Rock Lobster Autopsy Manual

Professor Louis H. Evans

Project No. 1999/202

FRDC 1999/202 Rock Lobster Autopsy Manual

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OBJECTIVE:
The objective of this project was to publish an autopsy manual for use by the rock lobster industry. The manual was to include details of the approach to be used in conducting an autopsy of rock lobster, descriptions of assay methodology, normal ranges for selected immune and physiological functions and photographs of gross and microscopic lesions and descriptions of pathogens observed in lobsters.

The publication was to be aimed primarily for use by fishers, processing factory staff and seafood business managers but was to also include information of relevance to veterinarians, fish health personnel and researchers.

NON TECHNICAL SUMMARY:

OUTCOMES ACHIEVED:
The rock lobster autopsy manual provides readily available information on how to perform a lobster autopsy, factors that predispose to disease, the methods used to quantify lobster health and the diseases reported in spiny and clawed lobsters. The style of writing of the various chapters ensures that the level of understanding required by both laymen and scientists is addressed. A publication titled " Rock Lobster Health and Diseases: A Guide for the Lobster Industry" has also been produced.

Data and information on lobster host defense responses, disease conditions, dissection procedures and laboratory investigation methods were obtained from the FRDC projects 94/134. 07, 96/344, 96/345 and 98/302 and other sources and collated into a publication on the nature and investigation of lobster diseases. Contributions to the book were sought from lobster researchers and fish health personnel from Australia and New Zealand. Following collation and editorial review, the individual chapters were circulated for review and comment. The publication, titled 'A review of lobster diseases, their investigation and pre-disposing factors' was published in 2003 and contains the following chapters:

1. Lobster health and disease concepts (Louis Evans)
2. Responses to environmental stressors (Louis Evans)
3. Lobster autopsy procedure and immune assay methods (Louis Evans, Seema Fotedar and Anne Barnes)

4. Collection and handling of blood samples from spiny lobsters (Brian Paterson, Patrick Spanoghe and Glen Davidson)
5. Reagents and methodology for immune function tests (Louis Evans & Seema Fotedar)
6. Review of adult lobster diseases (Frances Stephens, Louis Evans, Seema Fotedar and Brian Jones)
7. Disease conditions of larval and juvenile spiny lobsters (Ben Diggles and Judith Handlinger)
8. Biosecurity and food safety issues (Frances Stephens)

In chapter one, disease is described as an abnormal condition resulting from a pathophysiological response to harmful environmental stimuli. Diseases characteristically involve abnormal tissue and organ function arising from cell injury and death (necrosis) or dysfunction. Disease conditions may arise from infectious or non-infectious conditions. Infectious diseases of lobsters include those caused by viruses, bacteria, fungi, parasites or fouling organisms. Non-infectious diseases may result from nutritional deficiencies or from a range of physical or chemical factors. The occurrence and outcome of infectious disease outbreaks are influenced by such factors as the virulence of the infectious agent and the number of pathogens. Some pathogens will cause disease in healthy lobsters while others will only affect lobsters that are in a weakened health status. Predisposing factors other than the health status of lobsters that also influence the severity of a disease outbreak include nutritional status, the presence of an existing disease state, the presence of exoskeleton damage and previous exposure to environmental stressors such as physical handling, exposure to air or poor water quality.

Disease conditions found in Australian spiny lobsters are described. They are similar to those occurring in clawed lobsters from the northern hemisphere and include shell disease, vibriosis, other bacterial diseases and fungal infections. Parasites are also widely observed and include nemertean worms, microsporidiosis, free living nematode worms, ciliates and dinoflagellates which may result in external fouling of exoskeleton and/or clinical disease. Non-infectious conditions reported in Australian post harvest lobsters include the turgid lobster syndrome.

Two of the major disease conditions affecting commercial lobster industries in the northern hemisphere, gaffkemia and bumper car disease, have not been reported from Australian spiny lobsters.

Keywords: lobster, health, disease, autopsy, hemolymph, physiology, stress, *Jasus verreauxi*, *Jasus edwardsii*, *Panulirus cygnus*, *Panulirus ornatus*.

Acknowledgements
The production of this autopsy manual would not have been possible without the help and support of the staff of the Aquatic Science Research Unit, Curtin University who assisted with the final collation, editing and publication of the manual. Professor Bruce Phillips, leader of the Rock Lobster Post-Harvest SubProgram, gave valuable advice and editorial comment. Dr Brian Jones and Mr Richard Stevens provided valuable comments. Dr Patrick Spanoghe, Dr Judith Handlinger, Dr Alistair Brown and Dr Ian Anderson provided advice on the overall content of the manuals. Facilities and on-going support were provided by Lobster Australia Pty Ltd and by other rock lobster processing companies in Australia and New Zealand. The production and publication of the manual was made possible through funding provided by the Fisheries Research and Development Corporation, Canberra.

Background
Effective health management of rock lobsters held in captivity is of critical importance to the success of a rock lobster live processing operation or aquaculture operation. One of the principal requirements of good health management is the availability of data and information on the nature and prevention of disease conditions that might occur in lobsters, and of appropriate procedures to investigate lobster disease outbreaks. As interest in the aquaculture of spiny lobster increases, and water quality management systems used in facilities holding lobster become more sophisticated, the level of expertise needed to maintain the health of the lobsters is also expected to increase.

Need
When this project commenced, published information on disease conditions found in spiny lobsters from Australia and New Zealand, and of approaches to investigating lobster diseases was lacking. There was no published account of the recommended approach to conducting an autopsy on a lobster nor was information readily available on laboratory procedures that could assist in studying lobster health and disease. The need for collation of such information from published works, and from research reports from previous FRDC lobster research projects, was identified through industry consultation. This project aimed to address this industry need.

Objectives
The project aimed to publish a lobster autopsy manual comprising contributions from a team of scientists with expertise in lobster health and disease investigations. Information and data for the publication was to be drawn from previous FRDC lobster research projects (94/134. 07, 96/344, 96/345 and 98/302) and from other reports and publications. Particular emphasis was to be placed in the inclusion of photographs of autopsy procedures and of lobster disease conditions.

Methods
A team of researchers from seven organizations in Australia and New Zealand was formed and individual team members collaborated in writing the eight chapters of the autopsy manual. Photographs from various collections were sourced for inclusion in the publication. All chapters were peer reviewed prior to publication.

Results
Two rock lobster autopsy manuals have been produced. In the manual, the approach to dissecting a lobster and carrying out investigations that could assist in determining the cause of the disease conditions are described. Details are provided of the analytical procedures for measuring total hemocyte counts, differential hemocyte counts, clotting time, bacterial colony counts and for carrying out histopathological investigations. Normal ranges for the hemolymph parameters, obtained during the FRDC 96/344 study, are included as are photographs of common disease conditions. Diseases of juvenile spiny lobsters and adult spiny and clawed lobsters are reviewed and some issues relating to biosecurity and food safety are discussed.

Benefits
The manuals produced during this project allows people that handle rock lobsters to understand the factors that contribute to health and disease in lobsters and provides some background into the methods that can be used to further assess the health of lobsters. This includes photographs of how to perform an autopsy and the appearance of rock lobsters that are affected by various disease conditions.

Further development and planned outcomes
An industry guide is being prepared for launching in September 2003. It summarises the key points in the rock lobster autopsy manual in laymen's terminology for the use of rock lobster fishers and processing companies in Australia.

Conclusion
The manuals produced during this project provide a readily accessible resource that makes it possible for people in the rock lobster industry, as well as scientists, to dissect a lobster and to draw some conclusions as to the causes of health problems in rock lobsters. They provide a range of options for further investigation and testing, as well as a range of expected values for some key physiological and immunological parameters of normal lobsters.

Appendix 1.
There is no identifiable intellectual property arising from this project.

Appendix 2.
The following people have been involved in this project: Louis Evans, Seema Fotedar, Anne Barnes, Brian Paterson, Patrick Spanoghe, Brian Jones, Frances Stephens, Judith Handlinger, Ben Diggles, Glen Davidson, Bruce Phillips, Richard Stevens.

A REVIEW OF LOBSTER DISEASES, THEIR INVESTIGATION AND PRE-DISPOSING FACTORS

Fisheries Research and Development Corporation Report
FRDC Project No. 1999/202

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2003
ISBN: 1 74067 276 3

Preface

Effective health management of rock lobsters held in captivity is of critical importance to the success of a rock lobster live processing operation or aquaculture operation. One of the principal requirements of good health management is the availability of data and information on the nature and prevention of disease conditions that might occur in lobsters, and of appropriate procedures to investigate lobster disease outbreaks

Several research projects supported by the Fisheries Research and Development Corporation (FRDC) over the period 1994 – 2000 generated data and information on health assessment and health problems in Australian rock lobsters. The first of these, 'Rock Lobster Autopsy Study' FRDC 94/134. 07, aimed to investigate the causes of mortality in captive 'white' lobsters held in processing factories during the 1995/1996 Western Australian rock lobster season. The project was also aimed at developing procedures for autopsy of lobsters. The second project, 'Physiological Studies of Stress and Morbidity during Post harvest Handling of Western Rock Lobster *Panulirus cygnus*' FRDC 96/344 and 96/345, aimed to develop methodology for measuring physiological and immune system stress responses in lobsters, evaluate health and stress status using physiological and immune parameters and document a standard protocol for autopsy of lobsters. The outcomes of this project included the development of standard test procedures for a range of immune parameters, information on normal levels for physiological and immune parameters in baseline lobsters and in healthy, post harvest, lobsters and on likely deviations in these parameters in lobsters with reduced health status.

These three projects culminated in study of lobster disease conditions by a group of researchers from Western Australia, South Australia, Tasmania and Queensland using the autopsy procedures and laboratory assays developed in the previous studies (FRDC 98/302). An international symposium on lobster health management was convened and the proceedings published (www.curtin.edu.au/curtin/muresk/lhm). Following the completion of these various studies it was decided that the information regarding Australian rock lobster diseases and their investigation reported in these four different research reports should be collated into a single publication on rock lobster diseases and their investigation. A final project was funded by FRDC to conducted to produce the lobster autopsy manual.

The objective was to publish an autopsy manual on the nature and investigation of rock lobster diseases for use by the rock lobster industry. The manual was to include a review of the basic concepts of lobster health management, host defense reactions and responses to environmental stressors, descriptions of procedures to be followed in conducting a lobster autopsy and collecting hemolymph for analysis, details of physiological and immune system assay methodologies, a review of disease conditions in larval, juvenile and adult lobsters with photographs of gross and microscopic lesions, and a section on biosecurity and food safety issues.

The publication was to be aimed primarily for use by fishers, processing factory staff and seafood business managers but was to also include information of relevance to veterinarians, fish health personnel and researchers. Chapter 2 was primarily written for fishers and processing staff, while Chapters 6 and 7 are likely to be of more

interest to managers and fish health professionals. The remaining chapters contain information of relevance to both industry and fish health personnel.

Acknowledgements

The production of this autopsy manual would not have been possible without the help and support of the staff of the Aquatic Science Research Unit, Curtin University of Technology, who assisted with the final collation, editing and publication of the manual. Professor Bruce Phillips, leader of the Rock Lobster Post-Harvest Subprogram, gave valuable advice and editorial comment. Facilities and on-going support were provided by Lobster Australia Pty Ltd and by other rock lobster processing companies in Australia and New Zealand. The production and publication of the manual was made possible through funding provided by the Fisheries Research and Development Corporation, Canberra.

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CHAPTER 1

LOBSTER HEALTH AND DISEASE CONCEPTS

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1.1 Introduction

The term 'health' describes a physiological state of an animal. A 'healthy' animal is one in which the physiological processes underpinning growth, maintenance, disease defence and reproduction – such processes as respiration, osmoregulation, acid-base balance, digestion, metabolism, reaction to internal and external stimuli and locomotion – are functioning normally. In this context the term 'normal' refers to a state which is appropriate and adequate for the animal at that point in its development. Simply put, the animal is in a state of 'ease'.

A 'diseased' animal, on the other hand, is in a state of 'dis-ease'. It is experiencing some form of injury or threat to its survival, which results in damage to body tissues and/or abnormal physiological function. The injury or threat may be in the form of a sudden environmental change; for example, exposing a fish to air or to a chemical toxin, or the invasion of body tissues by an infectious organism. These different causes of injury are collectively referred to as disease agents. Exposure to disease agents stimulates a suite of physiological processes – the host defence and immune processes – to counteract the threat and to repair the damaged tissue.

Diseases are caused by many different biological, physical or chemical agents. Diseases of aquatic organisms are mostly due to exposure to pathogenic organisms such as viruses or bacteria, or to inadequate nutrition. Disease conditions can be mild, having few if any adverse effects on the animal, through to severe where the condition threatens survival. Whether or not a lobster will succumb to a disease depends on factors relating to the host, the environment and the invading organism. Decapod crustaceans exhibit a wide range of host defence reactions aimed at preventing tissue injury or infections (Smith & Chisolm 1992; Bachere *et al*. 1997; Evans *et al.* 2000a). The success of these defence reactions in preventing or overcoming the disease is strongly influenced by the existing health status of the host at the time of exposure. Health status is in turn affected by prior exposure to environmental stressors. Excessive stress responses to environmental stressors weaken lobsters and pre-dispose them to disease.

A healthy animal can withstand the challenge of a disease agent better than one in poor health, unless the agent has the capacity to cause disease regardless of health status. Some viruses and highly virulent bacteria, and some forms of nutrient deficiencies or chemical exposures, fall in the latter category.

In order to avoid disease outbreaks it is essential that stock health is optimised within the economic constraints of the production or holding system. However, while optimal stock health should be the aim of any processor or aquaculturist, this is difficult to achieve in practice. Under most rearing and holding conditions lobsters are exposed to various forms of environmental stressors, (for example, crowding and confinement, adverse water quality, handling and grading procedures) which have the potential to affect health. In well designed and maintained holding systems the level of stress experienced by the lobsters does not significantly compromise performance or product quality. The successful manager is able to balance the trade-off between operational costs and stock health in such a way as to maximize financial returns from the operation. In poorly designed or maintained systems, on the other hand, stock health is compromised resulting in reduced production, poor product quality and reduced profits.

The impact of disease on lobster health is of minor importance in lobster post-harvest handling as it is currently practiced in Australia but is of significance in overseas lobster industries where harvested stock are held for long periods in lobster pounds. The introduction of long-term storage of harvested lobsters in Australian fisheries will inevitably lead to disease problems as will the culture of lobsters in hatcheries and grow-out facilities. Disease prevention through effective health management should therefore be the goal of any commercial operation based on lobster wild-stock harvest or aquaculture.

1.2 Stress and disease

Lobster health is influenced by a range of factors, one of the most important of which is stress. Stress responses are normal physiological reactions to changes in environmental conditions. These conditions include a wide range of factors such as water quality parameters (oxygen levels, pH, salinity, temperature, presence of toxins), physical factors (handling, injury, air exposure), behavioral interactions and nutrient availability. Exposure to these stressors leads to short and long-term changes in cardiovascular and respiratory function, energy metabolism, fluid and ionic balance, acid-base balance and immunity (Selye 1973; Barton & Iwama 1991; Thompson *et al.*, 1993; McDonald & Milligan 1997; Iwama *et al.* 1997; Hall & van Ham 1998). The physiological disturbances is temporary if the stressor is mild and of short duration. However, if the stressor is extreme, or if there is prolonged exposure even to a mild stressor, detrimental long-term effects can occur. These include reduced resistance to disease, reduced growth, impaired reproduction and reduced survival (Pickering & Pottinger 1989; Lee & Wickins 1992; Iwama *et al.* 1997). Post-harvest handling of lobsters is likely to cause exposure to both mild and extreme stressors. In the latter case the physiology of the lobster may be so disturbed as to result in mortality.

Stress responses in aquatic organisms, in particular fish, are traditionally divided into three stages – primary (stage 1), secondary (stage 2) and tertiary (stage 3); the reactions in the previous stage inducing those in the subsequent stage (Mazeaud *et al.* 1977; Barton & Iwama 1991; Barton 1997). The primary stage comprises the nervous reactions to detection of the stressor along with the release of stress hormones, in the case of fish, adrenalin and cortisol. The secondary stage consists of the metabolic and ion regulatory

responses to these nerve and hormonal stimuli, while the tertiary stage comprises alterations in growth, disease resistance and reproduction which occur as a result of the changes in metabolism and ion-regulation processes. Both acute and chronic stress responses occur, the former comprising stages 1 and part or all of stage 2, and the latter mainly referring to the consequences of continued exposure to a stressor and characterized by the responses occurring in stage 3.

There has been a long and protracted debate on the definition of stress and the classification of the different stages of the response (see review, Moberg 1985). In his classic description of the sequence of events in stress responses in higher vertebrates, termed the General Adaptation Syndrome (GAS), Selye identified three different phases of the response – an alarm phase characterized by 'fight or flight'; a resistance phase in which the subject adjusts to the continual presence of the stressor and enters a new, sub-optimal homeostatic state; and the exhaustion phase in which the subject can no longer resist the stressor and from which pathological changes are likely to follow. Fish stress physiologists, while recognising the occurrence of alarm responses in fish (Shreck 1981; Shreck *et al.* 1997), had difficulty in applying Selye's classification to their observations of hormonal and biochemical changes occurring during stress responses in fish and preferred to divide the response into primary, secondary and tertiary stages as described above.

In addition to the recognition of the three stages of the stress response, the occurrence of 'simple' and 'compound' stress responses has been highlighted (McDonald & Milligan 1997). Simple responses are defined as those physiological disturbances directly provoked by the initial nervous response and stress hormone secretion. They normally result in little direct harm to the aquatic organism. Compound responses to stressors result from additional physiological disturbances evoked by the compounding effects of metabolic responses and stress hormone action as may occur in prolonged vigorous exercise (e.g. prolonged tail flipping by a lobster) or air exposure. These stressors induce anaerobic conditions and lead to extracellular acid-base disturbances through the release of lactic acid from muscle tissue. Acid-base disturbances are a major feature of compound stress responses in fish (see review, McDonald & Milligan 1997) and are thought to lead to intracellular acidosis, elevated ammonia levels and, if not reversed, the death of the animal (Wood *et al.* 1983; Riley *et al.* 1997).

Immune responses to environmental stressors have not been as widely studied as physiological changes, particularly in decapod crustaceans. In decapods these responses include changes in levels of circulating hemocytes, coagulation, phagocytic capacity or antibacterial activity (Evans *et al.* 2000b). Early reports of immune responses to an environmental stressor in crustacean species include those of Hamann (1975), who observed an increase in circulating hemocytes in freshwater crayfish following a handling stress, and Stewart *et al.* (1967), who noted that the total hemocyte counts in *Homarus americanus* declined when the lobsters were starved. More recently Jussila *et al.* (1997, 1999a, 1999b, 2000a, 2000b) and Evans *et al.* (1999a, 1999b, 2000b, 2000c) have reported alterations in a range of immune parameters in response to handling, air exposure, wounding and other environmental stressors in both lobsters and freshwater crayfish. The results of these studies clearly demonstrate a rapid increase in circulating

hemocyte counts following exposure to physical handling and other types of stressors, similar to the alarm response originally described in humans by Selye (1973).

Alarm responses have been reported in a number of different crustacean species. For example, rapid, short-lived changes in scaphognathite activity and heart rate of crustaceans to a variety of tactile, chemical and visual stimuli have been described (Larimer 1964; Ashby & Larimer 1965; Wilkens & McMahon 1972; Wilkens & Young 1975; Wilkens *et al.* 1985) and likened to a startle reaction (Wilkens 1976). Cardiac and ventilatory response of longer duration (hours to more than a day), as occur following the trauma of handling and surgery, have also been described (McDonald *et al.* 1977, 1980; McMahon & Wilkens 1977). The consistent demonstration of alarm responses in lobsters, as evidenced by increases in circulating hemocytes following exposure to handling (Jussila *et al.* 1999b, 2000a; Evans *et al.* 2000b), and of an exhaustion phase, characterized by a significant decrease in hemocyte numbers in moribund lobsters (Jussila *et al.* 1997; Evans *et al.* 2000b), lends weight to the application of Selye's terminology to the description of stress responses in these species. A suggested approach to describing the various phases of the stress response in lobsters is shown in Figure 1.

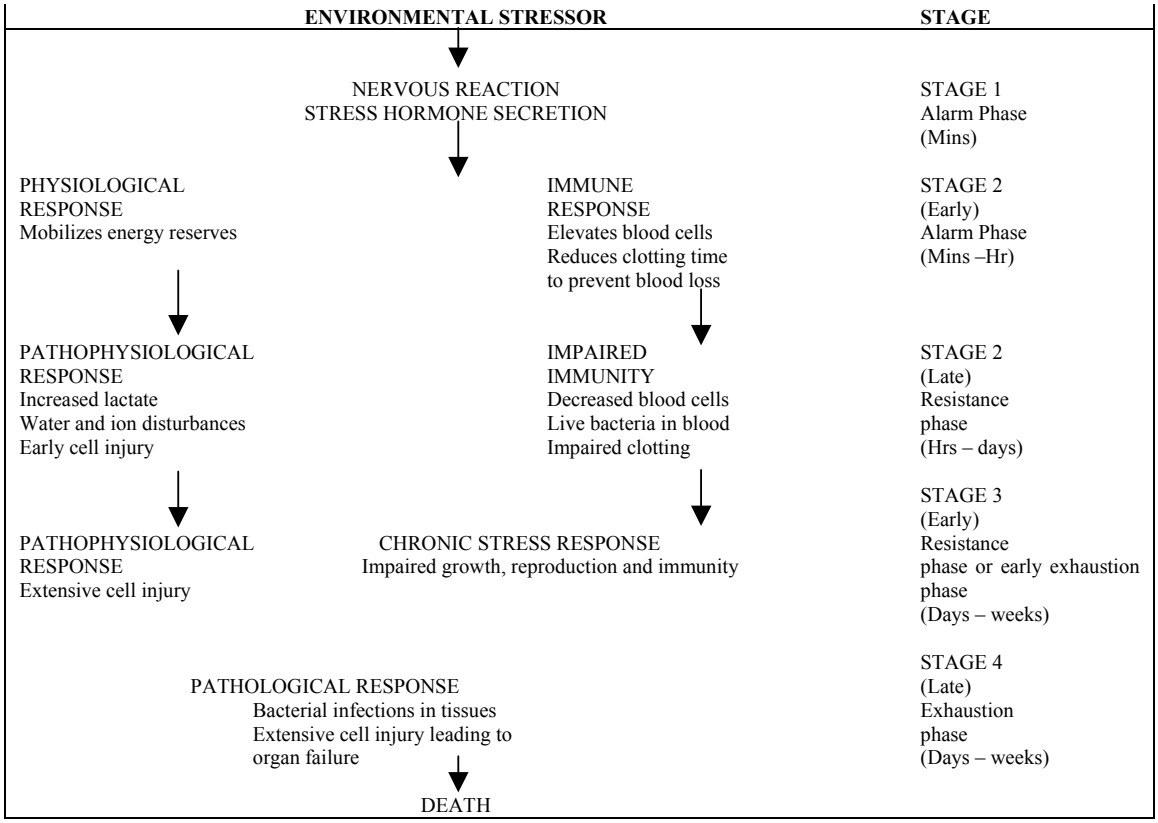


Figure 1. Classification of stages of stress response in lobsters

1.3 Physiological stress responses and likely causes of 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).

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 autonomy 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

The influence of such stressors on physiological processes, behavioural responses, immune parameters and survival in lobsters has been studied by a number of different authors (Table 2). Alterations in circulating oxygen content, ammonia levels and acid-base disturbances have been found to affect survival. The ability of lobsters to survive exposure to air during commercial shipment was influenced by a number of different factors including journey time, transportation temperature, disturbance levels, moult stage and the 'ponding' of post-harvest lobsters prior to transport to market. These factors affect vital physiological processes including immunity.

Assuming that lobsters 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, nitrogen metabolism or ion regulation and water balance.

