray treatment in both trials.

Table 85 Immunity Web areas for different treatment groups in factory trials 2 & 3[1].

Trial	Immunity Web Area (IWR)[2]				
	Flowsub	Recsub	Humid air	Flowspray	Recspray
November (Trial 2)	119	87	86	87	68
March (Trial 3)	115	103	85	78	69

[1] See Chapter 7, Section 5 for experimental design
[2] IWR – Immunity Web area calculated using bacterial colony ranks
Abbreviations: Flowsub – flow through, submerged; Recsub – recirculating, submerged; Flowspray – flow through, spray; Recspray – recirculating, spray

Comparisons of the IWR values with the % survival obtained in the different treatment groups for both factory trials are shown in Figures 61 and 62.

The pattern of survival in the different storage treatments showed a decreasing trend, indicative of a decrease in health status in the lobsters held in the humid air and in the spray systems compared to those held in the flow through and recirculating submerged systems. The pattern of change in IWR was similar to that of the %survival in both trials, particularly in factory trial 3. The lowest IWRs were obtained with the recirculating spray treatments in both trials and these groups exhibited the lowest survival. The highest survival was observed in the flow through submerged and the recirculating submerged groups and these groups displayed the highest IWR's in the factory 3 trial. In the factory 2 trial, the IWR was highest in the flow through submerged group but the recirculating submerged group had a similar IWR to the recirculating submerged and humid air groups.

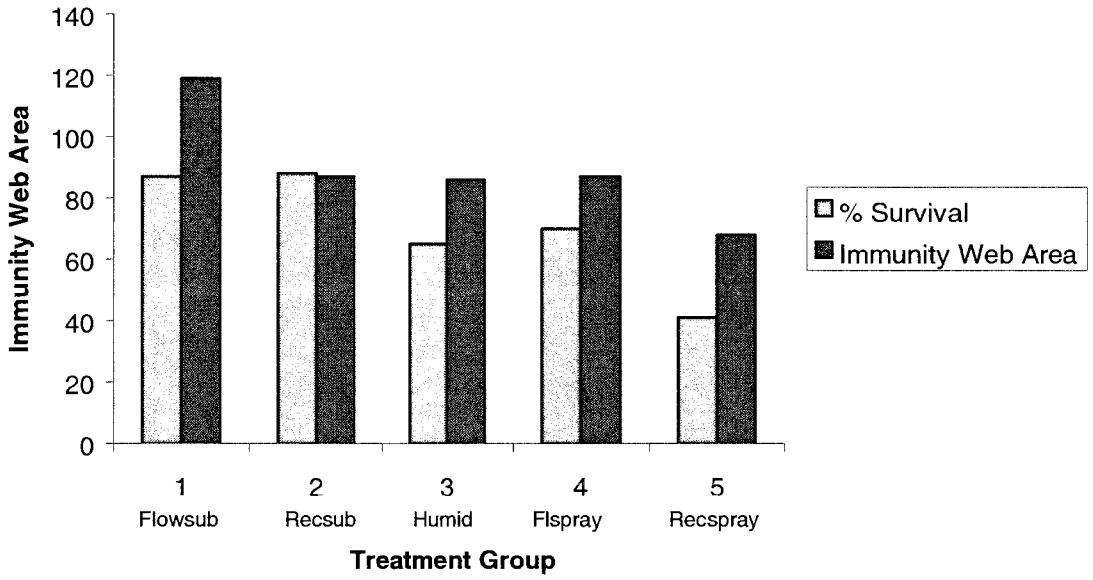


Figure 61 Comparison of percentage survival and Immunity Web areas (IWR) in lobsters held under the different storage conditions. Abbreviations - flow through submerged (Flowsub), recirculating submerged (Recsub), humid air (Humid), flow through spray (Flspray) and recirculating spray treatments (Recspray), in the factory 2 trial (November).

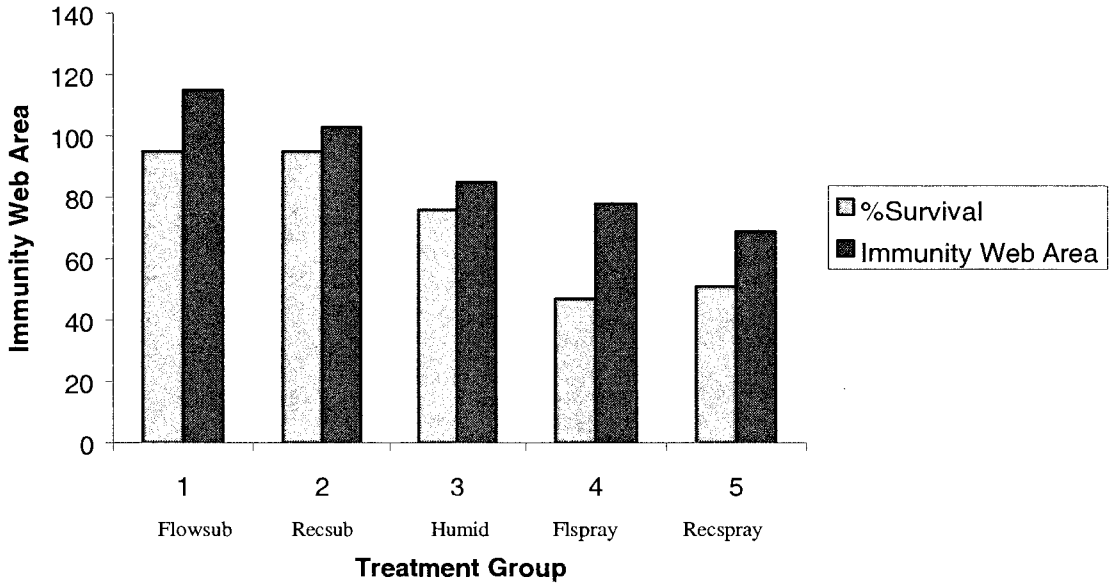


Figure 62 Comparison of percentage survival and Immunity Web areas (IWR) in lobsters held under the different storage conditions. Abbreviations - flow through submerged (Flowsub), recirculating submerged (Recsub), humid air (Humid), flow through spray (Flspray) and recirculating spray treatments (Recspray) in the factory 3 trial (March).

