

# **Investigation of Tail Fan Necrosis of Live-Held Southern Rock Lobsters**

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## Abstract

Tail fan necrosis (TFN) is a disease that affects southern rock lobsters during live-holding. The damage to affected tail fan uropods is seen as a major constraint in the development of a live-holding industry. A previous study has demonstrated that various *Vibrio* species are associated with diseased tissue (May, 2002. B.Sc. Honours Thesis, University of Adelaide). However, that study was restricted to an examination of TFN lesions that formed 8 weeks post infection for lobsters held under optimal growth conditions. Although damage to tail fan tissue by instruments contaminated by organisms isolated from TFN affected tissue was shown to result in formation of TFN-like lesions, the microbial community of lesions associated with TFN over time in terms of both the cultivable and non-cultivable communities was not identified. The extent of damage to tail fan tissue by bacteria and the response of lobster immune cells to infection was also not determined. Furthermore, the presence of potentially pathogenic *Vibrio* spp. within the diseased tissue was identified as a potential public health risk, particularly in food preparation facilities where live lobsters are handled. The work described in this thesis specifically examined the development and effect of TFN on the overall health of affected lobsters, as well a confirmation that the *Vibrio* spp. involved in establishment of TFN may represent a public health risk.

To answer these questions, a larger infection trial was set up. Uropod tissue of groups of lobsters were intentionally damaged with sterile instruments or instruments contaminated with a *Vibrio* spp. isolated from a TFN lesion. The lobsters were maintained in controlled environment aquaria and uropod tissue samples taken and subjected to microbiological, microscopic and molecular analysis.

Microscopic analysis of developing lesions demonstrated that several morphologically different bacterial cell types colonise the surface of TFN lesions. Bacteria involved in infection are essentially restricted to the surface of the lesions, but where significant damage to the uropod tissue occurs, these bacteria may invade the damaged tissue and penetrate deeper underlying tissue. Infection of tail fan tissue results in inflammation and concomitant loss of internal structure of the carapace and deposition of fibrous material within the soft tissue underlying the chitinous exoskeleton. In cases of severe inflammation, a central core develops within the fibrous tissue consisting of a

number of cell types, including hyaline cells, granulocytes and fibrocytes. However, there was no evidence of deep bacterial invasion into the underlying inflamed tissue.

Viable counts and identification of the bacteria associated with the diseased tissue demonstrated that the bacterial population of TFN lesions is dominated by *Vibrio* species. Whilst there was no significant increase ( $P < 0.05$ ) in the total viable bacterial counts associated with the diseased tissue compared with healthy tail fan tissue, *Vibrio* species were isolated more frequently from tissue samples from uropods subjected to simultaneous damage and infection. 8% of bacterial isolates recovered from lesions were identified as *V. vulnificus* and 27% of isolates were identified as *V. parahaemolyticus*. Isolates of *V. vulnificus* displayed colony morphology consistent with pathogenic strains. Similarly, all isolates of *V. parahaemolyticus* were *tdh* negative, but 41% were *trh* positive. The majority of these species were able to express cytolysins capable of lysing CHO cells. This data indicated that vibrios responsible for establishment of TFN may have potential to cause human infections and therefore lobsters with TFN lesions should be regarded as a potential health risk to consumers. The majority of isolates of *Vibrio* spp. recovered from infected tissue expressed extracellular lipase and/or chitinase, and this indicated that these enzymes may enable *Vibrio* spp. to induce TFN in damaged tail fan tissue.

The predominance of *Vibrio* spp. associated with lesions was confirmed by analysis of amplicons representative of genes encoding 16S rRNA prepared from lesion tissue DNA extracts. This was achieved by sequencing randomly selected clones of amplicons and by use of Denaturing Gradient Gel Electrophoresis to separate amplicons according to nucleotide sequence diversity.

Unlike other crustacean shell diseases, TFN does not induce changes in serum protein levels, lead to significant bacteraemia or changes in the circulating haemocyte population. Furthermore, TFN has apparently little effect on the overall health of affected lobsters. This observation may explain the lack of mortality associated with this disease. Only a non-specific activation of lobster phenoloxidase in response to TFN was observed and only limited activation of phagocytosis of *Vibrio* spp. *in vitro* could be demonstrated. This data suggested that the lobster immune system is unable to respond to infection and may explain why the bacteria are able to induce persistent infection resulting in formation of TFN lesions. Nevertheless, localised melanisation surrounding the wound site induced

by carapace degradation products is able to restrict bacterial invasion into the haemolymph. The impact of TFN on appearance and consumer acceptance is dependent on the extent of damage caused by TFN. Minor lesions are resolved during moulting, whereas more severe lesions are maintained across more than one moult cycle.

## Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

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Damian May

May, 2007

## **About Figures and Tables in this Thesis**

The figures and tables in this thesis have been placed at the end of each relevant chapter or section.

## Abbreviations

$\Omega$	Ohms
$^{\circ}\text{C}$	degrees Celsius
$\mu\text{F}$	microfarad
$\mu\text{g}$	microgram/s
$\mu\text{L}$	microlitre/s
g	relative centrifugal force
$A_{570}$	absorbance at 570 nm
aa	amino acid/s
bp	base pairs
CFU	colony forming units
CHO	Chinese hamster ovary
d	days
DGGE	denaturant gradient gel electrophoresis
DNA	deoxyribonucleic acid
EDTA	ethylene-diamine-tetra-acetic-acid disodium salt
FCS	foetal calf serum
$\text{g L}^{-1}$	grams per litre
h	hour/s
HCl	Hydrochloric acid
IP	intra-peritoneal
IPTG	isopropyl- $\beta$ -D-thio-galactopyranoside
Kb	kilobase/s
kDA	kilodalton/s
kg	kilogram/s
L	litre/s
LB	Luria Bertani broth
$\text{LD}_{50}$	lethal dose to 50% of the population
LPS	lipopolysaccharide



M	molar
mg	milligram/s
min	minute/s
mL	millilitre/s
mM	millimolar
nm	nanometres
NSW	New South Wales
NT	Northern Territory
OD	optical density
O/N	overnight
ONPG	o-nitrophenyl- $\beta$ -D-galactopyranoside
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
<i>p.i.</i>	post infection
PO	phenoloxidase
ppA	prophenoloxidase activating enzyme
ppm	parts per million
proPO	prophenoloxidase
QLD	Queensland
RT	room temperature
SA	South Australia
SDS	sodium dodecyl sulphate
s	second/s
SEM	scanning electron microscopy
SI	super integron
spp	species
TAE	tris-acetate EDTA buffer
Tas	Tasmania
TCBS	thiosulphate citrate bile salt sucrose agar

<i>tdh</i>	thermostable direct haemolysin
<i>tlh</i>	thermo-labile haemolysin
<i>trh</i>	thermostable direct haemolysin-related haemolysin
TEM	transmission electron microscopy
TFN	tail fan necrosis
TTSS	type three secretion system
TVC	total <i>Vibrio</i> count
USA	United States of America
UV	ultraviolet light
VBNC	viable but non-culturable
VCR	<i>Vibrio cholerae</i> repeat
Vic	Victoria
vol	volume/s
v/v	volume per volume
<i>vvh</i>	<i>Vibrio vulnificus</i> haemolysin
WA	Western Australia
w/v	weight per volume

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# Chapter 1: Literature Review

## 1.1 Overview

The southern rock lobster fishing industry is the single most valuable fishing enterprise in South Australia with an estimated annual turnover of around \$230 million, and export revenue valued at over \$110 million, with the gross value of production estimated at \$83 million (Table 1.1 and Table 1.2). This industry alone is responsible for the creation of over 2000 jobs in South Australia, mostly in regional areas (<http://www.pir.sa.gov.au>). Combined, this makes the rock lobster industry vital to South Australia's regional economy. In order to maintain the future of the rock lobster industry in South Australia, fishing strategies are planned and put into place with the aid of fishers themselves, the South Australian Government and the best available scientific advice.

A sustainable fishery in South Australia is based on limited entry into the southern rock lobster fisheries incorporated in 1966/67. The activity of licensed South Australian rock lobster fishers is now strictly controlled through catch quotas in the Southern Zone (for a comprehensive review of this fishery see Ward *et al.*, 2002a) and tight management of fishing time in the Northern Zone (for a comprehensive review of this fishery see Ward *et al.*, 2002b). Fishers are now also actively involved in projects aimed at the future sustainability of the industry by recording daily catches and participating in voluntary pot sampling programs in which the size, gender, colour and location of the catch is recorded. Further, any female lobsters carrying eggs are returned back into the sea.

Furthermore, industry is currently working closely with the South Australian Research and Development Institute (SARDI) on a research program aimed at monitoring and assessing the sustainability of wild stocks, population dynamics, growth rates and movements, with findings already being used to better the industry. This research has allowed the formation of one of the world's most extensive fishery databases, which is updated annually in order to allow the State Government and Resource Managers to access stock assessment reports and take necessary action to ensure the long term viability of the fishery.

In South Australia, southern rock lobsters (*Jasus edwardsii*) have been fished since the 1890's, but a commercial fishery did not develop until the late 1940's, at which time markets for frozen tails were established in the United States. As of 2006, over 90% of the annual commercial catch is exported live, mainly throughout Asia (<http://www.sardi.sa.gov.au>, ABARE, 2005). Over the period 1996 – 2002, demand for southern rock lobsters soared, especially in countries such as Japan, where live southern rock lobsters are particularly sought after. Other live export markets include Hong Kong, Taiwan, and to a lesser extent Europe, with Asian markets buying 95% of the exports. As a result of buoyant export markets, local prices of southern rock lobsters in South Australia have risen dramatically in recent years as fishers fetch the best prices overseas, with many now selling exclusively to overseas markets.

Fishers make use of airfreight to get their product to Asian markets within 30 h of packing to allow the export of live lobsters to overseas markets. The process starts with fishing vessels being equipped with wet wells or a tank that pumps through fresh seawater in order to keep the lobsters in the best possible condition until they reach port. On land, the lobsters are placed in holding tanks at 10-12°C in order to slow their metabolism. Immediately prior to export, the temperature is dropped further to 5-7°C in order to slow the lobster's metabolism sufficiently to survive freight to export markets. Lobsters are routinely packed in polystyrene boxes with wood shavings used as insulation, and sealed. The lobsters are then transported to Adelaide or Melbourne by road for immediate loading onto Asian bound aircraft.

Importantly, many fishers are investigating the potential for live holding of stock in the anticipation of market price rises or for purposes of fattening and conditioning in either on-shore tanks or in off shore leases (northern zone only) (Geddes *et al.*, 2001). However, initial investigations have shown that current handling practices place enormous stress on the lobsters held in live holding systems (Geddes *et al.*, 2001). Despite the initial spike in stress responses, such stress indicators including total haemocyte counts (THC), haemolymph clotting times, haemolymph pH, haemolymph protein concentration and the differential proportion of haemocytes during the handling and processing phase, within the lobster *Panulirus cygnus*, have been shown to decrease over time spent in holding tanks (Fotedar *et al.*, 2006). However, handling induced stress apparently results in a lowering of

the lobster's resistance to infection. Consequently, the time fishers can hold onto their stocks is limited before losses due to disease outweigh the gains from increased market prices.

One example of this is tail fan necrosis (TFN) of live-held southern rock lobsters. This disease is a recognised constraint on the advancement of the South Australian rock lobster live-holding industry due to the reduction in value of afflicted lobsters (Musgrove *et al.*, 2005). This disease was observed following a field trial aimed at determining optimum environment system requirements for adult rock lobster grow-out (Geddes *et al.*, 2001). Whilst the results from this study were promising for the industry, TFN was deemed to be a major obstacle to the future success of the live-holding industry. Furthermore, Geddes *et al.* (2000) suggested that the severity of TFN may be related to seasonal water temperatures, with warmer temperatures over summer leading to the most severe cases. Indeed, this relationship was thought to be related to bacterial numbers as at this time, bacterial infections, especially *Vibrio*, were identified in association with diseased tissue. Following this initial study, Musgrove *et al.* (2005), demonstrated that individually bagging lobsters upon capture significantly ( $P < 0.001$ ) reduced the incidence of TFN. Furthermore, this study was able to demonstrate significant concentrations of bacterial cells, particularly *Vibrio* spp., in association with diseased tissue by both S.E.M. and by growth on selected media. However, these initial studies were not conclusive in determining the true nature of the disease. Indeed, the mode of infection has not been fully elucidated, nor has the infective agent/s been conclusively determined. Furthermore, there has not been any research into whether affected animals are able to mount an effective immune response against TFN. A more concerning issue is that there has currently been no study to determine whether the infective agent/s responsible for TFN would pose any health threat to consumers should live-holding of commercial lobsters be initiated.

## **1.2 Stress Responses in Lobsters**

Stress responses in lobsters and other crustaceans appear to be similar. Consequently, this review will use the American lobster *Homarus americanus* as a model, except where otherwise stated.

For *Homarus americanus*, reduction in water salinity results in increased oxygen consumption due to an increased metabolic rate. This is caused by an increase in energy demand required to control osmoregulation. Aerial exposure on the other hand induces anaerobic reactions to occur within the lobster that result in accumulation of lactate within the haemolymph (Evans *et al.*, 1998). Behavioural factors (eg. events that frighten or disturb lobsters) have also been shown to elicit an increase in activated immunity and glucose elevation (Evans *et al.*, 1998). Importantly, aerial exposure is more detrimental to lobster health if behavioural stress is superimposed on the animal (Evans, 2003), as occurs during the catching and handling process.

### **1.3 Immunological Responses**

Lobsters, like most other arthropods, are capable of non-specific cellular and non-cellular responses. Circulating haemocytes, which are generally classified according to size, presence and size of granules, staining of the nucleus and granules and other parameters, are responsible for cellular immune responses. However, there does not appear to be any consensus as to the number and types of haemocytes present within the circulation of crustacean species. Despite this, it has been demonstrated that haemocyte counts vary according to parameters such as diet, moult cycle, disease and introduction of various injectants (Rabin, 1969). However, crustaceans are also able to recognize foreign protein and clear it from the haemolymph using precipitin via a non-adaptive response. Anti-bacterial and/or bacteristatic factors present in the haemolymph can also restrict the growth of many types of non-pathogenic bacteria (Rabin, 1969). However, some species of bacteria, for example *Aerococcus viridans* var. *homari*, are unaffected by these responses and hence are able to induce disease. Furthermore, stress responses such as those mentioned above, reduce the ability of lobsters to limit infection and hence animals are left extremely susceptible to disease during the catching and holding process.

#### **1.3.1 Cellular Defences**

In-depth studies of cellular defences have been described by Aono *et al.*, 1993; Battison *et al.*, 2003; Chisholm & Smith, 1995; Cornick & Stewart, 1978; Evans *et al.*, 1998; Hearing, 1967; Hose *et al.*, 1990; Mix & Sparks, 1979; Paterson *et al.*, 1976; Rabin, 1969; Smith &

Söderhäll, 1983; and Toney, 1958. Although there is conjecture about the number and classification of circulating haemocytes, there is sufficient consensus to indicate recognition of three different classes of circulating haemocytes among the higher order crustaceans, which includes rock lobsters. The cell types recognised are:

1. Hyaline cells (also known as halocytes or hyalocytes) account for *ca.* 24% of circulating haemocytes. A majority of these are spindle shaped, however some remain ovoid. They have fine granules in the cytoplasm, with a less condensed nucleus. The role of these cells appears to be predominantly in phagocytosis. Some research groups suggest these are also precursors to the following classes of cells.
2. Semi-granular cells (also known as eosinophilic granulocytes or small granulocytes) account for *ca.* 64% of haemocytes. They are ovoid or spindle shaped with numerous small granules, can be involved in phagocytosis, but appear mainly to be involved with encapsulation and other cellular defence reactions.
3. Granulocytes (also known as chromophobic granulocytes, large granulocytes or explosive refractile granulocytes) account for the remaining 12% of circulating haemocytes. The majority of these cells are spindle shaped, however a few are ovoid. All, however, are packed with large granules and a condensed nucleus. These cells, along with semi-granulocytes, degranulate spontaneously to induce cellular defence reactions in response to stimuli such as lipopolysaccharide (LPS). Granulocytes have also been shown to trigger clotting in the spiny lobster, *Panulirus japonicus*.

### **1.3.2 Innate Immunity**

Bactericidins are proteins that inhibit the growth of, or kill bacteria. Early literature on the topic of bactericidins in crustaceans was contradictory. Some studies found the presence of bactericidins in the haemolymph (Ueda *et al.*, 1994), whereas others found haemocytes responsible (Chisolm and Smith, 1995). However, it is now believed that micro-organisms are eliminated from haemolymph through phagocytosis by haemocytes and subsequent killing by bactericidal molecules, rather than soluble anti-microbial factors present in



plasma (Destoumieux et al., 2000). Reports of bactericidins within the haemolymph are likely to be the result of lysis of haemocytes during experiments due to the delicate nature of these cells.

### 1.3.3 Prophenoloxidase and Associated Defence Reactions

One of the key aspects of arthropod immunity (including higher order crustaceans) is the process of melanization. The key enzyme involved in this process is phenoloxidase (PO), which is present in the haemolymph as an inactive pro-enzyme prophenoloxidase (ProPO). The proPO system is found throughout the invertebrate world (Cerenius & Söderhäll, 2004). The location of the inactive proPO in arthropods is usually within the haemolymph. In crustaceans, however, it is usually found within semi-granular and granular cells. Furthermore, some invertebrates, such as the horseshoe crab *Limulus polyphemus*, do not possess the proPO system, but rather clotting systems similar to, and potentially evolutionarily related to, the proPO system (Levin, 1985).

A number of these proPO primary structures have now been determined (Aspan *et al.*, 1995; Fujimoto *et al.*, 1995; Hall *et al.*, 1995; Kawabata *et al.*, 1995). All have been demonstrated to possess two functional copper binding sites, but lack a signal peptide. They also share an overall similarity of about 40%, which is increased to 60-70% around the copper binding sites (Söderhäll & Cerenius, 1998).

ProPO is activated in a step-wise fashion in the presence of picomole concentrations of microbial cell-wall components such as  $\beta$ -1,3-glucans of fungi, or the LPS and peptidoglycans of Gram-negative and Gram-positive bacteria respectively (Figure 1.1). Phenoloxidase has also been shown to play a role in other immunological functions including stimulating several cellular defence reactions such as phagocytosis, nodule formation, encapsulation, and haemocyte locomotion (for comprehensive reviews, refer to Johansson & Söderhäll, 1989, Söderhäll & Cerenius, 1998, and Söderhäll & Smith, 1985).

In order for proPO to be activated, it must first be cleaved by prophenoloxidase activating enzyme (ppA). The ppA is a serine protease that is activated by the presence of bacterial cell wall components, namely peptidoglycan and LPS from Gram-positive and -negative bacteria respectively, as well as  $\beta$ -1,3-glucans of fungi (Figure 1.1). Other compounds such as endogenous factors produced by tissue damage may also initiate

activation (Cerenius & Söderhäll, 2004). However, how these components activate ppA in crustaceans is currently unknown (Cerenius & Söderhäll, 2004).

An interesting aspect of the proPO cascade is that a number of different activation pathways are used that mimic the complement pathways of vertebrates. The first of these, the “alternate” pathway, is induced by non-self molecules and is likely to be induced by microbial invasion. The “classical” pathway is induced by low levels of calcium (< 5mM) independently of foreign molecules and is likely to occur in response to physical damage to the cuticle of the animal (Söderhäll & Smith, 1986).

#### **1.3.4 Phagocytosis as an Immune Response in Crustaceans**

Phagocytosis is accepted as an important cellular defense mechanism in crustaceans (Bachère *et al.*, 1995). Furthermore, clearance efficiency is considered to be a major humoral defense activation marker for crustaceans. This process has been observed *in vivo* in a number of crustacean species, including Pacific rock shrimp, *Sicyonia ingentis*, when infected with *V. alginolyticus* (Martin *et al.*, 1993) and the American lobster *Homarus americanus* when infected with *Aerococcus viridans* var. *homari* (Paterson *et al.*, 1976).

An important aspect of crustacean immunity with respect to infection is that the rate of phagocytosis increases following microbial challenge. McKay & Jenkin (1970) demonstrated that following immunisation with alcohol killed *Pseudomonas* CP, the average number of circulating haemocytes in the crayfish, *Parachaeraps bicarinatus*, increased significantly. Furthermore, the rate of degradation of phagocytosed erythrocytes also increased following immunisation, indicating that while there is no adaptive immune response in crustaceans, there is likely to be priming of the immune system, as is seen in insect immunity (Rahman *et al.*, 2003).

It is important to note that there are components of the haemolymph that are able to opsonise foreign particles leading to increased phagocytosis rates in cells from immunised animals. Indeed, unless red blood cells are first treated with serum, they will not be phagocytosed *in vitro* by haemocytes (McKay & Jenkin, 1970). Whilst there is no increase in the opsonic properties in the haemolymph of these animals, there is a marked increase in phagocytosis rates of opsonized bacteria by haemocytes from challenged crayfish as opposed to normal crayfish. This indicates that there is either activation of components of

the existing haemocyte populations, or proliferation of more active cells from progenitor cells. However, there is no cross-reactivity between opsonins and haemocytes of different species (McKay & Jenkin, 1969).

These studies demonstrate that crustaceans have effective cellular and innate immunity, but lack adaptive immunity. Usually this is adequate, despite living in an environmental medium with at times high microbial content. However, there is still potential for infections to take hold, particularly if the integrity of the cuticle is compromised.

## **1.4 Lobster Exoskeleton: Structure, Moulting and Repair**

The lobster exoskeleton is made up of a number of different layers: epicuticle, exocuticle, endocuticle and epidermis (Figure 1.2). The first of these is an outer epicuticle. Underlying this is the hard exocuticle that is composed of  $\alpha$ -chitin microfibrils, which are embedded in a protein matrix and calcium salts, particularly calcite. This is all arranged into a helicoidal pattern (Arsenault *et al.*, 1984). Underlying this is the endocuticle that connects the entire structure to the underlying tissue through the epidermis, a layer of cells that are also responsible for deposition of new cuticle during moulting. A brief description of each compositional layer is outlined in Table 1.3.

### **1.4.1 Molt Cycle of Lobsters**

Due to the encapsulating and static nature of the crustacean exoskeleton, lobsters spend their lives in a continuous cycle of moult and moult-related events. This cycle can be broken into five general phases based on morphological, physiological and cuticular changes. These consist of post-moult (subdivided into stages A, B, and C<sub>1</sub> – C<sub>3</sub>), intermoult (C<sub>4</sub>), premoult (D), and ecdysis (E) (Aiken, 1973; Drach, 1939).

For *Homarus americanus*, where pre-moult can span months, the final preparation for ecdysis may extend over days or even weeks. During this time it is essential that normal mobility, agility and awareness of the environment be maintained in order to lower risk of predation, even while neuromuscular control of these functions is being transferred from the old cuticle to the new one forming underneath. This transition is accomplished so efficiently, that the lobster is incapacitated for only 20-30 minutes. In *Homarus*

*americanus*, as with most crustacean species, this vulnerable period is spent in the safety of some kind of shelter (Cobb & Phillips, 1980).

A number of techniques have been described for determining the moult stages of *Homarus*, but most have been inaccurate, unreliable or inconvenient to use (Cobb & Phillips, 1980). As such, a more convenient and reliable method has been provided using setal development (Aiken, 1973; Freeman & Bartell, 1975; Mills & Lake, 1975; Musgrove, 2000; Schafer, 1968; van Herp & Bellon-Humbert, 1978). In all crustaceans however, post-moult is distinguished by extreme flaccidity of the exoskeleton, and corresponding absence of cuticular thickening of the pleopods and setae. Stage B results in the exoskeleton becoming parchment-like, the setal walls thickening and the base of the setae are more distinct. However, stages B and C<sub>1</sub> cannot be distinguished using this method (Musgrove, 2000).

Stages B/C<sub>1</sub> and C<sub>2</sub> (inter-moult) may be separated on the basis of hardness of the carapace. At this stage, pleopods continue to undergo cuticular thickening, to the point where setal lumen may be completely occluded (Musgrove, 2000).

During pre-moult, a change in colour of the ventral surface of the abdomen may be observed. This is seen as an orange-pink colouration, which darkens as the lobster approaches the moult. This colour change is believed to be due to increasing astaxanthin levels within the haemolymph (Musgrove, 2001). Indeed, haemolymph pigment stages (PS) may be used to roughly determine the moult stage of individual lobsters (Figure 1.3). These have been characterized into 9 PS (0.5 – 4.5) for southern rock lobsters. PS 0.5 indicates post-moult, PS 1 to PS 2.5 indicates C<sub>4</sub> (intermoult), PS 3 indicates late inter-moult / early pre-moult, and the remaining stages follow as ecdysis approaches (Musgrove, 2001). As ecdysis approaches, a range of changes occur including epidermal retraction, setal development and invagination, and barbules appearing along the setal axis can be seen as the epicuticle folds and exocuticle softens (Musgrove, 2000).

#### **1.4.2 Wound Healing**

Whilst there have been very few studies examining the process of wound repair in crustaceans, work based on other arthropods, such as insects, can be used as a model. Nevertheless, the entire process is still being examined and is yet to be fully elucidated.

Despite this, researchers have shown that sterile wounding is sufficient to trigger an immune response in *Drosophila melanogaster* under the control of the Toll and JAK/STAT pathways leading to repair of damaged cuticle (Markus *et al.*, 2005). These are considered to be the main controllers of gene activation during immune responses in *Drosophila* (Agaisse & Perrimon, 2004).

Although it has not been determined whether the same insect pathways are used in crustacean cuticle repair, it has been demonstrated that the response is equally rapid. This is necessary as the crustacean environment is usually littered with bacteria and viruses. Indeed, viruses are extremely abundant in aquatic systems (Bergh *et al.*, 1989). The first observations by transmission electron microscopy (TEM) indicated that, typically, there are  $10^7$  virus particles  $\text{mL}^{-1}$  (Bergh *et al.*, 1989; Proctor & Fuhrman, 1990) and that abundance decreases with depth and distance from the shore (Cochlon *et al.*, 1993; Paul *et al.*, 1993). Similarly, bacterial loads in seawater can be as high as  $10^5$  to  $10^6$  CFU  $\text{mL}^{-1}$ .

Since the cuticle is the first line of defense, it is imperative that crustaceans seal off wounds quickly. Getchall (1987) used V-notched American lobsters, *H. americanus*, to demonstrate that wounds are sufficiently sealed off to prevent infection by *Aerococcus viridans* var. *homari* within 24 h. The wound response was linked to mass haemocyte invasion to the damaged cuticle and melanin deposition. Fontaine & Lightner (1972) also demonstrated a similar response in penaeid shrimp following tagging using Peterson disk tags that require the insertion of a stainless steel pin through the abdomen of the shrimp. In both cases, the infiltration of haemocytes was followed by encapsulation of foreign material and cellular debris within 96 h. This was followed by the sealing of the wound by epidermal cells and fibrocytes over the next seven days.

Anti-microbial peptides with chitin-binding affinity have been isolated from horseshoe crabs (titled tachystatins) and penaeid shrimp (titled penaeidins) (Munoz *et al.*, 2002). These potent anti-microbial peptides are released into the plasma from haemocyte granules in response to microbial challenge (Munoz *et al.*, 2002; Osaki *et al.*, 1999). Further, they are also believed to play a role in wound repair of the exoskeleton following structural damage or infection (Destoumieux *et al.*, 2000). It is believed the chitin binding domains initiate interaction between the peptides and the chitin of damaged exo-skeleton following exocytosis from the haemocytes. Similar peptides are expressed by many different species,

but the associated chitin-binding domain was originally identified from the hevein protein isolated from the rubber tree, *Hevea brasiliensis* (Asensio *et al.*, 2000). Consequently, the chitin-binding domain was termed the hevein domain and is a common feature in many arthropod defense proteins. As such, this domain displays high conservation of amino acid sequence (>40%) and 3 dimensional structure among similar peptides isolated from the haemolymph of many insects (Suetake *et al.*, 2000).

Furthermore, mature male snow crabs (*Chionoecetes opilio*) possess low levels of circulating ecdysteroids, usually involved in moulting, which are also involved in the activation of cuticle repair. Mature snow crabs lack a Y-organ and hence no longer moult (Halcrow & Steel, 1992). The precise role of these was not determined beyond identifying a potential link between the processes of wound repair and moulting.

Interestingly, a study by Vogan & Rowley (2002) demonstrated that shell disease has very little effect on the humoral and cellular defences of the crab *Cancer pagurus*. This study demonstrated that there were no clearly definable effects on haemocyte counts, prophenoloxidase activation or antibacterial activity of the haemolymph. Further, they also found a linear relationship between the bacterial load in the haemolymph and severity of shell disease. This indicates that these crabs are unable to respond to the infection in the carapace, even when this leads to an increased bacterial load in the haemolymph.

## **1.5 Diseases of Crustaceans**

Diseases have caused significant economic losses in crustacean fisheries. In North America, for example, post harvest losses in the lobster fishery are estimated at 10-15% due to opportunistic infections (cited by Cawthorn, 1997). Within South Australia, live-holding of wild-caught lobsters was identified as a potential means of post-harvest value-adding to the existing commercial catch of southern rock lobsters. This value-adding could potentially occur in two ways: strategic marketing, and product enhancement. Access to holding facilities in the future would allow fishers to strategically market their catch by holding lobsters to take advantage of high market prices. In addition, live-holding may also enable product enhancement, i.e. increased weight through growth, and improvement in condition through feeding and growth of damaged, sick, and white lobsters (<http://www.sardi.sa.gov.au/pdfserve/fisheries/sas/cap/rocklob/subprog.pdf>). However, as

pilot studies have shown, lobsters can only be confined to holding tanks for a limited time before losses become too substantial due to the onset of disease, in particular TFN (Geddes *et al.*, 2001).

### **1.5.1 Diseases in Crustacean Aquaculture**

Disease in aquaculture is also the limiting factor to the success of rearing hatchlings to a marketable product. This is especially the case in prawn and shrimp aquaculture where prawns are grown from hatchlings right through to adulthood. Diseases such as white spot syndrome are capable of destroying entire harvests, and as such pose a significant threat to the industry. Often, these diseases are initiated following mechanical damage to the exoskeleton, as often occurs during the catching and handling process, or as a result of poor water quality or inadequate diet. Indeed, the most important factor in the development of disease in crustaceans is damage to the exoskeleton that allows a portal of entry to pathogens. If damaged tissue does not heal quickly, as is often the case in the case within lobster impoundments used overseas, particularly for the American lobster, *Homarus americanus*, where fighting, inadequate diet and adverse water temperatures prevent healing processes, opportunistic infection often results in the underlying tissue, the haemolymph, or the damaged exoskeleton itself (Cobb and Phillips, 1988). Crustaceans are also extremely susceptible to disease during the moulting period when harvest losses can be as high as 90%. The large number and variety of these diseases highlights the need to investigate common causes and possible preventative strategies.

### **1.5.2 Shell Disease**

Shell disease (also known as “burned spot” disease) is a disease that results in progressive chitinolysis and necrosis of the exoskeleton of aquatic crustaceans. This disease affects all crustacean species in all aquatic environments (Cobb & Phillips, 1980). It was originally thought that a fungus was the primary causative agent, but infection is now believed to be initiated by Gram-negative bacteria, including *Vibrio* spp. The initial bacterial infection is then occasionally followed by an opportunistic fungal infection. Minor infections are often overcome during moulting but more severe cases may result in death (Cobb & Phillips, 1980). In terms of occurrence of shell disease in live held lobsters, a study by Getchell (1991) found that among Maine’s (USA) lobster dealers, twenty one percent observed shell

disease in 1 – 5% of their lobsters when first purchased. A further eighteen percent discovered shell disease after 3 – 4 weeks in storage. This number increased to forty percent after long term storage, with fifty percent reporting shell disease after four months of storage. Perhaps surprisingly, these pounds are affected by shell disease predominantly over the winter months. As a result of this study, a number of recommendations were introduced to Maine lobster pounds. These included limiting overcrowding, providing adequate feed, avoiding damage by harmful gear at harvest and avoiding rapid changes in salinity, temperature and oxygen levels.

Histological studies of shell disease concluded that the infection resulting in shell disease are generally external in character and do not affect living tissue (Sawyer & Taylor, 1949). The exocuticle can be extensively damaged but the epidermis is generally not penetrated. The chitinous layers of the gill filaments are often destroyed and this results in impaired or lost respiratory function contributing to death. Lesions can be characterised according to severity, ranging from shallow lesions restricted to the epicuticle and exocuticle through to severe deep erosions that penetrate into the uncalcified endocuticle and membranous layer (Smolowitz *et al.*, 1992, 2005). In more severe cases, granulomas and haemocytic inflammation may be associated with the underlying connective tissues. However, this may not interfere with the moult, as the new epicuticle is able to form internal to the eroded carapace and associated inflammatory cuticle (Smolowitz *et al.*, 2005). Further, examination of cuticular inflammation associated with shell disease of winter impounded American lobsters, *H. americanus*, revealed inflammatory mechanisms including melanization, inflammatory cell proliferation and pseudo-membrane formation (Smolowitz *et al.*, 1992).

Furthermore, it has been traditionally recognised that bacteria associated with various shell diseases of crustaceans express lipase and/or chitinase (Cipriani *et al.*, 1980; Cook & Lofton, 1973). However, these enzyme activities are commonly expressed by marine bacteria and hence, the presence of these enzymes may not be responsible for the development of shell disease (Noga *et al.*, 1994). However, it is likely that at least lipase is required for initiation of lesions (Cipriani *et al.*, 1980).

One study by Vogan *et al.* (2002) isolated and characterised the pathogenicity of the bacteria associated with shell disease syndrome lesions in the crab, *Cancer pagurus*. This



study demonstrated that the majority of bacteria cultured from the haemolymph and exoskeleton of affected *C. pagurus*, were Gram-negative rods that expressed chitinase, most of which were subsequently characterised as *Vibrio* spp. All isolates tested were able to use chitin as a sole nutrient source. Furthermore, two isolates tested for pathogenicity for the crabs by intra-haemocoelic injections induced 100% mortality. In addition, extensive cuticular erosion in the chitinous procuticle resulting from this disease was demonstrated. The conclusions from this study were that shell disease is unlikely to be caused by a single pathogen; rather there are numerous bacteria within the marine environment capable of degrading the chitin component of the crustacean cuticle. As such, it is likely that a collective effect from a number of bacterial species leads to the exoskeletal degradation typical of shell disease.

### **1.5.3 Epizootic Shell Disease**

Epizootic shell disease affects inshore populations of *H. americanus* (Smolowitz *et al.*, 2005). This disease presents as severe, erosive shell disease affecting the dorsal carapace. It is characterised by severe deep dorsal midline erosions that affect the cephalothorax and abdomen that extend over the dorsal carapace of affected lobsters (Smolowitz *et al.*, 2005). Epizootic shell disease afflicts *H. americanus* populations extending from eastern Long Island Sound through to southeastern Massachusetts (Castro and Angel, 2000, Estrella, 1991). There is also evidence that epizootic shell disease is not the result of a systemic infection or an immunocompromised condition, but rather a strictly dermal disease (Christoserdov *et al.*, 2005).

Preliminary, culture dependant characterization of the microbial community associated with epizootic shell disease lesions by Christoserdov *et al.* (2005) suggested that various types of bacteria are associated with the lesions of epizootic shell disease. These bacteria are characteristic of both healthy and diseased carapace, but are typically present at 3 – 4 log greater numbers in the lesions of diseased lobsters. There are two common groups of bacteria associated with the lesions; a species complex belonging to the family *Flavobacteriaceae* and a series of closely related, if not identical, strains of *Pseudoalteromonas gracilis* (Christoserdov *et al.*, 2005). Interestingly, *Vibrio* species were described as rarely associated with the lesions of this type of disease (Christoserdov

*et al.*, 2005). Indeed Christoserdov *et al.* (2005) conceded that challenge experiments are required to fully understand the role of these lesion associated bacteria in the onset and progression of epizootic shell disease.

#### **1.5.4 Tail Fan Necrosis**

In 1998, live-holding was identified as a potential means of post harvest value-adding of southern rock lobsters (*Jasus edwardsii*) through product enhancement. Following initial trials in open sea water suspended cages, a disease developed within tail fan tissue (termed tail fan necrosis) (Figure 1.4). Tail fan necrosis (TFN) apparently develops as a result of bacterial infection of damaged tail fans. However, TFN is not thought to commonly occur in natural open water environments. Nevertheless, wild lobsters may be caught with the disease in South Australian waters, although this is extremely rare (Musgrove *et al.*, 2005).

The onset of this disease is believed to be as a result of bacterial infection of the tail fan following damage incurred when the lobsters are caught. Such damage may be caused by flapping of the tail on the deck of fishing vessels, or from fighting and movement within crowded wells onboard fishing vessels. It is currently unclear whether the wound becomes infected during or after the process of tissue damage. Harvested lobsters kept in experimental sea-based cages for periods of weeks, or months, tail fans become blackened (or melanised), presumably as a result of an immunological response. Melanisation was generally accompanied by erosion or rotting (necrosis) of the tail fan and often continued to the point where individual tail fans were completely eroded (Geddes *et al.*, 2000). The disease makes the tail fan unsightly and hence lowers the market value. However, a more pressing issue associated with TFN is the impact of disease on public health is unknown.

Previous studies have shown that TFN can be almost completely eliminated by individually bagging each lobster upon capture (Geddes *et al.*, 2000). The bagging process was shown to minimize tail flapping and also prevents fighting within wells on board fishing vessels, hence reducing damage to the tail fans. As there is no damage to tail fans, there is subsequently no portal for the causative agent to infect the underlying tissue, hence preventing the onset of disease. Whilst this study showed that this method significantly ( $P < 0.001$ ) reduces the prevalence of TFN, individual bagging of lobsters is a very time

consuming process and, in the absence of suitable automation, is considered to be not a viable process by lobster fishermen.

It has since been shown that TFN cannot be induced by artificial damage to tail fans with sterile instruments (C. J. Thomas, R. Musgrove and M. Geddes, personal communication). This observation supports the contention that TFN is a result of bacterial infection of damaged tail fan tissue. Furthermore, it is possible that the bacteria responsible are introduced from the micro-flora that colonise the exoskeleton of the lobster itself.

To date, three bacterial species have been isolated from wild stocks of *Jasus edwardsii* displaying symptoms synonymous with TFN following experimental live-holding. These were tentatively identified as *Vibrio parahaemolyticus*, *Vibrio vulnificus* and *Vibrio* species strain L5 (May, 2002). Representative isolates of each of these species were then used to induce TFN in healthy lobsters by infection of induced tail fan damage using inoculated blades and nails. A sterile control group with damage induced by sterile instruments and a group using instruments inoculated with the usually non-pathogenic *Vibrio alginolyticus* (also isolated from lesions associated with TFN) were also included in the trial. Lobsters were subsequently held in the dark within individual, temperature controlled tanks to prevent any further stresses on the animals for a period of eight weeks. At the completion of the trial, lobsters infected with the three test species of bacteria all displayed symptoms typical of TFN. The negative control group however, developed negligible levels of TFN. Furthermore, the results also demonstrated that while *V. alginolyticus* was unable to prevent the onset of disease, it did not contribute to the development of TFN (May, 2002).

At the completion of that study, tissue samples were taken from lesions associated with TFN from each treatment group for microbiological and microscopic analysis. The microbiological analysis demonstrated that all of the test species used to initiate infection were able to persist within lesions for the duration of the trial. Thus it is likely that these species of *Vibrio* indeed play a role in the development and/or persistence of TFN.

Electron microscopic analysis of sections of TFN demonstrated that there is close interaction between bacteria and the surface of lesions. The surface of lesions was covered with micro-colonies of bacterial cells (Figure 1.5). Furthermore, the underlying tissue

appeared to be infiltrated by haemocytes, however this observation was made with a small number of samples and the results are therefore preliminary at best.

What was not investigated in that study was what threat, if any, isolates recovered from tissue demonstrating symptoms synonymous with TFN could pose to consumers of fresh lobster if live-holding was to become main-stream practice. Although lobsters are prepared for consumption by cooking in boiling water, there is considerable potential for cross contamination of other foods and food work surfaces during handling of uncooked lobsters. It is the potential for cross contamination of other foods that represents significant public health risk. The cooking process kills all *Vibrio* spp. and properly conducted, there should be no risk of cross contamination.

Raw lobster meat is often used in many dishes in Asia, which is a major export market for southern rock lobsters. Indeed, whole live product is the preferred method of purchase in most overseas markets. Consequently, in areas where hygiene standards may not be adequate to prevent cross contamination, there is potentially a serious risk of illness from the microflora of lesions associated with TFN. Therefore, it is imperative that the potential threat posed to consumers by the causative agent(s) of this disease be thoroughly investigated before live-holding can become common practice. Furthermore, the disease pathology of TFN has not been fully characterised. This is particularly the case with regards to the immunological response, as well as determining the role of non-culturable bacteria. Indeed, the developing micro-flora has not been characterised beyond identifying the presence of various marine *Vibrio* species within the affected tissue.

## **1.6 Marine *Vibrio* species**

*Vibrio* species are halophilic, facultative anaerobic, Gram-negative bacteria belonging to the *Gamma-proteobacteria* class of bacteria. They generally grow in saline aquatic environments. Vibrios are straight or curved rod-shaped bacteria, about 1.4-2.6µm long and are motile by a single polar flagellum (Tantillo *et al.*, 2004). They are able to grow on marine agar (with about 3% salt) and on the selective medium thiosulfate citrate bile salt sucrose agar (TCBS). Vibrios are usually oxidase positive (Murray *et al.*, 1984).

The genus *Vibrio* currently contains more than 50 species and its taxonomy is continuously updated as new techniques allow the detection of new species (Tantillo *et al.*, 2004). The most widely studied of these is *V. cholerae*, which was initially discovered in 1854 by the Italian physician Filippo Pacini (Blake, 1994). *V. cholerae* is responsible for millions of cases of cholera each year, mostly in developing countries that cannot afford to establish or maintain necessary hygiene and medical facilities (Reidl & Klose, 2002). *V. cholerae* is also a major cause of disease following natural disasters such as earthquakes and flooding that disrupt the ability of an area to produce a clean water supply (Chotray *et al.*, 2002; Siddique *et al.*, 1989; Sur *et al.*, 2000). Other *Vibrio* species are associated with gastroenteritis of varying severity, which is generally more acute than that associated with viral pathogens, as well as severe septicaemia, and many diseases associated with marine life, including mortality of economically important crustacean species.

*Vibrio* species are ubiquitous in marine environments (Arias *et al.*, 1999; Hervio-Heath *et al.*, 2002; Maxwell *et al.*, 1991; Urakawa *et al.*, 1999) and are found as planktonic cells and in association with sediments and other surfaces. However, there is an increasing awareness of an association between *Vibrio* spp. and various crustacean species (see Section 1.6.5). This association may be symbiotic, parasitic or have no effect on the health of the animal.

Interestingly, many studies have demonstrated that there is significant seasonal variation in the numbers of vibrios within the water column. Numbers are generally highest when the water temperature is between 20°C and 30°C. Vibrios of clinical interest are generally at their lowest when the temperature drops below 10°C (Tantillo, 2004). Yam *et al.* (1999) also demonstrated a link between salinity and the presence of pathogenic vibrios. Most are only found in water with salinities covering the range 5 – 30ppm. However, some observations indicate that they may survive outside of this range in the presence of high water nutrient content or high water temperature.

### **1.6.1 Association of *Vibrios* with Copepods**

An apparently important aspect of *Vibrio* ecology is their dependence on organisms within the water column to survive. Numerous studies have shown a relationship between the survival of *Vibrio* species in the environment and their ability to interact with copepods

and chitin substrates (Amako *et al.*, 1987; Huq *et al.*, 1982; Kaneko & Colwell, 1974; Karunasagar *et al.*, 1986). The mechanism by which the bacteria associate with these animals may also be similar to the mechanisms used to attach to the surface of higher order crustacean species such as lobsters.

*Vibrio* species can use copepods as a nutrient source as a means of survival (Kaneko & Colwell, 1974). That study demonstrated that *V. parahaemolyticus* are able to absorb onto chitin particles and live copepods. The level of absorption was dependant on salt concentrations with the highest absorptions seen in sterilized water from Chesapeake Bay compared to that of open seawater, when minimal interaction was observed. This effect was deemed to be due to higher salt concentrations in the open seawater, leading to decreased absorption of *V. parahaemolyticus* onto the chitin particles and copepods. Importantly, this effect could not be demonstrated for a number of other enteropathogens. Nevertheless, it was determined that this absorption effect was possibly a major factor in the seasonal variation in *Vibrio* numbers, as the level of copepods in the water increase over summer and decrease over the cooler months, mirroring the levels of *V. parahaemolyticus* in the water column. Furthermore, these copepods also provide a substratum for the bacteria to attach and grow, whilst at the same time providing an adequate nutrient source.

Indeed, chitin may be essential for the annual growth cycle of many *Vibrio* species. Studies by Karunasagar *et al.* (1986) and Amako *et al.* (1987) have demonstrated that chitin in the growth media enhances the survival of *V. parahaemolyticus* and *V. cholerae* respectively at low temperatures. This process is likely to assist in the persistence of *Vibrio* spp. during the colder winter months, allowing for regeneration over spring and summer.

A study by Huq *et al.* (1982) further supported earlier findings using *V. cholerae*. This study demonstrated that *V. cholerae* preferentially attached to the oral and egg sac regions of the copepods. This interaction was not seen when copepods were killed. Importantly, survival of *V. cholerae* in the water was increased by the presence of live copepods, but again, dead copepods did not induce this effect. Similarly, it has recently been demonstrated that *V. cholerae* is able to survive and grow intracellularly within the amoeba

*Acanthamoeba castellanii* (Abd *et al.*, 2005). As such, it is now considered that amoebae are also another potential reservoir for *V. cholerae*.

However, these findings are rebutted by a study by Dumontet *et al.* (1996) who found that only *V. cholerae* was able to adsorb to the surface of planktonic copepods. This study found that *V. parahaemolyticus*, *V. mimicus*, *V. alginolyticus*, *Aeromonas hydrophila* and *Pseudomonas* spp. were unable to colonise the surface of the 4 species of copepods tested in this study. However, on closer examination, this study differed from others on this topic in that they used sterile physiological saline instead of either sterile artificial seawater or sterile seawater samples, which may have impacted on the final results due to the effects of the concentration of various salts and ions highlighted by Kaneko & Colwell (1974). Further to this, a study by Dumontet *et al.* (1996) demonstrated that in the summer months there is a 70% infection rate of copepods by vibrios in the waters off southern Italy. This is reduced to 0% over the winter months. However, this study did not consider the influence of the viable but non-culturable (VBNC) state of these bacteria. Conversely, Maugeri *et al.* (2006) used molecular techniques to detect low levels of *V. vulnificus* attached to plankton in the Mediterranean Sea (Italy) all year round and demonstrated that direct culture is insufficient. However, this study did not assess the presence of other *Vibrio* species. Importantly, the mechanism of attachment of vibrios to higher marine invertebrates, including crabs and shrimp, is believed to be the same as for copepods (Castro-Rothas & Escartin, 2002). Furthermore, the numbers of vibrios attached to the carapace of crabs and shrimp was at levels of concern to public health.

Significantly, the mechanism for attachment to copepods and like substrates has been found to be the same as that used to attach to epithelial cell walls in the small intestine. A study by Zampini *et al.* (2004) that employed various *V. cholerae* mutants, demonstrated that a 53 kDa protein may contribute to adhesion to chitin-containing substrates in both the marine environment and in the human intestine. This finding supported a view by Zampini *et al.* (2004), that ability of *Vibrio* species to adhere to chitin substrates not only allowed greater survival of these bacteria in the water column, but also played a potential role in infection of human hosts.

### 1.6.2 The Viable But Non-Culturable State

The Viable Bt Non-Culturable (VBNC) state of bacteria is described as a failure to grow on routine bacteriological media on which they would normally grow and develop into colonies, but are nevertheless, alive and capable of renewed metabolic activity (Oliver, 2000). Cells in the VBNC state typically demonstrate very low levels of metabolic activity, but upon resuscitation are again culturable (Oliver, 2005). A large number of bacterial species have been demonstrated to be capable of entering the VBNC state, including human pathogens (for a comprehensive review, see Oliver 2005).

An important aspect of marine *Vibrio* physiology, particularly in terms of food safety, is their ability to enter a VBNC state. The first demonstration that *Vibrio* species are able to enter a VBNC state was performed by Xu *et al.* (1982). This study demonstrated, for the first time, that although *V. cholerae* remained viable, all ability to produce colonies on media routinely employed for its culture was lost following incubation in artificial seawater at 4 to 6°C. Based on this work, it has been hypothesised that entry into the VBNC state by this pathogen may account for the seasonal distribution of vibrios in regions of the world where cholera is endemic. Since this initial study, it has been determined that vibrios enter the VBNC state when the organism is exposed to a combination of low temperatures and a nutrient poor environment (Mizunoe *et al.*, 1999; Oliver, 1995; Wong & Wang, 2004). As a result of entering this state, sensitive methods of detection, such as PCR, may be necessary to detect the presence of *Vibrio* species in some fish food products.

Establishment of a VBNC state brings about changes in cell morphology and physiology. This can be observed microscopically as a process of reductive division where each rod-shaped cell transforms into a number of smaller coccoid cells (Ostling *et al.*, 1993). Later studies examined the ability of VBNC *V. cholerae* to cause human cholera. For example, Colwell *et al.* (1985) demonstrated VBNC state *V. cholerae* exhibited typical virulence responses using the rabbit ileal loop method. Following this, Colwell *et al.* (1995) showed reversion to the culturable state following ingestion of VBNC *V. cholerae* cells in human volunteers. Importantly, the retention of pathogenicity in the VBNC state has also been demonstrated in *V. alginolyticus*, and *V. parahaemolyticus* (Baffone *et al.*, 2003). This was done by inducing the VBNC state followed by intragastric infection of



Balb/C mice, followed by autopsy at 2, 4, 8 and 12 d post infection and recovery of isolates from intestinal tissue cultures. Results of this experiment showed that *V. alginolyticus*, environmental *V. parahaemolyticus* and clinical *V. parahaemolyticus* were recovered from 25%, 37% and 50% of infected mice respectively. Of the recovered isolates tested, pathogenicity was retained (as identified using the rat ileal loop model). This provided strong evidence that *Vibrio* spp. still pose a significant threat to the health of consumers when in the VBNC state.

More is known about the VBNC state of *V. vulnificus* than for any other organism (for a comprehensive review see Oliver, 1995). Researchers have discovered that, as with other *Vibrio* species, temperature is the deciding factor in this organism entering the VBNC state. Importantly, it has also been demonstrated that *V. vulnificus* will enter the VBNC state whether incubated in either artificial seawater (Nilsson *et al.*, 1991) or in heart infusion broth (Oliver & Wanucha, 1989). This temperature response has been found to occur at temperatures <15°C when cells are placed in membrane diffusion chambers in coastal waters. The reverse was seen when laboratory induced VBNC cells were placed in membrane diffusion chambers in warm coastal water (25°C), at which point cells were resuscitated to the original level of culturability (Oliver *et al.*, 1995).

### **1.6.3 Flagella Based Motility**

Flagella act as semi-rigid helical propellers to provide bacteria with efficient locomotion (McCarter, 2001). This is equally true for vibrios, which possess both single sheathed polar flagellum for motion through liquid environments and numerous unsheathed lateral flagella to move the cell over surfaces (McCarter, 2001). Whilst they are important for locomotion, they also play pivotal roles in pathogenesis, attachment and biofilm formation.

Indeed, in *V. vulnificus* the flagellum is deemed necessary for virulence in mice. This was a result of decreased adherence to cultured cells, as well as loss of biofilm formation ability in the absence of flagellum by this bacterium (Lee *et al.*, 2004). Furthermore, flagellum-deficient strains were less virulent than the wild type strain when tested using the mouse model. Consequently, flagella may serve as an adhesin, or as a mediator of invasion of host cells (Lee *et al.*, 2004). Indeed, flagella are also required for infection by *V. cholerae*. Non-motile mutants of the El Tor biotype have a 10-fold lower ability to

infect cultured mammalian cells compared with the wild type strain (Lee *et al.*, 2000). A model describing this effect can be seen in Figure 1.6. The fish pathogen *V. anguillarum* also requires motility for infection of trout. *motY* mutants show a 750-fold decrease in virulence when compared with wild type *V. anguillarum* (Ormonde *et al.*, 2000). This conclusion is supported by Milton *et al.* (1995) who demonstrated that *flaA* mutants of *V. anguillarum* display a 70 and 700 fold higher LD<sub>50</sub> than wild-type strains when used to immersion infect fish. However, it is the active motility rather than any individual components of the flagellum that is needed for entry into the fish host. Once the bacterium has invaded the fish, motility is no longer required (Milton *et al.*, 1995; Ormonde *et al.*, 2000).

#### **1.6.4 Gene Transfer: The Role of VCR's**

Gene transfer among bacterial species is an integral part of bacterial survival and evolution. *Vibrio* species have been demonstrated to possess a unique form of integron that functions as a gene transfer system as well as a mechanism to add or remove genetic material. Integrons are gene expression elements that acquire gene cassettes and convert them into functional genes, often involving antibiotic resistance (Recchia & Hall, 1995). The insertion of a gene cassette requires site-specific recombination between a circularized cassette and the recipient integron. This process requires a number of essential components, namely an integrase gene and an attachment site (*attI*). This core site is defined at the 3' end as G↓TTRRRY with recombination occurring at the nick site between the GT residues. *V. cholerae* and other *Vibrio* spp. have evolved a similar integration system on a larger scale, dubbed a super-integron (SI) (Figure 1.7). These SI's have been shown to contain as many as 150 individual gene cassettes, dwarfing those typically associated with antibiotic resistance, which usually contain less than five gene cassettes (Clark *et al.*, 2000).

Further analysis of the SI's of various *Vibrio* spp. has shown that each was at least 100kb in size. Comparative analysis of these demonstrated a wide range of origin of the trapped genes as well as an active cassette assembly in these species (Rowe-Magnus *et al.*, 2003). Furthermore, the signature *attC* sites of each species displayed conserved structural characteristics, indicating that symmetry rather than sequence is important in the

recognition of target recombination sequences. Such recognition sequences among *Vibrio* species were termed *Vibrio cholerae* repeats (VCR's). VCR's are 124 bp direct repeats, with imperfect dyad symmetry (Barker *et al.*, 1994). This study found them to occur at least 60 – 100 times in the *V. cholerae* O1 chromosome and account for approximately 10% of the entire genome. Furthermore, chromosomal mapping has indicated that these repeats are confined to a single super integron within the chromosome. These cassettes have also been discovered in many other *Vibrio* species using PCR amplification of VCR - like cassettes or by southern hybridisation. These include in *V. metschikovii* (Rowe-Magnus *et al.*, 1999), *V. anguillarum* (Manning *et al.*, 1999), *V. mimicus*, and *V. parahaemolyticus* (Mazel *et al.*, 1998). It has also been demonstrated that these SI's are used to gain an evolutionary advantage beyond antibiotic resistance and pathogenicity (Rowe-Magnus *et al.*, 1999). Due to variation within these cassettes, it may be possible to determine where isolates have originated, or even clonality between isolates, based on the genes present within their SI structures.

#### **1.6.5 Association of Vibrios with Marine Invertebrates**

Many studies have demonstrated a link between vibrios and higher marine invertebrates. These have included studies of gut microflora (Lau *et al.*, 2002; Oxley *et al.*, 2002), within the haemolymph (Welsh & Sizemore, 1985) and the carapace (Porter *et al.*, 2001) of various crustacean species.

Importantly, vibrios have been demonstrated to be consistently present within the gut of shrimp and prawns. A study by Lau *et al.* (2002) demonstrated that the hindgut of the shrimp *Neotrypaea californiensis* consists of a diverse microbial community. Whilst they found a high number of novel species, there was a high proportion of *Vibrio* species also associated with the hindgut tissue. Another study that investigated the culturable microflora from the gut of wild and cultured banana prawns (*Penaeus merguensis*) demonstrated that vibrios were quantitatively the dominant genus of this particular niche (Oxley *et al.*, 2002). Notably, in this study the known human pathogens *V. vulnificus*, *V. mimicus*, and *V. parahaemolyticus* were all recovered from the hindgut of both species.

Furthermore, a study by Porter *et al.* (2001) of both healthy and diseased carapace tissue associated with shell disease from the spiny lobster *Panulirus argus*, demonstrated

the majority of isolates in both healthy and diseased tissue belonged to the genus *Vibrio*. Thus, while *Vibrio* spp. are normally associated with the carapace (as seen in “healthy” tissue), they are also opportunistic pathogens when primary barriers, such as the carapace or gut lining, are compromised. This concept is illustrated by the work of Welsh and Sizemore (1985) who demonstrated that 75% of freshly caught blue crabs (*Callinectes sapidus*) have low level bacteremia (ca. 14 CFU mL<sup>-1</sup>). The majority of the isolates recovered were identified as *Vibrio* spp. It was the authors view that when these animals are stressed by handling and processing, that these bacteria are capable of causing infections that can potentially progress to mortalities.

The presence of these bacteria can lead to public health issues in fish and fish products. Baffone *et al.* (2000) demonstrated that thirteen percent of widely consumed fresh seafood products were contaminated with halophilic *Vibrio*'s. However this prevalence may be as high as 34-80% (Occhia *et al.*, 1978; Schintu *et al.*, 1994 as cited by Baffone *et al.*, 2000). *Vibrio* spp. isolated included TDH-positive *V. parahaemolyticus* and *V. cholerae* non-O1. Indeed, *Vibrio* species may be responsible for up to 50% of bacterial food poisoning cases in Asia (Lee *et al.*, 1995).

The presence of marine vibrios in fish and fish products has generated interest in the role of pathogenic vibrios in various diseases of crustaceans and the subsequent threat to consumer health. Of particular concern is the fact that more diseases in crustaceans, and occasionally humans, are being attributed to *Vibrio* species previously thought to be harmless. This is highlighted by the study by Lee *et al.* (1996) in which *V. alginolyticus* was identified as the cause of a disease outbreak causing serious mortality among cultured kuruma prawns (*Penaeus japonicus*). This species of bacteria is often used as a probiotic in crustacean aquaculture (Vershuere *et al.*, 2000), although the cause of this outbreak was of a new serotype. However, a greater concern is the association of pathogenic *Vibrio* species (eg. *V. vulnificus*) with crustaceans used as food, (for example, shrimp; see Nascimento *et al.*, 2001). That study and similar reports, highlight a need to investigate the association between commonly consumed seafood and *Vibrio* species.

There are also concerns about the health of marine animal species held in aquaculture systems. Many diseases are caused by *Vibrio* spp. However, in terms of food safety of

aquaculture products, the outstanding *Vibrio* species of concern include *V. parahaemolyticus*, *V. vulnificus*, and *V. harveyi*.