Table 2. Investigations of environmental stressors on physiological processes, behavioural responses, immune parameters and survival in spiny and clawed lobsters

Lobster Species	**Stressor(s)**	**Physiological process(es)**	**Reference**
Homarus americanus	Variations in temperature, salinity and oxygen levels	Survival	McCleese (1956)
	Aerial exposure	Survival	McCleese (1964)
	Storage and live shipment	Survival	McLeese & Wilder (1965)
	Physical handling	Blood sugar composition	Telford (1968)
	Food deprivation	Osmoregulation	Stewart *et al.* (1967)
	Salinity variations	Blood constituents and circulating hemocyte numbers	Dall (1970)
	Hypoxia	Respiration and circulation	McMahon & Wilkens (1975)
	Physical handing	Oxygen consumption	Winkler (1987)
	Ammonia exposure	Osmoregulation and survival	Young-Lai *et al.* (1991)
	Hypoxia	Cardiovascular function	Reiber *et al.* (1992)
	Progressive hypoxia	Respiration and cardiovascular function	Reiber & McMahon (1998)
Homarus gammarus	Aerial exposure and re-submersion	Oxygen transport and acid-base balance	Taylor & Whiteley (1989)
	Aerial exposure at different temperatures	Acid-base balance	Whiteley & Taylor (1990)
	Disturbance and aerial exposure at different temperatures	Oxygen consumption	Whiteley *et al.* (1990)
	Commercial transport	Behavioural responses	van der Meeren (1991)
	Commercial transport	Oxygen transport and acid-base balance	Whiteley & Taylor (1992)
Homarus vulgaris	Exposure to hypoxia	Respiration and circulation	Butler *et al.* (1978)
	Physical disturbance and exposure to hypoxia	Acid-base balance	McMahon *et al.* (1978)
Jasus edwardsii	Aerial exposure	Respiration, osmoregulation, ion regulation, acid-base balance	Taylor & Waldron (1997)
Nephrops norvegicus	Hypoxia	Respiration and circulation	Hagerman & Uglow (1985)
	Aerial exposure	Oxygen transport, energy metabolism	Spicer *et al.* (1990)

Table 3. Investigations of environmental stressors on physiological processes, behavioural responses, immune parameters and survival in spiny and clawed lobsters (cont)

Nephrops norvegicus (cont)	Hypoxia	Energy metabolism, hemocyanin metabolism, circulating ammonia and ammonia excretion	Hagerman *et al.* (1990)
	Aerial exposure	Hemolymph constituent levels and ammonia efflux rates	Schmitt & Uglow (1997a)
	Ammonia exposure	Blood ammonia, ammonia excretion and heart and scaphognathite rates	Schmitt & Uglow (1997b)
		Blood glucose levels	
	Exposure to LPS (lipopolysaccharide)		Lorenzo *et al.* (1997)
Panulirus argus	Aerial exposure	Desiccation rate, hemolymph chemistry and escape behaviour	Vermeer (1987)
Panulirus cygnus	Salinity variations	0smoregulation	Dall (1974a, 1974b, 1975)
	Commercial post-harvest handling - physical handling, aerial exposure, temperature variation	Behavioural responses, energy metabolism, acid-base balance and other physiological parameters	Spanoghe (1996)
	Aerial exposure, temperature variation	Energy metabolism, acid-base balance, hemolymph constituents, behavioural responses	Tod & Spanoghe (1997)
	Commercial post-harvest handling, aerial exposure & temperature variations	Morbidity and mortality	Spanoghe & Bourne (1997)
	Commercial post-harvest handling, aerial exposure, wounding, physical handling	Immune parameters, hemolymph constituents	Jussila *et al.* (1997, 1999b, 2000a, 2000b)
	Commercial post-harvest handling, aerial exposure, wounding, physical handling and other post-harvest stressors	Immune parameters, hemolymph constituents	Evans *et al.* (2000b, 2000c)
Panulirus japonicus	Hypoxia	Oxygen uptake	Nimura & Inoue (1969)
	Temperature variation	Heart rate	Nakamura *et al.* (1994)
	Commercial post-harvest handling – temperature, starvation, salinity and transport	Serum bactericidal activity	Sugita & Deguchi (1994)
	Salinity variation	Intracellular nitrogenous osmolytes	Shinagawa (1995)

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

- 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 (Figure 2). The actual cause of death is likely to be failure of vital organ function through cell injury caused by irreversible physiological dysfunction, cell exposure to elevated ammonia, intracellular acidosis or opportunistic bacterial infections arising from either physical injury or from an impairment in the lobster's host defence responses (La Via & Hill 1975; Wood *et al.* 1983; Riley *et al*. 1996; McDonald & Milligan 1997). Stress responses induced by exposure to environmental stressors during capture and handling can lead to death through any of these pathways and are undoubtedly an important factor in the development of weakness or poor health in post-harvest lobsters.

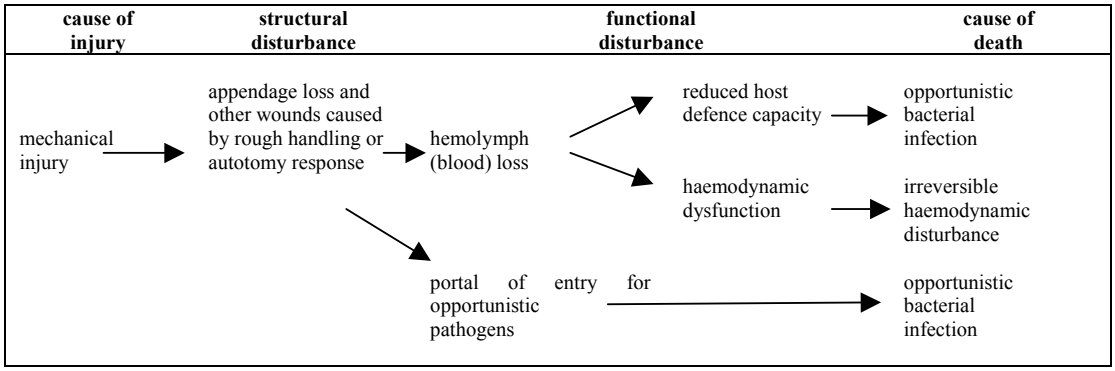
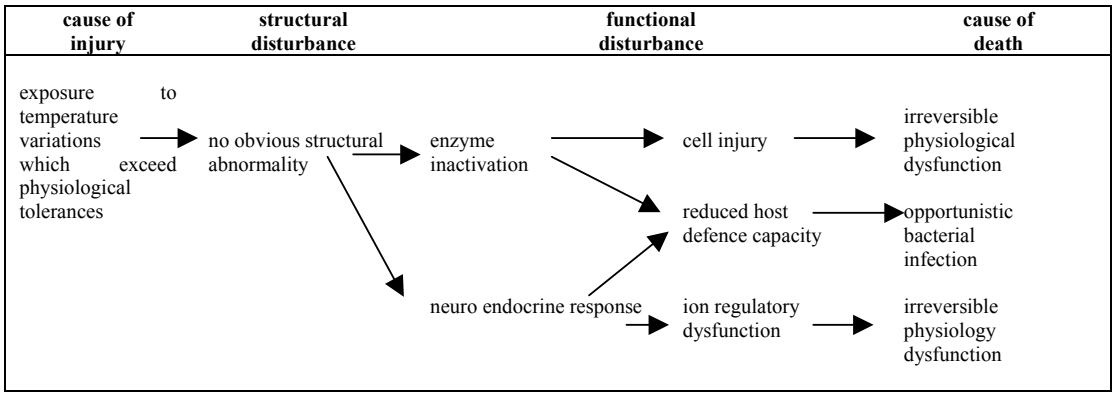
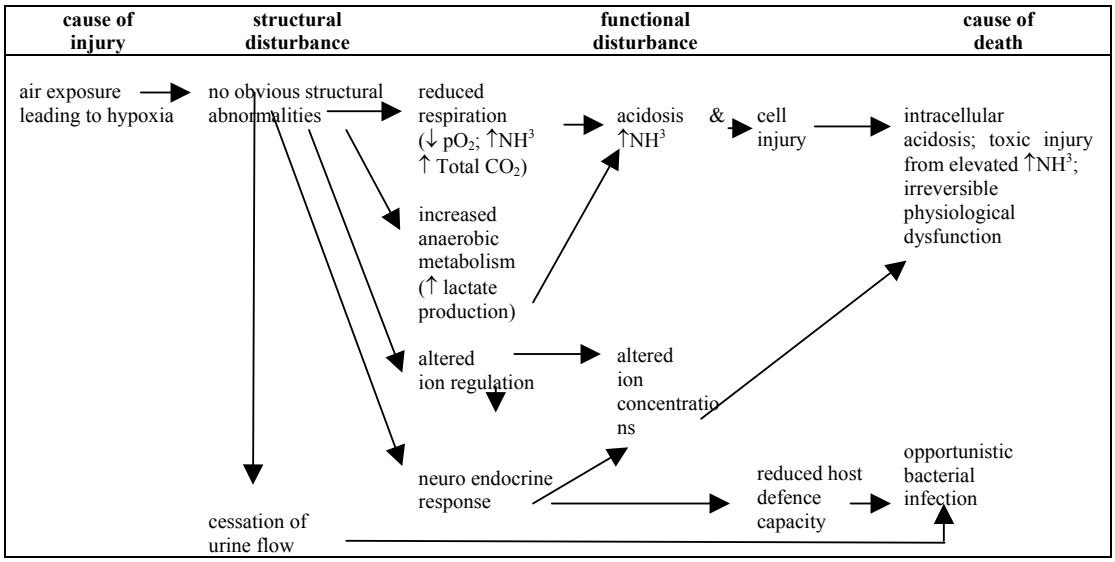


Figure 2. Pathogenesis of rock lobster post-harvest mortality

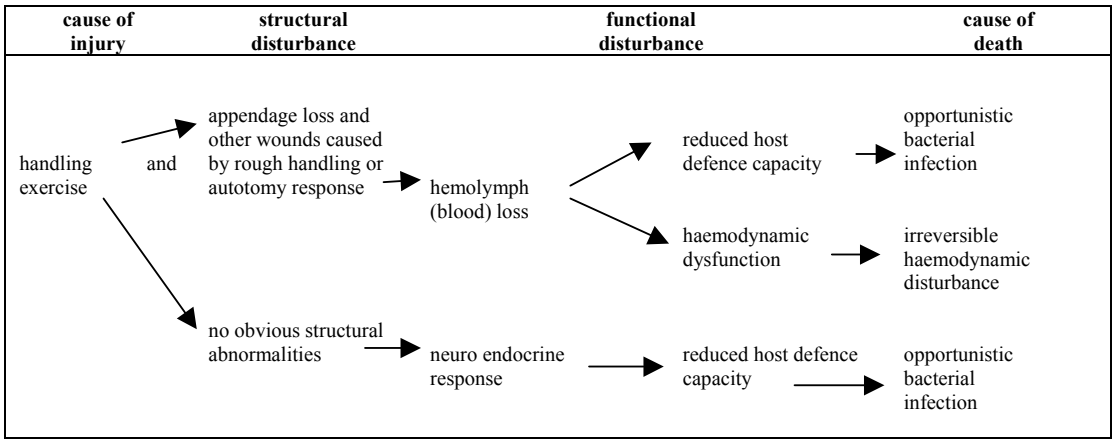
(a) Mechanical injury



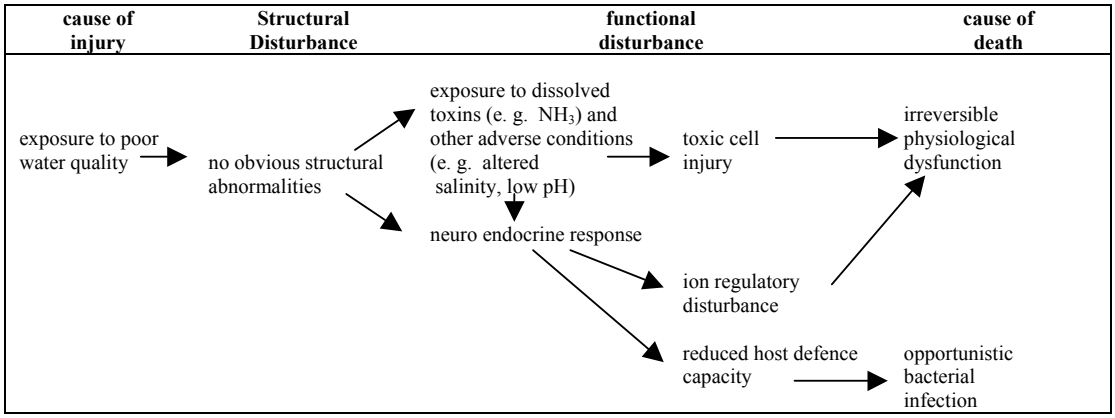
(b) Exposure to temperature variation that exceeds physiological tolerances



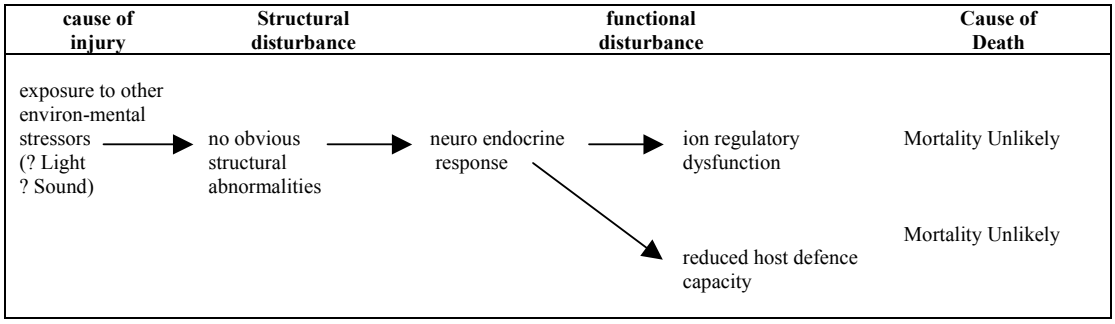
(c) Air exposure leading to hypoxia



(d) Handling and exercise



(e) Exposure to poor water quality



(f) Exposure to other environmental stressors

1.4 Lobster diseases

Diseases are caused by both infectious and non-infectious agents. Infectious diseases fall into five main categories – viral, bacterial, fungal, and those caused by protists (single cell organisms) and metazoans (multi-cell organisms). To date no viral diseases have been described in lobsters. There have been numerous reports, however, of diseases caused by other infectious agents (Evans *et al*. 2000). Non-infectious diseases include such conditions as nutritional deficiencies, genetic disorders, immune diseases and exposure of lobsters to toxins. There have been very few reports of non-infectious diseases of spiny lobsters. However, with the growth of aquaculture it is likely that more information on these types of conditions will become available.

Disease causing agents are called pathogens. Pathogens are often classified into primary and secondary or opportunistic pathogens. A primary pathogen will invade tissues and cause damage regardless of the health status of the host. Such organisms, if cultured in the laboratory and injected into a lobster, or administered by some other route, will invariably cause the lobster to become diseased. This is not the case with secondary or opportunistic pathogens. These organisms only cause disease if the lobster is in a weak condition and has a reduced host defence capacity. Weakness in lobsters can arise from a pre-existing disease condition, from exposure to environmental stressors or from some other cause. Post-harvest handling procedures can weaken lobsters thereby rendering them susceptible to infections by opportunistic pathogens.

Disease agents are sometimes called parasites. The term 'parasite' refers to organisms that spend part or all of their life cycle in the body of another, host organism. Parasites are found either in an inactive, resting phase, in which tissue damage is minimal, or in an active state in which their presence is debilitating and may kill the host.

1. 5 Host responses to infectious agents

Infectious diseases arise from the invasion of animal tissues by an infectious organism. The actual disease results from the subsequent growth, and in most instances, multiplication of the organism in the host tissues. These processes lead to tissue destruction, either through direct mechanical injury or through the release of harmful toxins released by the organism as part of its normal life processes. When the cells are damaged a host defence response is elicited. This response, if successful, destroys or inactivates the organism(s) and limits spread of the infectious agent into adjacent tissues. If the host defence response is successful, the agent is destroyed and wound healing takes place. If the infectious agent is not inactivated or destroyed, the infection is likely to spread and could eventually kill the host.

The shell of the lobster is an effective barrier to most infectious agents. It impedes the entry of bacteria and other pathogens and also protects the underlying soft tissues from mechanical damage. A population of non-pathogenic bacteria permanently resides on the external shell of a lobster. The presence of these bacteria limits the growth of other harmful bacteria and therefore plays an important role in host defence.

When the shell is damaged, as occurs during wounding or when a leg or antennae is lost, infectious agents may gain entry to lobster tissues. Furthermore, wounding leads to loss of hemolymph which coagulates and darkens upon exposure to air. Hemolymph loss weakens lobsters and predisposes them to disease. Excessive bleeding leads to the presence of large amounts of dark, translucent fluid in boxes or containers of post-harvest lobsters and is a sign of poor handling practices.

1. 6 Role of hemocytes in lobster host defence responses

Hemocytes are the cells found in the blood (hemolymph) of lobsters. There are three main types of hemocytes: agranular, semi-granular and granular. They differ in their size, internal morphology and function. The number of hemocytes found in lobster hemolymph varies with the stage of the moult cycle and with the stress and nutritional status of the lobster. Weak lobsters have been shown to have reduced numbers of hemocytes (Jussila *et al*. 1998).

Hemocytes are intimately involved in most host defence responses, either through a process of encapsulation, whereby large numbers of hemocytes migrate to the site of injury and cluster around the foreign organism, or through other processes such as hemolymph coagulation. They also play a role in detecting the presence of the infectious agent in the host tissues and initiating the host defence response. Examples of hemocytic

aggregations, a feature of inflammatory responses in lobsters, are shown in Plates 1 and 2.

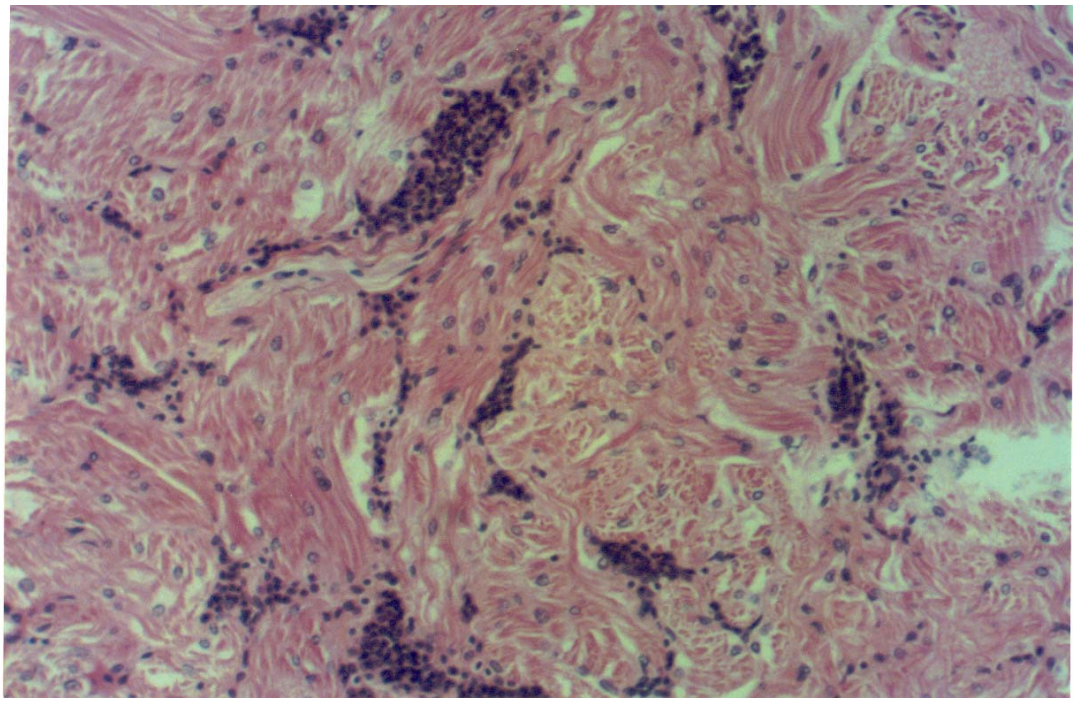


Plate 1. Hemocytic aggregation lesions in lobster heart.
Note multiple foci of hemocytic aggregations lying between muscle bundles.

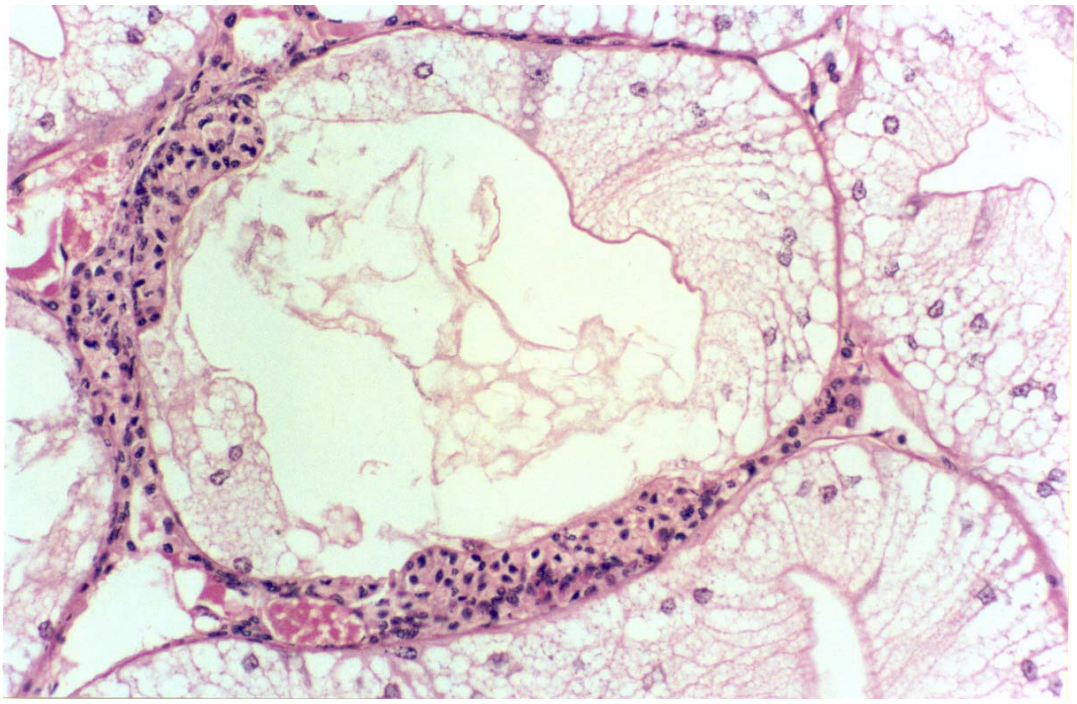


Plate 2. Hemocytic aggregation lesions in hepatopancreas.
Note aggregations of hemocytes in intertubular space.

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CHAPTER 2

PATHOPHYSIOLOGICAL REACTIONS TO ENVIRONMENTAL STRESSORS

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2. 1 Introduction

Top quality product is the goal of all lobster fishers. Quality is judged by the number of missing legs or antennae and by the condition of the lobster upon arrival at the factory. Appendage loss and condition are mainly determined by handling procedures on board the fishing vessel. Poor handling leads to stress responses and physical damage which in turn results in a loss of product quality.

Physical damage can be prevented by avoiding rough handling and using 'lobster friendly' gear. Minimising stress, on the other hand, is achieved by reducing exposure of lobsters to environmental conditions that cause stress reactions. While these conditions are mostly well known by fishers – air exposure, temperature extremes, poor water quality – the physiological reactions to these conditions are less well understood. The aim of this chapter is to explain in simple terms the underlying physiological responses that occur when a lobster is exposed to post-harvest environmental stressors.

2. 2 What is a stress reaction?

As explained in Chapter 1, a stress reaction is a physiological response to an environmental stressor. The reaction follows a set pattern, commencing with nervous responses to the stressor and culminating in either a return to the normal physiological state or a change to a new state – either ill health or, in the extreme, death.

2. 3 How can a stress reaction kill a lobster?

Lobsters, like any animal, are exposed to changes in environmental conditions all the time. Because the external environment is constantly changing (e.g. changes in temperature, pressure, light, sound, food intake, air or water composition) the lobster's physiology is geared to minimize the effect of these external changes on the state and composition of its internal environment – the cellular environment. Specialized cells within the body (mostly nerve cells) detect changes in the internal environment resulting from external environmental variations and initiate physiological responses to counteract these changes. This process is called homeostasis.