5.4 Assessment of stress status using immune parameters

The application of Immunity Web areas to the assessment of stress status was evaluated by calculating IWRs for the various treatments of the definitive stress trial described in Chapter 6 Section 7. In this study lobsters were held in a quiet, darkened room with minimal disturbance (undisturbed controls) and their immune parameters compared to those of lobsters held in a noisy room with bright lights and visual disturbance (disturbed controls) and lobsters exposed to a handling stress procedure and sampled 5 and 120 minutes after stressor exposure (stress 5 min and stress 120 min respectively). The results obtained are shown in Table 86.

Table 86 Immunity Web areas obtained in different treatment groups in the definitive laboratory stress trial[1]

Treatments	Undisturb Cont 0	Undisturb Cont 120	Disturb Cont 0	Disturb Cont 120	Stress 5 min	Stress 120 min
Health Web Area	51	74	63	77	86	106

[1] See Chapter 6, Section 7 for experimental design
Abbreviations: Undisturb Cont 0 (undisturbed controls at 0min); Undisturb Cont 120 (undisturbed controls at 120mins); Disturb Cont 0 (disturbed controls at 0min); Disturb Cont 120 (disturbed controls at 120mins); Stress 5 min (stressed lobsters, 5mins after stressor exposure); Stress 120 min (stressed lobsters, 120mins after stressor exposure)

The IWR values showed the predicted change in the various experimental groups. The lowest values were obtained with the undisturbed control lobsters at the commencement of the experiment (Undisturb cont 0). The value for the Distur Cont 0 group was higher than that of the Undisturb cont 0 but lower than that of the undisturbed controls after 120 minutes (Undisturb cont 120). The IWR for the disturbed controls after 120 minutes (Disturb cont 120) was the highest of all IWRs for the control lobsters but was lower than those of the stressed lobsters. The IWR for the stressed lobsters after 120 minutes was the highest value recorded in all treatment groups. The IWRs for the control lobsters were below the normal range (Table 84) and those of the stressed lobsters fell within the normal range.

6. Discussion

The major findings from this study of immune parameters in lobsters from different experiments performed during the course of this project were that the total number of circulating hemocytes and the proportion of granular cells present in the hemolymph were significantly reduced in lobsters with poor health status while the clotting time and levels of bacteremia were elevated. When combined into a single composite parameter, the Immunity Web area, highly significant differences were demonstrated between healthy and unhealthy lobsters sampled in both Fremantle and Geraldton trials. IW values for unhealthy lobsters were 49 – 93% lower than those obtained with the healthy controls and all values were less than the lower limit of the normal range for IW%. In addition, there was a strong correlation between the mean %prevalence of systemic inflammation in lobster groups and the level of IW% in the same groups.

Similarly, the predicted trend in Immunity Web values (decreasing trend with increasing mortality) were obtained from the analysis of results obtained in factory trials. Overall the results suggest that Immunity Web analyses show promise as an approach to assessing immune status, and, therefore, health status, in the spiny lobster *Panulirus cygnus*.

Similarly, the Immunity Web analysis of results obtained in the definitive stress trial showed the predicted trends, the IWR values increasing as the lobsters became more stressed, either through exposure to environmental stressors (visual disturbance, noise, light – control lobsters) or through handling (stressed lobsters). The IWR value obtained with the lobsters likely to be experiencing the strongest stress reaction (stress 120 lobsters) was the highest of all treatment groups. In contrast, the IWR value obtained in the group predicted to be least stressed (undisturbed controls, 0 min) was the lowest value recorded. These trends lend weight to the conclusion that IW analysis can be applied in the assessment of acute stress status in healthy lobsters, the higher the IW value the more stressed the lobster.

The IWR obtained with the undisturbed controls in the definitive stress trial, 51, was well below the normal range for this parameter (84 – 150), suggesting that the lobsters used in this study were in poor health. The demonstration of high levels of bacteremia in these lobsters, combined with the isolation of almost pure cultures of *Rhodococcus maris* from hemolymph samples taken from three lobsters used in the study (Chapter 4, Section 5.5), is in agreement with this conclusion and provides further evidence of the suitability of IW analysis as a measure of health status in *Panulirus cygnus*.

Unhealthy lobsters exhibited significantly lower levels of circulating hemocytes and significantly higher levels of systemic inflammatory reactions in lobster tissues. The low levels of circulating hemocytes could have been due to a shift of hemocytes out of the hemolymph channels and into lobster tissue spaces, to lysis of circulating hemocytes, or a combination of both. Smith and Ratcliffe (1980), studying the hemocytic and histopathological response of injected bacteria in the shore crab *Carcinus maenas*, showed that hemocyte clumps developed in body organs following the injection of bacteria. Similar lesions have been described in *Callinectes sapidus* (Johnson, 1976). A decrease in circulating hemocytes was seen in freshwater crayfish exhibiting inflammatory responses in muscle tissues (Evans et al., 1999a). From these reports, and the findings presented in this study, it would appear that the low levels of circulating hemocytes in unhealthy lobsters was caused, at least in part, by the formation of hemocytic aggregations in body tissues and a failure of the hemopoietic processes to restore normal circulating cell levels.

Only small numbers of infectious agents were observed in histological sections of body organs from healthy and unhealthy lobsters. The %prevalence of parasites was very low and organisms were seen in sections from both healthy and unhealthy lobsters. This observation suggests that parasitic infections do not play a significant role in the development of weakness in post harvest lobsters. Bacteria, on the other hand, appear to be important in the development of weakness in some post harvest lobsters. The unhealthy lobsters examined in the factory trials all exhibited low levels of circulating granular cells, bacteria in their hemolymph, increased prevalences of systemic inflammatory reactions in body tissues and, in some lobsters, histopathological evidence of bladder and antennal gland infections. These findings

provide evidence for a role of bacterial infection in the development of morbidity in these lobsters. However, the infections were probably opportunistic and unlikely to be due to the presence of a primary bacterial pathogen.

