Aspects of the biology of tail fan necrosis in spiny lobster

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A thesis submitted in complete fulfilment of the requirements for the degree of Doctor of Philosophy in Marine Science,

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Abstract

Tail fan necrosis (TFN) is a condition commonly found in some populations of commercially fished and cultured lobsters. The unsightly appearance of affected lobster tails significantly lowers their commercial value. Currently, knowledge about TFN is limited. In this current study, two immune parameters, total haemocyte count and phenoloxidase activity in the haemocyte lysate supernatant (HLS), were found to be significantly compromised in lobsters with TFN. No differences were found in the other immune parameters, i.e., haemocyte viability, haemolymph bacterial count, and the protein content of haemolymph plasma and HLS. The overall chitinolytic activity of bacteria present in lobsters with TFN was higher than in lobsters without TFN. The bacteria isolates with a combination of proteolytic, chitinolytic and lipolytic activities were more prevalent on the tail fans and in the haemolymph of lobsters with TFN. Biofilm formation was stronger in bacterial isolates from both haemolymph and tail fans of TFN affected lobsters compared to those from unaffected lobsters, while melanin and siderophore production were stronger in the isolates from tail fans of lobsters with TFN. By contrast, the other characteristics of isolates (morphology, antimicrobial activity, antimicrobial resistance, production of and ammonia) were similar in lobsters with and without TFN. The establishment of the condition at points of injury is consistent with the penetration of TFN through the cuticle and tissue layers of the affected tail fans, which is rarely seen in other forms of shell disease. Entry into these tissues was characterised initially by caseous necrosis and haemocyte accumulation, followed by the spread of these responses together with melanisation. Additional pathological changes to the tail fans included pseudomembrane formation, detachment of epidermis or cuticle, clotted haemolymph and fibrosis. A group of 21 bacterial OTUs were closely associated with TFN in spiny lobsters, six of which were most closely associated with TFN, i.e., *Aquimarina*, *Flavobacterium*, *Neptunomonas*, *Streptomyces*, *Flavobacteriaceae*, *Thiohalorhabdales*.

Collectively the results of this study suggest that TFN was associated with the changes in the immune status and pathology in *J. edwardsii*. Furthermore, some specific bacterial characteristics and a group of bacteria appear to be strongly associated with this condition.

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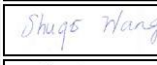
Shuqi Wang		06/03/18
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1. Chapter One - General introduction

1.1. Introduction

Tail fan necrosis (TFN) is a subtype of shell disease (Shields, 2011; Vogan et al., 2008). This condition usually appears in spiny lobsters (*Jasus edwardsii, Panulirus argus, P. elephas, P. cygnus* and *P. ornatus*) (Diggles et al., 2002; Geddes et al., 2004; Mancuso et al., 2010; Musgrove et al., 2005), and is typically characterised by necrosis and melanisation in the tail fans of lobsters (Mancuso et al., 2010; Musgrove et al., 2005). Precursors to the appearance of the condition include scratches, blisters, tears and holes (Musgrove et al., 2005).

In New Zealand and Australian waters, *J. edwardsii* is the most affected spiny lobster species (Freeman & MacDiarmid, 2009). It is reported that *J. edwardsii* in the New Zealand fishery had low levels of prevalence of TFN previously, but from around 2005 it became a major concern in some areas around New Zealand (Breen et al., 2005; Freeman & MacDiarmid, 2009). The prevalence of TFN was examined in *J. edwardsii* in a three-year investigation from 2003 to 2006 in one area on the east coast of the North Island of New Zealand (Freeman & MacDiarmid, 2009). In this study male lobsters accounted for on average of 90% of the catches. TFN was found in 17.1% of male and 1.9% female *J. edwardsii* in the southern part of the fished area (Turihaua Reef), and in 16.6% of male and 2.6% female *J. edwardsii* in the northern part (Whangara Reef) (Freeman & MacDiarmid, 2009). By contrast, only 1.8% of male and 0.4% of female lobsters within a nearby marine reserve were affected by TFN (Freeman & MacDiarmid, 2009).

An Australian questionnaire based survey of prevalence of TFN was conducted among key lobster fishing industry members in Queensland, Victoria, Tasmania, South Australia and Western Australia (Geddes et al., 2004). Most respondents indicated that the prevalence of TFN was below 5%, and they did not see TFN as a major concern in the wild fishery before the year 2004. The exception was from South Australia where one respondent reported that up to 10% of lobsters showed TFN in some years (Geddes et al., 2004).

The prevalence of TFN has been found to become elevated in captive spiny lobsters. Between 33 - 43% of *J. edwardsii* developed TFN during 29 - 30 weeks of holding in sea-cages in South Australia (Bryars & Geddes, 2005). A total of 41 - 47% of the lobsters have had visible damage in their tail fans at the beginning of the trial, while tail fan damage was found in 90 - 100% of lobsters at the end (Bryars & Geddes, 2005). These results were consistent with

other studies that have concluded that injuries sustained during repeated capture and handling of lobsters may play an important role in initiating TFN in spiny lobsters (Freeman & MacDiarmid, 2009).

Bacteria have been found to be associated with TFN in spiny lobsters (May, 2007; Musgrove et al., 2005). A group of bacteria were isolated from the TFN-affected tail fan lesions of spiny lobsters by culture method, and among them, *Vibrio* spp. were the more frequently isolated bacterial genus (Mancuso et al., 2010; May, 2007; Musgrove et al., 2005). *Vibrio alginolyticus, V. vulnificus* and *V. parahaemolyticus* were isolated from the TFN-affected lesions in wild *J. edwardsii* in Australia (May, 2002). In another study, *Aeromonas caviae, V. alginolyticus, V. vulnificus* and *V. parahaemolyticus* were isolated from TFN affected lesions of lobsters reared in the laboratory for six weeks in Australia (Musgrove et al., 2005). *Vibrio alginolyticus, V. anguillarum* and *V. parahaemolyticus* were isolated from the appendages and tail fan of *Palinurus elephas* with TFN (Mancuso et al., 2010). *Aeromonus caviae, A. hydrophila* and *V. alginolyticus* were also reported in TFN-affected lesions in *J. edwardsii* (Lorkin et al., 1999). In another study, bacteria belonging to the genera *Vibrio, Pseudoaltermonas* and *Shewanella,* were reported in both lesions and unaffected cuticle of *P. argus* with shell-disease-like (Porter et al., 2001). The bacterial isolates appeared to be evenly distributed on affected and unaffected cuticle, which lead to the conclusion that the normal bacterial flora of *P. argus* is likely to be in part responsible for shell-disease-like signs (Porter et al., 2001).

Some bacteria isolated from the TFN-affected lesions have been used to reproduce TFN in spiny lobsters. Different bacteria isolated from TFN lesions were added to an artificially made hole in the central tail fans of *J. edwardsii* and TFN subsequently developed around the inoculated injury (Geddes et al., 2004). The damaged tail fans that were inoculated with the bacteria (i.e., *A. caviae, V. alginolyticus, V. vulnificus* and *V. parahaemolyticus*) developed different degrees of melanisation and necrosis, while the control group (damaged but without bacterial culture inoculated) also demonstrated mild necrosis (Geddes et al., 2004), most likely due to the establishment of bacteria from the surrounding environment. *Vibrio alginolyticus, V. vulnificus* and *V. parahaemolyticus* were also found to be able to induce TFN after contaminating the knife-made injury in the distal region of the tail fan of *J. edwardsii* (May, 2007). In addition to the inoculated bacteria, some other bacteria were also found in the these treatments (injury plus bacteria) at the end of the experiments (May, 2007). However, both lobsters with and without initial artificial damage (no bacteria inoculation)

also had TFN-affected lesions at the end of the 12-week experiments (May, 2007). Interestingly, the lesions were found in the margin of the tail fans of the lobsters, but not found in the experimentally made holes and cuts in the cuticle (May, 2007).

Vibrio spp. were re-isolated from the experimentally induced TFN-lesions to test their characteristics that may be associated with virulence, including productions of extracellular chitinase, protease, lecithinase, urease, lipase as well as cytotoxicity to cultured mammalian cells (May, 2007). Most or all of the *V. parahaemolyticus* and *V. vulnificus* isolates produced these enzymes and cytotoxicity, as well as a small percentages of the *V. alginolyticus* and other *Vibrio* spp. isolates (May, 2007).

In the study by Geddes et al., (2004) three lobsters were selected for quantification of bacteria by plating the homogenised tail fan tissue from the unaffected tail fan cuticles and TFN-affected lesions of *J. edwardsii* onto marine agar plates. Greater numbers of bacteria were counted from the TFN-affected lesions than the unaffected tail fan cuticle of the affected lobsters, and a number of acid-producing isolates were found in TFN-affected lesions but not detected in the unaffected cuticles.

A preliminary investigation was undertaken as part of a student study of the immune response induced by TFN in five unaffected *J. edwardsii* (no damage) as a control treatment and five *J. edwardsii* with artificially induced TFN (*V. parahaemolyticus* inoculated to damaged tail fan) (May, 2007). Haemolymph samples were collected from these lobsters at one-week intervals over a period of six weeks, for the assessment of their immune status (May, 2007). Phenoloxidase activity in the haemocytes was found to be significantly higher in unaffected lobsters than the affected ones on the fourth week post infection, but it was reported that TFN had no effect on the different immune parameters, i.e., serum protein content, haemolymph bacterial count, total / differential haemocyte counts, phenoloxidase activity and in vitro phagocytosis by hyaline cells (May, 2007).

Seawater temperature was reported to have limited effect on TFN (Geddes et al., 2004; Musgrove et al., 2005). Lobsters were captured and reared in tanks at 15 °C and 23 °C, and the TFN erosion was observed at 2, 4 and 6 weeks afterwards (Geddes et al., 2004; Musgrove et al., 2005). Both 15 °C and 23 °C could induce TFN within 6 weeks, but there was no difference in the extent of TFN in lobsters between the two temperature treatments (Geddes et al., 2004; Musgrove et al., 2005).

Holding density and feeding frequency were tested for their association with TFN in *J. edwardsii* held in seacages (Musgrove et al., 2005). Three feeding frequency treatments (daily, weekly and starved) and two density treatments (10 lobsters and 20 lobsters per cage) were set up and conducted for four months to assess the occurrence of TFN at the start of experiment, and then after two and four months (Musgrove et al., 2005). In all the fed treatments, lobsters were allocated with 2% body weight per day of an artificial diet which comprised mainly of fish meal, wheat flour, crustacean meal and wheat gluten (Musgrove et al., 2005). It was found that lobster density and feeding frequency had no effect on TFN (Musgrove et al., 2005).

The tail fan pathology in spiny lobsters with TFN has been examined (Mancuso et al., 2010; May, 2007). In laboratory reared *J. edwardsii*, significant inflammation was found in the dorsal surface of their tail fans, typically showing a loss of internal structure in the affected tissue (May, 2007). In contrast the ventral surface of the tail fans also displayed a loss of structure, with less inflammation than the dorsal surface (May, 2007). Inflammation of the underlying epidermis was characterised by large amount of fibrous deposition in all affected cuticle (May, 2007). In tail fans with severe inflammation, a central core with a large amount of different cell types was observed within the epidermis, i.e., infiltrating haemocytes, granulocytes, hyaline cells and other fibrocytes (May, 2007). In wild *P. elephas*, histological examination of the affected lobsters revealed that melanisation in the cuticle accounted for the black/brown appearance in the lesions, while proliferation of the inflammatory membrane was reported to increase the depth of cuticle to prevent the further spread of the lesion (Mancuso et al., 2010).

One useful measure has been identified for presenting the development of TFN in *J. edwardsii* by protecting them from injuring the cuticle during periods of handling (Musgrove et al., 2005). Experimental lobsters were enclosed in fine nylon bags before transportation and then reared in individual tanks in laboratory for six weeks (Musgrove et al., 2005). The bagged lobsters were reported to have significantly less initial damage (i.e., holes, blisters, scratches, tears and erosions of the cuticle) in the beginning of the trial (during day 9 - 11) (Musgrove et al., 2005). Significant lower levels of TFN were found in the bagged lobsters than those not bagged at the end of the six week holding period, suggesting the bagging method is effective on lowering the occurrence of TFN whist handling captive spiny lobsters (Musgrove et al., 2005).

1.2. Research aims

These previous studies on TFN in spiny lobsters has provided some initial insights into the causes and progression of the condition and the possible identity of causative organisms. However, other bacteria may also be involved in contributing to TFN, and the characteristics of the bacteria associated with TFN need to be determined to better understand the nature of their association with TFN. Many of the previous studies have used small sample sizes for comparative analyses or have had limited control treatments. Consequently, larger comparative sample sizes including appropriate controls are needed for determining the immune status, pathology and the other morphology characteristics of TFN in spiny lobsters.

The goals of the research presented in this thesis are to more comprehensively identify the bacteria and the potential bacterial characteristics which are associated with TFN in spiny lobsters, and to examine the immune status and pathology associated with TFN in the affected lobsters.

The specific aims and structure of individual chapters in the thesis are as follows:

Chapter One: General Introduction (current chapter)

• This chapter provides some background information about TFN to the research presented in the thesis by summarising the results of previous studies. It also outlines the research aims and objectives for the studies that are subsequently presented in the thesis.

Chapter Two (literature review): Bacterial Infection in Lobsters with Shell Disease

• This chapter provides a literature review of shell disease in marine lobsters. As TFN is a subtype of shell disease (Shields, 2011), the aim of this chapter was to review the wider scientific literature in relation to the role of bacteria in shell disease in lobsters. This review critically reviewed the literature of bacterial association with shell disease and interprets the initiating and exacerbating factors for shell disease in marine lobsters. The conclusions of the review helped to provide background information and refine the research objectives presented in the research that follows in the thesis.

Submitted as: Zha, H., Lewis, G., Dong, Y. & Jeffs, A. 2018. Bacterial invaders in lobsters with shell disease, under review at time of thesis submission by *Reviews in Fisheries Science & Aquaculture*.

Chapter Three: Immune Status of the Spiny Lobster Jasus edwardsii with Tail Fan Necrosis

• Immune status of TFN-affected lobsters has not been comprehensively investigated. The research presented in this chapter examines whether TFN in wild *J. edwardsii* is associated with any differences in their immune status compared to that of unaffected spiny lobsters. Six immune parameters were assessed in a sample of wild-caught lobsters, i.e., total haemocyte count, phenoloxidase activity in the haemocyte lysate supernatant (HLS), haemocyte viability, haemolymph bacterial count, and the protein content of haemolymph plasma and HLS. Some morphological characteristics of this condition were also studied, as well as the association of tail fan injury and TFN.

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Chapter Four: Characteristics of Culturable Bacteria Associated with Tail Fan Necrosis in the Spiny Lobster, Jasus edwardsii

• The enzymatic activities of bacteria have not been investigated for their association with TFN. The research presented in this chapter compares the enzymatic activities (proteolytic, chitinolytic and lipolytic activities) of culturable bacteria in affected and unaffected lobsters, aiming to sorting out the possible association of these characteristics with TFN.

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Chapter Five: Potential Virulence Factors of Bacteria Associated with Tail Fan Necrosis in the Spiny Lobster, Jasus edwardsii

• The bacterial characteristics, such as the potential virulence factors, have not been reported in marine lobsters with shell disease. Based on the results of Chapter Four, the research presented in this chapter determined whether some further bacterial characteristics were associated with TFN in *J. edwardsii*, i.e., morphology, biofilm formation, antimicrobial activity, antimicrobial resistance, production of siderophores, melanin and ammonia. It intends to sort out the potential virulence factors associated with TFN for better understanding of TFN.

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Chapter Six: Pathology of Tail Fan Necrosis in the Spiny Lobster, Jasus edwardsii

• The pathology of TFN-affected lesions has been briefly reported (Mancuso et al., 2010; May, 2007), but the internal organ pathology has not been reported in TFN-affected spiny lobsters. This chapter determines the pathology of affected tail fan and the four selected internal organs (i.e., heart, gill, hepatopancreas and midgut) in *J. edwardsii* affected with TFN by external observation and histopathology. It intends to sort out the pathological changes in the lesions, and determine the association of internal organ pathology with TFN.

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Chapter Seven: Multiple opportunistic bacteria associated with tail fan necrosis in spiny lobster, Jasus edwardsii

• The bacterial community of shell disease lesions in American lobster have been well studied by culture-independent molecular methods (Chistoserdov et al., 2012; Feinman et al., 2017; Meres et al., 2012; Quinn et al., 2013a; Whitten et al., 2014). However, the bacterial community in TFN-affected lesions of spiny lobsters has not been reported yet. This chapter investigated the bacterial community associated with TFN in *J. edwardsii* by using Illumina sequencing, aiming to examining whether there were a characteristic suite of bacteria associated with TFN in spiny lobsters.

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Chapter Eight: General Discussion

• The chapter discusses the findings of the research chapters of this thesis and provides some overarching conclusions in relation to previously reported studies, as well as recommendations on future directions for research in this field.

2. Chapter Two - Literature review - Bacterial invaders in lobsters with shell disease

2.1. Introduction

Shell disease affects many aquatic crustaceans worldwide, such as lobsters, crabs and shrimp, frequently causing significant economic losses within affected fisheries (Cawthorn, 2011; Dyrynda, 1998; Myers & Tlusty, 2009; Rosen, 1967; Vogan et al., 2008; Vogan & Rowley, 2002). Marine lobster fisheries are generally of very high value (Jeffs & Hooker, 2000; Merella et al., 1998; Steneck et al., 2011), and shell disease has the potential to cause considerable economic losses (Chistoserdov et al., 2012; Meres et al., 2012; Vogan et al., 2008). The slower growth of affected lobsters and their unappealing appearance reduce their value in the market (Castro et al., 2006; Homerding et al., 2012; Maynard et al., 2016; Vogan et al., 2008; Wahle et al., 2009; Zha et al., 2017). The prevalence of shell disease in lobster populations varies by harvest area (Freeman & MacDiarmid, 2009; Landers et al., 2005), and there is an overall trend of increasing reports of the condition since the latter part of the 20th century which may be related to increasing prevalence of this condition within lobster populations (Freeman & MacDiarmid, 2009; Landers et al., 2005; Young & Pearce, 1975).

Shell disease is most typically characterized by necrosis and melanisation on the crustacean cuticle (Freeman & MacDiarmid, 2009; Vogan et al., 2008). Some damage to cuticle (i.e., scratches, blisters and holes) are regarded to be precursors of shell disease in spiny lobsters (*Jasus edwardsii*) (Musgrove et al., 2005). Captive lobsters (i.e., those in holding facilities) usually have a higher prevalence of shell disease than wild populations of lobsters (Bryars & Geddes, 2005; Freeman & MacDiarmid, 2009; Glenn & Pugh, 2006; Musgrove et al., 2005).

Shell disease has been found in different marine lobster species, including American lobsters, European lobsters, spiny lobsters and Norway lobster (Cawthorn, 2011; Davies et al., 2014a; Musgrove et al., 2005; Stentiford & Neil, 2011). Several different subtypes of shell disease have been classified in American lobsters from the east coast of North America (Chistoserdov et al., 2012; Davies et al., 2014b; Homerding et al., 2012; Hsu & Smolowitz, 2003; Quinn et al., 2013a), including epizootic shell disease (ESD), enzootic shell disease (EnSD), impoundment shell disease (ISD), diet-induced shell disease (DISD) and black spot shell disease (Chistoserdov et al., 2005; Musgrove et al., 2005; Quinn et al., 2013a; Shields et al., 2012). Tail fan necrosis (TFN) is another subtype of shell disease usually found in spiny

lobsters, such as *J. edwardsii* from Australasian coasts (Freeman & MacDiarmid, 2009; Musgrove et al., 2005; Shields, 2011; Vogan et al., 2008), *Panulirus argus* found from Brazil to Florida and through the coasts of the Caribbean Sea, *Panulirus elephas* in the Mediterranean Sea and *Panulirus cygnus* in Western Australian waters (Mancuso et al., 2010; Porter et al., 2001).

The lobster cuticle commonly consists of an outer epicuticle that covers an exocuticle layer and an endocuticle layer (Davies et al., 2014b; Smolowitz et al., 2005; Vogan et al., 2008), although different lobsters species have distinct shell structures (Davies et al., 2014b). Protein and lipid were reported to constitute the epicuticle, while largely chitin with a small proportion of protein are important components of the exocuticle and endocuticle layers (Davies et al., 2014b; Vogan et al., 2008).

Lobsters with artificially damaged cuticle were found to develop shell disease lesions (Geddes et al., 2004; Quinn et al., 2012a) probably because injuries to the cuticle could provide an entry point for bacterial infection that ultimately leads to shell disease (Musgrove et al., 2005; Quinn et al., 2012a; Shields, 2011; Vogan et al., 2001). A variety of bacteria have been identified from marine lobsters affected with shell disease (Chistoserdov et al., 2012; Musgrove et al., 2005; Quinn et al., 2013a; Whitten et al., 2014). Chitinolytic bacteria that are capable of breaking down cuticle have been thought to be closely associated with this condition (Costa-Ramos & Rowley, 2004; Li & Roseman, 2004; Mancuso et al., 2010; Vogan et al., 2002).

Some subtypes of shell disease (i.e., ESD and TFN), have been reported to be associated with the impaired health of marine lobsters (Homerding et al., 2012; Musgrove et al., 2005; Zha et al., 2017). Likewise, some affected lobsters were found to exhibit bacteraemia and pathogenic changes in some internal tissues (Chistoserdov et al., 2005; Comeau & Benhalima, 2009), which may result in mortalities (Castro et al., 2012). The ecological implications of shell disease may be broader than the direct impact on the population of affected lobsters because many marine crustaceans also often play important roles in the marine ecosystem as predators and prey (Blamey et al., 2013; Kristensen, 2008; Zhang & Chen, 2007), such that shell disease may impact the balance of an ecosystem by compromising these marine organisms.

This review focuses on shell disease in marine lobsters, including the prevalence, association with changes in health status, and the host response to the bacteria associated with shell

disease. It critically reviews the recent reports of bacteria associated with shell disease among different lobster species, and interprets the different associations of environmental factors with shell disease in marine lobsters, with an overall aim of improving the understanding of this condition.

2.2. The increasing prevalence of shell disease

High prevalence of shell disease has been frequently reported in crabs and shrimp populations (Comely & Ansell, 1989; Dyrynda, 1998; Gopalan & Young, 1975; King et al., 2014; Rogers et al., 2015a; Rogers et al., 2015b; Vogan et al., 1999). By contrast, the prevalence of shell disease in wild marine lobster populations is generally reported as being less than 5% of harvested lobsters (Ayres & Edwards, 1982; Iversen & Beardsley, 1976; Taylor, 1948; Young & Pearce, 1975). However, the prevalence of this condition in some populations of marine lobsters appears to have increased recently, and the condition has become a major concern in some areas (Freeman & MacDiarmid, 2009; Glenn & Pugh, 2006; Wahle et al., 2015).

The prevalence of ESD in American lobsters (*Homarus americanus*) from New England waters increased significantly from less than 1% of fished lobsters in 1995 to over 30% in 2003 (Castro et al., 2005). Similar increases in the prevalence of ESD have been reported in other fisheries including for wild American lobsters in Long Island Sound, Connecticut (from 0.85% during 1984 - 1999 to 19.27% in 2000 - 2005) (Landers et al., 2005). Between 2002 and 2003, over 25% of *H. americanus* were reported to have ESD in Buzzards Bay of coastal Massachusetts (Glenn & Pugh, 2006). From 2000 to 2004, the prevalence of ESD was consistently over 20% in *H. americanus* caught in the inshore area of Rhode Island (Castro et al., 2005). Up to 17% of male *J. edwardsii* (the males accounted for 90% of the total harvested lobsters) were affected by TFN in a commercially fished area on the east coast of the North Island of New Zealand over a period from 2003 to 2006 (Freeman & MacDiarmid, 2009). Such high prevalence of TFN has not been reported in previous studies, e.g., (Breen et al., 2005). High prevalence of shell disease (up to 28%) was also found in European lobsters (*Homarus gammarus*) from a no-take zone off the North Devon coast in Britain in 2010 - 2011 (Davies et al., 2014a).