How does homeostasis work? This is best explained by using an example. If the concentration of a particular component of the blood (e.g. blood glucose) is measured continuously, it will be found to oscillate around a mean value. This pattern of oscillation reflects the homeostatic process. The principle here is very similar to that which operates to maintain the temperature of a refrigerator at a constant level – when the

internal temperature rises above a certain set level, the compressor switches on and causes the temperature to fall. When the temperature falls below a set level the compressor switches off and the internal temperature begins to rise again. The constant switching off and on results in maintenance of temperature within a narrow, set range. A graph of the temperature over time will show an oscillating pattern.

Blood components, and other physiological parameters, show similar patterns of oscillation to that of the internal temperature of a refrigerator. Minor changes in the external environment cause fluctuations in the levels of the component or physiological process. Nerves and hormones respond to these fluctuations, just like the temperature sensors and the compressor in a refrigerator, and initiate reactions which result in the maintenance of the parameter within homeostatic levels.

If the animal is exposed to a larger than usual change in an environmental condition, the changes in the internal environment exceed those usually seen in a normal homeostatic variation. This excessive deviation in the physiological parameter initiates a *stress reaction* aimed at counteracting the internal environmental change. Two outcomes are then possible – if the deviation is not too extreme, the physiological parameter will, in time, return to the normal value. On the other hand, if the deviation is extreme, or if there are multiple environmental stressors superimposed, cell damage occurs.

Following the occurrence of cell damage two outcomes are likely. If cell damage is minimal, a new, sub-optimal steady state develops. In this new state the damaged cells, while remaining alive, cease to function at an optimal level. In this state the health of the animal is compromised and the lobster is said to be in poor health. Immunocompetence is affected and the lobsters are susceptible to disease. Mortality could follow, particularly if the lobster is exposed to further stress. Alternatively, if cell damage is extreme, the cells die (a process called necrosis). This can lead directly to organ failure or, if the nerves are affected, indirect organ failure due to nerve dysfunction. The end result is the death of the lobster.

Thus, stress kills lobsters either through an immediate effect on organ function caused by tissue damage or through a delayed effect resulting from compromised organ or immune system function.

2. 4 What does air exposure do to a lobster?

All animals need oxygen to fuel physiological processes. Oxygen is extracted from water at the gills and transported to the body cells via the blood (called hemolymph in lobsters). The cells use the oxygen to generate energy and produce carbon dioxide (CO_2) as a waste product. This process is called respiration. The carbon dioxide is transported back to the gills where it diffuses into the surrounding water.

It is important that CO_2 is removed from the body tissues as it can be harmful if allowed to accumulate. Increased CO_2 concentration causes an increase in hydrogen ions (i. e. decreased pH) due to the following chemical reaction:

$$CO_2 + H_20 \Leftrightarrow H_2CO_3 \Leftrightarrow H^+ + HCO3^-$$

Changes in pH affect enzyme reactions and can adversely affect physiological processes. If the pH becomes very acidic cellular damage and, ultimately, cell death can occur.

When a lobster is removed from the water the transfer of oxygen across the gills becomes less efficient. Similarly, the removal of CO_2 from the hemolymph is impeded. As a result, oxygen levels fall and CO_2 levels rise. The lack of oxygen results in a change in the way cells generate energy. A different physiological process, called anaerobic respiration, is used. The same change to anaerobic respiration occurs when the lobster flaps its tail. Anaerobic respiration results in the accumulation of lactic acid in the muscle and in the hemolymph. The overall result is a fall in hemolymph pH. This process is called acidosis.

Acidosis leads to lethargy in lobsters. The tail becomes limp, and leg and antennae movement is reduced. The term 'weak' is given to a lobster affected by acidosis. The condition is reversible providing the lobster is returned to the water before irreversible cell damage occurs. Once returned to the water the lobster is able to remove the excess acid from its tissues and normal aerobic respiration is restored. The length of time it takes for the lobster's physiology to return to normal depends on the degree of acidosis. This, in turn, is affected by the time period of air exposure and by how much exercise (tail flipping) took place during the air exposure.

2. 5 What happens when a lobster is exposed to high or low temperatures?

Physiological processes are governed by enzyme reactions. Enzyme reactions occur at different rates depending on the temperature. All animals have an optimal temperature range within which their enzyme reactions and, hence, physiological processes occur at optimum levels. If the temperature rises or falls outside this range the enzyme reactions, and the body functions they control, are adversely affected.

High temperatures are generally more detrimental than low temperatures. Prolonged exposure to high temperatures in fish (i.e. those that exceed the normal range but not high enough to cause immediate death) can lead to malfunction of the immune system, disease outbreaks and general poor health. The same processes probably occur in lobsters. If the temperature is returned to normal the lobsters are likely to continue in a poor health status for some time.

Low temperatures slow the enzyme reactions down, particularly those involved in muscle contraction, and the lobster becomes comatose. However, if the temperature returns to normal, high health status is quickly restored.

2. 6 What effect does a high level of dissolved ammonia have on a lobster?

Ammonia (NH_3) is a toxin and can damage cells and their components. The precise nature of the damage is not well understood. When ammonia is dissolved in water, ammonium ions (NH_4^+) are formed according to the equation:

$$NH_3 + H_2O \Leftrightarrow NH_4^+ + OH^-$$

Ammonium is not as harmful as ammonia but is a toxin, nonetheless. Ammonia is produced in lobster tissues as a result of a breakdown of proteins. It is a normal waste product and will accumulate in the hemolymph and in the water in which the lobsters are held. Bacteria in the water and in filtration systems convert ammonia to nitrite (NO_2^-) which is then converted to nitrate (NO_3^-). Nitrite is also a mild toxin but nitrate is only toxic in high concentrations. Collectively these three compounds are called nitrogenous wastes.

The levels of nitrogenous wastes must be kept low in lobster holding systems. In flow-through systems the rate of water replacement should be sufficient to prevent build-up of these toxins while in re-circulating systems the biological filter should have sufficient capacity to maintain ammonia levels below tolerance limits.

Lobsters can tolerate transient exposure to high ammonia levels but prolonged exposure will cause the lobsters to die. Mortality is presumably due to injury of cells in vital organs or in the nervous system.

2. 7 What causes swelling in lobsters?

Two types of swelling are commonly seen in lobsters. The first type is characterized by a puffing-out of the soft membranes, particularly the membrane at the junction of the cephalothorax and the tail (Plate 12). The other form of swelling is seen as a blister in the tail fan involving part or all of the tail fan (Plate 7).

In the former case the swelling is caused by an abnormal intake of water. The tissues become waterlogged and pressure builds up under the hard, inflexible shell or exoskeleton. This type of swelling is seen in several different situations in post-harvest lobsters; following drowning in fresh water; when lobsters are held in low salinity water; and, occasionally, in a disease condition associated with poor health and mortality.

The accumulation of water in lobster tissues in fresh or low salinity water results from a process called osmosis. Osmosis occurs when two solutions of differing solute concentration are separated by a semi-permeable membrane. Since the solute cannot pass through the membrane, water diffuses across the membrane, driven by the concentration differences. The net result is a movement of water from the area of low solute concentration to one of high solute concentration. The two solutions are said to have different osmotic pressures.

The solute concentration in lobster hemolymph is similar to that of seawater. When the lobster is placed in low salinity water, or in freshwater, the differences in osmotic pressure leads to a movement of water into lobster tissues. This process causes the tissues to become waterlogged and the membranes to bulge. If the lobster is returned to normal salinity it will completely recover, providing the period of exposure has not been too long.

Another, less well understood, condition is that in which the tissues become waterlogged when there is no history of exposure to low salinity water. This condition is occasionally observed in batches of lobsters in processing tanks or it may be seen in an isolated lobster, the other lobsters in the tank appearing to be in a healthy condition. The exact cause of this condition is not known. Presumably there has been a dysfunction in the processes controlling water balance in the lobster resulting from abnormalities in the rates of excretion or uptake of one or more of the hemolymph ions. This condition is discussed further in Chapter 6.

Blisters on the tail result from a different scenario to that which causes membrane swelling. The exact nature of the process is not completely understood but is likely to be similar to that which causes blisters in the skin of humans. In the latter case, local cell damage resulting from an injury or exposure to a bacterial toxin or some other toxic agent leads to a release of chemicals which affect the pattern of blood flow in the surrounding tissues. There is an increased blood flow to the area and a leakage of plasma into the tissue spaces. This leads to a localized accumulation of tissue water to form a blister.

2. 8 Why does a lobster get black or brown areas on its shell?

Lobsters often get lesions in their shell, particularly in the tail fan area, which appear black or brown and which are usually associated with tissue erosion (Plate 8). The lesions are due to physical damage or an infectious process. When cells are injured by either of these mechanisms a series of reactions occur which function to prevent further tissue damage and repair the areas of damaged tissue. These reactions are collectively referred to as inflammation and repair.

Inflammation in a lobster involves the accumulation of large numbers of hemocytes at the site of injury. Components of the hemocytes and the hemolymph react to produce melanin, a dark coloured pigment. Melanin has anti-bacterial and anti-fungal properties and is thought to help limit infections. Large amounts of melanin are deposited in the area of tissue damage. This pigment deposition causes the blackening of the wound or infection area.

2. 9 Why does the tail of a lobster turn white?

White tailed lobsters are occasionally observed by fishers. The lobster looks as if it has been cooked (Plate 11).

The whitening of the lobster flesh is caused by a parasite called a microsporidian. The parasite invades the muscle tissue and forms large numbers of microscopic spores within the muscle bundles. The presence of the spores and the consequent tissue damage affect the way light is reflected and absorbed by the muscle tissue and results in the white appearance of the tail. Further information on this disease is given in Chapter 6.

CHAPTER 3

LOBSTER AUTOPSY PROCEDURE

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3. 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.

3. 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 below.

Table 4. Determination of vigour index

Somatic response	**Classes**					
	0(d)	**1(m)**	**2(w)**	**3(h)**	**4(vh)**	**5(a)**
Defensive horn response	-	-	-	-	-	+
Vigorous tail flip	-	-	-	-	+	+
Appendage 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

from 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.

3. 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 (see Appendix 3).

3. 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 Chapters 4 and 5.

3. 5 Dissection procedure

Photographs and diagrams of he suggested dissection procedure and the position and appearance of the major organs are shown in the booklet, Rock Lobster Health and Diseases: a guide for the lobster industry, that is also being produced as part of this project.

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.

3. 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. The booklet, Rock Lobster Health and Diseases: a guide for the lobster industry, that has also been produced as part of FRDC Project 1999/202, illustrates correctly and incorrectly fixed tissues.

3. 7 Fixation and processing

Various fixatives are suitable for histopathological examination of lobster tissues (refer to Appendix 1). 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 (see Appendix 2).

CHAPTER 4

COLLECTION AND HANDLING OF BLOOD SAMPLES FROM SPINY LOBSTERS FOR ANALYSIS OF PHYSIOLOGICAL COMPONENTS

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4. 1 Introduction

On average, blood makes up thirty percent of a lobster's weight (Belman 1975), so sample volume is not usually an issue when sampling lobsters. A third of the body weight sounds like a lot of blood, but it isn't always easy to get. Perhaps the easiest place to bleed a lobster from is the blood space (or 'pericardium') that surrounds the lobster's heart.

To locate the heart of a spiny lobster, you need to take its head in your hand and find the thoracic carapace. If you observe the shell from above, you'll notice that the top of the shell is divided by a deep, curved groove. These and other grooves in the shells of crustaceans typically reflect anatomical features within the body and, with experience, these can be used as markers. The rearwards part, the part of the shell posterior to this major groove, is the thoracic carapace and it also has a minor groove on it (Figure 4.1). Mostly, the thoracic carapace extends down along the side of the lobster and protects the animal's gills. However, the heart is located along the back of the lobster, close below the thoracic carapace.

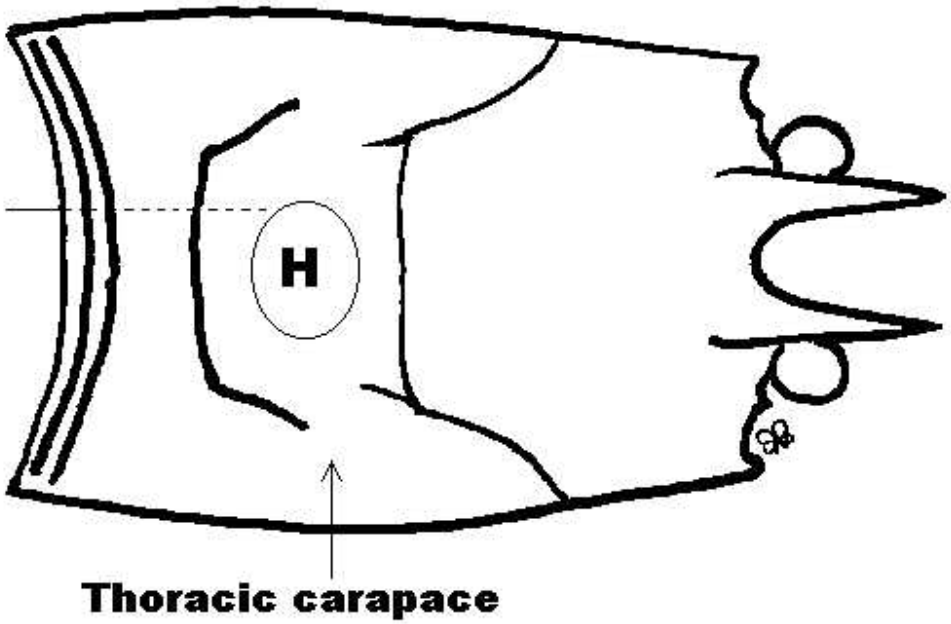

Figure 3. Collection of Hemolymph from the heart.
Diagram of the carapace ('shell') of a rock lobster viewed from above, showing the approximate location of the heart in relation to the thoracic carapace. Location of needle given by dotted line.

Oxygenated blood leaving the gills gathers in the blood space around the heart, where the beating heart is suspended by elastic threads or ligaments. When the heart relaxes, blood enters through numerous slit-like valves. When the heart contracts, the blood is propelled through a network of fine arteries to the body organs, tissues and extremities. The blood (or more correctly the hemolymph) then returns to the space around the heart, via the gills, along a series of broad blood sinuses.

The blood carries gases, substances, wastes and nutrients to and from the lobster's tissues. The principle behind sampling lobster blood is fairly straightforward. If the lobster's internal well-being is disturbed for whatever reason, the blood will be one of the first and often the simplest places to look for this disturbance. This is particularly true when studying the responses of lobsters that are handled in different ways, because the levels of some substances in the blood will change in ways that reveal how well the lobsters are coping with the treatment.

This chapter considers the simple steps involved in taking and processing blood samples from spiny lobsters when physiological measurements are being sought. While attempting to be comprehensive, it will not be possible to anticipate requirements and pre-conditions for all of the assays that could be performed on lobster blood. Controlling coagulation is a major issue. The general principles of rapid sampling and punctual chilling freezing will presumably hold for many tests; however, it is advisable technical

information accompanying each manual test or kit and, if possible, to consult others who have done similar work using crustacean blood.

In some cases, the pericardial sampling site (illustrated in Figure 4. 2) will not be appropriate. Sampling from the base of the walking legs, where blood is drawn from the animal before it passes through the gills, is also common. For further information about the circulatory anatomy and physiology of spiny lobsters, refer to studies of *Panulirus interruptus* and *Jasus lalandii* (Belman 1975; Paterson 1968). Stress indicators in the blood of spiny lobsters have been discussed recently (Paterson & Spanoghe, 1997).

4. 2 Sampling

First, take the lobster. A number of sampling points are available, depending upon the nature of the sampling. If the sample is required for measurement of substances such as glucose or lactate then the pericardial site is a straightforward option (Figure 4. 2). Blood is oxygenated in the gills prior to entering the pericardium, generally giving the sample drawn into the syringe barrel a variable blue-grey tint. This site certainly should be sampled when studying lobster respiration. There is an endocrine organ associated with the pericardium, so this site is not recommended when sampling for circulating neuro-hormones.

An alternative bleed point, for de-oxygenated blood entering the gills, is accessed via the base of the walking legs. This sample assists with interpretation of gill function and of course is favoured when sampling for these neuro-hormones. The locations of the pericardial and infra-branchial sinuses were confirmed by vascular corrosion casting of the blood spaces using Batson's #17 methacrylate casting resin. If pre-branchial blood is not required, the sample may simply be drawn from the sinus with the base of the leg itself.

Use luer tip disposable plastic syringes of 2.5 ml volume, with a 1.5 inch hypodermic needle (22-23 gauge). As the needles sometimes block, to avoid delay, assemble ample spare syringes and needles beforehand and ensure that they are ice-cold when needed to help retard coagulation.

To sample from the pericardial sinus (post-branchial sample), the lobster should be removed from the water and sampled immediately via the arthrodial membrane between the posterior margin of the carapace and the anterior margin of the tergum of the first abdominal segment. Push the needle carefully forward and at a slight downward angle slightly to the side of the midline. This works satisfactorily with *Panulirus cygnus*. However, we found that in this species cygnus if an animal was removed from the water for the purpose of obtaining post-branchial hemolymph samples for blood gas determinations, that post-branchial Po_2 declined rapidly (within 10s) following capture. We attribute this rapid decline to the close proximity of the gills to the pericardium. Due to the difficulty in catching and sampling an animal within a 10s period, an alternative method for obtaining post-branchial hemolymph samples to that described above was used for blood gas measurements (Paterson *et al.* 2001).

Briefly, lobsters were prepared in advance of sampling by catching them and drilling small holes through the cuticle overlying the pericardium using an engraving tool, but leaving the underlying epidermis intact. The lobsters were then returned to the water and allowed to recover for at least a day. When sampling, a hypodermic needle can be inserted carefully through the hole, cleanly piercing the epidermis to a distance of only around 1cm and a sample (0. 5 - 1. 0mL) could be taken without removing an animal from the water and with little apparent disturbance to the animal. After taking the post-branchial sample, the animal was then quickly removed from the water and a sample of pre-branchial hemolymph taken from the sinus at the base of the gills via the arthrodial membrane between the base of the 3rd and 4th legs.

It is possible to sample single-handedly by wearing a PVC apron and bracing the extended lobster tail against the sampler's thigh. Certain other basic protective measures are recommended: use safety glasses. A lobster can flick suddenly, particularly when being sampled from the walking leg base, launching the syringe free of the user. Gloves are important (they aren't called spiny lobsters for nothing) though you may need to expose the fingertips to give fine control of the syringe plunger.

Again, regardless of the sampling point, it is important to immobilize the animal and sample it as quickly as possible. Not only is there a danger that the blood will clot but prolonged sampling can itself change the things being assayed for. Still, it is feasible to draw up to 3mL of blood from a lobster in less than a minute. This is only a fraction of total blood volume so it not a significant impost on the lobster. We have sampled lobsters repetitively at weekly intervals with no diminution in blood protein level. Don't draw out the plunger of the syringe too abruptly. A slowly developed, gentle vacuum is enough. If necessary, rotating the syringe barrel helps to keep the needle free of obstructions.

The blood sample should be kept cold in the syringe and measured immediately (for example blood refractive index, pH, gas tensions, ammonia level enzyme activity); or refrigerated and allowed to coagulate (for example, determination of electrolytes in serum); or whole blood can be frozen and stored in liquid nitrogen, or added to perchloric acid to precipitate proteins and arrest biological processes prior to freezing, again in liquid nitrogen.

Discard any samples containing granular sediment (often yellow/brown in colour) pointing to contamination from gonads or midgut gland. This grainy contamination comes from damaged soft tissue and can't be confused with the yellowish or orange tint that the blood can acquire at certain times of the moult due to the presence of astaxanthin.

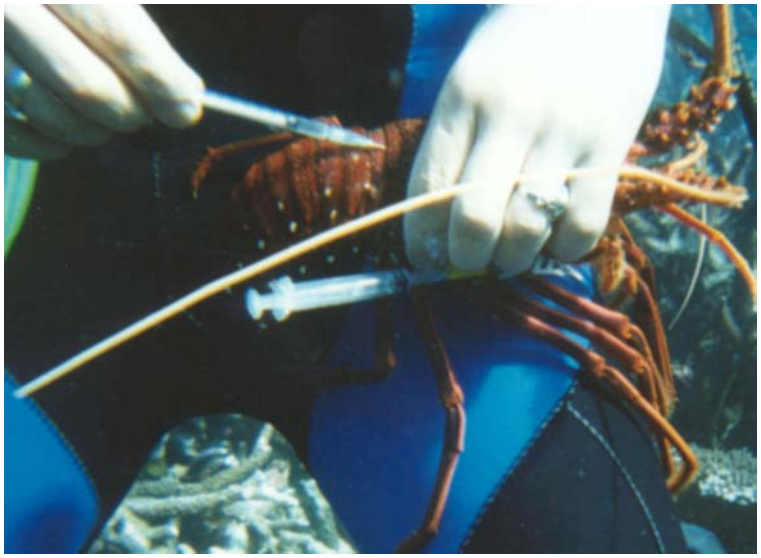

Plate 3. Sampling blood from the pericardium of a rock lobster
In this case, sampling was done underwater (Photo: P. Spanoghe)

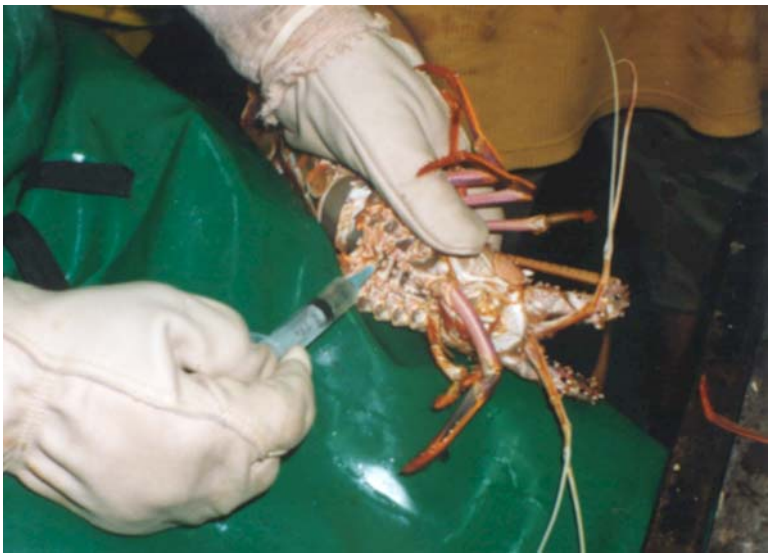

Plate 4. Sampling blood from the base of a walking leg.
(Photo: B. Paterson, Department of Primary Industries, Queensland)

4. 3 Coagulation and serum

Keep whole blood on ice to discourage clotting. The 'anti-coagulants' often used in crustacean hematology are not compatible with physiological analysis. They often contain buffers, acids or salts etc. and are often applied with a 1:1 dilution of blood in anticoagulant. As cells mediate coagulation, some delay is achieved by spinning down whole blood to remove cells from suspension.

Serum is prepared from whole blood samples by allowing the samples to clot, and then centrifuging under refrigeration (4ºC) at 9000 rpm for 10 min. The clot is then picked out and removed with a hypodermic needle. The serum can then be transferred to microcentrifuge tubes for storage at -20ºC. In this manner, about 30% of the original sample volume is recovered as serum. In this state, the sample lacks coagulability. However, given the drawn out nature of the process, this method is only suitable for measurement of substances not likely to change in blood isolated from the body (e.g. electrolytes). Volatile or reactive parameters should either be measured immediately on whole blood, or in neutralized perchlorate extracts.

4. 4 Extraction

When preparing extracts of blood for metabolic assays, it should first be deproteinized in ice-cold perchloric acid. For example, a 250 μL sample of blood can be mixed with 500 μL of 1 molar perchloric acid in an ice-cold Eppendorf vial. The acid extracted samples are then centrifuged at 9000 rpm to compact the fibrous white precipitate at 4ºC for 10 min. If a pink tint occurred in the whole blood sample (astaxanthin), this is now observed in the fibrous protein pellet.

The next step is to neutralize the perchloric acid prior to assays. For example, 500 μL of supernatant is removed and neutralized with 70 μL of 3 molar KOH. The vials are left on ice for 15 minutes while fine crystalline potassium perchlorate precipitates. The supernatant (the neutral perchlorate extracts) is stored at -25 ºC for subsequent analyses.