In the lobsters in which bacteremia and systemic inflammation were examined the presence of inflammation was almost always accompanied by the presence of bacteria in the hemolymph. However, the converse, lobsters with bacteremia consistently exhibiting systemic inflammation, was not the case. More than 50% of lobsters with bacteremia did not show any evidence of systemic inflammation. The lack of a consistent finding of systemic inflammation in lobsters with bacteremia suggests that either there is no causal relationship between the two processes, that there was systemic inflammation in bacteremic lobsters but the lesions were not displayed in the particular tissues sections examined, or that lobsters can experience a bacteremia that is of little health significance and does not lead to hemocytic aggregations in lobster tissues.

In contrast to the other three groups, the Fremantle Reds lobsters showed little evidence of bacterial infections. The levels of bacteremia and systemic inflammatory lesions were both low in the unhealthy lobster group. The cause of morbidity in these lobsters was not determined but is likely to have been organ failure resulting from post harvest handling stress.

7. Relevance to industry practices and recommendations

The study has demonstrated significant changes in immune parameters in lobsters with low health status. An approach has been developed to combine the results of immune parameter assays into a single measurement, the Immunity Web area and normal ranges for this parameter have been estimated. Immunity Web areas have significant potential in the assessment of health status in lobsters.

Further studies are required to improve this approach to measuring health status in *Panulirus cygnus*. For example, it is not clear whether clot rank or clotting time should be used in the derivation of Immunity Web areas. The two values differ in that the former includes data from lobsters exhibiting a 'no clot' reaction in their hemolymph (Chapter Chapter 4, Section 4.2). It is also not clear whether %prevalence bacteremia or bacterial colony rank is the better parameter for use in the derivation of IW area. Finally, further studies are required to confirm the range of IW values expected to be obtained with well acclimated, post harvest lobsters and in lobsters exposed to environmental stressors.

The significance of 'no clot' reactions to lobster health or stress status is not known. Clotting times were studied in 11 groups of lobsters in this project. Since the project was completed clotting time studies have been performed on 32 groups of post harvest lobsters, none of which were groups of unhealthy lobsters. The 'no clot' phenomenon was observed in 28 of the 43 groups of 8-12 lobsters. When present in a given batch of lobsters, the prevalence of occurrence was approximately 30%. The nature of the processes leading to prolonged clotting in lobsters is poorly understood and should be further studied.

Evidence has been presented to show that bacteria are likely to be have been involved in the development of morbidity in the majority of unhealthy lobsters examined in this study. Whether the presence of increased levels of bacteria in factory tanks, or a particular strain of bacteria in the tanks, contributed to the development of morbidity was not studied. An understanding of the relationship of bacterial levels in holding tanks, bacteremia in lobsters and the development of morbidity is presently lacking and is worthy of further investigation.

1. Achievement of project aims

1.1 Original project objectives

At the commencement of the research it was envisaged that the main outcome of the project would be the availability of a suite of simple stress tests which could be used by fishers or processors to measure stress and/or health status in a batch of lobsters. It was seen that this capability would be of value to fishers and processors in the improvement of post harvest handling procedures and in maximising the proportion of the catch that are 'fit for live'. The project was conducted in collaboration with another group of researchers who investigated physiological indicators of lobster stress status (Paterson et al., 2001). At the commencement of the project it was anticipated that the final stress or health status indices would combine both immune and physiological indicators.

A secondary aim was to determine common causes of mortality of lobsters in live holding tanks.

2. Achievement of project objectives

The original project objectives and outcomes achieved against these objectives were as follows:

2.1 ***To identify suitable immune system parameters which can be used to evaluate stress responses and health status in captive lobsters and to apply those parameters in a study of stress induced by post harvest handling procedures.***

Eight different immune system parameters were identified for study. Six tests, two more than specified in the project contract, were developed over the three year period and responses to post harvest stressors determined. All but one parameter (phagocytic capacity) were analysed in both 'accepted' (healthy) and 'reject' (unhealthy) lobsters and the variation from normal values which occurred in reject lobsters evaluated.

Significant findings were:

- Characteristic changes in the level of immune parameters occurred when lobsters became weakened through poor post harvest handling practices. This finding means that measurement of an appropriate suite of immune parameters in a batch of lobsters could provide a quantitative measure of the health (strength) of the lobsters.

- Physical handling, air exposure, wounding and other minor post harvest stressors caused reproducible changes in the levels of selected immune parameters in laboratory held lobsters. The degree of change appeared to reflect the level of stress effect. These findings suggest that measurement of an appropriate suite of

immune parameters could provide a measure of stress status of a batch of lobsters. However, this conclusion has yet to be confirmed through measurements of post harvest lobsters on boats and in factories.

- Factory based simulated truck and live shipment trials showed that a selected suite of immune parameters could accurately differentiate between holding conditions that resulted in high lobster survival (submerged storage) and those that resulted in high mortality (held in air with or without spray treatments at ambient temperatures). This finding means that these tests could be used to evaluate the efficacy of different post harvest practices.

- In the same factory trials, the immune parameters successfully predicted the outcome (classification of lobsters into those that would survive the simulated truck transport and live shipment and those that would not) in 66% of lobsters (trial 1) and 79% of lobsters (trial 2). Addition of the physiological parameters (Paterson et al., 2001) to the analysis improved the discrimination to 77% (trial 1) and 81% (trial 2).

- Variations occurred in some of the parameters with the moult stage. Seasonal and/or diurnal variations and variations with tidal movements or other physical phenomenon may also occur but these were not studied. No differences in the parameters have been observed with the sex of the lobster but sex differences in each of the parameters, if any, have still to be fully evaluated. This means that normal values with respect to moult stage, time of year, time of day and lobster sex will have to be determined if these parameters are to be used by processing factories.

- An approach to combining the results for four immune parameters, THC, %granular cells, bacteremia and clotting time, into one visual display and a single quantitative measure, was developed (Immunity Web analysis). An evaluation of the measure, Immunity Web (IW) area, by examining trends of changes in the calculated areas induced by predicted alterations in health and stress status, showed that the approach appears to be a reliable method of measuring acute stress and health status. However, further development work is required before the approach can be applied to health and stress management in lobster processing and lobster aquaculture.

In summary, technologies for assessing lobster stress and health status based on immune parameters were developed and used in studies of stress induced by post harvest handling procedures. The majority of these tests are simple to perform and could be carried out by trained factory staff. It is estimated that the suite of four immune system tests could be performed on 10 lobsters in two hours by an experienced operator. An approach to combining the results for these four parameters into a composite, quantitative measure was developed. This measure, IW area, could be used by lobster processors to improve lobster post harvest handling practices, in particular the long term holding of lobsters, to select batches of lobsters for live export, to improve live transport of lobsters to factories in trucks and in health management practices in lobster aquaculture.