In captive lobsters, the prevalence of shell disease usually increases with extended captive holding time (Bryars & Geddes, 2005; Theriault et al., 2008). For *J. edwardsii*, 33 - 42% of

lobsters developed TFN during 29 - 30 weeks of holding the lobsters in sea-cages in South Australia (Bryars & Geddes, 2005). Over half (52.4%) of 540 *H. americanus* were found to develop ISD after 120 days of holding in individual compartments in plastic storage trays in Nova Scotia, and the majority of new cases of affected lobsters were observed within 60 days (Theriault et al., 2008). Shell disease can be fatal to captive lobsters. Thirteen of 55 (24%) *H. americanus* died as a direct result of ESD in a one year study (Stevens, 2009).

Different types of injuries could be associated with the increasing trend of shell disease in different lobster species (Davies et al., 2014a; Freeman & MacDiarmid, 2009; Quinn et al., 2012a; Ziino et al., 2002). Physical interactions with lobsters occur during capture using trawls, traps and nets, which are all activities with the potential to contribute to the injuries to captured lobsters (Leland et al., 2013; Powrie & Tempero, 2009; Ridgway et al., 2006). Also, tagging lobsters by inserting dart or T-bar anchor tags was reported to cause necrosis in the abdomen of southern rock lobster (*J. edwardsii*) in south-east Tasmania (Wlnstanley, 1976). Injuries caused by interactions, including cannibalism, have been reported in wild and captive *J. edwardsii* and in Cape rock lobsters (*Jasus lalandii*) (Dubula et al., 2005; Jeffs & James, 2001; Pollock, 1986). Off the North Devon coast in Britain, agonistic behaviour was thought to be stimulated by the high density of territorial *H. gammarus* in marine reserves, which resulted in more injuries and a higher prevalence of shell disease in *H. gammarus* than in the nearby fished areas (Davies et al., 2014a).

Surveys of the prevalence of shell disease in lobster populations using commercial fishing devices, such as baited traps, may not be accurate. Affected *H. americanus* were found to have less exploratory behaviour and correspondingly more sheltering behaviour (Castro et al., 2005), suggesting that the affected lobsters are less likely to be captured in baited traps. Also, the avoidance of diseased individuals by social lobsters may also influence the numbers of lobsters entering traps, especially where juvenile lobsters are used to attract larger lobsters into traps (Behringer et al., 2006). In addition, affected lobsters in the ocean might experience poor health status or even mortality, which could make them absent in the fishing devices (Castro et al., 2012; Floreto et al., 2000; Hoenig et al., 2017; Musgrove et al., 2005; Stevens, 2009).

2.3. Multiple opportunistic bacterial invaders

A range of bacteria associated with shell disease in different marine lobster species have been identified at different locations throughout the world, including the coast near Long Island, Maine, Rhode Island, Massachusetts and Florida in America, Gisborne in New Zealand, Nova Scotia and Prince Edward Island in Canada, South Australia, Swansea in Britain, Sicily in Italy, and parts of the Mediterranean Sea (Chistoserdov et al., 2012; Chistoserdov et al., 2005; Mancuso et al., 2010; Meres et al., 2012; Musgrove et al., 2005; Porter et al., 2001; Quinn et al., 2013a; Quinn et al., 2012b; Shields et al., 2012; Whitten et al., 2014; Ziino et al., 2002).

Aquimarina homaria is closely associated with shell disease in *H. americanus* (Chistoserdov et al., 2005; Feinman et al., 2017), but subsequent studies have concluded that there are other bacteria also associated with this condition. *Aquimarina* sp. was found to be more abundant in *H. americanus* with ESD from Narragansett Bay of Rhode Island versus those lobsters not affected by ESD, which suggested the bacteria were likely to be part of normal bacterial flora on the cuticle and might be acting as opportunistic pathogens for ESD (Meres et al., 2012). In wild *H. americanus* with ESD, ISD and EnSD, *A. homaria* was detected in all the analysed shell lesions, while some other genera (i.e., *Tenacibaculum*, *Polaribacter*, *Maribacter*, *Cellulophaga*) were also detected and considered to have a particular association with shell disease lesions (Chistoserdov et al., 2012). Two bacteria belonging to *A. homaria* and *Thalassobius* sp. isolated from ESD lesions of wild *H. americanus* were found to persist in and act together as important components of lesion development on abraded surfaces of captive *H. americanus*, while *Pseudoalteromonas gracilis* did not (Quinn et al., 2012a).

Different bacterial associations have been found for shell disease in European lobsters (*H. gammarus*). In *H. gammarus* and *H. americanus* (reared together in USA) affected with lab-induced shell disease, *A. homaria* was at a much higher prevalence in *H. americanus* lesions than *H. gammarus*, and found to be associated with the exacerbation but not the initiation of lab-induced shell disease in *H. americanus* (Whitten et al., 2014). In the same study, *A. homaria* was also detected in *H. gammarus* (reared in UK) that were affected with shell disease, but the prevalence of this bacteria were similar (less than 5%) between affected and unaffected lobsters. In addition to *A. homaria*, bacteria from the genera *Arenicella* and *Maribacter* were also detected in the necrotic lesions of both *H. americanus* and *H.*

gammarus, while bacteria from the genus *Eoina* was detected only in *H. gammarus* lesions (Whitten et al., 2014).

There are limited reports of the bacteria associated with shell disease in Norway lobster (*Nephrops norvegicus*). *Pseudoalteromonas* sp. and *Enterobacter agglomerans* were isolated from shell lesions of affected Norway lobster in the Mediterranean Sea (Ziino et al., 2002). Histopathology of the affected lesions demonstrated erosion of the cuticle which was associated with bacterial colonisation (Ziino et al., 2002).

Different bacteria appear to be associated with TFN in different spiny lobster species living in different geographic locations (Mancuso et al., 2010; May, 2007; Musgrove et al., 2005; Zha et al., 2018b). In Florida, *Vibrio* sp., *Pseudoalteromonas* sp. and *Shewanella* sp. were isolated from both unaffected cuticle and affected lesions of *P. argus*, and they were assumed to be opportunistic pathogens (Porter et al., 2001). In South Australia, *Vibrio vulnificus*, *V. alginolyticus*, *V. parahaemolyticus* and *Aeromonas caviae* were isolated from the TFN lesions of captive *J. edwardsii* (Musgrove et al., 2005), and these bacteria were used to inoculate the experimentally damaged tail fans to successfully induce TFN in *J. edwardsii* (May, 2007). In Sicily, *V. anguillarum*, *V. alginolyticus* and *V. parahaemolyticus* were isolated from the TFN-affected lesions of *P. elephas* in an experimental aquaculture facility (Mancuso et al., 2010).

Among the investigated bacteria associated with shell disease in marine lobsters, some of their biological characteristics are likely to play an important role in shell disease. Chitinolytic bacteria are thought to be associated with shell disease in crustaceans (Li & Roseman, 2004; Mancuso et al., 2010; Vogan et al., 2002), including in some lobster species, i.e. *J. edwardsii* and *H. americanus* (Geddes et al., 2004; Quinn et al., 2012a). Some researchers believe that bacteria with proteolytic and/or lipolytic activities are also associated with shell disease in crustaceans, such as lobsters and crabs (May, 2007; Quinn et al., 2009; Vogan et al., 2008). This view is supported by the association of bacteria with proteolytic, chitinolytic and lipolytic activities with TFN in *J. edwardsii* (Zha et al., 2018a).

Some other characteristics of bacteria may be also associated with their role in shell disease in marine lobsters. The biofilm-forming ability of bacteria is likely to affect the bacterial abundance on the cuticle or in lesions on the cuticle and in doing so, and influence the bacterial communities that are formed. For example, *Aquimarina* sp. was found to develop shell disease in *H. americanus* at 15 °C and 20 °C, but failed to colonize the cuticle of

lobsters held at 10 °C, suggesting that the biofilm formation of *Aquimarina* was decreased due to the low temperature. In *J. edwardsii*, biofilm formation was commonly among bacteria cultured from TFN-affected lesions (Zha et al., 2018b).

Microbes, including bacteria, have been reported to be able to produce melanin in certain circumstances (Banerjee et al., 2014; Chatfield & Cianciotto, 2007; Valeru et al., 2009; Wang & Breuil, 2002). Melanisation in lobsters can be activated in damaged cuticle to prohibit bacterial invasion by occluding the margins of the wounded tissues, and is thought to be the mechanism for producing the blackened appearance of the cuticle in lobsters with shell disease (Chistoserdov et al., 2005; Porter et al., 2001; Powell & Rowley, 2005; Quinn et al., 2012a; Vogan et al., 2008; Wang, 2011). Bacteria are also likely to be associated with the accumulation of melanin in damaged tissues by taking advantage of melanin-related substrates, such as tyrosine (Floreto et al., 2000), from the host to form melanin. Bacterial melanin associated with TFN in *J. edwardsii* could provide some evidence for this view (Zha et al., 2018b). In addition, bacterial siderophores have also been found to be associated with TFN in *J. edwardsii* (Zha et al., 2018b).

2.4. The response of the lobster to bacterial colonisation

In crustaceans, the circulating haemocytes play vital roles in protecting the host against the invasive and opportunistic bacteria by contributing to different immune activities, such as immune recognition, phagocytosis, cytotoxicity and melanisation (Jiravanichpaisal et al., 2006).

Immunorecognition is thought to be mediated through the prophenoloxidase system, a cascade of serine proteases and prophenoloxidase present in the haemocytes which is activated by non-self molecules (Crawford et al., 2010; Söderhäll & Sritunyalucksana, 2002; Zhang et al., 2016c). Phagocytosis and cytotoxicity are of great importance in uptaking and eliminating foreign invaders (Cerenius et al., 2010a). Accumulation of necrotic haemocytes have been found in the eroded cuticle and the cuticular epidermis of shell disease affected *H. americanus*, which may provide evidence for the presence of these two immune responses to shell disease (Smolowitz et al., 2005).

Phenoloxidase activity, an important immune response of crustaceans including lobsters, usually results in the melanisation on the cuticle of lobsters (Perdomo-Morales et al., 2007; Shields, 2011; Vogan et al., 2008). Melanisation causes cross-linking of proteins in the

cuticle layers to form a wall, which is a mechanism to inhibit potential invasion of the tissues (Amparyup et al., 2013; Cerenius et al., 2008; Cerenius & Söderhäll, 2004; Jiravanichpaisal et al., 2006). This melanisation response was believed to be effective in assisting *H. americanus* to combat resist ESD (Homerding et al., 2012).

Antibacterial activity was found in the cuticle of *H. americanus*, and some bacterial isolates belonging to *Escherichia coli, Staphylococcus aureus, Vibrio parahaemolyticus* and *V. anguillarum* were inhibited by the shell extract, which is believed to be due to the antimicrobial peptides in the cuticle (Brisbin et al., 2015). This phenomenon has also been found in other crustaceans, such as shrimp and crabs, suggesting it is a common mechanism of defence against bacterial attachment to cuticle (Brisbin et al., 2015; Haug et al., 2002; Tsai & Hwang, 2004).

2.5. Affected health

Changes of some immune parameters were found to be associated with shell disease in *H. americanus* (Homerding et al., 2012; Prince, 1997). Lower levels of serum protein and circulating haemocytes were measured in *H. americanus* affected with shell disease compared with equivalent unaffected lobsters (Prince, 1997). Phenoloxidase activity was found to be higher in *H. americanus* with ESD than unaffected lobsters from one sampling site in eastern Long Island Sound (Homerding et al., 2012), while phagocytic activity and oxidative burst in the haemocytes of *H. americanus* were reported to be affected or suppressed by ESD (Homerding et al., 2012). In wild *J. edwardsii* in New Zealand, total haemocyte count and phenoloxidase activity in the haemocyte lysate supernatant were both lower in TFN-affected lobsters than those in unaffected ones (Zha et al., 2017). These suggest shell disease can be associated with impaired immunity in marine lobsters.

Changes of endogenous biochemical profiles were reported to be associated with shell disease in *H. americanus* (Floreto et al., 2000). A number of biochemical indices, (i.e., proximate composition, amino and fatty acid profiles) were found to be different in muscle, hepatopancreas, haemolymph and exoskeleton of lobsters affected with shell disease versus those unaffected lobsters (Floreto et al., 2000). These changes in biochemical profiles might be associated with shell disease. For example, increases in the mineral content of hepatopancreas and haemolymph of the affected lobsters possibly indicates problems in the transport and deposition of minerals to the cuticle (Floreto et al., 2000).

It is uncertain whether bacteraemia is a consequence of shell disease in lobsters. In two investigations, bacteraemia was reported not to be associated with shell disease in the affected lobsters (*H. americanus* and *P. elephas*) (Chistoserdov et al., 2005; Mancuso et al., 2010). By contrast, *H. americanus* affected with ESD in Long Island Sound demonstrated a positive correlation between bacterial counts in haemolymph and the severity of shell disease (Homerding et al., 2012). Although bacteraemia may not be an indicator of shell disease in all the lobsters, it is still possible that some bacteria associated with shell disease could access the haemolymph from the lesions, with the resulting bacteraemia potentially leading to mortality (Castro et al., 2012; Sindermann, 1989; Stevens, 2009; Taylor, 1948; Wahle et al., 2009). However, more definitive research is required to confirm the connection between shell disease and the possible progression to bacteraemia and further infection.

Shell disease influences the reproduction of *H. americanus* (Comeau & Benhalima, 2009). Pathological abnormalities of the reproductive organs were commonly found in male *H. americanus* with shell disease of unknown etiology (Comeau & Benhalima, 2009). Furthermore, over 50% of spermatozoa from the affected male *H. americanus* were found to be dead, while in contrast the spermatozoa were all alive in a comparative sample of healthy male *H. americanus*. The spermatozoa were thought to be compromised by changes in the chemical composition of the male lobster reproductive organs, from acidic to basophilic (Comeau & Benhalima, 2009). A higher prevalence of shell disease is usually found in egg-bearing *H. americanus* than among the females without eggs (Castro et al., 2006; Glenn & Pugh, 2006; Rycroft et al., 2012). This is believed to be a consequence of the extended inter-moult stage (Rycroft et al., 2012), which may result in mortalities of egg-bearing lobsters. Reduced egg production from egg-bearing lobsters can result from shell disease (Castro et al., 2006; Wahle et al., 2009). If the moult of egg-bearing lobsters occurs too early, the eggs will be lost with the moulted exoskeleton. If the moult occurs too late, it will cause a delay in subsequent egg production (Castro et al., 2006).

Whether internal organs, such as hepatopancreas and gills of lobsters affected with shell disease remains uncertain (Comeau & Benhalima, 2009; Shields et al., 2012). Shell disease was reported to affect the internal organs of 12 wild affected *H. americanus* in eastern Canada (Comeau & Benhalima, 2009). While no typical pathology was found in the hepatopancreas and gill of ESD-affected *H. americanus* (n = 80) in Rhode Island and Maine (Shields et al., 2012).

Shell disease can induce more frequent moult behaviour in affected *H. americanus*, which is thought to act as an effective strategy to remove the affected shell (Castro et al., 2006; Gomez-Chiarri & Cobb, 2012; Smolowitz et al., 2005). However, a number of *H. americanus* were found to be re-affected with shell disease after moulting (Landers et al., 2005), which suggests that moulting may not provide a permanent solution to overcome this condition (Castro et al., 2006). The affected *H. americanus* were found to have reduced growth and changed behaviour compared to the unaffected lobsters (Castro et al., 2006).

Shell disease was previously thought to be associated with moult death syndrome in *H. americanus* and in the hybrid of *H. americanus* and *H. gammarus* (Conklin et al., 1980). Although the condition is not fatal in its initial stages, death is known to result from adhesion of successive moult cuticles at the lesions leading to incomplete withdrawal from the exuviation at moult (Shields, 2013; Smolowitz et al., 1992; Vogan et al., 2001). From 1982 - 2015, wild *H. americanus* affected by different severities of ESD were found to have lower survival rates (i.e., 30 - 45%) compared with unaffected lobsters (Hoenig et al., 2017). Furthermore, high mortality in ovigerous females was assumed to be associated with ESD (Hoenig et al., 2017; Landers et al., 2005).

2.6. Environmental factors

Seawater temperature appears to be associated with the varying prevalence of shell disease in *H. americanus*. In Massachusetts coastal waters, a much higher ESD prevalence was reported in *H. americanus* from Buzzards Bay, which has a higher mean seafloor water temperature than for other sites from which lobsters were examined, suggesting that higher temperatures may increase the prevalence of ESD in lobsters (Glenn & Pugh, 2006). However, a higher prevalence of ESD in laboratory-reared *H. americanus* was reported to occur at moderate temperature (15 °C) rather than a lower (10 °C) or higher (20 °C) temperatures (Tlusty & Metzler, 2012), which might be explained by other physiological factors, such as shortened moult cycles at 20 °C which are replacing the damaged shell of juvenile lobsters more frequently (Kelly, 2001). A seafloor temperature range between 10 - 15 °C was associated with higher prevalence of shell disease in Long Island Sound compared to other temperatures (3.9 °C - 10 °C, 15 °C -22.1 °C) (Tanaka et al., 2017). In *J. edwardsii*, seawater temperature appears to have no association with the prevalence of TFN in captive lobsters, with TFN developing to a similar extent in lobsters held at 15 °C versus 23 °C (Musgrove et al., 2005).

Pollutants of various kinds are associated with varying prevalence of shell disease in lobsters. Some pollutants, such as alkylphenol, were assumed to be associated with higher prevalence of shell disease in *H. americanus* due to their negative influence in shell hardening (Shields, 2013; Tanaka et al., 2017), but in some studies the lowest prevalence of shell disease was found in waters with high concentrations of alkylphenol than the other less contaminated areas (Glenn & Pugh, 2006; Jacobs et al., 2012). The possible causal mechanism may be that alkylphenol could consume phenoloxidase which leads to less melanisation in the lobster cuticle (Laufer et al., 2012). There may have also been differences in the mix of contaminants (i.e., antibiotics) in the investigated areas, which could also explain the unexpected results.

The effects of food and nutrient intake on shell disease in lobsters have been investigated (Bryars & Geddes, 2005; Myers & Tlusty, 2009; Prince & Bayer, 2005; Tlusty et al., 2008). An artificial diet was suspected of inducing shell disease as it was thought to lack the nutrients required for building a lipid-based epicuticular layer (Fisher et al., 1978). Another study demonstrated that a 100 % fish diet could lead to increased prevalence of shell disease in *H. americanus*, potentially resulting in mortality in the longer term (Castro et al., 2012; Tlusty et al., 2008). This was thought to be due to the fish diet accelerating the lobster growth, which exacerbated the onset of the condition through a lack of protective response to bacteria or a structural deficiency in the newly formed cuticle (Tlusty et al., 2008). However, a study using $\delta^{15}N$ labelled fish bait found that the fish bait was not related to ESD severity and suggested a diet including fish bait did not contribute to ESD outbreaks in *H. americanus* (Bethoney et al., 2011).

Higher benthic salinity (31 ppt versus 25 ppt) was found to be associated with higher prevalence of shell disease in wild *H. americanus* in Long Island Sound (Tanaka et al., 2017). In the same area, lobsters living at a depth of around 20 m had lower prevalence of shell disease, while the prevalence was higher among lobsters living at < 10 m and around 40 m depth (Tanaka et al., 2017).

2.7. Control and prevention

Eliminating damage to lobster cuticle is a key preventative measure for shell disease. For example, placing captured lobsters in fine-mesh nylon bags was found to be effective for decreasing injuries to lobsters whilst undergoing handling, loading and transporting (Musgrove et al., 2005). Improving the effectiveness of escape gaps on lobster traps are

recommended as means for reducing the frequency of repeated catching, handling and return of undersize lobsters (Leland et al., 2013; Zha et al., 2017). Other possible mechanisms for alleviating or treating shell disease in captive lobsters, such as probiotic and antibiotic intervention, have not been reported.

2.8. Conclusions and future directions

Shell disease has been found in different wild and captive lobster species, and has become a major concern in some areas. To further improve the understanding of shell disease, some aspects need to be further studied in affected lobsters with different severities, i.e., the avoidance of affected lobsters by the unaffected lobsters, the mortality of the affected lobsters, the effectiveness of moulting in recovering from this condition, and possible differences in the rate of the affected lobsters entering fishing devices which are typically used for sampling lobster populations to measure prevalence of shell disease.

The compromised health of some lobster species with shell disease have been found to be associated with the presence of shell disease (i.e., ESD-affected *Homarus americanus* and TFN-affected *J. edwardsii*), but the situation is largely unknown for other lobster species (i.e., European lobsters and Norway lobster). The changes in immune response, antibacterial activity and the innate mechanisms for preventing any progression from this condition are recommended for the future studies. Several environmental factors seem to be associated with shell disease in different marine lobsters, but these associations could be further investigated based on laboratory work. For example, some selected pollutants can be used in animal experiments in laboratory to find out their association with the prevalence or severity of shell disease in lobsters, as well as their detailed mechanisms of causality.

Bacterial communities have been investigated in marine lobsters with shell disease, especially *H. americanus* (Chistoserdov et al., 2012; Quinn et al., 2013a; Whitten et al., 2014). A few specific bacterial species were thought to be causative agents responsible for shell disease, however, there is growing evidence that there are a group of bacteria are associated with this condition (Feinman et al., 2017; Meres et al., 2012; Quinn et al., 2012b; Whitten et al., 2014). More comprehensive investigations of bacteria in the affected lesions are recommended for improving our understanding of shell disease, especially for the lobster species not well studied, i.e., European lobster, spiny lobsters and Norway lobster. The potential contributory role of bacteria to the progression of shell disease have been reported (Feinman et al., 2017;

Whitten et al., 2014), however, the detailed mechanisms of the bacterial infection still remain poorly understood. Examining the range of bacterial characteristics associated with shell disease may provide some evidence for the mechanisms for bacterial involvement in shell disease (Zha et al., 2018a; Zha et al., 2018b). There is a need to confirm the causative organisms for shell disease which could be done via animal experiment. As shell disease is usually initially observed to occur on the surficial cuticle layers, the inoculation of bacteria onto the cuticle could be interfered with other bacteria that naturally present in the wild or captive environment (Geddes et al., 2004; May, 2007; Quinn et al., 2012a). More detailed attention to experimental design and closer monitoring of the succession of bacterial populations associated with shell disease is required to better understand the processes leading to the establishment of the condition in lobsters.

There is a lack of effective measures for preventing and treating shell disease in lobsters largely due to the limited understanding about this condition, such as the identity and transmission of the causative organisms and the possible mechanisms for triggering the condition. Future research could be directed at achieving more effective prevention and treatment measures for shell disease.

3. Chapter Three - Immune status of the spiny lobster *Jasus edwardsii* with tail fan necrosis

3.1. Introduction

Commercial fisheries and aquaculture of marine lobsters globally are worth well in excess of US$2B a year (Kough et al., 2013). An increasingly common condition in lobsters is a classical shell disease which encompasses a range of different conditions and etiologies that are externally apparent on the exoskeleton (Chistoserdov et al., 2005; Quinn et al., 2013a). Classical shell disease has been described in many lobster species, including the European lobster (*Homarus gammarus*), the American lobster (*H. americanus*), spiny lobsters (i.e. *Panulirus argus*, *P. cygnus* and *Jasus edwardsii*) and Norway lobster (*Nephrops norvegicus*) (Chistoserdov et al., 2012; Chistoserdov et al., 2005; Freeman & MacDiarmid, 2009; Mancuso et al., 2010; Quinn et al., 2013a).