The aim is to titrate the acid with the known amount of KOH to obtain a final pH close to neutrality. In practice, titrating such a small sample is difficult because addition of base changes the pH quite dramatically so the pH is unlikely to be exactly neutral. Modest deviations from neutrality may be acceptable for some tests as many enzymatic kits use buffers to ensure the pH stays in the optimal range. Check the specifications of each kit and the pH of the reaction mixture. One simple test to confirm that the extract is not interfering with the reaction is to add a small quantity of the substance being tested for. The reaction should re-start.

4. 5 'On the day' assays

Blood pH is preferably measured promptly using a radiometer micro-capillary pH electrode equilibrated to the ambient water temperature. Alternatively, blood pH can be

simply measured by placing a 'spear-tip' pH electrode into a conical Eppendorf vial containing a small volume of blood.

When measuring pH and especially gas levels there should be no gas bubbles in the syringe after sampling. The levels of blood gases can change if the sample is exposed to atmosphere inside of the syringe. Inverting and expelling the air through the tip can remove stray bubbles in the syringe. Ensure that the blood flows freely from the animal. It is important NOT to apply so much suction to the syringe that the blood de-gasses (bubbles appear on the surface of the syringe).

Blood oxygen and carbon dioxide measurements are a useful adjunct to studies of lobster handling, but require specialized equipment. Methods are available for determination of total oxygen and total carbon dioxide content of blood (Tucker 1967; Cameron 1971). HCO_3^- concentrations and PCO_2 of samples can be calculated from total CO_2 content using rearrangements of the Hendersen-Hasselbach equation. Values of the constants determined for *Carcinus maenas* at the appropriate salinity and temperature can be used (Truchot 1976).

Total protein content of the blood can be measured as refractive index using a serum protein refractometer and converted to grams per litre using published equations (Paterson *et al.* , 2000, Leavitt & Bayer, 1977). The relative hemocyanin concentration can be measured as the absorbance at 340nm of a solution of 50µL of hemolymph in 950µL of distilled water (Hagerman 1983). The hemocyanin content can be estimated using published extinction coefficients, but caution is required, particularly as it is possible for incorrect coefficients to give a hemocyanin content higher than the total protein content.

Blood ammonia assays are best conducted on the day of sampling. Ammonia is volatile, and care is recommended when neutralising perchlorate extracts prior to ammonia assays as addition of strong bases like KOH can drive ammonia gas from the sample (Bergmeyer & Buetler 1984). Measurements of ammonia can be carried out using simple colorimetric methods such as the Berthelot reaction, or using a spectrophotometric/enzymatic kit. A flow injection method for quantification of plasma ammonia in crustaceans has been described recently (Hunter & Uglow 1993).

4. 6 Assays on processed/ stored samples

If small numbers of assays are involved, then veterinary or clinical pathology laboratories can measure many of the parameters of interest (e.g. lactate, glucose, ammonia, sodium, calcium, magnesium, potassium and chloride), for a price.

Depending upon the user's analytical equipment, numerous clinical diagnostic kits are available off the shelf, either for manual assays or for use in automated analysers. However, it pays the user to investigate the specifications of the kit since none will have been designed with crustacean blood in mind. Take note of the operating range of the assay, since you might need to dilute your sample to get it within range. Alternatively, because crustaceans may have less than a 'normal' amount of a chemical in their blood,

you may find you have to take steps to maximize the sample absorbance to bring it up into the acceptable range for the technique.

Pocket meters or kits are also available for field measurements (Wells & Pankhurst 1999).

4. 7 Interferences and troubleshooting

Clinical diagnostic methods are usually designed for assay of human blood, plasma or serum. Interferences arising from unique characteristics of crustacean blood needs to be considered.

The respiratory pigment is a large molecular weight copper protein aggregate called hemocyanin. To make matters worse, this large protein is dissolved in solution in the plasma, and not carried in cells. Extraction of protein, to remove hemocyanin, is an essential step in preparing blood for spectrophotometric assays, though free copper is possibly liberated by the extraction process.

The presence of copper in blood extracts is believed to be the cause of unstable end-points in lactate assays that use hydrazine to favour pyruvate formation (Gutmann & Wahlefeld 1974) (e.g. Sigma 826 UV). This interference has been successfully eliminated by modifying the original method (Engel & Jones 1978; Graham *et al ,* 1983). However, another method (Noll 1974) (e.g. Boehringer 139 084) avoids the problem altogether as it effects the removal of pyruvate using the enzyme glutaminate-pyruvate transaminase.

Hemocyanin needs to be removed from the sample because it absorbs strongly in the ultraviolet. Specifications accompanying some clinical UV spectrophotometric methods allow plasma or serum to be used directly in assays. However, in these cases, a caveat must be placed on treating crustacean 'plasma' in this way. These enzymatic methods often exploit the absorption spectra of NADH and require the change in absorbance of light to be registered at 340nm. This coincides with the absorption maxima of oxygenated hemocyanin.

4. 8 Normal levels and other factors

Normal levels should be sought for each species. Literature values exist for a number of species (Dall 1974a, 1974b, 1975; Davidson *et al.* 2000; Evans *et al.* 2000; Malley 1977a, 1977b; Paterson *et al.* 1997; Travis 1955; Vermeer 1987) and more information is being published. Moult stage may have a major impact on results. Lobsters are readily moult staged by removing the tip of one pleopod on the first abdominal segment (Turnbull 1989; Lyle & McDonald 1983).

To remove the impact of moult stage during experiments, it is sometimes the practice to confine observations to animals in Stage C of the moult cycle. A method of quantifying the liveliness or the vigour of lobsters has been proposed (Spanoghe & Bourne 1997). By

this method animals are ranked on a scale from 0 - 5, with 5 being the most vigorous and 0 being a dead animal.

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CHAPTER 5

REAGENTS AND METHODOLOGY FOR IMMUNE FUNCTION TESTS

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5. 1 Total hemocyte count (THC)

Reagent

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.

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 hemocytometer 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.

Normal range for THC for acclimated *Panulirus cygnus*

4. 01 – 9. 01 x 10^6 cells/ml

5. 2 Differential hemocyte counts

Reagents

Sodium cacodylate anticoagulant

See section 5. 1 above for preparation method.

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 5. 1). 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 (Bancroft & Stevens 1977)(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 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 5.3 below).

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).

5. 3 Percentage granular cells (%GC)

Reagents

Sodium cacodylate anticoagulant
See Section 5. 1 above for preparation method.

Method

See Section 5. 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.

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).

5. 4 Bacteraemia

Reagents

Marine saline agar plates.

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^{o}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 Table 5 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 5. 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 bacteraemia for each group.

Normal range for bacteraemia 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 bacteraemia 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. 5 Clotting time

Reagents

Nil.

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. $1/3^{rd}$ of length of tube)(which has been rinsed clean with 70% ethanol prior to use). 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^{o}. 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 (Plate 5). 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'.

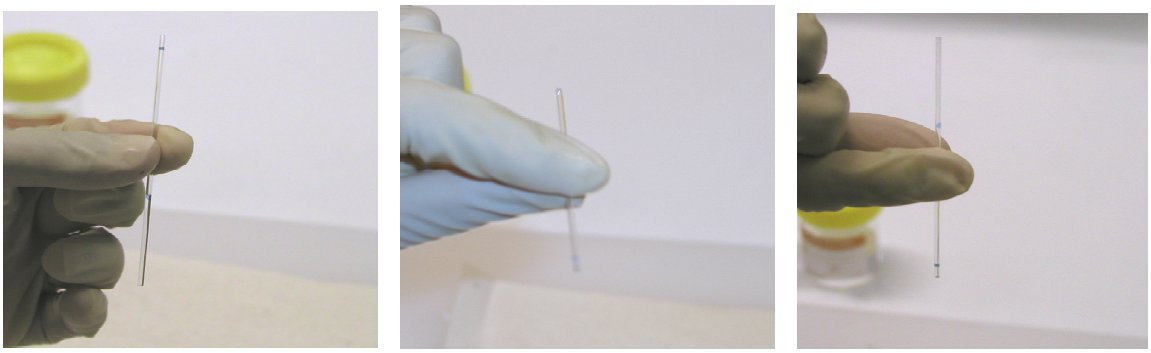

Plate 5. Method used to calculate clotting time of lobster hemolymph.
The series of photographs demonstrates inversion of a hematocrit tube filled with hemolymph to determine clotting time of lobster hemolymph.

Use Table 6 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 6. 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.

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%

5. 6 Normal range deviation

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. 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 bacteraemia 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.

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CHAPTER 6

DISEASES OF MATURE SPINY AND CLAWED LOBSTER

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6. 1 Introduction

This chapter summarizes disease conditions that have been reported in adult spiny and clawed lobster throughout the world. In many reported disease outbreaks, mortality occurred in lobsters that had been caught in the wild and held in commercial holding facilities. This suggests that, although some infectious disease agents are found in wild lobster, the majority of outbreaks of overt disease are the result of stress, poor water quality and nutrition following capture. Most diseases reported in lobster are the result of opportunistic pathogens that are ubiquitous in the marine environment and only cause overt disease in lobsters following injury or adverse conditions. Thus many of the conditions, especially those reported in lobster in Australia and New Zealand, should be regarded more as conditions resulting from stress rather than primary diseases. In the majority of cases, preventing or removing the pre-disposing stress factors by providing management and husbandry that suit the physiological and behavioural characteristics of lobster can successfully control health problems (Fisher *et al.* 1978). There is seldom a need to resort to the use of chemical treatments if optimal water quality, temperature, diet and stocking density is provided.

Diseases seen in adult spiny lobster in Australia are described in the first part of this chapter, followed by a description of the major diseases of lobster in other areas of the world. A discussion of health problems of aquatic animals in Australia is incomplete without some reference to the risk of introduction of disease by translocation from other regions of the world. Likewise, although treatment of diseased lobsters are not discussed in specific terms in this manual, there are some principles of treating animals, especially those destined for human consumption that should be noted. Chapter 8 of this manual outlines some of the important aspects of translocation and food safety risks of relevance to lobster disease.

Several conditions reported to affect lobster eggs, larvae and juveniles also occur in adult lobster. Fouling by epibionts (see Sections 7.4.1 and 7.5.2) may also be a problem in adult lobster held in captivity but it has not been recorded as a cause of high mortality in larger animals. Infection of lobster eggs with nemertean worms such as *Pseudocarcinomertes* spp. (Brattey & Campbell 1985), infection of larval and juvenile lobster with fungi such as *Haliphthorus* spp. (Section 7.5.5)(Diggles 1999; Fisher, Nilson, Shleser 1975; Overton 1983) and *Lagenidium* spp. (Nilson, Fisher, Shleser, 1976)

have not been reported in adult lobster and are not described in this chapter. Shell disease occurs in lobsters of any age (Section 7.5.5) but is a common in adult lobster and is discussed in this Chapter.

There have been a few reviews of diseases of lobster (Bower *et al.* 1994; Evans *et al.* 2000; Fisher *et al* 1978), however, the majority of available published information is in scientific journals. Increasingly there are reports of disease status available on websites; however, pathologists in diagnostic laboratories are likely to have many more case histories of diseases affecting lobsters in their local area. The few reports of sporadic outbreaks of disease or conditions affecting adult lobsters have not been included in detail in this manual. They include iron deposition on the gills of lobster held in bore or well water (Jansen & Groman 1993), myocarditis of undetermined aetiology (Wada *et al.* 1994) and paramoebiasis of lobster following infection with *Paramoeba perniciosa* (Bower *et al.* 1994). It is to be expected that the number of published reports of disease in lobster will increase as more study is done in this area.

Table 7. Diseases of spiny and clawed lobster reported in Australia and New Zealand

Disease agent	**Condition**	**Causative agents**	**Chapter**
Bacteria			
	Shell disease	*Vibrio* spp. ,	6.2.1, 7.5.1,
	Septicaemia	*Vibrio* spp.	6.2.2, 7.4.2,
Fungi			
	Fungal infection	*Fusarium solani*	6.2.3
	Black gill disease	*Haliphthorus* sp.	7.5.5
Protist			
	Microsporidiosis	*Ameson* sp.	6.2.4
Helminth			
	Nemertean	*Carcinonemertes* spp.	Campbell *et al*.,
Miscellaneous			
	External fouling	*Leucothrix*-like bacteria	7.4.1, 7.5.2
		Sessile ciliates	
		Free living nematodes	
		Detritus	
	Black		7.5.4
	Tail blister		6.2.1
	Turgid lobster		6.2.5
	Moult death		6.2.6
	Body deformities		6.2.8

6. 2 Diseases reported in Australia

6.2.1 Condition 1: Shell disease

Major pathological characteristics: Damage or necrosis of the exoskeleton with lesions having a black/brown or orange/red colour most commonly on the tail or perieopods of lobster. Lobster may be weak and lethargic (Floreto *et al.* 2000).

Causative agents: Gram negative bacteria such as *Vibrio* spp. that produce chitinase. Occasionally fungal hyphae, rather than bacteria, are found in lesions (Alderman 1973; Diggles 1999).

Diagnosis: Culture of Gram negative bacteria such as *Vibrio* spp., *Shewanella* spp., *Pseudoalteromonas* spp. (Porter *et al.* 2001), *Vibrio alginolyticus*, *Vibrio vulnificus*, *Vibrio parahaemolyticus*, *Plesiomonas shigelloides* (Reuter *et al.* 1999; Geddes *et al.* 2003) from lesions together with histopathological findings of fissures in chitin, associated with underlying hemocytes aggregations, haemorrhage, oedema and possibly thrombosis and the presence of bacterial colonies (Reuter *et al.* 1999).

Epizootiology: The prevalence of shell disease in wild lobster varies and is associated with location of the lobster and local water conditions. Surveys of wild lobster in North America indicated that the disease had a low prevalence, under 2% (Lavallée *et al.* 2001; Porter *et al.* 2001) except in lobster from the polluted waters where 20 to 70% of lobsters had shell disease (Castro & Angell 2000). Although these surveys probably under estimate prevalence of the disease, as affected lobsters are often weak and lethargic and unlikely to be caught in traps, they indicate that the disease is unlikely to be a major problem when unstressed lobsters are held in water of good quality that suits the requirements for the particular species of lobster.

The bacteria found in shell lesions of lobster affected by the condition are ubiquitous in the marine environment and are found on the exoskeletons of healthy as well as diseased lobster (Porter *et al.* 2001). They appear to be opportunistic pathogens that cause lesions after lobster are stressed by handling; holding in aquaculture facilities; injury from fighting; abrasions from cages or holding facilities; or high loads of organic matter, sewage or sludge in the environment (Getchell 1989). The disease is sometimes more prevalent when lobsters are held higher than normal water temperatures (Reuter *et al.* 1999).

Although lobsters affected by the condition usually continue to eat, there are severe alterations to the composition of hemolymph, hepatopancreas and muscle of affected lobsters (Floreto *et al.* 2000; Porter *et al.* 2001. Death of affected lobsters may be the result of bacterial septicaemia due to perforations of the exoskeleton or from failure to moult as a result of adhesions of the old and new exoskeleton (Fisher *et al.* 1978).

Plate 6. Shell disease in a western rock lobster, *Panulirus cygnus*. Note the discoloration and fissures in the chitin.

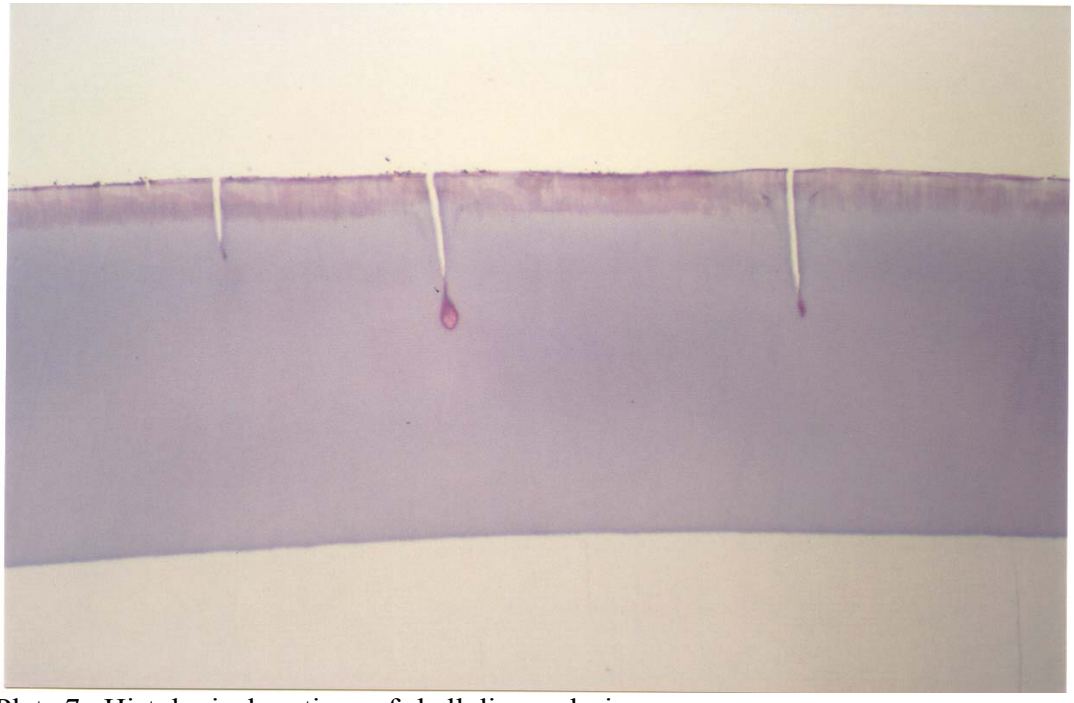
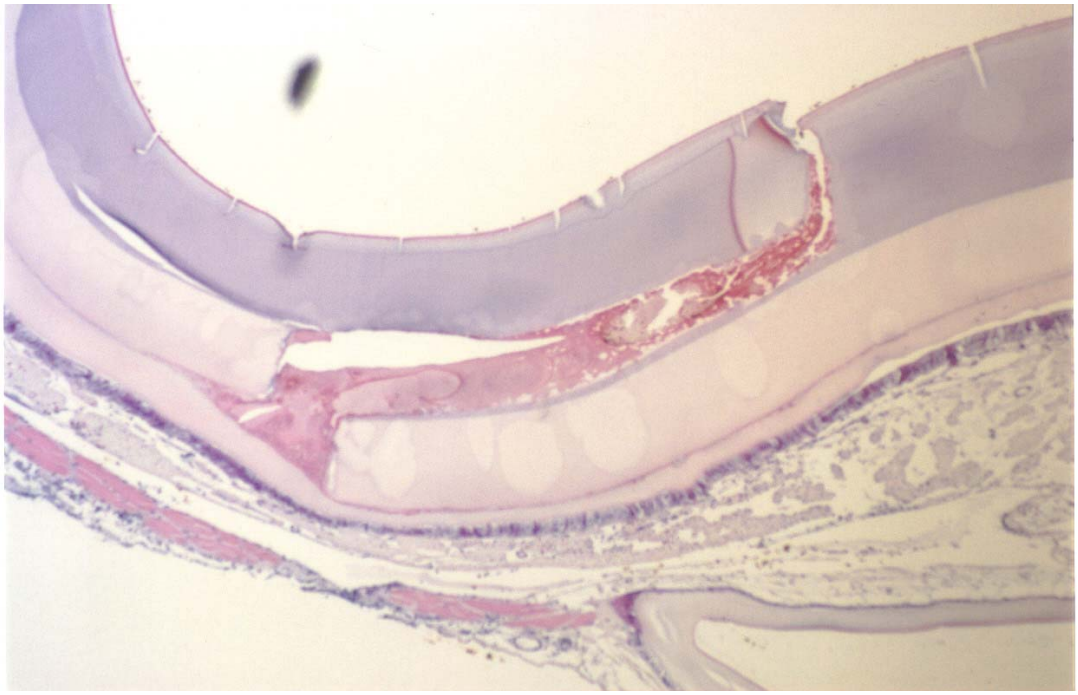
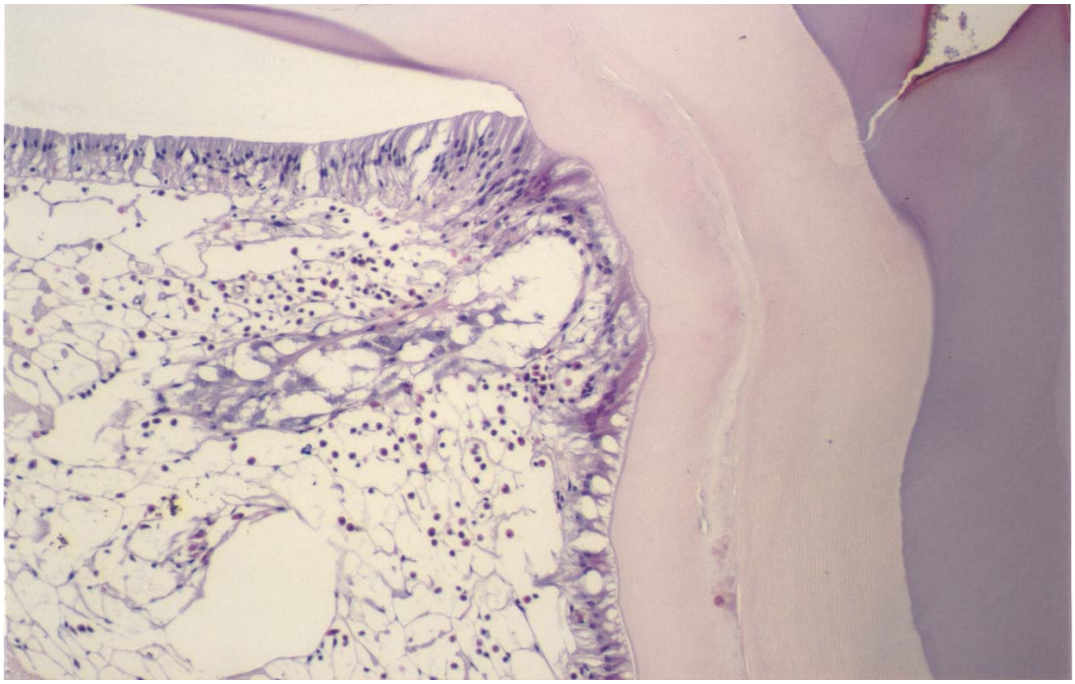


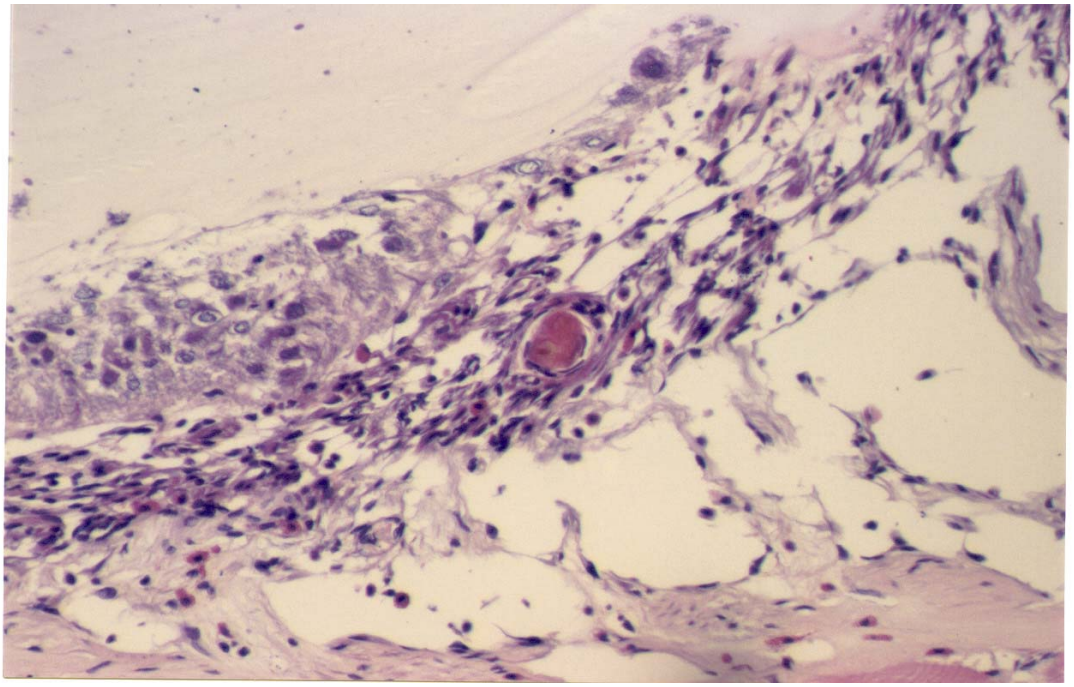
Plate 7. Histological sections of shell disease lesions
A: Early lesion. Section shows breaks in the chitin layer. The dark spots at the base of the crack are melanisation responses.