2.2 *To investigate the causes of mortality in captive lobsters held in processing factories. This study will focus on bacteriological and histopathological examinations and will result in the development of a standard protocol for autopsy of lobsters.*

Autopsies were conducted on 139 lobsters, 56 healthy lobsters (accepted - lobsters judged to be healthy by factory grading staff, stored in factory tanks for 24-48hr and judged to be healthy at the completion of storage), 49 unhealthy lobsters (rejects - lobsters judged to be healthy by factory grading staff, stored in factory tanks for 24-48hr and then judged to be unhealthy) and 34 lobsters of uncertain health status (fresh arrivals - lobsters sampled immediately upon arrival at factory prior to grading).

A small proportion of lobsters (6%) exhibited parasitic infections and cardiac myopathy was seen in one lobster. The low prevalence of parasitic or other pathological lesions (apart from inflammation of likely recent onset) eliminates pre-existing disease conditions as a main cause of post harvest mortality. As discussed below (Section 3.4) the most likely cause of mortality in post harvest lobsters are opportunistic bacterial infections and organ failure.

A standard protocol for autopsy of lobsters was developed and documented (Appendix 3). A full description of the autopsy procedure was provided in the final report for project FRDC 98/304 (Evans et al., 2001).

The results of this aspect of the study have made a significant contribution to the understanding of causes of mortality in post harvest lobsters. These data and information will be of benefit to processors evaluating approaches to reduce post harvest mortality. The findings could also assist in development of improved approaches to long term holding of lobsters.

2.3 To evaluate the influence of temperature change on immunological and physiological stress responses.

Following recommendations from the Steering Committee this objective was modified to a laboratory based study of the influence of physical handling with and without air exposure on immune stress parameters.

Air exposure and/or handling was shown to cause alterations in several immune system parameters. Of particular note was the finding that handling causes an alarm reaction in lobsters as evidenced by a rapid rise in total hemocyte counts (THC). A more delayed response in clotting time (decrease) was also observed as a reaction to physical handling. Air exposure without handling had no initial effect on THC but caused an elevation in this parameter after 24hr exposure. The likely significance of these findings is discussed in the next section.

These studies will contribute to the interpretation of results obtained in field studies and, hence, to improvement of post harvest handling procedures, particularly truck transport.

2.4 *To study the influence of hormonal secretions on immunological and physiological stress responses.*

Following recommendations from the Steering Committee this objective was changed to an investigation of the influence of holding conditions on immune stress parameters achieved through a series of simulated truck transport and live shipment trials conducted in a lobster processing factory (Geraldton Fishermen's Cooperative). Some studies on hormones produced by lobsters were commenced by the other research team in 1998 but the factory trials were given precedence over hormone investigations and this work was discontinued.

2.5 *To investigate innovative techniques which will boost immunocompetence but not adversely affect marketability of live product.*

Following recommendations from the Steering Committee this objective was changed to an investigation of the influence of holding conditions on immune stress parameters achieved through a series of simulated truck transport and live shipment trials conducted in a lobster processing factory (Geraldton Fishermen's Cooperative). However, studies in this area are seen to be important and should be conducted in the future.

3. Conclusions

3.1 Test development

Eight different approaches to measuring an immune system response to an environmental stressor were examined in this study - total hemocyte counts, differential hemocyte counts, clotting time, antibacterial activity, phagocytic capacity, bacterial colony count and quantitative histopathological measurements of histological features seen in fixed sections of lobster tissues (quantification of occurrence of clots in the heart and muscle myopathy).

Histopathological features, while quantifiable, are laborious and expensive to perform and subject to significant technical variation. Because of these problems it was decided to only investigate the hemolymph assays in detail. Of the six hemolymph assays developed, differential hemocyte counts were also abandoned early in the project. While technically achievable and reproducible, differentiation of the three cell types is difficult to perform and subject to observer error due to the ill defined nature of distinguishing cytological features of semi-granular cells and hyaline cells. To overcome this problem it was decided to measure the granular cells only and use full differential counts in specific investigations where information on the variation of all three types of hemocytes is required.

Test development of antibacterial activity assays were also only partially achieved, no normal ranges being established for this parameter. These assays, while clearly providing a measure of immunocompetence, involve lengthy laboratory procedures unlikely to be performed in a processing factory or under conditions where many samples have to be tested. For this reason further development of the assay was not carried out.

Work on the phagocytic capacity test did not commence until the final year of the project and full development of this test procedure was not achieved. The test was only used in two stress studies, although considerable effort went into achieving a reproducible assay procedure. The results obtained in the experiments on the influence of handling stress on phagocytic capacity were very encouraging – a marked fall in phagocytic capacity was observed in the disturbed controls after 120 mins and in the stressed groups of lobsters. It is possible that this parameter provides a very sensitive measure of acute stress responses and should be investigated further.

Full test development was achieved for the THC, %granular cells, bacteremia and the clotting time assays.

3.2 Normal ranges for selected suite of immune system tests

THC, %granular cells, bacteremia and the clotting time assays were used in the latter experiments performed in this project and in subsequent work conducted since the project was completed. An approach to presenting data from all four tests in the one visual display and in combining the results into a single quantitative measure was also developed.

Normal ranges were established for all four tests but, with the exception of THC assays, small sample sizes were used to calculate the normal ranges. Further work is required to establish reliable normal values for well acclimated lobsters (those that have been kept in a quiet environment with minimal visual disturbance) and for lobsters stored for different time periods in environments that are not likely to be conducive to full acclimation to base line levels (lobster processing factory tanks). Since it is likely that these hemolymph components are affected by seasonal changes, tidal variations and other physical or biological factors, the effects of these factors on normal ranges should also be evaluated.