### **1.6.6 *Vibrio parahaemolyticus***

*Vibrio parahaemolyticus* has been implicated as a major cause of foodborne illness around the globe (Yeung & Boor, 2004). Indeed, it is the leading cause of *Vibrio* associated gastroenteritis in the United States (Potasman *et al.*, 2002). In Australia, the median number of gastroenteritis cases due to *V. parahaemolyticus* per year is ~1,080 cases, mostly from food-borne sources (Hall *et al.*, 2005). Commonly associated with the consumption of undercooked seafood, the infective dose of this organism is usually greater than  $10^6$  cells. However, this may be markedly reduced by the consumption of antacids (<http://www.cfsan.fda.gov/~mow/chap9.html>). Infected persons present with watery diarrhea, but other clinical symptoms can include abdominal cramps, nausea, fever, vomiting, headache and bloody diarrhea. Antimicrobial treatment has not been shown to shorten the course of uncomplicated gastroenteritis associated with this pathogen (Potasman *et al.*, 2002).

The mechanism of infection used by this organism is distinct from that used by *V. cholerae*. A study by Makino *et al.* (2003), demonstrated that this organism uses a Type III secretion system (TTSS) similar to that used by other pathogens such as *Shigella*, *Salmonella* and enteropathogenic *E. coli*. *V. cholerae* on the other hand, lacks this system. The TTSS is a needle like structure composed of approximately 20 proteins (Blocker *et al.*, 2001) and in *V. parahaemolyticus* allows these bacteria to invade epithelial cells and/or intimately associate with them. The role for the TTSS in part explains the inflammation of *V. parahaemolyticus* induced disease (Makino *et al.*, 2003; Park *et al.*, 2004). This is distinct from non-inflammatory disease caused by *V. cholerae*.

Disease associated with *V. parahaemolyticus* infections is generally restricted to isolates possessing either the thermostable direct hemolysin (*tdh*) gene, or the thermostable direct hemolysin-related hemolysin (*trh*) gene. A study by Shirai *et al.* (1990) demonstrated that of 285 clinical strains of *V. parahaemolyticus* tested, 52.3% carried the *tdh* gene only, 24.3% carried the *trh* gene only and 11.2% carried both. Of 71 environmental strains, only 7% hybridised very weakly with the *trh* gene probe and none

hybridised with the *tdh* gene probe. These results strongly suggest a role for these genes in disease associated with ingestion of *V. parahaemolyticus*, and as such are widely considered as markers of pathogenicity in this species.

Tdh is a 46 kDa homodimer protein (Honda & Iida, 1993) that, due to its early identification as a virulence determinant of *V. parahaemolyticus*, has been extensively studied. One of the first recognised activities of Tdh was haemolysis, and this activity remains the most extensively studied. It acts as a pore-forming toxin (Honda *et al.*, 1992) that causes osmotic lysis of erythrocytes derived from different mammalian species (Huntley & Hall, 1994). In addition to its haemolytic activity, Tdh elicits lethal activity in small experimental animals (Takeda, 1988). More recently, Tdh has been shown to modulate cytoskeletal re-organization and calcium homeostasis in cultured intestinal cells leading to loss of viability (Fabri *et al.*, 1999). Both Tdh and Trh have also been implicated in chloride secretion in human colonic epithelial cells (Takahashi *et al.*, 2000), a key component of infections leading to diarrhea.

By comparison, very little is known about Trh. Nevertheless, it has been shown to possess significant homology (70%) to Tdh and as such is believed to function in a similar manner (Nishibuchi *et al.*, 1989). Indeed, Trh has been shown to stimulate fluid secretion in the rabbit ileal loop model, indicating a role in the induction of diarrhoea (Honda *et al.*, 1988), much like Tdh. However, unlike Tdh, Trh is heat labile to 60°C for 10 min (Honda *et al.*, 1988). Furthermore, a study by Kishishita *et al.* (1992) demonstrated two serotypes of *trh*, *trh1* and *trh2*. The genes encoding these possess 84% nucleotide sequence similarity and this study concluded that both should be considered virulence markers of this organism.

An additional haemolysin found in *V. parahaemolyticus* is the thermo-labile haemolysin (Tlh), which was identified by Sakurai *et al.* (1974). Whilst the role of this haemolysin in virulence is unknown (Shinoda *et al.*, 1991), *tlh* has been found in the genome of all *V. parahaemolyticus* isolates examined, regardless of clinical or environmental etiology. Consequently, this gene is often used as a method of confirming the identity of isolates of this species (Bej, 1999; McCarthy *et al.*, 1999).

Interestingly, pathogenic isolates are apparently very rarely found in nature, and are only ever recovered from clinical infections. There are two schools of thought to explain this observation. The first is that pathogenic strains may be at lower concentrations in the natural environment, but proliferate more readily in the host. The alternative is, as mentioned in Section 1.6.2, that pathogenic strains are more sensitive to low nutrient conditions and while in the aqueous environment readily enter the VBNC state and are hence not detected as efficiently as non-pathogenic strains. Support for this argument comes from a study by Pace *et al.* (1997), which showed that when *V. parahaemolyticus* was deprived of nutrients and subsequently grown in media containing bile, or bile derivatives, as would be seen as they pass through the small intestine, there was a direct increase in viable counts and colony counts among pathogenic strains, but not among the non-pathogenic strains of *V. parahaemolyticus*.

#### **1.6.7 *Vibrio vulnificus***

*V. vulnificus* is another *Vibrio* species subject to increasing scrutiny due to its role as both a human and fish pathogen. In humans, this organism causes two kinds of clinical manifestations; fatal septicemia following consumption of contaminated seafood harboring the bacterium, or severe wound infections following exposure to sea water or handling of contaminated seafood, which can also lead to septicemia. People with suppressed immune responses, or those with chronic diseases resulting in elevated serum iron levels, are particularly susceptible to this bacterium, but healthy individuals may also be affected (Hoyer *et al.*, 1995). The infective dose for *V. vulnificus* required to cause gastroenteritis in healthy individuals is currently unknown. However in susceptible individuals, septicemia can be induced by less than 100 infective organisms (<http://www.foodsafety.gov/~mow/chap10.html>). Two biotypes of *V. vulnificus* (Biotypes 1 and 2) are implicated in disease. Biotype 1 is considered to be a human pathogen, whilst Biotype 2 is considered an eel pathogen, although the latter is also considered an opportunistic human pathogen (Amaro & Biosca, 1995). Infections are generally acquired by the consumption of raw oysters harvested from warmer waters, however this organism has been responsible for human infections as far south as southern Victoria, Australia (Maxwell *et al.*, 1991).

Little is known about the mechanisms of virulence associated with *V. vulnificus* (Moreno & Landgraf, 1998). However, opaque colony morphology has been linked with virulence due to the presence of a capsule (Simpson *et al.*, 1987). This was tested by Biosca *et al.* (1993) who demonstrated that reversion to the translucent colony morphology (non-capsulated) resulted in a 1 - 3 log increase in the LD<sub>50</sub> of I.P. injected eels. This was seen to be due to the loss of the protective function of the capsule. Yoshida *et al.*, 1985, also observed this effect in mice. Apart from this, generic virulence factors are generally investigated to determine potential virulence of *V. vulnificus* isolates. For example, production of proteases, haemolysins, elastase and *in vitro* cytotoxicity to cultured mammalian cells are usually accepted as indicators of pathogenicity (Levin, 2005). Furthermore, changes in colony morphology, and hence capsular polysaccharide (CPS) expression, are caused by reversible phase variation (Wright *et al.*, 2001). Indeed, the operon responsible for this phenomenon is controlled by an epimerase gene that shares 75 and 85% identity at the nucleotide and amino acid levels respectively with the epimerase of *V. cholerae* (Zuppardo & Siebeling, 1998).

One of the key markers for identifying *V. vulnificus* is the presence of the *Vibrio vulnificus* haemolysin (*vvh*) gene. This gene is generally detected by PCR methods. However this haemolysin is also implicated in virulence, both *in vitro* (Grey & Kreger, 1985) and *in vivo* (Lee *et al.*, 2004). Both of these studies conclude that there is a potential role for *vvh* in pathogenesis of this organism due to its ability to induce lyses of red blood cells and cytolytic activity in cultured cells.

As mentioned earlier, iron levels in serum is one of the key host components that affect the outcome of *V. vulnificus* infections. Indeed, diseases such as liver cirrhosis that result in elevated serum iron levels are considered high risk factors for infection by this organism (Tacket *et al.*, 1984; Wright *et al.*, 1981). It is currently accepted that chronic liver damage may result in iron overload resulting in sufficient free iron levels in the blood to allow septicemic growth of *V. vulnificus*. Indeed, as many as 86% of cases may be attributable to individuals with chronic liver disease or other diseases leading to iron dysregulation (Shapiro *et al.*, 1998). This effect was confirmed by Stelma *et al.* (1992) who found the LD<sub>50</sub> of *V. vulnificus* for iron overloaded mice was reduced from 10<sup>6</sup> CFU to just a single CFU in non-compromised mice. Furthermore, Hor *et al.* (1999) investigated the survival



of *V. vulnificus* in whole blood of individuals with liver disease of varying severity. Importantly, this study concluded that bacterial numbers increased along with the severity of liver disease. Survival in whole blood by this organism also correlated with serum ferritin concentration, percentage of transferrin iron saturation and phagocytosis by neutrophils. Further confirmation of the role of serum iron levels in *V. vulnificus* infections is provided by Starks *et al.* (2000) who demonstrated that mice treated with iron dextran leading to serum iron overload had an infectious dose  $10^5$  fold lower than normal mice. This phenomenon has been attributed to the production of siderophores (microbial iron chelators), which are produced by this bacterium in low iron conditions (Simpson & Oliver, 1983).

#### **1.6.8 Other Vibrios of Significance**

*Vibrio mimicus* is another enteropathogenic *Vibrio* species that poses a significant health threat to consumers of raw and undercooked seafood. It is closely related to *V. cholerae*, so much so that it wasn't considered a separate species until 1981, when minor differences in biochemical characteristics, for example sucrose fermentation, were discovered between the two species (Davis *et al.*, 1981). As a consequence of the close relationship between *V. mimicus* and *V. cholerae*, they share pathogenic factors such as enterotoxins and hemolysins (Shi *et al.*, 1998; Spira *et al.*, 1984). However, unlike *V. cholerae*, *V. mimicus* has not been reported to produce severe epidemics of disease, but is often isolated from patients of sporadic diarrheal cases (Shandera, 1983).

*Vibrio harveyi*, on the other hand, is generally associated with diseases in marine animals, particularly farmed shrimp. Of particular interest is their association with diseases such as luminous vibriosis in rock lobsters (Diggles *et al.*, 2000) and outbreaks of high mortality in cultured kuruma prawns (Liu *et al.*, 1996). However, this species is not generally associated with human disease.

Most, but not all, other *Vibrio* species are not generally considered to be a threat to either humans or crustacean species, either due to their low frequency or usual lack of virulence. Nevertheless, many are still the cause of sporadic outbreaks of disease in either humans (Abbott & Janda, 1994, Maxwell *et al.*, 1991, Potasman *et al.*, 2002) or commercially important crustaceans (Lee *et al.*, 1996, Liu *et al.*, 1996). Importantly, little

is known about the association between *Vibrio* species and crustacean species. This is particularly true in Australian fisheries where few, if any, studies into this area have taken place. However, a major study of the incidence and impact of marine *Vibrios* in aquaculture systems has been developed<sup>1</sup>.

### 1.6.9 Chitin Utilisation and Chitinolytic Shell Diseases

Chitin utilization, particularly by marine *Vibrios*, is a key element required by any pathogen to induce infection of the exocuticle of crustaceans. By degrading the chitinous exoskeleton, bacteria are able to gain important metabolites essential for growth. Chitin utilization is also essential in the marine environment to recycle carbon and nitrogen from the form  $\beta,1,4$ -linked N-acetyl-D-glucosaminopyranosyl ((GlcNAc)<sub>n</sub>), otherwise known as chitin. Thus not surprisingly, chitinases play a key role in many crustacean diseases, such as shell disease, where bacterial chitinases solubilise the chitinous layers of the exoskeleton and gill filaments for use as a carbon and nitrogen source (Bassler *et al.*, 1991).

In marine *Vibrio* species, two parallel pathways for degradation and catabolism of the chitin substrate have been proposed (Figure 1.8). These comprise 6 – 10 enzymes and a number of chemotactic proteins. In the common part of the pathway, the bacteria adhere to chitin by chitin binding proteins. *V. furnissii* expresses one of the better understood chitin utilization systems. This has been shown to be a Ca<sup>2+</sup> dependent lectin (Wu & Laine, 1999). Extracellular chitinase and periplasmic chitinodextrinase then work in unison to form N-N'-diacetylchitobiose [(GlcNAc)<sub>2</sub>]. In one branch of the pathway, a glycosidase/PTS enzyme cleaves (GlcNAc)<sub>2</sub> to give 2-deoxy-2-acetamino-d-glucose (GlcNAc) in the periplasmic space via membrane bound chitobiase (Enzyme II<sup>Nag</sup>). PTS then phosphorylates and transports GlcNAc into the cytoplasm. The second branch of the pathway utilizes an as yet unidentified N, N'-diacetylchitobiose permease to transport (GlcNAc)<sub>2</sub> into the cytoplasm. It is then cleaved by a cytoplasmic chitobiase and phosphorylated by an ATP dependant N-acetyl-D-glucosamine kinase independently of the

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<sup>1</sup> See [http://www.utas.edu.au/tafi/TAFI\\_R&D\\_0Sections/TAFI\\_R&D\\_Prog\\_Aqua\\_Health.htm](http://www.utas.edu.au/tafi/TAFI_R&D_0Sections/TAFI_R&D_Prog_Aqua_Health.htm)).

PTS system (Wu & Laine, 1999). GlcNAc is then deacetylated and deaminated to give the final catabolites fructose-6-phosphate, acetate and ammonia (Bassler *et al.*, 1991).

This system of breaking down chitin to usable products by the bacteria allows them to occupy niches that would normally be inaccessible. This includes survival and amplification on the surface of organisms such as copepods and higher crustacean species. Colonization of the surfaces of the exoskeletons of these animals may subsequently lead to disease by the bacteria degrading the outer chitin layer of the exoskeleton, which in turn may lead to various forms of shell disease.

## **1.7 Aims**

1. Determine the cause of TFN and identify associated bacterial species.
2. Assess the microbial community dynamics within developing lesions associated with TFN.
3. Determine overall health affects of TFN on affected lobsters.
4. Investigate the immune response associated with lobsters affected by TFN.
5. Determine the health risks to consumers associated with southern rock lobsters affected by TFN.

## **1.8 Hypotheses**

1. TFN associated lesions will comprise a mixed population of bacteria.
2. Marine vibrios will dominate the microflora of TFN associated lesions but that a mixed population of bacterial types will comprise the microflora of the normal lobster carapace.
3. TFN will not adversely affect the overall health of affected lobsters.
4. There will be a continual activation of the immune response characterised by increased levels of phenoloxidase activity and increased in vitro phagocytosis in response to TFN.

5. *V. vulnificus* and *V. parahaemolyticus* will be present within lesions associated with TFN, hence there is a significant health risk associated with lobsters affected by TFN.

**Table 1.1: Overview of the South Australian southern rock lobster fisheries.**

<b>Fact</b>	<b>Northern Zone</b>	<b>Southern Zone</b>
Commercial Licences (1998)	69	181
Commercial Pots	3950	11923
Recreational Pots	Approximately 11,000 (for the state)	
Season	Nov 1 - May 31	Oct 1 - Apr 30
Minimum Size Limits	105 mm Carapace Length	98.5mm Carapace Length
Management Strategy	Controlled fishing effort	Controlled catch
Ave. Season Catch	940 tonnes	1680 tonnes
2001/2002 Average Price	Whole: \$52.00/kg*	Meat only: \$97.00/kg
Total Value (ex-vessel 1998)	\$47 million	\$90 million
Recreational Catch	Approximately 100 tonnes (for the state)	

\*prices on 28/03/02 were \$68.00/kg

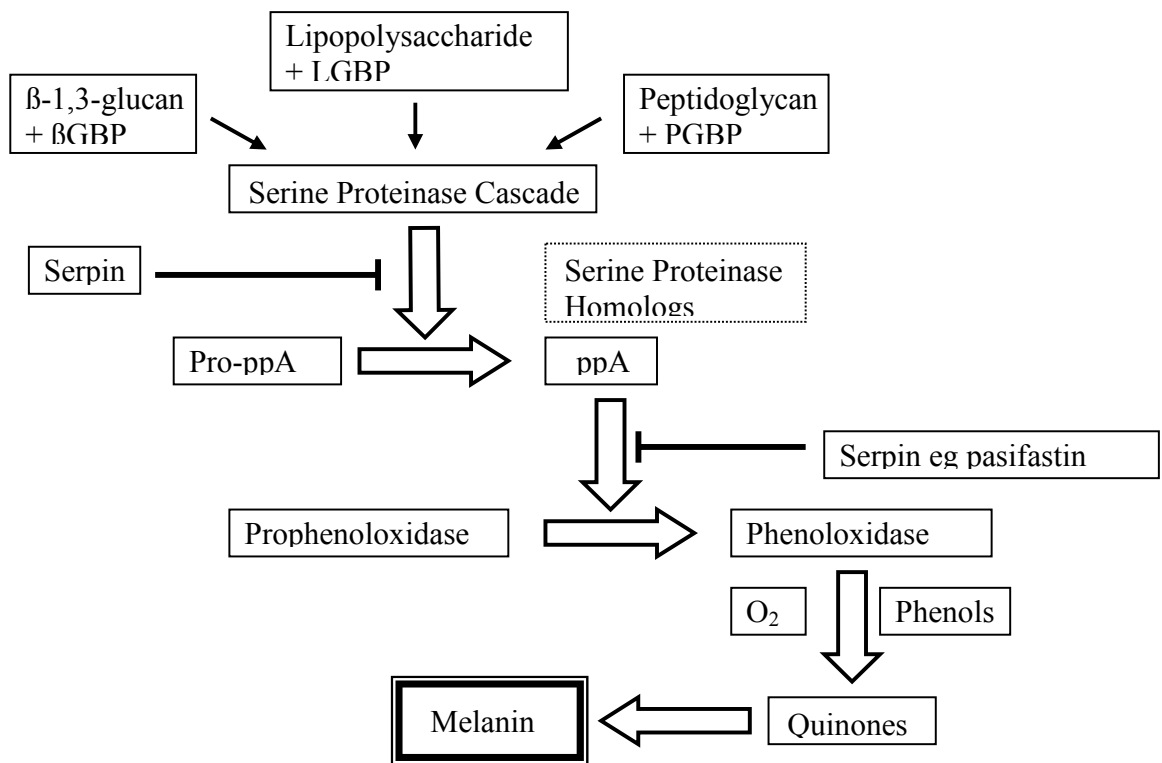
**Table 1.2: Gross Value of Australian Fisheries Production 1999-2000 (\$'000)**

Catch type	State							Commonwealth	Australia
	NSW	VIC	QLD	SA	WA	TAS	NT		
<b>Fish</b>									
Tuna	19,479	125	13,186	280,147	2,923	504	29	106,095	276,148
Other	67,492	54,631	102,826	58,337	53,872	146,620	25,850	160,935	550,296
<b>Total</b>	<b>86,971</b>	<b>54,756</b>	<b>116,013</b>	<b>338,484</b>	<b>56,796</b>	<b>147,124</b>	<b>25,879</b>	<b>267,030</b>	<b>826,444</b>
<b>Crustaceans</b>									
Prawns	27,834	731	184,076	43,423	50,475	-	48,108	135,684	354,872
Rock lobster	4,338	14,055	18,298	61,365	261,405	44,921	318	5,390	405,565
Other	7,576	614	31,090	4,925	8,970	1,658	4,786	2,457	60,833
<b>Total</b>	<b>39,135</b>	<b>19,615</b>	<b>145,801</b>	<b>128,636</b>	<b>470,582</b>	<b>47,634</b>	<b>12,062</b>	<b>143,530</b>	<b>1,006,995</b>
<b>Molluscs</b>									
Abalone	10,668	57,743	NA	32,394	35,310	99,513	-	-	235,628
Scallops	-	642	18,068	-	14,471	5,280	5	91	38,557
Oysters	28,813	-	650	9,309	-	13,176	-	-	51,948
Other	4,438	3,019	1,1853	5,466	205,032	3,137	3,353	2,095	228,363
<b>Total</b>	<b>43,919</b>	<b>61,404</b>	<b>20,571</b>	<b>47,169</b>	<b>254,813</b>	<b>121,106</b>	<b>3,358</b>	<b>2,186</b>	<b>554,526</b>
<b>Total value</b>	<b>115,034</b>	<b>101,761</b>	<b>231,757</b>	<b>401,638</b>	<b>767,590</b>	<b>258,962</b>	<b>86,684</b>	<b>412,749</b>	<b>2,322,305</b>

Data from ABARE, 2005.

**Table 1.3: Composition of crustacean exoskeleton layers.**

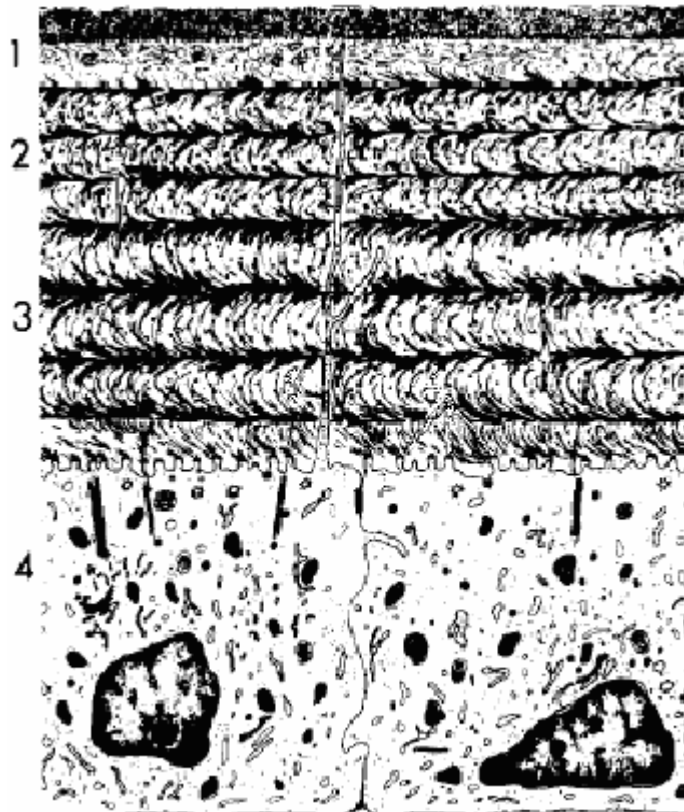
<b>Layer</b>	<b>Location</b>	<b>Thickness</b>	<b>Composition</b>	<b>Colour</b>	<b>Properties</b>
Epicuticle	Outer	Very thin (~ 7µm)	Lipid and protein	None	Composed of a cement like outer layer overlaying a wax layer consisting of lipids that determine permeability. This overlays a thin, tough insoluble, non-elastic lipid and protein layer and finally an inner, thicker mostly protein layer which limits expansion needed with growth
Exocuticle	Middle	May be variable, (usually ~30µm)	Carbohydrate and protein	Dark	Consists of 20-50% chitin along with protein and Calcium salts, principally calcite. This layer may be very thin in soft bodied insects or thick in hard bodied insects and crustaceans
Endocuticle	Inner	Variable (~200-400µm)	Carbohydrate and protein	Light	Similar in composition to the exocuticle, however it is softer due to a lower level of calcite and protein cross-linking with chitin, generally has lower chitin content and higher protein content than exocuticle



**Figure 1.1: Overview of the Prophenoloxidase activating cascade.**

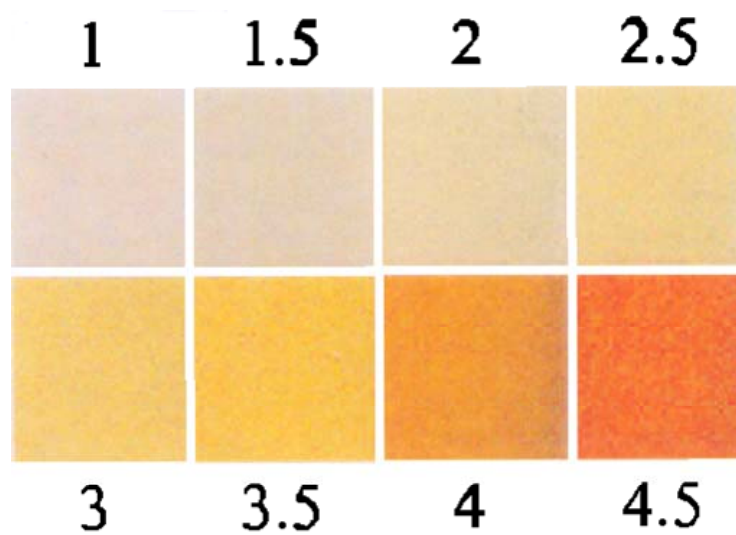
The cascade is initiated by pattern recognition proteins that have bound to  $\beta$ -1,3 glucans of fungi, lipopolysaccharide or peptidoglycan (ie  $\beta$ GBP, LGBP or PGBP activities), which may be carried out by several proteins or individual proteins with several recognition domains, depending on the species and protein, or by endogenous factors such as those produced upon cuticular damage. A serine protein cascade, the members of which are yet to be identified, then result in the activation of pro form of the prophenoloxidase activating enzyme (pro-ppA) into ppA. Some ppA's then require additional serine proteinase homologs to aid cleavage of pro-PO, whilst others do not. (Cerenius and Söderhäll, 2004)





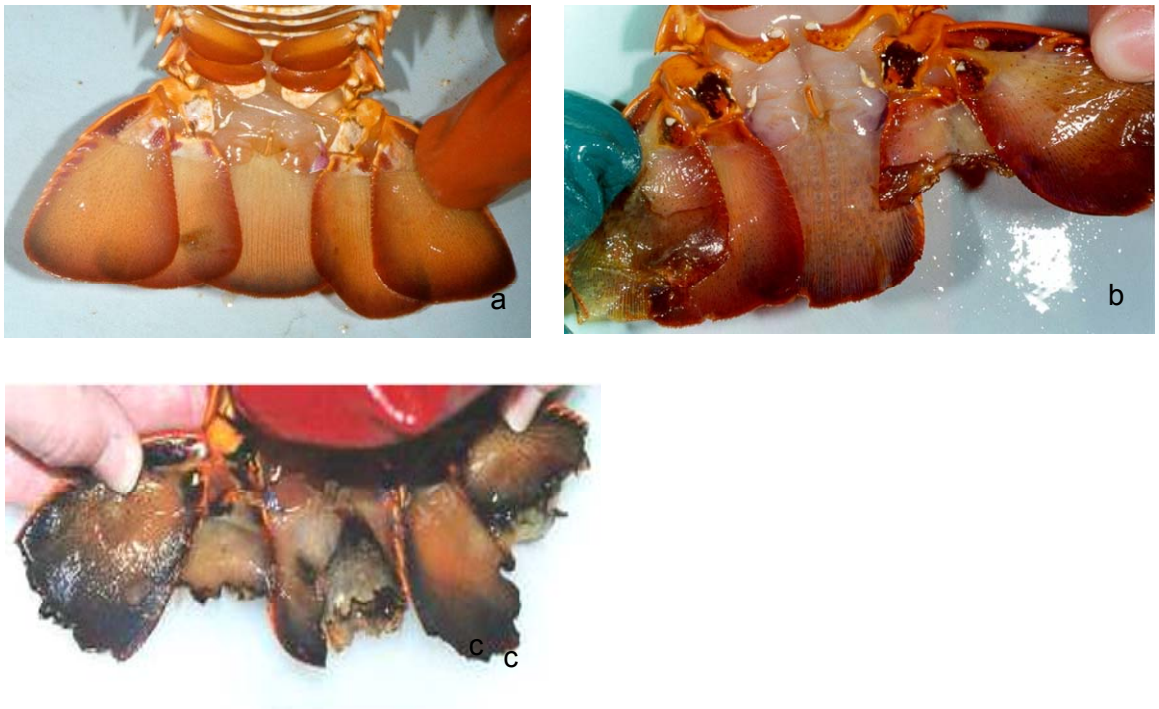
**Figure 1.2:** Schematic of the lobster exo-skeletal-epidermal complex.

Key to numbers: (1) epicuticle; (2) exocuticle; (3) endocuticle; (4) epidermis. Figure from: Arsenault *et al.*, 1984).



**Figure 1.3: Haemolymph pigment colour chart.**

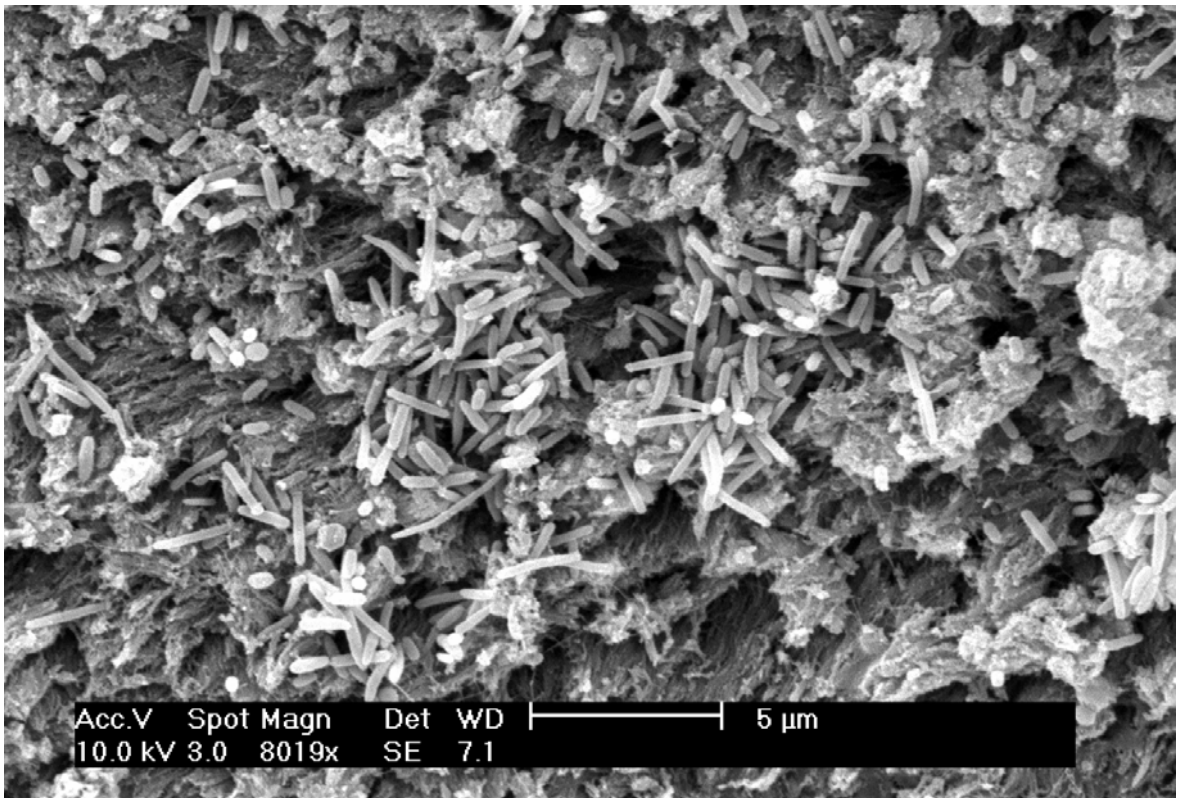
Chart depicts haemolymph colour at each stage of the moult cycle as per Musgrove (2001). PS 0.5 denotes post moult, PS 1 to PS 2.5 denotes inter moult, PS 3 denotes late inter-moult/early pre-moult, and the remaining stages follow as ecdysis approaches.



**Figure 1.4: Symptoms of TFN on tail fans of southern rock lobsters.**

a, Healthy tail fans;

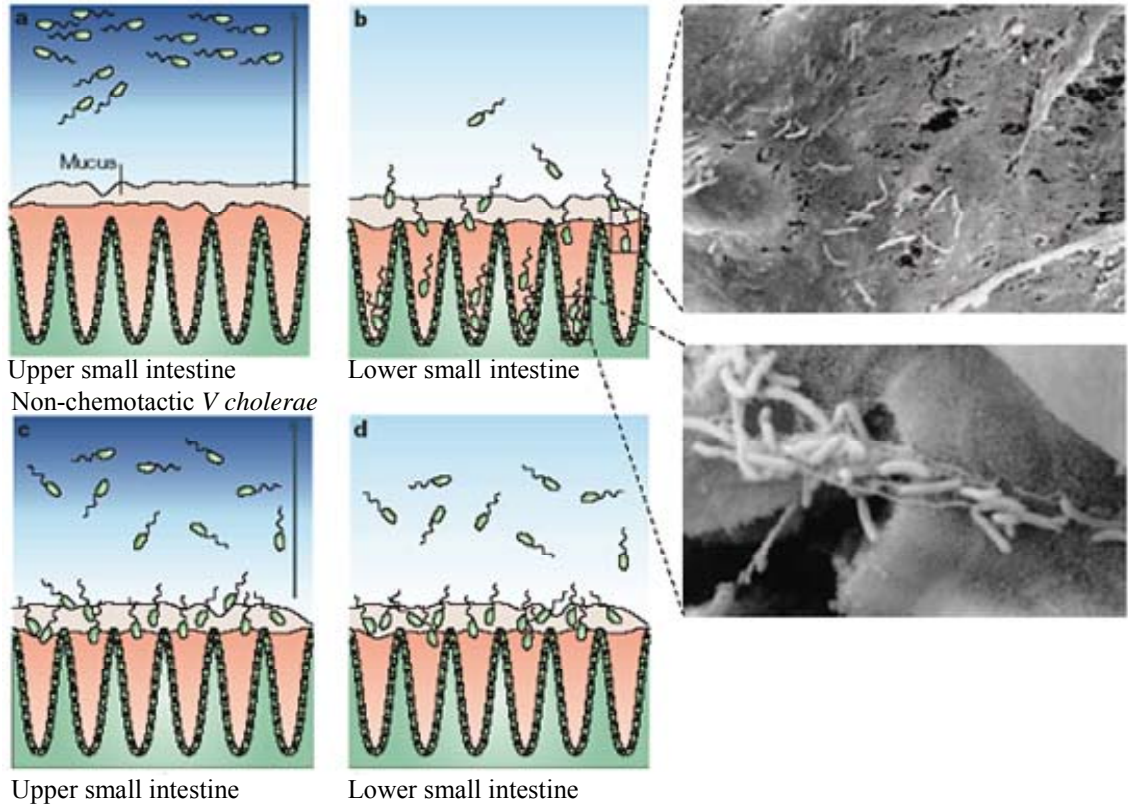
b and c, tail fan tissue affected by TFN. Note the extensive blackening (melanisation) and erosion (necrosis)



**Figure 1.5** Typical micro-colony associated with surface of TFN lesions

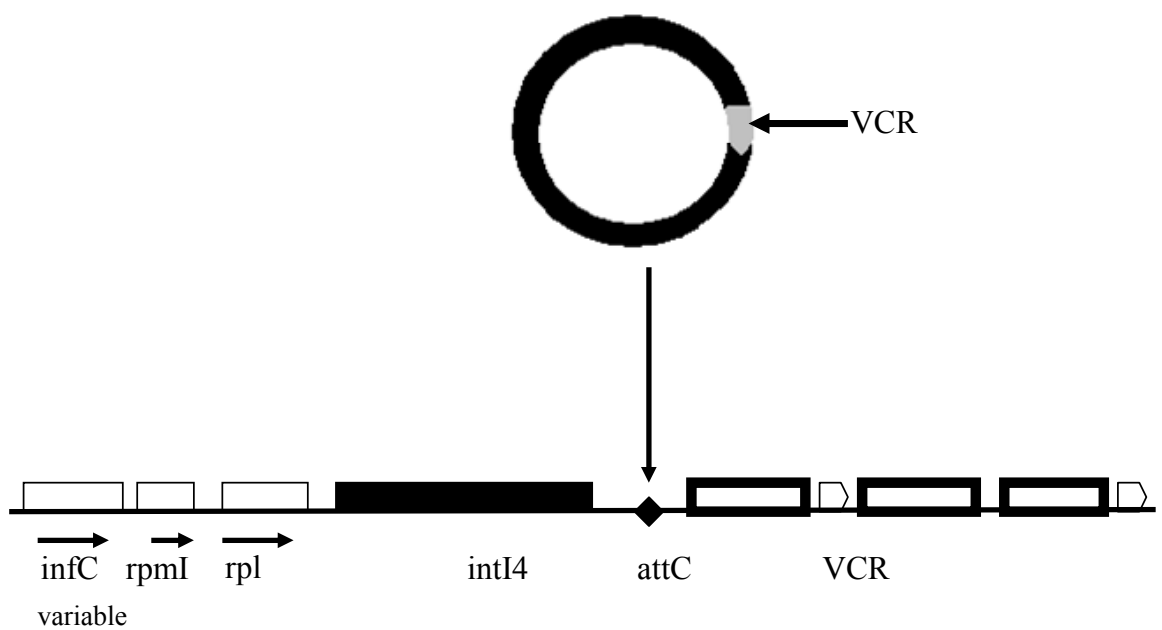
This micrograph shows the typical micro-colonies that were seen in association with the surface of TFN-like lesions by May (2002).

Wild type *V. cholerae*



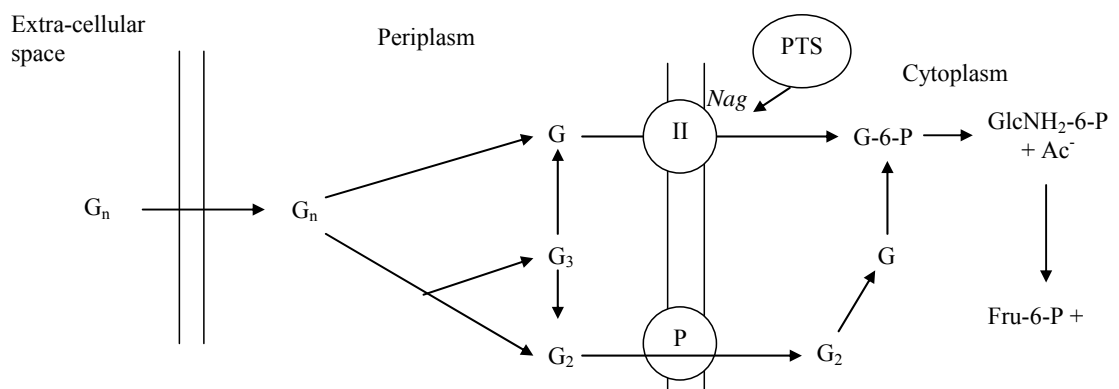
**Figure 1.6: Chemotaxis and colonization of the small intestinal epithelium by *V. cholerae*.**

A gradient of chemo-attractant is present in the lumen of the upper small intestine, oriented away from the villi (depicted as arrows in a and c). Using chemotaxis, wild-type *V. cholerae* respond to this gradient by concentrating within the lumen. However, this chemo-attractant gradient is absent from the lumen of the lower small intestine (b and d), allowing wild-type *V. cholerae* to respond to a different chemo-attractant gradient that directs them into the intervillous spaces and onto epithelial surfaces (see scanning electron micrograph panels). By contrast, non-chemotactic *V. cholerae* are blind to these gradients and therefore colonize both the upper and lower small intestine (panels c and d) (From: Butler & Camilli, 2005).



**Figure 1.7 Model for gene assimilation within super-integrans of *Vibrio* spp.**

Gene cassette insertion in vibrios is associated with the super integron (SI) and cassettes containing a 124 bp direct repeat unit dubbed *Vibrio cholerae* repeats (VCR's). Integration occurs at the *attC* site following recognition of the VCR by *IntI4* (Manning *et al.*, 1999).



**Figure 1.8:** Chitin utilisation pathway of *V. furnissii*

Extracellular chitinase and periplasmic chitinodextrinase work in unison to produce  $(GlcNAc)_2$ . In one branch of the pathway, a PTS enzyme (a) cleaves  $(GlcNAc)_2$  to give  $GlcNAc$  via Enzyme II<sup>Nag</sup>. PTS then phosphorylates and transports  $GlcNAc$  into the cytoplasm. In the second pathway (b), an as yet unidentified permease transports  $(GlcNAc)_2$  into the cytoplasm. Here, it is cleaved by a cytoplasmic chitobiase and phosphorylated independently of the PTS pathway.  $GlcNAc$  is then deacylated and deaminated to give the catabolites fructose-6-phosphate, acetate and ammonia (From: Bassler *et al.*, 1991).

## Chapter 2: Materials and Methods

### 2.1 Chemicals and Reagents

Unless otherwise stated, all chemicals were sourced from Sigma, Ajax Chemicals, BDH or Bio-Rad and were of AnalaR grade. All enzymes were from New England Biolabs unless otherwise stated.

### 2.2 Bacterial Cultures

During this study, a number of bacterial type strains were used. These were: *Vibrio parahaemolyticus* NCTL strains 10884 and 10885; *V. parahaemolyticus* strain L21 isolated by May (2002); *V. vulnificus* ATCC strains 27562 and 33148; *Escherichia coli* strain DH5 $\alpha$ ; *E. coli* strain XL10; *E. coli* ATCC strain 2120. Type strains of *V. vulnificus* strains or those that had been independently identified<sup>2</sup> used for this work were: *V. vulnificus* biovar 1 (host = barramundi); *V. vulnificus* biovar 1 (host = Dugong); *V. vulnificus* biovar 1 (host = green turtle); *V. vulnificus* biovar 1 (host = isopod); *V. vulnificus* biovar 1 ATCC 27562; *V. vulnificus* biovar 2 ATCC 33148 and one *V. parahaemolyticus* type strain NCTC 10884.

### 2.3 Growth Media for Bacterial Cultures

All isolates from rock lobster tissues were routinely cultivated on marine agar (MA) [1% peptone (w/v), 1% soluble yeast extract (w/v), 3% NaCl (w/v), 1.5% Agar (w/v)] or marine broth [1% peptone (w/v), 1% soluble yeast extract (w/v) and 3% NaCl (w/v)] with incubation overnight at 30°C. For long-term storage, isolates were suspended in glycerols (30% glycerol (v/v), 1% peptone (w/v)) and maintained at -70°C. Fresh cultures were prepared by streaking a loop full of glycerol stock for single colonies onto appropriate media, followed by incubation over-night as appropriate.

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<sup>2</sup> Strains provide by Dr J. Carsons, Fish Health Unit, Department of Primary Industries, Water and Environment, Kings Meadows, Launceston, Tasmania, Australia.



## **2.4 Counts of Bacteria Associated with Lobster Tissues and Holding Water**

### **2.4.1 Bacterial Load of Lobster Holding Tank Water**

Seawater samples (100  $\mu$ L) were surface spread onto plates of MA and thio-citrate bile sucrose (TCBS) media and inoculated plates incubated for 48 h at 30°C. Colony counts were expressed as numbers of bacteria per mL of seawater.

### **2.4.2 Counts of Bacteria in Lobster Haemolymph**

Haemolymph samples (100  $\mu$ L) taken by pericardial puncture using a 21 gauge syringe containing 2 mL of cold marine anti-coagulant buffer (3 mM tri-sodium citrate, 0.34 M NaCl, 10 mM EDTA, 0.12 M glucose, pH 7.55), were spread onto the surface of MA and TCBS media and inoculated plates incubated for 48 h at 30°C. Colony counts were used to estimate numbers of bacteria per mL of haemolymph.

### **2.4.3 Bacterial Load of Tissue Affected by TFN**

Tissue excised from uropod lesions from affected lobsters was homogenised in 10 mL sterile saline solution [3% NaCl (w/v)] using an Ultra-Turrax (IKA-WERK, Germany), serially diluted in sterile saline solution [3% NaCl (w/v)] and plated onto MA and TCBS agar. Inoculated plates were incubated at 30°C for 48 h. Colony counts were used to estimate total viable counts and total vibrio counts (TVC) per gram of tissue.

Counts of bacteria associated with deep tissue of inflamed lesions were obtained as follows. The uropod carapace surface was surface sterilised by swabbing with a solution of crystal violet (0.5% w/v). The sterilized carapace was aseptically removed to expose underlying tissue. Samples of tissue was then aseptically excised, weighed and homogenised in 10 mL of sterile saline solution [3% NaCl (w/v)] using an Ultra-Turrax homogeniser. The homogenate was serially diluted in sterile saline solution [3% NaCl (w/v)] and 100  $\mu$ L volumes of each dilution plated on MA and TCBS. Inoculated plates were incubated as described above and colony counts expressed as numbers of bacteria per gram of tissue.

## 2.5 Identification of *Vibrio* Isolates from TFN tissue

Pure cultures of isolates from TCBS plates were cultured on MA and used to assess Gram reaction, morphology and oxidase reaction (Maugeri *et al.*, 2000). Motility was examined by microscopy of overnight marine broth cultures. Gram negative, oxidase positive bacteria that grew on TCBS medium were subsequently identified as per Alsina and Blanch (1994). Confirmatory biochemical and physiological tests were carried out as per Gerhardt *et al.* (1994). Salt tolerance was measured by growth of isolates on nutrient agar [1% peptone (w/v), 1% yeast extract (w/v)] containing salt concentrations of 0% 3%, 6% or 8% NaCl (w/v).

Expression of lipase by bacterial isolates on agar containing peptone (10 g), CaCl<sub>2</sub>·2H<sub>2</sub>O (0.1 g), NaCl (30 g) and agar (15 g) were added to distilled water (1000 mL). This was sterilized, adjusted to pH 7.4 and allowed to cool to 55°C and Tween 80 added to a final concentration of 1% (v/v). Spot inoculated plates of this medium were incubated overnight at 30°C. Lipase positive isolates were characterised by a clear zone surrounding the colonies.

Expression of extra-cellular proteases was detected by growth on casein plates (1% (w/v) skim milk powder in MA). Streak plates prepared from pure isolates were incubated at 30°C for up to 14 d. Protease positive isolates were characterised by a clear zone surrounding the bacterial growth.

Expression of lecithinase was detected following growth of isolates on egg yolk agar plates [peptone (20 g), Na<sub>2</sub>HPO<sub>4</sub> (2.5 g), NaCl (15 g), MgSO<sub>4</sub> (0.5% w/v), glucose (1 g), agar (12.5 g), egg yolk (10% v/v) and distilled water (500 mL)], as described by Gerhardt *et al.* (1994). Inoculated plates were incubated at 30°C. Lecithinase positive isolates were characterised by a yellow precipitate surrounding the colonies.

Expression of chitinase by isolates was tested using a method described by Suginta *et al.* (2000). Briefly, chitin agar plates were prepared by addition of swollen chitin (1% w/v) to MA. Overnight MA cultures of each isolate were stab inoculated into the agar and inoculated plates were incubated at 30°C for 3 d followed by storage at 4°C for four weeks. Chitinase activity was determined by the presence or absence of a cleared zone around the colony growth at the stab inoculation site.

## **2.6 Isolation of DNA from Bacterial Cultures**

### **2.6.1 Isolation of Genomic DNA from Bacterial Cultures**

#### **2.6.1.1 Method 1**

DNA was extracted from bacterial cultures using the Promega Wizard Genomic DNA Purification System (Promega, Annandale, NSW, Australia) as per the manufacturer's instructions.

#### **2.6.1.2 Method 2**

Crude DNA extracts were collected from pure cultures as follows. Overnight culture (1.5 mL) was centrifuged (14,000 rpm, 1 min) and the cell pellet washed twice in sterile PBS, followed by resuspension in sterile MQ water (100  $\mu$ L) and boiling for 3 min. The mixture was then briefly vortexed, placed on ice for 10 min, then centrifuged (14,000 rpm, 10 min) at 4°C to remove cell debris. 1 to 2  $\mu$ L of supernatant was used as a source of DNA for PCR reactions.

### **2.6.2 Extraction of Genomic DNA from Lobster Tail Fan Tissue**

For some experiments (see Section 2.16) it was necessary to isolate total DNA from lobster tail fan tissue. For this purpose, a method described by Guthrie *et al.* (2000), was employed.

Briefly, tissue samples were cut up finely using a sterile scalpel. Approximately 200 mg of sample was added to 1 mL of lysis buffer [25% (w/v) sucrose in 50 mM Tris-HCl, pH 8.0, 1 mM EDTA and 1 mg ml<sup>-1</sup> lysozyme] and incubated at room temperature for 30 min with gentle shaking. Proteinase K solution (50  $\mu$ l; 20 mg ml<sup>-1</sup> in Tris-HCl, pH 7.5), EDTA (400  $\mu$ L, 0.25 M) and N-lauroyl sarcosine (10% w/v, 250  $\mu$ L) were added and incubated at 60°C with occasional gentle shaking for 1 h. An aliquot of 5 M NaCl (5 M, 300  $\mu$ L) was added and mixed thoroughly. CTAB-NaCl (240  $\mu$ L) was added, mixed and incubated for 10 min at 65°C. Samples were extracted with an equal volume of chloroform-isoamyl alcohol and centrifuged at 14 000 g for 5 min. The aqueous phase was extracted with phenol - chloroform-isoamyl alcohol and centrifuged at 14 000 g for 5 min.

Twice the volume of ethanol (20°C) was added to the aqueous layer, gently mixed, incubated at 20°C for 60 min and centrifuged for 20 min at 14 000 g. The pellet was washed with 70% ethanol, recovered by centrifugation for 10 min at 14 000 g, dried, resuspended in TE buffer and stored at 20°C.

## **2.7 Polymerase Chain Reaction**

All PCR reactions described in this study were formulated as follows: each reaction mixture consisted of Milli-Q H<sub>2</sub>O (22 µL), forward and reverse primer (1 µM each), template DNA (1 µL) and Amplitaq Gold PCR Mastermix (Applied Biosystems). Oligonucleotide primer pairs and thermal cycling conditions used are detailed in Table 2.1 and Table 2.2. All oligonucleotide primers used in this study were synthesised by Geneworks (Adelaide, South Australia, Australia). An MJ Research PTC100 Programmable Thermal Controller (MJ Research Inc, Wentworth, Massachusetts, USA) was used for all thermal cycling reactions.

## **2.8 Agarose Gel Electrophoresis**

Electrophoretic separation of DNA fragments was carried out at room temperature using 1% (w/v) agarose gels prepared in 1× TAE buffer (50 × TAE Buffer: 242 g Tris base, 57.1 mL Acetic acid, 100 mL 0.5M EDTA, ddH<sub>2</sub>O to 1 liter and adjusted pH to 8.5). A 2 - log DNA ladder (New England Biolabs) or *Eco*RI digested SPP1 bacteriophage DNA or the GeneRuler 100bp DNA Ladder (Fermentas Life Sciences) was used as molecular weight markers for DNA fragments separated in agarose gels. Gels were run at 100 V in 1 × TAE buffer and stained in distilled water containing 2 µg mL<sup>-1</sup> ethidium bromide. Gels were visualized using a transilluminator (UVP Inc., Upland California, USA) and photographed using a Tracktel GDS-2 Gel Documentation System (Vision Systems, Salisbury, South Australia, Australia).

## **2.9 Cloning of PCR Products**

PCR products were purified using the QIAquick PCR Purification System (QIAGEN, Doncaster, Victoria, Australia) as per the manufacturer's instructions. Subsequently, the

purified PCR products were cloned into pGEM-T (Promega) as per the manufacturer's instructions.

## **2.10 Transformation of *E. coli***

### **2.10.1 Preparation of Chemically Competent *E. coli***

A 10 mL overnight culture was diluted 1:20 in 20 mL of Luria broth (LB). This was then grown to an OD<sub>600</sub> of 0.6 at 37°C with constant shaking. The cell suspension was then placed on ice for 15 min. The cells were then harvested by centrifugation (7,400 × g, 10 min, in a JA14 rotor using a Beckman J2-21M ultracentrifuge) at 4°C and resuspended in ice cold MgCl<sub>2</sub> (100 mM). The cells were then pelleted by centrifugation (7,400 × g, 10 min), in a JA14 rotor using a Beckman J2-21M ultracentrifuge at 4°C and the pellet resuspended in 2 mL of ice cold MgCl<sub>2</sub> (100 mM). The cell suspension was then left on ice for 1 h. Glycerol was then added (to 15% v/v) and 100 µL volumes frozen in a dry ice/ethanol mixture were stored at -70°C.

### **2.10.2 Transformation of Chemically Competent *E. coli***

Chemically competent *E. coli* were thawed on ice prior to the addition of the plasmid DNA contained in a maximum volume of 10 µL H<sub>2</sub>O. These were then centrifuged (14,000 × g, 1 min) and the cell pellet resuspended in 200 µL cold CaCl<sub>2</sub> (100 mM). The cell suspension was then left on ice for 30 min. The DNA was then dropped onto the cells and the tube gently mixed. The cells were then returned to ice for 1 h. Following this, the cells were heat shocked at 42°C for 45 s and immediately placed on ice for 2 min. 750 µL of LB was then added to the cells and incubated at 37°C for 1 h. The cells were then centrifuged (14,000 × g, 1 min) and 100 µL of the supernatant used to resuspend the cells and appropriate dilutions plated onto selective media.

### **2.10.3 Preparation of Electrocompetent *E. coli***

NB (100 mL) was inoculated with 0.1 volume of an O/N *E. coli* culture and grown to middle to late log phase with agitation at 37°C. The culture was chilled for 20 min in an ice/water slurry and centrifuged (7,400 × g, 10 min, in a JA14 rotor using a Beckman J2-

21M ultracentrifuge) at 4°C. The cell pellet was consecutively washed in 100 mL and 20 mL sterile ice cold H<sub>2</sub>O, followed by a 20 mL ice cold 10% (v/v) glycerol wash. The bacteria were resuspended in 2 mL ice cold 10% (v/v) glycerol and 100µL aliquots frozen in a dry ice/ethanol bath and stored at -70°C.

#### **2.10.4 Electroporation procedure**

Electrocompetent *E. coli* were thawed on ice prior to the addition of the plasmid DNA contained in a maximum volume of 10 µL H<sub>2</sub>O. The *E. coli* Pulser™ transformation apparatus (Bio-Rad), set to 2000 V, 27 µF capacitance and 200 Ω resistance. Cold 0.2 cm electrode gap cuvettes (Bio-Rad) were used to pulse bacteria. Bacteria were immediately recovered in 1 mL of SOC medium [Bacto Tryptone (20 g), Bacto Yeast extract (5 g), NaCl (0.5 g), 1M KCl (2.5 mL), ddH<sub>2</sub>O to 1000 mL. Note: adjust pH to 7.0 with 10 M NaOH, autoclave to sterilize, add 20 mL of sterile 1 M glucose immediately before use] and incubated at 37°C for 1 h prior to plating appropriate dilutions onto selective media.

#### **2.11 DNA Sequencing**

DNA sequencing was performed by adding purified PCR product (1 µL) to Big Dye Version 3 (4 µL) (Applied Biosystems) with appropriate forward or reverse primers (1 µL) and Milli-Q water (to 20 µL). The reaction cycle consisted of 25 cycles of 95°C for 30 sec, appropriate oligonucleotide annealing temperature for 30 sec and 60°C for 4 min. Labelled DNA was washed with isopropanol (75%) to remove residual dye and centrifuged (14,000 rpm for 5 min) to pellet the labelled DNA. The pellet was then dried in a Speed Vac SVC100 (Savant Instruments Inc., Farmingdale, New York, USA) and sequenced using DNA Sequencing Facilities at the Institute for Medical and Veterinary Science (IMVS) (Adelaide, South Australia, 5005).

#### **2.12 Strain Typing of *V. vulnificus* Isolates**

##### **2.12.1 Detection of the *vvh* gene**

All isolates identified as *V. vulnificus* in this study by methods described by Alsina and Blanch (1992), were screened for the presence of this gene. Conditions and

oligonucleotide primers used were as per Table 2.1. ATCC strains 27562 and 33148 were used as positive controls.

### **2.12.2 Colony Opacity/Translucence**

Colony morphology of *V. vulnificus* isolates was assessed as opaque or translucent as per Simpson *et al.* (1987), to aid in determining the virulence of these isolates.

### **2.12.3 Strain typing PCR**

*V. vulnificus* isolates were also classified as clinical (C) or environmental (E) type based on the method described by Rosche *et al.* (2005) (Table 2.1). *V. vulnificus* ATCC strains 27562 and 33148 were used as positive controls.

## **2.13 Strain Typing of *V. parahaemolyticus***

### **2.13.1 Detection of the *tlh* gene**

All isolates identified as *V. parahaemolyticus* in this study by methods described by Alsina & Blanch (1992), were screened for the presence of this gene. Conditions and oligonucleotide primers used were as per Table 2.1.

### **2.13.2 Detection of the *tdh* and *trh* genes**

Isolates identified as *V. parahaemolyticus* were assessed for the presence of both the *tdh* gene and the *trh* gene, as a potential indicator of pathogenicity of these isolates as per Tada *et al.* (1992) (Table 2.1). NCTL strains 10884 and 10885 were used as positive and negative controls respectively for the presence of the *tdh* gene. A number of randomly selected non-*parahaemolyticus* vibrio strains were also screened for the presence of these genes.

### **2.13.3 Kanagawa Reaction of *Vibrio* spp.**

All isolates identified as *V. parahaemolyticus* were screened for  $\beta$ -haemolysis of red blood cells (Kanagawa phenomenon) on Wagatsuma agar as per Miyamoto *et al.* (1969).

## **2.14 In Vitro Cytotoxicity of *Vibrio* spp. isolated from TFN lesions**

The cytotoxicity of *Vibrio* spp. for Chinese hamster ovary (CHO) cells was used as an indicator of virulence. Cytotoxicity was assessed using a method based on that described by Baffone *et al.* (2000) and Oliver *et al.* (1986).

CHO cells were grown in RPMI 1640 tissue culture medium containing 10% foetal calf serum (FCS) at 37°C in the presence of 5% CO<sub>2</sub>. Cells were collected by trypsinization and cell concentration and viability determined using trypan blue and a haemocytometer. CHO cells were resuspended in growth medium containing FCS to a concentration of  $5 \times 10^5$  cells mL<sup>-1</sup>. The suspension (100 µL) was then added to wells of a 96 well flat bottomed tray and incubated at 37°C in the presence of 5% CO<sub>2</sub> for 6 h to allow attachment of cells to the tray.

Bacterial supernatant from overnight culture (in marine broth, 30°C) was then sterilised by centrifugation (14,000 rpm for 4 min) and the supernatant passed through a 0.22 µm pore sterile filter. Sterile culture supernatant was then serially diluted in RPMI medium to concentrations of 1:2, 1:4, 1:8, 1:16, 1:32 and 1:64 dilutions. A sample (100 µL) of each dilution was then added in triplicate to wells containing CHO cells prepared as above. The tray was incubated at 37°C overnight in the presence of 5% CO<sub>2</sub>. The supernatant was subsequently removed and the tray gently washed twice in PBS to remove cell debris. 70% (v/v) ethanol (100 µL) was then added to all wells and left for 1 min to fix the cell monolayer to the tray. The ethanol was removed by two immersions of the tray in tap water. Crystal violet (0.5% w/v) (100 µL) was added to all wells and left for 20 min. The stain was then removed by repeated immersion of the tray in water. Acetic acid (100 µL of a 33% w/v solution) was added to all wells and left at room temperature for 3 to 5 min to solubilize the stained monolayer into a transparent debris free solution. Absorbance of each well was measured at 570 nm in a Dynatech MR5000 plate reader (Dynatech Laboratories, Chantilly, Virginia). Results were expressed as percent cytotoxicity relative to a negative control. Positive cytotoxicity was considered to be > 50% reduction in absorbance at a supernatant dilution of 1 in 10 or greater, as per Baffone *et al.* (2000). The top row of each tray was left without the addition of supernatant as a negative control.