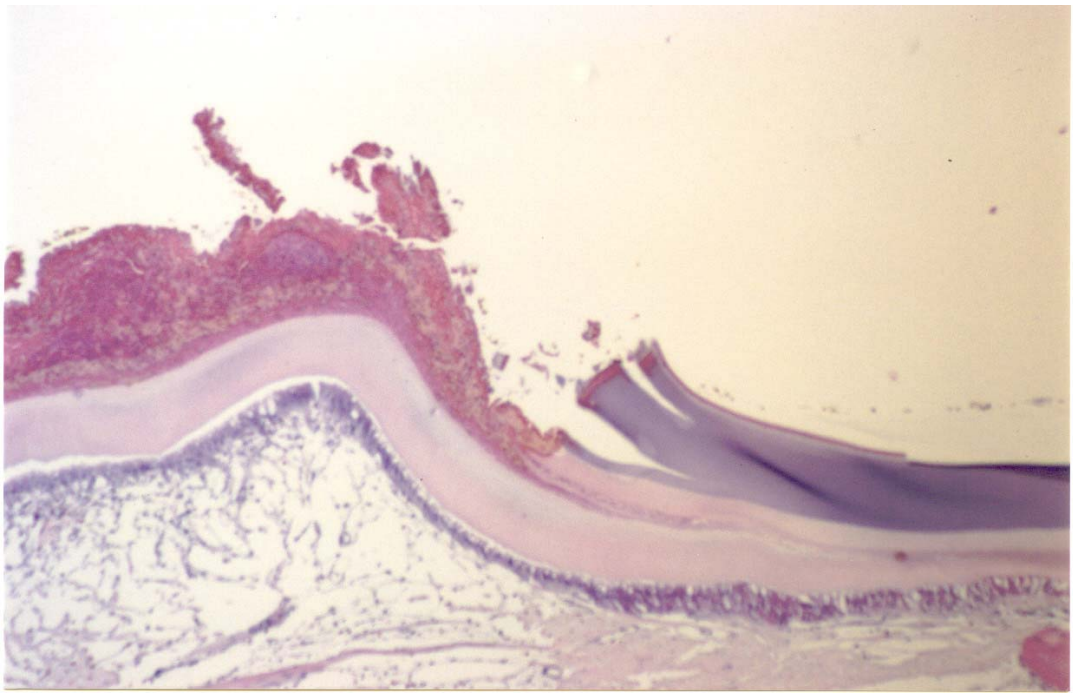
B: More advanced lesion. Section shows large hemocytic aggregation just beneath the crack in the chitinous layer.



C: Histological section showing mobilization of hemocytes in the tissue underlying the crack in the chitinous layer.



D: Histological section showing formation of a small nodule in area underlying shell lesion.



E: Histological section showing large hemocytic aggregation and loss of outer chitin layer.

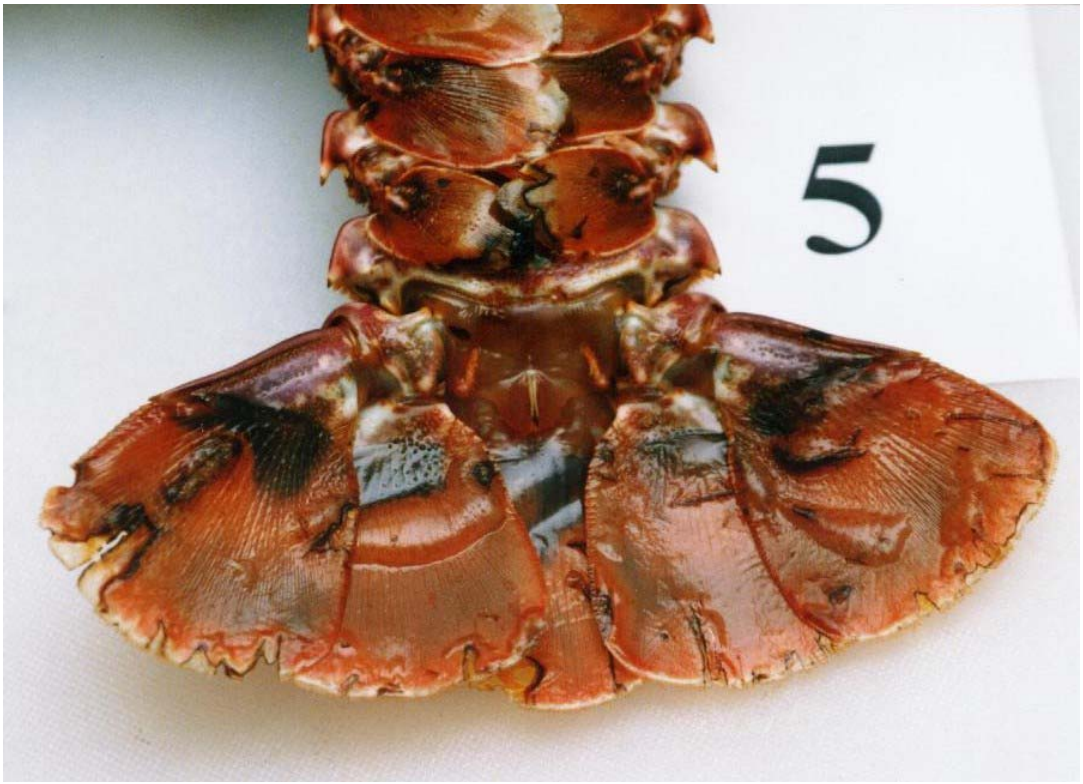
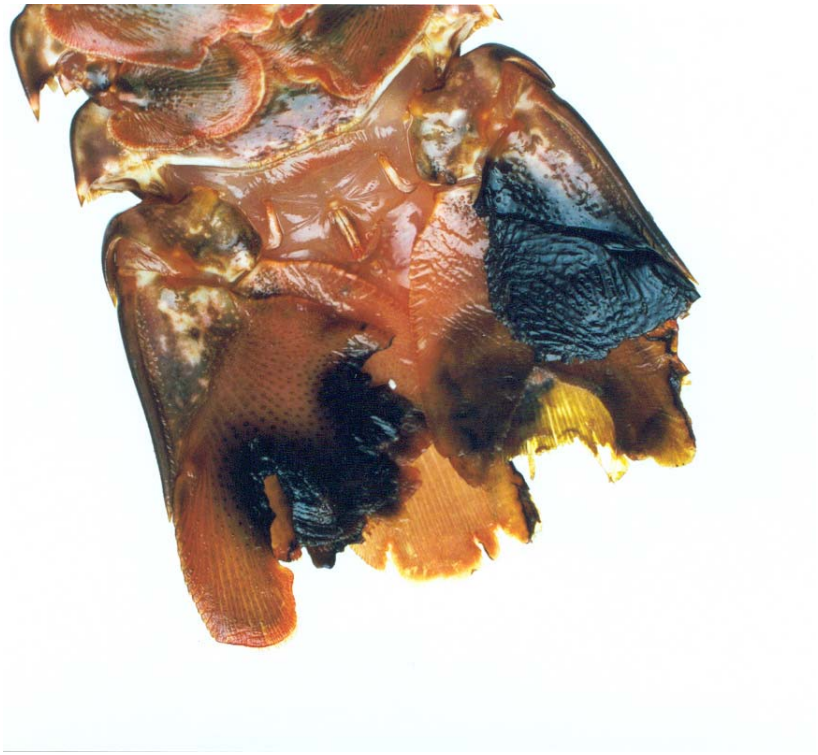


Plate 8. Tail blisters in a western rock lobster, *Panulirus cygnus*. Blister formation shown in the uropod and telson.

Plate 9. Tail necrosis in a western rock lobster, *Panulirus cygnus*.
A: Note the raised necrotic region on the uropod, suggestion of melanisation of previous blister.



B: Tail necrosis. Note the loss of tissue as a result of necrosis of the uropod tissues.

C: Tail necrosis. Note the significant loss of tissue as a result of necrosis of uropod tissues.

6.2.2 Condition 2: Bacterial septicaemia

Major pathological characteristics: Lethargy followed by death. Often no gross lesions are present although at times exoskeleton damage, shell disease or fungal infections may also be present.

Causative agents: Various species of *Vibrio*.

Diagnosis: Culture of hemolymph using standard techniques for isolating bacteria on marine animals. Histopathological findings include oedema and necrosis of the hepatopancreas, intestinal tract and muscle and rod-shaped, gram-negative bacteria are sometimes visible in tissues (Bower *et al.* 1994; Bowser *et al.* 1981; Hameed 1994).

Epizootiology: Bacteria such as *Vibrio* spp. are opportunistic pathogens of lobster that often infect lobsters during periods of stress such as poor nutrition (Bowser & Rosemark 1981), high water temperatures (*Brinkley et al.* 1976) or when the exoskeleton is damaged (Jawahar *et al.* 1996). They colonized western rock lobster, *Panulirus cygnus*, held in aquaria (Evans 1987) and it is to be expected that the condition is rarely reported in scientific literature as the bacteria are part of the normal marine bacterial flora and are often isolated from moribund lobsters.

Studies have indicated that many non-pathogenic bacteria are present in the hemolymph of apparently healthy lobster (Cornick & Stewart 1966; Stewart *et al.* 1966). Thus it may be invalid to conclude that ill health of lethargic or moribund lobsters was brought about by bacterial infection. A complete investigation including water analysis and necropsy including wet smears of hemolymph and histopathology should be performed to eliminate other contributing factors or pathogens.

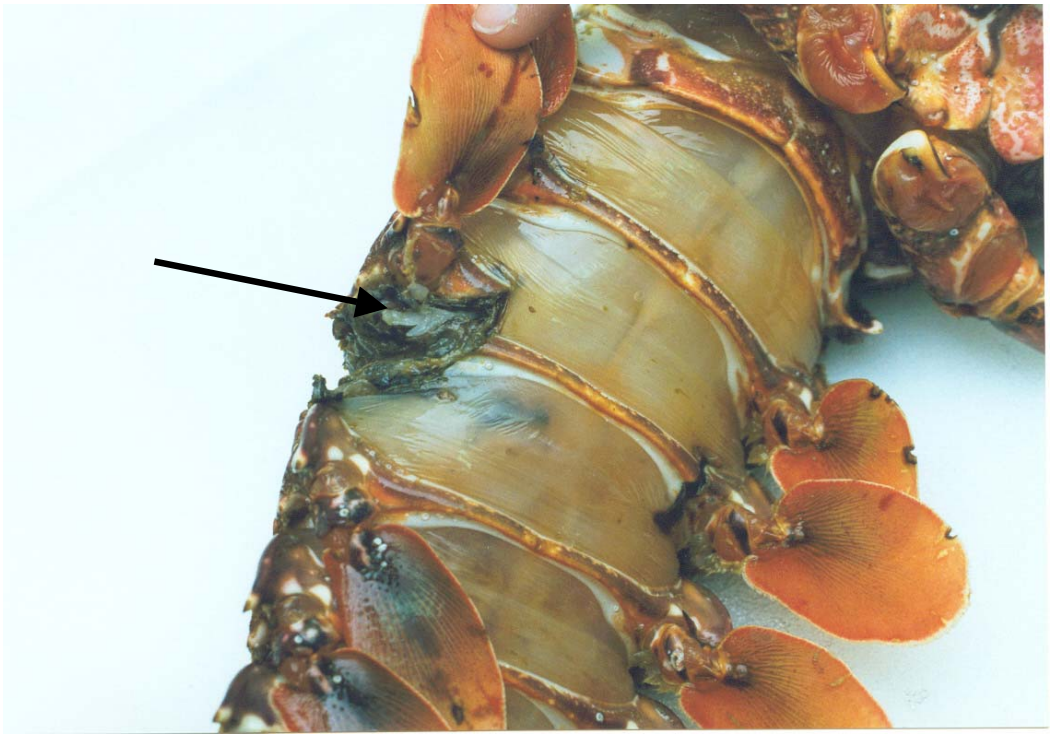


Plate 10. Wounding with associated inflammation.
A western rock lobster, *Panulirus cygnus*, with a lesion on the lateral side of the tail (arrow). The lesion extends through the full thickness of the exoskeleton and exhibits areas of black pigmentation resulting from melanin depositions. Pale discolouration of underlying tissues suggestive of inflammation. Penetrating wounds or infections such as the one on this lobster, can result in bacterial septicaemia, loss of large amounts of hemolymph and death.

6.2.3 Condition 3: Fungal infections

Major pathological characteristics: The appearance of white, orange, brown or black spots or patches on the exoskeleton of lobster.

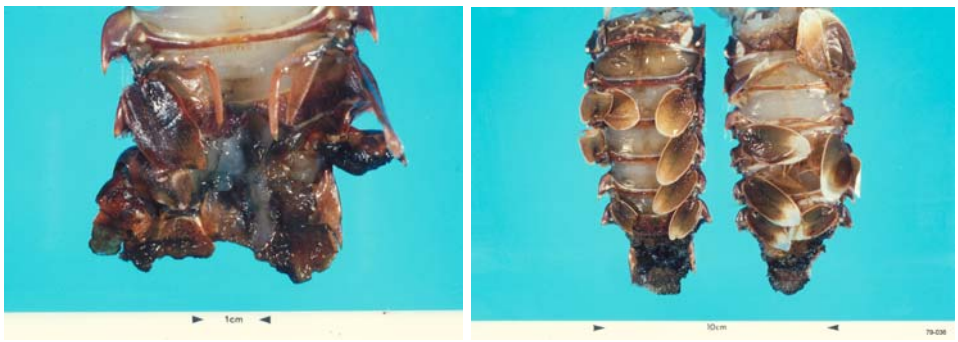
Causative agents: *Fusarium solani* and other unidentified fungi.

Diagnosis: Wet preparations of affected exoskeleton and subcuticular tissues reveal fungal hyphae. The morphology of the fungus can be described from its cultural characteristics on isolation media and from the appearance of hyphae in wet preparations and histological sections. *F. solani* hyphae are straight, occasionally branching hyphae of 2. 5. to 5. 0 µm width.

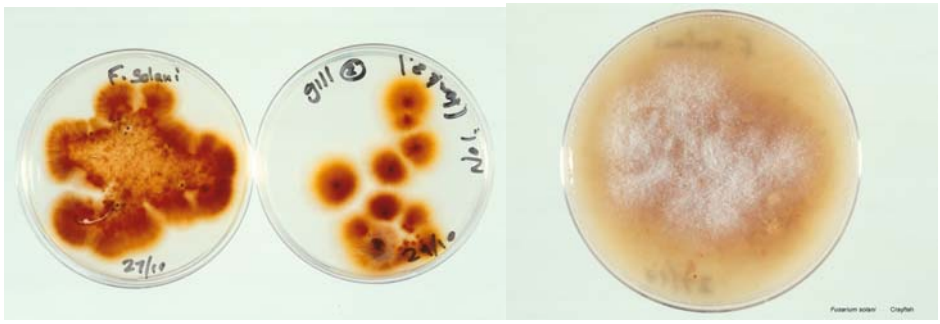
Epizootiology:
Fusarium solani is the most commonly reported fungus of adult lobster. Mortalities can be high and usually occur just before or during moulting (Lightner & Fontaine 1975). As well as the pigmented patches of exoskeleton, the gills of affected lobster may be brown and lobsters may autotomize infected appendages. Infected lobster usually are held in aquaculture facilities (Alderman 1981; Lightner *et al.* 1975) and are often associated with

the presence of wood in the environment (Dr David Alderman, personal communication), but an outbreak in the wild fishery of the western rock lobster, *Panulirus cygnus*, was reported in Western Australia in 1979 (McAleer 1982).

Erosive, brown or black pigmented areas of the affected exoskeleton are thickened. Fungal hyphae invade the underlying subcuticular tissue and muscle as well as the exoskeleton. Hyphae are surrounded by aggregations of hemocytes and melanisation which are features of the immune response of the crustacean to tissue injury and the presence of many disease agents (Alderman 1981; Lightner & Fontaine 1975). The pigmentation seen in histology slides of infected tissue is the result of host melanisation rather than pigmentation of fungal hyphae because the purple-brown pigment produced by fungal hyphae is water soluble and would not remain following embedding and preparation of tissue sections (Lightner & Fontaine 1975).



(A) (B)



(C) (D)

Plates 11. *Fusarium solani* infection in a lobster.
A and B: Western rock lobster, *Panulirus cygnus*, infected with the fungus, *Fusarium solani*. The telson and uropods are severely damaged and the black pigment, melanin, produced by the lobster's host defence mechanism is visible in the affected areas.
Plates C and D: *Fusarium solani* growing on isolation media. Fungal hyphae produce a brown pigment (C) and aerial hyphae producing spores are a whitish colour (D). The brown pigment produced by the fungus is different from the melanin that is visible on infected lobsters and produced by the lobster in response to infection.
(Photographs courtesy of School of Biomedical Science, Murdoch University)

6. 2. 4 Condition 4: Microsporidiosis

Major pathological characteristics: Uncooked muscles in the tail of affected lobsters are white, resembling those of cooked muscle.

Causative agents: *Ameson* sp. and other genera of Microsporidan

Diagnosis: Demonstration of typical lesions and refractile spores in distended muscle fibres in histological sections. Electron microscopy to demonstrate the morphology of the spores that measure 1.4-1.8 x 2.0-2.4μm.

Epizootiology: Prevalence of affected lobsters (*Panuliris cygnus* and *Panulirus ornatus*) in Australia is low (Dennis & Munday 1994). The morphology of the infective agent is similar to that of the widely distributed microsporidan of prawns, *Ameson nelsoni* (formerly *Nosema nelsoni*) (Sprague 1970; Sprague & Couch 1971). Infection has not been associated with morbidity in lobster in Australia, however, *Panulirus cygnus* suffered mortality when they are stressed.

Plate 12. White tail lobster.
The tail muscle of a western rock lobster, *Panulirus cygnus*, that is severely affected by microsporidian parasites appears white.

6. 2. 5 Condition 5: Turgid lobster disease

Major pathological characteristics: Soft parts of the uncalcified exoskeleton bulge and a clear fluid is visible especially at the base of perieopods and the dorsal carapace-tail junction. Lobsters resemble those that have been 'drowned' during processing.

Causative agents: Unknown

Diagnosis: The gross appearance of affected lobster is diagnostic, in the absence of other factors that result in lobster having a similar appearance such as holding in very low salinity water.

Epizootiology: The aetiology of the disease is not understood but does not appear to be of an infectious nature. The condition is sporadic and usually of low prevalence. Affected lobster stop feeding, become lethargic and may become moribund and die (Diggles 1999). Salinity fluctuations, handling and other stress factors are often not associated with the appearance of the condition.

The most likely reasons for initiation of events leading to the condition are physiological disturbances from starvation or disturbances of osmotic homeostasis during moulting or severe water quality problems such as high ammonia levels (Chen & Chen 1996).

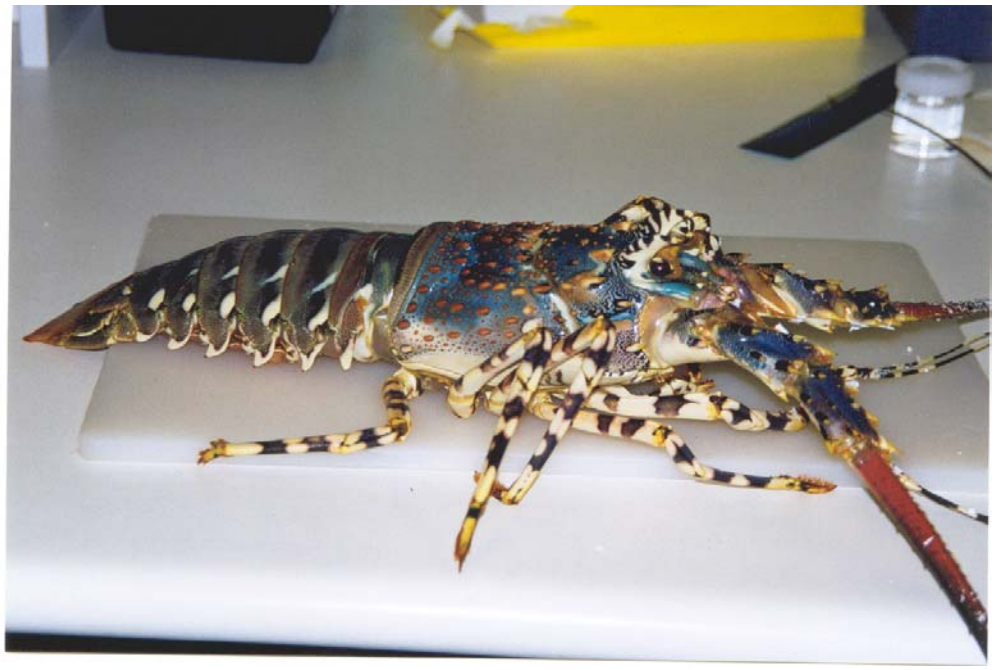


Plate 13. Turgid tropical rock lobster, *Panulirus ornatus*.
A: Note the swollen abdomen and the raised carapace.

13. B: Note the swelling causing protrusion of the arthrodial membrane.

6.2.6 Condition 6: Moult death syndrome

Major pathological characteristics: Death of lobster at or around moulting.

Causative agents: Multiple factors

Diagnosis: Examination of the exuvia, hemolymph and other organs by direct microscopy and histological techniques may reveal the reason for deaths.

Epizootiology: Lobster often dies just before, during or after moulting. The reasons for this phenomenon are varied and, at times, poorly understood. They may be associated with metabolic, homeostasis problems, binding of the old and new shell preventing completion of the moult or from the increased risk of damage to the soft exoskeleton which provides an entry point for pathogens. Deformities may also indicate problems with chitin development or shedding of the exuvia.

The cellular host response of the lobster is thought to prevent separation of the exuvia in conditions such as shell disease (Fisher *et al.* 1978; Floreto *et al* 2000), protozoan infections (Cawthorn 1997) and fungal infection (Lightner & Fontaine 1975). Some examples of death associated with moulting were noted in a nutritional trial (Bowser, Rosemark, 1981). These deaths involved failure to shed parts of the exuvia, most notably the appendages. In each case adhesion of the old and new exoskeleton appeared to cause mortality during or soon after moulting apparently due to globular mineral deposits containing calcium found between the old and new exoskeleton which appeared to have

prevented shedding of some appendages. The investigators concluded that physiological stress from an incompletely balanced diet had resulted in mortality.

6. 2. 7 Condition 7: Pathological lesions suggestive of bladder infection

An inflammatory condition of the bladder and antennal gland of unknown cause has been reported for Western rock lobster (Evans *et al.* 2002). Examples of the lesions are compared to the appearance of an unaffected organ in Plates 14 and 15. The dominant features of the lesions were the high proportion of granular cells sequestered into the tissue spaces and the presence of an eosinophilic precipitate, suggestive of coagulated hemolymph.