3.3 Variations in immune parameter values following stressor exposure

3.3.1 Experimental induction of stress responses

One of the major findings of this study was that two of the immune parameters, THC and clotting time, showed significant variation when a lobster was experiencing an alarm stress reaction, the first stage of acute stress. Significant alterations in these parameters were seen when lobsters were:

- removed from the tank, held and induced to flap their tails
- placed in a foam box and rocked backwards and forwards
- shaken in a net while submerged in an aquarium
- exposed to minor environmental stressors such as visual disturbance, strong light, loud noise

The changes in THC and clotting time occurred in a relatively short time after stressor exposure and were only demonstrated in well acclimated lobsters.

A particularly important observation was that the lobsters did not exhibit this alarm response in immune parameters when they were exposed to air without physical disturbance even though a classical physiological response to air exposure (elevation of blood glucose) occurred (Chapter 6, Section 4). This shows that measurement of the immune parameters THC and clotting time allows a differentiation between *behavioral stress responses* (those responses that occur when a lobster is agitated, disturbed, frightened) and *environmental stress responses* (homeostatic responses to changes in the external environment eg. decreased oxygen availability, altered water salinity, change in external temperature).

Both forms of stress reaction should involve release of hormones and consequent alterations of energy metabolism but the latter is regarded as being most often the cause of the development of a third stage stress response – reduced growth, reproduction and immunity. This accepted view is open to challenge. If an animal is constantly exposed to a behavioral stressor (eg crowding in aquaculture) it will become chronically stressed and exhibit the classical features of a third stage stress response. It is quite likely that lobsters react in the same way.

3.3.2 Environmental stressor exposure

The results of a survey conducted in 1990 on factors affecting appendage loss in *Panulirus cygnus* (Tod et al., 1990) suggested that these animals experience autotomy as a behavioral stress response to such environmental factors as large ocean swells, strong easterly winds and the presence of an octopus in the lobster pot. Autotomy is an easily demonstrated behavioral stress response to an environmental perturbation in *P. cygnus*. The present study has provided evidence of a second type of behavioral stress response involving changes in the numbers of circulating hemocytes and, presumably as a consequence, alteration in the dynamics of the clotting process.

If the conclusion that elevated THC levels are a consequence of exposure to a behavioral stressor is correct, it can be also concluded that the elevation of THC values seen during the acclimation trial and in lobsters transported in trucks could have been caused by an environmental perturbation that induces a behavioral stress response in *P. cygnus*. The close correlation between barometric pressure and THC values in well acclimated lobsters (Chapter 6, Section 2) provides some evidence that lobsters may be sensitive to barometric pressure. However, the interrelationship between barometric pressure and immune status was not studied in this project, apart from the one observation cited above, and further work is required to determine whether or not *P. cygnus* does exhibit an alarm stress response to low barometric pressure. Similarly, work in other species has shown that circulating hemocyte levels alter with the state of the tide (Truscott and White, 1990) but the influence of this variable on immunity in *P. cygnus* has not been studied. The nature of the stressors causing an elevation in THC in lobsters transported in trucks was also not elucidated in this study.

Overall, too little work has been conducted on the environmental stressors that induce a behavioural stress response in *P. cygnus* and on the physiological consequences of such responses. Research should be conducted in this area, behavioral stressors identified and their effect on lobster health and survival determined.

3.4 Variation in immune parameters in unhealthy lobsters

3.4.1 Immune parameter values in unhealthy lobsters

The results obtained in this study have shown that weak, moribund lobsters have a reduced THC, reduced proportion of granular cells and a prolonged clotting time, observations suggestive of a compromised immune system and findings of direct relevance to health assessment of post harvest lobsters. An approach has been developed to combine the results of immune parameter assays into a single measurement, the Immunity Web area and normal ranges for this parameter were estimated. Immunity Web areas have significant potential in the assessment of health status in lobsters.

3.4.2 Bacteremia

Bacteremia was present at higher prevalence in unhealthy lobsters but, as discussed in Chapter 8, Section 5.4, bacteremia was also present in apparently healthy lobsters. Alternative explanations for these findings are that 1) bacteremia is a consequence of exposure to environmental stressors; or 2) that bacteremia occurs when there is an inbalance between the numbers of bacteria in the water in which the lobsters are held and the strength of the immune processes that stop access and multiplication of bacteria in lobster hemolymph. If the lobsters are healthy and not being subjected to environmental stressors the hemolymph is likely to be sterile. If there are large numbers of bacteria in the water, the lobsters may exhibit a bacteremia regardless of their immune status. If the lobsters are experiencing an acute stress response they may develop a transitory bacteremia that will resolve once the stressor is removed.

3.5 Cause of mortality in moribund post harvest lobsters

Four possible causes of mortality in captive lobsters were hypothesized in this study:

- Cell injury and organ failure due to physiological disturbances - air exposure, rough handling and other stressors
- Opportunistic bacterial infections resulting from impaired immunity induced by above stressors
- Wounding - increased likelihood of bacterial infections
- Pre-existing disease conditions - weakens ability to resist environmental stressors

Only small numbers of infectious agents were observed in histological sections of body organs from healthy and unhealthy lobsters. The %prevalence of the parasites was very low and organisms were seen in sections from both healthy and unhealthy lobsters. This observation suggests that parasitic infections do not play a significant role in the development of weakness in post harvest lobsters.

Another possible cause of mortality, blood loss through wounding, was not investigated apart from recording the occurrence of missing appendages and exoskeleton lesions in accepted and reject lobsters at autopsy. Live export of wounded lobsters without obvious mortality occurs throughout the lobster industry. This suggests that wounding is unlikely to be a major contributing factor to lobster mortality in post harvest processing. However, whether appendage loss and the subsequent breaching of the protective exoskeleton predisposes lobsters to bacteremia should not be discounted as a contributory factor in the development of weakness in post harvest lobsters.

The contribution of bacterial infection as a cause of mortality was investigated by both bacteriological and histopathological investigations. No evidence was obtained to suggest that the post harvest lobsters were dying of an infection by a highly pathogenic bacterial species or strain, i.e. one that will cause disease in healthy lobsters. This observation confirms that bacterial infections in post harvest western rock lobsters are most likely to be due to opportunistic infections, i.e. infections by bacteria which will not cause disease in healthy lobsters. Seventeen to ninety percent of unhealthy (reject) lobsters had bacteria in their hemolymph and a high proportion of these lobsters had inflammatory reactions in their tissues, suggestive of bacterial infections. The level of bacteremia and inflammatory lesions in healthy lobsters was low. These observations suggest that bacteria could play a significant role in post harvest mortality in those lobsters which have been weakened due to prior stressor exposure. Further studies on the development of bacteremia as stress reactions in lobsters, and the relationship between bacteremia and tissue injury in post harvest lobsters should be conducted.