Among spiny lobsters, a commonly reported form of classical or endemic shell disease is tail fan necrosis (TFN) which is, characterized by erosion and blackening of the biramous uropods (i.e., each comprising an exopod and an endopod) and telson that make up the tail fan (Mancuso et al., 2010; Musgrove et al., 2005; Shields, 2011). This condition has been reported in a number of spiny lobster species from captive animals and wild populations (Ooi, 2014). For example, between 33% and 43% of wild-caught adult Australasian red spiny lobsters, *J. edwardsii*, developed TFN after 30 weeks of holding in sea-cages in south Australia (Bryars & Geddes, 2005). Also, in north-eastern New Zealand over 17% of adult male *J. edwardsii* sampled from a fished wild population over a three-year period of sampling were found to have TFN (Freeman & MacDiarmid, 2009). By comparison, only 2% of lobsters within a nearby unfished marine reserve had TFN, which strongly indicated that handling by commercial fishers was a likely cause of the TFN in the lobsters living outside the reserve. This conclusion is supported by prior evidence that TFN is initiated by external

physical damage to the exoskeleton, with subsequent invasion of the damaged tissues by opportunistic marine bacteria, such as *Vibrio* spp. (Musgrove et al., 2005). Physical damage has also been implicated in the initiation of classical shell disease in the American lobster (Quinn et al., 2012a). The reactions leading to wound repair in spiny lobsters include haemocyte aggregation and coagulation followed by melanisation of the wound area which produces the distinctive blackened appearance of the affected area of the exoskeleton (Cerenius et al., 2008; Theopold et al., 2004; Vafopoulou et al., 2007). Frequently, the blackened tissues associated with TFN extend beyond the site of the initial wound as further melanisation of tissues occurs as a result of an immune response which aims to isolate an advancing bacterial incursion in the infected tissues (Musgrove et al., 2005).

In addition to the physical barrier presented by the exoskeleton in spiny lobsters, the haemolymph and especially the haemocytes, are thought to play the main role in pathogen defence in crustaceans (Destoumieux-Garzón et al., 2016). The wound repair processes are mainly mediated by haemocytes or antimicrobial peptides, and other haemolymph components (Roch, 1999). The haemocytes are involved in many immune functions and wound repair, such as prophenoloxidase activation, deposition of melanin precursors and phagocytosis (Fotedar et al., 2006; Hernández-López et al., 2003; Theopold et al., 2004). As a consequence of the pivotal role haemocytes play in immune responses, total haemocyte counts and haemocyte viability are frequently used as measures of immune status in crustaceans. The recognition, inactivation and elimination of foreign organisms are effected through host defence responses involving circulating haemocytes, fixed phagocytes, agglutinins or lectins and antimicrobial factors present in the haemolymph of invertebrates (Cerenius et al., 2008; Vogt, 2012). Immunorecognition is thought to be mediated in part through the prophenoloxidase system, which involves a cascade of serine proteases and prophenoloxidase present in the haemocytes which is activated by non-self molecules

(Perdomo-Morales et al., 2007; Söderhäll & Sritunyalucksana, 2002). Consequently, these various immune responses provide the basis of a range of measures that have been used for determining the immune status of crustaceans (Fotedar et al., 2006; Fotedar et al., 2001; Hernroth et al., 2012; Verghese et al., 2007). Despite the ready availability of these measures for determining the immune status of lobsters, they have not been used previously to determine whether the immune status of lobsters affected with TFN is impaired as a result of actively responding to a TFN infection. The limitations of the crustacean immune response to acute bacterial infection would suggest that TFN has the potential to lead to systemic infection of lobsters by pathogenic bacteria with the potential to ultimately overwhelm infected individuals (Ooi, 2014). It is also possible that TFN develops after the host immunity has been compromised from the stress of handling and physical damage, a pathway which has been implicated in other types of classical shell disease (Tlusty et al., 2007). However, it is likely that a combination of both pathways contribute to the development of TFN in spiny lobsters.

In this study, we contrast the physical condition and immune status of lobsters affected with TFN with those unaffected to provide some insight into the aetiology of TFN in spiny lobsters. We applied commonly used measures of immune status to lobster sampled from a commercially fished population of red Australasian spiny lobsters *J. edwardsii*, in northeastern New Zealand characterised by relatively high prevalence of TFN (Freeman & MacDiarmid, 2009). Total haemocyte count (THC), haemocyte viability, total protein concentration in plasma and haemocyte lysate supernatant (HLS), phenoloxidase (PO) activity in the HLS, and haemolymph bacteria count by culture were used to assess the immune status of lobsters sampled from the fishery. Lobsters, both with and without TFN, were sampled and analysed for comparative purposes.

3.2. Materials and methods

3.2.1. Spiny lobster specimens

Wild adult and juvenile *J. edwardsii* were caught over two days in April 2016 using commercial lobster pots on the east coast of the North Island of New Zealand in commercially fished areas previously identified to have high prevalence of TFN among lobsters (178°13’ E, 38°37’ S, map included in Freeman & MacDiarmid, (2009)). A total of 44 lobsters with TFN and 38 without TFN were randomly selected from the entire catch, and immediately placed in chilled insulated boxes and transferred to the laboratory within two hours of capture for further processing. Other than initially categorising the captured lobsters into two groups (i.e., TFN present or absent) based on an initial examination of the lobsters immediately after capture, the lobster sample is representative of a typical lobster harvest from this location at this time.

3.2.2. Morphological measures and TFN assessment

The carapace length (CL) and tail width (TW) of all the lobsters were measured using calipers and the number of missing appendages (antennae, pereiopods and pleopods) on individual lobsters was recorded. Moult stage of the lobsters was also recorded according to Musgrove, (2000). For lobsters with TFN, the number of affected elements of the tail fan (i.e., exopods, endopods (uropods) and telson – Fig. 3-2A) of each individual lobster was counted, including whether the ventral and dorsal parts of tail fans were affected. The percentage of total areas of the ventral and dorsal surfaces of tail fans were separately estimated for each exopod, endopods and telson on each lobster that was affected by TFN. This estimate included an allowance for areas of each exopod, endopod or telson which were missing but for which the attached remaining margins were affected. The total area of the tail

fan affected by TFN for each lobster was then calculated as the mean of the affected ventral and dorsal surface areas.

3.2.3. Haemolymph collection

The base of the fifth thoracic legs of all the lobsters were disinfected with 70% alcohol and a sample of haemolymph was removed with a 26-ga needle attached to a 1-ml sterile syringe (Terumo, Inc.).

3.2.4. Total haemocyte count (THC) and haemocyte viability assay

Total haemocyte count and haemocyte viability of all the lobsters were assessed using a portable flow cytometer - Muse® Cell Analyser (Merck Millipore, Inc.) and Muse® Count & Viability Assay Kit (Merck Millipore, Inc.). An aliquot of 100 µl of haemolymph was transferred to an equal amount of Alsever's solution (Sigma-Aldrich, Inc.). Diluted haemolymph was then gently mixed twice to obtain a homogeneous suspension of haemocytes, and incubated at room temperature and then analysed on the instrument as per the manufacturer's instructions and as modified by Grandiosa et al., (2016).

3.2.5. Phenoloxidase (PO) activity in haemocyte lysate supernatant (HLS)

3.2.5.1. Preparation of HLS

HLS was prepared from lobster haemolymph as per Perdomo-Morales et al., (2007) and Safari et al., (2015). Briefly, a 700 µl aliquot of haemolymph was mixed with anticoagulant (sodium citrate 0.114 M, sodium chloride 0.10 M, pH 7.45) before centrifugation at 800 × g for 10 min at 4 °C. The cell pellet was washed twice with anticoagulant, suspended in Tris-HCl (pH 7.5) and homogenized using a glass piston homogenizer. The lysate was then centrifuged at 10000 × g for 10 min at 4 °C to obtain the clarified haemocyte lysate supernatant (HLS).

3.2.5.2. PO activity

The PO activity in the HLS of all the lobsters was measured using previously described methods, with some modification (Celi et al., 2015; Hernández-López et al., 1996; Perdomo-Morales et al., 2007). An aliquot of 25 µl of HLS was incubated with 50 µl trypsin (1 mg ml^{-1}) in 50 mM Tris-HCl buffer (pH 7.5) for 20 min at room temperature in 96-well polystyrene plates. A control consisting of only buffer was also included. An aliquot of 50 µl L-DOPA (3 mg ml^{-1} in distilled water) was added to each well, and the absorbance was measured at 490 nm using a microplate reader after 10 min of incubation at 25 °C. Enzyme activity was expressed as the change in absorbance at 490 nm per minute per mg of protein in 10 min.

3.2.6. Total protein concentration in haemolymph plasma and HLS

The total protein concentrations in haemolymph plasma and HLS from all the lobsters were determined by using a Qubit® 2.0 Fluorometer (Thermo Fisher Scientific, Inc.) and Quant-iT™ Protein Assay Kit (Thermo Fisher Scientific, Inc.) (Celi et al., 2015). Haemolymph was mixed with anticoagulant (sodium citrate 0.114 M, sodium chloride 0.10 M, pH 7.45). Plasma was extracted by centrifuging lobster haemolymph at 800 × g and the supernatant was collected for plasma protein assay. The HLS used for this assay was prepared in the manner described previously.

3.2.7. Haemolymph bacteria assessment

A 100 µl aliquot of haemolymph from each lobster and a ten-fold dilution (in autoclaved filtered seawater) were spread onto each of three replicate marine agar plates (BD Difco, Inc.), and incubated at 18 °C for seven days. Colony-forming units (CFU) were counted and CFU ml^{-1} was calculated for each sample.

3.2.8. Statistical analyses

A t-test and Welch's t-test were used to compare the mean CL and TW of lobsters with and without TFN, respectively. The Welch's t-test was used because of unequal variances between the two samples. A Pearson's chi-square test was used to compare the proportions of male lobsters that were over the legal size limit (i.e., TW = 54 mm) for those individuals with and without TFN. This test was used because it determines whether proportions of categorical data were independent (Sokal & Rohlf, 2012). A Pearson's chi-square test was also used to compare the proportion of lobsters with and without missing appendages for the lobsters with TFN versus those lobsters without TFN. A Mann Whitney test was used to compare the number of missing appendages between lobsters affected and not affected by TFN. The Mann Whitney test was used because it provides a statistical comparison of two independent groups of discrete nonparametric variables (Sokal & Rohlf, 2012). A Pearson's chi-square test was also used to compare the proportions of the different tail fan elements (i.e., exopod, endopod, telson) that were affected by TFN. The percentages of the ventral and dorsal surface areas of the tail fans of individual lobsters affected by TFN were compared with a paired t-test after the data were arcsine transformed to normalise their distributions (Sokal & Rohlf, 2012).

Welch's t-tests were used to compare THC, haemocyte viability, PO activity, protein content in haemolymph plasma, protein content of HLS and haemolymph bacteria counts for lobsters with and without TFN, respectively. Percentages of haemocyte viability were arcsine transformed for analysis. Haemolymph bacteria counts were $\log_{10}$ transformed to improve homogeneity of variance, normality of the residual errors and additivity of treatment effects for analysis.

All results are presented as mean ± S.E. unless otherwise stated. Data analyses were undertaken using SPSS Statistics 23 software.

3.3. Results

3.3.1. Morphological characteristics

The CL of the sampled lobsters ranged between 89 and 111 mm. Lobsters with TFN were significantly larger than those without TFN, i.e., mean CL 103 ± 0.6 mm versus 99.0 ± 0.7 mm respectively (t-test, $t = -4.37$, $P < 0.001$). The TW of the sampled lobsters ranged from 43 - 57 mm. Lobsters with TFN had significantly larger TW than those lobsters without TFN, i.e., mean TW 52.6 ± 0.2 mm versus 51.4 ± 0.4 mm (Welch's t-test, $F = 6.26$, $P < 0.02$). The legal TW size is 54 mm for males and 29.5% of male lobsters with TFN were legal size versus 18.4% for those male lobsters without TFN ($\chi^2 = 1.37$, $P = 0.24$). The majority of the sampled lobsters were male, with a total of only three female lobsters sampled, and all unaffected by TFN. All the lobsters were at the inter-moult stage.

Lobsters with TFN were more than four times more likely to have a missing or injured appendage (i.e., antenna, pereiopod or pleopod) than lobsters without TFN, 63.6% versus 18.4% ($\chi^2 = 17.04$, $P < 0.001$). Furthermore, lobsters with TFN had more missing or injured appendages than those without TFN (Mann-Whitney test, $U = -4.42$, $P < 0.001$) (Fig. 3-1A). For example, among the lobsters with TFN one lobster was missing a total of eight appendages whereas the maximum number of missing appendages for lobsters without TFN was only three. Whilst sampling lobsters it was observed that lobsters with more advanced TFN were generally less vigorous in response to handling than those without TFN in terms of antennae waving, tail flicking, pereiopod grasping and strength of their pereiopod grip, however, these parameters were not quantified.

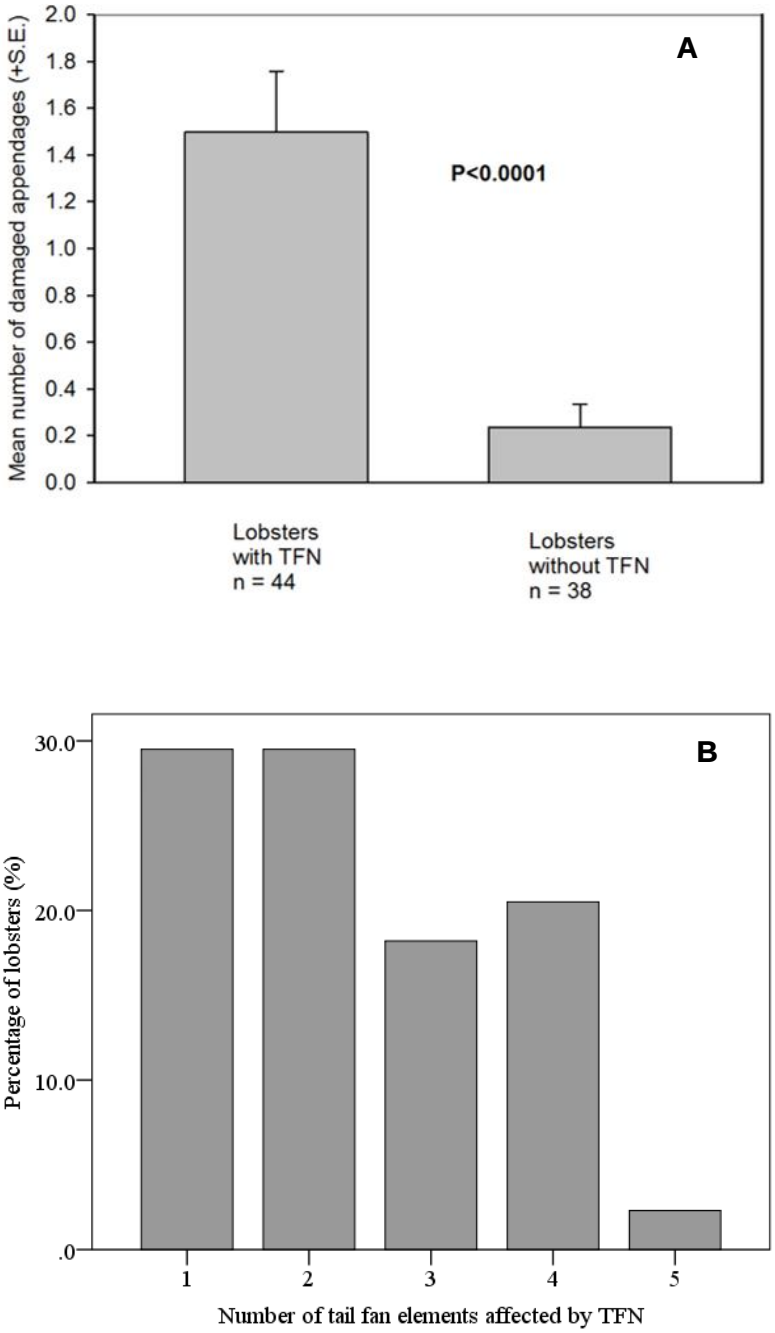


Fig. 3-1. Morphological characteristics of sampled lobsters. 1A) Mean number (±S.E.) of damaged appendages (i.e., antennae, pereiopods and pleopods) in sampled lobsters with and without tail fan necrosis (TFN); 1B) Number of individual elements of the tail fan, (i.e., either an exopod, endopod, or telson) affected by TFN in 44 affected lobsters.

Of the 44 lobsters with TFN, 29.5% had TFN present in only one element of their tail fan, i.e., either an exopod, endopod or telson. A total of 29.5% of the lobsters had TFN present in two tail fan elements, 18.2% in three elements, 20.5% in four elements and 2.3% in all five elements (Fig. 3-1B). Necrosis had extended into the peduncle and the base of abdomen in three TFN-affected lobsters. In one lobster, necrosis was present in the anterior portion of the abdominal somites. Of the lobsters with TFN, the affected area of the tail fans affected with TFN of individual lobsters ranged between 3% and 54%. Eighteen percent of lobsters had less than 10% of their tail fan affected by TFN (Fig. 3-2A); 36% of lobsters had TFN affecting between 10% and 20% of the tail fan (Fig. 3-2B and 3-2C), whereas 46% lobsters had more than 20% of their tail fans affected by TFN (Fig. 3-2D). TFN was more commonly found in endopods (59.1%) than in the exopods (43.2%) ($\chi^2 = 4.5$, $P < 0.04$) and in the telson (34.1%) ($\chi^2 = 7.3$, $P < 0.01$). The mean surface area of the tail fan of lobsters that was affected by TFN was greater on the ventral surface than on the dorsal surface, i.e., 25.7 ± 2.1% versus 20.0 ± 1.9% (paired t-test, $t = 10.1$, $P < 0.0001$) (Fig. 3-2B and 3-2C).

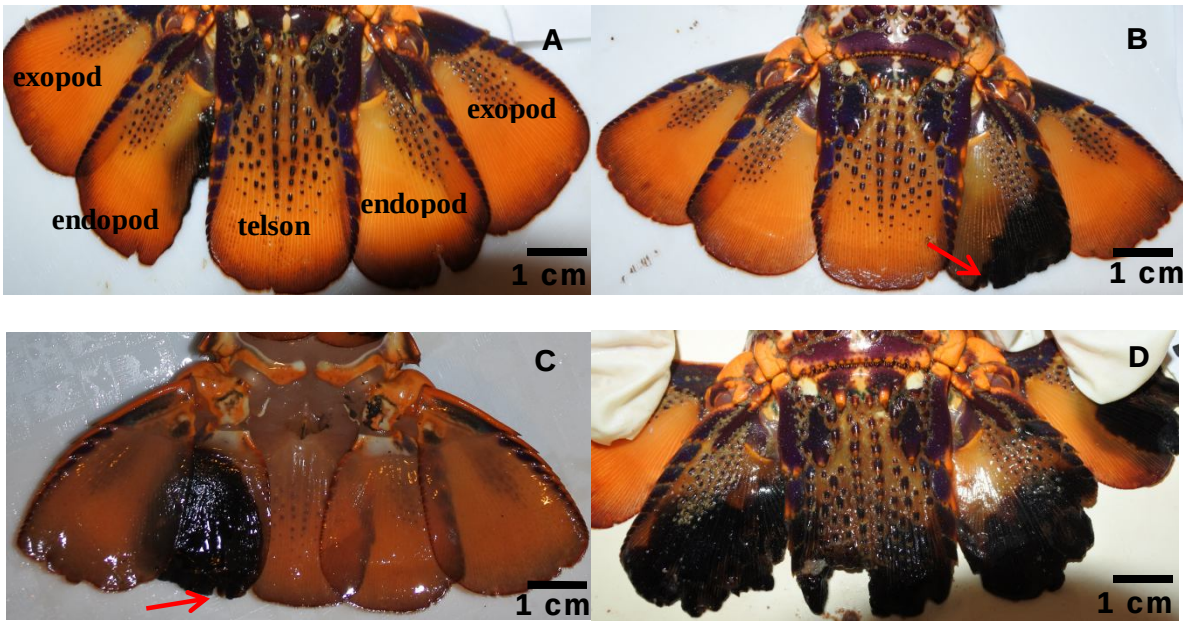


Fig. 3-2. Photographs of sampled lobsters showing typical appearance of tail fan affected with tail fan necrosis; A) lightly affected, <10% TFN, dorsal surface and showing the three

elements of the tail fan, B) moderately affected, 10-20%, dorsal surface –note the small notch in the endopod from which the TFN appears to have radiated from (arrowed); C) Same tail fan as in (B), showing difference in extent of TFN between dorsal and moderately affected ventralsurface (8 versus 18%, respectively).; D) severely affected, >20%, dorsal view. Photographs 3-2B and 3-2C are from the same lobster tail fan showing the difference in extent of TFN between dorsal and ventral surfaces, i.e., 8% versus 18% respectively.

3.3.2. THC and haemocyte viability assay

The mean THC of lobsters without TFN was $3.01 \pm 0.08 \times 10^6$ cells ml^{-1}, which was significantly higher than lobsters with TFN $2.77 \pm 0.06 \times 10^6$ cells ml^{-1} (Welch's t-test, $F = 6.02$, $P < 0.02$). The mean haemocyte viability in lobsters without TFN was $89.4 \pm 0.8\%$, which was not different to lobsters with TFN $89.7 \pm 0.3\%$ (Welch's t-test, $F = 0.04$, $P = 0.83$).

3.3.3. PO activity in the HLS

The mean PO activity in the HLS of lobsters without TFN was 1.11 ± 0.06 mg^{-1} min^{-1}, which was significantly higher than that of lobsters with TFN 0.71 ± 0.05 mg^{-1} min^{-1} (Welch's t-test, $F = 26.04$, $P < 0.001$).

3.3.4. Total protein concentration in haemolymph plasma and HLS

The protein concentration of haemolymph plasma ranged from 22 - 95.6 mg ml^{-1}. There was no difference in the mean protein concentration of haemolymph plasma in lobsters without TFN (70.41 ± 2.48 mg ml^{-1}) versus lobsters with TFN (67.55 ± 2.90 mg ml^{-1}) (Welch's t-test, $F = 0.56$, $P > 0.45$). The protein content in HLS ranged from 2 - 7.4 mg ml^{-1}. There was no difference in the mean protein concentration in HLS of lobsters without TFN versus those with TFN (4.40 ± 0.18 mg ml^{-1} and 4.40 ± 0.16 mg ml^{-1}) (Welch's t-test, $F = 0.01$, $P = 0.95$).

3.3.5. Haemolymph bacteria assessment

Counts of haemolymph bacteria (> 900 CFU ml^{-1}) were found to be highly variable in both lobsters with and without TFN, with a small number of lobsters both with and without TFN having counts exceeding 900 CFU ml^{-1}. There was no significant difference in the mean haemolymph bacteria counts for lobsters without TFN (2.75 ± 2.43 CFU ml^{-1}) versus those with TFN (1.97 ± 1.53 CFU ml^{-1}) (Welch's t-test, $F = 0.51$, $P > 0.22$).

3.4. Discussion

This study is the first investigation of the physical condition, in terms of injury, and the corresponding immune status in a population of *J. edwardsii* with endemic TFN infections as evaluated by applying a range of immunological tools commonly used in crustaceans (Fotedar et al., 2006; Grandiosa et al., 2016; Safari et al., 2015).