Culture supernatant prepared from *V. parahaemolyticus* Kanagawa positive strain NCTC 10884 was used as a positive control.

## **2.15 Characterisation of the VCR regions located on genomic DNA isolated from *Vibrio* Isolates**

VCR elements were amplified from *Vibrio* spp. genomic DNA by PCR using oligonucleotide primer pairs VCR1 and VCR2, and VCR3 and VCR4. Amplified DNA fragments were separated by agarose gel electrophoresis. Separated DNA fragments were excised, the DNA purified and subjected to nucleotide sequencing using the oligonucleotide primers VCR3 and VCR4.

## **2.16 16S rRNA Gene Sequence Analysis**

Genomic DNA isolated bacterial isolates and from uropod tissue prepared as described in Section 2.6. This DNA template was used with the universal primers 16sF and 16sR to amplify either 500 bp using oligonucleotide primer pair 27f and 519r or 1500 bp using oligonucleotide primer pair 16sF and 16sR fragments of 16s rRNA genes as per Table 2.2.

Amplified DNA fragments were routinely cloned into pGEM-T (Promega) as per the manufacturer's instructions and used to transfect chemically competent *E. coli* strains XL-10 or DH5 $\alpha$ . Randomly selected transformants were used to prepare plasmid clones and the inserts subjected to DNA sequencing as described in Section 2.11.

## **2.17 Denaturing Gradient Gel Electrophoresis**

Amplified DNA fragments (500 bp) of 16S rRNA genes were prepared using genomic DNA isolated from TFN lesions as a template (Table 2.2). A G-C clamp was then added to the product by PCR using the oligonucleotide primer pair 27f-GC and 519r and amplification conditions described in Table 2.2. Resultant PCR products possessing the G-C clamp obtained from three identical amplification reactions were pooled, purified and concentrated.

Amplified DNA was then separated by electrophoresis in 20% acrylamide denaturing gels containing 35% to 60% denaturant (100% denaturant consisted of 42% urea, 40%

formamide) as per Ji *et al.* (2004). Gels were electrophoresed at 70 V for 16 h in 1 × TAE, stained using ethidium bromide and single stranded DNA bands visualised by UV transillumination as per Section 2.8. Individual bands were excised and the DNA purified using a Gel Extraction Kit (Qiagen) as per the manufacturer's instructions. Semi-nested PCR was then used to re-amplify the DNA from each band minus the G-C clamp using oligonucleotide pair 27f and 519r and reaction conditions described in Table 2.2. PCR product was purified and subjected to DNA sequencing as described in Section 2.11.

## **2.18 Analysis of Nucleotide Sequence Data**

### **2.18.1 Identification of Bacterial Phyla Present Within Lesions**

DNA sequence data for PCR amplified fragments of 16S rRNA genes obtained from Sections 2.16 and 2.17 was subsequently used to identify what bacterial genera are present within TFN lesions. To do this, the generated nucleotide sequences were compared with known DNA sequences available in the GenBank database by use of the BLAST alignment algorithm available at [http://www.ncbi.nlm.nih.gov/sutils/genom\\_table](http://www.ncbi.nlm.nih.gov/sutils/genom_table).

The sequence data was also downloaded onto the 16S Ribosomal Database (Cole *et al.*, 2003). This allows direct comparison to sequences of your choice already located within the online database, as well as production of phylogenetic trees based on 16S rDNA sequences. This was done by selecting appropriate sequences and aligning them with the sequence data obtained as above using ClustalX multiple sequence alignment program. The alignment file was subsequently fed into JalView multiple alignment editor to produce phylogenetic trees based on 16S rDNA sequences based on average distance using percent identity.

### **2.19 Isolation and Purification of Bacterial Lipopolysaccharide**

LPS was purified from *V. parahaemolyticus* strain L21 and *E. coli* ATCC strain 2120 using methods described by Darveau & Hancock (1983). The quality of the LPS extracts were assessed by separation on an SDS-PAGE gel (15% acrylamide) electrophoresed at 200 V for 45 min using a Mini-Protean II system (BioRad) in electrophoresis buffer (25 mM Tris-

HCl, 192 mM glycine, 0.1% SDS in RO water). The LPS gel was then silver stained (Tsai & Frasch, 1982). LPS purity was determined as a single clear band.

## **2.20 Collection and Characterization of Haemolymph**

### **2.20.1 Haemolymph from Lobsters**

Haemolymph (1 mL) was taken by pericardial puncture from lobsters using a 21-gauge needle containing 2 mL of cold anti-coagulant buffer. Haemolymph was placed on ice and stored at 4°C until required (2-4 hours).

### **2.20.2 Lobster Moulting Stage and Haemolymph Serum Protein Levels**

Moulting stage and serum protein ( $\text{g L}^{-1}$ ) of lobster haemolymph was determined as per Musgrove (2001).

### **2.20.3 Haemocyte and Differential Haemocyte Counts**

Haemolymph taken as described in Section 2.20.1 was also used to assess the total and differential counts of haemocytes as per Evans *et al.* (1998).

## **2.21 Phenoloxidase Activation Assay**

The level of activation of haemocytes in the haemolymph of lobsters was assessed using methods adapted from those described by Otto Schmidt (School of Agriculture, Food and Wine, University of Adelaide, Adelaide, South Australia, personal communication) and Liu & Chen (2003). Diluted lobster haemolymph (1 mL) prepared as per Section 2.20.1 was centrifuged immediately at  $420 \times g$  for 20 min at 4°C. The cell pellet was then rinsed and gently resuspended in 450  $\mu\text{L}$  of cold anti-coagulant buffer. Aliquots (100  $\mu\text{L}$ ) of the haemocyte suspension were then used for phenoloxidase activation assays.

Phenoloxidase was routinely activated by incubation of haemocyte suspensions with 50  $\mu\text{L}$  trypsin ( $1 \text{ mg mL}^{-1}$ ). Samples of *V. parahaemolyticus* strain L21 LPS (final conc.  $10 \mu\text{g mL}^{-1}$ ) and *E. coli* LPS (final concentration  $10 \mu\text{g mL}^{-1}$ ) were used as specific and non-specific activators of phenoloxidase respectively. The mixtures were incubated at 18°C for 10 min, 50  $\mu\text{L}$  of L-DOPA (10 mM final concentration) added, followed by a

further incubation of 5 min. Anti-coagulant buffer (800  $\mu$ L) was then added and the optical density at 490 nm was measured every 10 min for 90 min. Non-specific phenoloxidase activity in haemocyte suspensions was determined following addition of anti-coagulant buffer in place of trypsin or LPS. Background phenoloxidase activity was determined by the addition of sterile marine anti-coagulant instead of LPS or trypsin.

## 2.22 Phagocytosis Assay

Hyaline cells were separated from washed suspensions of haemocytes using a method adapted from Hart *et al.* (1985) and Söderhäll & Smith (1983). Briefly, Percoll solutions were prepared as per Table 2.3. Gradients were then prepared in polycarbonate tubes. 2 mL of the 80% (v/v) Percoll solution was added to the bottom of each tube. A Pasteur pipette was used to then lay 1.5 mL of the 70% (v/v) Percoll mixture on top, followed by 1.5 mL of each of the 55% (v/v) and 50% (v/v) Percoll mixtures. Finally, 2 mL of the 45% (v/v) Percoll mixture was loaded as the top layer. 1 mL of the haemocyte suspensions prepared as per Section 2.20, was gently loaded on the top of the gradient. The tubes were then centrifuged in a swing out rotor at  $2,900 \times g$  for 10 min at 18°C. The gradient zones containing different cell populations were then collected using a Pasteur pipette (uppermost bands first). The zone containing the hyaline cell population was identified using phase contrast microscopy. Hyaline cells were collected and washed twice in anti-coagulant buffer ( $2000 \times g$  at 18°C, 10 min).

Bacterial suspension to be used in phagocytosis assays, were opsonised using lobster serum as follows. Lobster haemolymph (500  $\mu$ L) was collected and centrifuged ( $420 \times g$  for 5 min) to remove the cells. The serum was then filter sterilised (0.20  $\mu$ m sterile filter) and stored at 4°C. Overnight cultures of *V. parahaemolyticus* strain L21 (1 mL per haemolymph sample to be tested) were harvested by centrifugation at  $20,000 \times g$  for 1 min. The cell pellet was then washed twice in marine anti-coagulant. Washed cells were then resuspended in sterile lobster serum (1 mL) and incubated for 1 h at 20°C. The opsonised bacterial cells were then sedimented by centrifugation, washed in anti-coagulant buffer and the cells resuspended in a final volume of 1 mL for final use.

Hyaline cells were then resuspended to a final concentration of  $2 \times 10^5$  cells mL<sup>-1</sup> and mixed in a ratio of 1:2 with opsonised bacteria ( $4 \times 10^5$  cells mL<sup>-1</sup>). The hyaline and bacterial cells were then co-incubated at 18°C for 90 min. Serial dilutions were then plated onto marine agar and incubated overnight at 30°C. Changes in numbers of viable bacteria during the course of co-incubation with hyaline cells were then calculated.

Phagocytosis was confirmed visually using fluorescence microscopy. Following co-incubation of hyaline cells with opsonised bacteria for 1 h, the mixture was treated with gentamycin (20 µg mL<sup>-1</sup>) to remove all extracellular bacteria. The remaining hyaline cells were then subjected to cytospin (1000 rpm for 8 min). Slides were then stained and mounted using Vectashield Hard Set Mounting Medium with DAPI (1.5 µg mL<sup>-1</sup>) (Vector Laboratories, Burlingame, California, USA) and examined with an Olympus AX70 Fluorescence Microscope.

## **2.23 Light Microscopy of Inflammation Associated with TFN**

### **Lesions**

Uropod tissue affected by TFN was excised and immediately placed in fixation buffer (10% formaldehyde (ACE)) for a minimum of 2 d. Fixed tissue was then de-calcified by incubation in freshly prepared decalcification buffer [equal volumes of Solution A (sodium citrate (50 g) and distilled water (250 mL)) and Solution B (equal volumes of formic acid and distilled water)] for 2 d at 4°C. Samples were then dehydrated through a graded series of ethanol and embedded in paraffin wax. Sections (10 to 15 µm thick) of embedded tissue were cut using a sledge microtome and mounted on glass slides. Sections were then stained with haematoxylin and eosin as per standard technique (<http://www.bcm.edu/rosenlab>). Stained sections were cover slipped and visualized by light microscopy and photographed using a Fujix HC-1000 3CCD Image Computing Camera (Fuji, Tokyo, Japan).

## **2.24 Scanning Electron Microscopy (SEM)**

Samples of uropod tissues were placed in fixative [4% (w/v) paraformaldehyde, 4% (w/v) sucrose, 1.25% (v/v) glutaraldehyde in phosphate buffered saline, pH7.2] for a minimum

of 4 h. Fixed tissue was then washed twice in PBS containing 4% (w/v) sucrose for at least 30 min. Tissue was post fixed in 1% (w/v) osmium tetroxide (OsO<sub>4</sub>) in PBS for 2 h, followed by washing with PBS to remove residual OsO<sub>4</sub>. Fixed tissue was then dehydrated by passing the specimens through a graded ethanol series consisting of: 50% ethanol – 1 change 20 min ; 70% ethanol – 1 change 20 min ; 90% ethanol – 1 change 20 min ; 95% ethanol – 1 change 20 min; 100% ethanol – 2 changes of at least 1 h each.

Samples were then dried in a critical point drying unit, mounted on SEM stubs, coated with carbon and gold and viewed using a Philips XL20 Scanning Electron Microscope.

## **2.25 Transmission Electron Microscopy (TEM)**

Uropod tissue was excised and immediately placed in fixative (4% (w/v) paraformaldehyde, 1.25% (v/v) glutaraldehyde, 4% (w/v) sucrose in PBS pH 7.2) and left overnight at 4°C. The samples were washed in PBS with 4% (w/v) sucrose with two changes of ten min each. The samples were then post fixed in 2% (w/v) OsO<sub>4</sub> for 1 h. The samples were subsequently dehydrated through a graded ethanol series as per samples for S.E.M. The samples infiltrated overnight with a 50% ethanol resin mixture, followed by 3 × 8 h changes of 100% resin.

The samples were then embedded in fresh resin and the resin polymerised by heating at 70°C for at least 24 h. Thin survey sections (1 µm) cut from the embedded tissue, were stained with toluidine blue and viewed by light microscopy to choose regions for preparation of ultra thin sections. Ultra thin sections were cut using a diamond knife and stained with 4% (w/v) uranyl acetate and Reynolds lead citrate prior to viewing with a Philips CM100 Transmission Electron Microscope.

## **2.26 Data Analysis**

All values are expressed as arithmetic means ± standard deviation (SD) unless otherwise stated. All *n* values represent the number of individuals tested unless otherwise stated. Statistical significance was determined using one-way ANOVA with Dunnett's post test performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego California USA, <http://www.graphpad.com>).

**Table 2.1: Oligonucleotide primer pairs used for characterization of isolates of *Vibrio* spp.**

Target Gene	Sequence	Primer ID	Amplicon size	Reaction Protocol	Reference
<i>tdh</i>	5'-CCATCTGTCCCTTTTCCTGC-3'	tdhd1	335 bp	94°C for 1min, 55°C for 1 min, 72°C for 1 min, 35 cycles	Tada <i>et al.</i> (1992)
	5'-CCAAATACATTT-TACTTGG-3'	tdhd2			
<i>trh</i>	5'-GGCTCAAATGGTTAAGCG-3'	trh2	190bp	94°C for 1 min, 55°C for 1 min, 72°C for 1 min, 35 cycles	Tada <i>et al.</i> (1992)
	5'-CATTTCCGCTCTCATATGC-3'	trh6			
<i>tlh</i>	5'-AAAGCGGATTATGCAGAAGCACTG-3'	tlf	450bp	94°C for 1 min, 58°C for 1 min, 72°C for 1 min, 35 cycles	Bej <i>et al.</i> (1999)
	5'-GCTACTTTCTAGCATTTTCTCTGC-3	tlr			
VV0401 ORF	5'-AGCTGCCGATAGCGATCT-3'	P1*	277 bp	94°C for 1 min, 50°C for 1 min, 72°C for 1 min, 30 cycles	Rosche <i>et al.</i> (2005)
	5'-CTCAATTGACAATGATCT-3'	P2			
	5'-CGCTTAGGATGATCGGTG-3'	P3			
<i>vvh</i>	5'-TTCCAACCTCAAACCGAACTATGA-3'	F- <i>vvh</i>	205bp	94°C for 1min, 60°C for 1 min, 72°C for 1 min, 35 cycles	Panicker <i>et al.</i> (2004)
	5'-ATTCCAGTCGATGCGAATACGTTG-3'	R- <i>vvh</i>			

\* denotes combinations of primers selected from P1, P2 and P3. P1 and P3 detect clinical isolates of *V. vulnificus*, whilst primer pair P2 and P3 detect environmental isolates of this organism

**Table 2.2 Oligonucleotide primer pairs used for characterization of isolates of bacteria from lobsters.**

Target Gene	Sequence	Primer ID	Amplicon size	Reaction Protocol	Reference
16S rRNA	5' -GAGCGTTAATGGGAATTACT-3' 5' -ATTACCGCGGCTGCTGG-3'	27f 519r	492bp	94°C for 1 min, 55°C for 1 min, 72°C for 1 min, 35 cycles	Stackebrandt <i>et al.</i> , 1988
16S rRNA	5' -CGCCCGCCGCGCGCGGCGGGCGG GGCGGGGGCCCGGGG GAGCGTTAATGGGAATTACT-3' 5' - ATTACCGCGGCTGCTGG-3'	27f-GC 519r	532bp Includes GC Clamp	94°C for 1 min, 55°C for 1 min, 72°C for 1 min, 40 cycles	Adapted from Stackebrandt, <i>et al.</i> , 1988
VCR mobile cassettes	5' -TAACAAACGCCTCAAGAAGGGAC-3' 5' -TAACGCCCCGCCTAAGGGGC-3'	VCR1 VCR2		94°C for 1 min, 55°C for 1 min, 72°C for 1 min <u>or</u> 72°C for 4 min, 30 cycles	Mazel <i>et al.</i> , 1998
VCR mobile cassettes	5' -GTCCCTCTTGAGGCGTTTGTTA-3' 5' -GCCCCTTAGCGGGCGTTA-3'	VCR3 VCR4		As above	Reverse complement of primers described by Mazel <i>et al.</i> , 1998



**Table 2.3: Composition of Percoll gradients.**

<b>Percoll Concentration (%)</b>	<b>Volume of Percoll (mL)</b>	<b>Volume of 10 x Anti-Coagulant (mL)</b>	<b>Volume of Milli Q H<sub>2</sub>O (mL)</b>	<b>Total Volume (mL)</b>
81	14.4	1.8	1.8	18
70	9.45	1.35	2.7	13.5
55	7.425	1.35	4.725	13.5
50	6.75	1.32	5.4	13.5
45	7.2	1.8	9	18

## Chapter 3: Bacteriology of TFN Lesions of Live-Held Southern Rock Lobsters

### 3.1 Introduction

The microflora of lesions associated with TFN of live-held lobsters following artificial infection has been previously described to comprise *Vibrio* spp. (May, 2002). That study used isolates of *Vibrio* spp. recovered from naturally occurring lesions associated with TFN to induce the disease in previously healthy animals by simultaneous damage to the uropod and contamination of the wound with selected isolates of bacteria. For the purpose of that trial, 20 lobsters were separated into 5 different treatment groups thus:

1. Control lobsters: uropods were left undamaged.
2. *V. alginolyticus* infection group 1: four of the five tail fans of each lobster were nicked at the distal end with a knife dipped in overnight culture of *V. alginolyticus* strain 1 recovered from a naturally induced TFN lesion and had a hole induced using a nail dipped in the same overnight culture.
3. *V. vulnificus* infection group: tail fans were damaged as per Group 2 lobsters, only instruments were dipped in overnight culture of *V. vulnificus* recovered from naturally induced TFN lesion.
4. *V. parahaemolyticus* infection group: tail fans were damaged as per Group 2 lobsters, only instruments were dipped in overnight culture of *V. parahaemolyticus* strain L21 recovered from naturally induced TFN lesion.
5. *V. alginolyticus* infection group 2: tail fans were damaged as per Group 2 lobsters, only instruments were dipped in overnight culture of a second strain of *V. alginolyticus* recovered from naturally induced TFN lesion.

Whilst the full range of symptoms associated with TFN were not induced by this trial, RFLP analysis was used to show that isolates used to infect damaged tail fans persisted within infected tissue for up to eight weeks. Partial nucleotide sequence analysis of genes

encoding 16S rRNA was used as additional evidence to support the identity of isolates recovered from induced lesions.

However, a large pool of bacterial isolates recovered from induced and naturally occurring TFN lesions by May (2002) was not fully characterised. Furthermore, the potential health risks to consumers associated with the consumption of raw or undercooked lobsters displaying lesions associated with TFN and/or cross-contamination with food that has already been prepared is not understood. It was also necessary to further support the RFLP data with other methods to demonstrate clonality between the isolates used to induce disease, and those isolates later recovered from induced lesions.

The aim of this chapter was to identify the *Vibrio* isolates recovered by May (2002) from lesions associated with lobsters deliberately subjected to simultaneous damage and infection with specific strains of vibrios. Furthermore, the potential for these isolates to cause disease in humans was characterised using well described phenotypic and genotypic properties.

### **3.2 Experimental design**

Bacterial isolates recovered from lesions associated with TFN by May (2002) were tentatively identified as *V. parahaemolyticus*, *V. vulnificus*, *V. alginolyticus* and *Vibrio* spp. In this Chapter, the identity of each isolate, to species level where possible, was determined using methods described by Alsina & Blanch (1994). Isolates identified as *V. parahaemolyticus* were confirmed by amplification of a part of the species-specific *tlh* gene by PCR. Similarly, isolates identified as *V. vulnificus*, were confirmed by amplification of part of the *vvh* gene by PCR.

The potential health threat posed to consumers was assessed by examining the expression of known virulence factors associated with *Vibrio* species, including extracellular protease, urease, lecithinase and cytotoxicity towards cultured mammalian cells. Colony morphology of *V. vulnificus* isolates was also assessed as either opaque or translucent as per Simpson *et al.* (1987). *V. parahaemolyticus* isolates were also screened for the presence of the *tdh* and *trh* genes by PCR, as well as screened for the Kanagawa phenomenon on Wagatsuma agar as per as per Honda *et al.* (1988). Expression of the

extracellular factors lipase and chitinase known to be involved in the induction of shell diseases, was also assessed.

Following this, confirmation of the RFLP data obtained by May (2002) was attempted by sequence analysis of the mobile gene cassettes associated with VCR's of individual *Vibrio* isolates. This initially took an overall approach, but was narrowed down to just those genes immediately adjacent to the *attC* site as described later in this chapter.

### 3.3 Results

#### 3.3.1 Identification of *Vibrio* isolates recovered from uropod tissue

Isolates of vibrios to be identified were originally obtained from lesions that developed on tail fans of experimental animals at the site of simultaneous damage and infection with cultures of *V. alginolyticus*, *V. parahaemolyticus*, and *V. vulnificus* as well as those from control animals that were damaged with sterile instruments. Each isolate was identified using a combination of biochemical tests available from the Oxoid Microbact 24E Identification System (adapted for use with *Vibrio* spp. as per Myett *et al.*, 1988) and those described by Alsina & Blanch (1994).

Table 3.1 lists the proportion of specific *Vibrio* spp. identified from tail fan tissue obtained for each infection trial. Of all of the isolates recovered by May (2002), 34% were identified as *V. alginolyticus*, 31% as *V. parahaemolyticus*, 12% as *V. vulnificus* and a further 23% as various *Vibrio* spp. When isolates were separated into groups that corresponded to the treatment group from which they were recovered, the principal species present was found to correspond to the species of *Vibrio* used to simultaneously damage and infect lobsters. Lobsters that had tail fans damaged with sterile instruments were used as a control and for that reason, vibrio species associated with tail fan samples were regarded as “the normal micro-flora” associated with the carapace of southern rock lobsters. These included *V. alginolyticus* (30%), *V. parahaemolyticus* (30%), and *V. vulnificus* (10%), with a range of other *Vibrio* spp. comprising the remaining 30% of the population.

Isolates from a group of animals infected with second type of *V. alginolyticus* (1) strain were also predominated by *V. alginolyticus* (38% of isolates) at eight weeks post

infection; 31% of isolates were identified as *V. parahaemolyticus* and the remainder were identified as various *Vibrio* spp. (31%). No *V. vulnificus* were isolated from this group of lobsters. *V. alginolyticus* (56%) was the predominant isolate from lesions on lobsters eight weeks post infection with *V. alginolyticus* (2); 11% of isolates were identified as *V. parahaemolyticus*, 11% were *V. vulnificus* and the remainder were various other *Vibrio* spp. (22%).

By comparison, samples of tail fan tissue taken eight weeks post infection of lobsters that had tail fans infected with *V. vulnificus*, were dominated by isolates of *V. vulnificus* (60%), although *V. alginolyticus* also comprised 20% of isolates. Other *Vibrio* spp. comprised the remainder of isolates from this tissue. No *V. parahaemolyticus* was detected from tail fan tissue harvested from these lobsters.

The species composition of isolates from tissue samples taken from a group of lobsters that had tail fans artificially infected with a *V. parahaemolyticus* strain L21, was dominated by *V. parahaemolyticus* (93%). Only a small proportion of *V. alginolyticus* isolates were also detected (7%).

### **3.3.2 Molecular characterisation of *Vibrio* spp. isolated from lesions**

Isolates of *V. parahaemolyticus* and *V. vulnificus* obtained from TFN lesions were tested to determine whether they carried DNA that encoded species specific loci and loci that encoded known haemolysin genes. DNA from *V. vulnificus* isolates was also used in PCR assays previously used for epidemiological analyses that enabled the distinction of clinically significant isolates from environmental isolates of no clinical significance (Rosche *et al.*, 2005). In addition, these isolates were tested to determine expression of phenotypic characteristics commonly associated with clinically significant isolates.

#### **3.3.2.1 Confirmation of *V. parahaemolyticus* and *V. vulnificus* Isolates by PCR**

PCR was used to confirm the identity of suspected isolates of *V. parahaemolyticus* by amplification of a part (450 bp) of the species-specific *tlh* gene. A 450 bp product was amplified from DNA prepared from all isolates initially identified as *V. parahemolyticus* (Figure 3.1). Nucleotide sequence for amplicons obtained from randomly selected isolates was 100% similar to known *tlh* gene sequences available in the GenBank Nucleotide

Database (Appendix A, Page 213). No product was generated from genomic DNA isolated from a number of *V. vulnificus* or other *Vibrio* spp. isolated from lesions. Consequently, this data strongly supports the identification of this group of isolates as *V. parahaemolyticus*.

Similarly, isolates identified as *V. vulnificus*, were confirmed by PCR amplification of part (205 bp) of the *vvh* gene that is widely used as genetic a marker for this species. All DNA samples prepared from bacterial isolates tentatively identified as *V. vulnificus* resulted in amplification of a 205 bp product consistent with that of the *vvh* fragment (Figure 3.2). Nucleotide sequence data obtained for the product from ATCC strain 27562 and for randomly selected *V. vulnificus* isolates recovered from TFN lesions was 100% similar to other *vvh* sequences from the GenBank Nucleotide Database (Appendix B, Page 214). No amplicons were obtained when DNA from a range of other *Vibrio* species was used as a template for PCR. This data provides additional support for the identification of *vvh* positive isolates as *V. vulnificus*.

### 3.3.2.2 Expression of factors associated with pathogenicity

It is widely recognised that only a small proportion of environmental isolates of *V. parahaemolyticus* and *V. vulnificus* are likely to be clinically significant (Shirai *et al.*, 1990, Simpson *et al.*, 1987). In view of this, isolates of *V. parahaemolyticus* and *V. vulnificus* recovered from lesions in lobsters with experimentally induced TFN were potentially pathogenic, and consequently were tested for ability to express factors associated with virulence eg. production of extracellular protease, lecithinase, urease and lipase, as well as cytotoxicity to cultured mammalian cells. A summary of the results obtained is presented in Table 3.2.

Of the 19 *V. parahaemolyticus* isolates screened in this way, 18 produced proteases, all produced lecithinase, 12 produced lipases, 15 produced urease and 14 were cytotoxic to cultured CHO cells. Of the 10 isolates identified as *V. vulnificus*, 10 produced extracellular proteases, 8 produced lecithinase, 7 produced lipases, 9 produced urease and all were cytotoxic to cultured CHO cells.

Of the remaining *Vibrio* isolates, including all *V. alginolyticus* isolates, only 2 produced extracellular proteases, 2 produced, lecithinase, none produced lipases, 1 produced urease and only 3 were cytotoxic to cultured CHO cells.

Chitinase expression among marine *Vibrio* species is an important determinant of ability to colonise the surface of marine animals such as copepods and the carapace of higher marine invertebrates. Also, chitinase expression is believed to be intimately involved in carapace degradation characteristic of shell diseases of marine invertebrates (see Chapter 1.5.2). Given the extensive erosion of the chitin exoskeleton of lobster tail fans affected by TFN, chitinase is likely to be an important factor responsible for the progression of this disease. Consequently, all *Vibrio* isolates were screened for the ability to degrade chitin. All *V. parahaemolyticus* and *V. vulnificus* isolates expressed extra-cellular chitinase. However, only 17% of the remaining isolates expressed extra-cellular chitinase.

#### 3.3.2.3 Strain typing of *V. parahaemolyticus* isolates

*V. parahaemolyticus* isolates were tested for the presence or absence of genes encoding known hemolysins (*tdh* and *trh* genes) by PCR. Surprisingly, none of these isolates were *tdh* positive, but 68% were *trh* positive (Appendix C, Page 215). Typical results obtained by PCR are shown in Figure 3.3 and Figure 3.4. Nucleotide sequence data obtained for randomly selected *trh* positive amplicons was 100% similar to *trh* sequences in the GenBank Nucleotide Database (Appendix D, Page 216). Furthermore, all *trh* positive isolates also expressed molecules cytotoxic for CHO cells and together, this data indicates that *trh* positive isolates are likely to be pathogenic for humans. However, of the remaining *trh* and *tdh* negative isolates that were identified as *V. parahaemolyticus*, only 20% were cytotoxic. This suggests the latter isolates are probably not pathogenic.

#### 3.3.2.4 Strain typing of *V. vulnificus* isolates

In order to determine the potential virulence of the isolates identified as *V. vulnificus*, these isolates were assessed for ability to produce opaque (capsule expressing) or translucent (non-capsulate) colony phenotypes when grown on marine agar. All *V. vulnificus* isolates produced colonies with an opaque phenotype and hence are likely to produce a capsule that

is regarded as a requirement for virulence. Furthermore, all of these isolates were also cytotoxic to CHO cells.

#### 3.3.2.5 Virulence associated gene typing of *V. vulnificus* isolates

A study by Rosche *et al.* (2005) described the use of PCR to identify sequence differences within and open reading frame (VV0401, GenBank accession: NC\_005139) of *V. vulnificus* isolates that could be used to differentiate clinical and environmental isolates of this organism. This approach was subsequently trialled in an attempt to provide additional information that might assist in determining the clinical significance of the presence of *V. vulnificus* associated with TFN lesions. Preliminary experiments that used the oligonucleotide primer pairs described by Rosche *et al.* (2005) to group *V. vulnificus* isolates as either environmental or clinical strains gave inconsistent results. Type strains of *V. vulnificus* strains or those that had been independently identified used for this work were: *V. vulnificus* biovar 1 (host = barramundi); *V. vulnificus* biovar 1 (host = Dugong); *V. vulnificus* biovar 1 (host = green turtle); *V. vulnificus* biovar 1 (host = isopod); *V. vulnificus* biovar 1 ATCC 27562; *V. vulnificus* biovar 2 ATCC 33148) and one *V. parahaemolyticus* type strain (NCTC 10884. Typical PCR results are displayed as Figure 3.5. All strains, including the *V. parahaemolyticus* isolates, produced a PCR amplicon of the expected size, regardless of the oligonucleotide primer pair used (oligonucleotide primer pair P1 and P3 or oligonucleotide primer pair P2 and P3). Furthermore, two amplicons of slightly different sizes were produced following PCR amplification from DNA prepared from some strains. This observation indicated significant mis-priming using oligo-nucleotide pairs P1 – P3 and P2 – P3 using both *V. vulnificus* and *V. parahaemolyticus* isolates had taken place. On the basis of the inconsistent results as a consequence of this mis-priming, this section of the work was not continued.

#### 3.3.3 Characterisation of VCR elements

Previous work undertaken by May (2002) using RFLP analysis of PCR amplified DNA encoding 16s rRNA showed that isolates used to infect damaged tail fan tissue could persistently associate with lesions. RFLP patterns consistent with that of the infecting strain of *Vibrio* spp. were always obtained, although other *Vibrio* spp. isolated from the



same lesion produced different patterns. That preliminary data indicated the infecting strain was able to persist in lesion tissue and that clonally distinct populations of bacteria could contribute to lesion development. However, this approach was not corroborated by alternative methodologies. As *Vibrio* spp. are well known to harbour integrons (Mazel *et al.*, 1998), it is possible that variation within these structures could be used as markers for clonal populations of bacteria. In particular, analysis of the gene cassettes associated with VCR's of these structures could identify useful sequence dependent differences based on the ability of these structures to assimilate or lose cassettes.

Several PCR amplification strategies were employed to amplify either VCR elements or gene cassettes between VCR elements within individual integrons. A diagrammatic representation of generic integron structure, and the position and orientation of PCR oligonucleotide primers used is shown in Figure 3.6. Primers VCR1 and VCR2, as described by Mazel *et al.* (1998) are specific for conserved regions within the VCRs and are designed to amplify individual VCR elements and gene cassettes between two VCRs. Primers pair VCR3 and VCR4, are reverse complement oligonucleotides of VCR1 and VCR2 respectively. This primer pair was designed to amplify integrated gene cassettes in the same manner as VCR1 and VCR2. Oligonucleotide primers 2355 and 2583, as described by Clark *et al.* (2000), were paired with primers VCR2 or VCR3 to amplify the most recently inserted gene cassette in integrons ie. the cassette closest to the integrase gene. Similarly, primer int3, designed from an analysis of known *Vibrio* integrase genes sequences, was also paired with either VCR2 or VCR3 in order to amplify the most recently inserted gene cassette within integrons.

Of the described sets of oligonucleotide primers, only VCR3 and VCR4 resulted in the generation of amplicons from DNA isolated from all *Vibrio* isolates (total of 50) obtained from tail fan tissue. Interestingly, all PCR amplicon profiles obtained were identical, regardless of *Vibrio* spp. used (Figure 3.7). In each case, PCR amplified two fragments (450 bp and 550 bp). DNA sequence analysis of agarose gel purified amplicons demonstrated that all amplicons were essentially identical. No intra-species variation of the PCR amplicon profiles was noted. BlastX analysis of the 550 bp product showed the translated sequence had 93% amino acid similarity to *Vibro* spp. DAT722 inDAT722 integron (Appendix E, Page 217). Furthermore, this sequence has been identified to be

part of integrons carried by different *Vibrio* spp. (Boucher *et al.*, 2006). BlastX analysis of the nucleotide sequence for the 450 bp product indicated the translated sequence had 92% similarity to *Vibrio parahaemolyticus* RIMD 2210633 DNA. Based on the PCR amplicon profiles obtained and the sequence data of amplified DNA, use of gene cassettes associated with the integrons of *Vibrio* spp. is unlikely to be useful for identification of clonal groups or populations of vibrios within lesions on tail fans of TFN affected rock lobsters.

### 3.4 Discussion

The data described in this chapter confirm findings by May (2002) that *Vibrio* species are the predominant bacteria associated with artificially induced TFN lesions eight weeks post infection. In particular, the data confirmed that *V. parahaemolyticus*, *V. vulnificus* and at least one strain of *V. alginolyticus* were able to persist in lesion tissue for up to eight weeks. Furthermore, *V. parahaemolyticus* strain L21 used to induce infections in tail fan tissue typical of TFN dominated the bacterial population (comprised 93% of the population within tissue of induced lesions after eight weeks). In view of these experimental outcomes, *V. parahaemolyticus* L21 was selected for use in other artificial infection trials described elsewhere in this thesis.

These results clearly demonstrated that selected isolates of *Vibrio* spp. are able to induce TFN in artificially damaged tail fans of rock lobsters and that these can persist in association with the diseased tissue for up to eight weeks. A similar outcome has been reported for earlier studies of the natural carapace microflora of the spiny lobster, *Panulirus argus*, which established that the dominant species present were *Vibrio* spp. (Porter *et al.* 2001).

Significantly, data presented in this chapter, in addition to that already published by May (2002), has allowed a confirmation that Koch's postulates of disease (Prescott *et al.*, 1999) can be directly applied to studies of the role of vibrios in development of shell diseases of crustaceans. In particular:

**First Postulate:**            **Association of the microbe with lesions of the disease.**  
*Vibrio* spp. have been isolated from TFN lesions.

**Second Postulate:**     **Isolating the bacterium in pure culture.**

Pure cultures of vibrios from TFN lesions have been obtained.

**Third Postulate:**     **Show that the isolated bacterium causes disease in the animals.**

Damaged tail fan tissue of otherwise healthy rock lobsters infected with selected *Vibrio* spp. causes formation of TFN like lesions.

**Fourth Postulate:**     **Re-isolation of the bacterium from intentionally infected animals.**

May (2002) provided RFLP data to support the hypothesis that *Vibrio* spp. used to establish infection in rock lobsters are able to persist in TFN lesions and can be re-isolated from those lesions.

As is the case for bacteria implicated in other shell diseases of crustaceans, most isolates from TFN lesions expressed extracellular lipase and chitinase. This includes those identified as *V. alginolyticus*, a species normally not considered to be a human or fish pathogen. These characteristics have previously been identified as likely to be required for the initiation of shell diseases (Cipriani *et al.*, 1980, Cook & Lofton, 1973). Thus, the expression of these enzymes by isolates of vibrios associated with TFN lesions may contribute to the onset and progression of TFN. Expression of chitinase especially, may explain the tissue destruction typical of the pathology of TFN, as chitin is a major structural component of the lobster exoskeleton. This characteristic of TFN isolates and the implication for establishment of disease will be discussed further in Chapter 5 of this thesis.

Analysis of the gene cassettes associated with the VCR's of the *Vibrio* isolates indicated that there was no variation in either the number of amplified fragments or the nucleotide sequence of those fragments that could be used to identify of clonal groups of vibrios. Remarkably, the gene cassettes amplified from all strains were essentially identical. Consequently, analysis of VCRs and associated gene cassettes is unlikely be useful for confirming that the bacterial strains used to induce TFN by May (2002) are able to persist in association with the artificially infected tail fan tissue. Furthermore, this approach is unlikely to be suitable for identification of clonal groups in other settings such as to compare vibrios recovered from different niches. Nevertheless, this work confirmed

that the *Vibrio* species isolated carried VCRs and are therefore either likely to carry integron like elements or have the potential to acquire these at some time.

In terms of food safety, a major concern is raised by the presence of *trh* positive *V. parahaemolyticus* and *V. vulnificus* with the opaque colony morphology. Both of these phenotypes are characteristic of virulent forms of these organisms capable of inducing serious illness in humans. The fact that all *trh* positive *V. parahaemolyticus* and opaque *V. vulnificus* isolates were cytotoxic towards cultured mammalian cells, whereas very few other isolates were, provided evidence to support the potential for pathogenesis. Before any firm recommendations can be made on this point, it is prudent to further investigate the presence of these isolates associated with TFN lesions and as such, will be further investigated in Chapter 5 of this thesis.

As described in Chapter 1.6.6, the *trh* gene, along with *tdh*, encodes hemolysins normally associated with clinical strains of *V. parahaemolyticus*. Usually *tdh* positive isolates are found in greater abundance than *trh* positive isolates (Deepanjali *et al.*, 2005; Gopal *et al.*, 2004, Shirai *et al.*, 1990, Ward & Bej, 2005). Importantly, this study found that *V. parahaemolyticus* isolates associated with TFN lesions are predominantly *trh* positive. Whilst this observation means that the isolates are potentially pathogenic, it also indicated that *trh* positive *V. parahaemolyticus* isolates associated with the diseased tissue may have a selective advantage for lobster tissues compared with *tdh* positive isolates. However, it is also possible that different clonal groups of *V. parahaemolyticus*, capable of occupying various ecological niches, exist in the water column. Evidence to support this hypothesis has been previously reported by Robert-Pillot *et al.* (2003). In that study, the prevalence of strains encoding *trh* in two potentially different populations of *V. parahaemolyticus* along the coast of France was shown to 3% and 15% respectively.

Interestingly, during attempts to characterise the isolates identified as *V. vulnificus* as either clinical or environmental strains using the PCR based strategy outlined by Rosche *et al.* (2005), both sets of oligonucleotide primers produced amplification of the appropriate product from all isolates tested. This result indicated one of two things. The first is that these isolates have been potentially mis-identified. However, as part of the *vvh* gene was amplified by PCR from all isolates initially identified as *V. vulnificus*, it is likely that this identification is correct. On the basis of these results, it is likely that there are different

strains of *V. vulnificus* in different regions of the world (the isolates used in the study by Rosche *et al.*, 2005, were predominantly isolated from the United States of America) and that this approach may not necessarily be useful for characterizing clinical significance of *V. vulnificus* strains from different geographical locations. This contention is further supported by the data presented by Robert-Pillot *et al.* (2003) that showed there is likely to be different characteristics associated with vibrios recovered from different animal species, even within close geographical proximity.

### 3.5 Conclusions

This chapter has presented evidence that vibrios are able to induce TFN and persist in association with diseased tissue for up to eight weeks. Furthermore, the presence of potentially pathogenic *V. parahaemolyticus* and *V. vulnificus* strains in association with TFN lesions presents a significant health risk to consumers. In particular:

1. Previous work reported by Musgrove *et al.* (2005) showed that TFN like lesions developed on tail fan tissue subjected to simultaneous wounding and infection, but not on tail fan tissue subjected to wounding with sterile instruments. These results implicate bacteria in the process of development of TFN. In the present study, *Vibrios* isolated from TFN lesions were shown to also be implicated in initiation of TFN like lesions when introduced in association with simultaneous intentional damage to tail fans of normal healthy lobsters.
2. Vibrios associated with TFN lesions are likely to be able to persist in affected tissue for up to eight weeks post infection.
3. The majority of vibrios associated with TFN lesions are able to express chitinase and/or lipase. Expression of this phenotypic character may be a prerequisite for colonisation and persistence within lesions.
4. The majority of *V. parahaemolyticus* and *V. vulnificus* isolates from lesions expressed phenotypic characteristics and encoded genotypic markers typical of clinically important strains. Consequently, lobsters carrying TFN lesions are likely to represent a potential public health threat to consumers.

**Table 3.1: Proportions of various *Vibrio* spp. isolated from uropod tissue.**

Isolates associated with tail fan tissue samples were harvested from rock lobsters subjected to simultaneous damage and infection with an inoculum of selected lesion associated *Vibrio* isolates. All isolates were collected from an infection trial conducted in 2002 (May, 2002). Collected isolates were subsequently identified as per Alsina and Blanch (1994) due to irregularities experienced during the initial identification by May (2002) using the Microbact 24E Identification Kits.

Uropod inoculum	Proportion of <i>Vibrio</i> spp. isolated from uropod lesions			
	<i>V. alginolyticus</i>	<i>V. vulnificus</i>	<i>V. parahaemolyticus</i>	<i>Vibrio</i> spp.
<i>V. alginolyticus</i> (1)	6 (38%)	0	5 (31%)	5 (31%)
<i>V. alginolyticus</i> (2)	9 (56%)	2 (11%)	2 (11%)	3 (19%)
<i>V. vulnificus</i>	2 (20%)	10 (60)	0	4 (25%)
<i>V. parahaemolyticus</i>	0	0	15 (93%)	1 (7%)
Uninfected control	5 (31%)	1 (7%)	5 (31%)	5 (31%)
Total all treatments	27 (34%)	10 (12%)	25 (31%)	18 (23%)

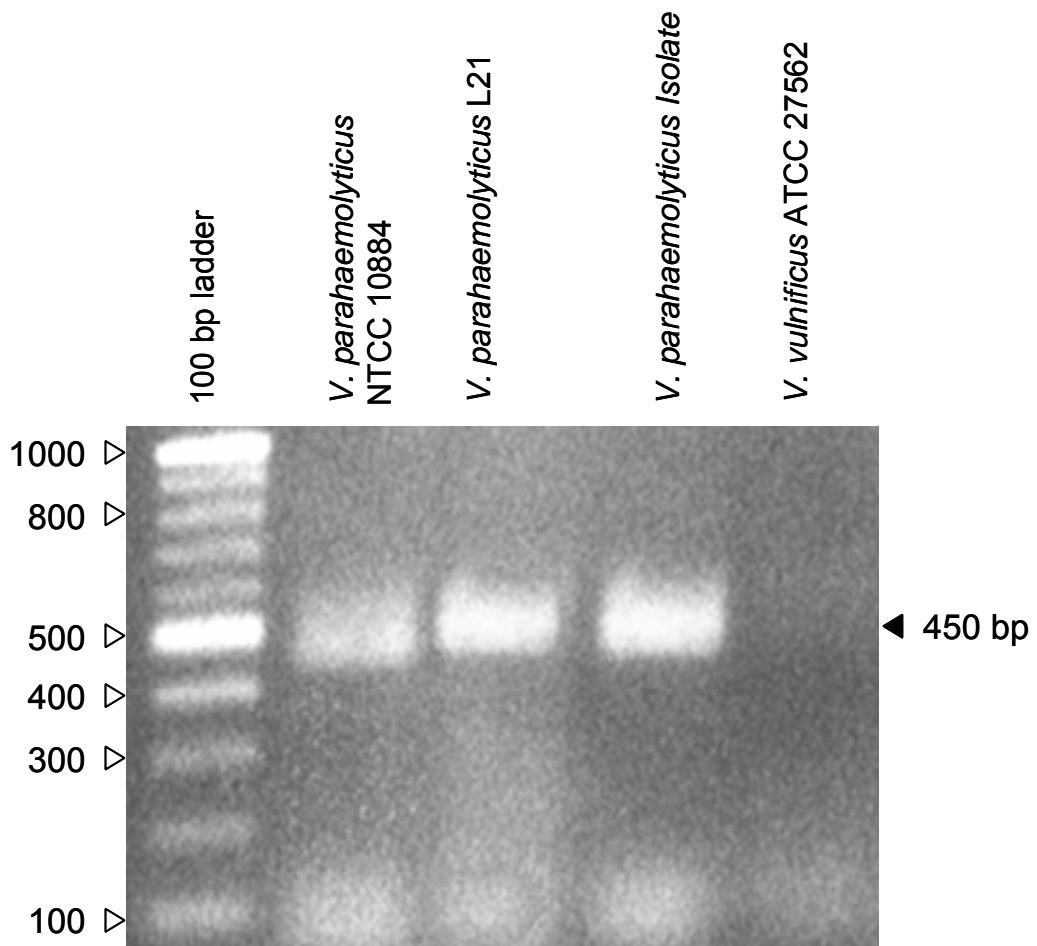
**Table 3.2: Virulence associated phenotypic characteristics of *Vibrio* isolates recovered from TFN lesions.**

Isolates collected from a study by May (2002).

Isolate Identity	Protease	Lecithinase	Lipase	Urease	Cytotoxicity to CHO cells
<i>V. parahaemolyticus</i> (19) <sup>1</sup>	18 (95%)	19 (100%)	12 (63%)	15 (79%)	14 (74%)
<i>V. vulnificus</i> (10)	10 (100%)	8 (80%)	7 (70%)	9 (90%)	10 (100%)
Other <i>Vibrio</i> spp. (20)	2 (10%)	2 (10%)	0 (0%)	1 (5%)	3 (15%)
Total (49)	20 (41%)	29 (59%)	19 (39%)	25 (51%)	27 (55%)

<sup>1</sup> Numbers in brackets are numbers of isolates tested.

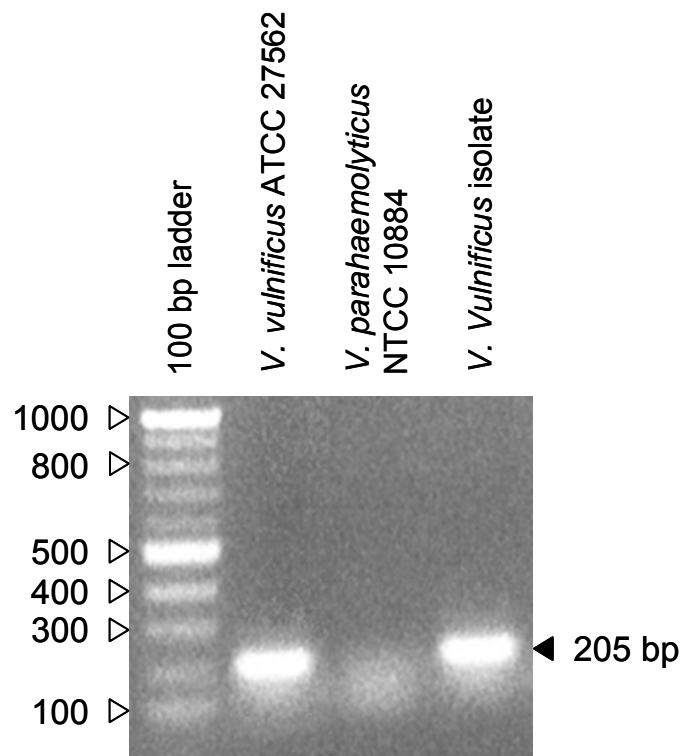
<sup>2</sup> Cytotoxicity of culture filtrates for CHO cells.



**Figure 3.1: Amplification of a 450 bp fragment of the *tlh* gene.**

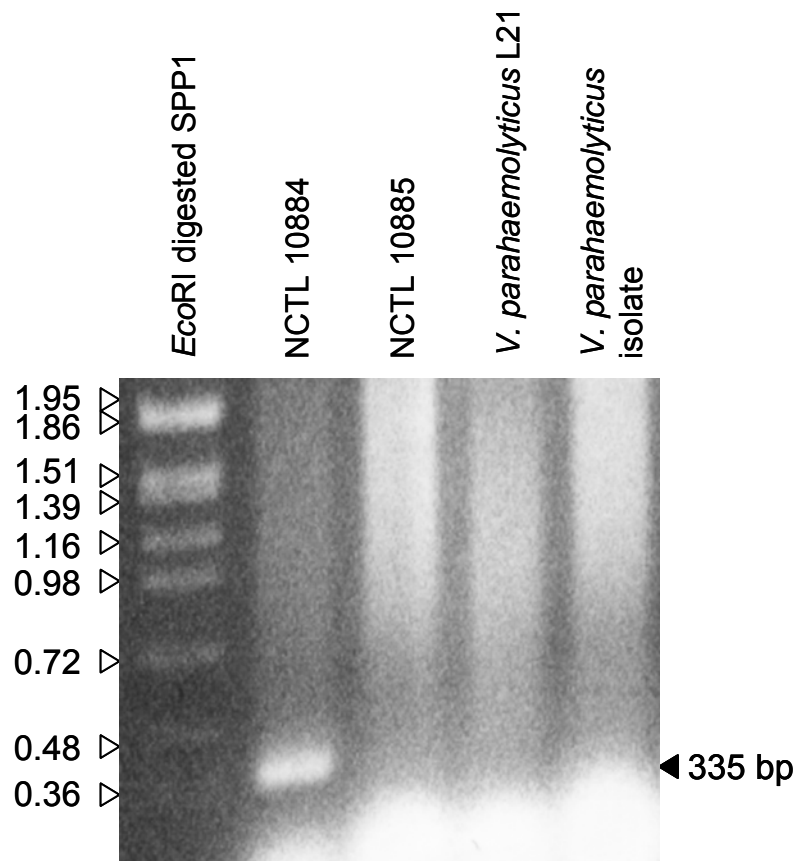
Template DNA was prepared from selected *V. parahaemolyticus* isolates from uropod tissue of rock lobsters. Typical results are shown. *V. parahaemolyticus* NTCC strain 10884 DNA was used as a positive control. *V. vulnificus* ATCC strain 27562 was used as a source of negative control DNA.





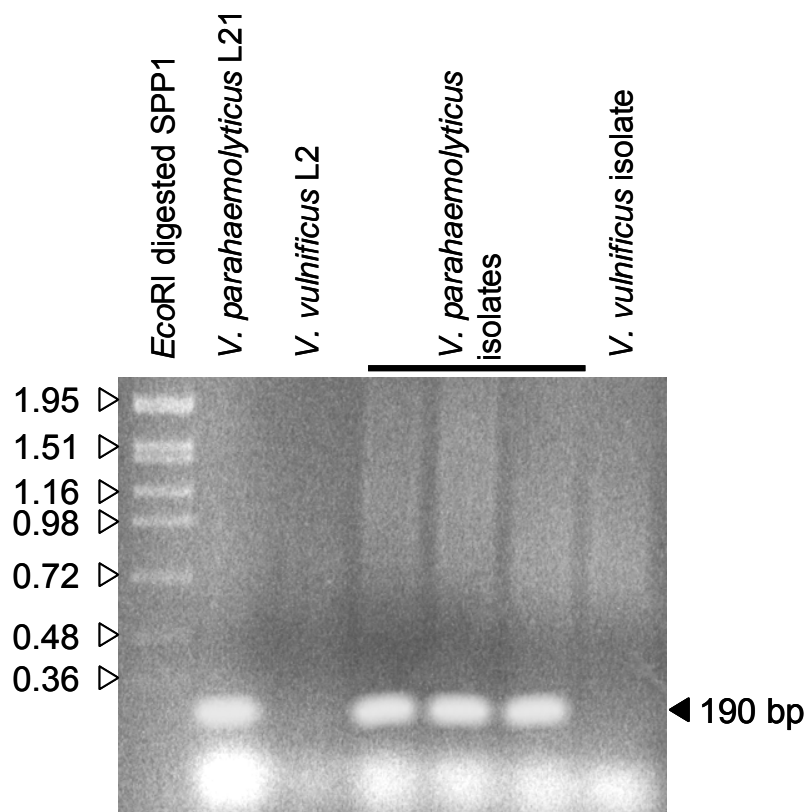
**Figure 3.2:** Amplification of 205 bp fragment of the *vvh* gene.

Template DNA was prepared from selected *V. vulnificus* isolates from uropod tissue of rock lobsters. Typical results are shown. *V. parahaemolyticus* NTCC strain 10884 was used as a source of negative control DNA.



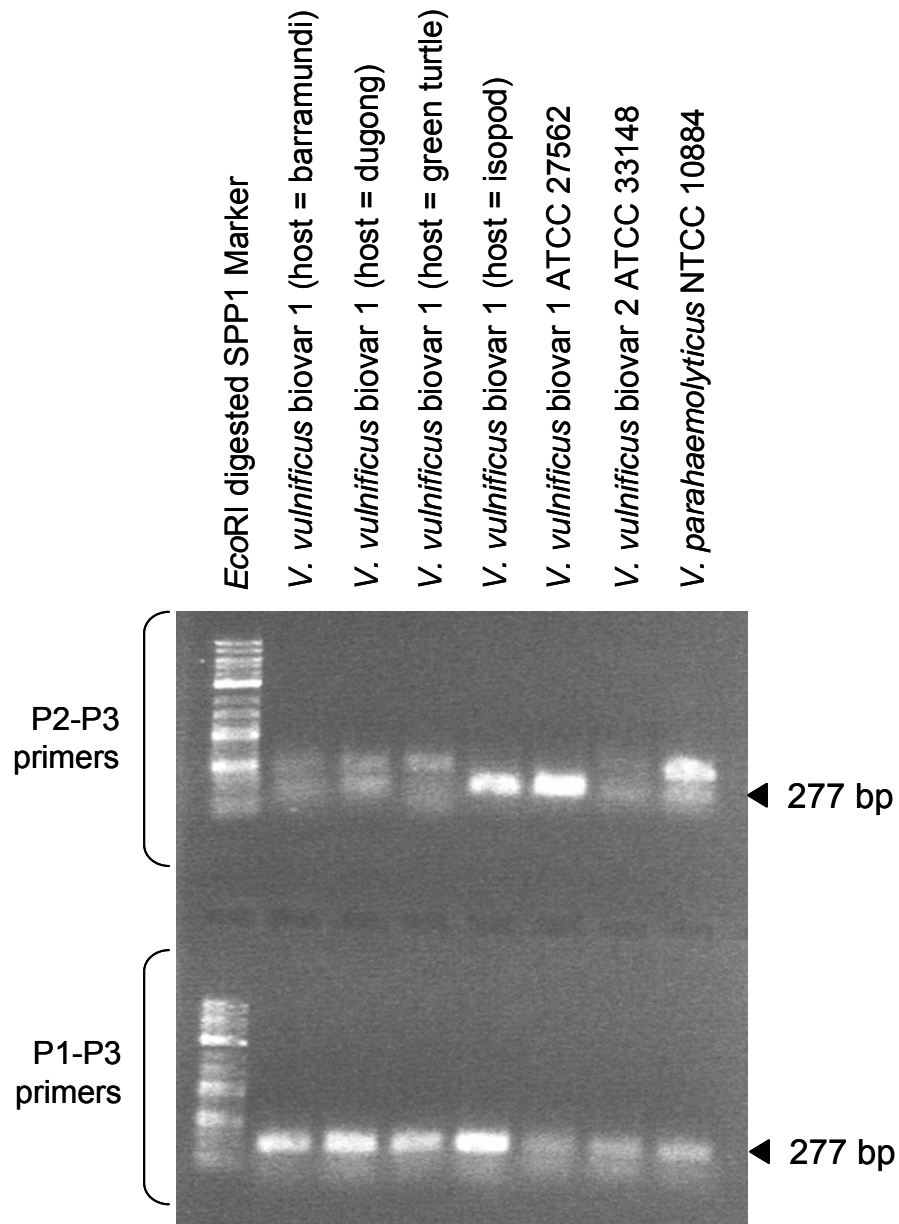
**Figure 3.3: Amplification of a 335 bp fragment of the *tdh* gene.**

Template DNA was prepared from selected *V. parahaemolyticus* isolates from uropod tissue of Rock Lobsters. *V. parahaemolyticus* NCTL strain 10885 DNA was used as a negative control. *V. parahaemolyticus* NCTL strain 10884 DNA was used as a positive control. NCTL 10884, but not other isolates of *Vibrios* pp tested, produced an amplicon of correct size and sequence.



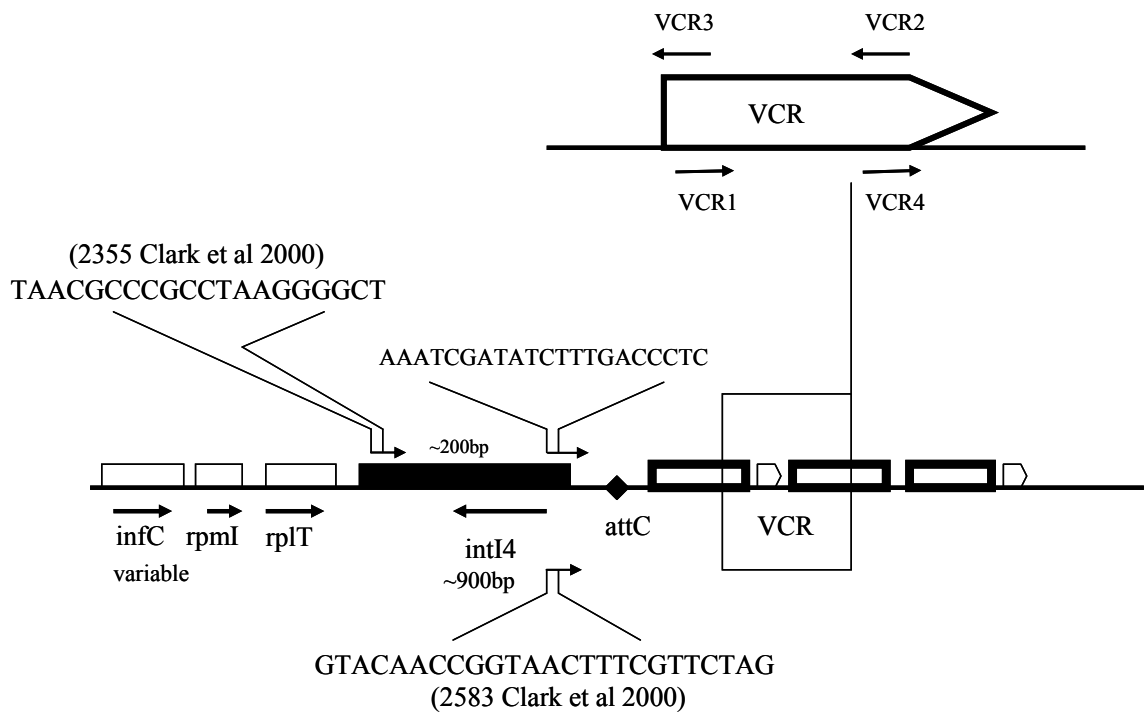
**Figure 3.4** Amplification of a 190 bp fragment of the *trh* gene.