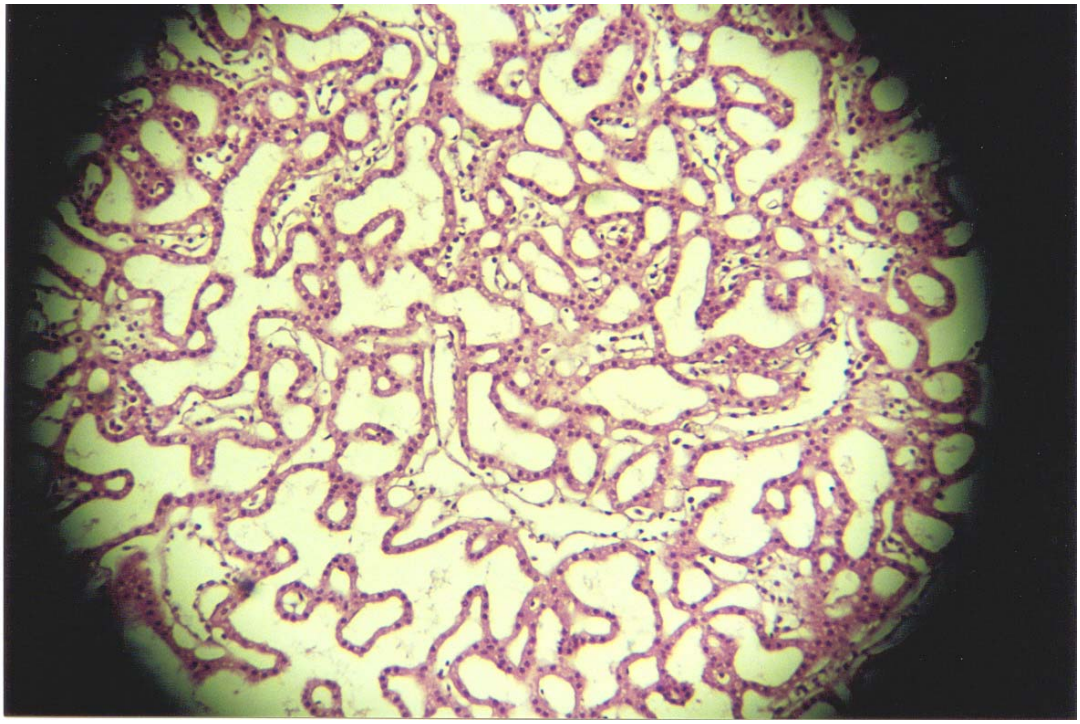
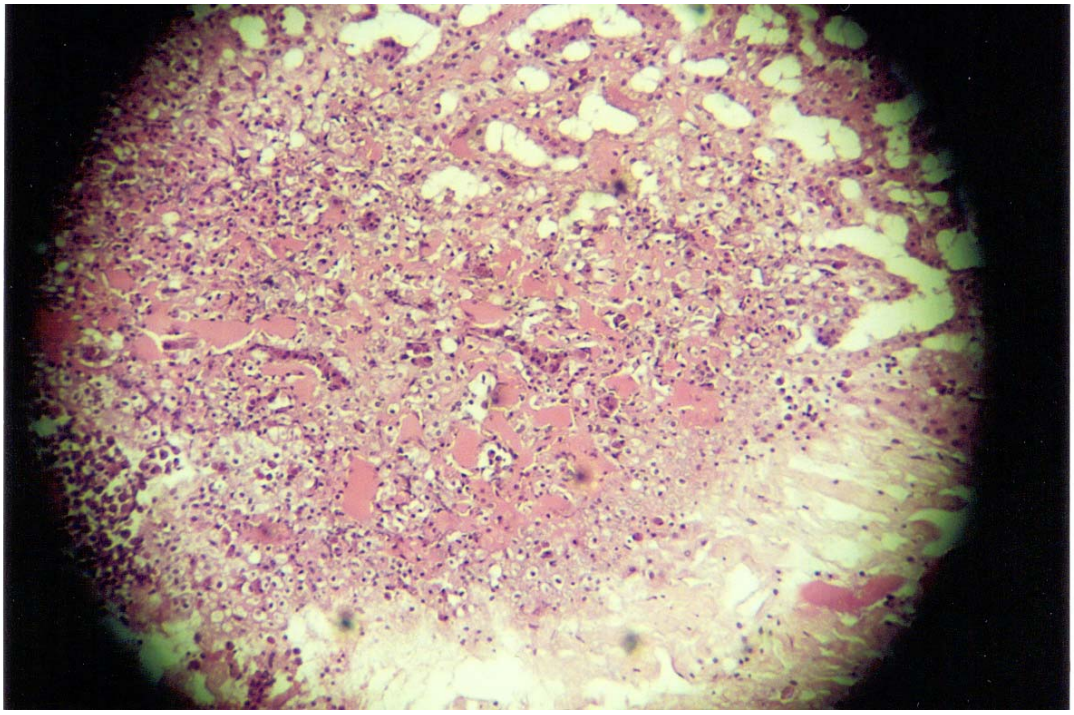


Plate 14. Histology of the antennal gland.
A: Normal histological appearance of antennal gland.



14. B: Histological section of antennal gland showing areas of marked inflammation with hemocytic aggregation and coagulated hemolymph.

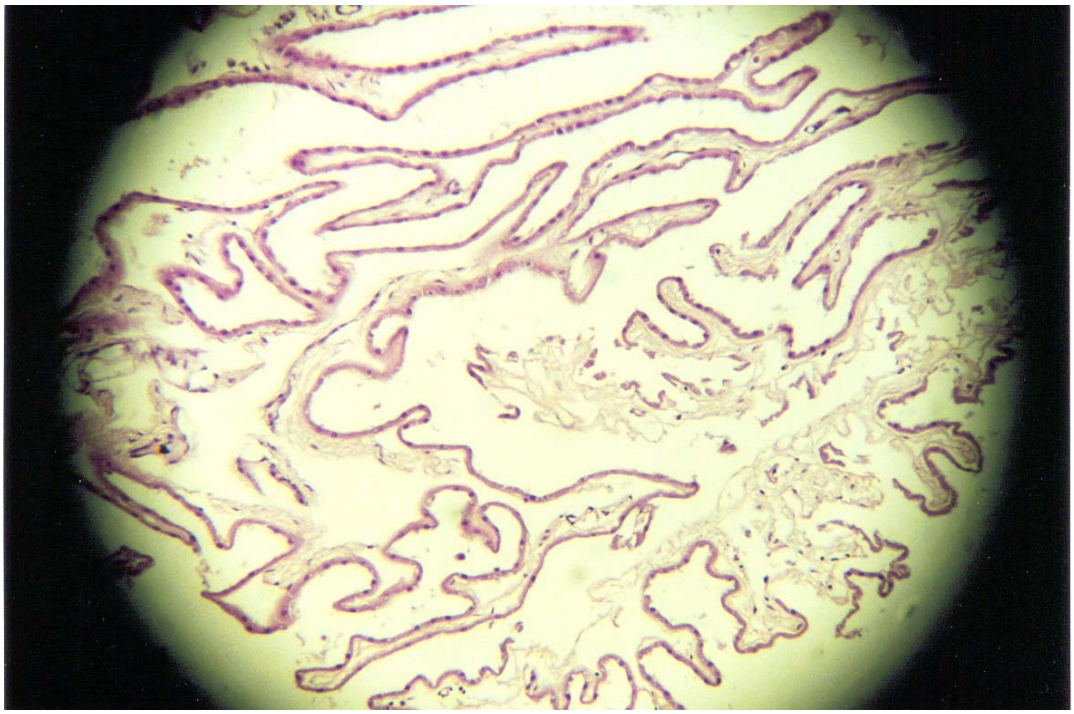
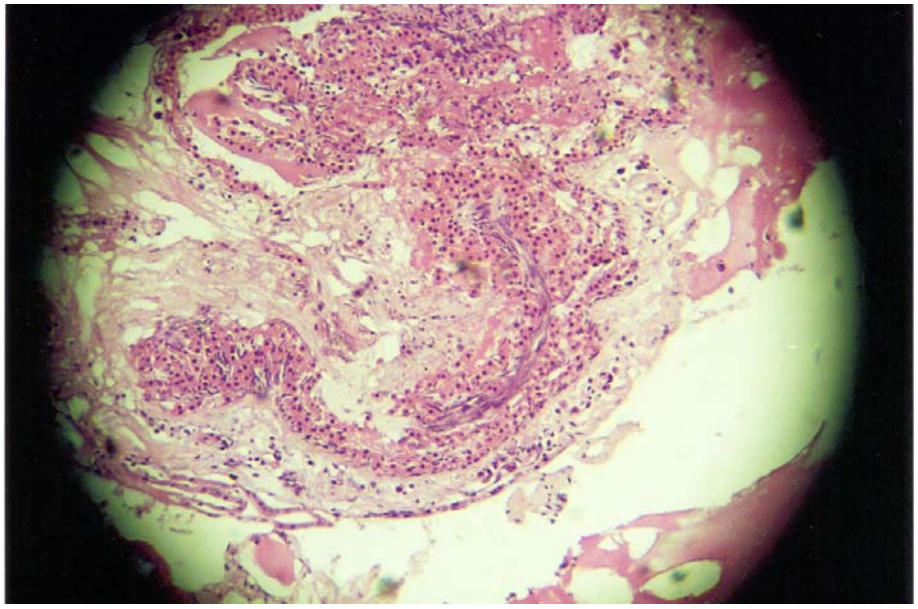


Plate 15. Histology of the bladder.
A: Normal histological appearance of bladder.



15. B: Histological section of bladder showing area of marked inflammation with hemocytic aggregation of predominantly granular cells.

6. 2. 8 Condition 8: Body deformities

Deformities such as that shown in Plate 16 are occasionally observed in Western rock lobster.

Plate 16. Antennal deformity.
A western rock lobster, *Panulirus cygnus*, with a deformed antenna. This type of deformity is likely to result from incomplete separation of the old and new exoskeleton at moulting. It may indicate inadequate nutritioadhesions caused by the cellular host response to infection with bacteria, protozoan parasites or fungi.

6. 3 Conditions not reported in Australia

6. 3. 1 Condition 1: Gaffkaemia (sometimes known as red tail disease)

Major pathological characteristics: Lethargy, anorexia, mortality (sometimes high), pink ventral abdomen. Moribund lobsters lie on their side in distress and may autotomize their chelipeds (Fisher *et al.* 1978; Snieszko & Taylor 1947).

Causative agents: *Aerococcus viridans* var. *homari* - coccoid, non motile, gram-negative bacteria that form tetrads and are 0. 8-1. 1um in diameter.

Diagnosis: There are three steps or methods that can be used in the diagnosis of gaffkaemia. First, clinical signs of infected lobster are consistent with the disease. Next, gram-stained hemolymph smears examined using oil immersion microscopy show characteristic bacteria in tetrad formations. Finally presumptive testing using selective enrichment media followed by confirmatory biochemical and other tests are used to reach a definitive diagnosis (Fisher *et al.* 1978; Wiik *et al.* 1987).

Epizootiology: The disease was first described in 1947 in American lobster, *Homarus americanus,* (Snieszko & Taylor 1947) and the bacterium causing the disease is only found on lobsters or in sediment in lobster habitats (Fisher *et al* 1978; Kellog *et al.* 1974; Rabin, 1965; Stewart & MacDonald 1962). Gaffkaemia affects North American and European lobsters of the *Homarus* genus and is endemic in Canada and eastern parts of the USA (Stewart *et al.* 1966). It has been introduced to Europe by importation of lobster from North America (Alderman 1996; Jørstad *et al.* 1999). The disease has also been reported or experimentally induced in *Panulirus interruptus* (Schapiro *et al.* 1974; Steenbergen & Schapiro 1974). Although prevalence in wild populations is generally low it can be as high as 40% (Rabin 1965; Stewart *et al.* 1966) and is more common in lobsters that are downgraded because of weakness, damaged exoskeleton damage or loss of appendages (Lavallée *et al.* 2001). *Aerococcus viridans* var. *homari* can survive in mud or detritus for several months and at temperatures as low as 1°C (Stewart 1984).

The disease can result from inoculation with as few as 5 bacteria (Cornick & Stewart 1968) and is more prevalent during summer months (Snieszko & Taylor, 1947). Mortality occurs more than 60 days after infection when lobsters are held at 3°C, but takes only 2 days in lobsters held at 20°C (Stewart *et al.* 1969).

The infectious agent is introduced into lobster through cracks or punctures in the exoskeleton, but once it has gained entry to the haemocoel of the lobster, the host response mechanisms are unable to limit and control the replication and spread of the infectious agent (Cornick & Stewart 1968; Stewart 1984). Bacteria colonize the hepatopancreas and heart (Johnson *et al.* 1981) and are phagocytosed by hemocytes. However, hemocytes are often destroyed whilst the infectious agent thrives in the nutrients within the lobster (Stewart 1984). The bacteria then spread to the hemolymph resulting in a septicaemia. The hemolymph of infected lobsters may be a pink colour, contain very few hemocytes, have a prolonged clotting time and low viscosity (Snieszko & Taylor 1947; Stewart 1984).

The causative agent is similar to the ubiquitous *Aerococcus viridans* (Fisher *et al.* 1978; Kelly & Evans 1974) that occurs in Australian waters (N. Buller, personal communication).

Vaccination has is effective in reducing mortality (Paterson & Keith 1992; Schapiro & Steenbergen 1974; Schapiro *et al.* 1974; Stewart & Zwicker 1974).

6.3.2 Condition 2: Bumper car disease

Major pathological characteristics: Weakness, anorexia and lethargy. Lobster may be 'spread-eagled'. Meat yield is reduced and meat has poor quality and flavour.

Causative agents: *Anophryoides haemophila*

Diagnosis: Wet preparations of hemolymph examined with a light microscope reveal the ovoid, scuticociliate which is approximately 35um in length. An indirect fluorescent antibody test using monoclonal antibodies has been developed and used in Canada (Cawthorn *et al.* 1996; Lavallée *et al.* 2001). The parasite consumes hemocytes (Loughlin *et al.* 1998) and sequesters in the heart, gill and abdominal muscle (Cawthorn 1997).

Epizootiology: The condition occurs in the eastern parts of the USA and Canada and is caused by a cold-water ciliated protozoan (Cawthorn *et al.* 1996). The ciliate is a facultative endoparasite (Speare *et al.* 1996) that multiplies by binary fission (Cawthorn 1997). Lobster are most often infected when water temperatures are 1-4°C, however, the disease occurs between 0-10°C (Cawthorn 1997). Lobsters often die during and shortly after moulting. The route of infection may be damage to the exoskeleton caused by fighting or the thin exoskeleton of the gills (Cawthorn 1997).

Prevalence in wild lobster varies, with up to 17. 8% of lobsters infected in one study (Cawthorn *et al.* 1996), but only 0. 4% in another (Lavallée *et al.* 2001).

Vaccination has been successful in reducing losses from gaffkaemia (Paterson & Keith, 1992).

6. 3. 3 Condition 3: Syndinean dinoflagellate infection

Major pathological characteristics: Heavily infected lobsters have a dull orange colour, are lethargic, have increased mortality, 'watery' muscles, milky-white hemolymph and a green shrunken hapatopancreas (Field *et al.* 1992).

Causative agents: *Haematodinium* sp. (Order: Syndiniales)

Diagnosis: Diagnosis is made following demonstration of typical protists in the hemolymph (Field *et al.* 1992) or indirect fluorescent antibody. The parasites have an irregular, roundish shape, are non motile in hemolymph and approximately 6-10um in diameter (Field *et al.* 1992).

Histological sections show distension of haemal spaces of the heart, gills, midgut and haematopoietic tissue and lysis of muscle and hepatopancreas. The haematopoietic tissue is enlarged and hemocyte as well as dinoflagellate numbers increase with severity of infection. Narrow haemal spaces such as those in the gills may be occluded by dinoflagellates or hemocyte aggregations (Field & Appleton, 1995).

Epizootiology: The disease is most prevalent in females *Nephrops norvegicus* in western Scotland in spring (Field *et al.* 1992). The background prevalence of the disease is thought to be 10-15%; however, up to 70% of sampled lobsters have been infected in spring in some areas. The number and velocity of tail flips are reduced in infected lobsters this is thought to reduce swimming activity and increase the risk of predation and possibly capture by trawling (Stentiford *et al.* 2000). Water temperatures of 8°C initiated a series of events that resulted in the production of flagellated dinospores *in vitro* (Appleton & Vickerman, 1998)and it is possible that once water temperature rises above 8°C there is increased numbers of parasites and an increased rate of transmission of infection to previously uninfected lobsters.

The disease is more prevalent in female and in post moult lobsters. As spring is the main annual moulting period for this species of lobster, with females moulting before males, it has been suggested that injuries to the soft cuticle or increased transmission by ingestion (because freshly moulted animals bury themselves in the sediment) may increase the infection rate in recently moulted individuals (Appleton & Vickerman, 1998).

Hemolymph of infected lobsters has a reduced copper content and hemocyanin has a lower oxygen-carrying capacity than that of uninfected animals (Field *et al.* 1992). The number of cells in the hemolymph is increased from both the presence of the parasite and increased numbers of hemocytes.

6.3.4 Condition 4: Lobster die-off (Maine-USA)

Major pathological characteristics: Lethargy followed by death with no obvious lesions.

Causative agents: Unknown

Diagnosis: Necropsy of moribund animals and histology, bacteriology, water quality analysis and virology are required to provide a differential and definitive diagnosis of the possible cause of mortality.

Epizootiology: Mortalities have occurred in summer months for several years in the Maine and New Brunswick areas of the USA. This has resulted in decline in catches and significant numbers of dead lobsters being found in pulled traps, and mortality in lobster pounds (Adams 1998). Several possible reasons have been suggested for the die-offs but no single aetiology has been consistently linked to mortality.

Some suggested causes are bacteraemia or viral infections (Pollack 1999). Pollution, low dissolved oxygen associated with algal blooms in water and gaffkaemia have been investigated as possible causes of the problem but investigations have not found any evidence to link these conditions with the occurrence of peaks of mortality (Pollack 1999). No lesions are seen on lobster but *Vibrio* spp. have been cultured from the hemolymph of some affected lobsters (Promed 1999).

6. 3. 5 Condition 5: Lobster Herpes virus

Major pathological characteristics: Lethargy and death, the hemolymph is white.

Causative agents: Herpes-like virus (HLV-PA)

Diagnosis: Hemolymph has a milky white appearance. Specialist laboratory techniques are required to identify the virus.

Epizootiology: The virus is reported to result in 100% mortality in infected Caribbean spiny lobster (*Panulirus argus*). It is spread by horizontal transmission. Prevalence is thought to reach 30% in juvenile lobster. The host range and predisposing factors are unknown at the present time (Promed, 2003).

6. 4 Treatment of rock lobster diseases

There are currently no treatments registered by the Australian Pesticides and Veterinary Medicines Authority for use on rock lobster. This means that chemotherapeutic substances used to treat rock lobster are used 'off label'. In many instances it is recommended that a veterinarian be consulted, who can then use his/her skills to ensure that the most effective and suitable treatment is used and that the dose and withholding period reduce the risk of toxicity to handlers and lobsters and minimizes the risk of residues occurring in lobster meat.

When a health problem occurs in a lobster holding facility, the first step in disease investigation should be a careful review of management practices and water quality. In many instances, rectification of some aspect of management of the lobsters will result in improvement of the health of the lobsters without the need to resort to expansive and potentially dangerous chemicals.

All treatments will stress lobster and the advantages and disadvantages of treatment need to be carefully considered. In some instance's, however, treatment may be the preferred option. In general terms, antibiotics or antibacterial disinfectants are used to treat bacterial infections, but they should only be used if the bacteria causing infection have been isolated and identified. A suitable antibiotic is selected by a veterinarian on the basis of the results of laboratory culture and sensitivity testing.

Non specific treatments such as formalin baths may be useful in removing external protozoan parasites but should only be used in consultation with a veterinarian after accurate diagnosis of the cause of the condition.

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CHAPTER 7

DISEASE CONDITIONS OF LARVAL AND JUVENILE SPINY LOBSTERS

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Methods

7. 1 Phyllosoma larvae

For gross examination, whole phyllosomas may be examined by direct microscopy in well slides or with a dissecting microscope if 4th instar or older. Examination of appendages or live whole larvae will visualize external fouling. Phase contrast examination of live larvae allows assessment of the adequacy of that hemolymph flow and hemocyte numbers. Heavy bacterial infection and focal granulomas may also be visualized.

External fouling bacteria are seldom cultured. For culture of internal bacteria, surface sterilize phyllosomas in 70% ethanol for 5 seconds, then blot dry before macerating the whole animal. If bacteriology and histopathology are required on the same animal, larger phyllosoma can be sterilized then sectioned medially (Figure 4) with fine, flame sterilized scissors. One-half can be fixed immediately, while the other can be utilized for bacteriology. If bacteriology is not required, phyllosomas can be fixed whole.

Fixative choice will depend on whether electron microscopy is anticipated, and on the processing method. Good histological preparation can be obtained using standard paraffin blocks and staining after double embedding smaller larvae (1st to 5th instar) in agar to facilitate handling during processing. For this purpose a range of fixatives appears suitable, including 10% formalin in seawater, Davidson's seawater fixative, and combined formaldehyde/glutaraldehyde fixative (4F:1G, see Appendix 1). Specimens fixed in Davidson's fixative should be processed or transferred to 70% alcohol after 24 hours. Prior to processing, decalcify larger phyllosoma (5th instar and older) with standard decalcification fluid diluted 1:10 with distilled water for approximately 10 minutes, then rinse with distilled water.

Larvae are embedded in agar by placing the fixed specimen into a suitably sized container such as a cavity slide, multiwell cell culture plate, or small petri dish. Melted but cooled agar (2%) is then dropped with a pipette over the larvae to enclose the entire specimen. Smaller larvae (up to 5th instar) can be embedded whole. Larger animals may need to be sectioned transversely between the abdomen and thorax then longitudinally

with perieopods (legs) trimmed, using a razor blade on a dental wax cutting surface. Once cooled the agar can be removed from the mould and then processed routinely with hematoxylin and eosin or other stains. For most routine purposes, orient the agar block to give flat whole-body sections (Plate 17.A). For detailed examination of gut, perpendicular orientation to give transverse sections (Plate 17.B) may be preferable but will require more sections to examine all organs.

For detailed study of cell changes and for electron microscopy, embedding in resin after fixation in fixatives containing glutaraldehyde may be preferred. Gluteraldehyde (2. 5%) in 0.22µm filtered seawater is suitable for electron microscopy, as are glutaraldehyde mixtures. The latter are preferable for light microscopy (see Appendix 1). Hard resins such as Spurr's resin are required for electron microscopy. With hard resins, good light microscopy sections may be difficult and time consuming to achieve. Where electron microscopy is not required, soft resins such as glycol methachrylate resin are more practical to use and give better light microscopy results (Johnson and Ritar, 2001).

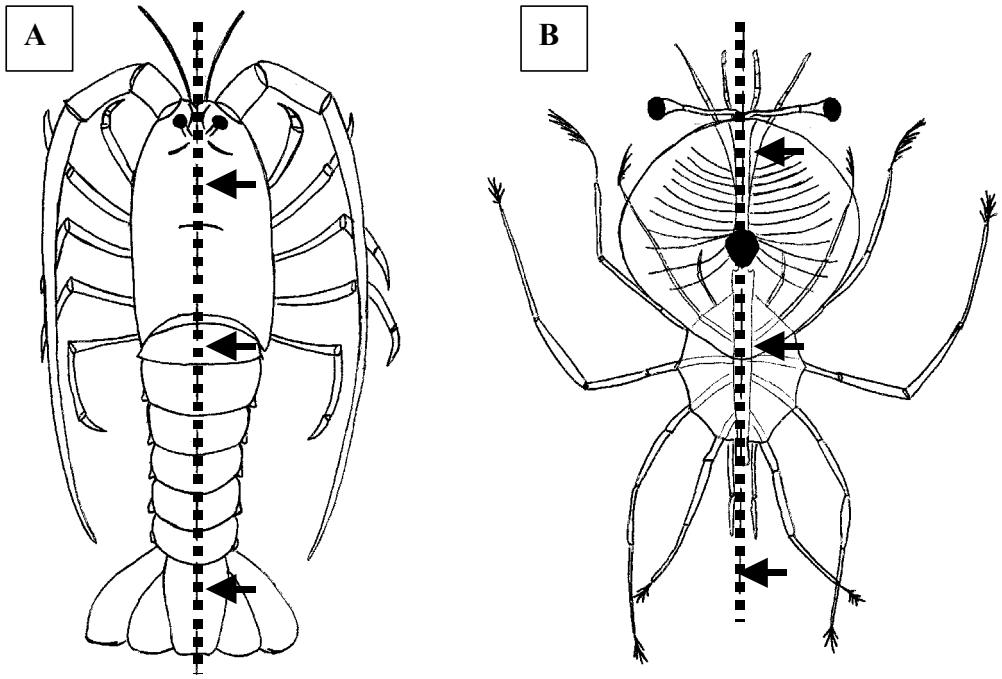


Figure 4. Orientation of medial/sagittal sections through a juvenile (A) and phyllosoma larvae (B) of a spiny lobster.
Figures not to scale.