The cause of death in lobsters dying in the simulated truck transport and live shipment trials was shown to be an infection in the bladder and antennal glands, presumably caused by either exposure to high levels of bacteria in the holding systems or to urinary stasis resulting from the cessation of urine output.

Approximately half of the unhealthy lobsters examined by histopathology had no evidence of tissue injury so the precise cause of mortality in this group was not identified. In these lobsters the cause of mortality was probably physiological failure caused by an irreversible loss of organ function. If physiological failure had occurred in these lobsters this should be reflected in the biochemistry of the hemolymph. A full analysis of biochemical parameters as well as immunological parameters in unhealthy lobsters should resolve this question.

3.6 Recommendations for future studies

Recommendations for future studies have been made in the concluding sections within the various chapters of this report and in the above summary. The major recommendations are:

- Physical and biological factors inducing behavioural stress responses in *Panulirus cygnus* should be identified and their contribution to the development of weakness in captive lobsters determined. This study would have direct relevance to improving health of lobsters transported to factories in trucks and to the holding of lobsters in factory tanks or in aquaculture facilities.

- The application of Immunity Web analyses to health management in *P. cygnus* should be evaluated. This study would be of value if processors or aquaculturists wish to use immune parameter analysis as a health management tool in their operations.

- The cause and health significance of clotting defects in *P. cygnus* should be determined. This study would have direct relevance to procedures used to process live export lobsters and to the selection and management of broodstock used in lobster aquaculture.

- Conditions inducing bacteremia in lobsters should be studied and approaches to reducing the occurrence of bacteremia identified. While not of immediate concern, it would be of value to know whether the presence of increased levels of bacteria in factory tanks, or a particular strain of bacteria in the tanks, contributes to the development of morbidity in post harvest lobsters. An understanding of the relationship of bacterial levels in holding tanks, bacteremia in lobsters and the development of morbidity is presently lacking and is worthy of further investigation.

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APPENDIX 1

REAGENTS AND METHODOLOGY FOR IMMUNE FUNCTION TESTS

1. TOTAL HEMOCYTE COUNT (THC)

1.1 Reagent

1.1.1 *Sodium cacodylate anticoagulant*

4.28g of sodium cacodylate added to 90 ml of distilled water, pH adjusted to 7.0 using 1.0M HCL, 400 µl of stock 25% glutaraldehyde solution added and volume adjusted to 100 ml with distilled water.

1.2 Method

Clean base of 5th walking leg with 70% alcohol. Withdraw 0.2ml of hemolymph from the venus sinus at the base of the 5th walking leg into a 1ml sterile syringe containing 0.2ml of ice cold anticoagulant. Dispense into an Eppendorf tube held in ice and mix thoroughly.

Hemolymph samples can be kept on ice for up to 24hr without change in THC providing the sample is well mixed immediately prior to the cell count.

Estimate total hemocyte count using a haemocytometer under x100 magnification. Count cells in both grids and use the mean value to calculate hemocytometer count.

THC = (cells counted x dilution factor x 1000)/volume of grid (0.1mm^3).

THC can also be determined using a Coulter counter. Information on this method is given in Cht 4, Section 2.8.

1.3 Normal range for THC for acclimated *Panulirus cygnus*

4.01 – 9.01 x 10^6 cells/ml (See Cht 4, Section 3.1) (see Section 6 for explanation of normal ranges).

2. DIFFERENTIAL HEMOCYTE COUNTS

2.1 Reagents

2.1.1 *Sodium cacodylate anticoagulant*

See above, 1.1.1

2.1.2 Cytological stain

May-Grunwald and Geimsa (Bancroft & Stevens, 1977)

2.2 Method

(Note: this test is usually done in conjunction with THC and the same hemolymph sample is used for both tests).

Collect hemolymph into a syringe containing ice cold anticoagulant as described above (Section 1.2). Mix thoroughly, place one drop of hemolymph/anticoagulant mixture on a microscope slide and make a smear according to standard hematological procedures.

Air dry smear and fix in 70% methanol for 10 min.

Stain smear with May-Grunwald and Geimsa (10 min. in each) and mount with coverslip.

Count a total of 200 cells on each slide.

Three cell types are distinguished – hyaline cells, semigranular cells and granular cells. The criteria for differentiating between these three cells types is given in Cht 4, Section 3 and is also described in Jussila et al. (1997). It should be noted that the classification of cell types into three basic categories is often difficult due to the presence of cells with features common to more than one cell type. For this reason it is preferable to determine the proportion of granular cells present in the sample, this cell type being distinctive and easy to identify (see Section 3 below).

2.3 Normal range for DHC for acclimated *Panulirus cygnus*

Hyaline cells: 7.9 – 35.5%
Semi-granular cells: 55.2 – 85.6%
Granular cells: 4.5 – 11.3%
(Note – these data were obtained on small numbers of group means and will need updating as more information becomes available).

3. PERCENTAGE GRANULAR CELLS (%GC)

3.1 Reagents

3.1.1 *Sodium cacodylate anticoagulant*

See above, 1.1.1

3.1.2 Cytological stain

May-Grunwald and Geimsa (Bancroft & Stevens, 1977).

3.2 Method

See Section 2.2. Granular cells are the largest of the three cell types, have a small, pale nucleus and a large number of eosinophilic granules in the cytoplasm.

3.3 Normal range for %GC for acclimated *Panulirus cygnus*

%GC: 4.5 – 11.3%
(Note – these data were obtained on small numbers of group means and will need updating as more information becomes available).

4. BACTEREMIA

4.1 Reagents

Marine saline agar plates.

4.2 Method

(Note: this test is usually done in conjunction with clotting time and the same hemolymph sample is used for both tests).

Withdraw 0.3ml aliquot of hemolymph. Place five drops of hemolymph around the periphery of the agar plate. Care should be taken to ensure that the surface of the drop does not contact the agar during application as this could introduce inaccuracies in volume delivery. Carefully invert the plate and leave at room temperature (20 – 22°C) for up to 5 days.