Results shown are typical of those described in the text. *V. vulnificus* DNA was used as a negative control.



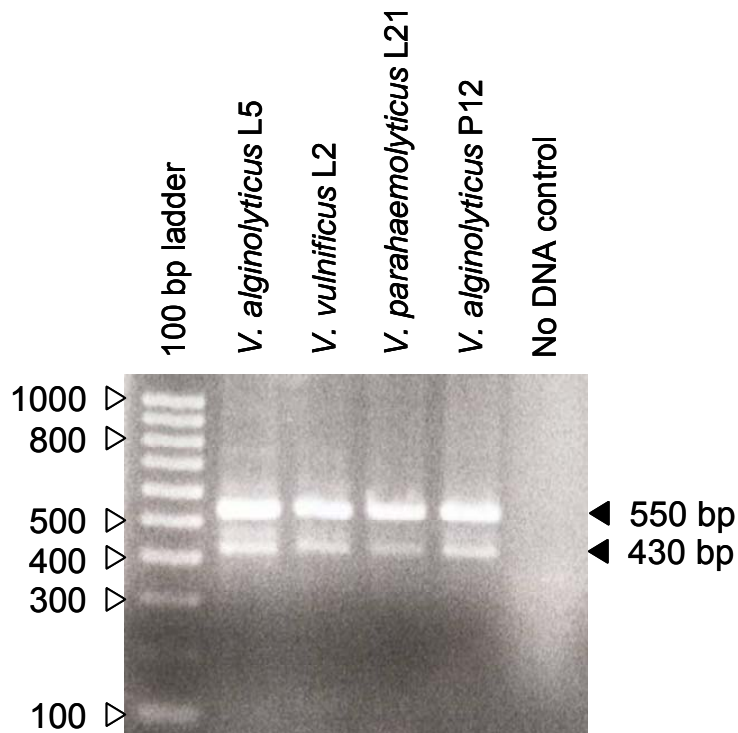
**Figure 3.5: PCR typing of clinical and environmental isolates of *V. vulnificus*.**

*V. parahaemolyticus* NCTC 10884 was used as a source of negative control DNA. Results shown are those obtained for reference cultures. Note significant mis-priming using both primer sets, in particular using primers P2-P3 from which multiple band sizes were produced. Furthermore, amplicons were produced using *V. parahaemolyticus* DNA, highlighting the inability of these primers to distinguish *V. vulnificus* isolates, let alone clinical significance of these.



**Figure 3.6: Schematic representation of a typical *Vibrio* integron.**

Locations of oligonucleotide primers used to amplify gene cassettes associated with super-integrans of *Vibrio* isolates are shown. VCR elements are shown as open box arrows. The *attC* sequence is shown as a closed diamond. Primer sequences are shown as black arrows. Filled and open boxes are integron associated genes or flanking genes. Primer sequences shown are those described by Clark *et al.* (2000).



**Figure 3.7: PCR amplification of VCR associated gene cassettes**

*Vibrio* isolates tested were those recovered from lobster uropod tissue lesions by May (2002). DNA extracted from these isolates was subjected to PCR using oligonucleotide primer pairs VCR3 and VCR4. For each isolate, PCR amplified 2 distinct fragments (arrows). Nucleotide sequence analysis (Appendix E) of the 550 bp products showed the translated sequence had 93% amino acid similarity to hypothetical proteins typical of the *Vibrio* spp. DAT722 integron. The nucleotide sequence analysis (Appendix E) of the 430 bp products showed the translated sequence had 93% similarity to hypothetical proteins typical of *Vibrio parahaemolyticus* RIMD 2210633 DNA.

## Chapter 4: Induction of TFN in Live-Held Southern Rock Lobsters

### 4.1 Introduction

A previous study by May (2002) of the microflora of lesions associated with TFN established that *Vibrio* spp. were the predominant bacteria associated with diseased tissue (For a more detailed description see Chapter 1.5.4). However, that study was restricted to lesions that developed on tail fans when lobsters were fed and maintained individually in aquaria at 18°C in the dark with constant aeration and water flow. The conditions used differ markedly from experimental live-holding facilities, where over crowding and water quality issues may be a concern. However, an important outcome of that study was that TFN-like disease symptoms can be induced in live-held lobsters as a result of deliberate damage to tail fan tissue by using instruments inoculated with *Vibrio* spp., but not with sterile instruments. This work suggested that tail fan injury is a prerequisite for establishment of TFN, but also that infection of the wound is necessary for establishment of disease. Nevertheless, the progression of TFN as a disease and the overall health of affected rock lobsters have not yet been established. Other types of shell diseases result in significant differences in haemolymph serum protein levels and establishment of bacteraemia within the haemolymph proportionate to the severity of disease (see Chapter 1.5.2).

The aim of the work described in this chapter was to monitor the:

1. Microbiology and pathology of developing lesions associated with TFN in lobsters artificially infected with a *Vibrio* spp. previously identified by May (2002),
2. Microbiological quality of aquaria water and,
3. Indicators of lobster health (serum protein levels, haemolymph bacteraemia, and total and differential cell counts).

The types of bacteria associated with lesion development and the epidemiology of this disease are described separately in Chapter 5.

## 4.2 Experimental Design

TFN lesions were induced in live lobsters as follows. An experimental design for the infection trial consisted of three treatment groups each comprising fifteen lobsters. Each treatment group was as follows:

Group I: Control lobsters that had all tail fans left undamaged.

Group II: Lobsters that had four of the five tail fans artificially damaged by puncture with a sterile nail, and by creating a 5 mm cut at the distal end of four of the five uropods with a sterile blade.

Group III: Lobsters that had four of the five tail fans artificially wounded as for Group II animals, but with instruments contaminated with a fresh overnight culture of *V. parahaemolyticus* strain L21.

Lobsters from separate treatment groups were housed in aquarium tanks (1.5 m × 1.5 m × 0.6 m deep; containing 500 L of seawater. Each tank was continuously aerated. Fresh filtered seawater was provided via an independent supply to avoid mixing of water between tanks) (Figure 4.1 and Figure 4.2). A maximum of 5 lobsters were held in each tank. Each tank contained cinder blocks as housing. Lobsters were fed a diet of mussels, squid and cockles 3 times per week throughout the period of the trial.

## 4.3 Sampling

Lobsters from each treatment group were sampled at four week intervals for a maximum of twelve weeks *post* infection (Table 4.1). At each sampling period, 5 lobsters per treatment group were physically assessed (Appendix H) and tissue and haemolymph samples taken for further analysis.

### 4.3.1 Physical Assessments

The weight of each animal was recorded and photographs of the tail fans were taken. Comparison of control photographs (including the presence of an appropriate scale) taken



prior to the commencement of the trial with photographs taken of the same lobster tail fans at the end of each sampling period, were used to assess percentage of tissue affected by TFN. The mean tissue loss per tail fan for each animal was calculated as percent change in tail fan area affected by TFN based on calculation of the total tail fan area calculated at the commencement of the trial with that at the appropriate sample point.

#### **4.3.2 Water Quality**

Water temperature and flow through the tanks, aeration (as dissolved oxygen levels), and the microbiological quality of tank inlet and outlet water (Total Viable Counts on Marine agar and counts of vibrios on TCBS agar) were monitored throughout the trial, with samples taken for analysis at each of the four sampling time points. The temperature and flow rate of water into holding tanks was monitored. Samples of holding water were also taken for microbiological analysis.

#### **4.3.3 Microbiology**

Two samples of damaged tail fan tissue were taken from each of five lobsters from each treatment group at four week intervals up to twelve weeks post infection. The samples were weighed, homogenised in 10 mL sterile saline (3% w/v NaCl) and homogenate plated on MA and TCBS to determine Total Viable counts and total *Vibrio* spp. counts.

#### **4.3.4 Microscopy**

A further four samples of damaged tail fan tissue from each treatment group were used for TEM and SEM analysis. At the completion of the trial, whole tail fans displaying various degrees of inflammation were removed and used for microbiological analysis, as well as light microscopy.

#### **4.3.5 Cell Biology**

Haemolymph samples were taken from all lobsters at the beginning of the trial as a control, as well as from each of five lobsters from each treatment group at four week intervals up to twelve weeks post infection. These samples were used to determine serum protein levels,

extent of bacteraemia and total and differential haemocyte counts. Differential haemocyte counts included counts of granulocytes, hyaline cells and semi-granulocytes (Figure 4.14).

## 4.4 Results

### 4.4.1 Water Quality within Live-Holding Tanks

The temperature of the tank water over the duration of the trial ranged from 11.5 to 18.5°C (Figure 4.3). However, during the last 8 weeks of the trial, the tank water temperature was consistently low (11.5 – 14.5°C). Tank inlet water flow rates ranged from 6 – 8.5 L min<sup>-1</sup> (mean of .13 L min<sup>-1</sup> with a standard error about the mean of 2.517) for all tanks for the duration of the experiment (Appendix F). The mean dissolved oxygen level was 11.53 ppm (with ranged from 10.5 ppm to 12.5 ppm) and standard error about the mean of 0.1856 ppm (n = 9) (Appendix G).

Total bacterial Viable Counts of tank inlet seawater were *ca.* 5 × 10<sup>4</sup> CFU mL<sup>-1</sup> and counts of vibrios on TCBS were *ca.* 1 × 10<sup>4</sup> CFU mL<sup>-1</sup>. Randomly selected colonies from TCBS agar plates were identified to species level. Approximately 50% of isolates were identified as *V. alginolyticus*, 25% as *V. parahaemolyticus* and the remaining 25% characterised as a mixture of other *Vibrio* species. No *V. vulnificus* isolates were isolated from any sample of tank feed seawater.

The total bacterial Viable Count (*ca.* 6 × 10<sup>4</sup> CFU mL<sup>-1</sup>) and counts of vibrios on TCBS (*ca.* 4 × 10<sup>4</sup> CFU mL<sup>-1</sup>) in tank effluent generally exceeded that of the tank inlet feed water. Furthermore, the predominant species of *Vibrio* isolated from tank effluent was *V. alginolyticus*, with few other *Vibrio* spp. detected.

### 4.4.2 Macroscopic Analysis of Tail Fan Tissue from Trial Lobsters

At the start of the trial, all lobsters were in good physical condition and tail fans were essentially complete with little or no damage to the margins. Figure 4.5 shows photographs of tail fans of lobsters from each trial group at 4, 8 and 12 weeks after the beginning of the trial. Some lobsters from all treatment groups developed symptoms of TFN, but notably, the margins of tail fans of control group (Group I) lobsters remained essentially intact throughout the entire 12 weeks trial period, whereas the margins of tail

fans of Group III lobsters developed significant erosion typical of TFN. Tail fans that developed TFN displayed symptoms that included erosion and melanisation of the tail fan margins, inflammation (thickening) of the tail fan, and several lobsters developed melanised holes within some tail fans. However, as a general observation, symptoms typical of TFN developed more frequently on lobsters intentionally damaged and infected with *V. parahaemolyticus* strain L21. In particular, these lesions developed at the site of mechanical damage on tail fans from Group III lobsters (see Figure 4.6 –Figure 4.8). Lesions were not observed to form at the site of damage (holes and marginal cuts) on tail fans of Group II lobsters. Lesions that formed on tail fans from Group I and group II lobsters usually formed at the tail fan margin.

Figure 4.9 presents data that shows the mean percentage loss in tail fan tissue during the entire period of the infection trial. No significant difference in tail fan tissue loss ( $P < 0.05$ ) was observed for lobsters from any treatment group at any individual sampling period. Nevertheless, significant differences ( $P < 0.01$ ) in tail fan tissue erosion in lobsters that were intentionally damaged with instruments inoculated with *V. parahaemolyticus* strain L21 was observed across all sampling points.

During weeks 10 to 12 of the trial, almost all lobsters underwent a moult. By 12 weeks post infection, most of the lobsters had completed moulting. Interestingly, following the moult of lobsters, most minor (generally  $<15\%$  uropod affected) tail fan lesions had healed by week 12 post infection. However, more serious lesions (generally  $>15\%$  of tail fan affected) remained as part of the new carapace tissue.

#### **4.4.3 Microbiology of Tail Fan Tissue from Experimental Lobsters**

To determine if the appearance of lesions typical of TFN were accompanied by increases in numbers of bacteria associated with lesion tissue, samples of diseased tail fans were homogenised and used to prepare plate counts on MA and TCBS culture media. TCBS medium was used to obtain estimates of numbers of vibrios associated with tail fan tissue.

Figure 4.10 illustrates mean count on MA per gram of tail fan tissue for lobsters from each treatment group for each of the four sampling periods of the infection trial. Tissue from lobsters in the control treatment group four weeks *post* infection carried on average  $1.4 \times 10^6$  total CFU  $g^{-1}$  of tissue. Interestingly, at 8 weeks post infection, counts of

bacteria growing on both MA and TCBS were generally one log lower than counts at 0 and 4 weeks *post* infection. The observed reduction in counts coincided with a gradual reduction in holding tank water temperature (final temperature <15°C) noted for the duration of the trial. Typically, the mean counts on MA and counts on TCBS agar of bacteria on tail fan tissue harvested from the control animals was  $2.9 \times 10^5$  CFU g<sup>-1</sup> and  $2.1 \times 10^5$  CFU g<sup>-1</sup> respectively. At 12 weeks post infection, mean MA counts for tail fan samples of control group lobsters were  $7.1 \times 10^5$  CFU g<sup>-1</sup> of tissue.

Figure 4.10 also illustrates changes in counts of *Vibrio* associated with tail fan tissue for lobsters at each sampling period over the entire infection trial. At 4 weeks post infection, the mean counts of vibrios were generally lower than counts obtained for the same tissue plated on MA, with a mean of  $3.3 \times 10^4$  *Vibrio* CFU g<sup>-1</sup> of tissue in tail fan tissue harvested from lobsters in the control group. At 8 weeks post infection, lobsters that had tail fans damaged with sterile instruments had an average of  $6.7 \times 10^4$  total CFU g<sup>-1</sup> of tail fan tissue. This count increased to an average of  $1.1 \times 10^5$  total vibrios per gram of tail fan tissue after 12 weeks post infection.

No significant differences ( $P < 0.05$ ) were detected in the TVC on MA per gram of tissue for tissue harvested from lobsters that had tail fans damaged with sterile instruments or those that had tail fans artificially infected, compared to the control lobsters.

Bacteriological analysis of tissue affected by severe inflammation was performed by aseptic removal of the contents of inflamed tissue (Section 2.4.3), followed by homogenisation in saline and plating onto MA and TCBS followed by incubation at 30°C for 48 h. No colonies of bacteria grew on either of the growth media used. This analysis indicated that this tissue was likely to have been sterile.

#### **4.4.4 Microbiology of the Haemolymph of Experimental Lobsters**

To determine if infection or damage of tail fan tissue resulted in systemic infection of lobsters, samples of haemolymph were tested for the presence of bacteria capable of growth on either MA or TCBS media (Figure 11, Appendix I). Whilst ANOVA indicated that there were no significant differences between counts of bacteria obtained for any of the treatment groups for the duration of the trial ( $P < 0.05$ ), counts of bacteria in the

haemolymph determined by plating on MA declined over the course of the trial, irrespective of treatment groups. Conversely, counts on TCBS gradually increased with time the lobsters spent in tanks. In particular, Group III lobsters had consistently higher counts on TCBS compared with haemolymph counts for lobsters from other treatment groups. ( $P > 0.05$ ). Randomly selected isolates from TCBS plates were identified as per Alsina and Blanch (1994) as *V. alginolyticus*; one isolate was identified as *V. hollisae*. None of these isolates were found to possess any pathogenic traits other than the expression of extra-cellular protease (see Chapter 5).

#### **4.4.5 Haemolymph Serum Protein Levels**

Serum protein levels have been used as a marker of disease or stress that impact directly on the health of lobsters (Leavitt & Bayer, 1977). Analysis of serum protein levels in haemolymph samples taken from all lobsters in the trial indicated there was no significant difference ( $P < 0.05$ ) between lobsters from the control group for the duration of the trial (Figure 4.12). Similar results were obtained for lobsters that had tail fans damaged with sterile instruments and for those that were damaged with instruments inoculated with *V. parahaemolyticus* strain L21. No correlation between either the onset or severity of TFN and haemolymph serum protein levels were identified. Furthermore, serum protein levels of lobsters from each of the treatment groups were not significantly different ( $P < 0.05$ ) at any stage of the trial. All serum protein levels were at the upper end of the scale outlined by Leavitt & Bayer (1977) ( $80 - 90 \text{ mg mL}^{-1}$ ) for normal healthy lobsters. This data indicated all lobsters were in good health throughout the trial.

#### **4.4.6 Haemolymph Total and Differential Cell Counts**

Changes in total and differential haemocyte numbers are known to occur in lobsters and other crustaceans following infection or environmental stress. Typically, this results in reductions in total numbers of cells, or variation in the differential counts of cells. In this study, little or no change in either the total cell count or the differential cell count of haemolymph samples from lobsters was noted, irrespective of the treatment group (see Figure 4.13 and Figure 4.14). No significant differences ( $P < 0.05$ ) were noted in the number of circulating haemocytes of lobsters between the start of the trial and subsequent sample points. For the duration of the trial, these counts remained within the normal

parameters established by Evans *et al.* (1998) and Stewart *et al.* (1967) (range of  $2.5 \times 10^6$  to  $16.8 \times 10^6$  cells mL<sup>-1</sup>) for normal healthy lobsters.

Differential cell counts of circulating haemocytes in haemolymph samples taken from lobsters in each of the three treatment groups also remained within the parameters set by Evans *et al.* (1998) (range of 29 – 37% for hyaline cells, 8 – 12% for granulocytes, 51 – 63% for semi-granulocytes) for each cell type. No significant differences ( $P < 0.05$ ) in differential cell counts were noted at any stage of the trial.

#### **4.4.7 Histology of Lesions Associated with TFN**

To characterise the morphological changes in tail fan histology associated with development of symptoms typical of the tail fan inflammation commonly associated with development of TFN, samples of affected tail fan tissue were fixed, decalcified and embedded in paraffin wax and sections (10 to 15  $\mu$ M) prepared for staining with Haematoxylin and Eosin, followed by examination under a light microscope. Figure 4.15 is a collage of micrographs of sections through inflamed tissue mapped on a schematic representation of tail fan tissue cross-section. Importantly, these micrographs showed no evidence of significant microbial invasion into the underlying inflamed tissue was observed in any section of tissue examined. This observation was consistent with the microbiological analysis of the same tissue (described in Section 4.4.3).

Figure 4.16 illustrates a cross section of healthy tail fan tissue, with all layers of the carapace clearly defined. These included the epicuticle, exocuticle, endocuticle and epidermis. A thin layer of connective tissue free of any cellular matter was observed between the dorsal and ventral carapace.

Microscopic observation of sections of inflamed tissue indicated three anatomically distinct regions of tissue were present. The first of these was the tail fan carapace tissue itself. Figure 4.17 presents a micrograph of a cross section of the dorsal surface of tail fan carapace that displayed significant inflammation. Typically, tissue affected by inflammation showed a loss of internal structure associated with the dorsal carapace, particularly at the boundaries between the exocuticle and endocuticle and between the endocuticle and the epidermis. Sections of the ventral side of the tail fan, also displayed

loss of structure, particularly at the boundaries between the exocuticle and endocuticle and between the endocuticle and the epidermis. However, the ventral surface of affected uropods usually displayed only mild inflammation of the carapace tissue when compared with the dorsal side (Figure 4.18). Inflammation of the underlying epidermis was characterised by a large deposition of fibrous matter in all lesions, regardless of severity (Figure 4.19).

Tail fan tissue affected by severe inflammation was characterized tissue pathology consisting of a central core consisting of a number of different cell types within the epidermis (Figure 4.20). Cell types identified in this region included large numbers of infiltrating haemocytes, with granulocytes and hyaline cells clearly visible, as well as other fibrocytes.

A more comprehensive histological analysis of this tissue was not possible because stains other than Haematoxylin and Eosin were not effective at staining the tissue sections. Staining techniques, such as Giemsa and Vital New Red did not adequately stain the tail fan tissue. Consequently, the composition of the fibrous matter described above or a definitive identification of potential cell types present eg. eosinophil-like cells, could not be determined.

#### **4.4.8 SEM of Tail Fan Tissue**

Examination of the surface of apparently healthy tail fan tissue by SEM indicated this tissue was essentially free of any significant biofilm containing bacterial cells. However, sporadic patches of tissue colonised by microbial cells were observed on this tissue (Figure 4.21A, B). This observation was typical of tissue from the control group for entire duration of the infection trial, and for tissue obtained four weeks post infection from lobsters damaged with sterile instruments. However, sections of tail fan tissue taken from lobsters deliberately damaged with instruments contaminated with *V. parahaemolyticus* L21, as well as lobsters damaged with sterile instruments at 8 and 12 weeks, contained large numbers of bacteria along the margins of lesions (Figure 4.21C – F). Typically, these bacteria included coccoid, short rods, and a few spiral shaped cells. These lesion surface-associated bacteria were generally arranged in micro-colonies, although isolated bacterial

cells were also observed. In addition, some bacterial cells were observed to be embedded within the surface of the lesion tissue (Figure 4.21G), as described by May, 2002.

#### **4.4.9 TEM of Tail Fan Tissue**

To obtain a detailed view of the interaction of bacteria and tail fan tissue, samples of tissue from control and experimental animals were embedded in resin and ultra-thin sections prepared for examination by TEM. Examination of sections through the surface of tail fan tissue from control group lobsters indicated that these surfaces supported low numbers of bacteria and this observation was consistent with that described in Section 4.4.8. The tail fan surface was apparently free of any significant microbial biofilm. Areas of tail fan surface colonised by bacteria usually only supported a thin biofilm and bacterial cells seemed to be organised into micro-colonies (Figure 4.22A, B). Similar observations were obtained for tissue from lobsters that had been damaged with sterile instruments 4 weeks prior (Figure 4.22C).

No bacterial cells were observed in sub-surface tissue of tail fans from lobsters from the control group. Tail fan tissue from control animals was characterised by a well defined structure, with clear boundaries between the different tissue layers ie. epicuticle, exocuticle etc.

By contrast, tail fan tissue from the lobsters damaged with sterile instruments and instruments inoculated with *V. parahaemolyticus* strain L21, was characterised by large numbers of bacterial cells in the sub-surface layers. Typically, this tissue showed significant damage to cell fine structure of the endocuticle. This damage was evident in tail fan tissue taken from lobsters 8 weeks after commencement of the trial (Figure 4.22D-H). This tissue morphology is consistent with SEM images that display discrete micro-colonies comprising a mixture of bacterial cell morphologies (see Section 4.4.8).

Where bacterial cells were present, significant destruction of the underlying tissue and breakdown of tissue structure was observed. This damage was apparent either as localised erosion of chitinous tissue that lay under surface associated bacteria (Figure 4.22E, F), or as erosion of deeper tissue by bacterial cells that had penetrated into the tissue at the site of lesion development (Figure 4.22G, H). In some instances, the surface of tail fan tissue



appeared to be relatively healthy but the underlying tissue contained large numbers of bacterial cells and concomitant loss of cell fine structure (Figure 4.22H – J).

Some sections of tissue showed surface-associated bacteria that were apparently attached to the surface by micro-fibrils (Figure 4.22K). No significant invasion of lesion associated tissue by haemocytes was observed.

## 4.5 Discussion

In a previous study, May (2002) provided experimental evidence to indicate that mechanical damage to uropod tail fan alone was insufficient to initiate an increased incidence of TFN in live-held lobsters compared to control animals. Furthermore, that study also showed that mechanical damage of tail fan tissue with instruments contaminated with *Vibrio* spp. isolated from TFN lesions resulted in formation of TFN-like lesions at the site of damage. However, the small scale of the experiments conducted limited statistical evaluation of the data obtained. Nevertheless, the results obtained indicated that the combination of mechanical damage plus deliberate infection of damaged tissue with isolates of bacteria obtained from TFN lesions did increase the incidence of TFN in experimental lobsters compared with control animals. Importantly, that study also provided experimental evidence to indicate that the strain of bacterium used to infect tail fan tissue could be isolated from developing TFN-like lesions associated with damaged tissue. This suggested that the bacteria used were responsible for both initiation of disease as well as maintenance and development of lesions.

Data presented in this chapter describes the outcome of larger scale experiments specifically designed to determine if simultaneous damage and infection of tail fans with *V. parahaemolyticus* strain L21 isolated from TFN lesions leads to increased incidence of TFN at the site of damage in rock lobsters under conditions used in this study. Significantly, the present study has shown that lobster tail fans simultaneously damaged and infected with *V. parahaemolyticus* L21 tended to develop a higher incidence of TFN compared with control animals. These lesions were typical of TFN and importantly, developed at the site of damage with contaminated instruments.

To determine whether bacteria other than those used to infect experimental animals could contribute to development of TFN-like lesions, the microbial flora of water flowing

into and out of housing tanks was monitored. Whilst there was a low level of bacteria present within the tank water, this population was dominated by *V. alginolyticus*, a vibrio that is generally considered of little significance to the health of crustaceans or humans. This contrasted with microbiological analysis of the microflora of lesions on tail fans that showed *V. parahaemolyticus* was the predominant microorganism present. Consequently, it is unlikely that tank water was a possible source of bacteria responsible for the onset of TFN within this study.

Interestingly, neither the method of housing nor the development of TFN-like lesions on tail fan tissue apparently had no significant impact on the overall health of the lobsters. When lobsters are placed under stress or affected by systemic bacterial infections, haemolymph protein levels and differential haemocyte counts are known to change. However, in this study, haemolymph protein and haemocyte populations remained essentially constant and well within levels known to be characteristic of healthy lobsters (Evans *et al.*, 1999, Stewart *et al.*, 1969). This is in contrast to a previous study by Vogan & Rowley (2002) which showed that haemolymph of freshly caught edible crab, *Cancer pagurus* affected by shell disease, was characterised by elevated serum protein levels and minor changes in the semi-granulocyte and hyaline cell populations of haemolymph.

The absence of significant bacteraemia in haemolymph also indicated that the experimental animals remained in good health for the majority of the trial. In fact, only low levels of bacteria (<100 per mL) were detected in most samples of haemolymph sampled from lobsters by the end of the trial. Interestingly, the counts of bacteria obtained by plating on marine agar decreased with time, whereas counts obtained by plating on TCBS increased over the same time period. This observation may reflect changes in seawater temperature from 8 – 12 weeks *p.i.* During this time, the water temperature was consistently below 15°C. At these temperatures, *Vibrio* species are reported to enter a viable but non-culturable (VBNC) state. VBNC state vibrios would not be detectable by culture on either MA. However, a study by Pace *et al.* (1997) demonstrated that the presence of bile salts in the growth medium is able to revive vibrios from the VBNC state. This may explain the higher counts obtained when samples were plated on TCBS as the bile salts present within this medium may have revived vibrios that had entered the VBNC state.

Despite the trends mentioned above, no statistical differences were found between the three treatment groups for the duration of the trial as per 2 – way ANOVA. Statistical comparisons were not possible because many counts obtained were below the minimum detectable limit of the method used to count bacteria present ( $<10 \text{ mL}^{-1}$ ).

Identification of randomly selected isolates indicated the micro-flora consisted primarily of *V. alginolyticus*, a species of *Vibrio* not normally known to be pathogenic to crustaceans. However, absence of any correlation between bacteraemia within the haemolymph of lobsters and severity of TFN suggested that other factors were responsible

The surface of tail fans of control animals was surprisingly free of a biofilm of micro-organisms. Isolated micro-colonies of bacteria were located on the surface of these animals, but no evidence of degradation of the chitinous surface or invasion of deeper tissues was observed. This observation was in stark contrast with microscopic analysis of TFN-like lesions, where the surface of tissue affected was extensively colonised by micro-colonies of bacteria. Importantly, this work showed that the bacteria were limited to the lesion surface and immediate subsurface layers of affected tissue. Given the extensive melanisation and necrosis surrounding lesions, it is likely that melanisation at least, is a host dependent response that limits the rate of spread of infection and this is sufficient to prevent other systemic effects that could lead to lobster morbidity.

Unlike other shell diseases that involve a breach of the carapace that allows bacteria direct access to the haemolymph, tail fan tissue is poorly vascularised and there is very little flow of haemolymph through the tail fan tissue. Consequently, lobster haemocytes may have limited ability to migrate to regions of the tail fan affected by damage and infection. This in turn may mean that the ability of the lobster to respond to and resolve infections like TFN is limited. However, the limited vascularisation of tail fan tissue does not necessarily eliminate the potential for haemocytes to migrate to regions of tail fans affected by some trauma or infection. In lobsters that displayed more serious TFN, the tail fan tissue became inflamed. Microscopic analysis of this inflamed tissue indicated development of significant histology characterised by a wide range of cell types normally not found in the deeper tissues of tail fans. These observations indicated that the swelling and inflammation may be a result of migration of cells into the tail fan tissue. Remarkably,

both the histological and microbiological analysis indicated this tissue was essentially sterile.

To investigate the macroscopic and microscopic structure of TFN lesions, sections of tail fan tissue were stained with histologically relevant dyes. Initially, Haemotoxylin and Eosin was used. However, this staining method cannot be used to fully distinguish all cell types. Two additional staining methods were also tried. However, tissue sections stained with either Giemsa or Vital New Red did not absorb dye sufficiently to provide any useful information. Vital New Red was specifically chosen as it is recognised as a method to distinguish eosinophil-type cells. The reasons for lack of tissue staining are not known.

The persistent nature of TFN infections suggested that even in the face of host responses (eg. Melanisation, migration of haemocytes to site of infection) the bacteria are able to slowly degrade tail fan tissue. The outcome of this activity is a progression in the size and severity of the TFN lesions. This is in contrast to other shell diseases where disease severity is directly correlated to the level of bacteraemia and subsequent mortality. Indeed, a study by Vogan *et al.*(2001) investigating shell disease syndrome in *Cancer pagurus*, showed an apparent correlation between disease severity and the number of viable bacteria in the haemolymph, which in turn led to infections in other organs, such as the gills and hepatopancreas, contributing to mortality. Since lobsters affected by TFN do not suffer significant bacteraemia, other biochemical changes typical of systemic infection, or infections of gill filaments and hepatopancreas typical of other shell diseases, it is not surprising that there is a lack of mortality associated with this disease.

The structure of the swollen/inflamed tissue noted for severe cases of TFN is similar to melanotic tumours seen in *Drosophila melanogaster* (Hanratty & Ryerse, 1981, Prof. Otto Schmidt, Department of Agriculture, Food and Wine, University of Adelaide, personal communication). These are a normal heritable response to some form of abnormal development (Sparrow *et al.*, 1978). During tumourgenesis, a haemocytic capsule forms around larval tissue, with melanin deposited within and around the capsule (Rizki & Rizki, 1986). This follows a mechanism similar to the normal mechanism responsible for responding to parasites, including bacteria, within the haemocoel of insects, as well as shell damage (Gotz, 1986). As there is no evidence to suggest the bacteria are getting into the deeper inflamed tissues, it is likely that this response is induced by damage to the cuticle.

Since no fluctuations in the haemocyte populations were observed, it is likely that the cells present in the inflamed tissue are not recruited to mount any major offensive against the infection. This then raises the question of whether any immune response is mounted against the disease at all. Whilst there is a localised melanisation response, as well as a response suggestive of immune activation due to either the bacterial infection or damage to the cuticle, the role of damage to the carapace, the invading organisms or a combination of both in establishment of this histology has yet to be demonstrated. Immunological evidence to show that the lobster is unlikely to mount an immune response to the bacteria at the periphery of TFN lesions will be presented in Chapter 6.

An unexpected outcome of the experimental trial was the observation that all but the most severe TFN-like lesions healed as a result of moulting between the eighth and twelfth week of the trial. Furthermore, additional moults may be sufficient to reduce the more severe lesions seen associated with TFN (Richard Musgrove, Personal Communication). This suggests that holding of lobsters until they had moulted prior to sale may be sufficient to dramatically decrease the prevalence of TFN.

## 4.6 Conclusions

This chapter has presented evidence to support the hypothesis that TFN lesions occur as a result of combined mechanical damage and infection of damaged tissue by bacteria. In particular:

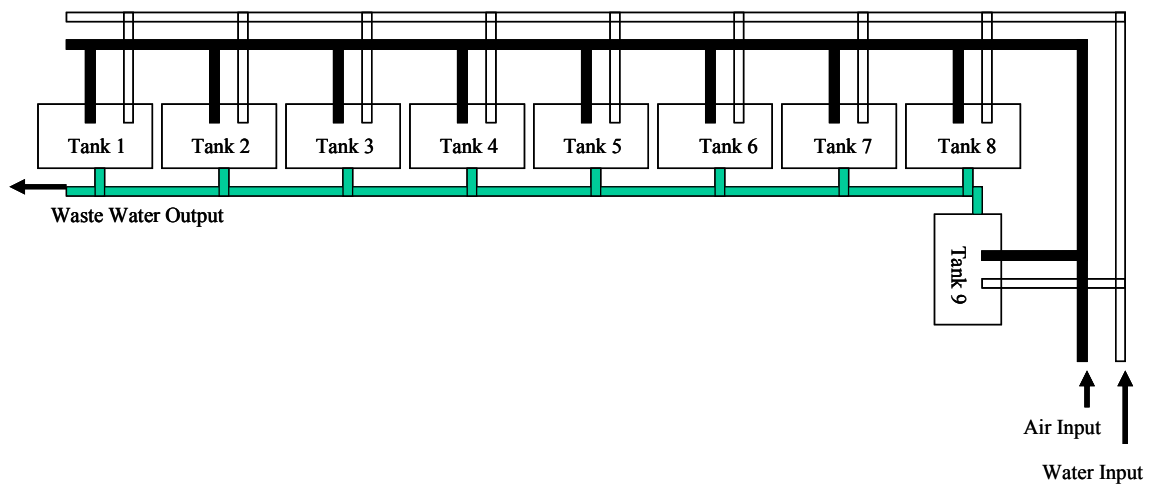
1. *V. parahaemolyticus* strain L21, originally isolated from a TFN lesion, is able to initiate and establish TFN-like lesions when used to infect intentionally damaged lobster tail fan tissue.
2. The extent of tail fan infection leading to TFN is restricted to the immediate boundaries of the lesions, although limited invasion of the underlying tissue occurs.
3. The microflora of lesions that develop at the site of infection by *V. parahaemolyticus* L21, is dominated by *V. parahaemolyticus* isolates.
4. The health of lobsters is not affected by TFN induced by infection of damaged tail fan tissue with *V. parahaemolyticus* L21. These animals do not develop

significant bacteraemia, display significant changes in haemocyte cell counts and composition, or changes in haemolymph protein levels that are typical of systemic infections. However, in severe cases of TFN, infiltration of affected tail fan tissue by haemocytes, may lead to significant inflammation and swelling.

5. Melanisation reactions at the periphery of TFN lesions indicate that host responses may restrict development of systemic infection.

**Table 4.1: Treatment and assessment date for lobsters in each tank.**

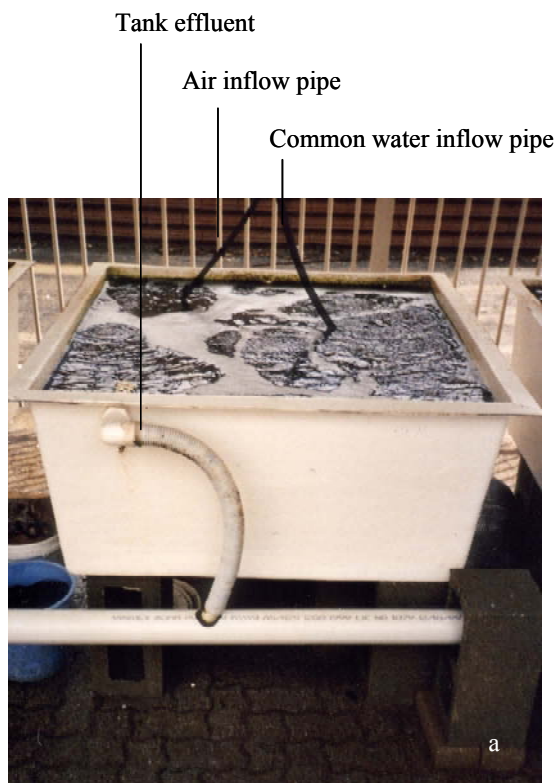
Tank	Tail Fan Treatment	Date assessed
1	Group I: Control	09/06/04
2	Group I: Control	07/07/04
3	Group I: Control	28/07/04
4	Group II: Damaged with sterile instruments	09/06/04
5	Group II: Damaged with sterile instruments	07/07/04
6	Group II: Damaged with sterile instruments	28/07/04
7	Group III: Damaged with instruments inoculated with <i>V.parahaemolyticus</i> strain L21	09/06/04
8	Group III: Damaged with instruments inoculated with <i>V.parahaemolyticus</i> strain L21	07/07/04
9	Group III: Damaged with instruments inoculated with <i>V.parahaemolyticus</i> strain L21	28/07/04



**Figure 4.1: Schematic holding tank design used for lobster infection trial one.**

All tanks were located under cover at ambient temperatures, were continuously aerated and included cinder blocks for lobster housing. Each tank was fitted with an independent, filtered sea-water supply fed from a common over-head pipe (water input) to prevent mixing of tank water with that of adjacent tanks. Effluent from each tank was removed via a pipe at the top of each tank, which fed down to a common external drain (waste water output).

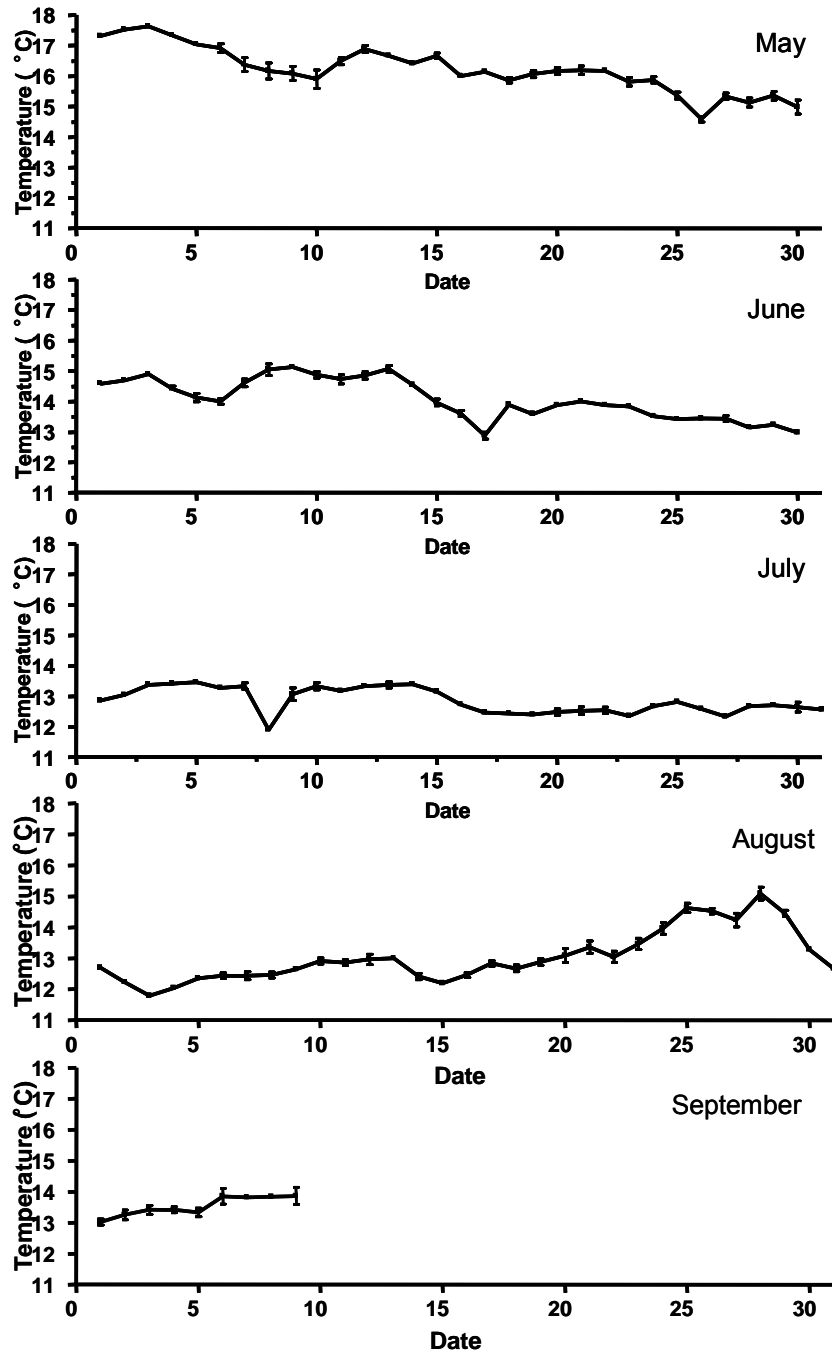




**Figure 4.2: Lobster holding tank arrangement.**

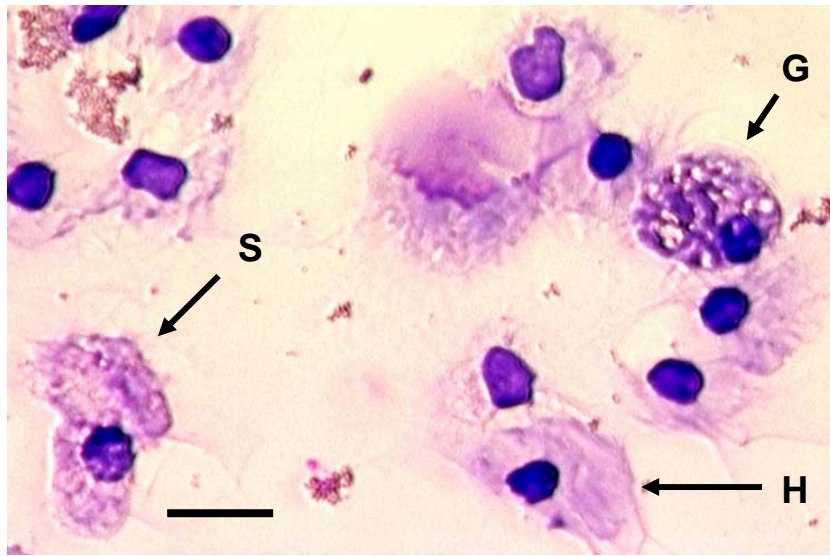
Panel A: Single tank used to house lobsters for the duration of the trial. Note the water inflow pipe providing an independent supply of filtered sea-water, the air inflow pipe and the tank effluent pipe leading to a common external drain

Panel B: Arrangement of all holding tanks used for the duration of the trial.



**Figure 4.3: Infection trial holding tank water temperature history.**

Data shown are mean temperatures. Error bars are the standard deviation about the mean temperature.



**Figure 4.4: Different cell haemocyte cell types within rock lobster haemolymph.**

Key to symbols:      G: granulocytes (large granules);  
                             S: semi-granulocytes (small granules);  
                             H: hyaline cells (no granules).

Scale bar represents 25  $\mu\text{m}$ .

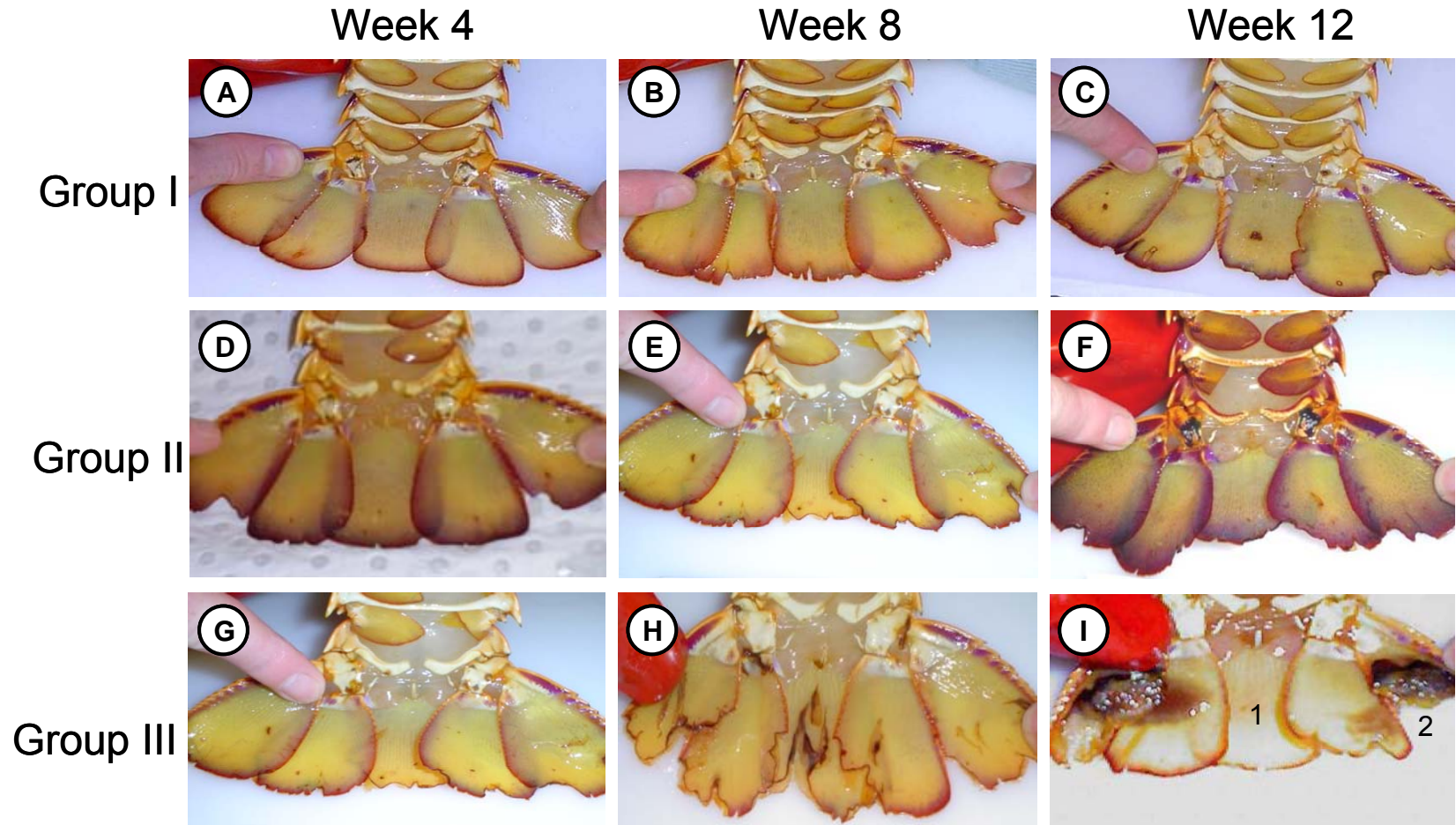
**Figure 4.5: Appearance of Tail Fans following damage and infection.**

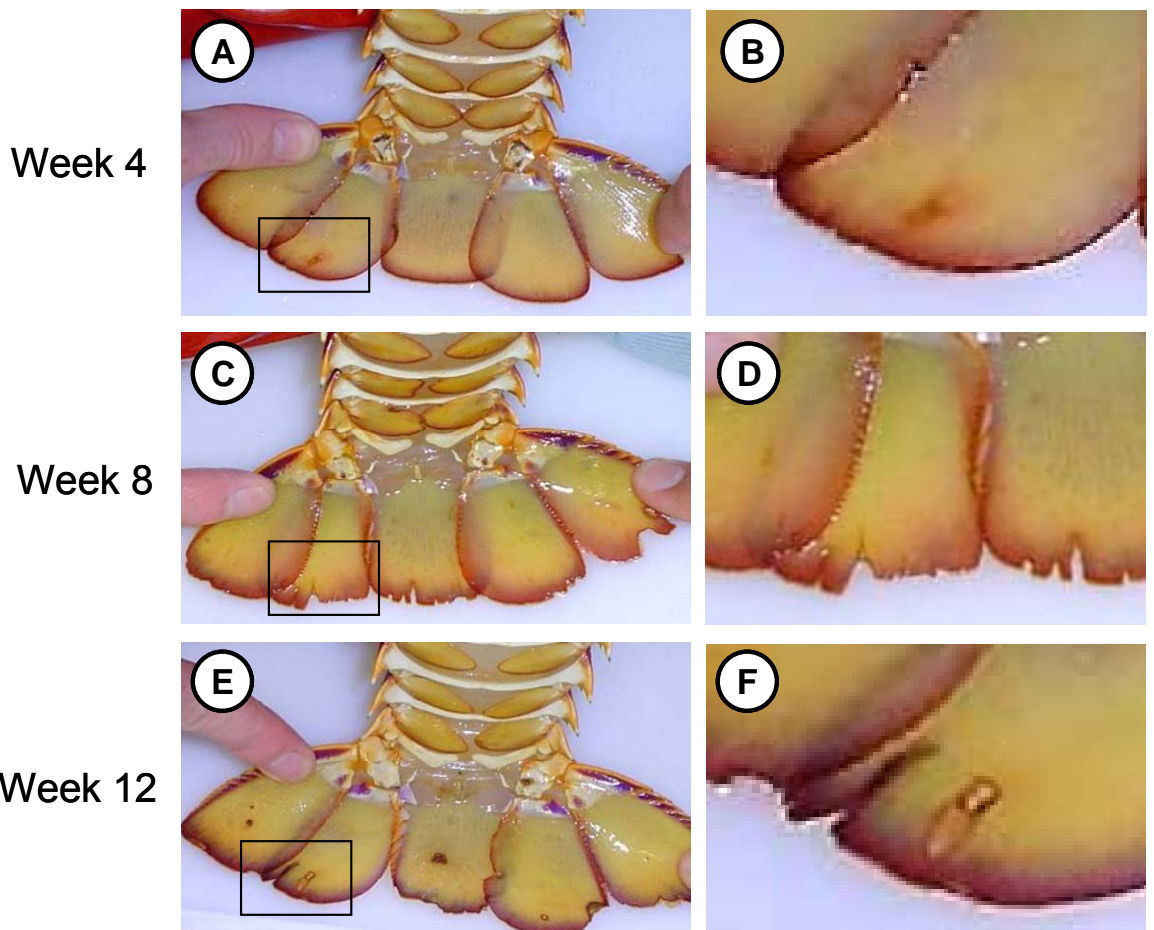
Images of tail fans after 4, 8 and 12 weeks of holding. Tail fans shown are typical of those observed from lobsters from infection trial Groups I (A, B and C), Group II (D, E and F) and Group III (G, H and I). Trial conducted over the period May-August, 2004.

Note the severe lesions associated with tail fans of group III lobsters. By week 12, severe lesions resulted in significant erosion of tail fan tissue (see I). By contrast tail fans from Group I and Group II lobsters remained essentially intact. Although some lobsters from these groups developed lesions typical of TFN, the lesions were in general not associated with deliberately inflicted cuts or holes (see D, E and F).

Key to symbols:

- 1 tail fan with minor lesions that were fully resolved upon moult;
- 2 tail fan tissue with severe lesions that were not resolved upon moult.

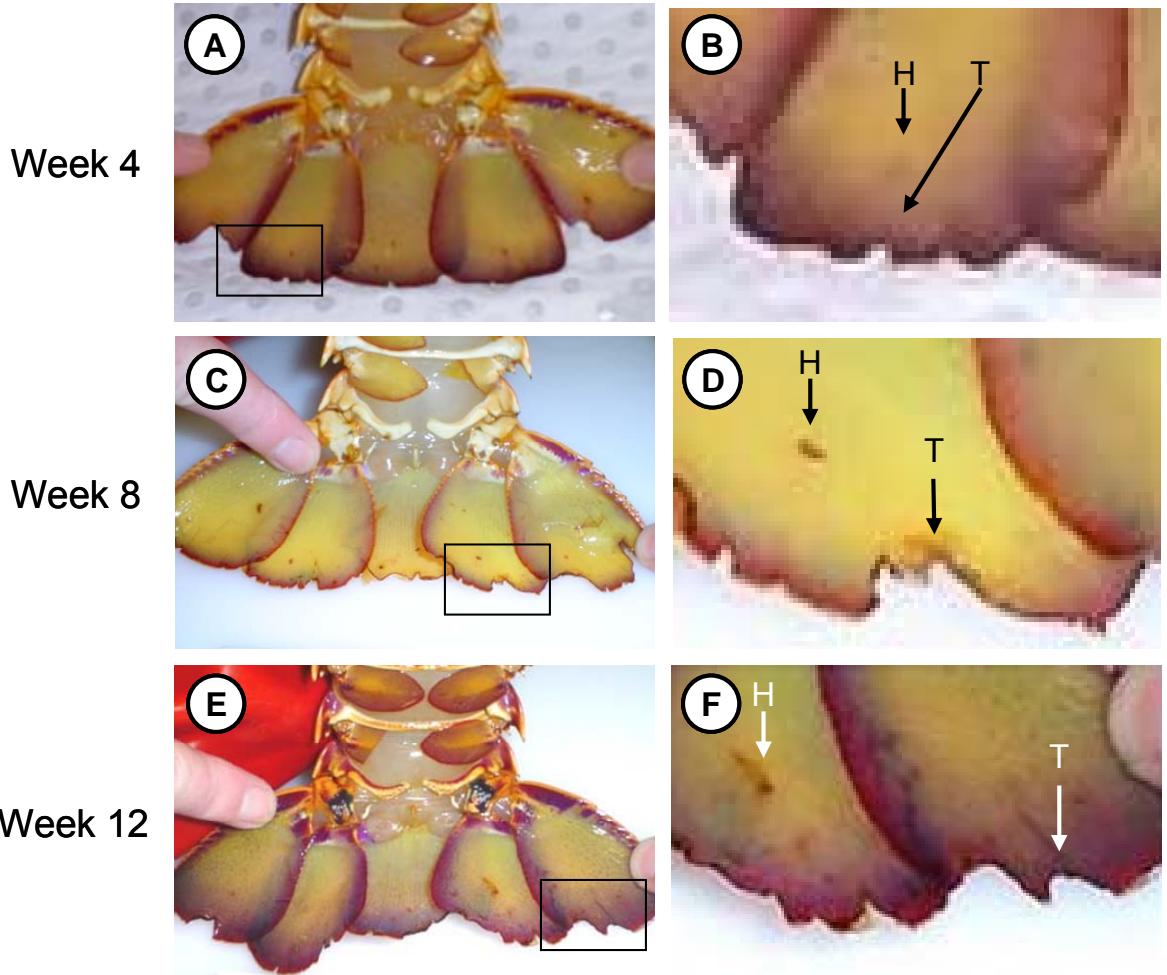




**Figure 4.6: Appearance of tail fans of Group I (control) lobsters after 4, 8 and 12 weeks holding.**

All Figures on the right hand panels are enlarged images of damaged tail fan tissue indicated by the rectangle in corresponding images shown on the left. Images are typical of those observed for all lobsters in this control treatment group.

Images show typical tears and holes in tail fans that result from normal lobster activity within holding tanks (see D, and F).

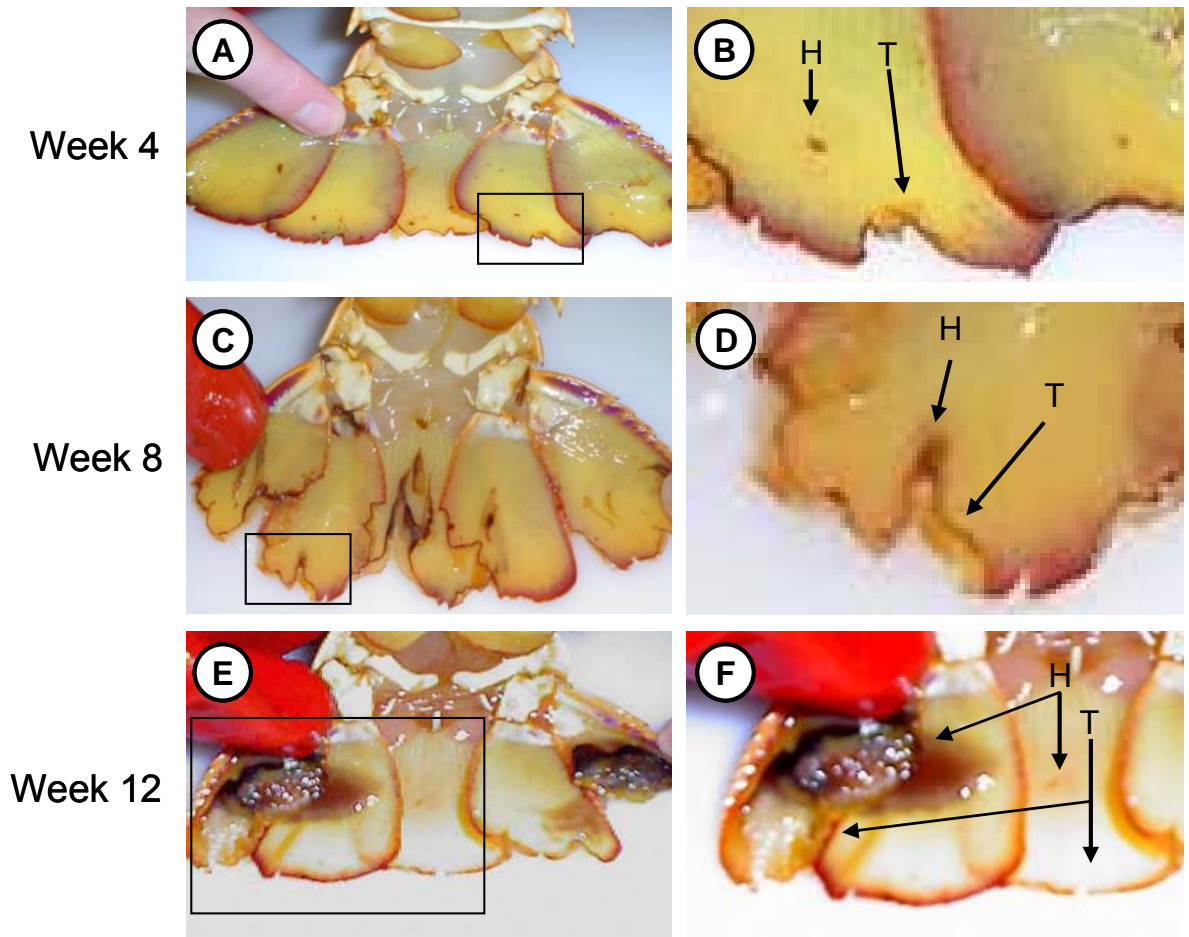


**Figure 4.7: Appearance of tail fans of Group II lobsters after 4, 8 and 12 weeks holding.**

Group II lobsters subjected to damage with sterile instruments. Position of holes and cuts inflicted are indicated. Note the progression of marginal damage to tail fans with holding time. Melanisation and erosion of tail fan tissue is not consistent with the site of damage.