7. 2 Juveniles

For juveniles up to around 25–30mm carapace length (CL), examination methods are similar to those for adult lobsters except that dissection of individual organs for fixation is unnecessary. Juvenile lobsters should first be examined for gross fouling, shell disease and moult stage, and hemolymph collected if required. For juveniles the most convenient site for hemolymph collection is the dorsal sinus. Over 25 mm CL the base of the 5th leg

can be used. For bacterial culture, hemolymph is generally the preferred sample, though culture from lesions may also be useful.

Tissues should be fixed immediately after euthanasia, which can be done by medial or slightly sagittal section of the cephalothorax (Figure 4) with an appropriate sized pair of sharp scissors. Fixation in the intact shell is unsuitable, as this will prevent rapid penetration of fixative. One-half is fixed immediately, to provide medial to sagittal whole body histological sections. The other half is used for the remaining microscopic examinations. Wet preparations are prepared on a standard area of gill (Plate 17.C), and any organs with lesions, and examined immediately. Additional material may be fixed for transverse sections of gills (Plate 17.D) and antennal gland.

A range of fixatives appears suitable, provided there is good tissue penetration. Davidson's fixative provides excellent tissue outlines but may result in poor differential staining compared to 10 % formalin in seawater. 4F:1G fixative produces excellent fixation and is more suitable for subsequent electron microscopy, but is more expensive for routine use. Electron microscopy is generally possible on material fixed in 10% formalin fixative, though the quality is reduced. Davidson's fixative is unsuitable for this purpose.

For most juveniles up to 25–30 mm CL, the medial/sagittal sectioned fixed half is usually decalcified and trimmed to provide slightly sagittal sections of the whole animal including the hepatopancreas (Plate 17.E), which is the major organ of interest, and other areas (Plate 17.F). Additional transverse sections may be needed of tail muscle and hind gut.

7. 3. Overview of normal microscopic anatomy of larval and juvenile spiny lobsters.

Plates 17 provide a brief overview of normal microscopic anatomy of phyllosoma larvae (Plates A and B) and juvenile (Plates C to F) spiny lobster.

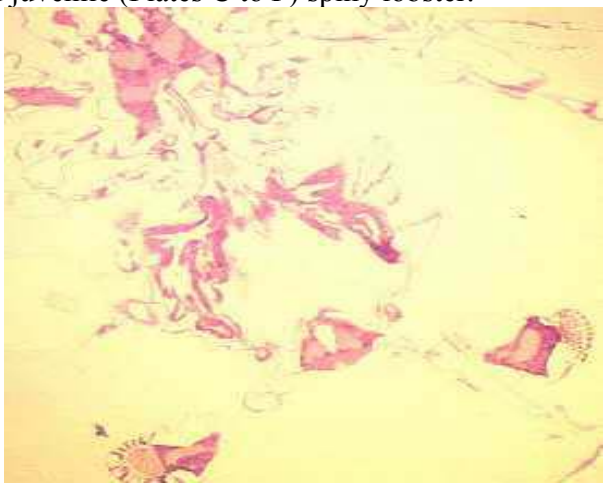
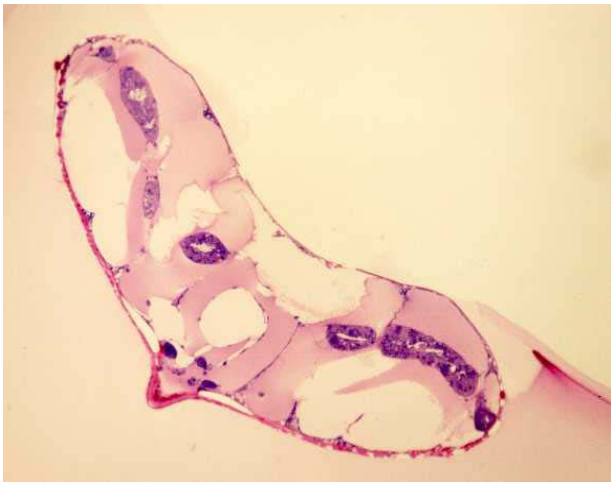
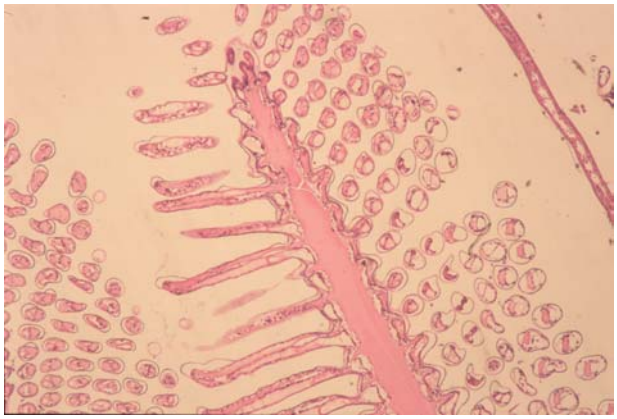


Plate 17. Histology of normal phyllosoma larvae of southern rock lobster, *Jasus edwardsii*.
A: Whole body section of phyllosoma larvae after double embedding in agar and standard processing. H&E stain, 20x magnification.

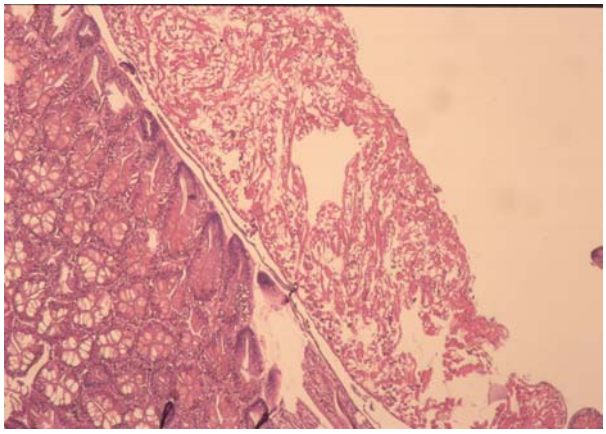


B: Transverse section of phyllosoma, embedded in soft resin, H&E stain, 20x magnification.

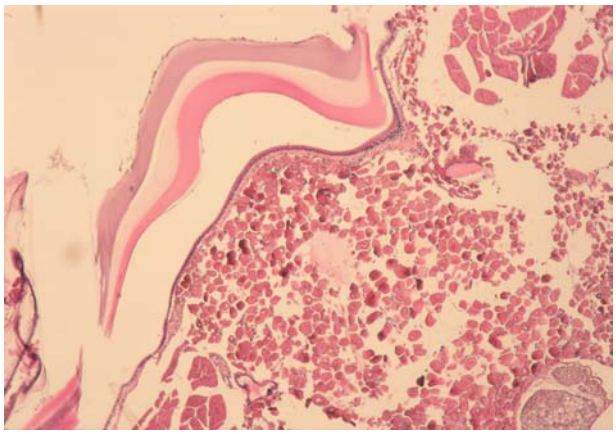
C: Wet preparation of normal gill of juvenile, 200x magnification.



D: Histology of normal gill, H&E stain, 100x magnification.



E: Histology of normal juvenile hepatopancreas (Hp) and heart (H), H&E stain, 100x magnification.



F: Histology of sub cuticular connective tissue showing cuticle (C) and large eosinophilic cells (R) thought to be reserve cells, H&E stain, 100x magnification

For a more detailed descriptions of gut morphology and development of phyllosoma larvae, see Johnson and Ritar (2001).

7. 4 Disease conditions recognized in phyllosoma larvae of spiny lobsters

Health monitoring has been carried out on phyllosoma larvae of *Jasus verreauxi* in New Zealand and *Jasus edwardsii* in Australia. All diseases detected to date have been bacterial in origin.

7. 4. 1 Condition 1: Epibiont infestation

Major pathological characteristic: External shell fouling.

Causative agent: Filamentous *Leucothrix* -like bacteria, sessile ciliates, associated flora

Diagnosis: Wet preparations of whole larvae can be observed with a light microscope. Numerous *Leucothrix*-like filamentous bacteria and sometimes sessile ciliates can be

observed attached to the perieopods and carapace in affected larvae (Plate 18). Fouling can also be detected in histological sections (Plate 19).

Epizootiology: Epibiont infestation of phyllosoma larvae with a *Leucothrix*-like filamentous bacterium has been recorded in intensive culture of *Jasus verreauxi* larvae in New Zealand. There is evidence that the filamentous bacteria are introduced with live food as heavy infestations of filamentous epibionts were found on *Artemia* cysts which were not completely separated from live *Artemia* prior to feeding to phyllosomas. Heavy epibiont infestations may adversely affect the growth and survival of phyllosomas due to increased energetic requirements for swimming and reduced oxygen uptake through the carapace. Heavy infestations may also contribute to death due to failure to completely cast off the moult.

Except for newly hatched larvae, *Leucothrix*-like bacteria were seen consistently throughout various instar levels of phyllosoma of *J. edwardsii* in Tasmania (Handlinger *et al*; 1999). This was part of mixed fouling, which was exacerbated by poor oxygen levels and high ammonia resulting from poor water flow patterns. Heavy fouling with *Leucothrix*-like bacteria and sessile ciliates (plate 19) was associated with focal erosion of the exoskeleton by non-*Leucothrix* bacteria, resulting in sub-dermal granulomas and ongoing low levels of mortality. *Vibrio* bacteria have been visualized in the granulomas. Various *Vibrio* species including *V. alginolyticus* and *V. tubiashi* have been implicated. Examination of *Artemia* fed to phyllosoma suggested these were not a likely source of fouling in this case.

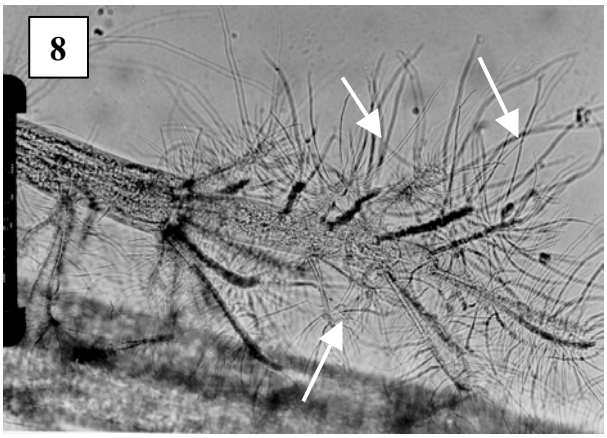


Plate 18. Fouling of perieopod.
Wet preparation of perieopod of a *Jasus verreauxi* phyllosoma larvae heavily fouled with *Leucothrix*-like filamentous bacteria (arrows), 100x magnification.

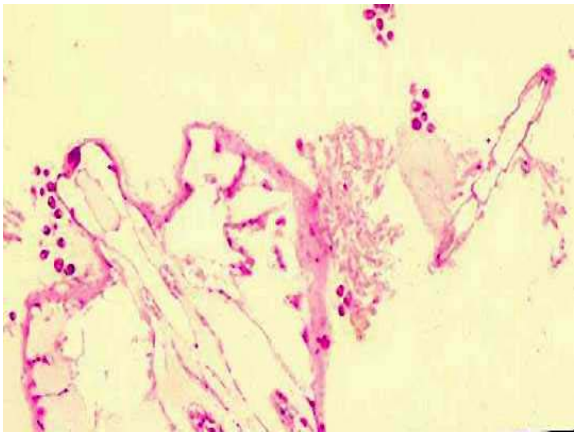


Plate 19: Histology section of *Jasus edwardsii* phyllosoma with heavy fouling with *Leucothrix*-like bacteria and sessile ciliates.
Double embedded in agar and paraffin, standard processing, H&E staining, 40x magnification.

7. 4. 2 Condition 2: General vibriosis

Major pathological characteristic: Enteritis and/or septicemia

Causative agent: Various *Vibrio* species, probably including uncharacterized species.

Diagnosis: Presumptive diagnosis can sometimes be obtained by observing larvae exhibiting a blackened hepatopancreas. Bacteria are evident in hepatopancreas tubules using wet preparations of whole larvae or histopathology (Plate 20.A), and there may be extensive erosion of hepatopancreas epithelium (Plate 20.B). Secondary septicemia may follow complete gut epithelial erosion.

Epizootiology: Bacterial disease associated with blackening of the hepatopancreas has been recorded in intensive culture of phyllosoma larvae of *Jasus verreauxi* in New Zealand. Bacterial disease has also been observed in *J. edwardsii* in Australia (Handlinger *et al.* 1999) though no gross observations of gut blackening were recorded in these instances. In New Zealand the disease has been observed in phyllosomas as early as the 5th instar stage when cultured at water temperatures of 20°C. Outbreaks of this disease in *J. verreauxi* phyllosomas are characterized by consistent low level mortalities of around 1 to 3 % per day, and may be associated with concurrent outbreaks of luminous vibriosis. Bacteria associated hepatopancreas erosion has been seen as early as the 2nd instar stage of *J. edwardsii* from which a variety of *Vibrio* species, including *V. anguillarum*, were isolated.

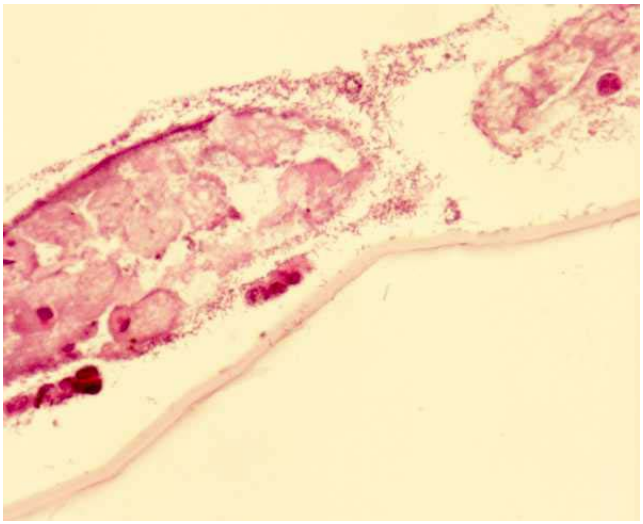
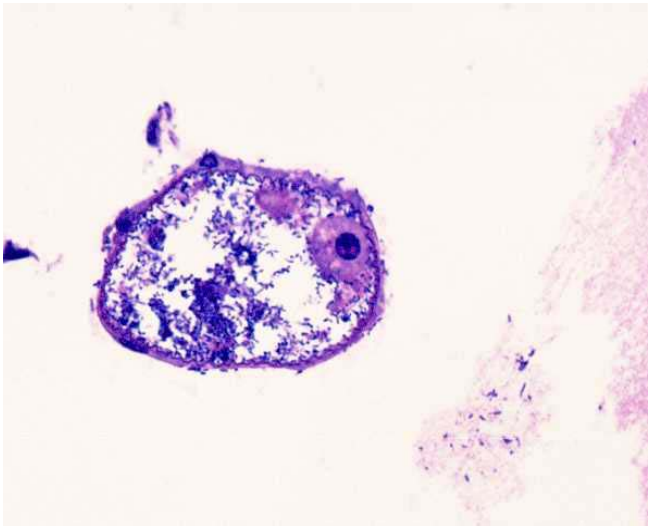


Plate 20. Bacterial infection in the hepatopancreas.
A: Hepatopancreas tubules of *Jasus edwardsii* phyllosoma larvae infected by large numbers of bacteria within and surrounding hepatopancreas tubules. 100x magnification, paraffin double embedded section.



B: Higher magnification of hepatopancreas tubule showing numerous bacteria *Vibrio* sp. in tubule lumen, extensive erosion of hepatopancreas epithelium and bacteria in hemolymph , soft resin section, 400x magnification.

7. 4. 3 Condition 3: Luminous vibriosis

Major pathological characteristic: Septicemia originating in the hepatopancreas due to luminous bacteria. Can be considered as similar condition to general vibriosis except that affected larvae are luminous and the disease spreads rapidly through the population.

Causative agent: *Vibrio harveyi*

Diagnosis: Presumptive diagnosis can sometimes be obtained by observing larvae in complete darkness. Those affected by *V. harveyi* can be faintly luminous in the dark. Massive bacterial plaques will be evident in the lumen of hepatopancreas tubules in wet preparations of whole larvae (Plate 21.A) or histopathology. This may be followed by septicemia (Plate 21.B). Definitive diagnosis requires culture of *V. harveyi* from internal organs on media such as thiosulphate citrate bile salt sucrose agar (TCBS), or tryptic soy agar with 2% NaCl (TSA+2), then incubated at 20°C overnight prior to biochemical characterization.

Epizootiology: Luminous vibriosis has been recorded in intensive culture of phyllosoma larvae of *Jasus verreauxi* in New Zealand (Diggles *et al.*2000). Onset of disease occurs as early as the 4th instar at water temperatures above 20°C. Disease outbreaks are characterized by consistent low level mortalities of around 3 to 5% per day. Total losses of up to 80% have been attributed to the disease. Mortality rate is increased if larvae are injured by excessive handling. There is evidence that infection can also be transferred by cannibalism of dead larvae as well as through the culture water. *Vibrio harveyi* may be introduced into larval culture tanks through the water or via live food such as *Artemia*.

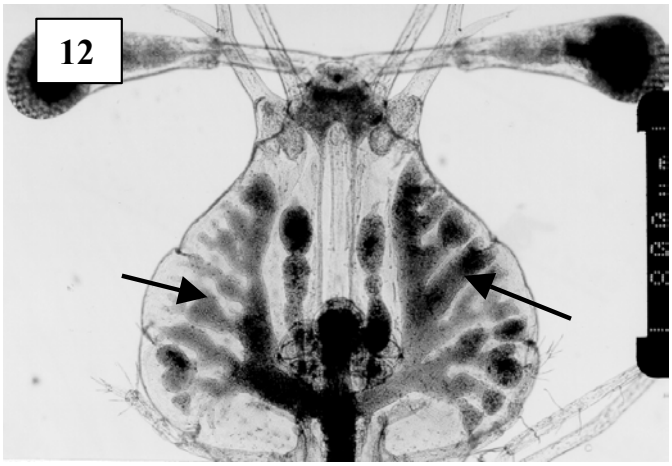
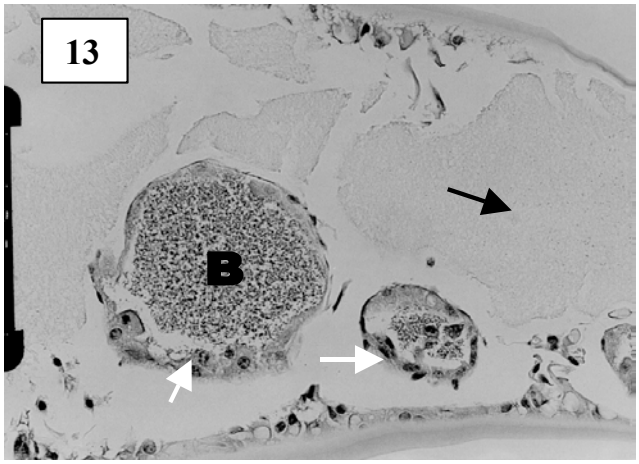


Plate 21. Luminous vibriosis of phyllosoma larvae of *Jasus verreauxi*.
A: Wet preparation of a 4th instar phyllosoma infected with *Vibrio harveyi*. Note the opaque hepatopancreas filled with bacteria (arrows), scale bar = 800 µm.



21. B: Luminous vibriosis of phyllosoma larvae of *Jasus verreauxi*.
Histopathology of two hepatopancreas tubules of a luminous phyllosoma. Note atrophy of hepatopancreocytes (white arrows), masses of bacteria (B) in tubule lumens and bacteria scattered in the hemolymph (black arrow), paraffin double embedded section, H&E, scale bar = 128 µm.

7. 5 Disease conditions recognized in juvenile spiny lobsters

Juveniles of *Jasus edwardsii* have been examined in New Zealand and Tasmania.

7. 5. 1 Condition 1: Shell disease

Major pathological characteristic: Erosion and blackening of the carapace, particularly the tail fan and walking legs.

Causative agent: Chitinoclastic bacteria, unidentified fungi.

Diagnosis: Erosion and/or blackening of affected areas of the cuticle are evident upon gross observation (Plate 22.A). Microscopic examination of tissue biopsies from the affected areas can be used to attempt to determine whether disease is associated with bacterial or fungal invaders. Both pathogens are likely to be present and may be visible in wet preparation smears (Plate 22.B).

Epizootiology: Shell disease has been recorded in wild and captive *Jasus edwardsii* and *J. verreauxi* in New Zealand, and *Jasus edwardsii* in Australia. Shell disease lesions are most obvious on the ventral part of the tail fan and other areas of the carapace in contact with bottom surfaces, or subject to injury. The primary cause is assumed to be infection by chitinoclastic bacteria. However some melanised fungal hyphae have been observed in shell disease lesions in adults of both species and following injury in a juvenile *J. edwardsii* from Tasmania.

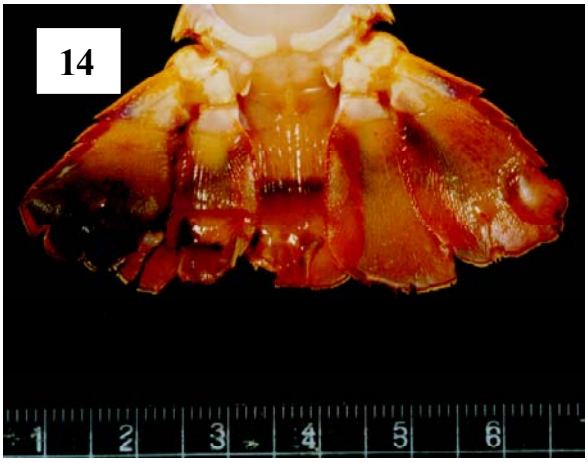
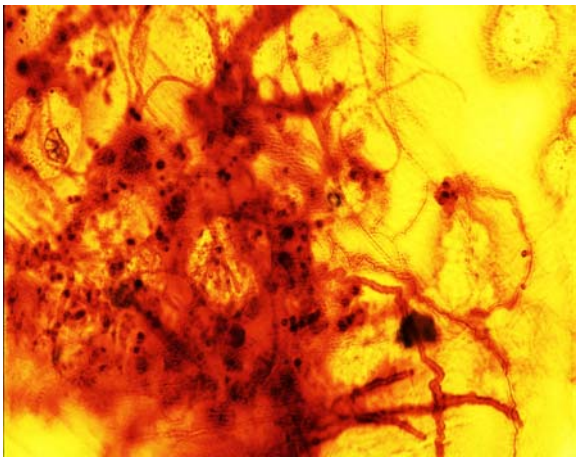


Plate 22. Shell disease.
A: Gross appearance of shell disease of tail fan of *Jasus edwardsii*, with cuticle blackening, erosion and a blistered appearance evident at the distal tips, scale in cm.



B: Wet preparation of a shell disease lesion in *Jasus verreauxi* showing melanisation at edges of circular lesions caused by chitinoclastic bacteria, and melanised tracks caused by fungal hyphae, 10 x magnification.

7. 5. 2 Condition 2: Epibiont infestation

Major pathological characteristic: Fouling of external surfaces, particularly gills, carapace.

Causative agents: Filamentous *Leucothrix* -like bacteria, sessile ciliates (*Carchesium* sp. , *Epistylis* sp. , *Zoothamnium* sp.), free living nematodes, organic detritus.

Diagnosis: Mixed growths of filamentous *Leucothrix*-like bacteria (Plates 23. A and B), sessile stalked ciliates (*Carchesium* sp., *Epistylis* sp. , *Zoothamnium* sp.) (Plate 24), and

sometimes organic detritus (dirt, faeces), free living nematodes (Plate 25), flagellates or amoebae, are evident when affected areas of gills are examined using wet squashes or histology. Heavy fouling may be associated with gill necrosis.

Epizootiology: Persistent low level mortalities of juvenile lobsters *Jasus edwardsii* in rearing systems utilising recirculated seawater were associated with moderate to heavy growths of epibionts (Diggles 1999). Affected animals showed sluggish behaviour and foci of light brown colouration in the gills. Most deaths of affected lobsters occurred just prior to or during the moult. It appears likely that heavy epibiont growth reduces respiratory effectiveness, as observed in penaeid shrimp (Lightner 1983). This may explain why animals died during the moult as oxygen demand in *J edwardsii* increases at night (Crear & Forteath 1998) when moulting usually occurs. Epibionts appear to be gradually accumulated over time. Scrapes of tank surfaces showed a buildup of organic detritus and moderate to heavy fouling with all of the types of epibionts found on lobster gills, indicating poor system hygiene as the cause of the condition. Poor oxygenation may not always be obvious. Fouling associated losses have been seen in a system where regular surface cleaning prevented heavy tank fouling and where oxygen measurement at the tank exit was adequate. However flow through hides was irregular and losses ceased within 48 hours of increasing water flow and aeration. High temperatures increased the need for adequate water flow.