Count the number of colony forming units (CFU) in each drop and calculate the total number of CFUs in the five drops. Calculate the CFU/ml for the sample based on a volume of 20µl for each drop (100µl total volume). Use the following table to calculate the colony rank. This approach provides numerical data for statistical analysis which takes into account those samples in which there are too many colonies in the individual drops and an accurate colony count cannot be obtained. If no samples in the group exhibit this phenomenon the CFU/ml results can be used to calculate mean values.

Table 1 Bacterial colony ranking scheme

CFU/ml	Colony Rank
<10	0
10 – 250	1
251 – 500	2
501 – 750	3
751 – 1000	4
1001 – 1250	5
1251 – 1500	6
1501 – 1750	7
1751 – 2000	8
2001 – 2250	9
2251 – 2500	10
2501 – 2750	11
2751 – 3000	12
>3000	13

If immune parameters in several groups of lobsters are being compared, it is also useful to calculate the %prevalence of lobsters with bacteremia for each group.

4.3 Normal range for bacteremia for acclimated *Panulirus cygnus*

Healthy, unstressed lobsters should not contain bacteria in their hemolymph. Post-harvest lobsters are not unstressed and may exhibit low levels of bacteremia which are of doubtful health significance. Based on a limited number of experiments the following criteria are used for the normal range:

Mean colony rank for group of ≥ 8 lobsters: ≤0.50
%prevalence for group of ≥ 8 lobsters: ≤50%

5. CLOTTING TIME

5.1 Reagents

Nil.

5.2 Method

Withdraw approximately 0.3ml hemolymph with a sterile syringe and place in an Eppendorf tube. Draw approx. 30µl aliquot into a plain soda lime glass capillary tube (inner diameter, 1.1-1.2mm; length, 75mm; Chase) or similar hematocrit tube (approx. 1/3rd of length of tube). Immediately after the hemolymph sample is inserted turn the tube into a vertical position with the sample at the upper end and start the stop watch. Allow the tube to remain in this position until the hemolymph column has moved to the lower end of the tube under the force of gravity and then invert the tube 180°. Care should be taken to avoid bubbles in the hemolymph column and also to avoid the

hemolymph spilling around the edge of the tube immediately prior to inversion.

Repeat inverting the tube until the hemolymph stops moving and note the time. The end point is defined as the point at which the flow of hemolymph ceases and the time taken for this to occur is defined as the clotting time. If a clot is not formed by the end of 90 secs (5 mins for *Jasus edwardsii*) the result is recorded as 'no clot'.

Use the following table to obtain the 'clot rank' value. This approach provides numerical data for statistical analysis that takes into account 'no clot' results. If no samples in the group exhibit this phenomenon the actual clotting times can be used to calculate mean values.

Table 2 Clotting time ranking scheme

Clotting time (secs)	Clot Rank
0 - 10	0
11 - 20	1
21 - 29	2
30 - 39	3
40 - 49	4
50 - 59	5
60 - 69	6
70 - 79	7
80 - 89	8
>90	9

If immune parameters in several groups of lobsters are being compared, it is also useful to calculate the %prevalence of 'no clot' reactions for each group.

5.3 Normal range for clotting time for acclimated *Panulirus cygnus*

Clotting time: 25 – 49 secs
Clot rank: ≤5
%prevalence of 'no clot' reactions in a group of 8 or more lobsters: ≤20%

6. NORMAL RANGE DERIVATION

Information on the derivation of normal ranges for *Panulirus cygnus* for these various immune parameters is provided in the main body of this report, in particular in Chapter 5. It should be noted that the normal ranges were obtained by determining the immune parameter in groups of 8 or more animals, obtaining the mean value for the group and using a series of these group mean values to determine the overall mean and standard deviation. The normal range was then defined as the average of the group means ± 2 SDs.

It is not unusual for the value for an immune parameter of a normal, healthy lobster to be outside the normal range for that parameter. Our approach to assessing health and/or stress status in individual lobsters has been to look at four parameters (THC, %granular cells, clotting time/rank and bacteremia rank) in the lobster. If two or more of these parameters are outside the normal range we have recorded the lobster as having poor health status or abnormal stress status, depending on the extent and nature of the deviation of the individual parameters. The preferred approach is to examine a suite of immune parameters in a group of 8 or more lobsters, calculate the mean value and compare this result to the normal range.

APPENDIX 2

DETERMINATION OF VIGOUR INDEX (Spanoghe, 1996)

Somatic response	Classes					
	0(d)	1(m)	2(w)	3(h)	4(vh)	5(a)
Defensive born response	-	-	-	-	-	+
Vigourous tail flip	-	-	-	-	+	+
Appendages movements	-	-	-	+	+	+
Firm tail	-	-	+	+	+	+
Eyestalk response	-	+	na	na	na	na

Legend:
a = defensive
vh = very healthy
h = healthy
w = weak
m = moribund
d = dead
na = not applicable

Spanoghe, P. 1996. An investigation of the physical and biochemical responses elicited by *Panulirus cygnus* to harvesting, holding and live transport. Doctoral Thesis. School of Biomedical Sciences, Curtin University, Perth, Western Australia. 378pp.

APPENDIX 3

LOBSTER AUTOPSY PROCEDURE

1. Lobster collection

It is preferable that lobsters on which an autopsy is to be conducted are held in an aquarium or a holding tank with good aeration and water quality conditions prior to the autopsy. Delivery to the laboratory in an esky or foam container with an ice bottle included to reduce temperature is an alternative collection procedure but it should be noted that prolonged air exposure will alter blood chemistry and vigour index results. Lobsters should be cooled down prior to dissection by placing in the freezer section of a refrigerator for at least 5 minutes.

2. Vigour index

Prior to collection of the hemolymph sample and dissection a vigour index should be performed. The method for this test is described in Appendix 2.

3. Gross observations and morphometric measurements

The following measurements and observations should be recorded on the results sheet: Sex, orbital carapace length, wet weight, moult stage. If the lobster is from an aquaculture facility, condition factors may also be of diagnostic significance. These are obtained by weighing the whole hepatopancreas and tail muscle, removing a 1 - 5g sample, recording the wet weight of the sample, drying the sample and calculating conditions factors as described in Appendix 2.