Key to symbols:

- H: site where a hole through the tail fan was produced using a sterile nail
- T: site where sterile scissors were used to cut a 5mm nick at the distal end of the tail fan in order to mimic tears produced when lobsters are caught and impounded.



**Figure 4.8: Appearance of tail fans of Group III lobsters.**

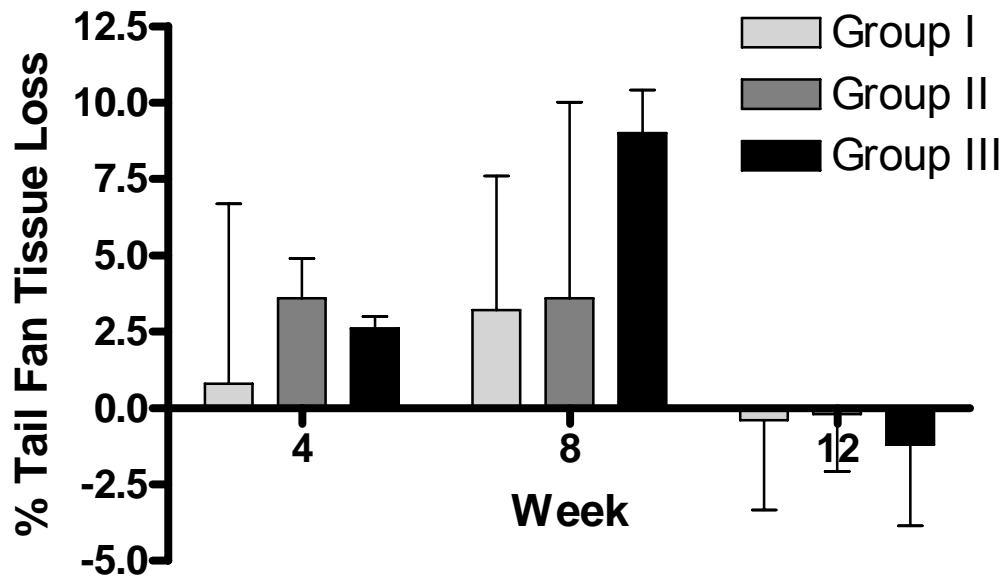
Group III lobsters subjected to damage with instruments contaminated with *V. parahaemolyticus* L21. Images show tail fans after 4, 8 and 12 weeks post commencement of the trial.

Position of holes and cuts inflicted are indicated. Note the progression of damage to tail fans with holding time. Melanisation and erosion of tail fan tissue is consistent with the site of intentionally inflicted damage.

Key to symbols:

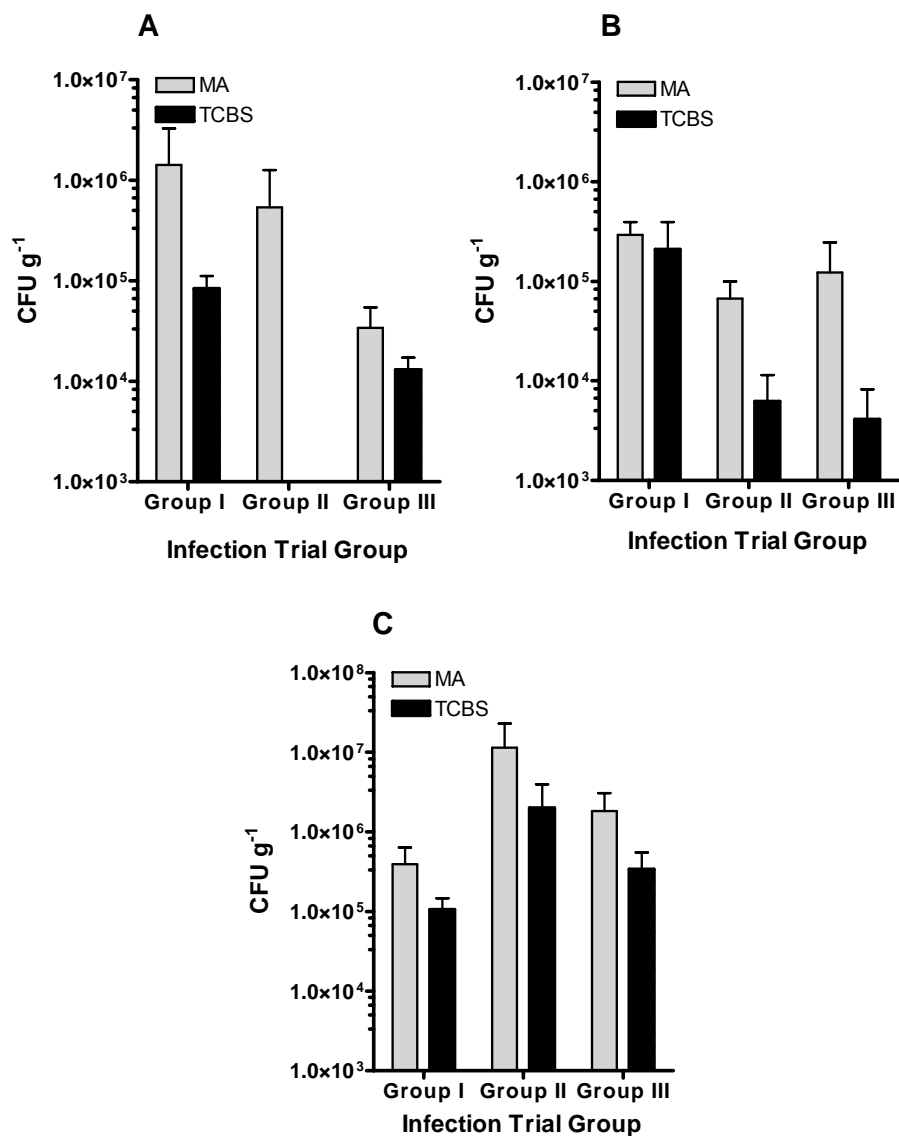
- H: site where a hole through the tail fan was produced using a sterile nail
- T: site where sterile scissors were used to cut a 5mm nick at the distal end of the tail fan in order to mimic tears produced when lobsters are caught and impounded.





**Figure 4.9: Percentage of tail fan tissue affected by TFN**

Data shown is the mean area of tissue affected by TFN lesions. Five lobsters from each experimental group were sampled at each of the three sampling times. Estimates were obtained at 4 weekly intervals over a 12 week period (14.05.04 to 28.07.04). Bar heights represent mean values, and the error bars represent the standard error about the mean (n = 5). Lobsters from Treatment Group III (tail fan tissue damaged with instruments inoculated with *V. parahaemolyticus* strain L21) demonstrated the greatest area of tail fan tissue affected by lesions. The extent of tail fans affected was greatest between week 4 and week 8 of the trial ( $P < 0.01$ ). However, there were no significant differences ( $P < 0.05$ ) between treatment groups for each sample point as per two-way ANOVA analysis. All statistical analyses were carried out using GraphPad Prism software.



**Figure 4.10: Counts of bacteria associated with tail fan tissue.**

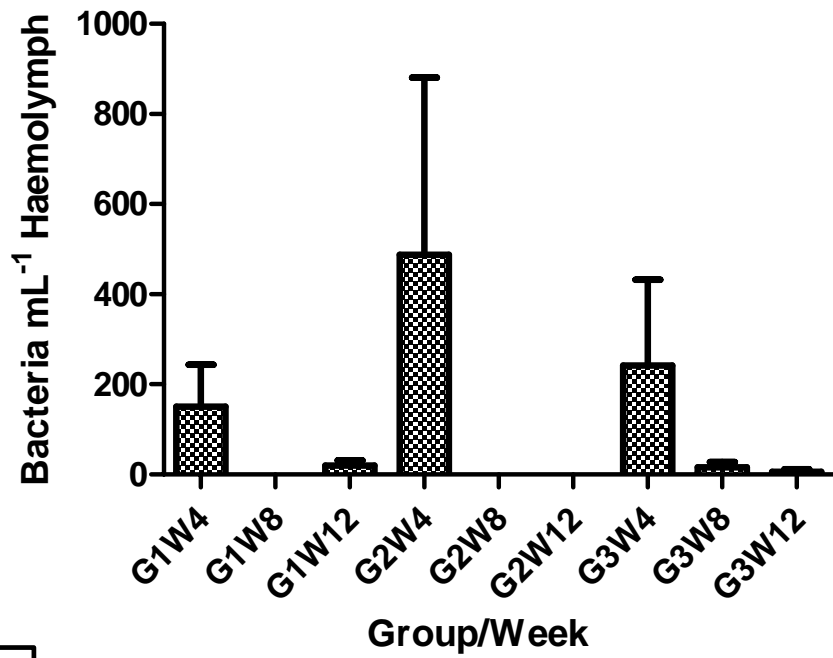
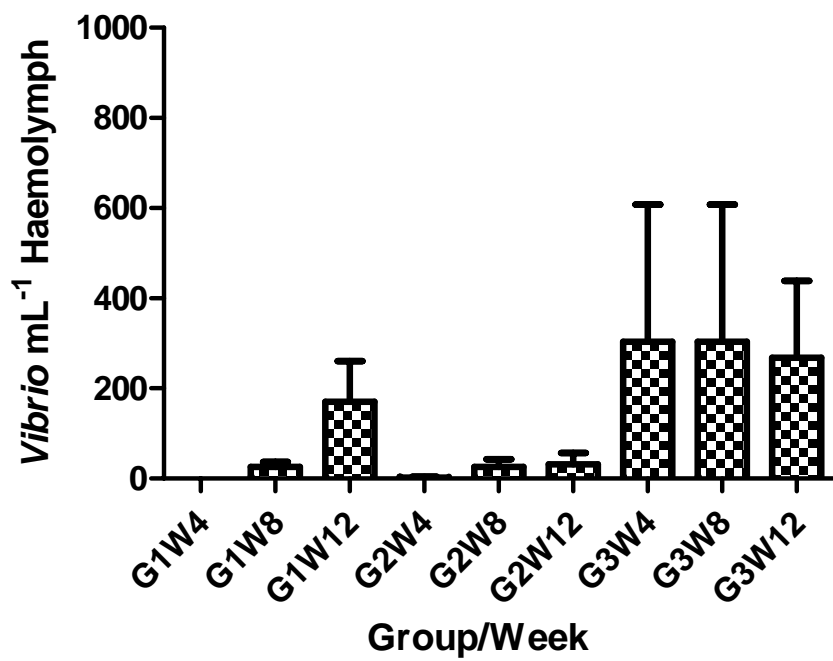
Data shown represents mean counts of bacteria associated with tail fan tissue for all groups of lobsters included in infection trial 1 that were sampled over a 12 week period (14.05.04 to 28.07.04). Five lobsters from each experimental group were sampled at each of the three sampling times. Error bars represent standard error about the mean values. No significant differences ( $P < 0.05$ ) in C.F.U. g<sup>-1</sup> on M.A. or T.C.B.S agars were observed for lobsters from any treatment group or sampling times, as determined by two-way ANOVA. All statistical analyses were carried out using GraphPad Prism software.

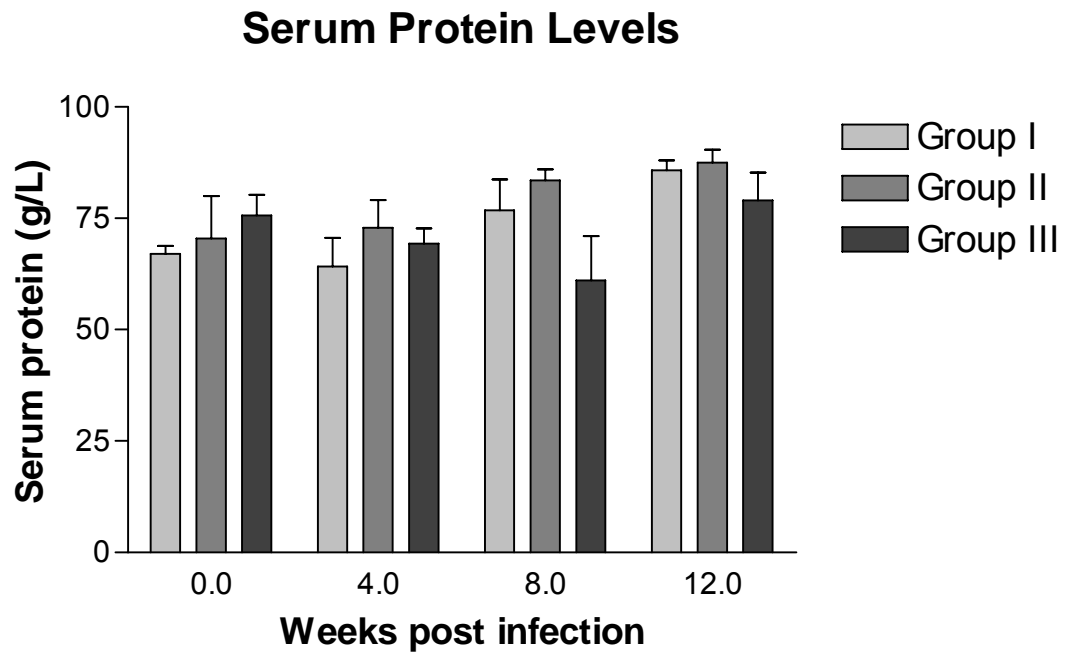
Panel A: 4 weeks post trial start  
Panel B: 8 weeks post trial start  
Panel C: 12 weeks post trial start

**Figure 11: Total culturable bacterial and Vibrio counts per mL of haemolymph.**

A: Total viable bacterial counts per mL of haemolymph for the infection trial for all groups of lobsters included in infection trial 1 over the period 14.05.04 to 28.07.04 as a standard mean ( $n = 5$ ). Error bars represent standard error. No significant differences ( $P < 0.05$ ) in C.F.U.  $g^{-1}$  on M.A. agar was observed for lobsters from any treatment group or sampling times, as determined by two-way ANOVA. All statistical analyses were carried out using Graphpad Prism software.

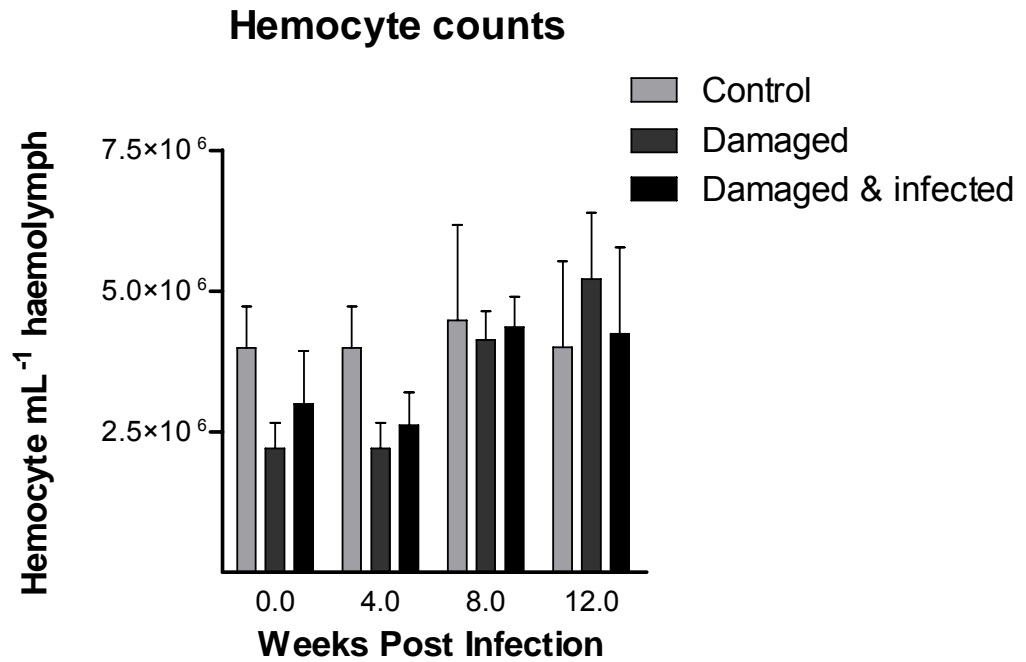
B: Total Vibrio counts per mL haemolymph for all groups of lobsters included in infection trial 1 over the period 14.05.04 to 28.07.04 as a standard mean ( $n = 5$ ). Error bars represent standard error. No significant differences ( $P < 0.05$ ) in C.F.U.  $mL^{-1}$  on T.C.B.S agar was observed for lobsters from any treatment group or sampling times, as determined by two-way ANOVA. Furthermore, there were no significant differences ( $P < 0.05$ ) in C.F.U.  $mL^{-1}$  between marine agar and TCBS, as determined by two-way ANOVA. All statistical analyses were carried out using GraphPad Prism software.

**A****B**



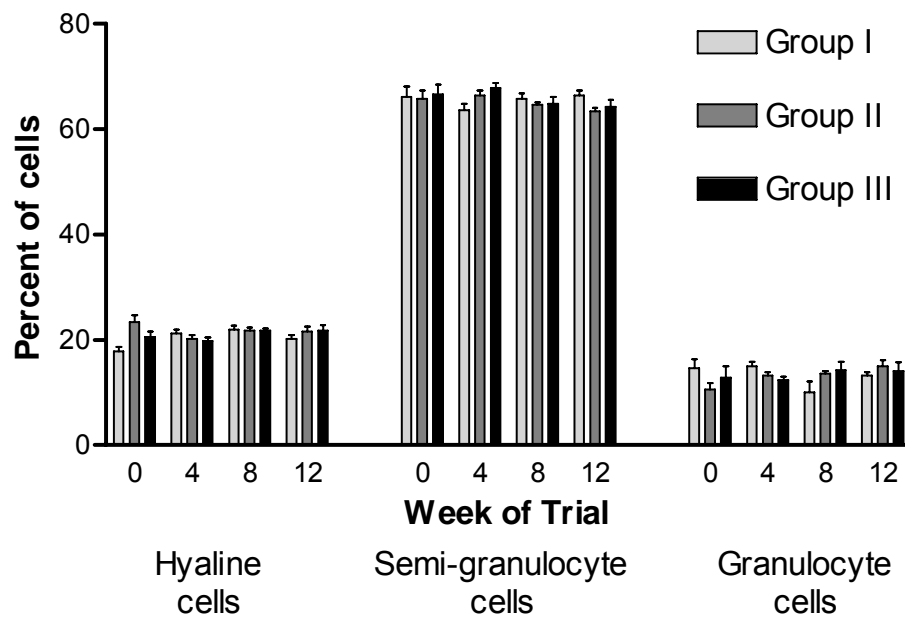
**Figure 4.12: Haemolymph serum protein levels for lobsters.**

Data shown represents mean serum protein levels for all groups of lobsters included in infection trial 1 over the period 14.05.04 to 28.07.04 as a standard mean. Five lobsters from each experimental group were sampled at each of the three sampling times.. Error bars represent standard error about the mean values. No significant differences ( $P < 0.05$ ) in haemolymph protein levels were observed for lobsters from any treatment group or sampling times, as determined by two-way ANOVA. All serum protein levels were on the upper end of the scale described by Leavitt and Bayer (1977). All statistical analyses were carried out using GraphPad Prism software.



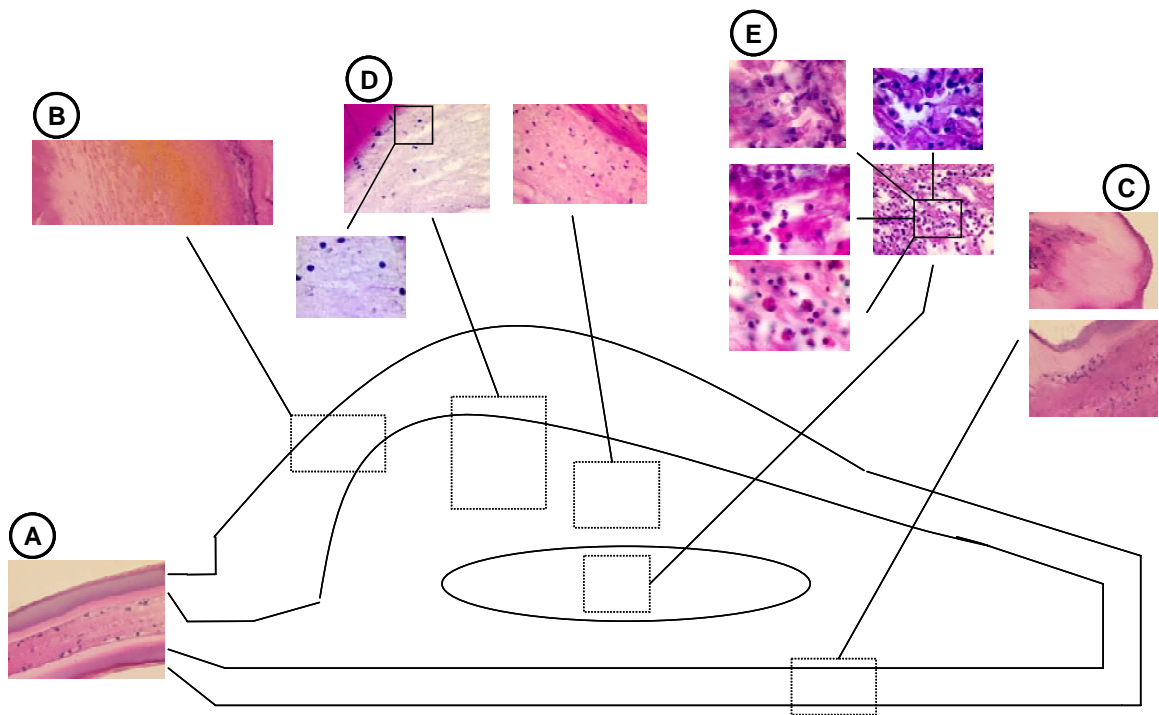
**Figure 4.13: Mean haemocyte counts in haemolymph of lobsters.**

Data represents mean haemocyte counts per mL of haemolymph ( $n = 5$ ) for lobsters included in infection trial 1 over the period 14.05.04 to 28.07.04. Error bars represent standard error about the mean values. For haemolymph samples from Group I (control) lobsters, the number of circulating haemocytes remained within the normal parameters set by Evans *et al.* (1998) and Stewart *et al.* (1969). There were no significant differences in haemocyte counts obtained at each sampling time for this group of lobsters. Haemocyte counts were also recorded for lobsters with tail fans that had been damaged with sterile instruments and for those that had tail fans damaged with instruments inoculated with *V. parahaemolyticus* strain L21. Haemocyte counts for lobsters from any treatment group at any stage of the trial were not significantly different ( $P < 0.05$ ), as determined by two-way ANOVA. All statistical analyses were carried out using GraphPad Prism software.



**Figure 4.14: Distribution of cell types in haemolymph of lobsters.**

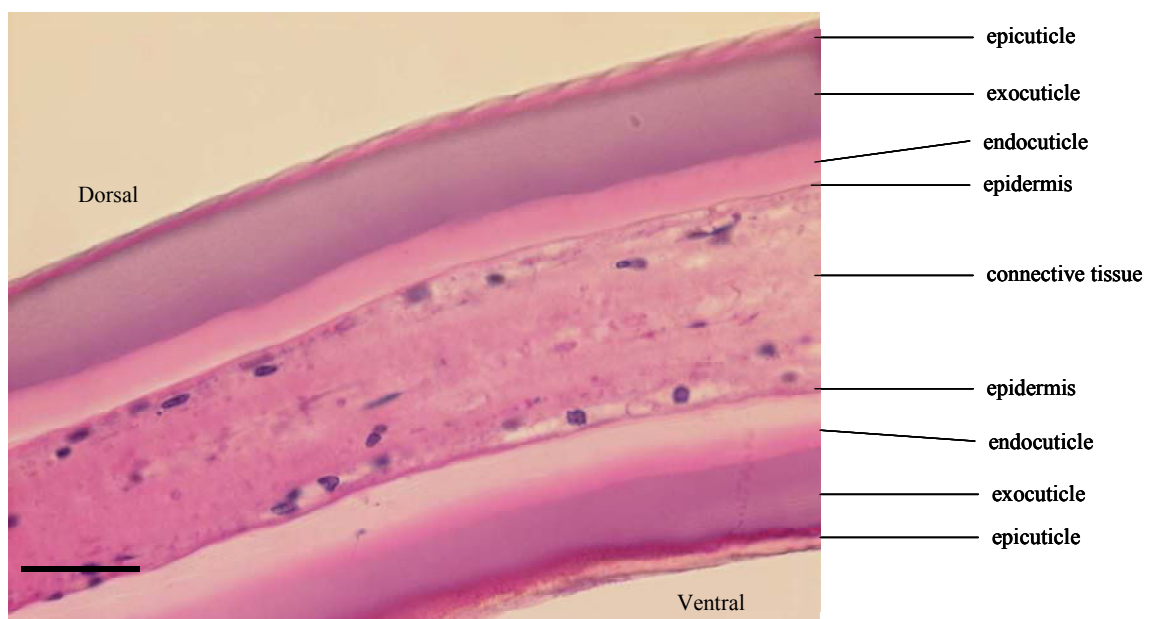
Data represents mean cell counts ( $n = 5$ ) for lobsters included in infection trial 1 over the period 14.05.04 to 28.07.04. Error bars represent standard error. There were no significant differences between time points for this group. Differential haemocyte counts for lobsters from any treatment group at any stage of the trail were not significantly different ( $P < 0.05$ ), as determined by two-way ANOVA. All statistical analyses were carried out using Graphpad Prism software.



**Figure 4.15: Histology of lobster tail fan affected by TFN.**

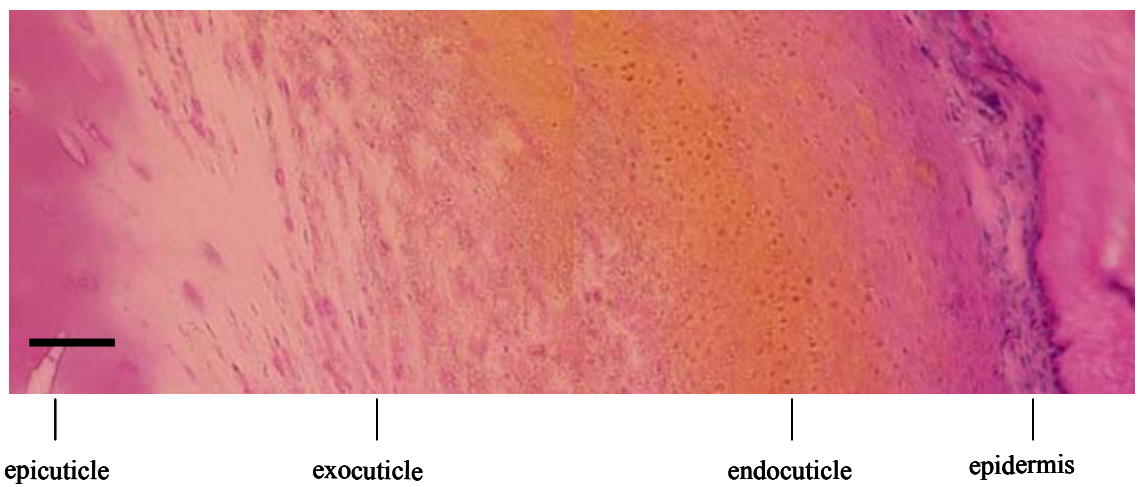
Schematic cross sectional view of a tail fan affected by TFN showing the tissue cell types associated with significant inflammation that often accompanies TFN. All micrographs of tissue sections were stained by Hemotoxylin and Eosin. Note the various regions displaying different features, ie healthy tissue adjacent to the lesion (A); the dorsal cuticle is inflamed (B); the ventral cuticle loses structure (C); there is a build up of fibrous material underlying this (D); in severe inflammation there is also a mass infiltration of various cell types (E). All sections were stained with Haemotoxylin and Eosin.





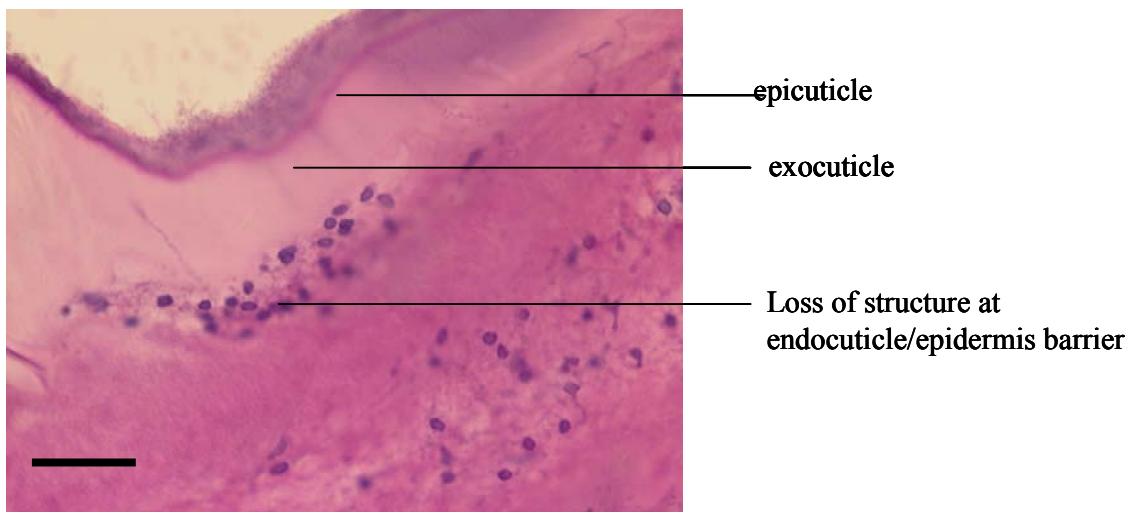
**Figure 4.16: Histology of normal tail fan tissue.**

Section stained with Haematoxylin and Eosin (H and E). Note the defined layers of the epicuticle, exocuticle, endocuticle and epidermis with a thin layer of cell-free connective tissue between the ventral and dorsal sides. The tissue was stained with Haematoxylin and Eosin. Scale bar denotes 200  $\mu\text{m}$ .



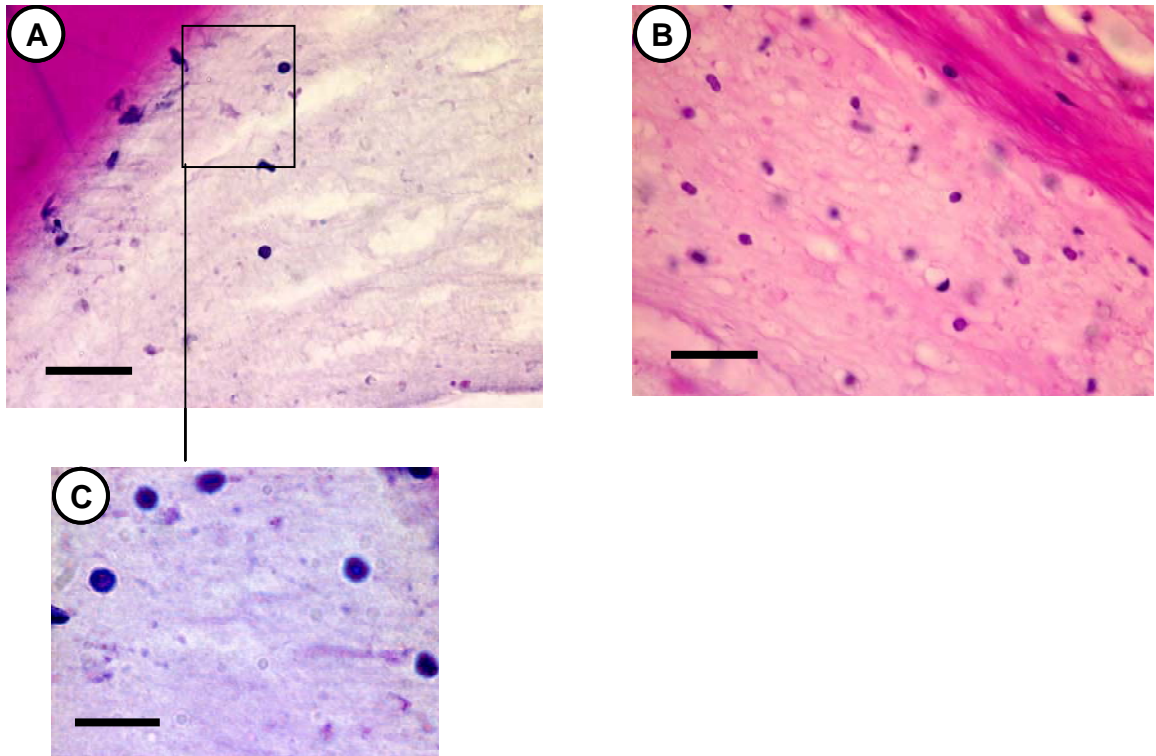
**Figure 4.17: Histology of the dorsal cuticle of a TFN lesion.**

Section stained with Hamotoxylin and Eosin. Note the associated inflammation of each of the layers of the cuticle compared to the normal healthy tail fan tissue in Figure 4.11, as well as the blurred boundaries between the respective layers of the cuticle. The tissue was stained with Haemotoxylin and Eosin. Scale bar denotes 200  $\mu$ m.



**Figure 4.18: Histology of the ventral cuticle of a TFN lesion**

Section stained with Hamotoxylin and Eosin. Note the loss of internal structure of the cuticle. Once again, note the indistinct boundary between the exocuticle and the endocuticle, as well as the endocuticle and epidermis. The tissue was stained with Haemotoxylin and Eosin. Scale bar denotes 200  $\mu\text{m}$ .

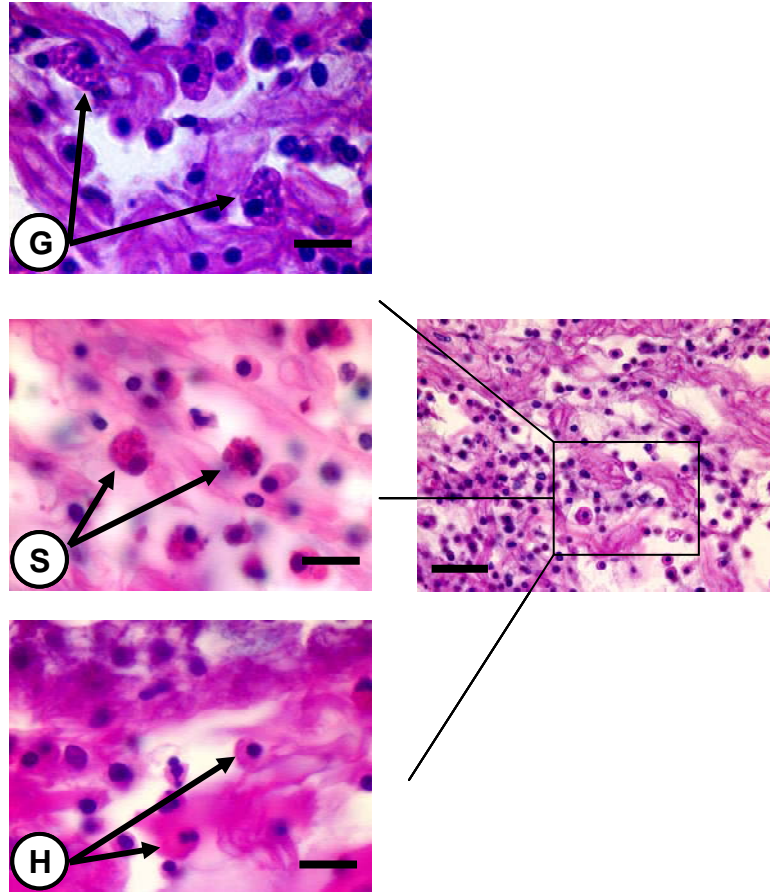


**Figure 4.19: Histology of tail fan connective tissue affected by inflammation.**

Light micrograph of stained sections of tail fans affected by TFN that display significant inflammation. All tissues were stained with Haematoxylin and Eosin.

Panels A & B: Sections stained with Haematoxylin and Eosin. Scale bars denote 100  $\mu\text{m}$ .

Panel C: Fibrous tissue typical of inflamed tissue interspersed with globules that may represent either calcium deposits or eosinophil-like cells. Scale bar denotes 25  $\mu\text{m}$ .



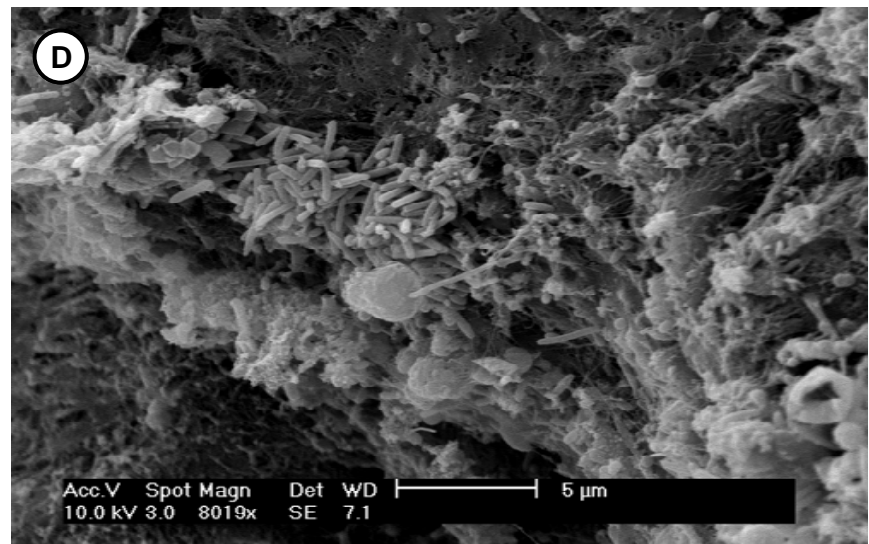
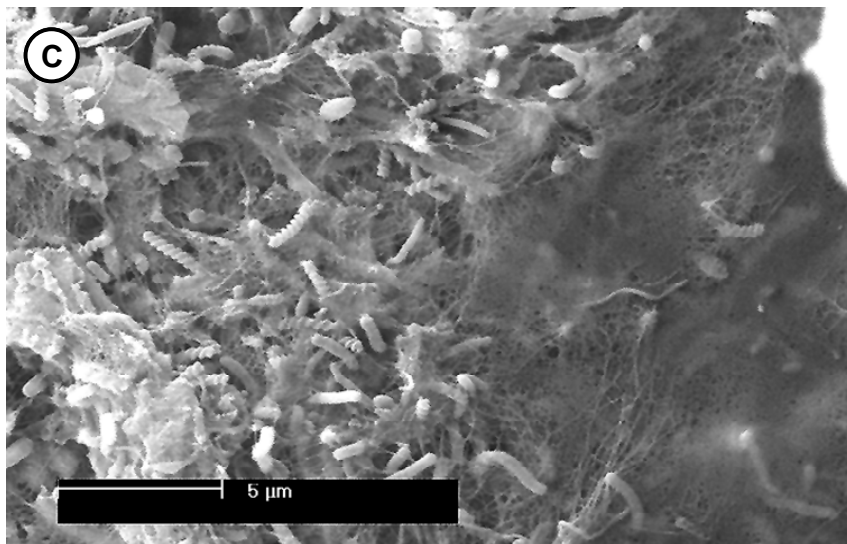
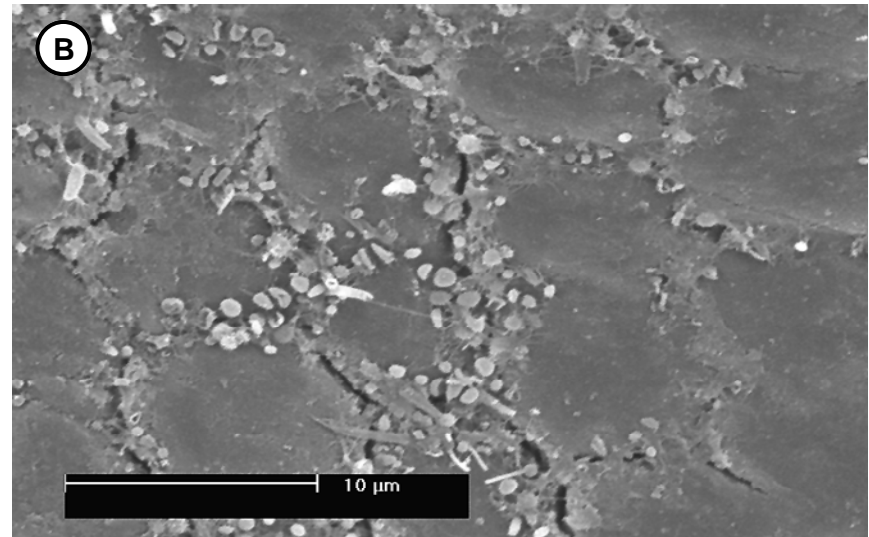
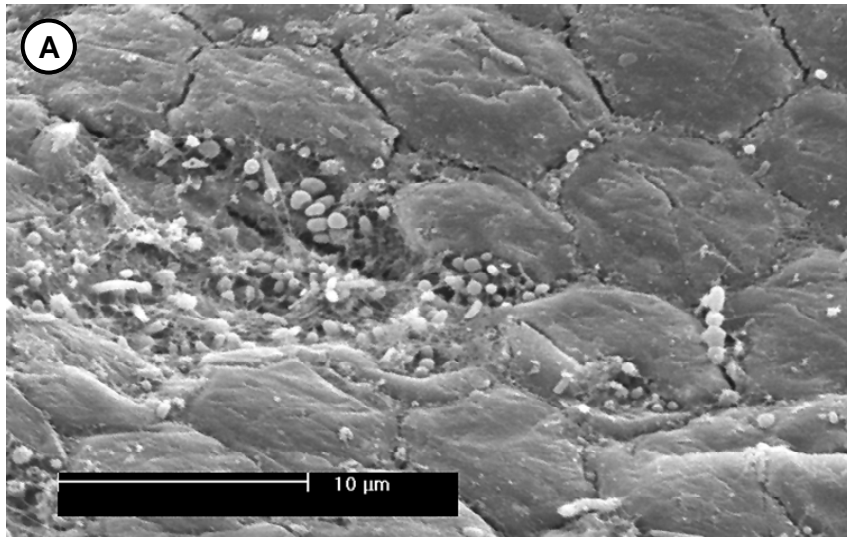
**Figure 4.20: Haemocytes within inflamed tissue associated with TFN.**

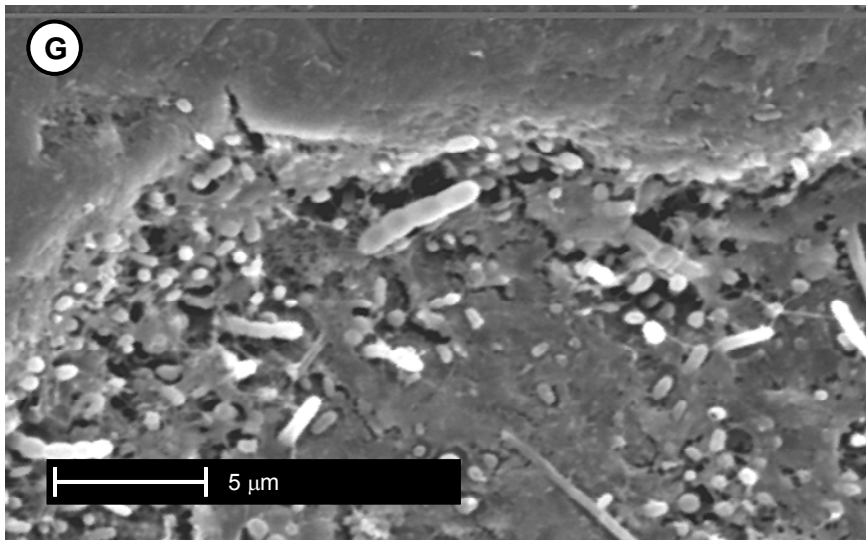
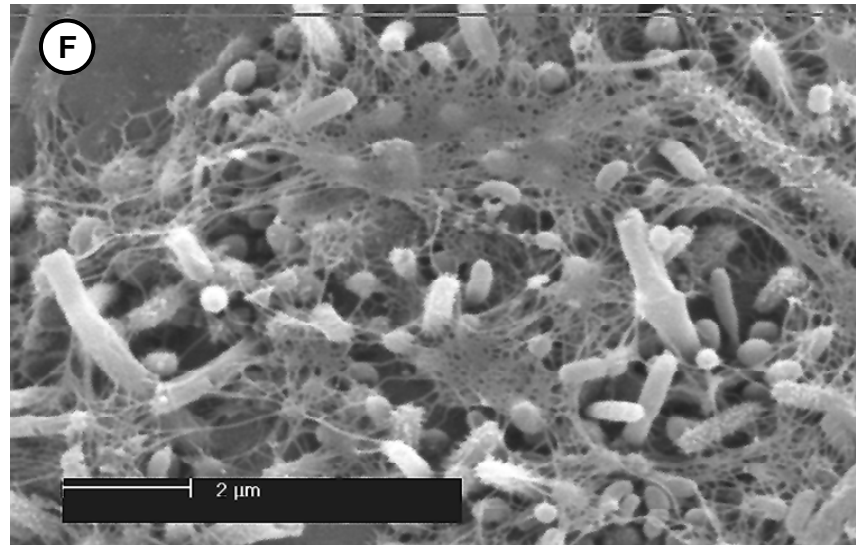
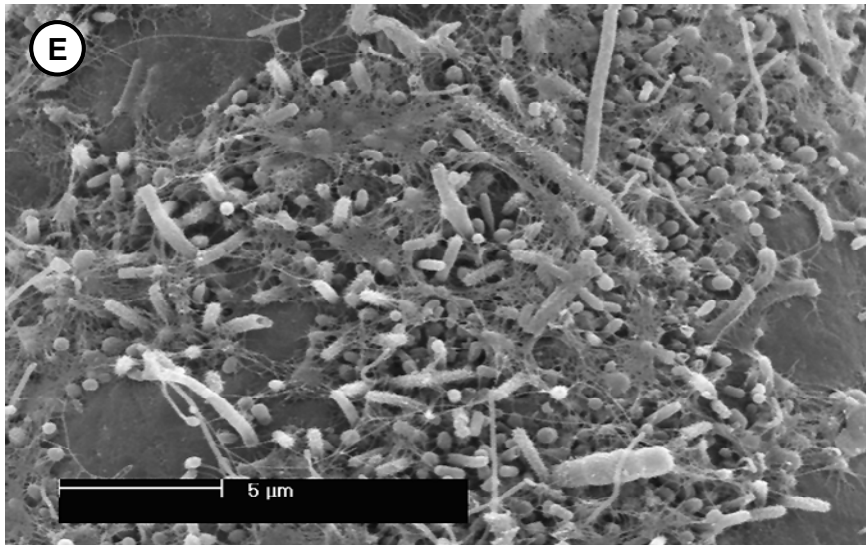
Inflamed tissue contained an accumulation of haemocytes and fibrocytes. These cells typically formed a central “core” within connective tissue associated with uropod tissue that showed moderate to severe inflammation. Note the presence of granulocytes (G), semi-granulocytes (S) and hyaline cells (H). Tissue was stained with haematoxylin and Eosin.

Scale bars in the left hand panels denote 25  $\mu\text{m}$  and the scale bar in the right hand panel denotes 100  $\mu\text{m}$ .

**Figure 4.21: SEM micrographs of tissue associated with TFN lesions.**

- Panel A: Surface of tail fan from a Group I (control) lobster, at the commencement of the infection trial. There were only sporadic patches of bacteria on the surface of uropods, generally associated with micro-colonies.
- Panel B: Surface of tail fan from a Group I (control) lobster, 4 weeks after the commencement of the infection trial.
- Panel C: Surface of tail fan from a Group III (damaged plus infected) lobster, 8 weeks after the commencement of the infection trial. Note the various cellular morphologies of the bacteria associated with the tissue
- Panel D: Surface of tail fan from a Group III (damaged plus infected) lobster, 8 weeks after the commencement of the infection trial. Note the formation of a micro-colony by the bacteria with few bacteria associated with the surrounding tissue.
- Panel E: Surface of tail fan from a Group III (damaged plus infected) lobster, 12 weeks after the commencement of the infection trial. By this stage the micro-colonies had increased in size and the bacteria appear to be burrowing into the tissue.
- Panel F: Panel F shows a higher magnification of Panel E.
- Panel G: Surface of tail fan from a Group III (damaged plus infected) lobster, 12 weeks after the commencement of the infection trial. This panel shows the leading edge of the lesion with bacteria eroding the surrounding tissue and “burrowing” into the surface, as described by May (2002).

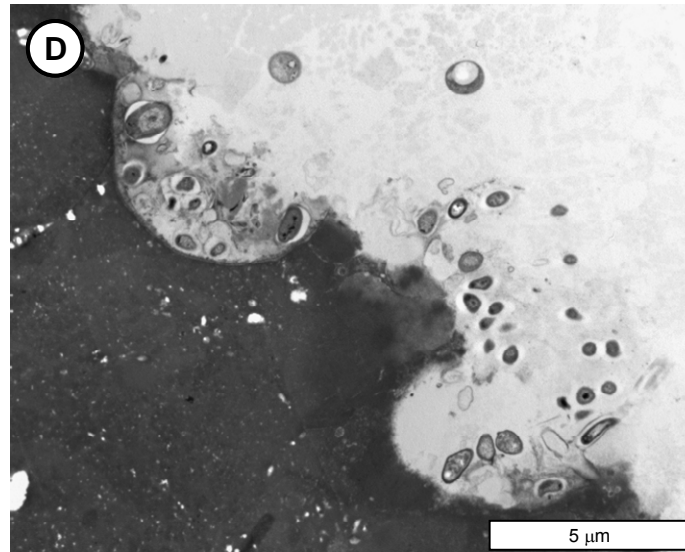
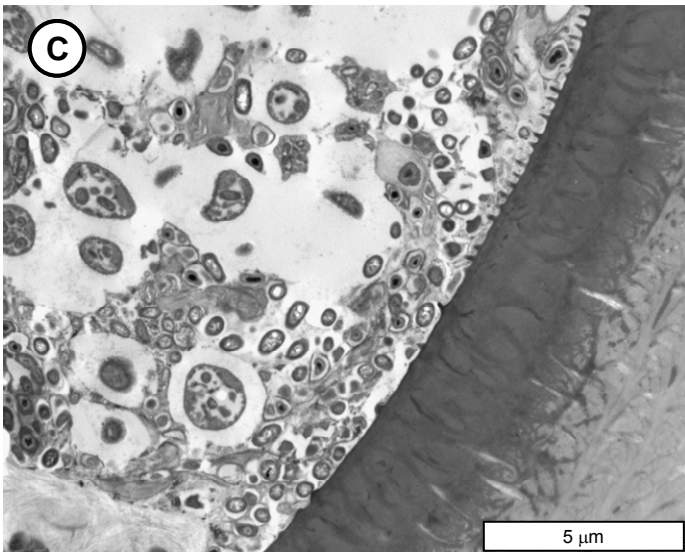
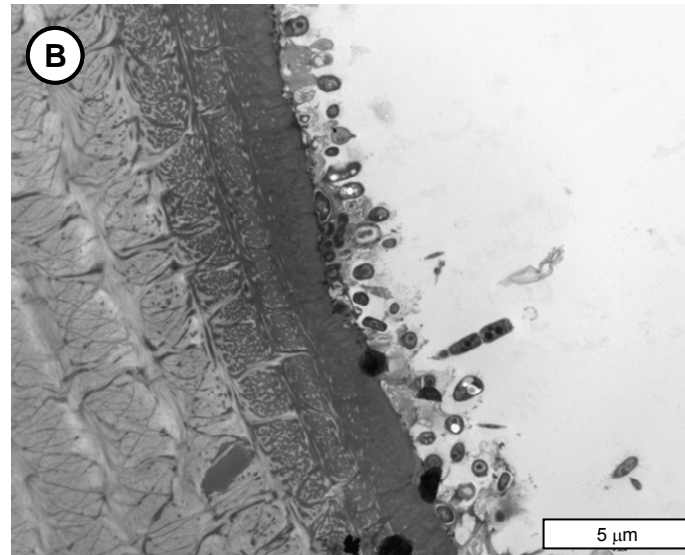
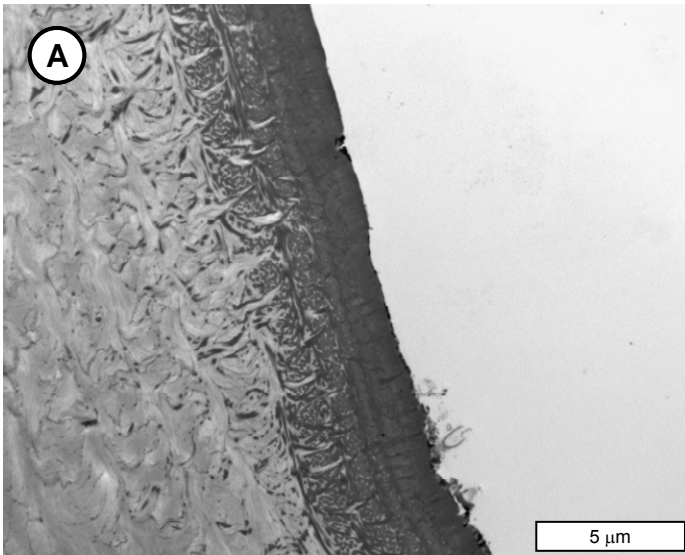


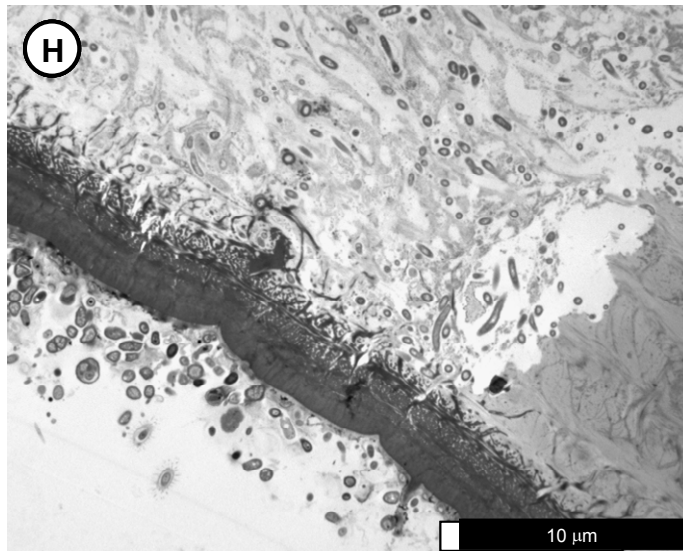
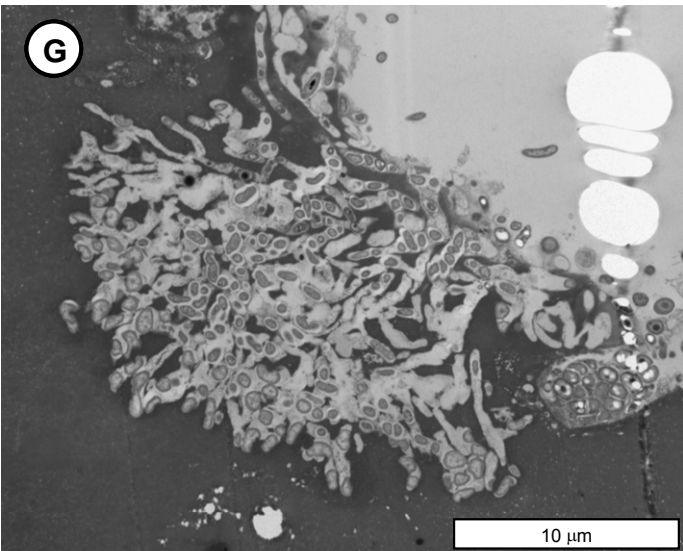
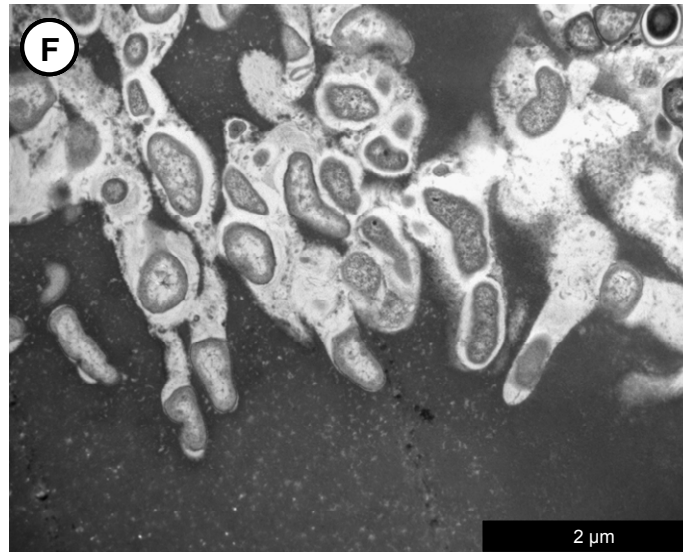
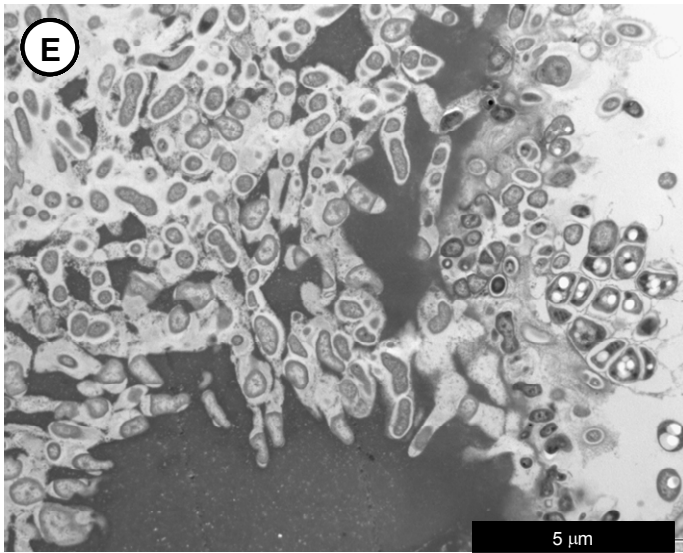


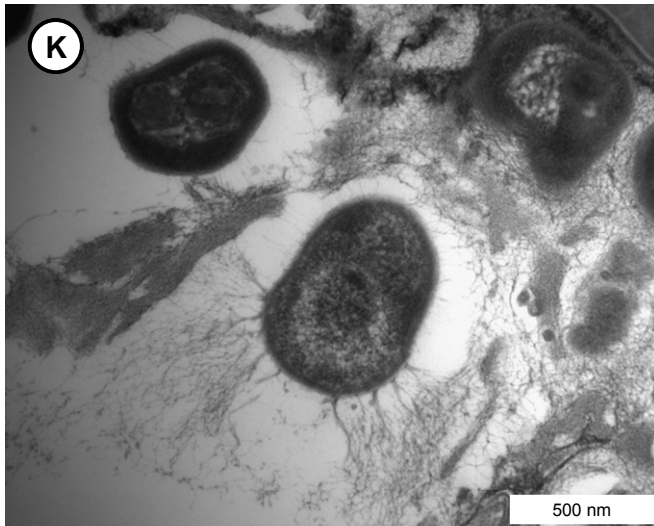
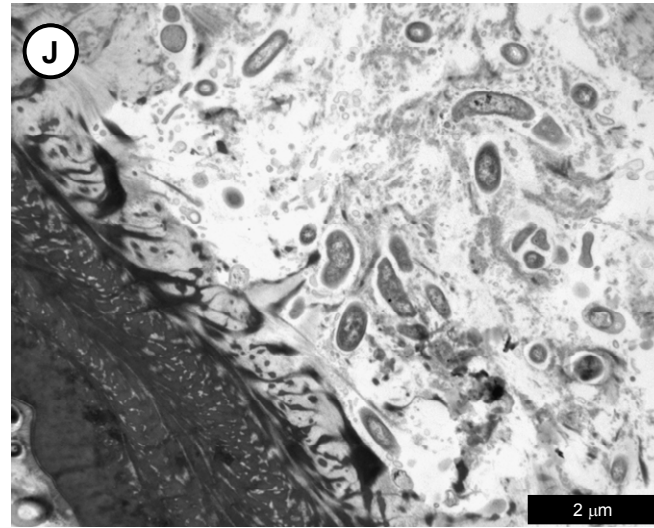
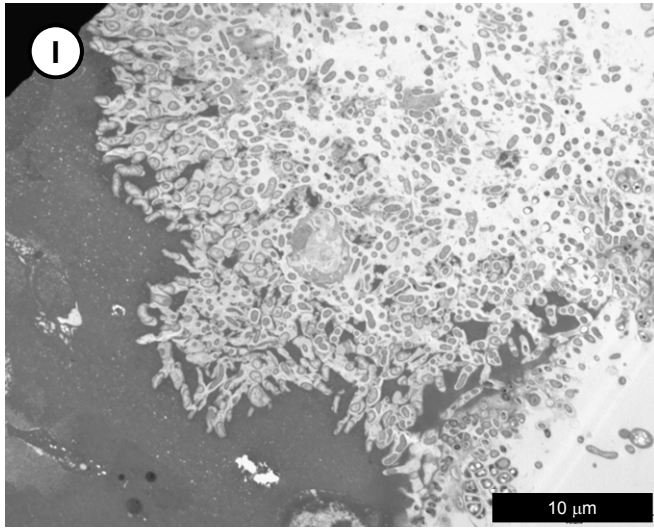


**Figure 4.22: TEM micrographs of sections of tissue associated with TFN lesions.**

- Panel A: Section of tail fan surface of a sample from a Group I (control) treatment lobster after holding for 4 weeks. Note the absence of detritus.
- Panel B: Section of tail fan surface of a sample from a Group I (control) treatment lobster after holding for 8 weeks. Note the fouling of the tail fan surface and bacterial cells.
- Panel C: Section of tail fan surface of a sample from a Group I (control) treatment lobster after holding for 12 weeks. Note the extensive fouling of the tail fan surface by bacterial cells.
- Panel D: Section of tail fan surface of a sample from a Group III (damage plus infection with *V. parahaemolyticus* L21) treatment lobster after holding for 4 weeks post infection. Note the extensive degradation of the tail fan tissue surface and presence of bacterial cells within pits of degraded tissue.
- Panel E: As for Panel D, except that bacteria in these panels are degrading the tissue at the leading edge of the lesion.
- Panel F: Higher magnification of Panel E showing the bacteria eroding the tissue creating pits
- Panel G: Section of tail fan surface of a sample from a treatment lobster after holding for 4 weeks post infection. This panel shows the typical micro-colony formation seen at this stage of the infection trial on the surface of tail fans of lobsters in this Group III (damage plus infection with *V. parahaemolyticus* L21) after 4 weeks post infection.
- Panels H – J: Section of tail fan tissue from Group III damage plus infection with *V. parahaemolyticus* L21) treatment lobster after holding for 12 weeks post infection. Note that the hard epicuticle remains intact, while the underlying exocuticle and epicuticle are eroded.
- Panel K: Bacterial cell embedded in carapace surface associated material. Note the strands of stained material connecting the cell to the carapace surface.