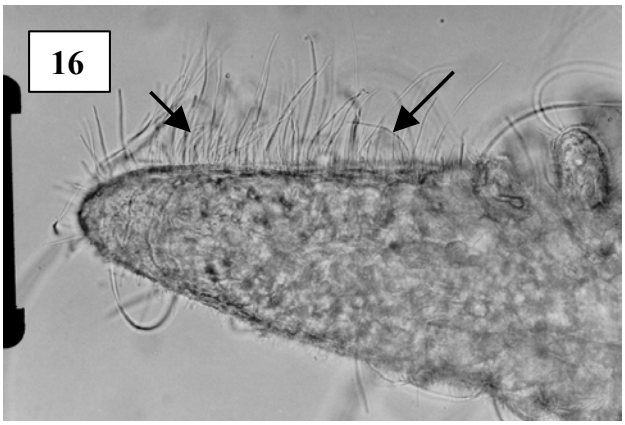
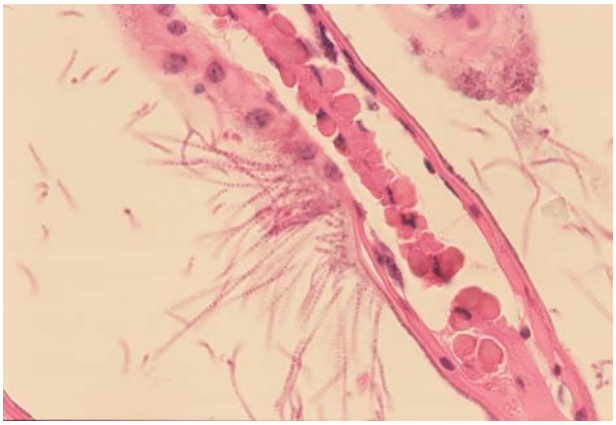


Plate 23. Fouling of the gill with *Leucothrix*-like filamentous bacteria.

A: Wet preparation of moderate infestation of *Leucothrix*-like filamentous bacteria (arrows) on gills of juvenile *Jasus edwardsii*, 200 x magnification.



B: Histology of juvenile *Jasus edwardsii* gill showing striated *Leucothrix*-like filamentous bacteria and associated erosion of the gill cuticle, H&E, 200 x magnification.

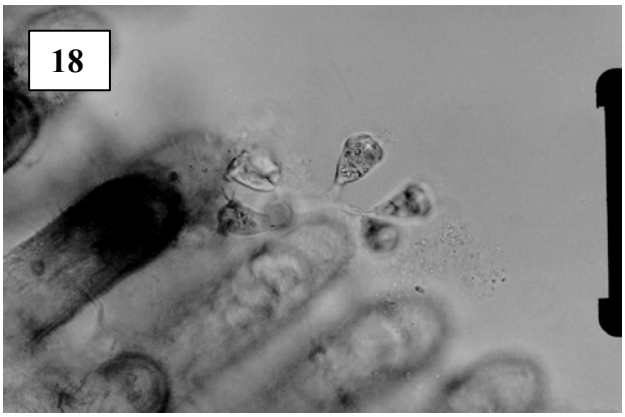


Plate 24. Sessile ciliate (*Carchesium* sp.) attached to juvenile *Jasus edwardsii* gill cuticle.
Wet preparation, 200 x magnification.

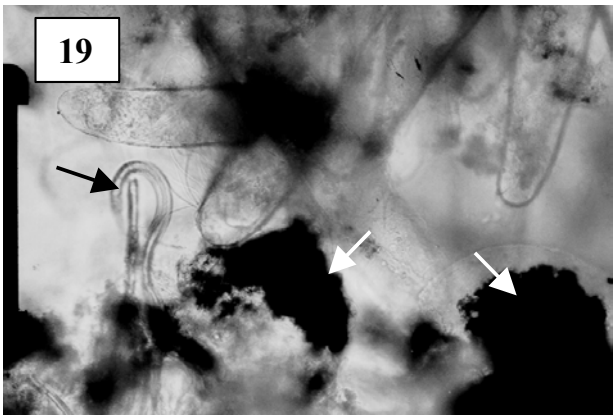


Plate 25. Fouling of the gills of juvenile *Jasus edwardsii*,
Organic detritus (white arrows), free living nematode (black arrow) and moderate *Leucothrix*-like infestation, wet preparation, 100 x magnification.

7. 5. 3 Condition 3. Bacterial enteritis

Major pathological characteristic: Enteric bacterial erosion, usually without septicemia.

Causative agent: Possibly *Vibrio* sp.

Diagnosis: Focal erosions of hepatopancreas epithelium associated with bacterial clumps, usually detected by histopathology (Plate 26). Complete erosion may elicit a granulomatous reaction (Plate 27).

Epizootiology: These lesions have been seen in *J. edwardsii* in both New Zealand and Australia. In New Zealand bacterial enteritis has been recorded in heavily fouled moribund lobsters which had ceased feeding. It is suggested in that case the bacterial enteritis was secondary and related to cessation of feeding. In Australia gram negative bacteria were seen within typical degenerate tubules and associated granulomas in a group of newly collected juveniles. Mixed bacteria including *V. harveyi* were recovered from some animals in this group.

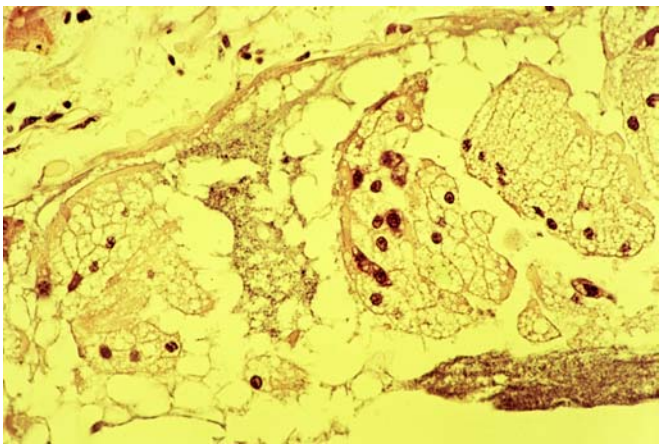


Plate 26. Focal erosion of hepatopancreas epithelium in juvenile *Jasus edwardsii* associated with clumps of bacteria.
H&E, 400 x magnification.

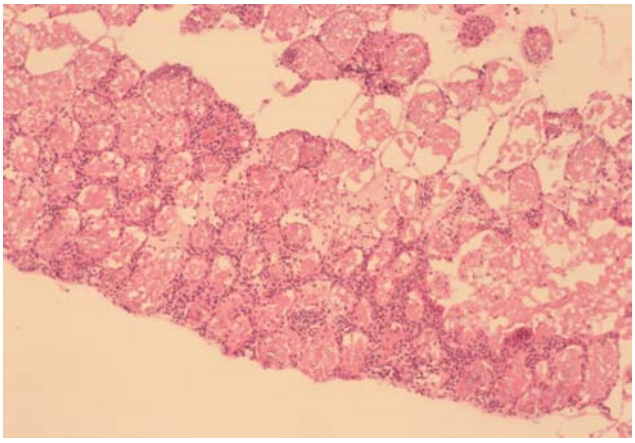


Plate 27. Histology of hepatopancreas of juvenile *Jasus edwardsii.*
There is a granulomatous response associated with complete erosion of the hepatopancreas tubules due to bacterial infection. H&E, 40 x magnification.

7. 5. 4 Condition 4: Black hepatopancreas disease

Major pathological characteristic: Large, blackened necrotic lesions in the hepatopancreas.

Causative agent: Unknown, possibly related to diet and/or infection with bacteria and flagellate-like protozoa.

Epizootiology: A hepatopancreas disease was observed in a small percentage of sub-adult *J. edwardsii* used in a dietary experiment in New Zealand. The experimental food source comprised a high percentage of abalone (*Haliotis* sp.) viscera. Affected lobsters were reportedly lethargic, stopped feeding and eventually died.

Diagnosis: Upon dissection affected lobsters exhibit large blackened, necrotic areas in the hepatopancreas (Diggles 1999). Histopathology showed complete necrosis of affected hepatopancreas tubules which were surrounded by a melanised layer with a granulomatous response in interstitial areas composed mainly of granulocytes and fibrocyte-like cells (Plate 28). Some tubules contained a mass of gram negative bacteria. Most also showed small protozoa (Plate 29), possibly flagellates, often peripheral to the bacterial masses, in closer approximation to the tubule wall. These were also observed within residual epithelium where this was present, indicating they may have been the primary causative agent. The histopathology of the disease resembled that of bacterial necrosis and mummification of hepatopancreas tubules of *Cherax tenuimanus* caused by failure to digest an inadequate diet (Langdon *et al.* 1992). Whether the presumptive protozoa or bacteria play a primary or secondary role is at this stage unclear.

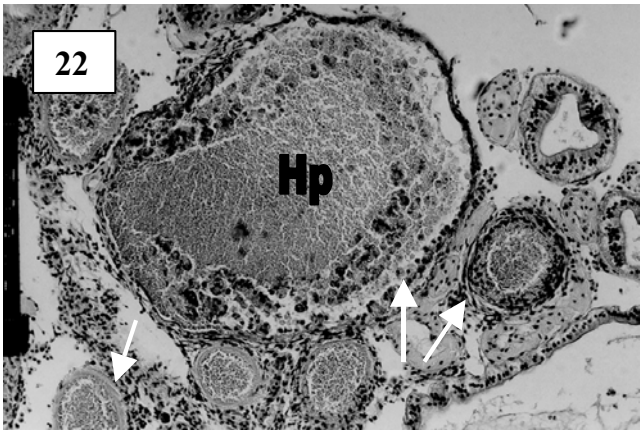


Plate 28. Histology of black hepatopancreas disease of juvenile *Jasus edwardsii*.
Hepatopancreas showing complete necrosis of hepatopancreas tubules (Hp) which are surrounded by infiltrating hemocytes and melanin (arrows), 100 x magnification.

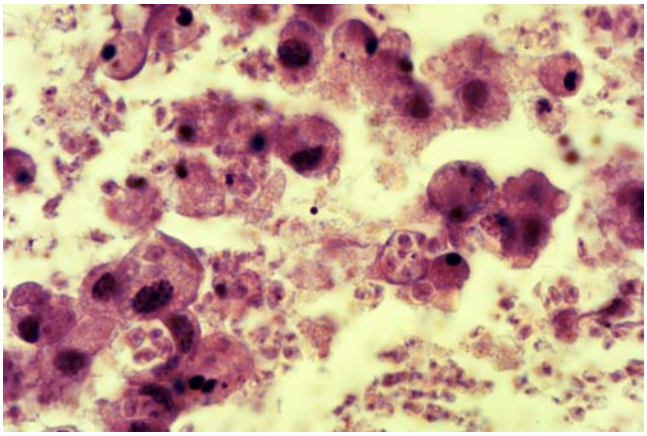


Plate 29. Histology of contents of necrotic tubule.
Clumps of flagellate-like protozoans amongst bacteria (b), necrotic hepatopancreocytes and hemocytes (h), 1000 x magnification.

7. 5. 5 Condition 5: Black gill disease

Major pathological characteristic: Invasive mycosis

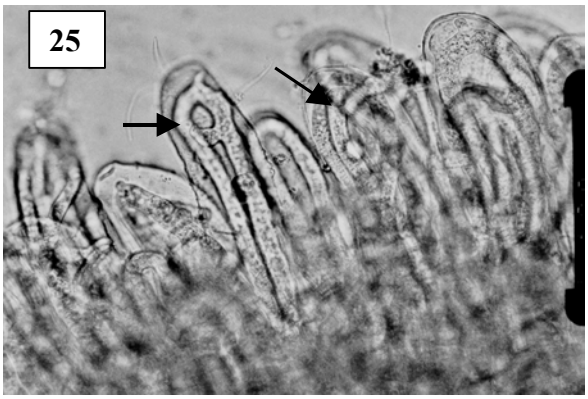
Causative agent: *Haliphthoros* sp.

Epizootiology: Significant mortalities have been observed in puerulus and juvenile *Jasus edwardsii* held for ongrowing in onshore experimental facilities in New Zealand at water temperatures between 10 and 18°C (Diggles 2000). Gross signs of disease included brown/black lesions at the base of the gills near insertion of the walking legs (Plate 30. A). Up to 30-50% of animals were affected in poorly maintained systems. An invasive fungus identified as a member of the genus *Haliphthoros* was observed inside infected

gill filaments adjacent to the blackened areas. The disease generally does not affect lobsters greater than 25 to 30 mm carapace length, as recorded for the fungus *Haliphthoros milfordensis* in American lobsters (Fisher *et al.* 1975; Fisher & Nilson 1977). Death of affected *J edwardsii* usually occurred just prior to or during the moult, perhaps from restriction of ecdysis due to extensive melanisation (Fisher *et al.* 1975; Fisher & Nilson 1977), though secondary bacterial infection may also be implicated in some cases.

Diagnosis: Wet mounts of gill lesions showed the presence of invasive fungal mycelia (Plate 30. B). Histology of gill lesions showed the presence of fungal mycelia inside the gill cuticle, and massive hemocyte infiltration and melanisation at the base of the walking leg adjacent to infected gill filaments. The fungus can be cultured for identification by placing infected gill filaments in marine agar 2216 (Difco) containing antibiotics to reduce bacterial contamination.

Plate 30. Black gill disease of juvenile *Jasus edwardsii* caused by the fungus *Haliphthoros* sp.
A: Gross appearance of gills (G) with blackened lesion at base of walking leg (arrow).



B: Black gill disease of juvenile *Jasus edwardsii* caused by the fungus *Haliphthoros* sp. Wet preparation of gill adjacent to lesion showing loops of fungus (arrows) inside the gill cuticle, 100 x magnification.

ACKNOWLEDGEMENTS

The New Zealand component of this work was funded by an augmenting PGSF-funded programme CO1606. The Australian component was carried out as a component of research on propagation and culture of larval and juvenile rock lobsters at the Tasmanian Aquaculture and Fisheries Institute, University of Tasmania, and supported in part by Department of Primary Industries, Water and Environment, Tasmania.

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CHAPTER 8

BIOSECURITY AND FOOD SAFETY ISSUES

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8. 1 Introduction

Commercial rock lobster production in which live lobsters are held in cages or pens for lengthy periods prior to sale can increase the potential for lobster to carry high numbers of potentially disease-causing organisms. This is the result of the stresses of capture and transport, combined with holding at higher stocking densities and sometimes less than ideal water quality conditions. Such events reduce the immunocompetence of the lobster and increase the risk of clinical disease.

Although the main end result of this process may be economic loss to the industry, it may also have other adverse effects that should be considered by the lobster industry and individual processors. There is a risk that disease agents may be translocated to lobster in a different geographical location. There is also the potential for residues in lobster meat following infection and/or treatment of diseased lobster, and the possibility for food poisoning to occur under certain circumstances. Although the risk of food safety problems in Australian rock lobster is low because of the nature of the industry and the high value of the end product, it should nevertheless be mentioned. In addition, there is at least one zoonosis reported in Australia that results from handling marine animals such as lobster, as well as from contact with other sources of infection such as farm animals and soil.

In this chapter the potential risks are discussed in greater detail.

8. 2 Translocation of lobster diseases

The policy of globalisation and the increasing ease of translocation of live aquatic animals throughout the world in jet aircraft pose an increased threat of introduction of infectious agents to aquatic animals that have had no prior exposure to the specific disease agent. This can cause serious disease outbreaks and considerable economic and ecological damage, as has already occurred with several diseases of crustacea:

- Crayfish plague of freshwater crayfish in Europe following translocation of resistant carrier crayfish from North America (Alderman 1996)
- Gaffkaemia spread to European lobster *Homarus grammarus* by *Homarus americanus* imported from North America to Europe (Alderman 1996)
- White spot virus of prawns. This parasite has a wide host range and has spread to many species in several areas of the world (Wang, Lo, Chang & Kou 1998)
- Taura syndrome of prawns imported with *Penaeus vannamei* from South America to Asia

Commensals of lobster in some regions of the world may cause few, if any, signs of disease in lobster native to the area, however, once these lobster, together with their associated bacterial, fungal, viral, protozoan and metazoan populations are introduced to a naive lobster population in a another region, disease outbreaks may result. This is the cause of many epizootics of disease because the host/parasite relationship does not have the features of long term relationships whereby the host and parasite co-exist with only the occasional sporadic outbreak of disease. Following long-term co-existence, the pathogen and host evolve in such a way that the populations of both species remain in sustainable relationship.

The aim of zoning policies, movement restrictions and quarantine protocols in many regions of the world is to minimize the risk of disease agents entering disease-free areas. It should be noted, however, that such measures are not always effective, as outbreaks of previously unknown diseases sometimes occur. There are 2 major reasons for such events:

- disease agents constantly evolve and their host range or pathogenicity may increase
- disease agents may not cause overt disease in infected animals of one species and thus are not recognized as potential disease agents, but they may cause disease in another population of animals

Live or chilled, uncooked lobster imported from other regions pose the greatest threat of introducing disease to Australian lobsters, especially if the imported animals come from areas with a similar climate and water conditions. Pathogens such as bacteria, viruses and protozoa are likely to survive in similar conditions and their introduction to Australia could have serious and far-reaching consequences. In Australia, Biosecurity Australia[1] conducts research and implements policies that aim to protect Australian species from the introduction of such diseases.

8. 3 Public health and food safety issues

Lobster is a delicacy consumed in many areas of the world and increasingly there is greater emphasis being placed on ensuring consumer safety. HACCP (Hazard Analysis Critical Control Point) is a key aspect of food safety programs that are designed to ensure that food is free of potentially dangerous chemical, biological or physical residues or material. Lobster that have been held as live lobster by marketing companies for lengthy periods, particularly if water quality and other management strategies cause stress to the rock lobsters, pose two potential food safety risks:

- the possibility of lobster harbouring viable micro-organisms that may cause food poisoning in humans when the lobster is consumed

[1] http://www.affa.gov.au

- the possibility that residues may remain in lobster following periods of suboptimal water quality or disease treatment.

These risks are greater in lobsters that have been stressed by handling and holding in crowded conditions than in lobsters harvested and processed directly after capture in the wild.

Overseas, captive lobsters sometimes require treatment to control two major diseases, gaffkaemia and 'bumper car disease'. Antibiotics are sometimes used to treat the bacterial disease, gaffkaemia, and formalin or other compounds may be used to treat 'bumper car disease' caused by the ciliate *Anophryoides haemophila.* Although similar, serious endemic diseases do not occur in Australian lobster at the present time, it must be remembered that any treatments of lobster or water may leave potentially dangerous residues in lobster meat.

In addition some bacteria that have been isolated from lobster hemolymph may also cause food poisoning when infected food in consumed by humans. An example is *Vibrio parahaemolyticus*, a bacterium that can be pathogenic for lobster and can also cause food poisoning in humans (Brinkley, Rommel & Huber 1976; Jay 1992). Fortunately the potentially dangerous seafood-borne pathogens are readily inactivated by heating to pasteurization temperatures and are unlikely to occur in food that has been adequately heat treated and not recontaminated (Jay 1992). Careful control of temperature and hygiene procedures in processing plants is required for uncooked seafood as harmful bacteria may readily multiply at ambient temperature. Toxins from dinoflagellates and diatoms may also cause food poisoning in humans, and algal blooms or disease conditions caused by dinoflagellates or diatoms are potential food safety risks in filter-feeding molluscs, however, they have not been reported in lobster meat.

'Crayfish handler's finger' is an ailment that sometimes affects personnel working in the rock lobster industry. The condition is caused by various ubiquitous bacteria, including *Erysipelothrix rhusiopathiae.* Bacteria gain entry through skin punctures or scratches and causes painful reddening and swelling (Brooke & Riley 1999)

The National Registration Authority for Agricultural and Chemicals (NRA)[2] controls registration of water and aquatic animal treatments, including those that may be used for edible lobster in Australia. At present, there are no chemicals registered for use on lobster. Minor Use Permits or off label prescription by registered veterinarians are possible methods by which certain treatments may be used to treat diseased lobsters or their holding water. However, before treatments are undertaken, the risks to the lobsters, people handling lobster, future consumers and the environment need to be carefully considered.

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[2] http://www. nra. gov. au

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Appendix 1: Reagent Requirements

10% seawater formalin for standard processing, light microscopy

Add 100 ml of formalin (37-40% formaldehyde) to 900 ml of filtered seawater

Davidson's fixative for standard processing, light microscopy

110 ml Acetic acid
330 ml 95% ethanol
335 ml tap water
220 ml formalin

Use 10x volume of fixative to volume of tissues, fix for 24-48 hrs at room temperature. After fixation, rinse the tissues in tap water and store in 70% ethanol.

IG4F solution for light microscopy and electron microscopy

Working IG4F solution (1% Gluteraldehyde: 4% Formaldehyde), prepared just prior to use:
500 ml Filtered seawater
500ml stock IG4F solution

Stock IG4F solution (may be held at 4°C for up to 3 months)
120 ml 37-40% buffered formalin solution
40 ml 25% gluteraldehyde
340 ml tap water

Agar for pre-embedding phyllosoma larvae for paraffin blocks

2% Bacteriological agar (Media Makers; BM 49) solution in distilled water

Decalcification Fluid

(RDO, Lomb Scientific and Co. ©) for decalcification of tissues containing calcium (use undiluted).

Combined fixative for soft resin embedding (Johnson and Ritar, 2001)

4% paraformaldehyde with 5% gluteraldehyde (1:1) made up in buffer comprised of 0. 2M sodium cacodylate, 0. 2 M NaCl, and 15% sucrose (W/v)

Appendix 2: Techniques for preparation of histological slides

1. Fixation (alternative fixatives can be used, see Appendix 1)

1.1 Davidson's Alcohol Formalin Acetic Acid Fixative

330 ml	95% Ethyl Alcohol (Ethanol)
220 ml	100% Formalin (saturated aqueous solution of formaldehyde, 37-39% solution)
115 ml	Glacial Acetic Acid
335ml	Distilled water

Store at room temperature.

1.2 Dissect the animal. Remove the required tissue quickly and place in fixative (minimum volume ratio 1: 10) for 24 hours.

1.3 Transfer to 70% Ethanol for 1 hour (can be kept for longer periods without spoiling).

2. Infiltration and Embedding

Tissues are processed in the following solutions

70% Ethanol	Two separate 0. 5h baths
80% Ethanol	Two separate 0. 5h baths
90% Ethanol	Two separate 0. 5h baths
100% Ethanol	Two separate 1h baths
Methyl benzoate	Till tissues sink to the bottom of the vial
Benzene	One dip, then blot between folds of filter paper

Transfer to molten paraffin in oven for 1h

Use vacuum infiltration, with 3 changes of wax for 10 min. each, then block tissues using embedding moulds.

3. Sectioning

Cut 5-6 μm thick sections and mount on slides.

4. Staining

Deparaffinize by passing through the following solutions:

Xylene- two changes

100% Ethanol-two changes

90%, 80%, 70% and 50% Ethanol- 2 min. in each

Stain in Hematoxylin- 10 min.

Rinse in tapwater

Acid alcohol (1ml Conc. HCl in 100ml 70% Ethanol)- two dips

Rinse in tapwater

Ammonia water (0. 2 ml Ammonia in 100ml Distilled water)- two dips

Running tapwater- 5 min.

70% Ethanol-five dips

Stain in Eosin solution-2min.

Dehydrate through 95%, 100% Ethanol- two changes and xylene- three changes, for 2 min. each

Mount in DPX

5. **Eosin Solution**
   Eosin Y- 1. 0g
   Distilled water-20ml
   95% Ethanol-80ml
   Filter and add 0. 5ml glacial Acetic Acid

Appendix 3: Calculation of condition indices

Hepatopancreas Moisture Content (%) = $(W_0-W_1)/ W_0 \times 100$,

where W_0- Hepatopancreas wet weight
W_1- Hepatopancreas dry weight

Moist Hepatosomatic Index(%) = (Hepatopancreas wet weight/ Body weight)x 100

Dry Hepatosomatic Index (%) = (Hepatopancreas dry weight/ Body weight)x 100