The lobsters sampled from the population using commercial pot methods were dominated by males (96.3%) of a size close to the legal size limit (54 mm TW) which is a characteristic of this coastal fishery where females seasonally migrate into deeper waters (Celi et al., 2015; Freeman & MacDiarmid, 2009; Linnane et al., 2015). Male lobsters around legal size are the focus of commercial potting with all captured lobsters handled for measuring and if undersized returned to the water (Starr et al., 2013). Sampled lobsters with TFN were larger in size (both CL and TW) which may be the result of TFN-affected animals being returned by commercial fishers who routinely sort their catch and only retain the highest value animals and return the remainder to the sea (known as "high grading") in order to maximise the value from their catch quota (Sykes, 2017). Three commercial fishers confirmed this behaviour because lobsters with TFN reportedly attract lower market prices due to their unsightly appearance and their reduced ability to withstand live transport to international markets. The effect of handling and returning diseased animals to the natural population may have

important implications for the aetiology of TFN within the lobster population, especially for providing increased potential for cross infection whilst lobsters are corralled in pots, collectively handled by fishers, and whilst cohabitating shelters in the wild, as have been observed to be important for transfer of diseases in fished populations of other lobster species (Campos et al., 2015; Emery et al., 2016; Leland et al., 2013; Milligan et al., 2009; Parsons & Eggleston, 2005). This is a possible explanation for the marked difference in the prevalence of TFN previously observed on the same coastline between an unfished lobster population in a marine reserve (< 2%) versus the adjacent fished population (17%) (Freeman & MacDiarmid, 2009).

Missing appendages were more commonly found in the TFN-affected lobsters, which is consistent with physical damage to the tail fan caused by capture in pots and post-capture handling initiating TFN (Campos et al., 2015; Milligan et al., 2009; Musgrove et al., 2005), and is also consistent with the findings of Freeman & MacDiarmid, (2009) for lobsters in this area of coast of New Zealand. In addition, TFN was also more prevalent on the ventral surfaces of tail fan elements than on the dorsal surface which may be due to differences in thickness of the carapace on the ventral surface, or due to physical damage sustained while lobsters use the tail flip escape response.

For those lobsters with small areas affected by TFN, it was frequently possible to identify the extent of physical damage (i.e., loss and tears) to the tail fan from which the TFN appeared to have been initiated. In most cases this consisted of a tear from the anterior margin of the tail fan element (i.e., telson, endopod or exopod) and extending into, and through, the element. Less commonly, pieces of tail fan would have been removed in their entirety or there would be clear signs of a puncture wound. Collectively these types of injury are characteristic of lobsters exposed to handling and holding in mesh enclosures (Musgrove et al., 2005). In contrast, in lobsters with more extensive TFN, it was frequently difficult to determine the

point of initiation of TFN because of the extensive loss of tail fan tissue, suggesting that the condition had continued to advance well beyond the site of initial injury. The TFN-affected lobsters may be able to replace their affected tail fans during a moult, but previous studies indicate that increasing severity of TFN rather than recovery is more common in lobsters affected with TFN (Musgrove et al., 2005). In the current study there was no sign of recovery from previous TFN among the sampled lobsters, because moult scars are commonly observed in crabs and lobsters that have recovered from injuries (Deangelis et al., 2012).

Immune parameters of an animal may vary in response to different diseases or environmental challenges (Shields, 2011). In the current study, of the seven commonly used parameters to evaluate immune status in crustaceans only two showed significant differences between lobsters with TFN and those without. The circulating haemocytes found in lobsters, contribute to a series of primary immune actions, such as phagocytosis, coagulation, encapsulation and melanisation (Cerenius & Söderhäll, 2012; Hernroth et al., 2004; Hernroth et al., 2012; Smith et al., 2003). THC was around 9% higher in lobsters without TFN versus those with TFN, while there was no difference in haemocyte viability. However, this would result in a higher relative number of viable haemocytes in the haemolymph of lobsters without TFN. This difference may be due to the loss of haemocytes in responding to the TFN infection. The lower abundance of haemocytes in the haemolymph of lobsters with chronic TFN has the potential to make them more prone to further invasion by the TFN infectives and any other invasive micro-organisms.

PO activity in the HLS was markedly higher (56%) in lobsters without TFN versus those with TFN in this study which is likely to be the result of responding to TFN. PO activity is a key step of the prophenoloxidase activating system, which results in the production of highly reactive and toxic quinone intermediates and finally the melanisation of infected tissues (Cerenius et al., 2010b; Cerenius et al., 2008). During this process, intruding protozoans and

bacteria are frequently attacked and melanised in many invertebrates, including lobsters (Cerenius et al., 2010b). Therefore, PO activity is an important measure of the primary immune response of a lobster to physical damage and microbial incursion arising from such damage. A lower PO activity in TFN-affected lobsters indicates that this PO response appears to be substantially compromised in lobsters with TFN, most likely as a result of active ongoing deployment against the chronic TFN condition resulting in the outward appearance of melanisation of the tissues.

Apparently healthy crustaceans, including lobsters, frequently have bacterial flora in their haemolymph (Chistoserdov et al., 2005; Fotedar et al., 2001; Meng et al., 2010; Quinn et al., 2013b; Shields et al., 2012; Wang, 2011), although there is also a study showing the haemolymph in a small sample of lobsters were aseptic (Mancuso et al., 2010). Bacteria were also found in the haemolymph of healthy lobsters, which is consistent with previous studies. The overall bacteria counts in the haemolymph of healthy lobsters and those with TFN were similar, which suggests that the bacteria count in haemolymph is not associated with TFN in lobsters. Bacteria counts from lobster haemolymph make no distinction between normal bacterial flora and potential pathogens. The limitation of this technique is further compounded by the inability of many bacteria to be cultured which can result in underestimates in bacteria counts (Stewart, 2012). The further application of molecular genetic methods would provide the opportunity to reliably identify the bacteria present in haemolymph that may not be culturable and that may also be associated with TFN.

There are a variety of proteins in haemolymph plasma and HLS, including haemocyanin protein, lectin, coagulogen, macroglobulin, subtilisin-inhibitor, trypsin inhibitor, clotting protein and phenoloxidase (Cerenius et al., 2008; Cerenius & Söderhäll, 2012; Söderhäll & Cerenius, 1992; Theopold et al., 2004), which are all known to contribute to a series of immune actions in crustaceans, including lobster species (Cerenius et al., 2010b; Clark, 2014;

Fotedar et al., 2006; Söderhäll & Cerenius, 1992). Consequently, haemolymph protein concentration has been used as a proxy measure of health and immune status (Behringer et al., 2008; Fotedar et al., 2006; Oliver & MacDiarmid, 2001; Shields, 2011; Verghese et al., 2007). For example, haemolymph protein concentration has been found to be significantly lower (by 4 - 5 mg l^{-1}) in Caribbean lobsters (*Panulirus argus*) infected with *Panulirus argus* virus 1 (PaV1) compared with uninfected lobsters (Behringer et al., 2008). However, the lack of any apparent differences in protein levels in haemolymph plasma and HLS due to TFN infection suggests the affected lobsters may not be greatly compromised by the presence of this condition as it is usually a marker for near-death condition in crustaceans.

It seems likely that physical damage to lobsters associated with fishing is an initiator of TFN in the population of lobsters in this study as firstly suggested by Freeman & MacDiarmid, (2009). If this is the case, then a number of measures can be taken to prevent and control the disorder. Commercial traps can be modified to have larger mesh sizes and softer surfaces, such as plastic-coated mesh, to minimise the physical damage to lobsters (Butler & Matthews, 2015; Leland et al., 2013; Matthews, 2001; Shields, 2012). Likewise, less damaging handling systems can be set up on fishing vessels for landing and removing the catches from traps (Davidson & Hosking, 2004). Handling gloves and commercial traps could be disinfected with weak chlorine bleach solution before use in fishing to decrease the possibility of cross contamination as lobsters with TFN are likely to come into contact with both sets of surfaces raising the potential for transfer of infective bacteria. Removal of the strict size limit might be another effective means for decreasing the damage to lobsters that is likely to result from repeated handling from intensively fishing to a size limit (DiNardo et al., 2002; Emery et al., 2016). Removal of diseased sublegal animals from the fishery could also decrease the chance of transmission among the lobster population (Shields, 2012). Lobster fishing could also be curtailed during the peak lobster moulting period when exoskeletons are

still hardening and more vulnerable to fishing-induced lacerations (Jeffs et al., 2013). Lobsters with TFN could be required to be landed rather than returned to the fishery in order to help prevent the possible persistence and transfer of the disorder through ongoing contact with other lobsters in traps and in natural shelters (Behringer et al., 2008; Musgrove et al., 2005; Shields, 2012).

Only relatively recently has the significance of crustacean diseases emerged as a major concern to the global production of crustaceans (Behringer et al., 2012; Evans et al., 2000; Shields, 2011; Stentiford et al., 2012; Wu et al., 2014). For example, infections of PaV1 in the population of *P. argus*, which supports the largest spiny lobster fishery in the world, can cause high mortality (up to 60%) of juvenile spiny lobsters *P. argus* (Behringer et al., 2011). PaV1-infected lobsters are also more vulnerable to co-infections, having almost 50% higher gill infestation by *Epystilis* sp. and *Zoothamniun* sp. compared to lobsters without PaV1 infection (Jiménez et al., 2012). Likewise, the prevalence of epizootic shell disease remains high (up to 40%) in American lobsters off the coast of southern New England, and the disease has played a major role in the decrease of the fished lobster stock in this area and has even had a catastrophic effect on the American lobster fishery (Castro & Somers, 2012; Hoenig et al., 2017; Wahle et al., 2009).

Injuries to lobsters are a common outcome of a variety of harvesting methods and are assumed to have relatively minor consequences to undersized animals returned to the wild, such as retarded growth whilst the lost appendages are replaced (Emery et al., 2016; Frisch & Hobbs, 2011; Leland et al., 2013; Powrie & Tempero, 2009). Furthermore, minor tissue damage, such as small tail fan lacerations that appear to have led to TFN infection that were observed in the present study (e.g., Fig. 3-2B) are not normally measured in previous studies or even considered to be of concern. However, if this damage leads to the establishment and

persistence of disease in a lobster stock, then the ultimate consequences for the harvestable stock could be significantly greater than anticipated.

3.5. Conclusion

Two immune parameters, total haemocyte count and phenoloxidase activity in the haemocyte lysate supernatant (HLS), were significantly compromised in lobsters with TFN. No differences were found in the other immune parameters, i.e., haemocyte viability, haemolymph bacterial count, and the protein content of haemolymph plasma and HLS. The results are consistent with injury sustained during prior capture and handling that initiates TFN in these natural caught lobsters.

4. Chapter Four - Characteristics of culturable bacteria associated with tail fan necrosis in the spiny lobster, *Jasus edwardsii*

4.1. Introduction

Shell disease is a common condition in both wild and captive lobsters, including American lobster, European lobster, spiny lobsters and Norway lobster, and has different etiologies (Davies et al., 2014a; Musgrove et al., 2005; Quinn et al., 2013a; Ziino et al., 2002). Shell disease has several etiologies in American lobsters (*Homarus americanus*), including epizootic shell disease, impoundment shell disease, enzootic or classical shell disease, diet-induced shell disease, and black spot shell disease (Chistoserdov et al., 2012; Chistoserdov et al., 2005; Quinn et al., 2013a; Shields, 2011). Tail fan necrosis (TFN) is commonly reported in spiny lobsters, and is characterized by erosion and blackening of the uropods and telson that make up the tail fan (Mancuso et al., 2010; Musgrove et al., 2005; Shields, 2011). This condition has been reported in different spiny lobster species from wild and captive populations (Mancuso et al., 2010; Ooi, 2014), and is the predominant shell disease reported in spiny lobsters in New Zealand and Australia (Diggles et al., 2002). Over 17% of adult male Australasian red spiny lobsters (*Jasus edwardsii*) in a wild population in north-eastern New Zealand were found to have TFN when sampled over a three years using commercial potting methods (Freeman & MacDiarmid, 2009). TFN was also observed in 33 - 43% of wild-caught *J. edwardsii* after 30 weeks of holding in sea-cages in South Australia (Bryars & Geddes, 2005).

The specific causes of shell disease have been extensively studied. Studies of shell disease in *H. americanus* have found that bacterial infection is often initiated, or associated with, physical damage to the cuticle, but not always (Quinn et al., 2013a; Quinn et al., 2012a)). With epizootic shell disease in *H. americanus*, a specific marine bacteria *Aquimarina*

homaria was commonly found in the lesions and was regarded as the primary causative pathogen of epizootic shell disease (Quinn et al., 2013a; Quinn et al., 2012a), although other bacteria including *Thalassobius* sp. and *Pseudoalteromonas* sp. were also reported to contribute to the disease (Chistoserdov et al., 2012; Quinn et al., 2012a; Whitten et al., 2014). These organisms appear not to be associated with shell disease in other lobster species. For example, these bacteria were not frequently present in the lesions of the European clawed lobster (*H. gammarus*) and the European spiny lobster (*Palinurus elephas*) (Mancuso et al., 2010; Whitten et al., 2014). *Vibrio parahaemolyticus, V. alginolyticus, V. vulnificus, V. anguillarum,* and *Aeromonas caviae* have been identified from the tail fan lesions of *J. edwardsii* affected with TFN and assumed to be the causative organisms (Mancuso et al., 2010; Musgrove et al., 2005). However, the consistent lack of negative controls to TFN in these studies casts some doubt on the conclusions that were drawn given that many of the bacteria that have been identified are ubiquitous in the marine environment (Mancuso et al., 2010; Musgrove et al., 2005). Hence, the causative bacteria involved in TFN remain to be elucidated.

TFN in spiny lobsters is thought to be associated with initial physical damage to the integrity of cuticle with subsequent infection with opportunistic bacteria (Freeman & MacDiarmid, 2009; Musgrove et al., 2005). The cuticle of lobsters usually consists of protein, lipid and a large percentage of chitin (Davies et al., 2014b; Smolowitz et al., 2005). Some researchers hold a view that shell disease is associated with chitinolytic bacteria capable of breaking down the major biochemical component of cuticle (Li & Roseman, 2004; Mancuso et al., 2010; Suginta, 2007). Other researchers postulate shell disease is also associated with proteolytic and lipolytic bacteria through their ability to degrade other important components of the cuticle, including the waxy protective outermost layer of the cuticle (Quinn et al., 2013a; Vogan et al., 2008). Regardless, in addition to some representative causative bacteria,

such as *A. homaria* in *H. americanus* (Quinn et al., 2017; Quinn et al., 2012a; Shields, 2012), it appears that a range of opportunistic bacteria are likely to be associated with shell disease in lobsters. Different bacterial communities have been identified in lobsters with different types of shell disease, such as for epizootic shell disease, enzootic shell disease and trauma-induced shell disease (Chistoserdov et al., 2012; Meres et al., 2012; Quinn et al., 2013a). A number of bacteria associated with different diseases have also been identified from the haemolymph of marine crustaceans, including lobsters (Bartlett et al., 2008; Defer et al., 2013; Nunan et al., 2010; Shields, 2011; Wang & Wang, 2015). For example, *Pseudomonas fragi*, *Brochothrix thermosphacta*, *Vibrio* spp. and *Delftia acidovorans* were found in the haemolymph of lobsters with epizootic shell disease (Chistoserdov et al., 2005; Quinn et al., 2013b; Shields et al., 2012). Overall, it appears that shell disease is associated with a variety of bacteria in different studies on different crustacean species (Chistoserdov et al., 2005; Cook & Lofton, 1973; Quinn et al., 2013b).

These previous findings suggest that in addition to some abundant representative bacteria (Feinman et al., 2017), bacteria with specific characteristics, i.e., proteolytic, chitinolytic and lipolytic activities, are possibly associated with shell disease, including TFN in spiny lobsters. While the presence of TFN results in externally obvious lesions of the cuticle that can extend into the deeper tissue layers (Mancuso et al., 2010), it is unclear whether the bacterial infection may progress to systemic infection, with the results of one study suggesting it is unlikely (Comeau & Benhalima, 2009). The examination of the haemolymph of nine spiny lobsters (*P. elephas*) with TFN failed to identify the presence of any bacteria (Mancuso et al., 2010). However, the potential for the initiation of bacteraemia via chronic TFN would appear to be a probable outcome and warrants further investigation.

In this study, the culturable bacteria from the tail fans and haemolymph of lobsters both with and without TFN were isolated and their proteolytic, chitinolytic and lipolytic activities

measured as an indicator of their possible role in the development of TFN. Bacteria possessing extensive enzymatic capabilities that were isolated from tail fans and haemolymph of lobsters with TFN were also identified using 16S rDNA sequencing and compared in an effort to identify those bacteria that are associated with TFN.

4.2. Materials and methods

4.2.1. Spiny lobster specimens

Wild adult and juvenile *J. edwardsii* were caught over two days in April 2016 using commercial lobster potting methods on the east coast of the North Island of New Zealand in commercially fished areas with a high prevalence of TFN among lobsters (Freeman & MacDiarmid, 2009). A total of 39 lobsters with TFN and 38 without TFN were randomly selected from the entire catch and the extent of the TFN quantified (see Zha et al. 2017). Upon landing, each lobster was loaded into separate sterile plastic bags and placed in chilled insulated boxes, then transferred to the laboratory for further processing within two hours of capture. This research was conducted under University of Auckland's approval #001471 under New Zealand's Animal Welfare Act 1999.

4.2.2. Haemolymph and tail fan swab collection

The base of the fifth thoracic leg of each lobster was disinfected with 70% alcohol. A 400 µl aliquot of haemolymph was removed with a 26 gauge needle attached to a 1-ml sterile syringe (Terumo, Inc.).

The affected area of tail fan in individual lobsters with TFN was swabbed using a sterile cotton swab and the same area of tail fan in lobsters without TFN was swabbed as unaffected samples. Each tail fan swab was transferred to 300 µl filtered and autoclaved seawater and vortexed for around ten seconds before being serially diluted and plated out (see below).

4.2.3. Bacterial isolation and culture

A 100 µl aliquot of haemolymph and a 10-fold dilution in filtered (0.2 µm, cellulose acetate membrane, Millipore) and autoclaved seawater were spread onto each of three marine agar plates (BD Difco, Inc.) and incubated at 18 °C for seven days. Tail fan swab samples were serially diluted in filtered and autoclaved seawater. The 10^3-fold, 10^4-fold and 10^5-fold diluted samples were spread in triplicate onto marine agar plates, and incubated at 18 °C for seven days. Bacterial colonies were randomly selected and subcultured from plates showing well-separated colonies. Either a total of 20 randomly selected colonies or all colonies were used from a single plate of a single dilution for any sample. When colony numbers were low, isolates were selected from all the plates at a single dilution. Each of the isolated bacteria was then streaked onto a marine agar and incubated at 18 °C for ten days. Cultures were re-streaked twice on marine agar each with incubation at 18 °C until well grown to achieve pure isolates which were stored at 18 °C ready for the subsequent experiments.

4.2.4. Proteolytic, chitinolytic and lipolytic activities

All isolates were tested for proteolytic, chitinolytic and lipolytic enzyme activities. Proteolytic activity of bacterial isolates were tested by streaking the bacterial isolates on marine agar containing 0.2% casein (Sigma-Aldrich, Inc.) (Shivaji et al., 2013). Chitinolytic activity of bacterial isolates was tested by the method of Zhang et al., (2016a). The bacterial isolates were streaked on marine agar plates containing 0.2% colloidal chitin which were prepared using chitin powder (Sigma-Aldrich, Inc.) as previously described (Reichenbach, 1992), with some minor modifications. Positive and negative controls were set up in each plate to avoid false positive and false negative results. The plates were incubated at 18 °C for 14 days. Lipolytic activity was tested on marine agar with 1% Tween-80 (Fisher Scientific, Inc.) (Slifkin, 2000). Positive and negative control strains were set up in each plate for

proteolytic and lipolytic assays to avoid false positive and false negative results. The plates were incubated at 18 °C for seven days.

Proteolytic and chitinolytic activities of bacterial isolates were scored as positive if a clear zone was observed around the colony. Enzymatic activity was ranked 0 - 4 according to the extent of the clear zone (Rank 0: 0 mm clear zone; Rank 1: 1-2 mm; Rank 2: 3-4 mm; Rank 3: 5-6 mm; Rank 4: 7-8 mm). Lipolytic activity of bacterial isolates were scored as positive if a turbid zone was observed around the colony, and activity was ranked 0 - 4 according to the extent of the turbid zone around each bacterial colony (i.e., from 0 - 8 mm).

4.2.5. Identification of bacteria by sequencing

A total of 35 bacterial isolates were found to have a broad enzyme profile (i.e., proteolytic, chitinolytic and lipolytic) from the haemolymph (16 isolates) and tail fan (19 isolates) of six lobsters with TFN were identified using PCR and sequencing methods. DNA extraction of the selected bacterial isolates used a boiling method (Bjornsdottir-Butler et al., 2016; Ngamwongsatit et al., 2008). A conserved region of the bacterial 16S rDNA gene was amplified by PCR with the universal bacterial primers PB36 (5'-AGR GTT TGA TCM TGG CTC AG-3') and PB38 (5'-GKT ACC TTG TTA CGA CTT-3') (Lear et al., 2009; Vega et al., 2011). PCRs were performed using Gotaq Green DNA polymerase (Promega, Inc.) as follows: (i) 95 °C for 5 min; (ii) 35 cycles of 95 °C for 45 s, 55 °C for 45 s, and 72 °C for 90 s; and then (iii) 72 °C for 10 min. PCR products were purified using a DNA Clean and Concentrator-5 kit (Zymo Research, Inc.). The nucleotide sequences were obtained using a contract sequencing facility (Macrogen, Inc.). The sequencing results were analyzed by using the NCBI BLAST database to identify the most closely matching sequences, and the sequences blasted to Genbank with > 97% similarity required for presumptive identification (Drancourt et al., 2000).

4.2.6. Statistical analyses

Comparisons were made of the mean proportions of bacterial isolates from the haemolymph, and from the tail fan, expressing enzyme activities (i.e., proteolytic, chitinolytic and lipolytic) for lobsters with TFN versus lobsters without TFN (Table 4-3). The means were derived from proportions calculated for each lobster as the number of bacterial isolates expressing an enzyme activity divided by the total number of bacterial isolates from the lobster for which enzyme activity could be determined (i.e., the total excluded all isolates for which enzymatic activity could not be determined). Statistical comparisons of the mean proportions used Welch's t-tests after the data were arcsine transformed to ensure compliance with the underlying assumptions for this parametric statistical method.

Chi-square tests were used to compare the overall proportions of the presence of proteolytic, chitinolytic and lipolytic activity in the bacterial isolates from the haemolymph, and from the tail fans, for lobsters with and without TFN (Table 4-4). The overall proportions were determined as the total number of bacterial isolates expressing an enzymatic activity for all sampled lobsters within a grouping (i.e., with TFN or without TFN), divided by the total number of bacterial isolates from the group of lobsters for which the enzyme activity could be determined.

Mann-Whitney tests were used to compare the difference in the measured scores of enzymatic activity (i.e., proteolytic, chitinolytic and lipolytic) of the bacterial isolates from the haemolymph, and of the tail fans, for lobsters with and without TFN for which the enzymatic activity could be determined (Table 4-4).

The enzymatic profile of each bacterial isolate was expressed as a code in which P (positive) represented some level of enzymatic activity present in a bacterial isolate, while N (negative) represented no enzymatic activity was present. Each bacterial isolate could show one of the

following eight enzymatic profiles; PPP, PPN, PNP, PNN, NPP, NPN, NNP, NNN (Table 4-4).

Table 4-1. The definition of the eight possible enzymatic profiles of bacterial isolates where P = positive and N = negative

	Enzymatic profile							
Enzymatic Activity	PPP	PPN	PNP	PNN	NPP	NPN	NNP	NNN
Proteolytic	+	+	+	+	-	-	-	-
Chitinolytic	+	+	-	-	+	+	-	-
Lipolytic	+	-	+	-	+	-	+	-

Note: "+" represents presence of enzymatic activity; "-" represents absence of enzymatic activity.