## Chapter 5: Bacteriology and Molecular Characterisation of Artificially Induced TFN lesions

### 5.1 Introduction

A previous study by May (2002) provided evidence that *Vibrio* species are the major component of culturable bacterial microflora of lesions associated with TFN of live-held southern rock lobsters in experimental sea cages. The identity and pathogenicity trait(s) of isolates recovered from lesions was confirmed by data presented in Chapter 3: of this thesis. Although that work provided experimental evidence to implicate *Vibrio* spp. in TFN, the bacterial population within lesions was only assessed at the completion of the eight-week infection trial used in the study and therefore did not account for any dynamic changes in microflora composition or size. May (2002) only focused on the total culturable bacterial population and as such missed potentially important components of the microbial community associated with developing lesions. Other studies of crustacean microbiology have established *Vibrio* spp. as an important part of the microflora of shell diseases. For example, Porter *et al.* (2001) showed marine vibrios were dominant in the microflora of the carapace of healthy and shell disease affected *Panulirus argus*. However, this study showed there were a number of other bacterial species present that were not detected using standard culture techniques (eg.  $\gamma$ -*Proteobacteria*). Furthermore, Christoserdov *et al.* (2005) have some preliminary results to suggest that there are two common groups of bacteria present in association with epizootic shell disease lesions; a species complex belonging to the family *Flavobacteriaceae* and a series of closely related, if not identical, strains of *Pseudoalteromonas gracilis*. However, these authors concede that challenge experiments are required to fully elucidate the role of these bacteria in the onset and progression of epizootic shell disease.

Since there is no published information available that describes the range of types of bacteria present in developing shell lesions, the aim of this chapter was to identify changes in the types of bacteria present in both the culturable and non-culturable microbial community of lesions during development of TFN on the tail fans of rock lobsters

subjected to simultaneous damage and infection with a *Vibrio* spp. originally isolated from TFN lesion tissue.

## 5.2 Experimental Design

Randomly selected *Vibrio* isolates recovered from all treatment groups of experimental animals described in Chapter 4:, were identified using biochemical keys as described by Alsina & Blanch (1994) (see Appendix G). *V. parahaemolyticus* isolates were confirmed by PCR amplification of part of the *tlh* gene, and *V. vulnificus* isolates were confirmed by amplification of part of the *vvh* gene. To assess the public health risk posed to consumers by lobsters affected by TFN affected tail fans, presence of genotypic (*tdh* and *trh* genes) and phenotypic traits of *V. parahaemolyticus* and *V. vulnificus* isolates consistent with a pathogenic phenotype were also assessed (Baffone *et al.*, 2004, Oliver, 1986).

To independently confirm the types of bacteria that comprise the microflora of TFN lesions, two culture independent methods that rely on the nucleotide sequence of DNA encoding 16S rRNA were employed. Firstly, PCR was used to amplify DNA encoding 16S rDNA from DNA template extracted from TFN lesion tissue by using generic oligonucleotide primers based on conserved 16S rDNA sequences across all bacterial genera. The amplified products were then cloned and randomly selected clones sequenced. Sequence data obtained was then used to identify bacterial genera present within lesions by identification to the genus level based on sequence similarity using BLASTn and by comparison to known 16S rDNA sequences in the Ribosomal Database Project II website (<http://rdp.cme.msu.edu/>).

Secondly, denaturing gradient gel electrophoresis (DGGE) of GC-clamped PCR fragments amplified from DNA encoding 16S rDNA was employed to investigate the range of types of bacteria associated with lesions. This method allows discrimination of amplicons that differ by as little as 1 or 2 nucleotide bases. When combined with sequence analysis of separated fragments, this method represents a powerful tool for investigating the species diversity of microbial communities. Furthermore, this approach minimises the probability that particular species are missed by sequencing randomly selected 16S rDNA clones.

## 5.3 Results

### 5.3.1 Identification of *Vibrio* Isolates recovered from TFN Lesions

Data presented in Chapter 4 provided good evidence that there is a large population of vibrios associated with healthy tail fan tissue and tissue from TFN lesions. In this section, the identity of *Vibrio* spp. present in lesion tissues obtained from lobsters included in the infection trial is described in order to identify differences between the microflora of the different treatment groups and potentially, the main types of bacteria involved in lesion development.

Initially, the identity to the species level for all *Vibrio* isolates recovered from lobster tail fan tissue was attempted using the Microbact 24E Identification System as per Myatt *et al.* (1992). However, this system proved to be unreliable for a number of reasons *viz*:

1. Type strains that were included in the analysis were mis-identified by the system.
2. Inconsistent results were obtained when single isolates were put through the system on multiple occasions.
3. The key amino-acid decarboxylase tests were unreliable.
4. Consistent mis-identification of *Vibrio* spp. as belonging to other genera.

Data obtained from these kits was to have been used to assist phylogenetic analysis based on the biochemical profiles obtained. However, as the results obtained from these kits were not reliable, this was not possible. Consequently, the keys outlined by Alsina & Blanch (1994) were used to identify all *Vibrio* isolates from lobster tail fan tissue. All tests required for the identification of individual isolates were carried out as per Murray *et al.* (1984). As different tests were required for the identification of individual isolates, it was not possible to use the data obtained from this process to undertake phylogenetic analysis based on biochemical characteristics as initially planned.

Table 5.1 lists the principal types of vibrios associated with lobster tissues obtained throughout the entire infection trial described in Chapter 4 (See also Appendix F, Page 225 for a full list of identified isolates). *V. alginolyticus* (45%) was the dominant species isolated from tail fan tissue, while *V. parahaemolyticus* isolates comprised 30% of the microflora, *V. vulnificus* comprised 9%, and other *Vibrio* species accounted for the remaining 16%.

However, when the microflora of lesions from individual treatment groups was assessed, different trends were identified (Table 5.1). The microflora of tail fan carapace tissue from control group (Group I) lobsters, was dominated by *V. alginolyticus*. This species comprised 59% of recovered isolates, whereas *V. parahaemolyticus* and *V. vulnificus* accounted for 12% and 3% of the isolates respectively.

By contrast, the microflora of tail fan tissue from lobsters that had tail fans damaged with sterile instruments (Group II) comprised a lower proportion of *V. alginolyticus* isolates (46% vs 59%). The proportion of *V. vulnificus* isolates recovered from lesions within this treatment group was 23% compared to 3% in the control group (Group I) and 0% for tail fan tissue from lobsters simultaneously damaged and infected with *V. parahaemolyticus* (Group III treatment group). Isolates identified as *V. parahaemolyticus* represented 8% of the *Vibrio* spp. isolated from the Group II treatment group tail fan tissue samples, with a range of other species comprising the remaining 23% of isolates.

As expected, of *Vibrio* spp. isolated from TFN lesions from the Group III lobsters that had tail fans artificially infected with *V. parahaemolyticus* strain L21, 70% of isolates were identified as *V. parahaemolyticus*. Very little species diversity occurred within the lesions sampled from this group; *V. alginolyticus* isolates comprised 30% of all isolates and was the only other type of vibrio isolated at any stage of the infection trial.

### **5.3.2 Molecular Characterisation of *Vibrio* spp.**

Data presented in Chapter 3 of this thesis indicated that vibrios recovered by May (2002) from lesions associated with TFN are potentially pathogenic and hence may present a particular health hazard to consumers if live-holding of lobsters is to proceed. This work was repeated for isolates recovered from the infection trial described in Chapter 4 of this thesis. In particular, isolates of *V. parahaemolyticus* and *V. vulnificus* were screened for genotypic and phenotypic characteristics linked to virulence.

All *V. parahaemolyticus* and *V. vulnificus* isolates were confirmed by PCR. DNA from isolates identified as *V. vulnificus* were PCR positive for a 205bp *vvh* amplicon when the oligonucleotide primer pair F-*vvh* and R-*vvh* was used to prime PCR (Figure 5.1). Similarly, DNA from all isolates identified as *V. parahaemolyticus* in this study, was PCR



positive for a 450 bp *tlh* amplicon when the oligonucleotide primer pair, *tlf* and *tlr*, was used (Figure 5.2). This data provided confirmation that these isolates were all correctly identified using the methods described by Alsina & Blanch (1994).

Table 5.2 lists the key phenotypic characteristics of the representative isolates selected for testing. Of the 27 *V. parahaemolyticus* isolates recovered during this trial, 100% expressed lipase, 89% expressed lecithinase, 100% expressed protease, and 39% expressed urease. Furthermore, 56% of these isolates were cytotoxic to cultured CHO cells. Interestingly, none of the *V. parahaemolyticus* isolates were positive for the *tdh* gene as tested by PCR (Figure 5.3), although 41% of these isolates were *trh*<sup>+</sup> (Figure 5.4) (Appendix G, Page 234). Furthermore, 100% of the *trh*<sup>+</sup> isolates were cytotoxic to cultured CHO cells, whereas only 17% of the *trh*<sup>-</sup> isolates were cytotoxic to cultured CHO cells.

Of the 8 *V. vulnificus* isolates tested, none expressed lipase, 87% expressed lecithinase, and 100% expressed both protease and urease. Surprisingly, none of these isolates were cytotoxic to cultured CHO cells. All of the *V. vulnificus* isolates recovered from tail fan tissue during the infection trial expressed a translucent colony morphology and hence would not be expected to express a capsule.

All 41 *V. alginolyticus* isolates expressed lipase and protease, but only 24% expressed lecithinase, 7% expressed urease and 25% were cytotoxic to CHO cells. Of the remaining *Vibrio* spp. isolated, 61% expressed lipase, 30% expressed lecithinase, all expressed protease, 21% expressed urease, and only 4% were cytotoxic to cultured CHO cells, including one *V. mimicus* isolate.

Expression of extracellular chitinase was also assessed for these isolates to identify potential candidates capable of destroying the lobster carapace tissue. All of the isolates that were identified as either *V. parahaemolyticus*, *V. vulnificus* or *V. alginolyticus* expressed extracellular chitinase. However, only 58% of the remaining *Vibrio* isolates expressed this enzyme.

### 5.3.3 Analysis of 16S rDNA amplified from Lesion Tissue DNA

Based on work described by May (2002), the microbiological analysis of TFN lesions described in this thesis was biased to examination of culturable bacteria, and in particular, marine vibrios. However, the microflora analysis alone does not explain the variety of morphological cell types of bacteria identified by electron microscopy of lesion tissue (See Chapter 4.4.8 and 4.4.9). Those observations indicated a range of different bacteria may have colonized this tissue and therefore a diverse microbial population may be present throughout the development of TFN associated lesions. To unequivocally confirm that marine vibrios are the major species of bacteria associated with lesions, methods that allowed analysis of gene(s) encoding 16S rRNA were deployed. Sequence analysis of DNA encoding 16S rDNA has long been regarded as a key approach to phylogenetic analysis of complex communities, and as a means of molecular identification of bacteria to genus level (Staley, 2006). As mentioned in Section 5.2 of this chapter, two independent methods for analysis of 16S rDNA amplicons were used. Both methods relied on recovery of DNA from TFN lesions and PCR amplification of DNA encoding 16S rRNA gene using generic oligonucleotide primers. The amplified DNA was then either cloned and randomly selected clones sequenced, or the GC-clamped amplicons were subjected to DGGE analysis, followed by sequencing of separated heterogeneous amplicons.

#### 5.3.3.1 Analysis of 16S rDNA clones

DNA isolated from lesion tissue using the method described in Chapter 2.6.2 was used as a template for PCR reactions that used generic primers (27f and 519r) specific for DNA encoding a region of the 16S rRNA gene. PCR products obtained were purified to remove any unincorporated nucleotides and cloned by ligation of the purified PCR product to pGEMT-TA cloning vector. Cloned DNA was used to transform chemically competent *E. coli* DH5 $\alpha$  cells. Transformed cells were then plated on NA containing IPTG, X-Gal and ampicillin. White, ampicillin resistant transformants containing plasmid vector with insert DNA were selected and stored as glycerol cultures at -70°C. DNA inserts in plasmids extracted from randomly selected transformants were sequenced using the M13 forward and reverse primers. Nucleotide sequence data obtained was compared with other nucleotide sequences in the GenBank Nucleotide Database by BLASTn analysis and by

comparison of the sequence data with 16S rDNA sequences in the Ribosomal RNA Project II Database. The Ribosomal Database Project II classifier was used to assign 16S rRNA sequences to the taxonomical hierarchy proposed in release 6.0 of the nomenclatural taxonomy of Garrity and Lilburn (see Garrity *et al.*, 2004). Hierarchical taxa used by this online resource are based on a naïve Bayesian rRNA classifier. This trained classifier allows classification of both bacterial and archaeal 16S rRNA sequences.

Analysis of the 16S rDNA nucleotide sequences obtained for 90 randomly selected transformants, indicated that the majority of sequences (65) were similar (95 to 100% over 502 nucleotide residues) to, or identical to, 16S rDNA nucleotide sequences for known *Vibrio* spp. However, 8 sequences obtained were 98% similar (over 502 nucleotide residues) to sequences for uncultured Vibrionaceae. Interestingly, a number of clones also possessed 99% nucleotide sequence similarity with marine *Psychrobacter* spp. Table 5.3A presents a summary of the genus assignments of 16S rDNA sequences by treatment group. Sequences typical of *Vibrio* spp. are predominant for samples from all three treatment groups. Notably, sequences typical of *Psychrobacter* spp. were most often detected in tissue samples harvested from lobsters in the control treatment group (Group I). When treatment group specific libraries of the sequence data were compared using the Ribosomal Database Project II Library Compare tool to determine whether the likelihood that the frequency of membership in a given taxon is the same for any of the treatment groups, no significant differences ( $p < 0.05$ ) were apparent for *Vibrio* spp. for any treatment group pair analysed. However, a significant difference ( $p < 0.001$ ) in the frequency of sequences typical of *Psychrobacter* spp. in Group I samples compared with other treatment group (Group II and III) samples was obtained. For sequences from any one treatment group, the number of sequences assigned to either *Vibrio* spp. or *Psychrobacter* spp. from each of the sampling times (4, 8 or 12 weeks) was essentially the same. Nevertheless, although the sample size of sequences compared is relatively small, the analysis does suggest that the microflora of control lobster (Group I) tail fan carapace is altered by damage with sterile tools (Group II) or tools intentionally contaminated with *V. parahaemolyticus* L21 (Group III).

Phylogenetic and molecular evolutionary analysis of the sequence data was conducted using MEGA version 3.1 (Kumar *et al.*, 2004). Reference sequences obtained from the

GenBank nucleotide database for *V. vulnificus* (4), *V. parahaemolyticus* (4), *V. alginolyticus* (4), *V. harveyi* (3), *V. campbellii* (1), *V. cholerae* (2) and *V. fischeri* (1) were included in the analysis. A boot-strapped phylogenetic tree was constructed from aligned sequence data using a UPGMA method and a Kimura 2-parameter nucleotide model. A compressed version of the tree is shown in Figure 5.5. Groups of sequence were arbitrarily identified by collapsing branches with distances  $\leq 0.01$  units. All the *Vibrio* sequences grouped within two main clusters (*Vibrio* Groups A and B), with the *Psychrobacter* and other Vibrionaceae sequences forming other distinct clusters. Similarly, *V. cholerae* and *V. fischeri* sequences formed distinct clusters. Interestingly *Vibrio* Group B sequences comprised only Treatment Group III sequences (14 of 30 sequences), those of *V. vulnificus*, *V. parahaemolyticus*, *V. harveyi* and *V. campbellii* reference sequences and the infecting strain *V. parahaemolyticus* L21. This suggested that pathogenic strains of vibrios predominated Treatment Group III tissues; a result consistent with the species analysis described in Section 5.3.1 and the molecular characterization studies described in Section 5.3.2. By contrast *Vibrio* Group A sequences comprised the majority of sequences as well as *V. alginolyticus* reference sequences. This data suggested that most isolates associated with lobster tail fan surfaces may be similar to *V. alginolyticus*, a result consistent with the species analysis described in Section 5.3.1 of this Chapter.

#### 5.3.3.2 DDGE Analysis of 16S rDNA Amplicons

DNA was extracted from lesion tissue samples taken from lobsters 4, 8 and 12 weeks after commencement of the infection trial using the method described in Chapter 2.6.2. Purified DNA was used as template for PCR reactions that used generic primers (27f and 519r) specifically designed to allow amplification of a 492 bp region of the gene(s) encoding 16S rRNA. PCR product was purified to remove any unincorporated nucleotides, then used as DNA templates for second rounds of PCR using the G-C clamp primer 27f-GC and primer 519r. Amplicons (532 bp) from 3 separate PCR amplifications for the same sample were combined and purified as described previously. The purified product carrying the G-C clamp was separated in a DGGE apparatus. Separated DNA fragments were stained with ethidium bromide and visualized with a UV transilluminator. Individual, partially denatured fragments were excised and extracted from the polyacrylamide gel. Extracted

DNA was then used as a template for PCR amplification using the primers 27f and 519r. PCR product obtained was then subjected to nucleotide sequencing. Nucleotide sequence data obtained was compared with other nucleotide sequences in the NCBI GenBank Nucleotide Database by BLASTn analysis and by comparison of the sequence data with 16S rDNA sequences in the Ribosomal Database Project II. The Ribosomal Database Project II classifier facility was used to assign 16S rRNA sequences to the taxonomical hierarchy proposed in release 6.0 of the nomenclatural taxonomy of Garrity and Lilburn (see Garrity *et al.*, 2004).

Figure 5.6 shows the results obtained for the DGGE analysis for GC-clamped PCR products derived from the nine sets of uropod tissue tested. For each tissue sample tested, DGGE separated different numbers of unique amplicons. For example, 2 different amplicons were separated from tissue taken from Group III lobsters at week 12 of the infection trial, whereas 11 different amplicons were isolated from tissue taken from Group I lobsters at week 8.

Nucleotide sequence data obtained for each amplicon separated by DGGE was classified against the taxonomy proposed for Release 6.0 of the nomenclatural taxonomy of Garrity & Lilburn (Garrity *et al.*, 2004). Most sequences (39 of 48 sequences) were similar to those for *Vibrio* spp. (95 – 100% similarity). Four sequences were similar to sequences obtained for uncultured Vibrionaceae (98% similarity) and another 5 sequences were similar to those obtained for marine *Psychrobacter* spp. (>99% similarity) (Table 5.3B). Assignment of sequences to taxonomic groups was limited to genus level (Fox *et al.*, 1992).

Table 5.3B is a summary of the genus assignments of 16S rDNA sequences by Treatment group. As was the case for 16S rDNA clone sequences (Section 5.3.3.1), sequences typical of *Vibrio* spp. were the most frequent sequences obtained for samples from each of the three treatment groups. Similarly, *Psychrobacter* spp. specific sequences were amplified from tissue samples harvested from lobsters in the control treatment group (Group I) and Group II animals damaged with sterile instruments. In particular, amplicon sequences characteristic of *Psychrobacter* species were primarily amplified from Group I lobster tissue (control group) sampled at weeks 8 and 12 when the housing tank water temperatures were within the range 11.5 – 14.5°C. Within each treatment group, similar

numbers of unique amplicons were obtained for each of the sampling times. Furthermore, the small number of unique amplicons obtained indicates that the species diversity of the tail fan sample microflora is small.

A dendrogram that illustrates the relatedness of different sequences to one another is shown as Figure 5.7. Based on an arbitrary distance measure of 0.01 units, sequences assigned to the genus *Vibrio* formed two distinct clusters (*Vibrio* Groups A and B), while those classified as typical of *Psychrobacter* and Vibrionaceae formed two other distinct clusters. *Vibrio* Group B consisted of sequences amplified only from uropod tissue harvested from lobsters in Treatment Group III, along with reference sequences for *V. vulnificus*, *V. parahaemolyticus*, *V. harveyi* and *V. campbellii* and the infecting strain *V. parahaemolyticus* L21. This indicated that the predominant types of bacteria associated with tissue from these lobsters are likely to be pathogenic marine vibrios; a result consistent with the species analysis described in Section 5.3.1 and the molecular characterization studies described in Section 5.3.2. Sequences in *Vibrio* group A comprised the majority of all sequences obtained (36/48) as well as *V. alginolyticus* reference sequences. This data suggested that the majority of bacteria associated with tail fan tissue from uninfected lobsters may be *V. alginolyticus*, a result also consistent with the analysis described in Sections 5.3.1 and 5.3.3.1.

#### 5.3.3.3 Combined Analysis of 16S rDNA sequences

To compare the sequence data obtained from independent clones of 16S rDNA and that obtained from DGGE analysis, all sequence data was collectively subjected to phylogenetic and molecular evolutionary analysis as per Section 5.3.3.1. A dendrogram that illustrates the relatedness of all of the sequences is shown as Figure 5.8. As for Figure 5.5 and Figure 5.7, and based on an arbitrary distance measure of 0.01 units, sequences assigned to the genus *Vibrio*, formed two clusters (*Vibrio* group A and B). Sequences assigned to Vibrionaceae and *Psychrobacter* formed separate clusters. Importantly, this analysis showed that the clone sequences and sequences obtained from the DGGE analysis formed the same groupings previously identified when these sequences were analysed separately eg. sequences obtained by PCR amplification of tail fan tissue harvested from lobsters in Group III formed a distinct cluster along with those of *V. vulnificus*,

*V. parahaemolyticus*, *V. harveyi* and *V. campbellii* reference sequences and the infecting strain *V. parahaemolyticus* L21, whereas the remaining *Vibrio* sequences all clustered together in *Vibrio* group A along with a number of *V. alginolyticus* type strains.

## 5.4 Discussion

The results outlined in Section 5.3.1 of this Chapter confirmed the finding in Chapter 3 of this thesis that *V. parahaemolyticus* strain L21 is likely to be involved in induction of TFN in previously healthy lobster tail fans, and to persist in association with the diseased tissue for up to twelve weeks. Furthermore, this strain is able to out-compete other *Vibrio* spp. naturally present in association with the lobster carapace.

Unlike the microflora of Group I lobsters, only *Vibrio* spp. were detected within lesions from tail fan tissue harvested from lobsters in Group III. Importantly, the main species detected throughout the trial, and in particular at week 12 post-infection, was *V. parahaemolyticus*. This outcome demonstrated that *V. parahaemolyticus* strain L21 is capable of inducing TFN and persisting in association with diseased tissue for up to 12 weeks when introduced to tail fan tissue at the time of wounding, a finding similar to that by May (2002). Furthermore, this experiment also demonstrated that this particular strain was capable of dominating the bacterial population within TFN lesions when introduced at the time of tail fan wounding.

Interestingly, when sterile instruments were used to damage tail fan tissue of Treatment Group II lobsters, a clearly identifiable change in the types of *Vibrio* spp. associated with damaged tissue was also observed. Within Treatment Group II, there was a much higher proportion of *V. vulnificus* isolates recovered from tail fan tissue compared to the other two treatment groups. This indicated that when tissue was damaged, this type of bacteria may have colonised the damaged tissue and played a role in induction and onset of TFN. As lesions that developed on tail fan tissue of these Group II lobsters took longer to initiate, and, based on the electron micrographs shown in Chapter 4 of this thesis, which demonstrated that these bacteria took longer to colonise the wound site, it is likely that the lobster immune system may have been able to at least partially seal off the wound before the bacteria had a chance to colonise the exposed softer layers of the carapace. As such, bacteria associated with the carapace that had the ability to colonise these areas more

efficiently would have had a selective advantage. This may explain the observation that *V. vulnificus* was isolated more often from tail fan tissues of these lobsters compared to lobsters from the other treatment groups. However, as wounding can occur at any stage of the catching and holding stages of live-held southern rock lobsters, it is more likely that the microbiology of the surface that causes damage will dictate which species of vibrios are involved in initiation and establishment of TFN lesions.

Combined use of DGGE and cloning/sequencing of 16S rDNA PCR amplicons has proven to be an effective tool for evaluation of non-culturable populations of bacteria associated with a range of environmental niches including salad (Handschr *et al.*, 2005), composting garbage (Takaku *et al.*, 2006) and heavy metal contaminated soil (Ellis *et al.*, 2003). However, of greater relevance to this study, is the use of this technique in marine environments to determine the microbial diversity associated with the colonial ascidian *Cystodytes dellechiajei* (Martinez-Garcia *et al.*, 2007), coral (Bourne & Munn, 2005) and cultured *Panullirus ornatus* phyllosoma (Payne *et al.*, in press). However, these techniques have limitations. For example, 16S rDNA sequence analysis is not useful for separation of bacteria at the species level (Fox *et al.*, 1992); although it is possible to separate isolates to genus groupings. Secondly, it is possible to miss groups of bacteria using either system in isolation (Takaku *et al.*, 2006). As DGGE, separates PCR amplicons according to melting point, amplicons with similar melting points may appear as a single band after electrophoresis and hence specific bacterial groups may be missed. Furthermore, when sequencing clones it is possible to overlook minor sub-groups when randomly selecting a limited number of clones for sequence analysis. However, combining the two techniques limits these observed problems (Takaku *et al.*, 2006, Ellis *et al.*, 2003). When DGGE and sequence analysis of random 16S rDNA clones was used to evaluate the microbial flora of the tail fan carapace of rock lobsters, a range of marine vibrio types were shown to naturally associate with these surfaces. This finding supports earlier work published by Porter *et al.* (2001). That study investigated the natural microflora of the carapace of the spiny lobster, *Panullirus argus*, and reported that *Vibrio* species were the predominant type of bacteria present.

When rock lobsters are placed in crowded impoundments within boat wells, wounding of tail fans is likely to occur. The wounds are predominantly self-inflicted or caused by



other lobsters (Geddes *et al.*, 2000). The bacteria that comprise the normal carapace micro-flora are likely to be the source of bacteria that enter wounds and initiate TFN. As most of the *Vibrio* spp. isolates from lobster tissues were able to express the extracellular enzymes lipase and chitinase, these organisms may have significant potential to induce tissue degradation at the site of infection. In that event, it is conceivable that tissue destruction could allow the rapid colonization of damaged tissue in a manner that compromises the ability of the lobster to seal the wound (outlined in Chapter 1.4.2). Nevertheless, the extent of damage may well be limited by an immune response. The potential for the immune response to limit infection of the tissue underlying the initial wound, the haemolymph and ultimately other organs, has yet to be determined. An examination of aspects of the immune response to infection of lobsters will be described in Chapter 6 of this thesis.

Ability of *Vibrio* spp. isolates to express chitinase and lipase may explain aspects of disease pathology identified by TEM observation of tissue sections. In particular, many micrographs of tissue sections examined showed bacterial cells located within pits on the surface of tail fan carapace as well as sub-surface lesion tissue. These pits, which were not usually observed on tissues from control group lobsters, may be the result of degradation of lobster carapace and other tissue by extracellular enzymes secreted by bacteria. This feature has been well described by studies of other environmental niches as varied as spoilage organisms associated with food surfaces (Thomas & McMeekin, 1981) to the degradation of carapace leading to shell diseases of crustaceans (See Chapter 1.5.2). Since the majority of the *Vibrio* spp. isolated from these tissues were capable of expressing chitinase and other enzymes, it is likely that discrete expression of these enzymes on lobster tissues could have lead to formation of the pits noted in micrographs. Indeed, diffusion of these enzymes into the softer endocuticle is also likely to be responsible for the break down of the carapace that was observed by examination of ultra-thin sections of tissue by TEM (see Chapter 4).

Interestingly, but perhaps not surprisingly, isolates of *V. parahaemolyticus* were the predominant type of bacteria isolated from lesion samples from Group III lobsters that had tail fans artificially infected with *V. parahaemolyticus* strain L21 at the time that mechanical damage was induced. This observation was supported by DGGE analysis of

the 16S rDNA amplified from lesion tissue. The fact that only 2 unique amplicons were identified from lesion tissue obtained twelve weeks post infection indicated that the microflora is predominated by only a few species of bacteria. Indeed, sequence analysis of these amplicons indicated that *V. parahaemolyticus* and *V. alginolyticus* were the major species present.

The DGGE and the 16S rDNA analysis provided evidence to support the hypothesis that *Vibrio* species are the dominant type of bacteria associated with TFN lesions. That data also directly corresponded with the microflora analysis of isolates of bacteria obtained from lesion and carapace tissues. Indeed, the only other genera implicated in TFN were various Vibrionaceae and *Psychrobacter* spp. *Psychrobacter* spp. are common in a range of environments including human sources, as spoilage bacteria in foods, the skin of poultry, the skin and gills of fish and the open sea (Juni, 1992), as well as Antarctic soil (Bowman *et al.*, 1996). However, to the author's knowledge, this is the first instance in which *Psychrobacter* spp. have been associated with the carapace of rock lobsters or indeed any crustacean species. Bacterial analysis of the incoming seawater demonstrated that the filtering process only removes particulate matter without removing bacterial contamination. As evidenced by DGGE and 16S rDNA clone sequence analysis, these bacteria were apparently not present in detectable numbers in association with tail fan tissue at the beginning of the trial. When the lobster holding water temperature was below 15°C, these types of bacteria may have been better adapted to grow to numbers that were detectable by the PCR methods used.

Although TEM of tail fan tissue allowed identification of a range of morphologically different types of bacterial cells, only a limited number of different genera of bacteria were detected by DGGE analysis. This discrepancy may simply reflect the effects of environmental factors on rates of growth of bacteria and ultimately the morphology adopted under those growth conditions (Chaiyanan *et al.*, 2001, Tangwacharin *et al.*, 2006, Wainwright *et al.*, 1999). Thus the predominance of coccoid shaped bacterial cells identified by TEM of tissue sections may be explained by any of the following:

1. 16S rDNA signatures typical of *Psychrobacter* spp., indicated a coccoid cell morphology (Juni and Heym, 1986).

2. Holding tank water temperatures below 15°C are known to influence cell morphology of *Vibrio* spp. At water temperatures below 15°C, vibrios enter a VBNC state. VBNC cells have been reported to adopt cellular morphologies that range from short fat rods to coccoid shaped cells. Indeed, the cell morphologies observed by SEM and TEM examination of tail fan tissues strongly resembled the morphologies of VBNC state *V. cholerae* cells reported by Chaiyanan *et al.* (2001).

Thus the predominance of coccoid cell morphologies identified by microscopy, when taken together with bacteriological and 16S rDNA based evidence that showed *Vibrio* spp. are predominant bacterial types present, most likely reflects the growth conditions of cells associated with tail fan tissue.

The data presented in this chapter also confirmed the findings presented in Chapter 3 that indicated there are potentially pathogenic vibrios present within lesions associated with TFN. This highlighted the potential risk to consumers. It is important to note that it is now accepted that pre-enrichment prior to plating onto TCBS may be necessary for of detection of pathogenic vibrios (Desmarchelier, 2003). Without an enrichment step, the levels of these species in association with tail fan tissue may have been underestimated. As such, future work investigating TFN should follow procedures such as pre-enrichment in alkaline peptone water prior determining vibrio numbers via the most probably number technique (Harwood *et al.*, 2004). Nevertheless, pathogenic vibrios were routinely isolated from tail fan tissue samples by direct plating on TCBS and incubation at 30°C for 48 h instead of the standard 24 h. Furthermore, independent use of culture-independent techniques demonstrated that vibrios were the dominant type of bacteria associated with TFN lesions, confirming their role in the disease.

Of particular concern is the presence of *trh*<sup>+</sup> *V. parahaemolyticus* isolates in association with TFN lesions. The incidence of this gene among lobster tail fan isolates (39%) is much higher than that normally associated with environmental isolates (range of 1 to 5%) (Hervio-Heath *et al.*, 2002; Robert-Pillot *et al.*, 2004). However, the high incidence of *trh*<sup>+</sup> compared to *tdh*<sup>+</sup> isolates is similar to the finding reported by Parvathi *et al.* (2006) following an investigation of *V. parahaemolyticus* isolated from oysters in Mangalore, India. Nevertheless, outbreak-associated *V. parahaemolyticus* isolates from Texas, New

York and Asia are predominantly *tdh*<sup>+</sup> (DePaola *et al.*, 2003). This may suggest that *trh*<sup>+</sup> *V. parahaemolyticus* may occupy different niches throughout the world to those that are *tdh*<sup>+</sup>.

Another concern relevant to food safety was the observation that *V. vulnificus* isolates that expressed opaque colony morphology were isolated from lesions in Chapter 3 of this thesis. This genotype/phenotype is considered by many to be characteristic of clinical isolates of this species (Simpson *et al.*, 1987). Indeed, *V. vulnificus* with this phenotype is one of the leading causes of seafood related deaths in the United States (Potasman *et al.*, 2002).

Although vibrios are efficiently destroyed by cooking regimes used for lobsters and other crustacean foods [eg. Cooking clam meat at 55°C for 30 seconds is sufficient to kill 99.9% of *V. parahaemolyticus* (Delmore & Chrisley, 1979), heating *V. vulnificus* in buffered saline to 50°C for 1.15 min will kill 99.9% of the bacteria (Cook & Ruple, 1992) and heating crayfish homogenate to 82°C for 30 seconds will kill 99.9% of *V. cholerae* present in the meat (Grodner & Hinton, 1985)], live lobsters displaying TFN lesions could represent a source of pathogenic vibrios. In the absence of good hygiene practices in food preparation areas, pathogenic marine vibrios could be transferred to other foods. Thus there is a high risk of illness when lobsters displaying symptoms of TFN are not prepared appropriately. This includes the sale of raw, or undercooked lobsters, or cross contamination with food that has already been prepared. This is particularly the case for consumers that are immuno-compromised or suffering illness resulting in elevated serum iron levels. As a consequence, it is the author's view that sale of affected lobsters should be restricted to pre-cooked lobsters only, with no mixing of preparation surfaces between uncooked and cooked lobsters if live-holding is to become common place.

## 5.5 Conclusions

1. 16S rDNA sequence based methods indicated the microbial flora of tail fan tissue of normal healthy rock lobsters is predominated by *Vibrio* spp, *Psychrobacter* spp. and other bacteria belonging to the Family Vibrionaceae.

2. Bacteriological analysis of isolates of *Vibrio* spp. indicated that although a range of different species of vibrios are present in association within TFN lesions on tail fan tissues of rock lobsters, the predominant species are *V. parahaemolyticus*, *V. alginolyticus* and *V. vulnificus*.
3. Simultaneous wounding/damage to tail fans and infection of wounds by *V. parahaemolyticus* strain L21 leads to induction of TFN-like lesions at the site of wounding. Furthermore, the types of vibrios isolated from lesions that develop at the wound site are predominantly *V. parahaemolyticus*. This suggests the infecting species is able to persist in TFN lesions and may out-compete other types of marine bacteria capable of colonizing the lesion tissue.
4. Wounding of tail fan tissue with sterile instruments leads to changes in the microflora of tissue around the wound site compared with undamaged control tail fans.
5. The majority of isolates of *Vibrio* spp. from tail fan tissue and TFN lesions were able to express and secrete chitinase and lipase. When this evidence is taken together with electron microscopic evidence of tissue erosion associated with bacterial cells, expression of these enzymes is likely to be responsible for the onset of destruction of the tail fan tissue underlying TFN lesions. Continued secretion of these enzymes is expected to allow the lesion-associated bacteria to establish chronic lesions that may eventually destroy the entire tail fan.
6. Potentially pathogenic isolates of *trh*<sup>+</sup> *V. parahaemolyticus* and *vvh*<sup>+</sup> *V. vulnificus* were consistently isolated from intentionally damaged tail fan tissue samples. The presence of these types of vibrios indicates rock lobsters affected by TFN lesions may present a significant public health risk in the absence of sufficiently stringent food preparation and storage practices designed to eliminate cross-contamination of other foods.

**Table 5.1 Proportion of *Vibrio* spp. recovered from tail fan tissue samples from each treatment group.**

Data shown are compiled from each of the four sampling periods for each treatment group. Isolates were recovered from TFN lesions affecting lobster from all treatment groups during the 12 weeks of the infection trial (covering the period 14.05.2004 – 28.07.2004). Total number of isolates per treatment group included in the analysis: Group I (control), 31; Group II (Damage only), 30; Group III (Damage plus infection), 30. Isolates identified as per Alsina and Blanch (1994) (see Appendix G).

\* Isolates were identified as per Alsina and Blanch (1994), as described in Chapter 5.2.

<b>Treatment Group</b>	<i>V. parahaemolyticus</i>	<i>V. vulnificus</i>	<i>V. alginolyticus</i>	<b>Other <i>Vibrio</i> spp.</b>
Group I	12% (4)	3% (1)	59% (18)	27% (8)
Group II	8% (2)	23% (7)	46% (14)	23% (7)
Group III	70% (21)	0%	30% (9)	0%
<b>Total all Groups</b>	<b>30% (27)</b>	<b>9% (8)</b>	<b>45% (46)</b>	<b>16% (15)</b>

**Table 5.2: Expression of phenotypic characteristics associated with virulence by *Vibrio* isolates.**

Isolates were recovered from TFN lesions affecting lobster in all treatment groups during the infection trial over the period 14.05.2004 – 28.07.2004.

Identity (Number of isolates tested)	Protease	Lecithinase	Lipase	Urease	Cytotoxicity to CHO cells
<i>V. parahaemolyticus</i> (27)	100%	89%	100%	39%	56%
<i>V. vulnificus</i> (8)	100%	87%	0%	100%	0%
<i>V. alginolyticus</i> (41)	96%	24%	100%	7%	25%
Other <i>Vibrio</i> spp. (15)	25%	30%	61%	21%	4%
Total (102)	80%	41%	86%	24%	24%

**Table 5.3: Composition of microbial flora of lobster tail fan tissue based on analysis of 16S rRNA gene sequence data.**

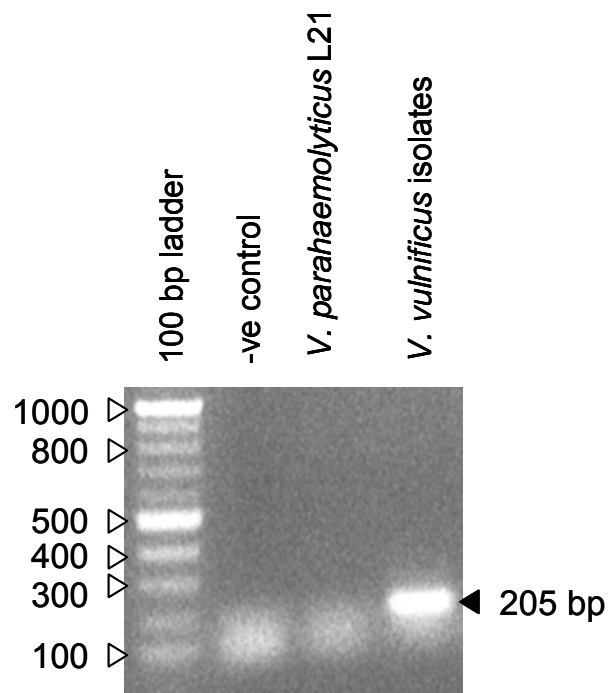
A: Composition based on nucleotide sequence data derived from 16S rDNA clones prepared from tail fan tissue from each treatment group

Lineage	Genus Name	Number of sequences analysed per Treatment Group		
		Group I	Group II	Group III
Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales;Moraxellaceae;	<i>Psychrobacter</i>	13	3	0
Bacteria;Proteobacteria;Gammaproteobacteria;Vibrionales;Vibrionaceae;	<i>Vibrio</i>	16	23	27
Bacteria;Proteobacteria;Gammaproteobacteria;Vibrionales;Vibrionaceae;	unclassified Vibrionaceae	1	3	4
Bacteria;Proteobacteria;Gammaproteobacteria;	unclassified Gammaproteobacteria	0	1	0
Total Number of 16S rDNA clone sequences		30	30	31

B: Composition based on number of unique amplicon nucleotide sequences identified by DGGE analysis.

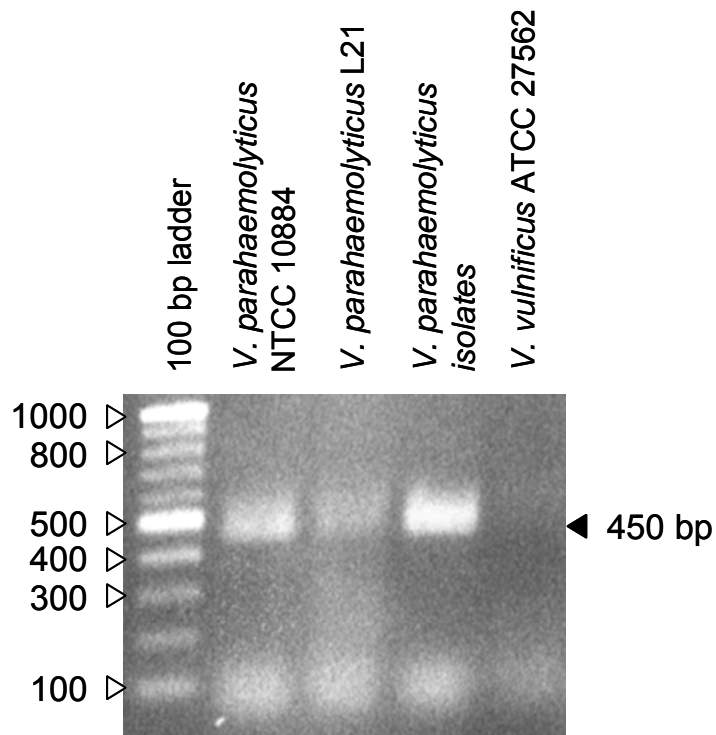
Lineage	Genus Name	Number of unique amplicons analysed per Treatment Group		
		Group I	Group II	Group III
Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales;Moraxellaceae;	<i>Psychrobacter</i>	3	2	0
Bacteria;Proteobacteria;Gammaproteobacteria;Vibrionales;Vibrionaceae;	<i>Vibrio</i>	16	12	10
Bacteria;Proteobacteria;Gammaproteobacteria;Vibrionales;Vibrionaceae;	unclassified Vibrionaceae	1	3	0
Bacteria;Proteobacteria;Gammaproteobacteria;	unclassified Gammaproteobacteria	0	0	0
Total Number of Unique Amplicon Sequences		20	17	10





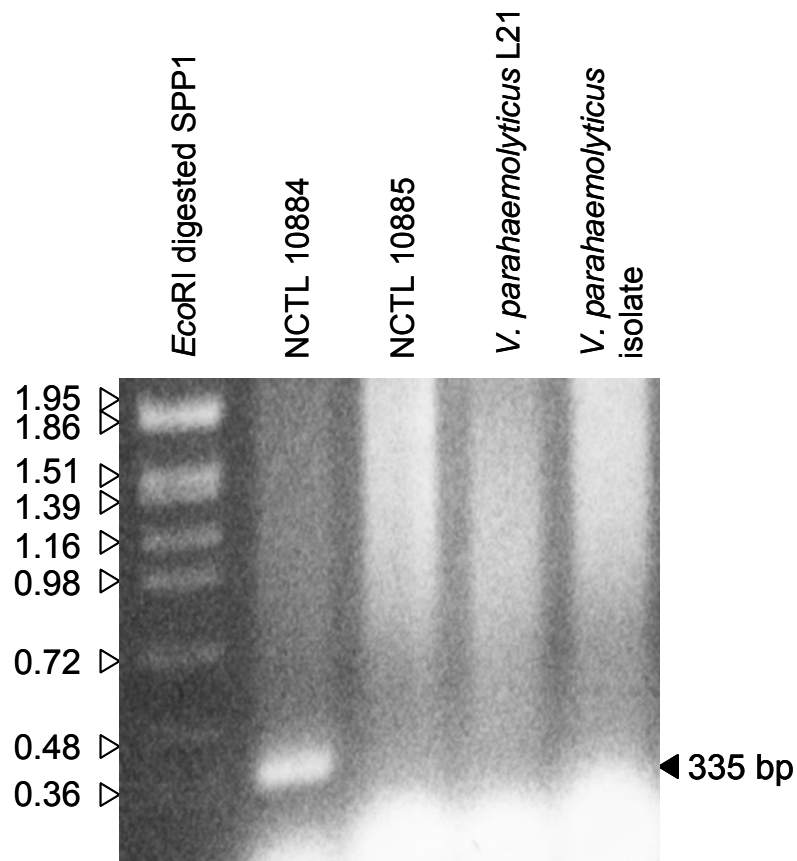
**Figure 5.1: PCR amplification of a 205 bp fragment of the *vvh* gene of isolates identified as *V. vulnificus*.**

The figure shows typical results obtained for all *V. vulnificus* isolates identified from tail fan tissue. Size marker units are base pairs. The negative control was Milli-Q water instead of DNA suspensions.



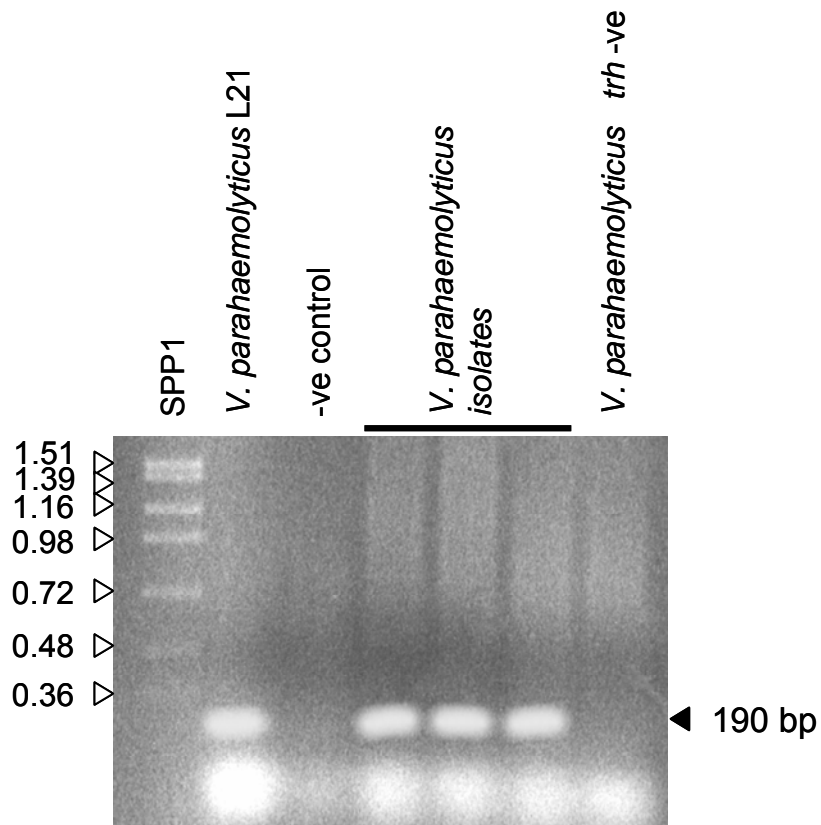
**Figure 5.2:** PCR amplification of a 450 bp fragment of the *tlh* gene of isolates identified as *V. parahaemolyticus*.

*V. parahaemolyticus* NTCC 10884 was used as a positive control. *V. vulnificus* ATCC 27562 was used as a negative control. The figure shows typical results obtained for all *V. parahaemolyticus* isolates identified from tail fan tissue. Size marker units are base pairs.



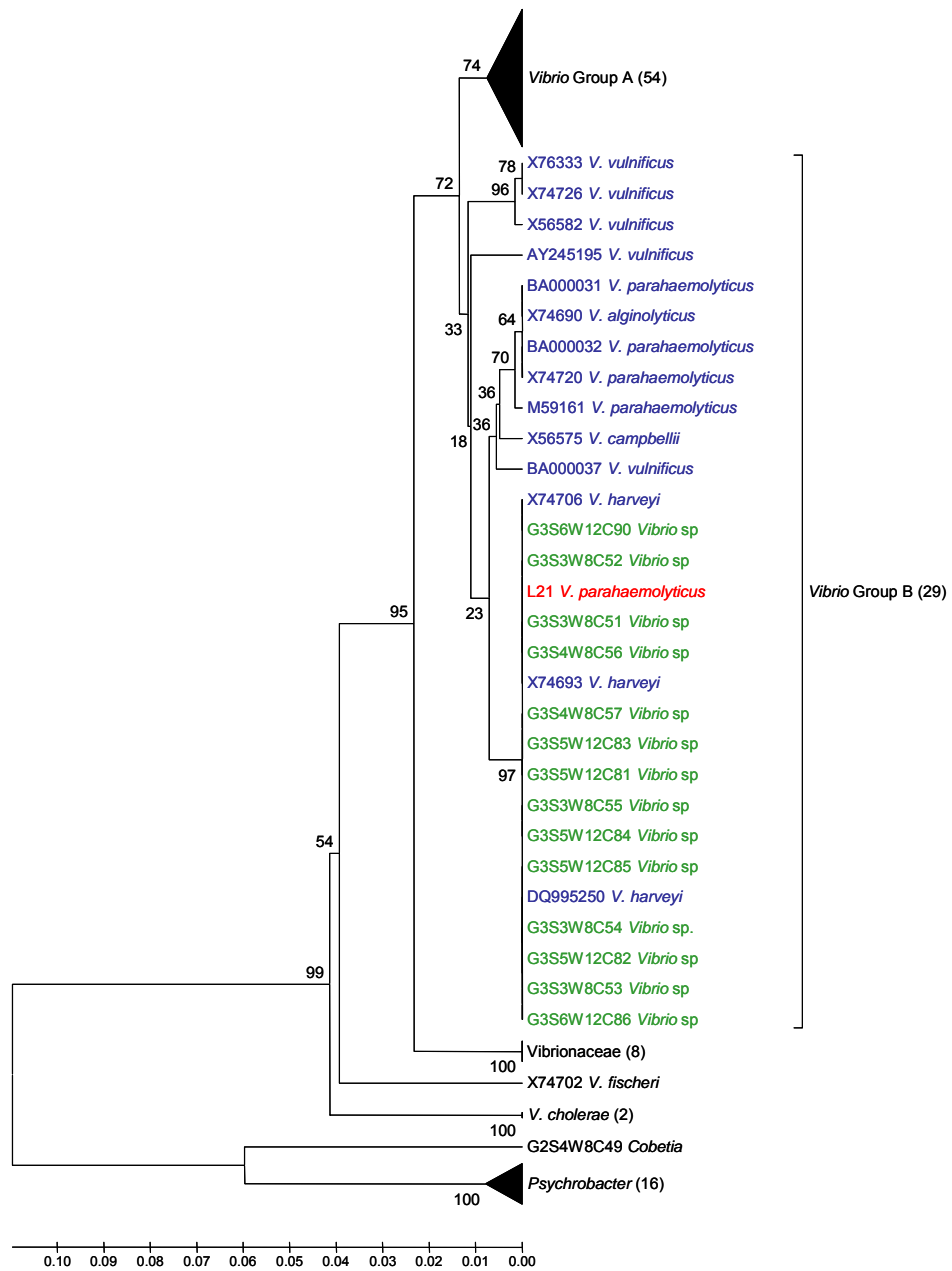
**Figure 5.3: PCR amplification of a 335 bp fragment of the *tdh* gene of isolates identified as *V. parahaemolyticus*.**

The figure shows typical results obtained for all *V. parahaemolyticus* isolates identified from tail fan tissue. Strain NCTL 10884 was used as a *tdh* positive control. Strain NCTL 10885 was used as a *tdh* negative control. All lobster *V. parahaemolyticus* isolates tested were *tdh* negative. SPP1 marker size units are kilobase pairs.



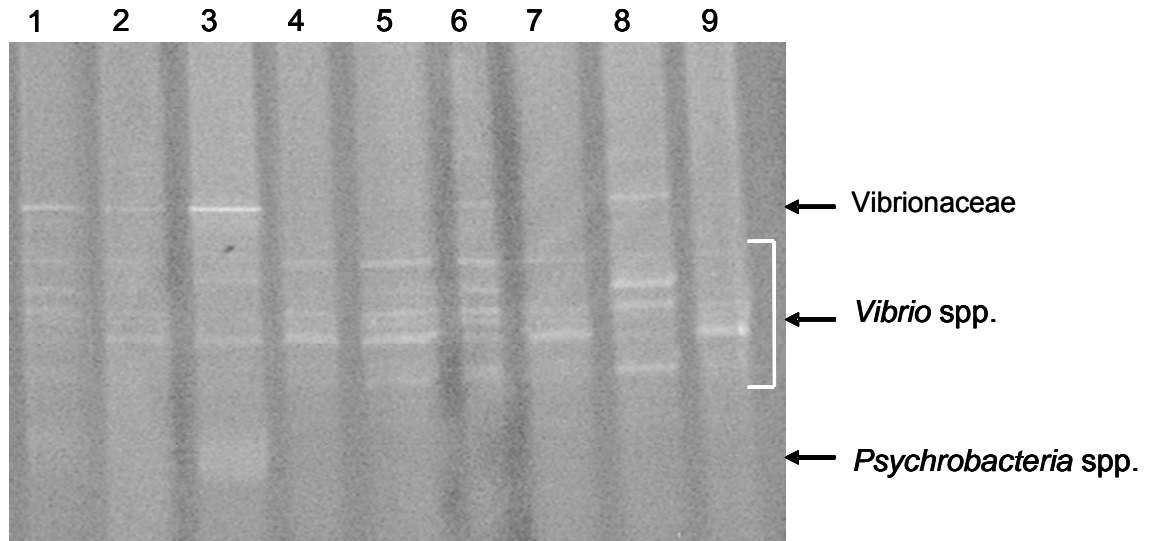
**Figure 5.4:** PCR amplification of a 190 bp fragment of the *trh* gene of isolates identified as *V. parahaemolyticus*.

The figure shows typical results obtained for all *V. parahaemolyticus* isolates identified from tao; fam tissue. The *trh* negative *V. parahaemolyticus* isolate was recovered from a lesion associated with TFN. SPP1 size marker units are kilobase pairs. The negative control was *V. vulnificus* DNA.