4. Hemolymph collection

Hemolymph should be collected from the base of the fifth walking leg. Three samples are required, one for hemocyte counts, one for hemolymph protein and bacteriology and the other for biochemistry tests. The surface of the membrane between the dorsal area of the carapace and the tail should be swabbed with 70% ethanol prior to hemolymph collection. Hemolymph is collected with a sterile syringe with a 24 gauge needle, both of which should be stored on ice or kept in the refrigerator prior to use. The various samples are processed for hemocytology, bacteriology and biochemistry as described in Appendix 1.

5. Dissection Procedure

The following dissection procedure is recommended:

Remove the lobster from the aquarium and place it on a dissection board. Heavy duty rubber gloves should be worn to avoid cuts or wounds. If skin damage is accidentally sustained it is recommended that the area be immediately washed and swabbed with a suitable disinfectant (e.g. betadene).

Using a sharp knife remove the proximal area of the carapace just behind the antennae and then dissect away all walking legs. Cut along the junction of the carapace and the abdomen to remove the tail. The specimen is now ready for removal of body tissues.

Cut through the thoracic region just internal to the two branchiostegal lines (i.e. two longitudinal cuts from the distal edge of the carapace to the proximal cut surface, about 1cm on either side of the centre of the carapace). Carefully cut away the exoskeleton from underlying tissues to reveal the heart, hepatopancreas and gonads. If the lobster is close to moult there may be a thin sheet of unhardened chitin underlying the exoskeleton – this should be stripped off with forceps. Remove the left brachiostegite to reveal the gill chambers by cutting along the line. Follow the same procedure to remove the right brachiostegite.

6. Tissue sample collection

The removal of tissues should be performed as rapidly as possible as to avoid autolysis. The most sensitive tissues with respect to autolysis are the hepatopancreas and the antennal glands. These should be the first tissues to be removed. Tissue samples should be removed from the following organs and placed in the fixative: hepatopancreas (two samples, one from the proximal lobe on one side of the body and the other from the distal lobe on the other side of the body), antennal gland (two samples, comprising one half of each gland), gills (one podobranch from each branchial cavity), heart (two samples, obtained by cutting the heart in half and then taking one half of each section), midgut (one sample taken close to the junction of the midgut and the hindgut), hindgut (one sample taken approximately half way down the length of the organ), ventral nerve (one sample taken from the proximal region of the tail) and abdominal muscle (two samples taken from the abdominal muscle immediately adjacent to the carapace). Each tissue piece should be approximately 2-3 cm^2 and not more than 5 cm^2. The volume of fixative to tissue should be at least 20:1.

7. Fixation and processing

Various fixatives are suitable for histopathological examination of lobster tissues. Buffered seawater formalin is not an ideal fixative but provides good visualization of granular cells and should therefore be used. It is suggested that tissue sections are mounted in two or more paraffin blocks.

APPENDIX 4

PUBLICATIONS ARISING FROM STUDIES PERFORMED IN THIS PROJECT

Conference Abstracts

Jussila, J., Jago, J., Tsvetnenko, E., Fotedar, S. and Evans, L.H. (1999). Total hemocyte counts in western rock lobster (*Panulirus cygnus*) under post-harvest handling conditions – indicator of stress? (abstract). Proceedings Annual International Conference and Exposition of the World Aquaculture Society, 26 April – 2 May, Sydney, p. 380.

Evans, L.H., Fotedar, S. Jussila, J. McBride, S. and Tsvetnenko, E. (1999). Immunological measures of stress in spiny lobsters (*Panulirus cygnus* George) (abstract). Proceedings International Symposium on Lobster Health Management, 19-21 September, Adelaide, pp. 21-22.

Tsvetnenko, E., Brown, J. and Evans, L.H. (1999). Measures of condition in dietary studies on western rock lobster post-pueruli. (abstract). Proceedings International Symposium on Lobster Health Management, 19-21 September, Adelaide, p, 13.

Fotedar, S., Barnes, A., Geddes, M., Reuter, R. and Evans, L. (1999). Investigations of bacteremia in spiny rock lobsters. (abstract). Proceedings International Symposium on Lobster Health Management, 19-21 September, Adelaide, p. 29.

Evans, L. (1999). Lobster Health Management. (Forward) Proceedings International Symposium on Lobster Health Management, 19-21 September, Adelaide, pp. 3-4.

Evans, L., Chan, V., Fotedar, S., Jussila, J. & Tsvetnenko, E. (2000c) Influence of environmental stressors on immunocompetence in the spiny lobster *Panulirus cygnus*: Implications for lobster aquaculture. (abstract) In: *AQUA 2000. Responsible Aquaculture in the New Millennium* (Ed. by R. Flos & L. Creswell), p. 206. European Aquaculture Society Special Publication, 28, Oostende, Belgium.

Refereed conference papers and journal articles

Jussila, J., Jago, J., Tsvetnenko, E., Dunstan, R. & Evans, L. H. (1997) Total and differential haemocyte counts in western rock lobster (*Panulirus cygnus* George) under post-harvest stress. *Marine and Freshwater Research*, **48**, 863-7.

Jussila, J., Jago, J., Tsvetnenko, E. & Evans, L.H. (2001). Effects of handling or injury disturbance on total hemocyte counts in western rock lobster (*Panulirus cygnus* George). In: *Proceedings, International Symposium on Lobster Health Management*, Adelaide, 1999. (Ed. by L. H. Evans, J. B. Jones). Muresk Institute of Agriculture, Curtin University Publication. pp 52-63.

Fotedar, S., Tsvetnenko, E. & Evans, L. (2001). Effect of air exposure on immune parameters of the rock lobster *Panulirus cygnus*. Marine and Freshwater Research 52:1351-1355.

Jussila, J., McBride, S., Jago, J. & Evans, L.H. (2001). Hemolymph clotting time as an indicator of stress in western rock lobster (*Panulirus cygnus* George). Aquaculture 199: 185-193.

Tsvetnenko, E., Fotedar, S. & Evans, L.H. (2001) Antibacterial activity in the hemolymph of western rock lobster, *Panulirus cygnus*. Marine and Freshwater Research 52:1407- 1412.

APPENDIX 5

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