An isolate was not included in the categorisation if one or more enzymatic activity could not be determined. The numbers of isolates for each enzymatic profile was recorded for the haemolymph and tail fans of lobsters with and without TFN, and these numbers were used to generate proportions which were compared among each of the eight different categories of isolates using Chi-square tests (i.e., isolates from haemolymph (Fig. 4-1a), and for tail fans (Fig. 4-1b) for lobsters without TFN versus lobsters with TFN, and for isolates from haemolymph versus those from tail fans, for lobsters without TFN (Fig. 4-2a) and for lobsters without TFN (Fig. 4-2b). Where a significant overall result was found with a Chi-square test, exact binomial tests were applied to frequency of isolates assigned to each of the eight categories to determine the source of differences. A sequential Holm correction was applied to control the familywise error rate associated with multiple testing on the same data set.

Data was tabulated in Microsoft Excel and analysed using the statistical package SPSS (IBM, Armonk, New York).

4.3. Results

4.3.1. Isolation and subculture of bacteria

A total of 2436 bacterial colonies were selected from the bacteria cultured from haemolymph and tail fans of all the lobsters, i.e., both lobsters with and without TFN. Bacteria were cultured from the haemolymph of 39 out of 39 TFN affected lobsters and 36 out of 38 unaffected lobsters. From the initial 2436 colonies, a total of 1678 bacterial isolates were successfully isolated, including 282 bacterial isolates from the haemolymph of 34 lobsters without TFN, 283 bacterial isolates from the haemolymph of 39 lobsters with TFN, 599 bacterial isolates from the tail fans of 37 lobsters without TFN and 524 bacterial isolates from the tail fans of 35 lobsters with TFN. There was no significant difference in the number of culturable bacteria in the haemolymph of lobsters with and without TFN.

4.3.2. Enzymatic activities of bacterial isolates

While there are a number of bacteria that did not express any enzymatic activity (i.e., proteolytic, chitinolytic or lipolytic), a relatively high overall proportion of all of the 1678 bacterial isolates from the tail fan and haemolymph of lobsters with and without TFN expressed some enzymatic activity (Table 4-2).

Table 4-2. Presence and absence of proteolytic, chitinolytic and lipolytic activity of all bacterial isolates taken from haemolymph and tail fan of lobsters with and without tail fan necrosis (TFN).

Bacterial isolates	Without TFN						With TFN					
	Haemolymph n = 282 (34 lobsters)			Tail fan n = 599 (37 lobsters)			Haemolymph n = 283 (39 lobsters)			Tail fan n = 524 (35 lobsters)		
Enzymatic activity	+	-	ND	+	-	ND	+	-	ND	+	-	ND
Proteolytic (n)	97	149	36	285	290	24	124	138	21	215	234	75
(%)	34.4	52.8	12.8	47.6	48.4	4.0	43.8	48.8	7.4	41.0	44.7	14.3
Chitinolytic(n)	50	228	4	117	478	4	111	171	1	176	339	9
(%)	17.7	80.9	1.4	19.5	79.8	0.7	39.2	60.4	0.4	33.6	64.7	1.7
Lipolytic (n)	178	86	18	357	231	11	161	117	5	311	197	16
(%)	63.1	30.5	6.4	59.6	38.6	1.8	56.9	41.3	1.8	59.4	37.6	3.1

Note: "+" represents presence of enzymatic activity; "-" represents absence of enzymatic activity; "ND" represents enzymatic activity not determined.

The mean proportions of chitinolytic bacteria isolated from both haemolymph and tail fans for each lobster with TFN were greater than that for lobsters without TFN (Table 4-3). In contrast, the mean proportions of proteolytic and lipolytic bacteria isolated from haemolymph or tail fan of lobsters with TFN were similar to those without TFN (Table 4-3).

Table 4-3. Comparisons of the mean proportions of the bacterial isolates from the haemolymph, and from the tail fan, expressing enzymatic activity (i.e., proteolytic, chitinolytic, and lipolytic) when individual lobsters are grouped as lobsters with TFN versus lobsters without TFN.

Enzymatic activity	Source	Lobster TFN status	
		Without TFN (%) Mean ± SE	With TFN (%) Mean ± SE
Proteolytic	Haemolymph	36.6 ± 5.0	41.7 ± 5.1
	Tail fan	50.8 ± 2.6	49.5 ± 2.8
Chitinolytic	Haemolymph	12.9 ± 2.9**	28.1 ± 4.9**
	Tail fan	20.3 ± 3.5*	35.3 ± 4.0*
Lipolytic	Haemolymph	66.1 ± 5.1	58.0 ± 5.6
adef	Tail fan	62.4 ± 2.9	59.4 ± 3.6

Note: The proportions are calculated for individual sampled lobsters as the number of bacterial isolates expressing an enzymatic capability as a proportion of the total number of bacterial isolates for which enzymatic activity could be determined. Mean ± SE value is the mean of the proportions for individual lobsters within one group, i.e., without TFN or with TFN. Data were analysed with a Welch's t-test following data transformation where * indicates a significant difference ($0.01 < P < 0.05$), and ** ($P < 0.01$), between with TFN and without TFN groups of lobsters.

4.3.3. Enzymatic capabilities and frequency of occurrence in bacterial isolates

There was no difference in the frequency of occurrence or activity of proteolytic bacteria in lobsters with and without TFN for either haemolymph or tail fan isolates (Table 4-4).

Chitinolytic bacteria were more than twice as commonly isolated from the haemolymph and tail fan of lobsters with TFN as compared to lobsters without TFN, and the overall

chitinolytic activity of the bacteria was also significantly higher in the TFN lobster isolates for both tail fan and haemolymph (Table 4-4).

Lipolytic bacteria were more frequently isolated from the haemolymph of lobsters without TFN compared to those with TFN, but there was no difference in enzyme activity. Bacterial isolates from tail fan of lobsters without TFN showed no difference in lipolytic bacteria occurrence, but greater overall lipolytic activity compared to those from tail fans of lobsters with TFN (Table 4-4).

4.3.4. Haemolymph bacteria versus tail fan bacteria

The proportion of proteolytic bacteria and the proteolytic activity among all bacteria isolated from the surface of the tail fans of lobsters without TFN were higher than for all those isolated from the haemolymph of lobsters without TFN (Table 4-4).

The proportion of chitinolytic bacteria and the chitinolytic activity of all the bacteria isolated from the surface of the tail fan of lobsters without TFN were both similar to those isolated from the haemolymph of those lobsters (Table 4-4).

A similar proportion of lipolytic bacteria were found on the surface of the tail fan and haemolymph of lobsters without TFN, but the lipolytic activity was greater in haemolymph (Table 4-4).

In contrast, no significant difference was found in any type of enzymatic activity of bacteria isolated from tail fans and haemolymph of lobsters with TFN (Table 4-4).

Table 4-4. Comparisons of the overall proportion of bacterial isolates expressing enzymatic activity, and the mean scores of bacterial enzymatic activity for isolates from haemolymph, and from tail fan, compared between lobsters with and without TFN.

		Haemolymph		Tail fan	
Enzymatic occurrence/activity		Without TFN Mean ± SE	With TFN Mean ± SE	Without TFN Mean ± SE	With TFN Mean ± SE
Proteolytic	% occurrence[1]	39.4‡‡	47.3	49.6‡‡	47.9
	Activity score[2]	0.77 ± 0.07‡	0.87 ± 0.07	0.95 ± 0.05‡	0.86 ± 0.05
Chitinolytic	% occurrence[1]	18.0 **	39.4 **	19.7 **	34.2 **
	Activity score[2]	0.24 ± 0.03**	0.54 ± 0.04**	0.25 ± 0.02**	0.49 ± 0.04**
Lipolytic	% occurrence[1]	67.4*	57.9*	60.7	61.2
	Activity score[2]	0.98 ± 0.05‡‡	1.00 ± 0.06	0.79 ± 0.03$^{** ‡‡}$	0.97 ± 0.04**

Note: 1 - Chi-square test; 2 - Mann-Whitney test.

‡ indicates a significant difference ($0.01 < P < 0.05$), and ‡‡ ($P < 0.01$), between bacterial isolates taken from haemolymph versus those taken from the tail fan for groups of lobsters either without TFN or with TFN.

* indicates a significant difference ($0.01 < P < 0.05$), and ** ($P < 0.01$), between bacterial isolates without TFN versus with TFN for either isolates from haemolymph or from the tail fan.

4.3.5. Enzymatic profiles of bacterial isolates

The proportions of the bacteria exhibiting each of the eight enzymatic profiles were significantly different for bacteria isolated from the haemolymph of lobsters with TFN versus those without TFN (Chi-square test, $\chi^2 = 58.6$, $P < 0.001$), with a lower proportion of PPP bacteria in lobsters without TFN and a higher proportion of PPP bacteria in lobsters with

TFN (Fig. 4-1a). Also, there was higher proportion of PNP bacteria in lobsters without TFN, and a lower proportion of PNP bacteria in lobsters with TFN (Fig. 4-1a).

The tail fan of lobsters with TFN showed elevated proportions of PPP and NPN bacteria, whilst the tail fan of lobsters without TFN had lower proportions of PPP and NPN bacteria (Chi-square test, $\chi^2 = 56.0$, $P < 0.001$) (Fig. 4-1b).

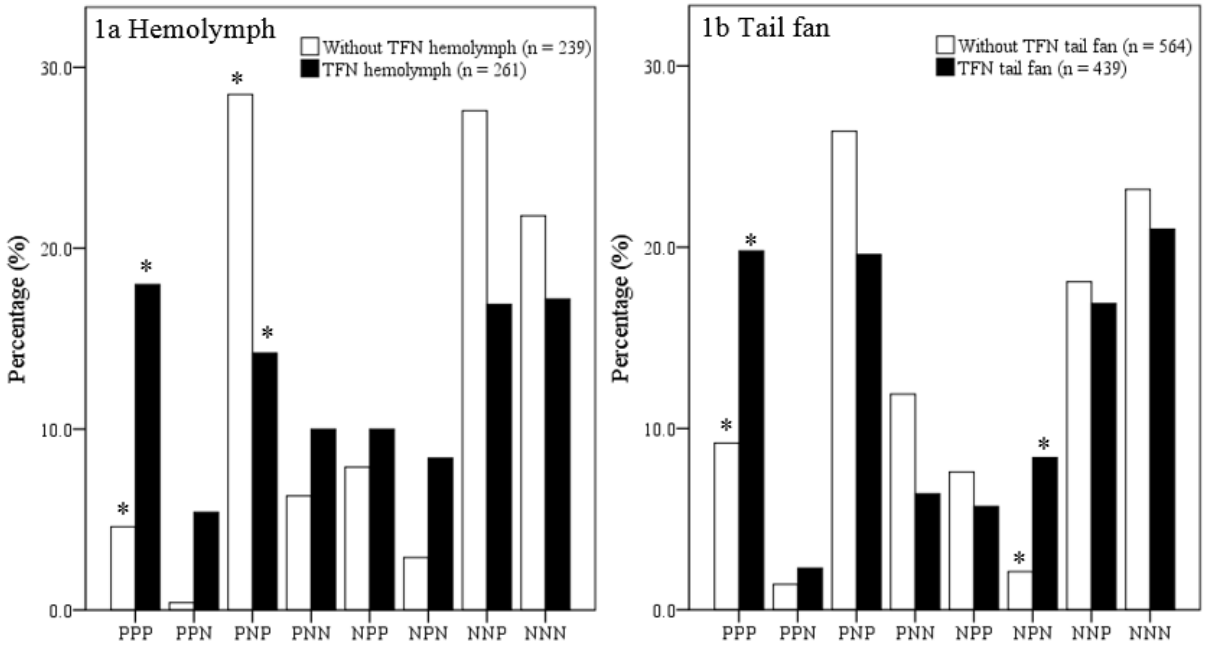


Fig. 4-1. Proportion of all of the successfully cultured bacterial isolates exhibiting each of the eight possible enzymatic profiles (see Table 4-4) from lobsters with TFN versus those without TFN for their; 1a) haemolymph, and 1b) tail fan. Proteolytic:chitinolytic:lipolytic. N = absent, P = present. * indicates a significant difference ($P < 0.05$) for the individual category represented by the bar below the asterisk.

Comparison of the proportions of the eight enzymatic profiles in bacteria isolated from haemolymph and tail fans for lobsters without TFN showed higher NNP proportions in the haemolymph (Chi-square test, $\chi^2 = 19.4$, $P < 0.008$) (Fig. 4-2a).

There was no significant difference in the proportions of the bacteria exhibiting each of the eight enzymatic profiles among all of the bacteria isolated from the haemolymph versus the tail fan of lobsters with TFN (Chi-square test, $\chi^2 = 12.0$, $P > 0.10$) (Fig. 4-2b).

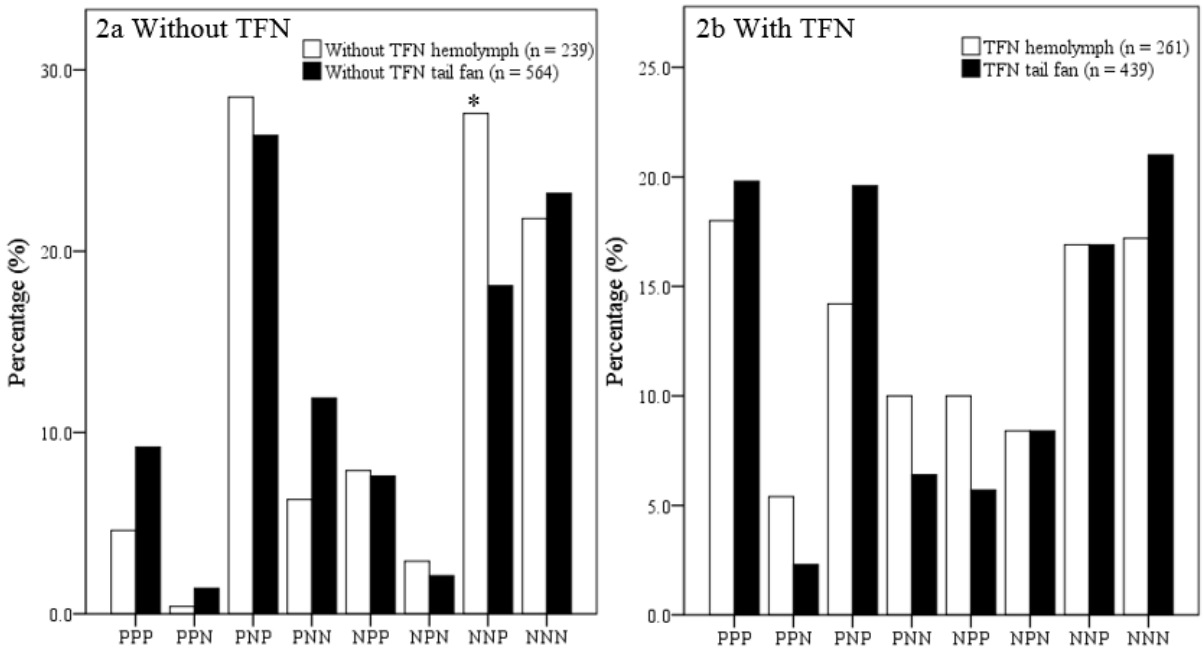


Fig. 4-2. Proportion of all successfully cultured bacterial isolates exhibiting each of the eight possible enzymatic profiles (see Table 4-4) from the haemolymph versus the tail fan of lobsters; 2a) without TFN, 2b) with TFN. Proteolytic:chitinolytic:lipolytic. N = absent, P = present. * indicates a significant difference ($P < 0.05$) for the individual category represented by the bar below the asterisk.

4.3.6. Identification of bacterial isolates

Thirty five bacterial isolates exhibiting all three targeted enzymatic capabilities from six of tail fans and haemolymph of the sampled lobsters with TFN were identified by 16S rDNA sequencing to species level. The isolates from haemolymph and tail fan in six lobsters were resolved to 14 species (Table 4-5).

Vibrio crassostreae was the most common species found in both the haemolymph and tail fans of lobsters with TFN (13 isolates). *Vibrio atlanticus* (6 isolates) occurred only on the tail fans and *V. gigantis* only in the haemolymph. All the *Vibrio* isolates belong to the Splendidus clade (Table 4-5).

Table 4-5. Identity of 35 bacterial isolates by 16S rDNA sequencing (> 97% identity) showing their respective proteolytic, chitinolytic and lipolytic activities. Isolates prepared from haemolymph and tail fan of six lobsters with TFN.

Closest Identification	Sequence Accession Number (% identity)	n (Total)	n (Haemolymph)	n (Tail fan)
Aliivibrio sifiae	NR112824 (99%)	1	0	1
Photobacterium lutimaris	NR043902 (98%)	1	1	0
Photobacterium sanguinicancri	GQ454934 (99%)	1	1	0
Photobacterium swingsii	HE584802 (100%)	2	1	1
Pseudoalteromonas spiralis	NR114801 (99%)	1	1	0
Ruegeria mobilis	HQ338132 (100%)	1	0	1
Shewanella vesiculosa	NR042710 (99%)	1	1	0
Stenotrophomonas rhizophila	EU977698 (100%)	1	1	0
Vibrio atlanticus	NR116067 (99%)	6	0	6
Vibrio crassostreae	KY229826 (98%-99%); LN832968 (99%); LN832963 (99%)	13	5	8
Vibrio cyclitroplicus	KT023534 (99%)	2	0	2
Vibrio gigantis	KR270224 (100%); JF412220 (99%)	3	3	0
Vibrio splendidus	FM954972 (99%)	1	1	0
Vibrio toranzoniae	LN832986 (99%)	1	1	0

4.4. Discussion

Chitin, protein and lipid are the three predominant materials constituting the cuticle of crustaceans, including lobsters (Davies et al., 2014b; Smolowitz et al., 2005). As a consequence, some researchers have proposed that proteolytic, chitinolytic and lipolytic activities are likely to be characteristics of those bacteria implicated in causing shell disease. However, shell disease has predominantly been found to be associated with chitinolytic bacteria (Gomez-Chiarri & Cobb, 2012; Klompmaker et al., 2016; Vogan et al., 2002; Vogan & Rowley, 2002), as chitin is the dominant component of the cuticle of crustaceans (Vogan et al., 2008). The culture methods for bacteria used in this study provided the opportunity to characterise their enzymatic capabilities, by determining which of these three key organic substrates present in crustacean cuticle they can utilize (Joint et al., 2010; Lin et al., 2005; Nguyen et al., 2014; Ruiz-Rodríguez et al., 2012; Zhang et al., 2016b).

4.4.1. Bacterial enzymatic activities

In the enzymatic analyses of this study, proportionately more chitinolytic bacteria were found on the tail fan and in the haemolymph of lobsters with TFN compared to those lobsters without TFN. Furthermore, more culturable chitinolytic bacteria, and with greater chitinolytic activity, were found to occur on the tail fan and in the haemolymph of lobsters with TFN compared to lobsters without TFN. In contrast, the proportions and occurrence of bacteria which had proteolytic or lipolytic enzymatic activities and the extent of these activities were in the most part similar on the tail fan and in the haemolymph of lobsters with and without TFN. This pronounced presence of chitinolytic bacteria among lobsters affected with TFN is consistent with previous studies of bacteria associated with other forms of shell disease (Gomez-Chiarri & Cobb, 2012; Klompmaker et al., 2016; Vogan et al., 2002; Vogan & Rowley, 2002). However, bacteria with the full complement of enzymatic capabilities (i.e.,

PPP - proteolytic chitinolytic and lipolytic) were dominant in both the haemolymph and tail fans of lobsters with TFN. Apart from the chitinolytic activity, the additional proteolytic and lipolytic activities of these bacteria are likely to be important co-contributors to TFN and possibly other types of shell disease (Hsu & Smolowitz, 2003; Vogan et al., 2008).

The presence of bacteria in the haemolymph has been found in some crustacean species without any outward apparent signs of infection suggesting that some bacteria may be tolerated in some instances (Fotedar et al., 2006; Kaizu et al., 2011; Ponprateep et al., 2012; Wang et al., 2014b). However, the presence of bacteria in the haemolymph can also be the result of bacteraemia often associated with the presence of disease (Bartlett et al., 2008; Defer et al., 2013; Nunan et al., 2010; Shields, 2011; Wang & Wang, 2015). Some previous studies have shown that bacteraemia can occur in lobsters with shell disease, however, the causality of this association is uncertain (Chistoserdov et al., 2005; Quinn et al., 2013b). In this study, there were differences in both the frequency of bacteria producing the targeted three enzymes and the overall enzymatic activity of the isolates found in the haemolymph and the tail fan of lobsters without TFN. In contrast, both of these two factors were similar in the haemolymph and the tail fan of lobsters with TFN (Table 4-4). Likewise, significant differences were found in the proportions of enzymatic profiles of the bacteria isolated from the haemolymph and the tail fan of lobsters without TFN, but not in lobsters with TFN (Fig. 4-2a versus Fig. 4-2b). These results suggest that opportunistic bacteria associated with the TFN lesions may access the haemolymph and lead to bacteraemia, causing the observed changes of bacterial flora and their corresponding enzymatic activities in haemolymph.

4.4.2. Bacterial taxonomy

There are examples of some types of shell disease in lobsters being caused by specific bacterial species or by a specific suite of bacteria (Musgrove et al., 2005; Quinn et al., 2013a;

Quinn et al., 2012a; Quinn et al., 2013b; Whitten et al., 2014), but the results of the current study suggest that no single bacteria is responsible for the TFN found in the wild spiny lobsters sampled in New Zealand. Some of the bacterial species that were identified, such as *Vibrio parahaemolyticus, V. alginolyticus* and *V. vulnificus*, have been previously reported from the tail fan of lobsters with TFN and were thought to be associated with the disease (Musgrove et al., 2005), while researchers have previously failed to detect or isolate bacteria from the haemolymph of spiny lobsters with TFN (Mancuso et al., 2010). Nonetheless, in the present study, a grouping of bacterial isolates characterised by possessing proteolytic, chitinolytic and lipolytic activities appeared to dominate the flora of lobsters with TFN. Over half (51.4%) of the 35 identified bacterial isolates belonged to four species in the Splendidus clade of Vibrio genus which are known aquatic pathogens, i.e., *V. splendidus, V. crassostreae, V. gigantis* and *V. toranzoniae* (Declercq et al., 2015; Gay et al., 2004; Gómez-León et al., 2005; Le Roux et al., 2009; Lemire et al., 2015).

Vibrio crassostreae was commonly found on both the tail fan and in the haemolymph of the six selected individual lobsters with TFN (Table 4-5). However, the group of PPP bacteria identified from the haemolymph (9 species) was largely distinct from those identified from the tail fan (6 species) suggesting different opportunistic bacteria are involved in occupying these two distinct habitats. However, the presence of bacteria in the haemolymph may rely upon bacteria gaining initial access via an injury that penetrates the lobster's cuticle to, and that injury site may subsequently become affected by TFN. It is also possible, that bacteria access to the haemolymph might occur at a later time following an injury, once TFN has developed and facilitates access for bacteria to the haemolymph due to the resulting suppression of the haemolymph immune response caused by the TFN (Zha et al., 2017).

There is already strong evidence that physical injuries resulting in the loss of the integrity of the carapace, from handling and housing lobsters, is the primary initiator of TFN in spiny

lobsters (Musgrove et al., 2005; Zha et al., 2017). The presence of TFN in the wild lobsters sampled for this study have previously been associated with the presence of injuries that are consistent with prior handling and return of undersized lobsters in a commercial potting fishery (Freeman & MacDiarmid, 2009; Zha et al., 2017). It appears that the damage to the carapace favours bacteria with enzymatic activities that are well suited to the substrate opportunities provided by the wound, and may also provide opportunistic bacteria access to the haemolymph, whether simultaneously, or after TFN has become established at the site of the wound.