**Figure 5.5: Phylogenetic affiliation of partial 16S rDNA sequence data obtained from independent clones of amplicons prepared by PCR amplification from DNA isolated from lobster tail fan tissue.**

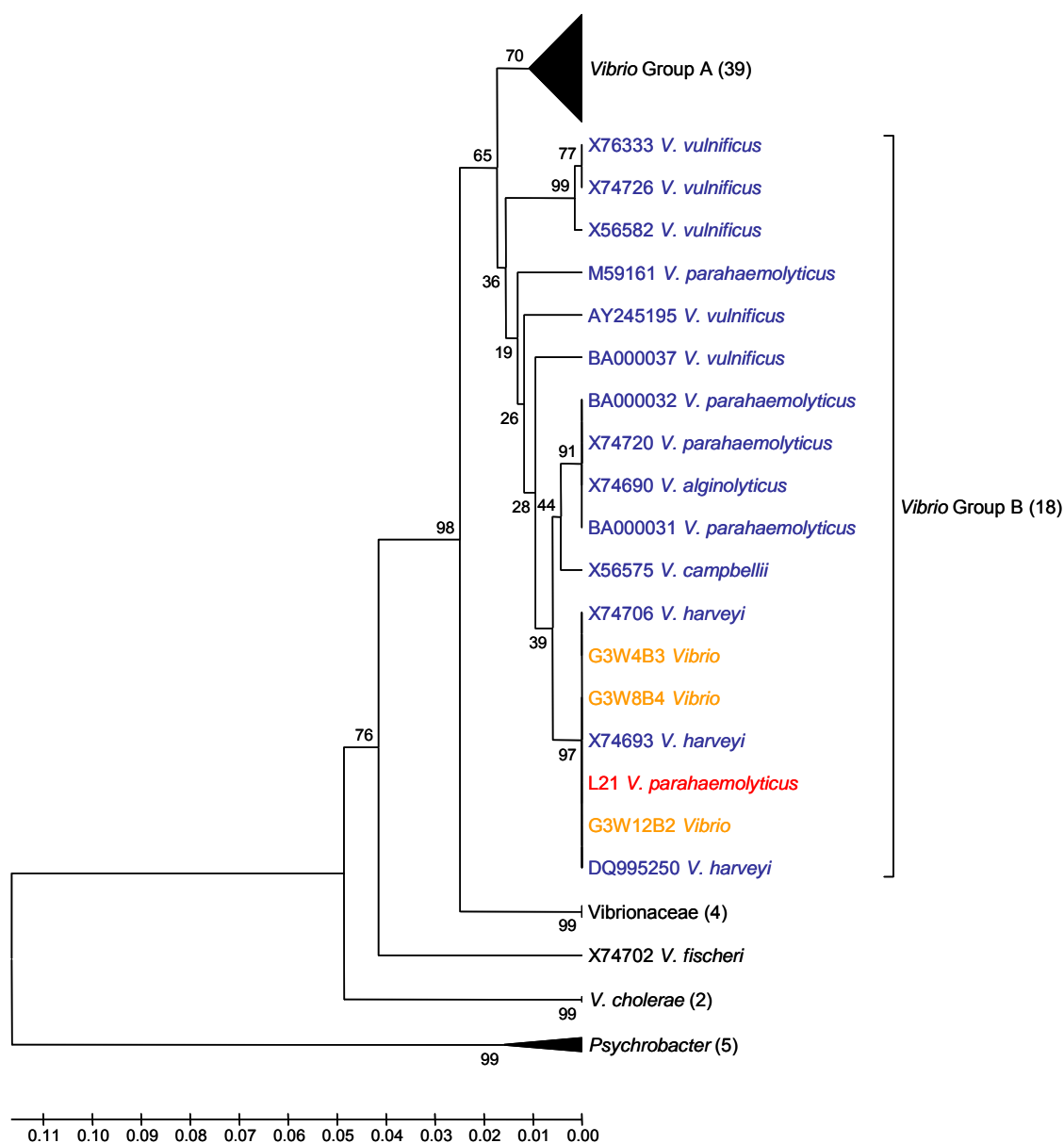
The dendrogram was constructed from a pairwise distance matrix by using a UPGMA method and a Kimura 2-parameter nucleotide model. The scale for the tree indicates inferred evolutionary distance. Bootstrap values were derived from 500 sampling events of the aligned sequence data. Reference sequences and source organism are shown in blue. Sequence data from the infecting strain L21, is shown in red. Sequences for independent clones, and proposed source organism, are shown in green. *Vibrio* Group A, *Vibrionaceae*, *V. cholerae* and *Psychrobacter* sequences have been collapsed to assist interpretation. A full list of sequences in each of the groups is presented in Appendix M, Page 235.



**Figure 5.6: Denaturing gradient gel electrophoretic (DGGE) separation of DNA fragments encoding 16S rRNA sequences.**

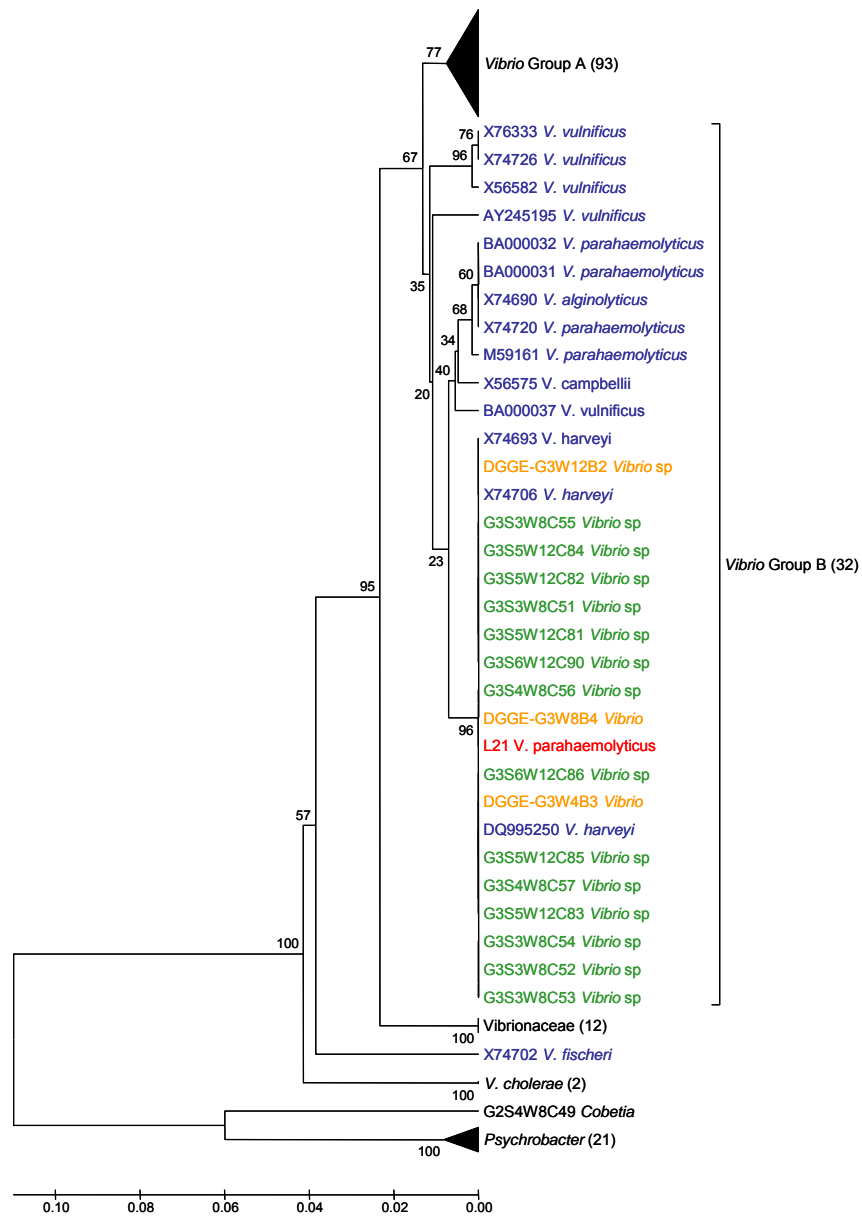
The DNA fragments were prepared by PCR amplification of DNA template isolated from lesion tissue. Each band represents an amplicon from an individual bacterial type.

- Lane 1: Control animal four weeks from commencement of infection trial;
- Lane 2: Control animal eight weeks from commencement of infection trial;
- Lane 3: Lesion after twelve weeks p.i.;
- Lane 4: Lesion from animal that had tail fans Damaged with sterile instruments after four weeks p.i.;
- Lane 5: Lesion from animal that had tail fans damaged with sterile instruments after eight weeks p.i.;
- Lane 6: Lesion from animal that had tail fans damaged with sterile instruments after twelve weeks p.i.;
- Lane 7: Lesion from animal that had tail fans damaged with instruments inoculated with *V. parahaemolyticus* strain L21 after eight weeks p.i.;
- Lane 8: Lesion from animal that had tail fans damaged with instruments inoculated with *V. parahaemolyticus* strain L21 after four weeks p.i.;
- Lane 9: Lesion from animal that had tail fans damaged with instruments inoculated with *V. parahaemolyticus* strain L21 after twelve weeks p.i



**Figure 5.7: Phylogenetic affiliation of partial 16S rDNA sequence data obtained from DGGE separated amplicons prepared by PCR amplification from DNA isolated from lobster tail fan tissue.**

The dendrogram was constructed from a pairwise distance matrix by using a UPGMA method and a Kimura 2-parameter nucleotide model. The scale for the tree indicates inferred evolutionary distance. Bootstrap values were derived from 500 sampling events of the aligned sequence data. Reference sequences and source organism are shown in blue. Sequence data from the infecting strain L21, is shown in red. Sequences for independent DGGE separated amplicons, and proposed source organism, are shown in orange. *Vibrio* Group A, *Vibrionaceae*, *V. cholerae* and *Psychrobacter* sequences have been collapsed to assist interpretation. A full list of sequences in each of the groups is presented in Appendix N, Page 236.



**Figure 5.8: Phylogenetic affiliation of all partial 16S rDNA sequence data.**

Sequence data was obtained from DGGE separated amplicons and independent clones of 16S rDNA prepared by PCR amplification from DNA isolated from lobster tail fan tissue. The dendrogram was constructed from a pairwise distance matrix by using a UPGMA method and a Kimura 2-parameter nucleotide model. The scale for the tree indicates inferred evolutionary distance. Bootstrap values were derived from 500 sampling events of the aligned sequence data. Reference sequences and source organism are shown in blue. Sequence data from the infecting strain L21, is shown in red. Sequences for independent DGGE separated amplicons, and proposed source organism, are shown in orange and those of clones are shown in green. *Vibrio* Group A, Vibrionaceae, *V. cholerae* and *Psychrobacter* sequences have been collapsed to assist interpretation. A full list of sequences in each of the groups is presented in Appendix O, Page 237.



## Chapter 6: Preliminary Study of Immunological Responses Induced by TFN

### 6.1 Introduction

Very few studies have investigated the immunological responses to shell diseases in crustaceans. As outlined in Chapter 1.4, sterile wounding of the cuticle is sufficient to induce an immunological response in various crustacean species. That response is characterised by mass infiltration of haemocytes into tissues surrounding the wound site.

In freshly caught crabs, the humoral and cellular response to shell disease involves changes in total and differential haemocyte counts, bacteraemia and pro-phenoloxidase (pro-PO) activation in the haemolymph (Vogan *et al.*, 2002). Furthermore, it is of interest that in crabs, the level of bacteraemia has been shown to be affected by the severity of shell disease. However, Vogan *et al.* (2002) did not establish whether any of the host responses mentioned above can occur during the early stages of infection; that study focused only on freshly caught crabs with established shell disease. Furthermore, phenoloxidase (PO) activity in haemocytes from affected crabs was only assessed using trypsin as an activator. Trypsin is known to be a universal, non-specific activator of all potential PO activity. However, where bacterial pathogens have either known, or suspected roles in establishment and maintenance of disease, it is important that the role of bacterial factors be assessed as potential activators of PO activity. Since bacterial LPS is a long established specific activator of PO, a broader approach that assesses PO activation specifically in response to bacterial antigens such as lipopolysaccharide (LPS), may aid understanding of the immune response directed toward shell disease. In particular an understanding of any immune response to bacteria associated with lesions may allow a better understanding of the factors that lead to establishment of chronic disease.

It is also of interest that Vogan *et al.* (2002) did not report whether circulating phagocytic haemocytes were able to actively phagocytose chitinolytic bacteria implicated in shell disease of crabs, even though activity is widely considered to be a marker of immune activation in crustaceans (Bachere *et al.*, 1995).

As TFN is apparently a newly described disease that is predominantly associated with experimental live-holding of rock lobsters, it is not surprising that there have not been any studies that describe immune activation in terms of the number and nature of circulating haemocytes and other immune activation markers in response to TFN. Indeed very few studies have investigated these parameters for shell diseases of other crustacean species. Although the total and differential haemocyte counts of lobsters affected by TFN were measured as part of work previously described in this thesis (Chapter 4.4.6), activation of haemocytes in response to TFN was not assessed. In view of the lack of information about the immune response of crustaceans affected by shell diseases in general, the aim of work described in this chapter was to determine whether:

- (a) establishment of TFN in rock lobsters results in activation of PO activity in circulating haemocytes in the presence of non-specific (trypsin and *E. coli* LPS) and potentially specific (*V. parahaemolyticus* LPS) activators
- (b) phagocytic haemocytes from haemolymph of TFN affected lobsters are able to specifically recognise *V. parahaemolyticus* or other types of bacteria.

Activation of PO by *V. parahaemolyticus* and phagocytosis of cells of this bacterium would indicate that the lobster immune system of animals affected by TFN had been primed to respond to the presence of cells of this bacterium. Furthermore, these properties are known markers of arthropod and crustacean immune activation (McKay & Jenkin, 1970, Söderhäll & Cerinius, 1998). However, there is no consensus as to the location of PO in crustaceans, with PO found in either the plasma (Hernandez-Lopez, 2001) or haemocytes (Hernandez-Lopez, 1996, Smith & Söderhäll, 1991, Söderhäll & Smith, 1983). Consequently, an understanding of the immune response mounted, if any, may lead to an understanding of how it is that the infecting organisms responsible for TFN are able to induce and maintain such persistent (chronic) infections.

## 6.2 Experimental Design

To specifically address the aims of this Chapter, a second, smaller infection trial was carried out using live-held lobsters. This trial involved lobsters housed in individual temperature controlled tanks in the dark at 18°C with constant aeration and a constant

supply of filtered sea-water. All lobsters were fed a rotating diet of mussels, squid and cockles every 2 days for the period of the trial. Lobsters were maintained individually in temperature controlled environments to minimize the impact of environmental factors on the lobster health and more particularly, immune activation markers. The latter aspect was considered particularly important as a study by Evans *et al.* (1998) established that a range of lobster immune responses, including phagocytosis rates, may be affected by environment.

This trial consisted of two treatment groups, each comprising 5 lobsters. The control group consisted of lobsters with undamaged tail fans, while the experimental group consisted of lobsters that had tail fans deliberately damaged with instruments inoculated with *V. parahaemolyticus* strain L21 to artificially induce TFN, as described previously (See Chapter 4). Samples of haemolymph were collected from all individual animals at one-week intervals over a period of 6 weeks. All haemolymph samples were then centrifuged to separate the haemocytes from the plasma. Both the haemocytes and the plasma were then assessed for PO activity and *in vitro* phagocytosis by hyaline cells isolated from lobster haemolymph.

In an attempt to assess the specificity of any PO activation, three treatments were used:

- Trypsin as a non-specific response activator,
- *V. parahaemolyticus* LPS as a specific response activator and,
- *E. coli* LPS as another alternative non-specific response activator.

*In vitro* phagocytosis of *V. parahaemolyticus* strain L21 by hyaline cells from lobster haemolymph was also assessed.

## **6.3 Results**

### **6.3.1 Preparation of Bacterial LPS**

LPS was purified from both *E. coli* ATCC 2120 and *V. parahaemolyticus* strain L21 specifically for use in determining the level and specificity of phenoloxidase activation in response to TFN. LPS from each organism was prepared using the method described by Darveau & Hancock (1983). Purified LPS was then separated along an acrylamide gel and

silver staining used to detect any protein impurities, as described in Chapter 2.19 of this thesis. Figure 6.1a, b are photographs of SDS PAGE gels of LPS preparations. Preparations from both *V. parahaemolyticus* strain L21 and *E. coli* ATCC strain 2120 were determined to be clear of protein impurities.

### **6.3.2 Induction of TFN**

The level of TFN associated with all individual tail fans from all lobsters used in this study was assessed on a weekly basis. From this, the percentage of tail fan tissue affected by TFN was determined as per Chapter 4 of this thesis. This analysis demonstrated that while there was no significant difference ( $P < 0.05$ ) between the two treatments, the lobsters in the control group consistently had less tail fan tissue that was affected by lesions associated with TFN than those in the infected group (Figure 6.2). This result agreed with data reported in Chapter 4 of this thesis, in which no statistically significant difference in the level of TFN induced on tail fans of different treatment groups was identified, although lobsters that had tail fans damaged with instruments inoculated with *V. parahaemolyticus* strain L21 developed consistently higher levels of tissue erosion.

### **6.3.3 PO-Activation in response to TFN**

In most arthropods, PO activity is generally regarded as a marker that can be used to determine the level of an immune response generated in response to infection. To investigate the level of PO activation in response to TFN within the haemolymph of trial lobsters, haemocytes and plasma were harvested from control and experimental group lobsters followed by assay of the level of PO enzyme activity after exposure to a range of activators. The agonists used included trypsin, as a general PO activation marker, purified *V. parahaemolyticus* strain L21 LPS as a specific activator and *E. coli* LPS as a non-specific activator. During the first three weeks of the trial, plasma of lobsters that had tail fans deliberately damaged did not elicit any response to any of the agonists used. It was therefore concluded that the phenoloxidase activity was restricted to the haemocytes.

#### **6.3.3.1 Trypsin induced activation of PO**

Figure 6.3 presents results that compare trypsin inducible PO activity associated with haemocytes and haemolymph plasma isolated from experimental and control groups of

lobsters. No significant PO activity was detected in plasma from haemolymph of lobsters during the first 3 weeks of the trial. PO activity was detected in haemocytes obtained from lobsters and during weeks 3 and 4 post infection. At this stage of infection, a significant ( $P < 0.05$ ) increase in trypsin inducible PO was detected in experimental animals. The level of PO activity associated with haemocytes harvested from experimental lobsters (with tail fans intentionally damaged and infected with *V. parahaemolyticus* L21) was >10 fold higher than the PO activity detected in control animals. Trypsin inducible PO activity in haemocytes from experimental group lobsters increased relative to PO activity of haemocytes from control group lobsters until 6 weeks post infection. After 6 weeks of holding, the PO activity of both groups of lobsters was nearly identical. This data indicated that the PO activity of haemocytes from experimental lobsters was likely to be affected by establishment of infections leading to TFN.

#### 6.3.3.2 Activation of PO by Bacterial LPS

Figure 6.4 presents PO activity of haemocyte samples following activation with *E. coli* LPS. As expected, a significant increase ( $P < 0.05$ ) in PO activity was observed for haemocytes harvested from experimental group lobsters 4 weeks post infection compared with the PO activity of haemocytes from control group lobsters. However this effect was only transitory. By the end of the trial, only low levels of PO activation were detected for samples from both the experimental and control group animals. Levels of PO activity associated with the plasma fraction of haemolymph was <10 fold lower than that observed for haemocytes.

Figure 6.5 presents the PO activity of haemocytes following activation by *V. parahaemolyticus* LPS. Interestingly, PO levels in haemocytes, was essentially the same as that recorded for the plasma fraction of haemolymph for both experimental and control lobsters. Indeed *Vibrio* LPS inducible PO levels remained more or less constant across the entire period of the trial

### **6.3.4 Phagocytosis by Hyaline Cell Populations in Response to Artificially Induced TFN**

In crustaceans, as with most other arthropods, phagocytosis of bacterial cells by haemocytes is widely considered to be a marker of immune activation. As such, phagocytosis of opsonised *V. parahaemolyticus* strain L21 was examined using hyaline cells from lobsters affected by TFN compared to those recovered from healthy animals. This analysis demonstrated that phagocytosis of opsonised *V. parahaemolyticus* L21 by hyaline cells harvested from control and infected animals, were almost identical ( $P < 0.05$ ) for the duration of the trial (Figure 6.6). Ability of hyaline cells to phagocytose these bacteria increased markedly over the first 2 weeks of the trial (0% to ~70% phagocytosis). Over the remaining 4 weeks of the trial, the ability of these cells to phagocytose bacteria gradually declined to levels similar to those at the commencement of the trial. *In vitro* phagocytosis was confirmed by fluorescence microscopy (see Figure 6.7). To achieve this, hyaline cells and opsonised bacterial suspensions were combined as described above and incubated for 1 h. Free bacteria that had not been phagocytosed, were eliminated by addition of gentamicin so that only internalised bacterial cells could be visualised. The preparations were then stained with DAPI, and examined by fluorescence microscopy.

To confirm that bacterial cells did not survive within intraellular compartments of haemocyte cells, extracellular bacterial cells were eliminated by the addition of gentamicin. Gentamicin was then removed by washing the cells in PBS. The haemocytes were then lysed with Triton X100 and the resultant lysate plated onto marine agar and incubated overnight at 30°C. No colonies of bacteria were observed for any of the lysates tested.

## **6.4 Discussion**

In this study, TFN had no effect on a range of haemolymph parameters tested to assess immune activation within the haemolymph of affected animals. These included serum protein levels, bacteraemia, changes in total and differential haemocyte counts and the immune response markers PO activity and *in vitro* phagocytosis rates by hyaline cells. Previous reports that describe the effect of shell disease in crustaceans on haemolymph properties, such as serum antibacterial activity (Noga *et al.*, 1994), haemocytes and

humoral defenses (Vogan & Rowley, 2002), as well as subsequent bacteraemia (Vogan *et al.*, 2001) have been described. This chapter also aimed to investigate the immune activation as a result of TFN infections by examining PO activation and *in vitro* phagocytosis. These have been demonstrated to be markers of arthropod and crustacean immune system activation, as mentioned previously.

Phagocytosis of *V. parahaemolyticus* strain L21, which was used to induce disease during each of the infection trials, by hyaline cells was examined by incubating opsonised bacterial cells with enriched hyaline cell populations. This analysis demonstrated that in both control and infected animals, there was an initial rise in phagocytosis rates within the first two weeks post infection. However, this rapidly dropped off in the following weeks. Whilst there was no significant difference between the two groups for the duration of the trial, phagocytosis rates by hyaline cells harvested from lobsters that had TFN induced by damaging the tail fans with instruments inoculated with *V. parahaemolyticus* tended to be marginally elevated relative to the control for the final four weeks of the six week trial. As the initial increase in ability of haemocytes to phagocytose bacteria that occurred in the first two weeks of the infection trial was the same for both experimental groups of lobsters, this effect was likely caused by handling processes inflicted on the lobsters rather than factors related to the infecting strain of bacterium used. Future experiments that use live lobsters should pay particular attention to the potential for stress related immune effects caused by transport and re-housing. A period of more than two weeks within the holding tanks may be required to allow the lobster immune system to recover from these stressors. Alternatively, a study investigating the most appropriate transport could be undertaken to assess the relative stresses induced by transport in air compared to transport within tanks.

It is also of note that no viable intra-cellular bacteria were detected in haemocytes following experiments designed to examine phagocytosis of bacteria by haemocytes. This observation is important as it suggests that opsonised *V. parahaemolyticus* are successfully cleared from the haemolymph and subsequently killed by lobster haemocytes. An alternative conclusion of the results of this experiment is that haemocytes did not phagocytose bacteria from suspension under the experimental conditions used. However this is unlikely as a marked reduction in bacterial counts in suspension were noted over the incubation period used for this assay.

PO activation was separated into three treatment groups; following treatment with trypsin to analyse the total PO activation within the haemolymph, non-specific activation via *E. coli* LPS and specific activation by *V. parahaemolyticus* LPS. This differed significantly from the only previous study that specifically examined this aspect of immunity to shell disease elicited by *C. pagurus* (Vogan & Rowley, 2002). That study only examined freshly caught crabs with cuticular lesions already present and only examined the total PO activation induced by the addition of trypsin to haemocyte suspensions only. Under these conditions, no significant difference was detected in the phenoloxidase activation of diseased and healthy lobsters. Furthermore, to the author's knowledge, no study has been conducted on any shell disease to assess immune activation parameters when shell disease is initiated, nor whether any response is specific for the organism/s causing disease. Within the present study however, TFN was induced by mechanical damage and simultaneous infection to tail fans and the level of PO activation measured as the lesions developed, as well as examining the specificity of the response.

Interestingly, Hernandez-Lopez *et al.* (2001) found the PO activity of spiny lobsters was located in the plasma fraction of haemolymph and not haemocytes. However, Hernandez-Lopez *et al.* (1996) and Smith and Söderhäll (1991) reported that PO activity of brown shrimp and various marine invertebrates respectively was located exclusively in haemocytes. In view of these conflicting reports, this study included preliminary experiments to confirm the location of PO activity in haemolymph of lobsters. That work demonstrated that PO activity was restricted to haemocytes. Low, or undetectable levels of PO activity were found in the plasma fraction of haemolymph. This outcome was in agreement with reported work for brown shrimp.

It is of interest that no detectable PO response was measured for any sample of haemocytes when treated with *V. parahaemolyticus* L21 LPS, even though a measurable response was observed when LPS from *E. coli* was added to haemocyte preparations. A similar observation was reported by Vogan & Rowley (2002). In that study, the PO activity of haemocytes from normal crabs and crabs displaying shell disease caused by chitinolytic bacteria. However, while the PO activity of haemocytes from lobsters was increased by addition of the non-specific activator trypsin, this was not the case for crabs (as reported by Vogan & Rowley, 2002). In lobsters, a significant increase ( $P < 0.05$ ) in

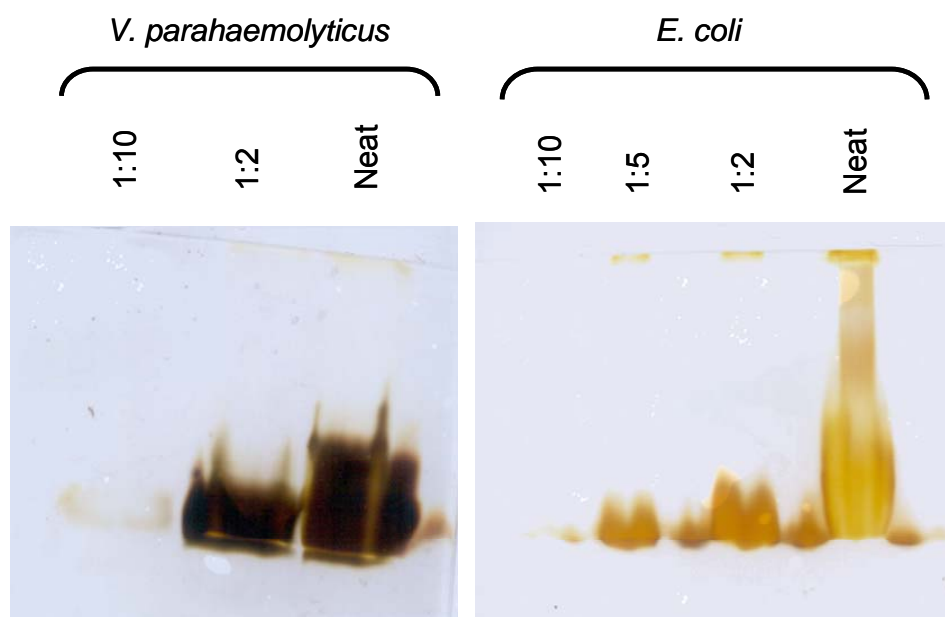


levels of PO activation within the haemocytes of lobsters that had tail fans damaged with inoculated instruments was detected compared with control animals. A similar response was observed when *E. coli* LPS was used as an activator of PO. The different results obtained for *V. parahaemolyticus* L21 LPS therefore suggested that the lobster immune system was unable to recognize, and hence respond to *Vibrio* induced infections taking place within the tail fan tissue. Nevertheless, the fact that the margins of developing lesions become heavily melanised is an indication that the lobster immune system does respond to either the presence of the bacteria responsible for damage, or some other elicitor released by damaged tissue. In view of the inability of *V. parahaemolyticus* L21 or LPS derived from this strain to elicit significant response, this suggested that molecules released by tail fan tissue degradation may be responsible for this response, rather than the infecting organisms. The melanisation characteristic of the margin of TFN lesions may also explain how the bacteria are able to induce persistent infections within the tail fan tissue without themselves being recognised and destroyed by the lobster humoral and cellular immune systems. It is possible that localised melanisation reactions that take place, result in formation of barriers that are sufficient to prevent lesion-associated bacteria from accessing the haemolymph and inducing bacteraemia typical of other shell diseases. Furthermore this observation may also explain the absence of mortality associated with TFN, as secondary bacterial infection of other tissues, particularly the hepatopancreas and gills, are unlikely to occur in the absence of bacteraemia.

## 6.5 Conclusions

1. The *V. parahaemolyticus* L21 strain used to establish TFN in experimental animals does not elicit activation of phagocytosis.
2. Infection of tail fans and establishment of TFN leads to limited activation of phenoloxidase activity. Any PO activity is unlikely to be the result of activation by vibrios associated with lesions. Results of the work in this chapter suggested that any PO activity is a response to degradation products associated with the progressive necrosis of uropod tissue typical of TFN.
3. Although no specific PO response to *V. parahaemolyticus* LPS could be demonstrated, PO activity is evident in tail fan tissue affected by TFN as melanin

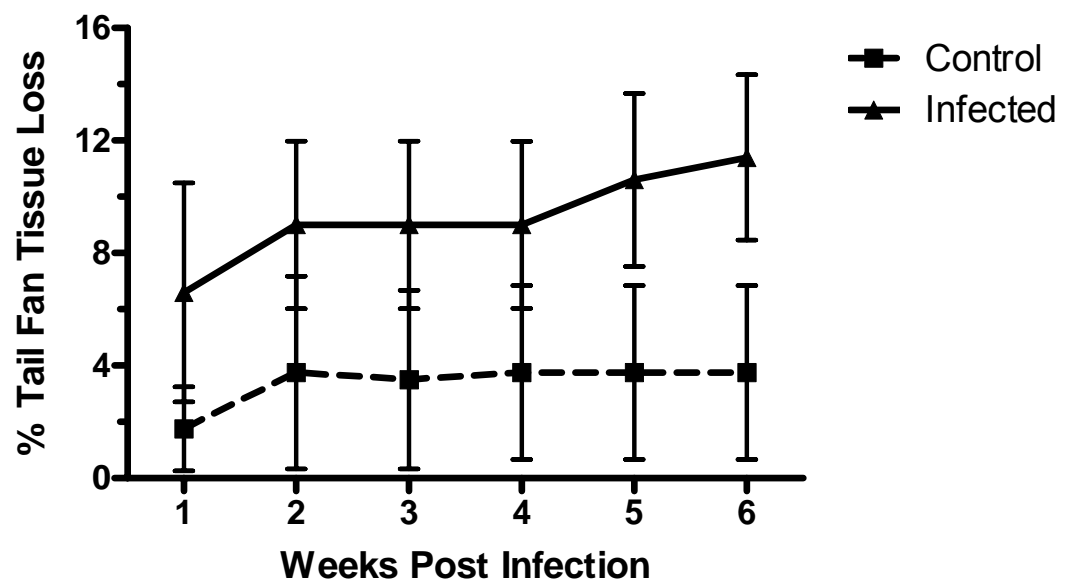
deposition at the wound site occurs. This data suggested that the PO activity and related melanisation may be sufficient to prevent bacteria from invading the haemolymph and other tissues from lesions.



**Figure 6.1: SDS PAGE separation of purified LPS recovered from *V. parahaemolyticus* and *E. coli*.**

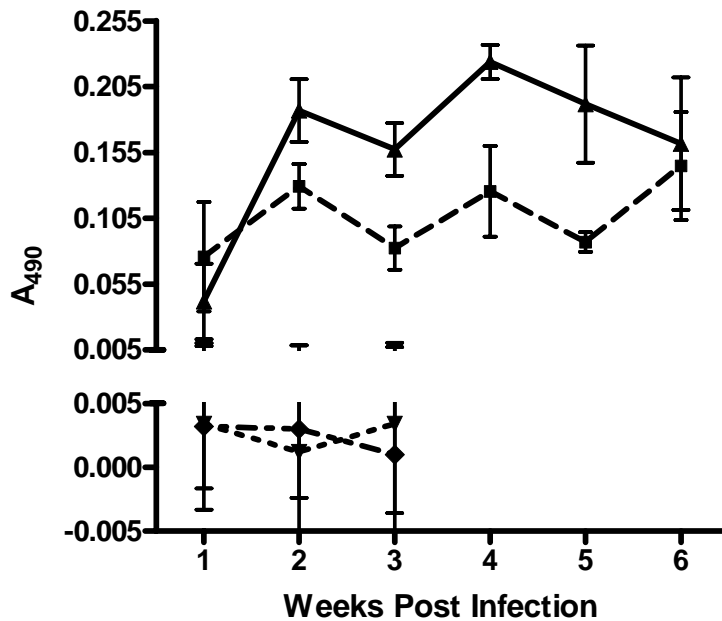
Dilutions of extracts loaded onto each gel are indicated.

### Percentage of Tail Fan Tissue Affected by TFN for Trial Number 2



**Figure 6.2:** Percentage of tail fan tissue affected by TFN.

Data shown are estimates for uninfected control lobsters and experimental lobsters that had tail fans infected with *V. parahaemolyticus* L21. There was a significant ( $P < 0.05$ ) difference in percentage of tail fan tissue loss between infected and control lobsters between weeks 3 – 5 post infection, as determined by 2-way ANOVA. However, there was a trend towards more severe TFN in lobsters that had tail fans damaged with inoculated instruments.

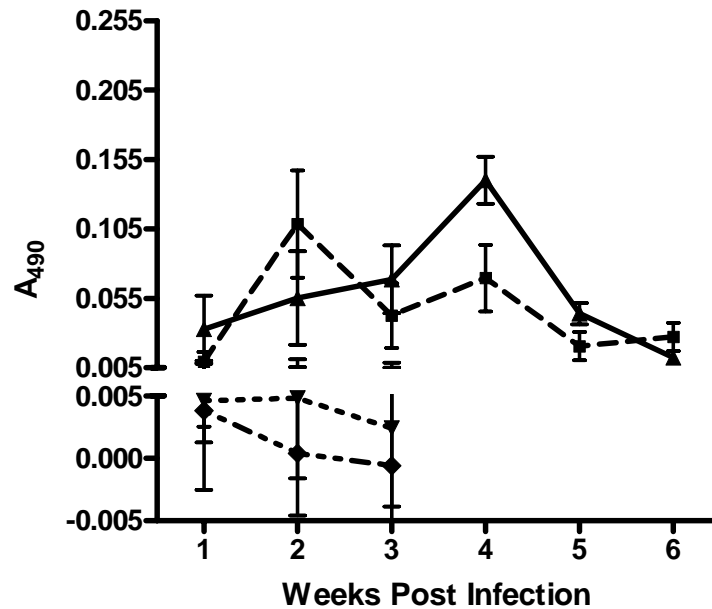


**Figure 6.3: PO activity associated with haemocytes following addition of trypsin.**

Data shown are the mean of 5 replicates. Error bars are the standard error about the mean. There was a significant ( $P < 0.05$ ) difference in PO activation measured for samples from infected and control lobsters over weeks 3 – 5 post infection, as determined by 2-way ANOVA. All statistical analyses were carried out using Graphpad Prism software.

Key to data symbols:

- Haemocytes of Control Lobsters
- ▲— Haemocytes of infected Lobster
- ◆- Plasma of Control Lobsters
- ▼- Plasma of Infected Lobsters

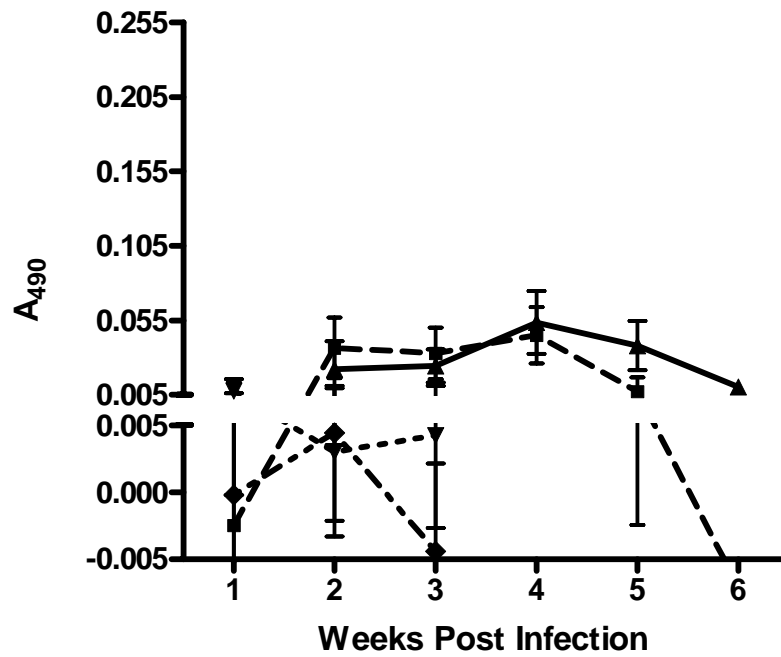


**Figure 6.4: Haemocyte associated PO activity in response to *E. coli* LPS.**

Data shown are the mean of 5 replicates. Error bars are the standard error about the mean. There was a significant ( $P < 0.05$ ) difference in PO activation noted for samples from control and infected lobsters at 6 weeks post infection, as determined by 2-way ANOVA. Statistical analysis was carried out using GraphPad Prism software. Background levels of PO activity were determined following addition of water *in lieu* of LPS or trypsin for each haemocyte sample. This estimate was then subtracted from the experimental estimates of PO activation obtained when LPS or trypsin was added to haemocyte suspensions to give the final PO activity registered due to the addition of the stimulant.

Key to data symbols:

- Haemocytes of Control Lobsters
- ▲- Haemocytes of infected Lobster
- ◆- Plasma of Control Lobsters
- ▼- Plasma of Infected Lobsters

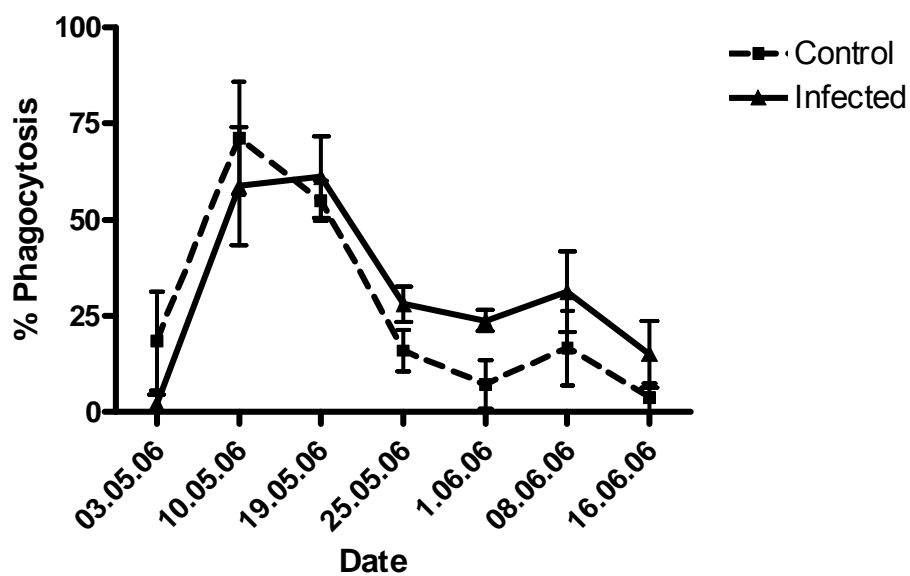


**Figure 6.5:** Haemocyte associated PO activity in response to *V. parahaemolyticus* L21 LPS.

Data shown are the mean of 5 replicates. Error bars are the standard error about the mean. There were no statistical differences ( $P < 0.05$ ) between the two treatment groups at any stage of the infection trial.

Key to data symbols:

- Haemocytes of Control Lobsters
- ▲— Haemocytes of infected Lobster
- ◆- Plasma of Control Lobsters
- ▼- Plasma of Infected Lobsters



**Figure 6.6: Phagocytosis of *V. parahaemolyticus* strain L21 by hyaline cells.**

Hyaline cells were separated from haemolymph recovered from control (uninfected) lobsters and lobsters infected with *V. parahaemolyticus* L21. Data shown are the mean of 5 replicates. Error bars are the standard error about the mean. There was no statistical difference between the two treatment groups at any stage of the trial, as determined by 2 - way ANOVA.





**Figure 6.7:** *In vitro* phagocytosis as demonstrated by fluorescence microscopy.

DAPI was used as a general fluorescence stain for DNA. The large circles are hyaline cell nuclei, the small dots (arrow) are phagocytosed bacterial cells within the cytoplasm of hyaline cells.

## Chapter 7: General Discussion

### 7.1 Introduction

Tail fan necrosis (TFN) is a disease that affects live-held southern rock lobsters in experimental live-holding facilities. Manifestation of symptoms typical of TFN represents a major constraint in the development of a live-holding industry in South Australia. The likely cause of TFN is infection of damaged uropod tissue by bacteria that are either present in the holding water column or associated with carapace surfaces. A previous unpublished study that demonstrated TFN lesion tissue is colonised by various *Vibrio* species provided good experimental evidence to support this hypothesis (May, 2002 B.Sc Honours Thesis, University of Adelaide). Indeed, that study showed unequivocally that damage to tail fan tissue by instruments contaminated by organisms isolated from tissue affected by TFN, resulted in formation of TFN-like lesions. Nevertheless, limitations associated with that study meant that:

- only lesions that formed 8 weeks post infection for lobsters held individually in temperature controlled aquaria conditions were examined,
- changes in culturable and non-culturable microbial community associated with TFN-like lesions were not identified and,
- the extent of damage to tail fan tissue by bacteria and the response of lobster immune cells to infection was not measured.

However, the presence of *V. parahaemolyticus* and *V. vulnificus* within diseased tissue was identified as a potential public health risk, particularly for food preparation facilities where live, uncooked or under-cooked lobsters are handled together with other foods without adequate food hygiene practices that limit cross contamination.

The purpose of work described in this thesis was to specifically examine the development and effect of TFN on the overall health of affected lobsters, as well as confirming that the *Vibrio* spp. involved in establishment of TFN may represent a public health risk. In particular, the aims of work described in this thesis were to:

1. Determine the cause of TFN and identify associated bacterial species.

2. Assess the microbial community dynamics within developing lesions associated with TFN.
3. Determine overall health affects of TFN on affected lobsters.
4. Investigate the immune response associated with lobsters affected by TFN.
5. Determine the health risks to consumers associated with southern rock lobsters affected by TFN if live-holding were to become common practice.

## **7.2 Role of Bacteria in Initiation and Development of TFN Lesions**

A requirement of the research described in this thesis was to confirm the role of marine vibrios in establishment of tail fan tissue infections that lead to establishment of TFN. To clarify the role of marine vibrios, an infection trial was used to establish whether (a) injury to tail fan tissue that results in tissue damage was sufficient to induce disease, or (b) infection of wounded/damaged tissue was required to establish TFN. Bacteriological and molecular methods were used to describe the types of bacteria (culturable and non-culturable) associated with developing lesions typical of TFN. These studies were complemented by a study of the histology of lesion development using light and electron microscopic methods to determine the extent of tissue damage and microbial invasion.

A key outcome of the described infection trial was that lobsters that had tail fans artificially infected at the time of damage had consistently higher prevalence of TFN. This confirmed the results of an earlier unpublished study (May, 2002). Thus, it is likely that wounding/damage and concomitant infection of damaged tissue is required for initiation of TFN lesions. However, studies by Getchall (1987) and Fontaine & Lightner (1972) indicated that wounds to the exoskeleton of crustaceans are rapidly sealed by an immune response. Therefore, there may be limited opportunity for micro-organisms to establish an infection within the softer layers of the cuticle beneath the carapace epicuticle and exocuticle, unless they are introduced at the time of damage. Indeed, that observation may well explain why TFN lesions are less likely to become established on tail fans where that tissue is wounded without concomitant deep tissue infection. Furthermore, as a consequence of the tail fan having little to no vascularisation, there is no opportunity for haemocytes to normally get direct access to the wound site. Therefore, it is likely that immune cells are detected degradation products from the gradual breakdown of the

carapace tissue and activates phenoloxidase in response in an attempt to create a seal around the wound. Because of this, it is unlikely that the haemocytes will encounter *V. parahaemolyticus* derived agonists and hence will not direct their response towards its presence.

Several different strategies were used to identify the types of bacteria associated with TFN lesions as well as assess any changes in the microflora that occurred during lesion development. Standard bacteriological identification methods were used to identify the *Vibrio* spp. isolated from two independent infection trials. In addition, sequence analysis of sections of 16S rDNA genes were used to independently validate the role of vibrios in TFN, as well as to better assess the culturable and non-culturable microflora. A critical outcome of this work was the finding that the type of marine vibrio used to infect damaged tail fan tissue was also the principle type isolated from developing lesions. For example, damaged tissue infected with a *V. parahaemolyticus* isolate from naturally occurring lesions was colonised primarily by that species of *Vibrio*. Given that the infecting species of *Vibrio* could apparently be isolated from lesions at different stages of development suggested that as a guiding principle, the infecting species is able to persist as a major component of lesions. Interestingly, *V. alginolyticus* also formed a significant fraction of the isolates of vibrios from developing lesions. That trend was consistent for the two independent infection trials described in this thesis and may be a reflection of the fact that *V. alginolyticus* was the most common isolate from tail fan surfaces of control lobsters. The fact that *Vibrio* spp. were isolated from lobster carapace surfaces and TFN lesions is consistent with work by Porter *et al.* (2001) on other lobsters. Put together, this evidence suggests that the source of the bacteria responsible for TFN, predominantly *Vibrio* spp., is the lobster carapace itself. Therefore, when lobsters damage their own, or others tail fans, the normal commensal bacteria on their carapace is likely to be introduced to the wound site at the time of damage. Establishment of a chronic, localized infection in tail fan tissue then leads to formation of TFN lesions.

Plate counts of homogenized lesion tissue on TCBS medium and Marine agar provided corroborating evidence to confirm that vibrios were the most common type of bacteria associated with lesions. Since counts of vibrios on TCBS were similar to total counts on Marine agar, vibrios accounted for a major part of the total culturable population

associated with the lobster carapace, as well as in association with TFN lesions. However, it is important to note that it is now accepted that pre-enrichment prior to plating onto TCBS may be necessary for detection of pathogenic vibrios (Desmarchelier, 2003). Therefore, the levels of these species in association with tail fan tissue may have been underestimated. As such, future work investigating TFN should follow procedures such as pre-enrichment in alkaline peptone water prior to determining vibrio numbers via the most probably number technique (Harwood *et al.*, 2004). However, detection of these species by direct plating on TCBS by growth at 30°C for 48 h instead of the standard 24 h was possible, and indeed, using this method, pathogenic vibrios were routinely detected in tail fan tissue samples. Furthermore, use of culture-independent techniques demonstrated that vibrios were the dominant type of bacteria associated with TFN lesions, confirming their role in the disease.

Nucleotide sequence analysis of clones of partial 16S rDNA obtained by PCR amplification of genomic DNA isolated from artificially induced lesions also showed that vibrios were the predominant type of bacteria present in TFN lesions. To ensure that non-culturable bacteria that might be associated with lesions were not excluded from the analysis, DGGE analysis of 16S rDNA amplicons confirmed that *Vibrio* spp. were the main types of bacteria present. 16S rDNA fragments consistent with *Psychrobacter* spp. were also identified as a minor component of the microflora of lesions. However, DGGE analysis of lesion tissue from lobsters 12 weeks post artificial infection with *V. parahaemolyticus* L21 showed those lesions to be dominated by *Vibrio*. In view of the corroborating evidence from each of four different approaches to microflora analysis of lesion associated bacteria, it seems reasonable to conclude that marine vibrios play an important role in establishment and development of TFN lesions.

The fact that bacteria isolated from naturally occurring lesions can be used to establish TFN-like lesions by simultaneous damage and infection of tail fan tissue with that isolate, unequivocally satisfies the first three guiding principles of Koch's postulates for infectious disease. Since species of vibrios that are the same as that used to initiate infection in trial animals are isolated from TFN lesions that develop in those experimental animals, it is implied, although not proven experimentally, that the data presented also satisfies Koch's fourth postulate. Conclusive proof will require experimental evidence that establishes that

the isolates from experimentally induced lesions are genetically identical to the infecting strain.

Future work should be undertaken to compare the 16S rDNA sequence data from 16S rDNA amplicon clones described in this thesis with that obtained from naturally induced TFN lesions associated with lobsters held in experimental sea based cages. That work would fill an important data gap and provide additional confirmation of the broader role of vibrios in establishment of TFN in wild caught and subsequently live-held rock lobsters. Although early data from work on TFN suggested that vibrios were the main bacteria associated with diseased tissue associated with lobsters held in sea-cages (Geddes *et al.* 2000), no work has previously been done to investigate the non-culturable population of naturally occurring lesions. In addition to the methods used in the present work, molecular probes, such as those described by Ward & Bej (2006) for detection of total and pathogenic *V. parahaemolyticus* and Panicker & Bej (2005) for detection of *V. vulnificus*, could be used to confirm the presence of pathogenic vibrios within TFN lesions.

Interestingly, counts of vibrios and total counts on Marine agar for samples of lesion tissue did not differ significantly for any of the infection trial treatment groups. One possible explanation for this observation is that lesion associated bacteria are restricted to the lesion surface and hence only small changes in numbers of bacteria are produced. TEM and SEM micrographs of sections of tissue support this view and show that the lesion associated organisms are present as micro-colonies on the lesion surface.

The majority of *Vibrio* isolates recovered from TFN-like lesions expressed extra-cellular degradative enzymes, including chitinase and lipase when cultured on suitable laboratory media. Significantly, these are considered the key enzymes required for bacteria to induce shell disease (Cipriani *et al.*, 1980; Cook & Lofton, 1973). Coupled with the fact that vibrios represent the principle component of lesion microflora, it is likely that these enzymes degrade the softer layers of the cuticle surrounding the periphery of lesions. Progressive destruction caused by secreted chitinase and lipase would eventually lead to a breach of the hard outer epicuticle. Indeed, micrographs of ultra-thin sections of lesion tissue show lesion-associated bacterial cells within pits on the surface of lesions, and the softer tissue underlying the uropod cuticle.

Although SEM and TEM of sections of tail fan lesion tissue identified a range of morphologically different types of bacterial cells, only a limited number of different genera of bacteria were detected by bacterial identification methods, nucleotide sequence analysis of 16S rDNA clones as well as DGGE analysis. This discrepancy may simply reflect the effects of environmental factors on rates of growth of bacteria and ultimately the morphology adopted under those growth conditions (Chaiyanan *et al.*, 2001, Tangwatcharin *et al.*, 2006, Wainwright *et al.*, 1999). In particular, *Psychrobacter* species are of a coccoid cellular morphology (Juni & Heym, 1986) and *Vibrio* cells that are normally short, fat, curved rods (Tantillo *et al.*, 2004) take on a coccoid morphology under temperature and nutritional stress (Chaiyanan *et al.*, 2001). Furthermore, this data, together with the bacterial growth experiments and 16S rRNA gene analysis of lesions, provided unequivocal proof that bacteria are associated with the onset and development of TFN. Indeed, the experiments described in thesis clearly demonstrated that bacteria are intimately involved in the establishment and progression of TFN. Whilst the growth experiments were biased towards the detection of vibrios, the analysis sequence data for 16S rDNA amplified from TFN-like lesions, by both sequencing of random clones and by DGGE analysis, demonstrated that these bacteria are likely to dominate the micro-flora associated with lesions.

### **7.3 Lobster Response to Infection**

Lobsters, like many other types of animals, are able to respond to infection using non-specific and specific mechanisms, although unlike mammals, they do not possess an adaptive immune mechanism. The aim of these immune mechanisms is to prevent systemic infection that might otherwise lead to loss of animal health and even death. Clearly, the carapace is the principle mechanism/barrier responsible for preventing access to the haemolymph, internal organs and other tissues of lobsters. Once this is breached however, humoral and cell mediated immune responses represent the next line of defense. The key processes involved in lobster immunity are phenoloxidase activation leading to melanisation (Cerenius & Söderhäll, 2004) and phagocytosis by haemocytes (Bachere *et al.*, 1995).

In view of the fact that establishment of TFN clearly involves a breach of the primary defense barrier (the carapace), followed by infection and colonization of wound tissue by bacteria, it is reasonable to expect some sort of humoral or cell mediated response to either the damage, the bacteria proliferating at the wound site, or both. Consequently, an important aim of this study was to determine firstly, whether an effective immune response mounted by affected lobsters is responsible for limiting infection to the surface of lesions typical of TFN and secondly, whether establishment of TFN affects the overall health of affected lobsters.

To investigate whether an immune response is mounted by lobsters affected by TFN, the level of phenoloxidase activation by specific (*V. parahaemolyticus* LPS) and non-specific (trypsin and *E. coli* LPS) agonists following artificial infection of tail fans versus control animals was assessed. The ability of enriched hyaline cells to phagocytose *V. parahaemolyticus* strain L21 opsonised with cell free haemolymph from infected and uninfected lobsters, was also determined. However, no activation of phagocytosis towards the infecting organism/s was observed. Those results indicated that the lobster immune system is probably unable to recognise the presence of an infection of the softer layers of the carapace. However, microscopic analysis of inflamed uropod tissue associated with lesions indicated that there was a recruitment of circulating haemocytes and other cells to affected tail fan tissue as a result of some form of immune activation.

Interestingly, no significant activation of haemolymph phenoloxidase by bacterial LPS was recorded. In view of this result, it is likely that non-specific phenoloxidase activation and deposition of melanin around the wound site occurred in response to degradation products associated with the enzymatic break down of the carapace rather than the bacteria-derived agonists. Indeed, this is likely to be the cause of inflammation associated with TFN, especially since underlying tissue is sterile with no evidence of bacterial contamination. The response may also explain why establishment of lesions does not lead to substantial changes in the number of bacteria associated with lesion tissue compared with counts on normal carapace tissue. The limited supply of freely available nutrients at the lesion site together with non-specific deposition of melanin to wall off the wound area, is therefore apparently sufficient to limit bacterial growth and prevent bacterial access to the haemolymph.



Microscopic analysis of developing lesions demonstrated that several morphologically different bacterial cell types colonise the surface of TFN lesions. Bacteria involved in infection are essentially restricted to the surface of the lesions, but where significant damage to the tail fan tissue occurs, these bacteria may invade the damaged tissue and penetrate deeper underlying tissue. Infection of tail fans results in inflammation and concomitant loss of internal structure of the tail fan and deposition of fibrous material within the soft tissue underlying the chitinous carapace. In cases of severe inflammation, a central core develops within the fibrous tissue consisting of a number of cell types, including hyaline cells, granulocytes and fibrocytes. However, there was no evidence of deep bacterial invasion into the underlying inflamed tissue. It was also noted during this study that minor lesions are resolved during moulting, whereas more severe lesions are maintained across more than one moult cycle.

Another key pathology associated with TFN is that in more severe cases, tail fans become inflamed. Bacteriological analysis and microscopy both demonstrated that the inflamed tissue is sterile. However, in severe cases of inflammation, a central core develops within the tail fan consisting of a range of lobster haemocytes and fibrocytes, characteristic of an immune response. Since the lobster immune system may not be able to recognise bacteria associated with lesions, and given that the inflamed tissue is effectively sterile anyway, it is likely that this response is due to the leaching of degradation products into the tissue adjacent to TFN lesions.

An assessment of the impact of TFN on the overall health of lobsters achieved by determining whether there was any correlation between the onset or severity of TFN and levels of bacteraemia, changes in serum protein levels or changes in circulating haemocytes. These indicators were selected because all have been previously described as markers of crustacean health (Vogan & Rowley, 2002). In contrast to responses in other reported shell diseases (Vogan *et al.*, 2001), onset and/or the severity of TFN was found not to lead to bacteraemia within the haemolymph of affected lobsters. Counts of culturable bacteria in the haemolymph of animals displaying TFN were no different than counts recorded for the haemolymph of control animals. This was also the case for serum protein levels and circulating haemocytes. This is in agreement with other studies involving shell diseases of crustaceans (Vogan and Rowley, 2002). Importantly, all total

haemocyte counts, as well as differential counts, were within the normal range described by other authors (Evans *et al.*, 1998; Stewart *et al.*, 1969). Consequently these results suggest that there is no effect of TFN on the overall health of affected animals. This also goes some way in explaining the lack of mortality associated with this disease, as a lack of bacteraemia prevents other organs such as the hepatopancreas and gills from succumbing to secondary infections.

## **7.4 Model of Events leading to TFN**

Based on the preceding discussion of the microbiology of naturally occurring and induced TFN lesions and associated immunological analysis, the following model of the sequence of events involved in TFN is proposed.

1. **Tail fans become damaged as a result of tears or puncture wounds inflicted by either their own or other lobster's leg and tail spines.** Tissue damage can occur during capture of southern rock lobsters, placement within high density tanks either on fishing vessels or in experimental holding cages. Tail fan wounds or tears can occur as a result of fighting, abrasion with other lobsters, or as a result of the catching process when lobsters flap their tails against contact surfaces.
2. **Damage is accompanied by simultaneous infection of deep tail fan tissue layers by either the normal commensal bacterial microflora of carapace surfaces or by bacteria from the water column or other marine surfaces.** Given the predominance of marine vibrios associated with the carapace surface of lobsters, these bacteria are likely to be involved in infection of damaged tissue, although other types of microorganisms may also be involved in the initiation of disease.
3. **Growth of bacteria on nutrients released from wound tissue leads to colonization of the wound.** As the bacteria become established in the softer underlying layers of the carapace, such as the endocuticle, they begin to secrete proteases and chitinase that assist progressive degradation of tail fan tissue around the wound site. The fact that most vibrios isolated from TFN lesions are able to degrade chitin and are able to secrete proteases lends support to this part of the model of disease.

4. **The lobster immune system responds in a non-specific manner to the wound damage and this response leads to melanisation of tissue surrounding the wound site.** This is characterised by non-specific activation of pro-phenoloxidase and infiltration of tail fan tissue by haemocytes leading to inflammation. In this way, the lobster immune system is able to restrict proliferation of bacteria at the wound site and prevent access to the haemolymph, and hence other organs such as the hepatopancreas and gills. This response is not directed towards the bacteria themselves, but rather the degradation products produced by their gradual destruction of the tail fan tissue leaching into the surrounding tissue. A lack of significant vascularisation is likely responsible for the non-specific nature of the response, as the haemocytes would have limited direct access to the wound site. Furthermore, the non-specific immune response, together with a lack of significant vascularisation within tail fan tissue, limits bacterial access to the haemolymph and internal organs. Thus the infection is largely restricted to superficial wound tissue.
5. **Expansion of the developing lesion is aided by progressive degradation of tail fan tissue at the lesion surface by bacteria.** As mentioned above, bacteria associated with TFN lesions are able to secrete chitinase and proteases that break down the carapace tissue. This in turn leads to progressively larger lesion size and tail fan erosion.
5. **Progression of lesion development leads to extensive inflammation of the tail fan tissue.** Inflammation is characterised by thickening of the carapace tissue and deposition of fibrous material within the tail fan. Severe inflammation also results in haemocyte infiltration into the tail fan. The haemocytes, together with fibrocytes, form a central core within the tail fan. As this inflamed tissue is sterile, it is likely that the inflammation represents a response to carapace degradation products that leach into the tissue surrounding the wound/lesion rather than a specific response to the bacteria responsible for the damage.