4.5. Conclusion

Overall, the results of this study demonstrate that a suite of bacteria with a combination of bacterial proteolytic, lipolytic activities and especially chitinolytic activity are strongly associated with the development of TFN in the spiny lobster, *Jasus edwardsii*. Furthermore, it appears that different combinations of individual species of bacteria from the suite of bacteria with broad enzymatic capabilities are associated with TFN, as well as bacteraemia associated with TFN.

5. Chapter Five - Potential virulence factors of bacteria associated with tail fan necrosis in the spiny lobster, *Jasus edwardsii*

5.1. Introduction

Shell disease is a common condition in both wild and captive lobsters which compromises lobster health and impacts on the market value of the affected lobsters (Chistoserdov et al., 2005; Homerding et al., 2012; Quinn et al., 2013a). Different types of classical shell disease have been described in American lobsters (*Homarus americanus*), including epizootic shell disease, enzootic shell disease, impoundment shell disease, diet-induced shell disease, and black spot shell disease (Chistoserdov et al., 2012; Chistoserdov et al., 2005; Quinn et al., 2013a; Shields, 2011). In many instances, bacterial infections have been associated with classic shell disease in lobsters, especially bacteria possessing chitinolytic activity, often in combination with proteolytic and lipolytic activities (Arias-Barrau et al., 2004; Mancuso et al., 2010; Quinn et al., 2013a; Suginta, 2007; Vogan et al., 2008; Zha et al., 2018a).

Tail fan necrosis (TFN) is a commonly reported form of classical shell disease found in both wild and captive holding populations of several species of spiny lobsters. TFN in spiny lobsters is most typically characterized by the erosion and blackening of the uropods and telson that make up the tail fan (Freeman & MacDiarmid, 2009; Musgrove et al., 2005; Shields, 2011). TFN is reported to compromise the overall health and immune status of spiny lobsters (Musgrove et al., 2005; Vogan et al., 2008; Zha et al., 2017).

TFN in spiny lobsters is thought to be initiated by physical damage to the tail fan followed by bacterial invasion of the injury (Freeman & MacDiarmid, 2009; Meres et al., 2012; Musgrove et al., 2005; Quinn et al., 2013b). Bacteria with a combination of chitinolytic, proteolytic and lipolytic activities appear to play a particularly important role in TFN most likely using lobster cuticle and underlying tissues as substrate that also facilitates the further breakdown

of the cuticle and the advance of the necrosis at the site of the initial injury (Zha et al., 2018a).

The possession of further bacterial attributes, such as motility, biofilm formation, siderophore production and ability to suppress host antimicrobial agents, would selectively enhance the association of certain organisms with TFN. Motility is associated with the virulence and adherence of bacteria (Chaban et al., 2015; Josenhans & Suerbaum, 2002). Mixed bacterial biofilms are thought to be associated with persistent external infections in compromised hosts such as marine organisms (Costerton et al., 1999) and diverse bacterial floras have been found on the cuticle and shell disease lesions of American lobsters in previous studies (Chistoserdov et al., 2012; Chistoserdov et al., 2005; Feinman et al., 2017; Quinn et al., 2013a). As TFN is a chronic condition and exacerbated by bacterial infection (Bryars & Geddes, 2005), those bacteria associated with TFN may be able to form biofilms and colonize on the surface of tail fans, which is possibly associated with the persistence of the infection in spiny lobsters.

TFN in spiny lobsters is characterised by blackening of the affected tissues as a result of the deposition of melanin which can be produced through melanisation by the lobster as an immune response intending to block the progression of pathogens beyond the damaged carapace and adjacent tissues (Perdomo-Morales et al., 2007; Verghese et al., 2007). However, melanin can also be produced by numerous bacteria such as *Azotobacter chroococcum*, *Legionella pneumophila*, *Pseudomonas aeruginosa*, *P. putida*, *Ralstonia solanacearum* and *Vibrio cholerae* (Arias-Barrau et al., 2004; Banerjee et al., 2014; Chatfield & Cianciotto, 2007; Ernst et al., 2003; Hernández-Romero et al., 2005; Valeru et al., 2009). The melanin the bacteria produce provides some protection to the bacteria from unfavourable environmental conditions (Garcia-Rivera & Casadevall, 2001; Moeller et al., 2005; Rosas & Casadevall, 1997; Tada et al., 2010), including antimicrobial compounds and other immune

responses from the hosts (Jacobson, 2000; Nosanchuk & Casadevall, 2006). Furthermore, melanin producing bacteria can be expected to resist the presence of melanin whether derived from bacterial sources or from lobsters as an immune response. In most organisms, melanin production starts with an enzymatic reaction of L-tyrosine via L-3,4-dihydroxyphenylalanine to 3,4-dihydroxyphenylalanine-quinone (Plonka & Grabacka, 2006).

Many bacteria actively produce siderophores (Cordero et al., 2012; Luján et al., 2015), which are thought to enable these bacteria to compete more effectively with other bacteria which have obligate iron requirements (Skaar, 2010). Consequently, the bacteria with strong siderophore effects are frequently more virulent (Buckling et al., 2007; Holden & Bachman, 2015; Russo et al., 2011) during the stages of colonization and infection (Bachman et al., 2009; Caza et al., 2011; Fischbach et al., 2006). Therefore, this bacterial characteristic may be of distinct advantage for a marine pathogen, such as those are associated with TFN.

The cuticle of crustaceans has been shown to have antimicrobial activity (Brisbin et al., 2015; Haug et al., 2002; Hwang, 2004). For example, the peptides in the shell extract of the American lobster, have been found to have some antimicrobial activity when tested against four different bacteria (i.e., *Escherichia coli*, *Staphylococcus aureus*, *V. parahaemolyticus* and *V. anguillarum*) (Brisbin et al., 2015). Therefore, those bacteria which are resistant to any antimicrobial activity of tail fan extract or have the ability to take advantage of tail fan extract would be well predisposed to being associated with TFN in lobsters.

Antimicrobial activities among bacteria are common (Nguyen et al., 2014; Sant'Ana et al., 2012; Smaoui et al., 2010; Zeng et al., 2014), but unreported in bacteria associated with classical shell disease, including TFN. Among the bacteria on the tail fan of lobsters, the bacteria associated with TFN may be advantaged by the ability to inhibit other bacteria.

Alternatively, a mix of bacterial species may co-exist generating a synergistic effect that is associated with chronic TFN.

Ammonia is a potentially toxic compound that can be produced by bacteria in marine organisms that can potentially impact host health and immunity (Carvalho & Phan, 1997; Kemp et al., 2009; Randall & Tsui, 2002). For instance, the phenoloxidase activity in the haemolymph of juvenile Pacific white shrimp significantly decreased when the shrimps were exposed to different levels of ammonia (Zhang et al., 2012). As our previous study has indicated that TFN is associated with diminished immune status in lobsters (Zha et al., 2017), any release of ammonia by the bacteria in infected tissues is likely to be associated with TFN and also impact lobster health.

In a previous study 1678 bacterial strains were successfully isolated from haemolymph and tail fans of a sample of 76 wild lobsters (*J. edwardsii*) captured on the east coast of the North Island of New Zealand with around half of the lobsters affected with TFN and the remainder without TFN (Zha et al., 2018a). The proteolytic, chitinolytic and lipolytic activities of 1678 bacteria isolates were examined, and those bacteria possessing all three enzymatic capabilities (i.e., PCL+) were found to be more frequent in the lesions of lobsters with TFN than for the equivalent locations of those lobsters without TFN (Zha et al., 2018a). PCL+ isolates were identified by 16S rDNA sequencing and included *Aliivibrio sifiae, Photobacterium lutimaris, P. sanguinicancri, P. swingsii*, *Pseudoalteromonas spiralis, Ruegeria mobilis, Shewanella vesiculosa, Stenotrophomonas rhizophila, Vibrio atlanticus, V. crassostreae, V. cyclitroplicus, V. gigantis, V. splendidus, V. toranzoniae* (Zha et al., 2018a).

The aim of the current study was to investigate the PCL+ isolates for other characteristics that may play a role in mediation of TFN in lobsters. These bacterial characteristics include their morphology, ability to form biofilms, melanin, ammonia and siderophore production,

antimicrobial activity, as well as the ability to surmount any antimicrobial activity from the extract of lobster tail fan.

5.2. Materials and methods

5.2.1. Identification of bacterial isolates

A total of 69 bacterial isolates were used in this study which possessed the combination of proteolytic, chitinolytic and lipolytic capabilities. These were subsequently called PCL+ isolates. These PCL+ isolates were sourced from a previous study of 76 wild *J. edwardsii* (including 39 TFN affected and 37 unaffected lobsters) which were captured off the east coast of the North Island of New Zealand (Zha et al., 2018a). A total of 35 PCL+ bacterial isolates from haemolymph and tail fan surfaces of six wild lobsters with TFN were identified in this study using PCR and sequencing methods as previously described (Zha et al., 2018a), while the remaining 34 PCL+ bacterial isolates from ten lobsters without TFN were identified as described below.

Bacterial DNA extraction of the selected PCL+ bacteria isolates used a boiling method (Bjornsdottir-Butler et al., 2016; Ngamwongsatit et al., 2008; Zhi et al., 2016). A conserved region (V1-V9, 1500 bp) of the bacterial 16S rDNA gene was amplified by PCR with the universal bacterial primers PB36 (5'-AGR GTT TGA TCM TGG CTC AG-3') and PB38 (5'-GKT ACC TTG TTA CGA CTT-3') (Lear et al., 2009). PCRs were performed using Gotaq Green DNA polymerase (Promega, Inc.) as follows: (i) 95 °C for 5 min; (ii) 35 cycles of 95 °C for 45 s, 55 °C for 45 s, and 72 °C for 90 s; and then (iii) 72 °C for 10 min. PCR products were purified using a DNA Clean and Concentrator-5 kit (Zymo Research, Inc.). The nucleotide sequences were obtained using a contract sequencing facility (Macrogen, Inc.).

The sequencing results were analyzed by using the NCBI BLAST database to identify the most closely matching sequences, and the sequences blasted to Genbank with > 97% similarity required for presumptive identification (Drancourt et al., 2000).

5.2.2. Bacterial morphology

The cell morphology and motility of the 69 selected PCL+ bacterial isolates were observed using a phase-contrast microscope. Gram staining and motility was conducted as previously described (Zheng et al., 2015).

5.2.3. Biofilm forming ability

Bacterial biofilm forming ability was measured using previously described methods (Kjærgaard et al., 2000), with some modifications. Briefly, PCL+ bacterial isolates were inoculated into marine broth (BD Difco, Inc.), and incubated overnight at 18 ℃ on an orbital shaker (180 rpm). A 1-ml aliquot of bacterial culture was added into each well of a 12-well plate, and incubated at 18 ℃ for 24 h.

All of the liquid was pipetted out of each well taking care not to disturb the attached biofilm. To each well, 1 ml of 0.1% crystal violet solution was added and allowed to stain the biofilm for 5 min. The 12-well tray was tilted gently and any residual staining solution was carefully removed with a pipette. Filtered autoclaved seawater (1 ml) was added and excess stain was washed off by gently shaking, this step was repeated three times. Then the crystal violet stain remaining in the biofilm was removed by adding 1 ml of 96 % ethanol and the released crystal violet was homogenized by vortex. Absorbance of crystal violet was measured at 650 nm with three replicates and blank control. Biofilm forming ability was defined as positive if the Abs_{650} mean value of isolate was over three times higher than that of the blank control.

5.2.4. Melanin production

All PCL+ bacterial isolates were tested for the production of melanin using previously described methods (Coyne & al-Harthi, 1992), with some modifications. Briefly, bacteria were inoculated onto both marine agar (BD Difco, Inc.) plates and marine agar containing 5 mM L-tyrosine (Sigma-Aldrich, Inc.), and incubated at 28 °C for 120 h. Melanin production was defined as positive if dark pigment was observed around the colony on marine agar with L-tyrosine, but not around the colony on marine agar. Melanin production was ranked from 0.5 - 3 according to the extent of the dark pigment around the colony (i.e., Rank 0.5 - 1: light colour; Rank 2: dark brown; Rank 3: black).

5.2.5. Siderophore production

All the PCL+ bacterial isolates were assayed for siderophore production on the chrome azurol S agar media using previously described methods (Ahmad et al., 2008). The chrome azurol S agar was prepared according to the procedures of Louden et al., (2011). Briefly, bacterial isolates were inoculated on the chrome azurol S agar which were divided into three equal sectors, and incubated at 28 °C for 72 h. Development of yellow-orange zone around bacteria was considered as positive for siderophore production. Siderophore production was ranked 1 - 3 according to the extent of yellow-orange zone.

5.2.6. Antimicrobial activity of tail fan extract

Eleven unaffected lobsters were collected from the east coast of North Island of New Zealand and their tail fans were removed, thoroughly washed with filtered autoclaved seawater, dried and stored at - 80 °C. A water-soluble extract of the pooled tail fans was prepared based on the procedures of Brisbin et al., (2015). Briefly, equal quantities of the marginal area of the uropods and telsons were excised from 11 lobsters without TFN. They were frozen with liquid nitrogen and quickly ground to fine powder with grinder, and 6 g of the powder was

then added to 14 ml of filtered autoclaved seawater and kept on ice while mixed for 3 h. After centrifugation at 2500 × g at 4 °C for 1 h, the supernatant was sterilized by filtration (0.22 µm, cellulose acetate membrane) and stored at - 80 °C until use (within one week).

All 69 PCL+ bacterial isolates were tested for their ability to surmount any antimicrobial activity of the tail fan extract as previously described (Brisbin et al., 2015), with some modifications. Briefly, bacterial suspensions were prepared by inoculating sterile marine broth with a single colony of a bacterial isolate (grown on marine agar), and incubated overnight at 18 °C on an orbital shaker (180 rpm). Bacterial cells were rinsed three times by spinning the suspensions at 3000 × g, at 18 °C for 10 min, discarding the supernatant and resuspending the pellet with filtered autoclaved seawater. After rinsing, 10 µl of the bacterial suspension was mixed with 20 µl of tail fan extract and 40 µl of filtered autoclaved seawater in sterile 1.5 ml Eppendorf tubes. A positive control treatment consisted of 10 µl of bacterial suspension and 60 µl filtered autoclaved seawater. Two controls were; 1) 20 µl of tail fan extract and 50 µl of filtered autoclaved seawater; 2) 70 µl filtered autoclaved seawater. All tubes were incubated at 18 °C for 2 h before adding 430 µl of marine broth. The tubes were then incubated for a further 18h, 24 h and 48 h before aliquots of 100 µl from four replicates of each isolate were transferred into separate wells of clear, flat-bottomed, 96-well plate and an absorbance at 570 nm was measured using an EnVision plate reader (Perkin Elmer, Inc.).

Bacterial growth in the presence of tail fan extract was defined as positive (enhanced) when: [Abs_{570}(bacteria+extract) - Abs_{570}(extract control)] - [Abs_{570}(bacteria only) - Abs_{570} (seawater control)] - Abs_{570}(Standard deviation sum of the two treatments and two controls) was over 0.01 at any time point (18, 24 or 48 h). An inhibition effect was defined when the value was less than -0.01 at any time point, while no-effect result was defined when the values were between -0.01 and 0.01 at all time points.

5.2.7. Antimicrobial activity

The PCL+ bacterial isolates from lobsters with TFN (14 isolates from 14 different species) and without TFN (17 isolates from 17 different species) were tested for antimicrobial activities against each other in a pairwise manner. Bacterial isolates were streaked onto a plate of sterile marine agar such that pairs of isolates directly abutted but did not touch each other. Each pair was tested in triplicate. Plates were then incubated at 18 °C for 24 - 48 h with any subsequent inhibition of one bacteria isolate by another bacteria being defined as positive if bacteria did not grow next to the challenge isolate.

5.2.8. Ammonia production

Ammonia production of 69 PCL+ bacterial isolates was determined using a method modified from Ahmad et al., (2008). Briefly, freshly grown colonies were inoculated in 3 ml marine broth in a 15 ml tube and incubated at 28 °C for 72 h, with tubes containing only marine broth as blank controls. A 150 µl aliquot of Nessler's reagent was added to each tube with the immediate subsequent development of yellow (+) to orange (++) colour providing a positive test for bacterial ammonia production.

5.2.9. Genotype comparison using ERIC-PCR

Amplification reactions were carried out in 25-µl volumes, using universal ERIC-PCR primers: ERIC-1R, 5'-ATG TAA GCT CCT GGG GAT TCA C-3'; and ERIC-2, 5'-AAG TAA GTG ACT GGG GTG AGC G-3' (Versalovic et al., 1991). PCRs were performed using Gotaq Green DNA polymerase as follows: (i) 95 °C for 5 min; (ii) 35 cycles of 95 °C for 45 s, 55 °C for 45 s, and 72 °C for 90 s; and then (iii) 72 °C for 10 min (Chokesajjawatee et al., 2008).

The gel image was further analysed with BioNumerics 6.6 (Applied Maths, Belgium), using a pair wise comparison with a UPGMA method generating dendrograms and similarity matrix.

5.2.10. Statistical analyses

Mann-Whitney tests were used to compare the extent of the capabilities of the PCL+ bacterial isolates for producing melanin, ammonia, siderophore and forming biofilms for those isolates from haemolymph of lobsters with TFN versus those without. Likewise, the same statistical approach was used for the PCL+ bacterial isolates from tail fan of lobsters with TFN versus those without.

Mann-Whitney tests were also used to compare the extent of the above capabilities of the PCL+ bacterial isolates from haemolymph versus tail fans in either lobsters with TFN or those without.

A Chi-square test was used to compare the frequency of PCL+ bacterial isolates from haemolymph of lobsters with TFN versus those without for each of the following capabilities; forming biofilms, producing melanin, producing siderophores, and their proliferation of being promoted by the tail fan extract. Likewise, the same statistical approach was used for the PCL+ bacterial isolates from tail fan of lobsters with TFN and from those without TFN.

A Chi-square test was also used to compare the frequency of PCL+ bacterial isolates showing above capabilities from haemolymph versus tail fans in either lobsters with TFN or those without TFN.

A Chi-square test was used for comparing the frequency of PCL+ bacterial isolates with a combination of biofilm formation, melanin and siderophore production from haemolymph of affected lobsters and those from unaffected lobsters. The same approach was used for the PCL+ bacterial isolates from tail fan of affected versus unaffected lobsters.

Data was tabulated in Microsoft Excel and analysed using the SPSS software (IBM, Inc.).

5.3. Results

5.3.1. Identification of bacteria and their morphology

The 34 PCL+ bacterial isolates from lobsters without TFN were identified as belonging to 17 species (Appendix A), including *Paracoccus rhizosphaerae*, *Photobacterium sanguinicacri*, *Photobacterium swingsii*, *Pseudoalteromonas espejiana*, *Pseudoalteromonas haloplanktis*, *Pseudoalteromonas issachenkonii*, *Sulfitobacter delicates*, *Vibrio atlanticus*, *V. crassostreae*, *V. cyclitrophicus*, *V. kanaloae*, *V. pomeroyi*, *V. splendidus*, *V. tapetis*, *V. tasmaniensis*, *V. toranzoniae* and *Vitellibacter vladivostokensis*.

The previous 35 PCL+ bacterial isolates from TFN-affected lobsters belonging to 14 bacterial species (Appendix A), including *Aliivibrio sifiae*, *Photobacterium lutimaris*, *P. sanguinicancri*, *P. swingsii*, *Pseudoalteromonas spiralis*, *Ruegeria mobilis*, *Shewanella vesiculosa*, *Stenotrophomonas rhizophila*, *V. atlanticus*, *V. crassostreae*, *V. cyclitroplicus*, *V. gigantis*, *V. splendidus*, *V. toranzoniae* (Zha et al., 2018a).

Of the 14 bacterial species (35 PCL+ isolates) from lobsters with TFN and the 17 bacterial species (34 PCL+ isolates) from lobsters without TFN, a total of seven species were found in common between the two groups of lobsters, i.e., *P. sanguinicacri*, *P. swingsii*, *V. atlanticus*, *V. crassostreae*, *V. cyclitrophicus*, *V. splendidus* and *V. toranzoniae*. Among them, *V. crassostreae* was the most common, comprising the largest percentage in both lobsters with TFN (37.1%) and lobsters without TFN (29.4%).

All of the 69 PCL+ bacterial isolates were gram negative, and most of them were motile (95.7%) and rod-shaped (97.1%).

5.3.2. Biofilm forming ability

There were significantly more biofilm forming PCL+ bacterial isolates from the tail fans of lobsters with TFN, and an overall greater biofilm forming ability by PCL+ bacteria from the tail fans of lobsters with TFN than those from the tail fans of lobsters without TFN (Table 5-1). In haemolymph, there was no significant difference in frequencies of PCL+ bacterial isolates from lobsters with and without TFN, but an overall stronger biofilm forming ability was found in PCL+ bacterial isolates from lobsters with TFN than those without TFN (Table 5-1).

In the lobsters with TFN, the occurrence of biofilm forming PCL+ bacteria from the tail fans was higher than those from haemolymph. But for lobsters without TFN, the frequencies of biofilm forming PCL+ bacteria were similar in tail fans and haemolymph (Table 5-1). The PCL+ bacterial isolates from the tail fans and haemolymph had similar biofilm forming ability, in either lobsters with TFN or those without TFN (Table 5-1).

5.3.3. Melanin production

There were significantly more melanin producing PCL+ bacterial isolates from the tail fans of lobsters with TFN, and an overall higher amount of melanin produced by PCL+ bacterial isolates, than for the PCL+ bacterial isolates source from the tail fans of lobsters without TFN (Table 5-1). There was no significant difference in the occurrence of melanin producing PCL+ bacteria or the overall amount of melanin produced by PCL+ bacteria from the haemolymph of lobsters with TFN versus those without TFN (Table 5-1).

There was no difference between the frequencies of melanin producing PCL+ bacteria or the overall level of melanin produced by PCL+ bacteria when comparing haemolymph and tail fans isolates from either lobsters with TFN or those without TFN (Table 5-1).

5.3.4. Siderophore production

The occurrence of siderophore producing PCL+ bacterial isolates was similar in the tail fans of lobsters with TFN and those without TFN, but an overall greater siderophore producing ability was found in PCL+ bacterial isolates from lobsters with TFN than those without. For isolates from haemolymph, there was no significant difference between the frequencies of siderophore producing PCL+ bacteria or the overall amount of siderophore produced by PCL+ bacteria from the lobsters with TFN and those without TFN (Table 5-1).

There was no difference between the frequencies of siderophore producing PCL+ bacteria or the overall level of siderophore produced by PCL+ bacteria when comparing isolates from haemolymph versus those from tail fans for either lobsters with TFN or those without TFN (Table 5-1).

Table 5-1 Bacterial biofilm formation, melanin and siderophore production comparison of PCL+ bacterial isolates from haemolymph and tail fan of lobsters with tail fan necrosis (TFN) and lobsters without TFN.

		Haemolymph		Tail fan	
Occurrence/activity		Without TFN (N = 10) Mean ± SE	With TFN (N = 16) Mean ± SE	Without TFN (N = 24) Mean ± SE	With TFN (N = 19) Mean ± SE
Biofilms	% occurrence[1]	30	69 ǂ	25 **	95 **ǂ
Biofilms	Activity score[2]	0.11 ± 0.02 **	0.29 ± 0.05 **	0.11 ± 0.02 **	0.23 ± 0.04 **
Melanin	% occurrence[1]	90	87	62 *	89 *
Melanin	Activity score[2]	1.05 ± 0.23	1.31 ± 0.21	0.92 ± 0.19 *	1.61 ± 0.21 *
Siderophores	% occurrence[1]	40	69	38	63
Siderophores	Activity score[2]	0.90 ± 0.38	1.69 ± 0.34	0.67 ± 0.19 *	1.47 ± 0.29 *

Note: 1 - Chi-square test; 2 - Mann-Whitney test for significance.

* indicates a significant difference (0.01< P < 0.05) between Without TFN and With TFN isolates; ** indicates a significant difference (P < 0.01) between Without TFN and With TFN isolates.

‡ indicates a significant difference (0.01 < P < 0.05) between tail fan and haemolymph isolates.

5.3.5. Antimicrobial activity of tail fan extract

The growth of over half (51.4%) of the PCL+ bacterial isolates from lobsters with TFN were promoted by tail fan extract, while the balance of the isolates were not influenced by tail fan extract. The growth of 44.1% of the PCL+ bacterial isolates from lobsters without TFN was promoted by tail fan extract, while 52.9% of the isolates were not influenced by the extract. Only one bacterial isolate from the tail fans of lobsters without TFN was inhibited by tail fan extract. There was no difference in the proportions of PCL+ bacteria isolated from haemolymph or from tail fan surfaces for which their growth was promoted by tail fan extract when comparing lobsters with and without TFN (Chi-square test, $\chi^2 = 0.39$, P = 0.53, and $\chi^2 = 0.001$, P = 0.98 respectively). There was no difference in the proportions of PCL+ bacteria isolated from haemolymph comparing with those from tail fan surfaces for which their growth was promoted by tail fan extract for either lobsters with TFN or those without TFN (Chi-square test, $\chi^2 = 1.45$, P = 0.23, and $\chi^2 = 0.20$, P = 0.66 respectively).

5.3.6. Antimicrobial activities among bacterial species

Most of the PCL+ bacteria from lobsters with TFN did not inhibit each other, with only *R. mobilis* being able to inhibit some isolates of other species from lobsters with TFN, i.e., *A. sifiae, P. lutimalis, P. spiralis, S. vesiculosa, S. rhizophila, V. atlanticus* and *V. cyclitrophicus*. The isolate belonging to *R. mobilis* from lobster with TFN also inhibited

isolates from lobsters without TFN belonging to *P. rhizosphaerae*, *P. sanguinicancri*, *P. espejiana*, *P. issachenkonii*, *V. atlanticus*, *V. cyclitrophicus*, *V. tapetis*, *V. tasmaniensis*, *V. toranzoniae* and *V. vladivostokensis*. By contrast, the PCL+ bacterial isolates belonging to different species from lobsters without TFN did not inhibit each other, and no isolate from unaffected lobsters inhibited any isolate from affected lobsters.

5.3.7. Ammonia production

All of the 69 PCL+ bacterial isolates secreted ammonia, and there was no significant difference in the abilities to produce ammonia between the PCL+ bacterial isolates from the haemolymph or for those from the tail fans of lobsters with TFN compared to those without TFN (Mann-Whitney test, U = -1.60, P = 0.11, and U =-0.76, P = 0.45 respectively). The PCL+ bacterial isolates from the haemolymph and tail fans have similar ammonia production in either lobsters with TFN or those without (Mann-Whitney test, U = -1.55, P = 0.12, and U = -0.86, P = 0.39 respectively).

5.3.8. Comparison of *V. crassostreae* by ERIC-PCR

Vibrio crassostreae was the most common species from the identified PCL+ bacterial isolates, and were selected for ERIC-PCR, including 13 isolates from TFN affected lobsters and 10 isolates from no-TFN lobsters. The majority (12 out of 13) of the *V. crassostreae* from TFN affected lobsters had similar genomic patterns, while the *V. crassostreae* from no-TFN lobsters had relatively diverse patterns (Fig. 5-1).

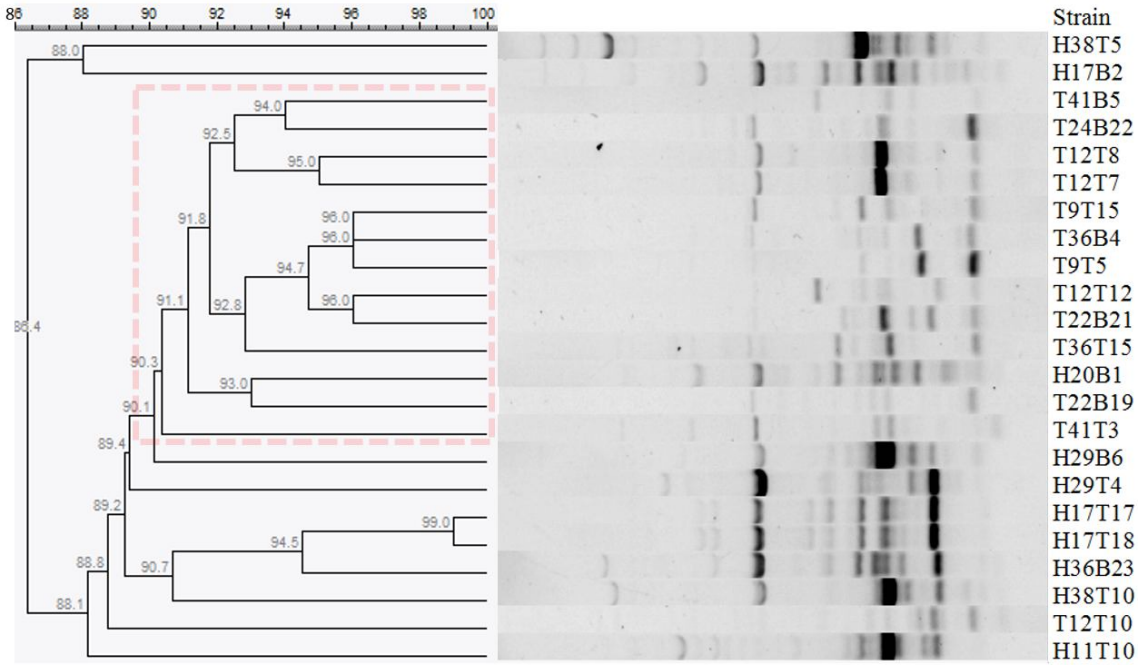


Fig. 5-1 Genomic fingerprints of selected *V. crassostreae* isolates. The scale bar showed similarity 86% - 100%. The code of strains consisted of no-TFN/TFN (H/T) + lobster number + haemolymph/tail fan (B/T) + isolate number. Note: the sequences within the pink dotted lines were similar, and the majority of them belonged to PCL+ *V. crassostreae* from TFN-affected lobsters.

5.3.9. Comparison of key characteristics combination

The frequency of PCL+ bacterial isolates which showed all of biofilm formation, melanin and siderophore production from haemolymph of affected lobsters was higher than that of the unaffected lobsters (Chi-square test, $\chi^2 = 9.77$, $P < 0.002$). Likewise, the frequency of tail fan PCL+ isolates exhibiting all the three characteristics was higher than that of unaffected lobsters (Chi-square test, $\chi^2 = 18.24$, $P < 0.001$).

5.4. Discussion

Bacteria are thought to be closely associated with classical shell disease, including TFN in spiny lobsters (Chistoserdov et al., 2012; Chistoserdov et al., 2005; Quinn et al., 2013a; Quinn et al., 2013b; Shields, 2012). For example, the bacterium *Aquimarina homaria* is ubiquitous in lesions on the carapace of American lobsters associated with epizootic shell disease, and consequently it is considered to be the causative pathogen (Quinn et al., 2013a; Quinn et al., 2012a). However, recent studies have shown that other bacteria such as *Thalassobius* sp. and *Pseudoalteromonas* sp. may also contribute to the disease (Chistoserdov et al., 2012; Quinn et al., 2012a; Whitten et al., 2014). Likewise, in other types of classical shell disease, no specific causative bacteria have been identified, rather a suite of bacteria frequently appear to be involved (Musgrove et al., 2005; Quinn et al., 2013a).

Classical shell disease has been found in both freshwater crayfish and seawater lobsters (Edgerton et al., 2002), suggesting that this disease is less likely to be caused by specific bacteria or bacterial communities. Also, if classical shell disease was consistently caused by specific bacteria, it could be expected that classical shell disease would reliably produce typical symptoms in the lobsters. However, the differences in the bacterial infection of haemolymph (Chistoserdov et al., 2005), pathological changes of internal organs (Comeau & Benhalima, 2009; Shields et al., 2012), the gross symptoms of lobsters with classical shell disease (Musgrove et al., 2005) suggest that no specific bacteria or communities are responsible for the disease, but more likely by an assemblage of bacteria with specific characteristics, such as strong enzymatic activities for breaking down the chitinous exoskeleton (Mancuso et al., 2010; Vogan et al., 2008; Zha et al., 2018a). In the present study, further characteristics of the bacterial isolates that are associated with TFN were investigated, and contrasted with a group of bacteria with strong enzymatic activities isolated from unaffected lobsters (Zha et al., 2018a).

The 69 PCL+ bacterial isolates from the haemolymph and tail fan of lobsters both with and without TFN were found to consist of 24 different species. Among them, isolates belonging to seven species occurred in both lobsters with and without TFN, i.e., *P. sanguinicacri*, *P. swingsii*, *V. atlanticus*, *V. crassostreae*, *V. cyclitrophicus*, *V. splendidus* and *V. toranzoniae*. This suggests that these bacteria may cause TFN opportunistically, such as when the tail fans sustain physical damage (Freeman & MacDiarmid, 2009; Musgrove et al., 2005). Although a few bacterial species were only found in TFN-affected lobsters in the current study, it is uncertain whether these species also exist in the unaffected lobsters and were not represented in the samples for the current study.

The majority of the PCL+ bacterial isolates from lobsters with and without TFN were motile. As motility is commonly associated with virulence and adherence among bacterial pathogens (Chaban et al., 2015; Josenhans & Suerbaum, 2002), the result suggests that these bacteria have the ability to migrate towards the injured tail fan cuticle and potentially into tissues to colonize new sites in a manner that would enable the initiation of TFN in lobsters.

Stronger biofilm-forming ability were found in the PCL+ bacterial isolates from the tail fans of lobsters with TFN than those from the tail fans of lobsters without TFN. As bacterial biofilm formation contributes to higher colonization ability and stronger antimicrobial resistance (Allewell, 2016; Hall-Stoodley et al., 2004; Kavanaugh & Horswill, 2016; Patel, 2005), this result suggests that the PCL+ bacterial isolates from the tail fans of lobsters with TFN have a greater capacity to colonize the surface of the tail fan against the surrounding antimicrobial agents and cause persistent infection. Likewise, a stronger biofilm forming PCL+ bacterial isolates in the haemolymph of lobsters with TFN suggests that these PCL+ bacteria may have originated from the tail fan where they may have previously established biofilms at the site affected by TFN. Alternatively, they may have opportunistically accessed

the haemolymph through an open lesion in the carapace, and persisted after resisting the host immune response.

More melanin-producing PCL+ bacterial isolates and the overall higher melanin production were found in the tail fans of lobsters with TFN than those from tail fan of lobsters without TFN, suggesting the bacterial melanin may also be responsible for the visible melanin deposits in the tail fan sites affected by TFN. Melanisation is an important immune response of lobsters to eliminate pathogens (Cerenius et al., 2008; Theopold et al., 2004), and the melanin in the tail fans of lobsters affected with TFN is generally thought to be a sign of an effective immune response (Perdomo-Morales et al., 2007; Wang et al., 2014a). The relevant result suggested that an alternative hypothesis is that the microbes on the tail fans may produce melanin and contribute to the characteristic melanised tissues associated with TFN. The bacterial melanin can protect bacteria from unfavourable conditions (Garcia-Rivera & Casadevall, 2001; Moeller et al., 2005; Tada et al., 2010), and the ability to tolerate melanin may exacerbate the TFN by protecting bacteria from the immune response and antimicrobial activities of lobsters (Brisbin et al., 2015; Perdomo-Morales et al., 2007). Furthermore, the ability to tolerate melanin may also help bacteria survive and gain access into the haemolymph through sites of melanised TFN, resulting in the possible subsequent bacterial infection in haemolymph (Zha et al., 2018a) and a decreased immune response of lobsters (Zha et al., 2017), which is likely to further exacerbate the TFN.

The PCL+ bacterial isolates from the tail fan of lobsters with TFN produced more siderophores than those isolates from the tail fans of lobsters without TFN. As the bacterial siderophore production contributes to greater iron accumulating capacity (Skaar, 2010), a higher siderophore production may enable the PCL+ bacterial isolates to obtain the iron for their metabolic functioning and outcompete other tail fan bacteria. Also, many of the PCL+ bacteria isolated from the haemolymph of both lobsters with and without TFN were also

found to produce siderophores. As bacteria producing siderophores are frequently virulent (Buckling et al., 2007; Holden & Bachman, 2015), some of these may potentially be pathogenic bacteria that can cause subsequent infection, i.e., haemolymph infection (Bartlett et al., 2008; Chistoserdov et al., 2005) and internal organ infection (Comeau & Benhalima, 2009).

Both tail fan PCL+ bacteria and haemolymph PCL+ bacteria with all three key characteristics (i.e., biofilm formation, melanin and siderophone production) from affected lobsters had significantly higher frequencies than those in unaffected lobsters, suggesting that the combination of the three characteristics are associated with TFN in spiny lobsters.

The majority of the PCL+ bacterial isolates from both the lobsters with and without TFN were not influenced by the antimicrobial activity of tail fan extract, while many of the other PCL+ bacterial isolates were able to take advantage of the extract by proliferating faster. Antimicrobial activity has been reported in the cuticle of a variety of marine crustaceans, including lobsters (Brisbin et al., 2015; Haug et al., 2002; Hwang, 2004) and it is thought to be an important attribute for helping resist bacterial infection of the cuticle in lobsters. The similar growth promoting effects of tail fan extract were present among many of the bacterial isolates from both lobsters with and without TFN, suggesting that these PCL+ bacterial isolates are not inhibited by the antimicrobial activities of the tail fan, which could be another important factor associated with TFN (Zha et al., 2018a).

One PCL+ bacterial isolate belonging to *R. mobilis* from lobsters with TFN inhibited the growth of isolates belonging to seven other species isolated from the same group of lobsters with TFN. This was consistent with previous studies, which reported that *R. mobilis* could produce the antibacterial compound, tropodithietic acid (D'alvise et al., 2014). By contrast, most of the PCL+ bacterial isolates appear capable of co-existing on the tail fans. As the

bacteria which are inhibited by other bacteria are less likely to survive in an open environment, such as on a lobster tail fan, while those bacteria capable of co-existing or inhibiting others are more likely to be persistent and capable of colonizing hosts (Nguyen et al., 2014; Sant'Ana et al., 2012; Zeng et al., 2014). The relevant result suggests that a number of the PCL+ bacterial isolates may be collectively associated with TFN.

In this study, all the PCL+ bacterial isolates could produce ammonia, which is likely to be toxic to tissues and potentially contributing to localised cell death in tissues at sites of bacterial infection (Crear & Forteath, 2002; Kemp et al., 2009; Kır et al., 2004), and may indirectly induce more severe TFN when infection initiates.

The overall similar genomic patterns of the *V. crassostreae* isolates from the TFN affected lobsters and the relatively diverse patterns in *V. crassostreae* from the no-TFN lobsters, suggesting that the conditions in TFN affected lobsters may have a selection effect for bacteria with a similar genomic patterns. For example, certain nutrient source may have a selection effect on the bacteria with similar level of pathogenicity (Jaiswal et al., 2016; Pettersen et al., 2016; Weng et al., 2016), and long-term provisioning (i.e., sufficient nutrition) may select more harmful pathogens (Becker et al., 2015; Bhadauria et al., 2015; Miller et al., 2006).

An overall enhanced activity of biofilm formation, melanin and siderophore production were found in PCL+ bacterial isolates from tail fan of lobsters with TFN versus those without, suggesting the three characteristics in combination may contribute to the outer bacterial invasion associated with TFN. An overall similarity in the these bacterial characteristics between haemolymph and tail fans of either lobsters with TFN or those without, however, suggested that the three characteristics might be influenced by either a different haemolymph environment or host immune response.

The bacteria associated with TFN appear to possess many of the bacterial capabilities examined in this study that would provide advantages for inhabiting sites affected by TFN, including; cell motility, biofilm formation, enzymatic activities (i.e., proteolytic, chitinolytic and lipolytic activity), melanin and siderophore production, possible antimicriobial activity and ammonia production.

5.5. Conclusion

The results of this study suggest that a group of PCL+ bacterial isolates with similar suite of biological characteristics are likely to be operating as opportunistic invaders following injuries to cause TFN in spiny lobsters. The extent of some characteristics was different between the PCL+ isolates from lobsters with and without TFN, i.e., melanin and siderophore production, and biofilm forming ability. A number of key bacterial virulence factors (i.e. biofilm formation, melanin and siderophore production), as well as their previously identified broad enzymatic capabilities (proteolytic, chitinolytic and lipolytic) (Zha et al., 2018a), appear to be strongly associated with TFN.

6. Chapter Six - Pathology of tail fan necrosis in the spiny lobster, *Jasus edwardsii*

6.1. Introduction

Shell disease syndrome is a common problem amongst many crustaceans, such as shrimp, crabs and lobsters (Jayasree et al., 2006; Smolowitz et al., 2014; Wang, 2011). The presence of shell disease in lobsters compromises their health and greatly impacts on their market value (Homerding et al., 2012; Mancuso et al., 2010; Vogan et al., 2008). Shell disease is found in different species of lobsters, such as the American lobster (*Homarus americanus*) and the European clawed lobster (*H. gammarus*) and Norway lobster (*Nephrops norwegicus*) (Shields, 2011; Stentiford & Neil, 2011). There are several different etiologies of shell disease in *H. americanus*, such as epizootic shell disease, enzootic shell disease, diet-induced shell disease, impoundment shell disease and black spot shell disease (Chistoserdov et al., 2012; Meres et al., 2012; Quinn et al., 2013a; Shields, 2012).

Tail fan necrosis (TFN) is a specific form of shell disease in lobsters, characterised by erosion and blackening of the uropods and telson that make up the tail fan. The disease has been commonly identified in captive and wild spiny lobsters (*Jasus edwardsii*) in New Zealand and Australian waters (Freeman & MacDiarmid, 2009; Musgrove et al., 2005). TFN has been identified in some wild populations of spiny lobsters, such as in an area on the east coast of the North Island of New Zealand, where up to 17% of male lobsters had TFN (Freeman & MacDiarmid, 2009). The prevalence of TFN can be higher in captive spiny lobsters. For example, 33 - 42% of wild-caught adult lobsters were found to have contracted the condition after 29 - 30 weeks of holding in sea cages in South Australia (Bryars & Geddes, 2005).

Shell disease is thought to be initiated by injuries to the lobster cuticle with subsequent bacterial invasion, as the outermost layer of the epicuticle consists of an extruded protective

waxy layer which is thought to be difficult for bacteria to degrade (Baross et al., 1978; Kunkel et al., 2012; Quinn et al., 2012a). Injuries such as abrasion and lacerations are considered to be precursors of TFN, as they provide the opportunity for bacteria to gain entry and subsequently degrade the chitin, protein and lipid in the inner cuticle layers, which are much thinner in the tail fan region than in other parts of the lobster carapace (Musgrove et al., 2005; Vogan et al., 2008).

The pathology of the exoskeleton has been reported in lobsters affected by different types of shell disease such as epizootic shell disease, enzootic shell disease, impoundment shell disease and black spot shell disease (Davies et al., 2014b; Smolowitz et al., 2005; Smolowitz et al., 2014). Some common pathogenic changes that have been observed include physically damaged and eroded cuticle layers, melanisation in different layers of the cuticle, formation of pseudomembranes and the accumulation of haemocytes around the affected site (Comeau & Benhalima, 2009; Davies et al., 2014b; Mancuso et al., 2010; Smolowitz et al., 2005; Smolowitz et al., 2014; Smolowitz et al., 1992). Different hypotheses about the initiation and progression of various forms of shell disease have been reported, with some detailed pathological evidence suggesting possible causality and progression of the condition (Mancuso et al., 2010; Quinn et al., 2012a; Vogan et al., 2008).

Lobsters with shell disease are frequently found to have impaired immunity, such as reduced phenoloxidase activity and total haemocyte count (Homerding et al., 2012; Zha et al., 2017). The biochemical profiles of some tissues (i.e., muscle, hepatopancreas, haemolymph and carapace) have also been found to be impaired in lobsters affected by shell disease (Floreto et al., 2000). In addition, bacteraemia has been identified in the haemolymph of some lobsters affected by shell disease, most likely as a result of bacterial infection due to damaged carapace and compromised immunity (Chistoserdov et al., 2005; Homerding et al., 2012).

Chronic shell disease caused by bacterial incursion, combined with compromised immune response and the possibility of bacteraemia raises the prospect of systemic bacterial infection.

One study has found that shell disease is likely to affect the internal organs such as gill, hepatopancreas, testis and ovary in a sample of 12 American lobsters with shell disease of unknown etiology (Comeau & Benhalima, 2009), while another study observed minimal pathology in the internal organs of American lobsters (n = 80) with epizootic shell disease (Shields et al., 2012). However, this situation has not been previously investigated for spiny lobsters with TFN. Therefore, in this study, the pathology of tail fan and some internal organs (i.e., mid-gut, hepatopancreas, heart and gill) in TFN affected spiny lobsters was investigated to determine the range of pathologies in tail fans affected by TFN, and whether any internal organ pathology might be associated with the condition.

6.2. Materials and methods

6.2.1. Sample collection

Wild adult and juvenile *Jasus edwardsii* were caught in April 2016 using commercial lobster trapping methods on the east coast of the North Island of New Zealand in an area previously identified to have high prevalence of TFN among lobsters (Freeman & MacDiarmid, 2009). Upon capture lobsters were visually inspected for presence of the condition and retained until a sufficient sample size was reached. A total of 43 lobsters were brought to the laboratory for pathology analysis, including 29 lobsters with TFN and 14 unaffected lobsters. The status of caught lobsters included no TFN, light TFN, moderate TFN and heavy TFN.

6.2.2. Gross observations

In the laboratory each lobster was photographed and detailed information recorded of their moult stage, sex and loss of appendages (i.e., pereiopod, pleopod and antennae). The

carapace length and tail width of the lobsters were measured with callipers. The region affected by TFN was photographed on the ventral and dorsal surfaces while the uropods on the tail fan were spread out. . The degree to which the sampled lobsters were affected by TFN was assessed as four categories which were visually obvious and therefore fast in practice to assess visually; none – no signs of TFN, light – up to 10% of tail fan area affected, moderate – 10 - 30% of tail fan area affected, heavy – greater than 30% of tail fan area affected

In addition, the apparent external extent of necrosis, melanisation and loss of tissues on the tail fan associated with TFN was assessed visually and recorded. Likewise, notes were made on the presence of signs of abrasion, scratches and lacerations on the tail fans.

6.2.3. Necropsy

Haemolymph was removed from the bases of the fifth thoracic legs of all lobsters with a 26 gauge needle attached to a 1-mL sterile syringe (Terumo, Inc.) for subsequent bacteria culture. The area was disinfected with 70% alcohol prior to sampling haemolymph. Lobsters were euthanized by plunging into iced seawater for five minutes followed by injection of a 1-mL of sterile ice-cold KCl solution into the region of the ventral nerve ganglia at the base of the second thoracic leg (Battison et al., 2000). The mid-gut, hepatopancreas, heart, gill and tail fans were dissected from both affected and unaffected lobsters with scalpel and tweezers. Internal organs were visually examined and any externally apparent gross abnormalities were recorded. Tissue samples from each of the sampled organs were then placed in tissue cassettes and immediately transferred to Davidson's solution for initial fixation.

6.2.4. Tissue processing

After 24 h fixation in Davidson's solution, the tissues were transferred to neutral buffered formalin for further fixation. The most typical blackened tail fan regions of the affected lobsters were selected and subsequently transferred to 10% formic acid solution for

decalcification. All the tissue samples were then dehydrated and processed in paraffin using standard histological methods. The samples were sectioned (around 3 µm thickness), with transverse sections being taken through the tail fan elements and gill branchia. The sections were stained with Gill's hematoxylin and eosin stain, and mounted on microscope slides for later examination (Bensadoun et al., 1996).