## 7.5 Public Health Significance of TFN

Practices that ensure that animals used for human food are free of contaminating pathogenic bacteria represent a cornerstone of quality control in the food industry. In the meat industry, animals that are diseased are excluded for use as food because of the risk to public health. Animals from aquaculture enterprises should be/are no exception to this rule. In view of the involvement of bacteria in the development of TFN in lobsters, it is proper that some assessment of the risk associated with the use of TFN affected lobsters for food is undertaken if live-holding of southern rock lobsters is to move into the commercial setting. The routine isolation of *V. parahaemolyticus* and *V. vulnificus* from TFN lesions associated with tail fan tissue is of particular concern. Both of these species of marine vibrio are known to be pathogenic for humans and other animals and have been implicated in infections resulting from consumption of contaminated seafood. However, not all isolates of these bacteria are necessarily pathogenic. Current thinking indicates that the broad majority of environmental isolates do not represent a clinically significant risk. However, most of the isolates obtained from lesions of TFN affected lobsters displayed many of the genotypic/phenotypic hallmarks of clinical isolates. A significant proportion of *V. vulnificus* isolates secreted capsular material widely accepted as the principle virulence determinant (Simpson *et al.*, 1987), in addition to carrying DNA encoding the *V. vulnificus* haemolysin (*vvh*) gene. Similarly, isolates of *V. parahaemolyticus* were *tdh*<sup>-</sup>, but importantly, were shown to be *trh*<sup>+</sup>. Whilst *tdh* is considered more common among clinical strains of *V. parahaemolyticus*, *trh* is also considered to be a marker of virulence for this organism as it is thought to function in much the same way and is also commonly found in clinical isolates, but very rarely in environmental isolates (Nishibuchi *et al.*, 1989, Shirai *et al.*, 1990). Further highlighting the potential pathogenicity of these isolates, the majority of these species were able to express cytolytins capable of lysing CHO cells. This data indicated that vibrios responsible for establishment of TFN may have potential to cause human infections and therefore lobsters with TFN lesions should be regarded as a potential health risk to consumers.

Consequently, if live-holding is to become a common practice, it is the author's view that great care will need to be taken when handling lobsters affected by TFN. Firstly, wounds infected with *V. vulnificus* may occur in people handling these lobsters prior to

processing. Furthermore, it is essential that proper food safety procedures are put in place when processing affected lobsters. This includes adequate cooking of lobsters to ensure total killing of the bacteria and to prevent potential for cross-contamination with foods that have already been prepared. Furthermore, it may also be necessary to restrict the sale of live-lobsters affected by TFN to prevent potential food chain contamination problems, particularly in over-seas markets where hygiene standards may be reduced and lobsters are often consumed raw.

Even though studies of TFN have involved a limited number of lobsters in experimental holding facilities, the fact that a number of potentially pathogenic vibrios were associated with the carapace tissue warrants further investigation of the wild fishery. Indeed, it may be of great value to the industry to conduct a longitudinal study to investigate the association of pathogenic vibrios with the carapace of wild stock. Such a study would necessarily involve a large sampling effort across the entire fishery at various time points throughout the fishing season. Ideally, such a study would also take into account factors which may affect detection of vibrios, such as VBNC state cells.

## **7.6 Potential Preventative Measures**

Live-holding of southern rock lobsters is not currently used in the South Australian lobster fishery, however it was identified as a potential means of post harvest value-adding of southern rock lobsters (*Jasus edwardsii*) through product enhancement. To date, the potential of open water suspended cages have been investigated for use in the Northern Fishing zone of South Australia, but TFN has been found to be a major constraint on the future potential of live-holding. Using the open water system, it is not possible to expose lobsters post harvest with treatments aimed at preventing the onset of TFN. Indeed, it may well be too late by that stage anyway, as damage to tail fans often occurs at the time of capture, or within wet wells of fishing vessels. As the results presented in this thesis suggest, concomitant infection at the moment of damage to tail fans is likely to be required for the onset of TFN. Importantly, the likely source of this infection is the lobster carapace itself. Although it has already been determined that immobilization by individually bagging lobsters immediately upon capture can significantly ( $p < 0.001$ ) reduce the incidence of TFN (Geddes *et al.*, 2000), attempts at automating the bagging process have

so far been unsuccessful and is not considered a viable option for fishers (Richard Musgrove, personal communication). Alternative methods to prevent the onset of TFN may include disinfection of the lobster carapace immediately after capture. In this way, although the lobsters are still likely to damage themselves and each other, there will be limited opportunity for subsequent infection allowing the immune system to adequately seal off the wound before an infection can take hold in the damaged carapace. Alternative approaches to achieve this are described below.

## 7.7 Future Directions

There are a range of possible mechanisms to reduce the incidence of shell disease in crustaceans. These include individually bagging lobsters in plastic mesh oyster spat bags upon capture. Geddes *et al.* (2001) have already shown that this approach can significantly ( $p < 0.001$ ) reduce the incidence of TFN. However, this mechanism could not be automated and hence was abandoned. Nevertheless, the key finding that limitation of damage to tail fans by preventing fighting etc within crowded impoundments within boat wet wells was sufficient to reduce the incidence of TFN. Consequently other approaches aimed at preventing damage to tail fans should be investigated. However, most of these would not be feasible in a sea cage system like the ones used in pilot studies in which large numbers of lobsters must be live-held in small spaces.

Nevertheless, there are at least two mechanisms worthy of investigation. The first involves an assessment of the affect of diet on incidence of TFN. A study by Prince *et al.* (1995) demonstrated that the incidence of shell disease could be reduced in winter-impounded American lobsters through the use of a better optimised diet. However, a field study by Bryars & Geddes (2005) found that regardless of feeding regime, TFN was identified as a serious outcome of live-holding of southern rock lobsters. Furthermore, Geddes *et al.* (2005) demonstrated that a frequent feeding regime during live holding leads to increases in the nutritional status of the surrounding water. Even with adequate flow of fresh seawater, the increased nutritional status of the water supported larger populations of bacteria associated with the surfaces of cages. In view of these findings, cleaner cage surfaces and concomitantly improved health of lobsters may be achieved through

optimized feeding as a means of minimizing growth of bacteria that may contribute to infection of damaged carapace tissues.

A second possibility involves an investigation of the use of chemical treatments that reduce the bacterial load on the carapace of freshly caught lobsters before infections can take hold. Fisher *et al.* (1988) were able to demonstrate that dipping lobster larvae in malachite green (20 ppm) for 8 min every other day, was sufficient to prevent onset of shell diseases. However, use of malachite green in any food product is now restricted to the point where detectable quantities within meat for example, is sufficient to prevent sale in Australia, USA, and most European and Asian countries. Consequently dye treatments are not currently a viable option.

However, sodium metabisulphite, a chemical already widely used by the prawn industry in South Australia for the reduction of black spot, has recently been shown to lower the survival of *V. cholerae* on prawns (Januario & Dykes, 2005). Thus, sodium metabisulphite dips could partially kill the natural micro-flora on the lobster carapace upon capture and disinfect wounded tissue to an extent that is sufficient to (a) reduce the bacterial load and (b) allow the lobster immune system sufficient time to seal wounds induced during the catching process.

Many methods for controlling microbial pathogens prevalent in other aquaculture systems are unlikely to be effective in the open sea-cages such as those proposed for live-holding southern rock lobsters. However, the implementation of closed cages on land could allow the introduction of such control measures as the use of probiotics, as seen in shrimp aquaculture (for a review, see Farzanfar, 2006). In this instance, other methods could also be investigated to control pathogenic bacteria associated with the carapace, such as the use of bacteriophage, which is being trialed in shrimp aquaculture (Pasharawipas *et al.*, 2005) and to control luminous vibriosis (Vinod *et al.*, 2006). However, this will not be possible without state and federal legislative changes that allow on-shore holding of live lobsters. Importantly, it was also observed by Geddes *et al.* (2005) that the normal feeding regime allows the build up of large populations of bacteria on the surfaces of tanks, despite adequate flow of fresh water within current model holding cages. Therefore, the use of materials that prevent biofilm formation within cages to reduce this bacterial build up within pens may also be beneficial.

The effective implementation of one or more of these methods may drastically reduce the incidence of TFN in live-held southern rock lobsters. This in turn will result in a greatly increased profit, as well as much safer product for the consumer that could potentially lead to the implementation of a live-holding industry. This will also have benefits for researchers attempting to close the life-cycle of southern rock lobsters for commercial scale aquaculture.

## 7.8 Overall Conclusions

1. TFN is caused by bacterial infection of superficially damaged lobster tail fan tissue initiated during commercial capture, by tail flapping or by fighting within crowded impoundments within boat wells.
2. The normal commensal bacteria associated with the lobster carapace and/or tail fan (predominantly *vibrios*), are likely to directly infect tissue at the time of damage, as the lobster itself is usually the source of such damage to tail fans.
3. Infection of wounded/damaged tissue at the time is likely to allow rapid growth of bacteria. Growth of these bacteria in wounds is likely to occur before the lobster immune system can effectively seal the wound.
4. Bacteria that gain access to wounded tissue are likely to degrade the tissue in a manner that leads to establishment of TFN lesions during experimental live-holding. Since vibrios isolated from lesions can express extra-cellular proteases and chitinases, these enzymes probably assist the bacteria to degrade uropod tissue.
5. A non-specific immune response is mounted due to the leaching of degradation products into the tissue surrounding the infection. This prevents the bacteria from gaining access to the haemolymph and other organs. This response is characterised by melanisation around the wound site. Furthermore, whilst this response is not directed towards the bacteria themselves, it does prevent the bacterial population from increasing beyond the levels of normal carapace commensal bacteria.
6. TFN lesions do not significantly impact on the overall health of lobsters.



7. Minor lesions can be resolved by moulting, however, more severe lesions may require subsequent moults before being resolved.
8. Severe lesions may also result in inflammation of the uropod. However, inflamed tissue is sterile and is likely to be a secondary response to the leaching of carapace degradation products into the surrounding tissue. Inflamed tissue is characterised by thickening of the uropod and deposition of fibrous matter within the uropod tissue. More severe inflammation also results in recruitment of haemocytes and fibrocytes not normally present within healthy uropod tissue.
9. Lobsters with TFN can present a potential health hazard to consumers due to the presence of potentially pathogenic strains of both *V. parahaemolyticus* and *V. vulnificus* within lesions.

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## Appendix A

### Sequence of Thermo-Labile Haemolysin PCR Amplicons

Typical PCR amplicon nucleotide sequence data of thermo-labile haemolysin from lobster isolates. Data cited in Chapter 3.3.2.1 (page 66) and Chapter 5.3.2 (page 133) of this thesis.

Nucleotide sequence data was aligned with *Vibrio parahaemolyticus* thermolabile hemolysin gene (GenBank Accession AY289609). Numbers refer to nucleotide base position relative to the first base of the *tlh* open reading frame of AY289609. The first 720 bases of AY289609 are not shown. Underlined sequence denotes forward and reverse oligonucleotide primers as described by Bej *et al.* (1999). Asterisks indicate nucleotide identity.

AY289609 TTTACGCTTGAGTTTGGTTTGAATGACTTCATGAACTACAACCGTGGCGTTCCAGAAGTG 780  
Amplicon -----

AY289609 AAAGCAGATTATGCAGAAGCACTGATTTCGTTTGACGGACGCAGGTGCGAAGAACTTCATG 840  
Amplicon -----ACTTCATG 8  
\*\*\*\*\*

AY289609 TTGATGACACTGCCAGACGCGACGAAAAGCGCCTCAGTTTAAAGTACTCAACACAAGAAGAG 900  
Amplicon TTGATGACACTGCCAGACGCGACGAAAAGCGCCTCAGTTTAAAGTACTCAACACAAGAAGAG 68  
\*\*\*\*\*

AY289609 ATCGACAAAATTCGTGCGAAAGTGCTTGAGATGAACGAGTTCATCAAGGCTCAAGCGATG 960  
Amplicon ATCGACAAAATTCGTGCGAAAGTGCTTGAGATGAACGAGTTCATCAAGGCTCAAGCGATG 128  
\*\*\*\*\*

AY289609 TACTACAAAGCGCAAGGTTACGACATCACGTTGTTTGATACTCACGCCCTTGTTTCGAGACG1020  
Amplicon TACTACAAAGCGCAAGGTTACAACATCACGTTGTTTGATACTCACGCCCTTGTTTCGAGACG 188  
\*\*\*\*\*

AY289609 CTAACTTCTGCGCCAGAAGAGCACGGTTTCGTGAACGCGAGCGATCCTTGTTTGGACATC1080  
Amplicon CTAACTTCTGCGCCAGAAGAGCACGGTTTCGTGAACGCGAGCGATCCTTGTTTGGACATC 248  
\*\*\*\*\*

AY289609 AACCGCTCATCGTCTGTGCGACTACATGTACACCCACGCATTGCGCTCTGAGTGTGCGGCG1140  
Amplicon AACCGCTCATCGTCTGTGCGATTACATGTACACCCACGCATTGCGCTCTGAGTGTGCGGCG 308  
\*\*\*\*\*

AY289609 TCTGGTGCTGAGAAGTTTGTGTTCTGGGATGTCACGCACCCAACAACAGCAACTCACCGC1200  
Amplicon TCTGGTGCTGAGAAGTTTGTGTTCTGGGATGTCACG----- 344  
\*\*\*\*\*

AY289609 TATGTTGCAGAGAAAATGCTAGAAAAGTAGCAAACAACCTTAGCCGAGTACCGTTTCTAA 1257  
Amplicon -----



## Appendix B

### Sequence of *Vibrio vulnificus* *vvh* PCR Amplicons

Typical PCR amplicon nucleotide sequence data of *Vibrio vulnificus* haemolysin from lobster isolates. Data cited in Chapter 3.3.2.2 (page 67) and Chapter 5.3.2 (page 133) of this thesis.

Nucleotide sequence data aligned with that of *Vibrio vulnificus* cytolysin (*vvhA*) gene (GenBank Accession Number AY046900). Numbers refer to nucleotide base position relative to the first base of the *vvhA* open reading frame of AY046900. Final 261 bp of AY046900 sequence are not shown. Underlined sequence denotes position of forward and reverse oligonucleotide primers as described by Panicker *et al.* (2004). Asterisks indicate nucleotide identity.

```
AY046900 GGCTGGGTATTTGATAAGACGAAGTTCAACCCTATCTCTTATTTCCAACCTCAAACCGAAC 60
Amplicon -----

AY046900 TATGACGTTTTGTACGAAGCGCCCGTGTCTGAAACCGGCGTAACGGATTTTGAGATGGGC 120
Amplicon -----CGCCC-TGTCTGAAACCGGCGTAACGGATTTTGAGATGGGC 40
                *****

AY046900 GTGAAACTCAACTATCGTGCACGCTTTGGTACCGTTCTTCCTTCAGCGCTGTTTTCGGTT 180
Amplicon GTGAAACTCAACTATCGTGCACGCTTTGGTACCGTTCTTCCTTCAGCGCTGTTTTCGGTT 100
                *****

AY046900 TACGGCTCTGCGGGCTCGTCAACCAACAGCAGTACTGTGAAACAACGTATTCGCATCGAC 240
Amplicon TACGGCTCTGCGGGCTCGTCAACCAACAGCAGTACTG----- 137
                *****

AY046900 TGGAATCACCCACTGTTTGAAGCGGAAGCACACGTTACACTACAGTCACTGAGCAACAAC 300
Amplicon -----
```

## Appendix C

### Characterisation of *V. parahaemolyticus* isolates

Data refers to isolates of *V. parahaemolyticus* isolated by May (2002) from a small scale infection trial. Data cited in Chapter 3.3.2.3 (page 68) of this thesis.

Isolate	<i>tlh</i> +/-	<i>trh</i> +/-	<i>tdh</i> +/-
<b>Control Group isolates<sup>1</sup></b>			
3NA2	+	-	-
<b><i>V. alginolyticus</i> Infection Group isolates</b>			
7NA1	+	-	-
8NA2	+	-	-
<b><i>V. parahaemolyticus</i> Infection Group isolates</b>			
13NA1	+	+	-
13NA2	+	+	-
13NA3	+	+	-
14NA1	+	+	-
14NA2	+	+	-
15NA1	+	+	-
15NA2	+	+	-
15NA3	+	+	-
15NA4	+	+	-
16NA1	+	+	-
16NA2	+	+	-
16NA3	+	+	-
16NA4	+	+	-
<b><i>V. alginolyticus</i> Infection Group isolates<sup>3</sup></b>			
20NA1	+	-	-
20NA2	+	-	-
20NA3	+	-	-
<b>TOTAL</b>	<b>19 (100%)</b>	<b>13 (68%)</b>	<b>0 (0%)</b>

<sup>1</sup> Control group isolates had uropods left undamaged

<sup>2</sup>*V. parahaemolyticus* infection group lobsters had uropods damaged with instruments inoculated with o/n culture of *V. parahaemolyticus* strain L21 recovered from naturally induced TFN lesion

<sup>3</sup>*V. alginolyticus* Infection Group 2 lobsters had uropods damaged with instruments inoculated with o/n culture of a *V. alginolyticus* strain recovered from naturally induced TFN lesion

## Appendix D

### Sequence of Thermo-Stable Direct Haemolysin-Related Haemolysin PCR Amplicons

Typical PCR amplification sequence data of thermo-stable direct haemolysin – related haemolysin from lobster isolates. Data cited in Chapter 3.3.2.3 (page 68) and Chapter 5.3.2 (page 133) of this thesis:

Nucleotide sequence data aligned with that of *Vibrio parahaemolyticus* strain GCSL28 thermostable direct hemolysin-related hemolysin-like () gene, (GenBank Accession number DQ359749). Numbers refer to nucleotide base position relative to the first base of the *trh1* open reading frame of DQ359749. First 240 bp and last 31 bp of DQ359749 not shown. Underlined sequences denote forward and reverse oligonucleotide primers as described by Tada *et al.* (1992). Asterisks indicate nucleotide identity.

```
DQ359749 TATTTTCACGACTTCAGGGCTCAAAATGGTTAAGCGCCTATATGACGGTAAATATTAATGG 300
Amplicon -----ATGG 4
                                     ****

DQ359749 AAATAACTACACAATGGCTGCTCTTTCTGGCTATAAAGATGGCCTTTC AACGGTCTTCAC 360
Amplicon AAATAACTACACAATGGCTGCTCTTTCTGGCTATAAAGATGGCCTTTC AACGGTCTTCAC 64
*****

DQ359749 AAAATCAGAAAAACAAGCCTAAATCAGAACTATTCTTCTGTTAGTGATTTTCGTTGGTGAA 420
Amplicon AAAATCAGAAAAACAAGCCTAAATCAGAACTATTCTTCTGTTAGTGATTTTCGTTGGTGAA 124
*****

DQ359749 AATGAAGAATCATTGCCAAGTGTAACGTATTTGGATGAAACGCCAGAATATTTTCGTCAAT 480
Amplicon AATGAAGAATCATTGCCAAGTGTAACGTATTTGGATGAAACGCCAGAAT----- 173
*****

DQ359749 GTCGAAGGCATATGAGAGCGGAAATGGGCATATGTTTGTATGTGTATTTCCAATAAATCA 540
Amplicon -----
```

## Appendix E

### Nucleotide Sequence of VCR-Associated PCR Amplicons

Typical PCR amplification sequence data of *Vibrio cholerae* repeat integron genes from lobster isolates. Data cited in Chapter 3.3.3 (page 70) and Figure 3.7 of this thesis.

#### Sequence of the 550 bp PCR fragment:

```
TTGTCCCTCTTGGAGGCGTTTGTTAGTCGCGAGGCACCGAGCGACAATTTTAAGCCGAACT
AAACTTTGCGAACAACAAACTTTATGTGCTTTGATGCATTTAAGTCAGGCGTAGTCTCAA
GCGGCTAGTCAATGAGACCACTTGGAAACAAACAATGTCCCAGAAAACGGACTGACAACACC
AAAAAGCGCTTTTGGAAAGCCAATCTCACTGTGGGGACTTTCAACAAAGTCACTGAAACCA
CGTGGAACTTACAACACCAGAGAAGACGTCATTTTCAGCGAGTAAATCGGCTTTTGTACCCA
ACAAACTCACCGAGCGGAGTTGCAACCCAAAACAGACTCTCGATTACCCTTCTCTATCAGC
ACCAATGGACTAAACAACCGCTAGGACAGTTGCAAAAACCTTTGGACTTGAAACAGTTGAAAC
TGAGTTTTGAGCATCGTTT
```

#### Summary of BLASTx comparison (Translated query vs protein database).

Sequences producing significant alignments <sup>1</sup>	Gene information	Score (Bits) <sup>2</sup>	E Value <sup>3</sup>
NP_798194.1	hypothetical protein VP1815 [ <i>Vibrio parahaemolyticus</i> RIMD 2210633]	159	7e-51
ABA55884.1	hypothetical protein [ <i>Vibrio</i> sp. DAT722]	132	4e-41
ABA55866.1	hypothetical protein [ <i>Vibrio</i> sp. DAT722]	152	2e-36
ABA55946.1	hypothetical protein [ <i>Vibrio</i> sp. DAT722]	102	8e-36
ABA55874.1	hypothetical protein [ <i>Vibrio</i> sp. DAT722]	96.7	3e-32
ABA55920.1	hypothetical protein [ <i>Vibrio</i> sp. DAT722]	133	1e-30
ABA55903.1	hypothetical protein [ <i>Vibrio</i> sp. DAT722]	113	1e-24
ABA55865.1	hypothetical protein [ <i>Vibrio</i> sp. DAT722]	110	8e-24
ABA55919.1	hypothetical protein [ <i>Vibrio</i> sp. DAT722]	80.9	7e-22
ZP_01260474.1	hypothetical protein V12G01_21103 [ <i>Vibrio alginolyticus</i> 12G01]	95.9	3e-19

### Sequence of the 430 bp PCR fragment:

TTTTGCAAACAACAAATTTTATGTGCTTTTGATGCATTTAAGTCAGGCGCAGTCTCAAAGCG  
GCTACTCAATGAAACCACTTAGAACAAACAACGCCGTAGAAAACGGAGTGGCAACACCAAAA  
AAGCGCTTTTGGAAAACCAATCTCACAGTGC GGACTTTCAACAAAGTCACTGAAACCACGT  
GGAACCTTACCACATCAGAGAAAAAGTCACTTCAGCGAGTAAATCGGCTTTTGTACCAACAA  
ACTCACCGAGCGGAGTTGCAACCAAAAAGCAAACCTCTCGATTGCCCTGCTATGTCAGCACCA  
AAGAATAAAACAACCACGAGGACACTTGCAAAACTTTGGACTTAAAACAGCTGAAA

### Summary of BLASTx comparison (Translated query vs protein database).

Sequences producing significant alignments <sup>1</sup>	Gene information	Score (Bits) <sup>2</sup>	E Value <sup>3</sup>
ABA55866.1	hypothetical protein [ <i>Vibrio</i> sp. DAT722]	159	7e-51
ABA55884.1	hypothetical protein [ <i>Vibrio</i> sp. DAT722]	132	4e-41
NP_798194.1	hypothetical protein VP1815 [ <i>Vibrio parahaemolyticus</i> RIMD 2210633]	152	2e-36
ABA55920.1	hypothetical protein [ <i>Vibrio</i> sp. DAT722]	102	8e-36
ABA55874.1	hypothetical protein [ <i>Vibrio</i> sp. DAT722]	96.7	3e-32
ABA55946.1	hypothetical protein [ <i>Vibrio</i> sp. DAT722]	133	1e-30
ABA55903.1	hypothetical protein [ <i>Vibrio</i> sp. DAT722]	113	1e-24
ABA55865.1	hypothetical protein [ <i>Vibrio</i> sp. DAT722]	110	8e-24
ABA55919.1	hypothetical protein [ <i>Vibrio</i> sp. DAT722]	80.9	7e-22
ABA55884.1	hypothetical protein [ <i>Vibrio</i> sp. DAT722]	87.8	7e-17

<sup>1</sup> GenBank Accession number

<sup>2</sup> An indication of how good the alignment is; the higher the score, the better the alignment.

<sup>3</sup> An indication of the statistical significance of a given pairwise alignment and reflects the size of the database and the scoring system used.

## Appendix F

### Water Flow Rates for Tanks Used in the Infection Trial

Trial Date 14.05.04 to 28.07.04 as per Chapter 4.4.1 (page 87).

<b>Tank Number</b>	<b>Water Flow Rate</b>
--------------------	------------------------

1	6.6 L min <sup>-1</sup>
2	7.1 L min <sup>-1</sup>
3	8.0 L min <sup>-1</sup>
4	6.6 L min <sup>-1</sup>
5	6.0 L min <sup>-1</sup>
6	7.2 L min <sup>-1</sup>
7	6.9 L min <sup>-1</sup>
8	8.5 L min <sup>-1</sup>
9	7.3 L min <sup>-1</sup>

Flow rate measurements were taken at the commencement of the trial and were not altered for the remainder of the trial. The mean flow rate was 7.13 L min<sup>-1</sup> with a standard error about the mean of 2.517 (n = 9).

## Appendix G

### Dissolved oxygen levels for Tanks Used in the Infection Trial.

Trial date 14.05.04 to 28.07.04 as per Chapter 4.4.1 (page 87).

<b>Tank</b>	<b>Dissolved Oxygen</b>
-------------	-------------------------

1	11.9 ppm
---	----------

2	11.3 ppm
---	----------

3	10.5 ppm
---	----------

4	11.7 ppm
---	----------

5	11.1 ppm
---	----------

6	11.8 ppm
---	----------

7	12.5 ppm
---	----------

8	11.5 ppm
---	----------

9	11.5 ppm
---	----------

Dissolved oxygen levels were measured on 15.07.04. Further measurements were not possible due to the equipment not being available. The dissolved oxygen level was 11.53 ppm with a standard error about the mean of 0.1856 (n = 9).

## Appendix H

### Lobster carapace length and weight.

Infection trial dated 14.05.04 to 28.07.04 as per Chapter 4.3 (page 85).

Lobster	Treatment	Sample Date	CL(mm)		Weight (g)	
			(t = 0 weeks)	(t = 0 weeks)	(t = 4, 8 or 12 weeks)	(t = 4, 8 or 12 weeks)
T1LB	1	9.6.04	113	719.1	112	718.9
T1RB	1	9.6.04	112	690.5	111	685.2
T1LW	1	9.6.04	104	576.7	104	557.5
T1RW	1	9.6.04	109	647.2	105	646.9
T1-	1	9.6.04	105	590.7	105	593.9
T2LB	1	7.7.04	104	516.4	112	522.2
T2RB	1	7.7.04	107	607.4	112	618
T2LW	1	7.7.04	111	684.1	114	699.9
T2RW	1	7.7.04	109	598.9	109	607.3
T2-	1	7.7.04	104	570.7	104	577.9
T3LB	1	28.7.04	115	678.1	114	690.2
T3RB	1	28.7.04	109	604.5	106	615.6
T3LW	1	28.7.04	110	668.8	109	691.6
T3RW	1	28.7.04	106	574.9	103	587.1
T3-	1	28.7.04	112	651	110	660.9
T4LB	2	9.6.04	119	774.8	117	783
T4RB	2	9.6.04	110	540.6	109	640.6
T4LW	2	9.6.04	117	755.8	116	756.8
T4RW	2	9.6.04	107	579	110	570.2
T4-	2	9.6.04	106	535.5	104	535.8
T5LB	2	7.7.04	115	752.5	103	533.4
T5RB	2	7.7.04	114	722.9	113	722
T5LW	2	7.7.04	105	525	115	763.7
T5RW	2	7.7.04	115	770.5	115	784.7
T5-	2	7.7.04	110	673.8	110	692.2
T6LB	2	28.7.04	114	711.6	113	733.5
T6RB	2	28.7.04	110	644.4	111	650.5
T6LW	2	28.7.04	112	711.1	110	727.5
T6RW	2	28.7.04	105	591.2	105	599.5
T6-	2	28.7.04	110	616	109	620.6



T7LB	3	9.6.04	110	646.9	111	656
T7RB	3	9.6.04	117	804.3	118	821
T7LW	3	9.6.04	117	787	118	787.1
T7RW	3	9.6.04	115	694.9	114	697.3
T7-	3	9.6.04	122	887.1	123	881.3
T8LB	3	7.7.04	115	758.8	115	754.1
T8RB	3	7.7.04	114	764.4	118	908.8
T8LW	3	7.7.04	109	617.1	109	681.1
T8RW	3	7.7.04	103	572.3	103	575.9
T8-	3	7.7.04	113	809.1	116	809.2
T9LB	3	28.7.04	105	548.9	104	560.8
T9RB	3	28.7.04	104	593.2	103	599.9
T9LW	3	28.7.04	108	605.3	118	623.3
T9RW	3	28.7.04	111	727.9	112	738.8
T9-	3	28.7.04	104	507.7	104	526.9

## Appendix I

### Total viable counts of bacteria in lobster haemolymph.

Data refers to Chapter 4.4.4, page 89.

Total viable counts per mL of lobster haemolymph on Marine Agar

G1W4	310.	440.	<10.	<10.	<10.
G1W8	<10.	<10.	<10.	<10.	<10.
G1W12	20.	60.	20.	<10.	<10.
G2W4	30.	2030.	380.	<10.	<10.
G2W8	<10.	<10.	<10.	<10.	<10.
G2W12	<10.	<10.	<10.	<10.	<10.
G3W4	1000.	70.	50.	10.	80.
G3W8	60.	20.	<10.	<10.	<10.
G3W12	30.	<10.	<10.	<10.	<10.

Total Vibrio counts per mL of lobster haemolymph on TCBS

G1W4	<10.	<10.	<10.	<10.	<10.
G1W8	20.	60.	10.	40.	<10.
G1W12	500.	210.	120.	20.	<10.
G2W4	10.	<10.	<10.	<10.	<10.
G2W8	90.	10.	30.	<10.	<10.
G2W12	20.	130.	10.	<10.	<10.
G3W4	<10.	1520.	<10.	<10.	<10.
G3W8	1520.	<10.	<10.	<10.	<10.
G3W12	210.	930.	10.	180.	10.

Counts shown are CFU per mL on both marine agar and TCBS. Counts of <10 were below the detectable level for this assay. These values contributed to the statistical analysis being inconclusive.

## Appendix J

**Relative percentages of *Vibrio* isolates for the infection trial dated 14.05.04 to 28.07.04.**

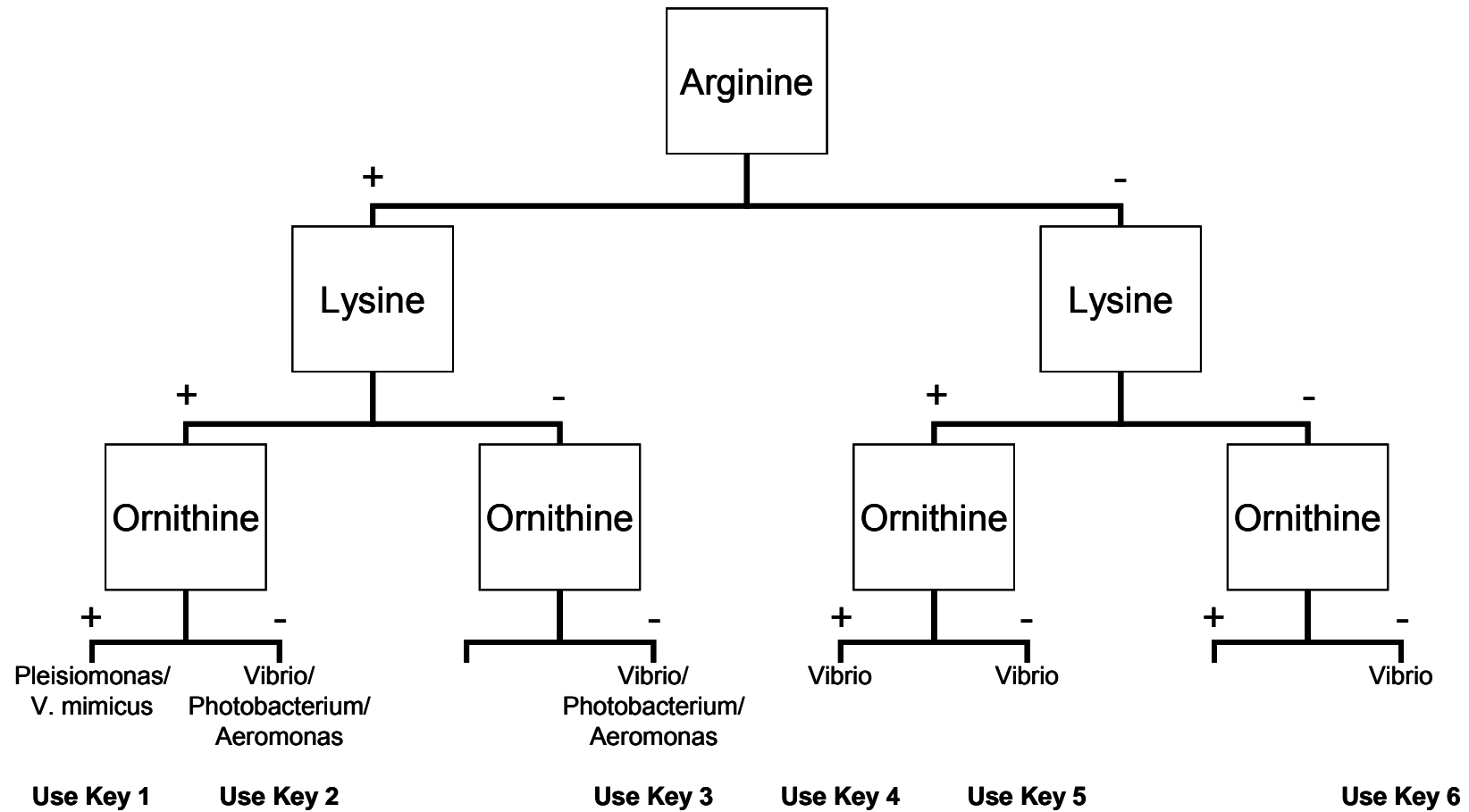
Group	<i>V. parahaemolyticus</i>	<i>V. vulnificus</i>	<i>V. alginolyticus</i>	Other <i>Vibrio</i> spp.
Group I, Wk 4 <i>p.i.</i>	29%	0%	29%	42%
Group II Wk 4 <i>p.i.</i>	0%	25%	37%	37%
Group III Wk 4 <i>p.i.</i>	70%	0%	30%	0%
Group I Wk 8 <i>p.i.</i>	0%	0%	63%	37%
Group II Wk 8 <i>p.i.</i>	14%	29%	42%	14%
Group III Wk 8 <i>p.i.</i>	90%	0%	10%	0%
Group I Wk 12 <i>p.i.</i>	33%	8%	17%	42%
Group II Wk 12 <i>p.i.</i>	0%	25%	67%	8%
Group III Wk 12 <i>p.i.</i>	100%	0%	0%	0%

## **Appendix K**

### **Identification keys for *Vibrio* spp.**

The following sets of keys for biochemical identification of environmental *Vibrio* spp. have been reproduced from Alsina & Blanch (1994).

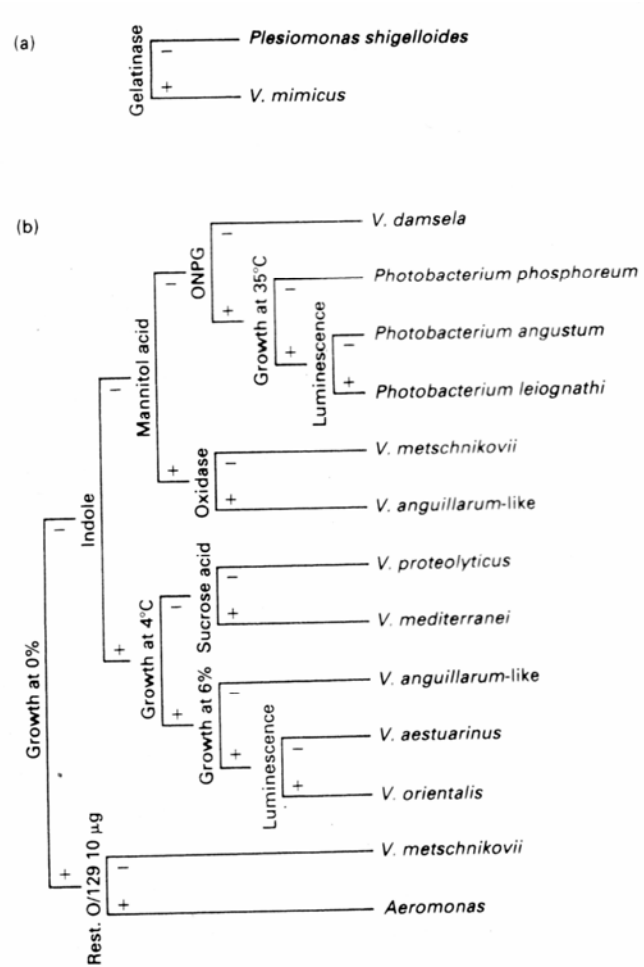
The ID code and identity of lobster isolates determined using these keys in Chapter 4 of this thesis are arranged in tables at the end of this Appendix.



Primary key to differentiate *Vibrio* spp. based on arginine dihydrolase, lysine decarboxylase and ornithine decarboxylase reactions. Key redrawn from Alsina & Blanch (1994).

Key 1

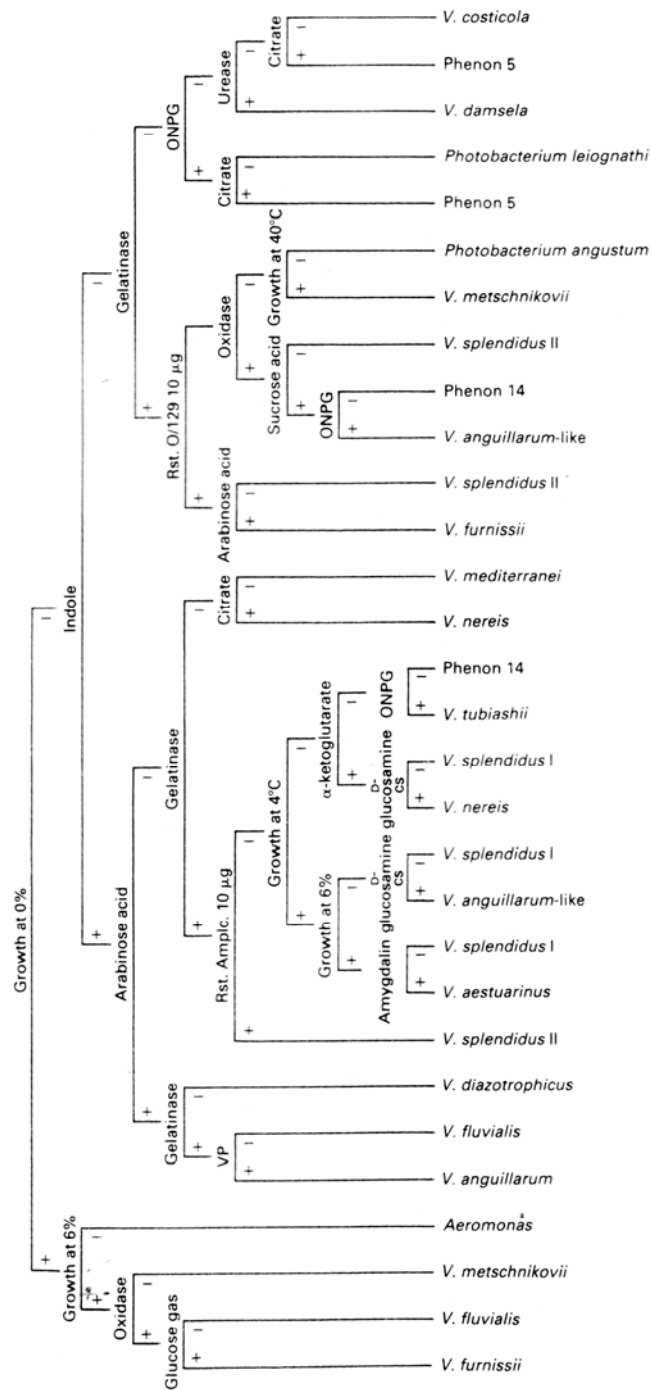
Key 2



**Identification Keys 1 and 2.**

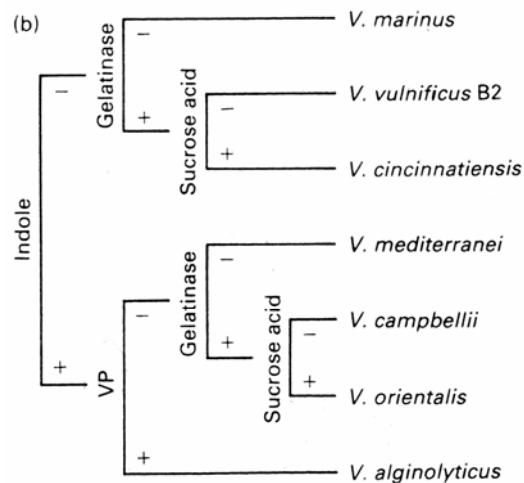
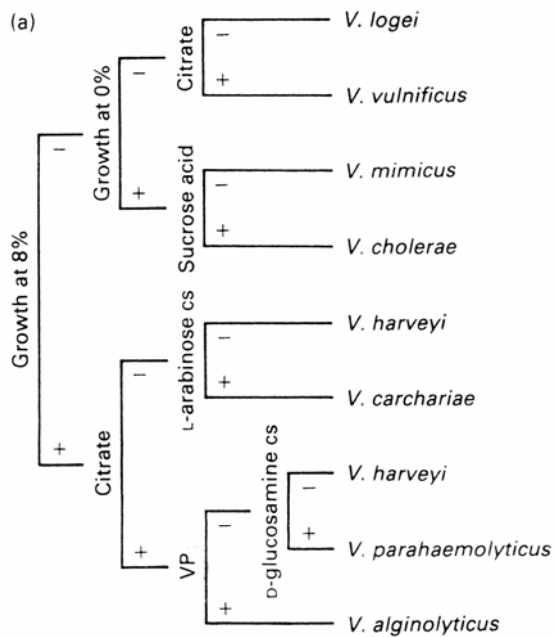
(a) Key 1: Cluster Arginine dihydrolase +ve, Lysine decarboxylase +ve, Ornithine decarboxylase +ve

(b) Key 2: Cluster Arginine dihydrolase +ve, Lysine decarboxylase +ve, Ornithine decarboxylase +ve



**Identification Key 3:**

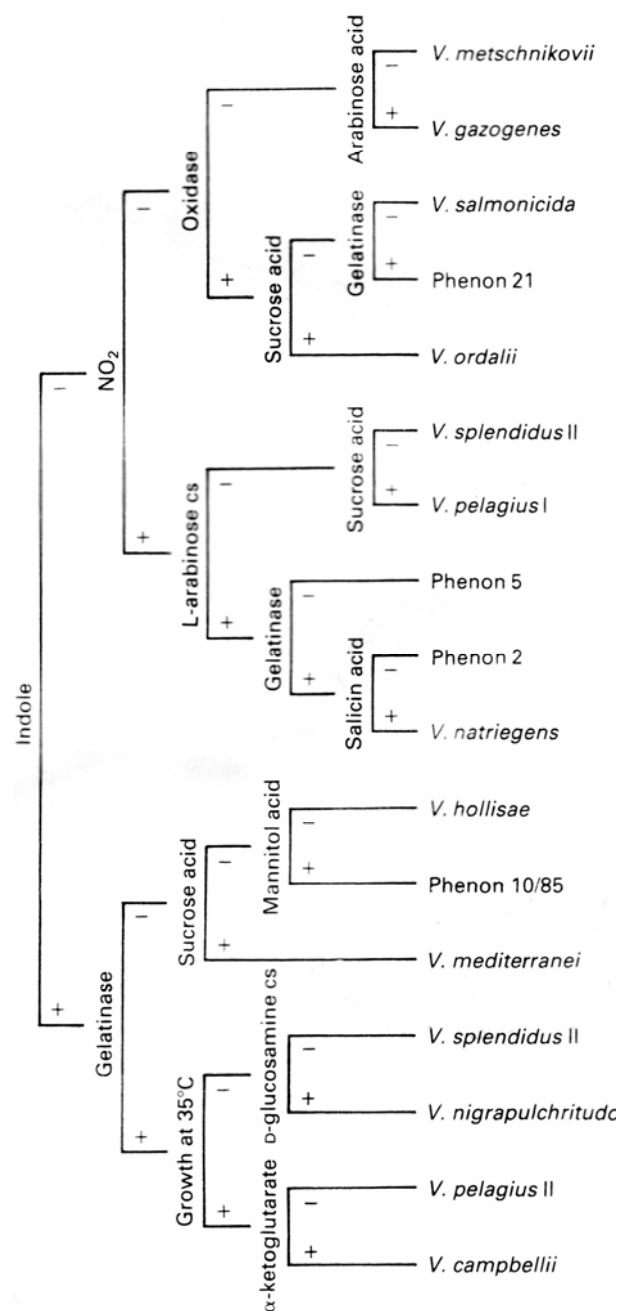
Cluster is Arginine dihydrolase +ve, Lysine decarboxylase -ve, Ornithine decarboxylase -ve.



#### Identification Keys 4 and 5

(a) Cluster is Arginine dihydrolase -ve, Lysine decarboxylase +ve, Ornithine decarboxylase +ve.

(b) Cluster is Arginine dihydrolase -ve, Lysine decarboxylase +ve, Ornithine decarboxylase -ve.



#### Identification Key 6

Cluster is Arginine dihydrolase -ve, Lysine decarboxylase -ve, Ornithine decarboxylase -ve.



## ID and Identity of lobster isolates

Isolates identified using Key 1. Isolates are arranged by Infection Trial Groupings.

Key 1	Lobster Group I	Lobster Group II	Lobster Group III
	<i>V. mimicus</i> T3-2NAC		

Isolates identified using Key 2. Isolates are arranged by Infection Trial Groupings.

Key 2	Lobster Group I	Lobster Group II	Lobster Group III
	<i>V. metschnikovii</i> T3-47	<i>V. damsela</i> T4LBNA48	

### Key to isolate identifier symbols: Scheme Tn(L/R/-)(B/W)(NA/A)nn

Tn = Tank number;

L = left antenna; R = right antenna; B = Lobster black tag identifier; W = Lobster white tag identifier; - = no lobster identifier.

NA = sucrose -ve on TCBS; A = sucrose +ve on TCBS;

nn = an isolate identifier.

**Isolates identified using Key 3. Isolates are arranged by Infection Trial Groupings.**

<b>Key 3</b>	<b>Lobster Group I</b>	<b>Lobster Group II</b>	<b>Lobster Group III</b>
	<i>V. costicola</i> T1LBNA1	<i>V. fluvialis</i> TT4LBNA3	
	<i>V. damsela</i> T1LBNA3	<i>V. fluvialis</i> T5RWNA4	
	<i>V. mediterranei</i> T1LBNA19, T2LB10	<i>V. mediterranei</i> T5RWNA44	
	<i>V. damsela</i> T3RBNAC		

**Key to isolate identifier symbols: Scheme Tn(L/R-)(B/W)(NA/A)nn**

Tn = Tank number;

L = left antenna; R = right antenna; B = Lobster black tag identifier; W = Lobster white tag identifier; - = no lobster identifier.

NA = sucrose -ve on TCBS; A = sucrose +ve on TCBS;

nn = an isolate identifier.

Isolates identified using Key 4. Isolates are arranged by Infection Trial Groupings.

Key 4	Lobster Group I	Lobster Group II	Lobster Group III
	<p><i>V. alginolyticus</i></p> <p>T1LBA16, T1LBA11, T2-A13, T2-A14, T2-A45, T2LBA47, T3RBA1, T3RBA1, T3RBAA, T3RBAB, T3LW48, T3RBA14, T3LB4, T3LB47 T3RW24, T3RBA27, T3RBA50</p>	<p><i>V. alginolyticus</i></p> <p>T5RBA34, T5RBA35, T5RWA23 T6LWA19, T6LWA17, T6LBA5, T6LBA12, T6LBA26, T6LBA42, T6LWA19, T6LWA21</p>	<p><i>V. alginolyticus</i></p> <p>T7LWA32, T7LWNA3, T7LWA3, T7LWA4, T7RBA3, T7RBA4, T7RBA7, T8RWNA47</p>
	<p><i>V. parahaemolyticus</i></p> <p>T3RB34, T3-2AA, T3LW19, T3LW33</p>	<p><i>V. parahaemolyticus</i></p> <p>T5RBNA17</p>	<p><i>V. parahaemolyticus</i></p> <p>T7LWNA40, T7RBNA36, T7RBNA22, T8-NA1, T8-A33, T8-NA24, T8-NA17, T8-NA18, T8RWNA6, T8RWNA19, T8RWNA36, T8RWNA27, T9LWNA1, T9LWNA2, T9LWNA19, T9LWNA27, T9LWNA44, T9RWNA3, T9RWNA12, T9RWNA24, T9RWNA34, T9RWNA39</p>
	<p><i>V. fluvialis</i></p> <p>T3LW39</p>	<p><i>V. vulnificus</i></p> <p>T6LWNA45, T6LWNA34, T6LWNA14, T5LWNA48</p>	
		<p><i>V. campbellii</i></p> <p>T4-NA5</p>	
		<p><i>V. harveyi</i></p> <p>T6LBNA49</p>	

**Isolates identified using Key 5. Isolates are arranged by Infection Trial Groupings.**

Key 5	Lobster Group I	Lobster Group II	Lobster Group III
	<i>V. alginolyticus</i> T2-A5	<i>V. alginolyticus</i> T4-A18, T4-A27, T4-A40	
	<i>V. vulnificus</i> B2 T3-NA13	<i>V. vulnificus</i> B2 T5RWNA27, T4-NA2, T4-NA7	

**Isolates identified using Key 6. Isolates are arranged by Infection Trial Groupings.**

Key 6	Lobster Group I	Lobster Group II	Lobster Group III
	<i>V. splendidus</i> T3LBNA36	<i>V. marinus</i> T4LBNA34	

**Key to isolate identifier symbols: Scheme Tn(L/R/-(B/W)(NA/A)nn**

Tn = Tank number;

L = left antenna; R = right antenna; B = Lobster black tag identifier; W = Lobster white tag identifier; - = no lobster identifier.

NA = sucrose -ve on TCBS; A = sucrose +ve on TCBS;

nn = an isolate identifier.

## Appendix L

### Haemolysin genotype of *V. parahaemolyticus* isolates.

Data cited in Chapter 5 of this thesis.

Isolate Source and Isolate ID	<i>tlh</i> +/-	<i>trh</i> +/-	<i>tdh</i> +/-
<b>Group I</b>			
T3RB34	+	-	-
T3-2AA	+	-	-
T3LW19	+	-	-
T3LW33	+	-	-
<b>Group II</b>			
T5RBNA17	+	-	-
<b>Group III</b>			
T7LWNA40	+	-	-
T7RBNA36	+	-	-
T7RBNA22	+	-	-
T8-NA1	+	-	-
T8-A33	+	+	-
T8-NA24	+	+	-
T8-NA17	+	-	-
T8-NA18	+	-	-
T8RWNA6	+	-	-
T8RWNA19	+	-	-
T8RWNA36	+	+	-
T8RWNA27	+	-	-
T9LWNA1	+	+	-
T9LWNA2	+	-	-
T9LWNA19	+	+	-
T9LWNA27	+	+	-
T9RWNA3	+	+	-
T9LWNA44	+	+	-
T9RWNA12	+	-	-
T9RWNA24	+	+	-
T9RWNA34	+	+	-
T9RWNA39	+	+	-
<b>TOTAL</b>	<b>27 (100%)</b>	<b>11 (41%)</b>	<b>0 (0%)</b>

## Appendix M

### Identity codes for cloned 16S rDNA sequences

Data cited in Figure 5.5 (page 154) of Chapter 5 of this thesis. Sequence data for each clone is presented in a FASTA formatted file on the CD accompanying this thesis.

<i>Vibrio</i> Group A		<i>Vibrionaceae</i>	<i>Psychrobacter</i>
G1S1W4C1	G1S1W4C2	G2S1W4C12	G1S1W4C5
G1S1W4C4	G1S1W4C3	G2S1W4C14	G1S2W4C6
G1S2W4C7	G1S2W4C8	G2S1W4C15	G1S2W4C9
G1S2W4C10	G2S2W4C16	G1S3W8C31	G2S1W4C11
G2S2W4C17	G2S2W4C18	G3S4W8C59	G2S1W4C13
G2S2W4C19	G2S2W4C20	G3S4W8C60	G1S3W8C32
G3S1W4C21	G3S1W4C22		G1S3W8C33
G3S1W4C23	G3S1W4C24		G1S3W8C34
G3S1W4C25	G3S2W4C26		G1S3W8C35
G3S2W4C27	G3S2W4C28		G1S4W8C37
G3S2W4C29	G3S2W4C30		G2S4W8C50
G1S4W8C36	G1S4W8C38		G1S5W12C64
G1S4W8C39	G1S4W8C40		G1S5W12C65
G2S3W8C41	G2S3W8C42		G1S6W12C66
G2S3W8C43	G2S3W8C44		G1S6W12C67
G2S3W8C45	G2S4W8C46		G1S6W12C68
G2S4W8C47	G2S4W8C48		
G3S4W8C58	G1S5W12C61		
G1S5W12C62	G1S5W12C63		
G1S6W12C69	G1S6W12C70		
G2S5W12C71	G2S5W12C72		
G2S5W12C73	G2S5W12C74		
G2S5W12C75	G2S6W12C76		
G2S6W12C77	G2S6W12C78		
G2S6W12C79	G2S6W12C80		
G3S6W12C86	G3S6W12C89		

#### Key to amplicon identifier symbols: GxSxWxCx

Gx: Group Number tissue sample was harvested from

Sx: Sample number for that group

Wx: Week of trial that tissue sample was harvested

Cx: Clone number

## Appendix N

### Identity codes for 16S rDNA amplicons separated by DGGE.

Data cited in Figure 5.7 (page 156) in Chapter 5 of this thesis. Sequence data for each clone is presented in a FASTA formatted file on the accompanying CD.

<i>Vibrio</i> Group A		Vibrionaceae	<i>Psychrobacter</i>
G1W4B1	G2W4B4	G1W8B2	G1W4B7
G1W4B2	G2W8B2	G2W4B1	G1W8B7
G1W4B3	G2W8B3	G2W8B1	G1W12B6
G1W4B4	G2W8B4	G2W12B2	G2W8B6
G1W4B5	G2W8B5		G2W12B7
G1W4B6	G2W12B1		
G1W8B1	G2W12B3		
G1W8B3	G2W12B4		
G1W8B4	G2W12B5		
G1W8B5	G2W12B6		
G1W8B6	G3W4B1		
G1W12B1	G3W4B2		
G1W12B2	G3W8B1		
G1W12B3	G3W8B2		
G1W12B4	G3W8B3		
G1W12B5	G3W8B5		
G2W4B2	G3W12B1		
G2W4B3			

### Key to amplicon identifier symbols: GxWxBx

Gx: Group Number tissue sample was harvested from

Wx: Week of trial that tissue sample was harvested

Bx: Band number

## Appendix O

### Cloned and DGGE 16S rRNA nucleotide sequence identity codes.

Cloned and DGGE 16S rRNA nucleotide sequence identity codes, as per Figure 5.8 (page 157) in Chapter 5 of this thesis. Nucleotide sequences corresponding to each code is located on the accompanying CD.

<i>Vibrio</i> Group A	Vibrionaceae	<i>Psychrobacter</i>	
G1S1W4C1	G2S5W12C75	G2S1W4C12	G1S1W4C5
G1S1W4C2	G2S6W12C76	G2S1W4C14	G1S2W4C6
G1S1W4C3	G2S6W12C77	G2S1W4C15	G1S2W4C9
G1S1W4C4	G2S6W12C78	G1S3W8C31	G2S1W4C11
G1S2W4C7	G2S6W12C79	G3S4W8C59	G2S1W4C13
G1S2W4C8	G2S6W12C80	G3S4W8C60	G1S3W8C32
G1S2W4C10	G3S6W12C86	DGGE-G1W8B2	G1S3W8C33
G2S2W4C16	G3S6W12C89	DGGE-G2W4B1	G1S3W8C34
G2S2W4C17	DGGE-G1W4B1	DGGE-G2W8B1	G1S3W8C35
G2S2W4C18	DGGE-G1W4B2	DGGE-G2W12B2	G1S4W8C37
G2S2W4C19	DGGE-G1W4B3		G2S4W8C50
G2S2W4C20	DGGE-G1W4B4		G1S5W12C64
G3S1W4C21	DGGE-G1W4B5		G1S5W12C65
G3S1W4C22	DGGE-G1W4B6		G1S6W12C66
G3S1W4C23	DGGE-G1W8B1		G1S6W12C67
G3S1W4C24	DGGE-G1W8B3		G1S6W12C68
G3S1W4C25	DGGE-G1W8B4		DGGE-G1W4B7
G3S2W4C26	DGGE-G1W8B5		DGGE-G1W8B7
G3S2W4C27	DGGE-G1W8B6		DGGE-G1W12B6
G3S2W4C28	DGGE-G1W12B1		DGGE-G2W8B6
G3S2W4C29	DGGE-G1W12B2		DGGE-G2W12B7
G3S2W4C30	DGGE-G1W12B3		
G1S4W8C36	DGGE-G1W12B4		
G1S4W8C38	DGGE-G1W12B5		
G1S4W8C39	DGGE-G2W4B2		
G1S4W8C40	DGGE-G2W4B3		
G2S3W8C41	DGGE-G2W4B4		
G2S3W8C42	DGGE-G2W8B2		
G2S3W8C43	DGGE-G2W8B3		
G2S3W8C44	DGGE-G2W8B4		
G2S3W8C45	DGGE-G2W8B5		
G2S4W8C46	DGGE-G2W12B1		
G2S4W8C47	DGGE-G2W12B3		
G2S4W8C48	DGGE-G2W12B4		
G3S4W8C58	DGGE-G2W12B5		



Appendix O continued.

	<i>Vibrio</i> Group A	Vibrionaceae	<i>Psychrobacter</i>
G1S5W12C61		DGGE-G2W12B6	
G1S5W12C62		DGGE-G3W4B1	
G1S5W12C63		DGGE-G3W4B2	
G1S6W12C69		DGGE-G3W8B1	
G1S6W12C70		DGGE-G3W8B2	
G2S5W12C71		DGGE-G3W8B3	
G2S5W12C72		DGGE-G3W8B5	
G2S5W12C73		DGGE-G3W12B1	
G2S5W12C74			

Sequence data associated with each clone ID shown is presented in the accompanying CD. DGGE derived sequences are prefixed by DGGE.

**CD containing sequence data is included with the print copy held in the University of Adelaide Library.**