6.2.5. Microscopic evaluation

Tail fan sections were examined for pathology commonly associated with shell disease, such as melanisation, haemocyte accumulation, pseudomembrane formation and the loss of cuticle. The sites and extent of the pathogenic changes and the area adjacent to the affected sites were also observed.

Histological sections of the internal organs were examined for abnormalities, such as haemocyte accumulation, as well as abnormal necrotic areas (in gill) and detached connective tissue (in hepatopancreas) (Comeau & Benhalima, 2009).

6.2.6. Haemolymph bacteria count

A 100 µl aliquot of haemolymph from all the lobsters and a ten-fold dilution (in autoclaved filtered seawater) were spread onto each of three replicate marine agar plates (BD Difco, Inc.), and incubated at 18 °C for seven days. Colony-forming units (CFU) were counted and CFU ml^{-1} was calculated for each sample.

Haemolymph bacteria counts were $\log_{10}$ transformed and compared for lobsters with and without TFN using a Mann-Whitney test. The same approach was used to compare the haemolymph bacteria counts from those for TFN affected lobsters with internal organ pathology versus all other TFN affected lobsters to determine if organ pathology may be associated with bacteraemia.

6.2.7. Comparisons of parameters

A Chi-square test was used to compare the proportions of lobsters with internal organ pathology for lobsters with TFN versus those unaffected.

6.3. Results

6.3.1. Lobster sample information

All the lobsters for this pathology study were in inter-moult stage and male. The carapace length of the TFN affected lobsters was from 93 to 111 mm, while the tail width was between 49 and 55 mm.

Of the 29 TFN affected lobsters, seven had missing or damaged antennae, eight had missing or damaged pereiopods, 10 had missing or damaged pleopods that had occurred prior to their collection for this study judging from the extent of the occlusion of the wound. By contrast, of the 14 unaffected lobsters, two had missing antenna, one had a missing pereiopod and no missing or damaged pleopods were found.

The degree to which TFN affected the sampled lobsters ranged from – light to heavy, with eight lobsters with light TFN, 12 lobsters with moderate TFN and nine lobsters with heavy TFN. All 14 lobsters without TFN had no signs of TFN.

6.3.2. Gross observation

Necrosis, melanisation and loss of tissues were consistently observed as the external signs of TFN among all 29 affected lobsters. Blackened lesions were observed in the burst blisters, lacerations and scratches that were present in the tail fans of lobsters with TFN (Fig. 6-1). Some areas of damaged tail fan in lobsters with TFN did not have the blackened appearance typically associated with melanisation of tissues (Fig. 6-1A).

In lightly affected lobsters, the lesions were more frequently observed on the posterior margin of one or two tail fan elements (i.e., telson and/or uropods) (Fig. 6-1A), while the blackened appearance was also observed in the base of the tail fan in one lobster. More extensive blackened melanised areas were found in the tail fans of moderately affected lobsters, including around burst blisters and lacerations. In the heavily affected lobsters, the blackened lesions were extensive around the base and central regions of the tail fans (Fig 6-1C, 6-1D). In many cases, external TFN signs were found in the corresponding dorsal and ventral regions of the tail fan, although most frequently the affected ventral region was visibly larger in extent (Fig. 6-1A, 6-1B).

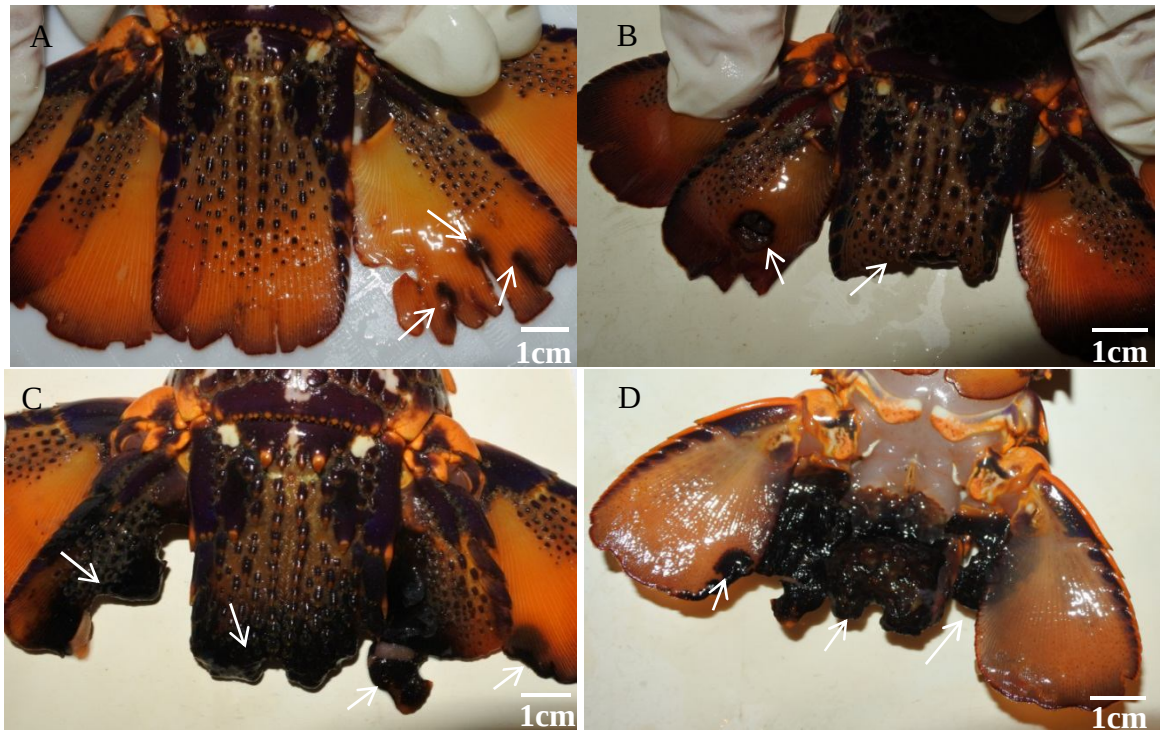


Fig. 6-1. Photographs of the sampled lobsters showing varying extent of external tail fan pathology of tail fan necrosis (TFN) from lightly to heavily affected. (A) Dorsal view of a lightly affected lobster showing initial melanisation around tears and scratches. (B) Dorsal view of a moderately affected lobster showing an eroded telson and necrosis around a burst blister on the uropod. (C) Dorsal view of a heavily affected lobster, showing melanisation,

necrosis and loss of tissue. (D) Ventral view of the same heavily affected lobster in Fig. 6-1A, showing more extensive TFN than the corresponding dorsal surface.

6.3.3. Necropsy

TFN affected tail fans were observed to be thicker than the unaffected ones. Dissection of these tail fans revealed the additional thickness was due to oedema in some tail fans, and solid black-brown melanin enriched material in most lobsters. In some affected tail fans where TFN signs were found in the underlying tissues, it was indurated with granulomatous foci and caseous necrosis compared to the unaffected tissues. In some cases, the lesion was limited to one side of the tail fan (mostly ventral), however, examination of the transverse sections showed the necrosis also typically appeared in the underlying tissues than was apparent from external examination alone.

Some softer black melanin enriched materials were also found on the TFN affected region of the tail fans (such as Fig. 6-1B) which was more frequently found on the ventral surface of the tail fan. Slightly brown-coloured hepatopancreas was found in two heavily affected lobsters, which was associated with an unusual odour.

6.3.4. Histology of tissues

6.3.4.1. Tail fan pathology

The pathological changes that were consistently observed in tan fan regions affected by TFN were; melanisation, haemocyte accumulation, pseudomembrane formation, fibrosis, proteinaceous oedema, detachment or loss of the epidermis and cuticle (Fig. 6-2).

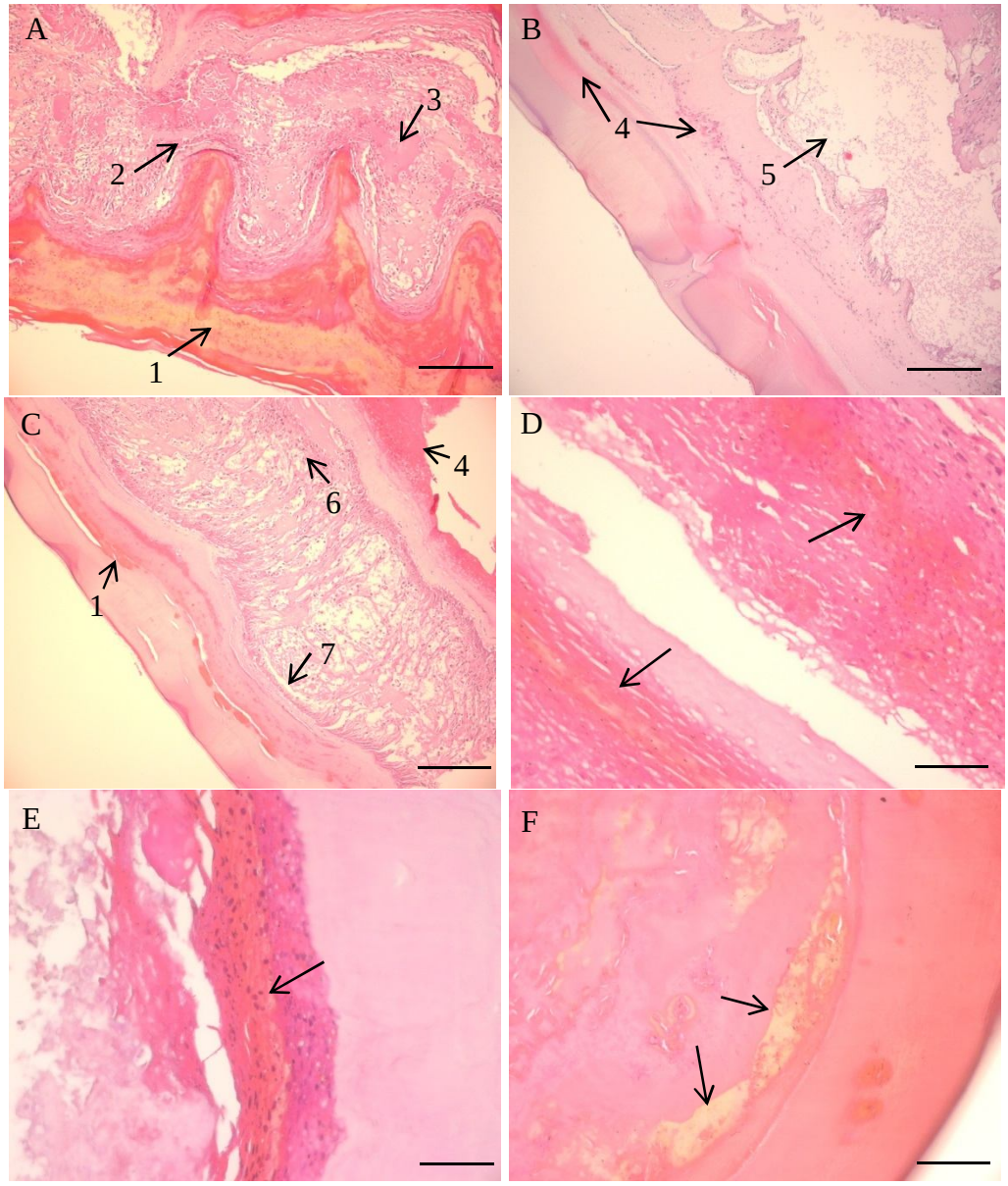


Fig. 6-2. Histological changes in transverse sections of spiny lobster (*Jasus edwardsii*) tail fans affected with TFN. (A) Section of affected tail fan showing externally apparent lesions extend to the subepithelial connective tissues (100×, moderate TFN) with extensive melanisation in the exocuticle and endocuticle (1), haemocyte accumulation (2), andhaemolymph clot (3). (B) Section of tail fan lesion showing caseous necrosis with cellular infiltrates (4), and detached connective tissue with fibrosis (5) (100×, moderate TFN). (C) Section of tail fan lesion showing melanisation (1), severe caseous necrosis (4), fibrosis in the subepithelial connective tissue (6), and detached epidermis (7) (100×, light TFN). (D) Section of affected tail fan showing melanisation in the underlying tissue (400×,

moderate TFN). (E) Section of affected tail fan showing melanisation around pseudomembrane (400×, moderate TFN). (F) Section of affected tail fan showing melanisation with no haemocyte or haemocyte granule (400×, heavy TFN). Scale bar: 200 µm (100×); 50 µm (400×).

6.3.4.2. Development of TFN

From the lightly affected lobsters, indications of TFN were most commonly visible as incursions around the margins of lacerations of tail fans (Fig. 6-3A, 6-3B), with the accumulation of proteinaceous material through the inner layers of the tail fan, such as the endocuticle and underlying connective tissue. While this was the most commonly observed pathology at this stage of the condition, other less commonly observed pathology included haemolymph clots in the underlying connective tissue, fibrosis, detached epidermis, endocuticle or epicuticle. In the lightly affected tail fans there was limited melanisation in cuticle layers and limited haemocyte accumulation.

There were no obvious differences in the tail fan lesion pathology between lobsters moderately and heavily affected with TFN. In moderately and heavily affected lobsters, TFN signs were more commonly found in the underlying connective tissue, and typically showed extensive melanisation in the cuticle, as well as haemocyte accumulation in the underlying tissue.

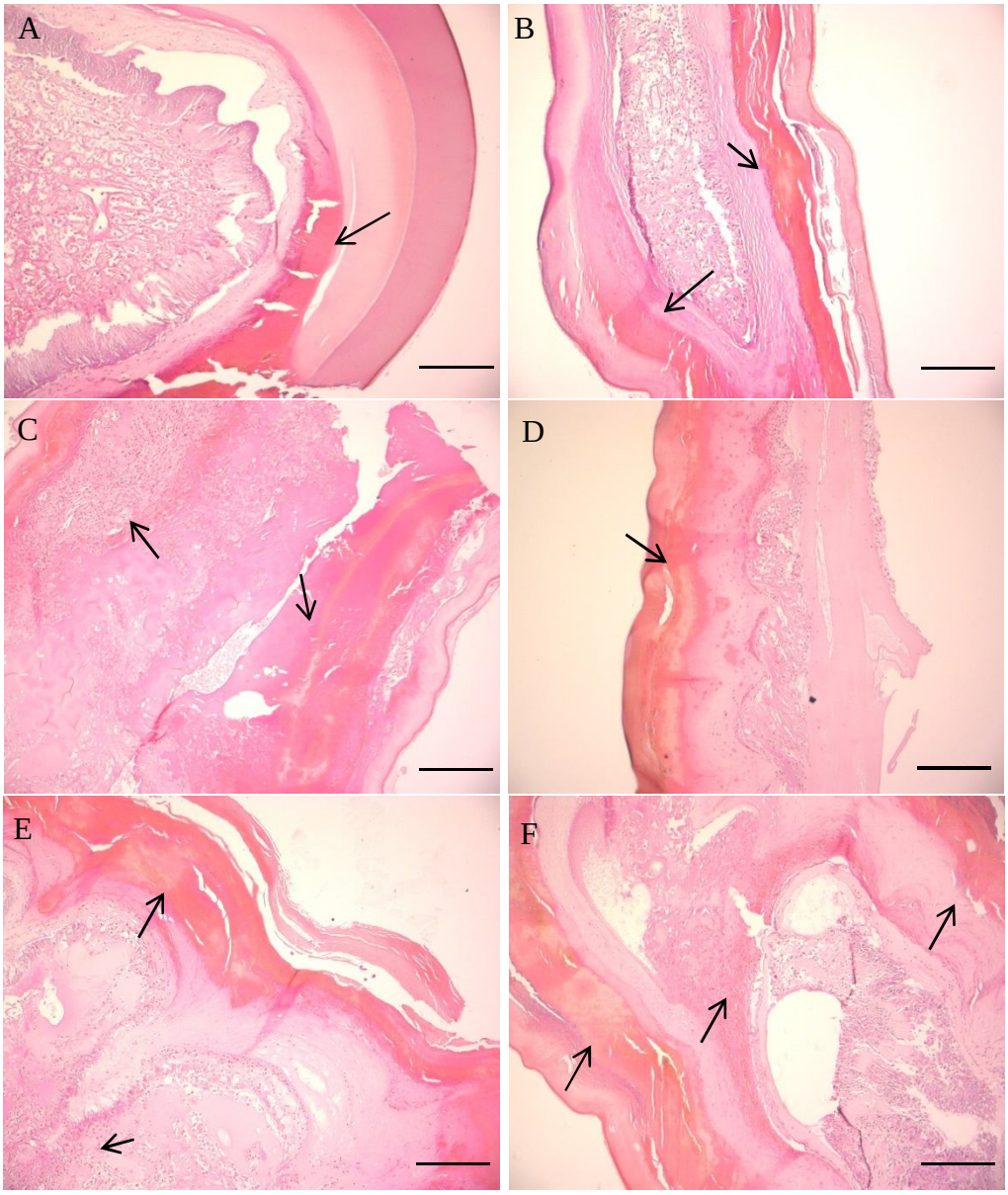


Fig. 6-3. Histology of tail fans of affected spiny lobster, *Jasus edwardsii* affected by TFN to different degrees. (A) Section of affected tail fan showing caseous necrosis in the epidermis and endocuticle (100×, light TFN). (B) Section of affected tail fan showing caseous necrosis in the healthier tail fan region (100×, light TFN). (C) Section of affected tail fan showing haemocyte accumulation in the subepithelial connective tissue with melanisation in the cuticle layers (100×, moderate TFN). (D) Section of affected tail fan showing loss of cuticle with associated melanisation (100×, moderate TFN). (E) Section of affected tail fan showing apparent haemocyte accumulation in tissue with melanisation in the damaged cuticle (100×, heavy TFN). (F) Section of affected tail fan showing apparent haemocyte accumulation in

tissue with melanisation in endocuticle (100×, heavy TFN). Scale bar: 200 µm. Arrows indicate the sites of typical pathological changes.

6.3.4.3. Internal organ pathology

Pathological changes were found in the mid-gut of one moderately affected and one heavily affected lobster, the heart of three lightly affected and one moderately affected lobster, and the gill of one moderately affected lobster. The pathological changes included cell debris, elevated abundance of pyknotic cells and large eosinophilic reserve cells (Fig. 6-4). The presence of bacteria was not visually apparent in the regions of the tissues of the internal organs showing pathology, however, this may have been a consequence of the Gill's hematoxylin and eosin stain. A subsequent attempt was made to visualise any bacteria in those histological sections of internal organs which revealed pathology by re-staining with the Brown and Hopps staining method (Brown & Hopps, 1973). However, this failed to reveal the presence of bacteria, although this may have been a result of the status of the samples. No apparent pathology was found in any of the internal organs of the 14 lobsters unaffected by TFN.

The extent of tail fan site affected by TFN was not associated with internal organ changes, as most of the affected lobsters with internal organ pathology (seven out of eight) were either lightly or moderately affected. More TFN affected lobsters were found to have internal organ pathology than observed in unaffected lobsters ($\chi^2 = 4.51$, $P < 0.034$).

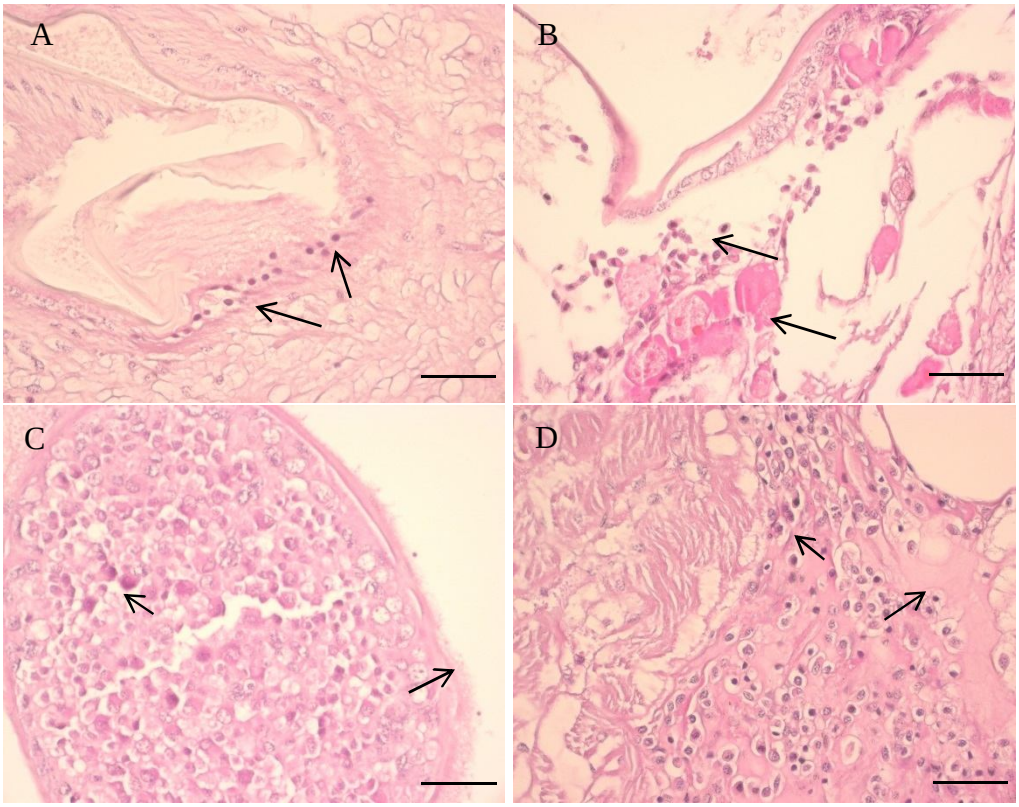


Fig. 6-4. Histological pathology in internal organs of the spiny lobsters *Jasus edwardsii* affected by TFN. (A) Section of affected gut showing pyknotic nucleii under the chitin layer (400×, moderate TFN). (B) Section of affected gill showing large eosinophilic reserve cells and scattered haemocytes above (400×, moderate TFN). (C) Section of affected pericardium showing pyknotic cells and cell debris in the surrounding tissue of heart (400×, moderate TFN). (D) Section of affected heart showing pyknotic nuclei with embedded clotted haemocytes in heart (400×, light TFN). Scale bar: 50 µm. Arrows indicate the locations of typical pathological changes.

6.3.5. Haemolymph bacteria count

Bacteria was cultured from haemolymph in 93.1% of 29 affected lobsters and 92.8% of 14 unaffected lobsters. The haemolymph bacteria count in TFN-affected lobsters ranged from 0 - 1247 CFU ml^{-1}, and from 0 - 163 CFU ml^{-1} in unaffected lobsters. There was no overall difference in the haemolymph bacteria count between lobsters affected with TFN versus

unaffected lobsters (Mann-Whitney test, $U = -0.68$, $P > 0.49$). The eight TFN-affected lobsters expressing pathology of their internal organs had less bacteria in their haemolymph when compared to the other TFN-affected lobsters (Mann Whitney test, $U = -2.06$, $P < 0.04$).

6.4. Discussion

Tail fan necrosis (TFN) has been observed to impact spiny lobsters in New Zealand, Australia and in parts of Europe (Freeman & MacDiarmid, 2009; Mancuso et al., 2010; Musgrove et al., 2005). It causes diverse pathological changes in the tail fan of spiny lobsters, some of which are similar to the changes in lobsters affected by other types of shell disease. The common appearance of black-brown melanin in the affected tissues is thought to be associated with melanisation by the host, although it may also be derived from bacterial sources directly (Zha et al., 2018b). A caseous necrosis in the cuticle of lobsters affected with TFN is commonly seen, which is thought to be a response for alleviating bacterial infection (Nyholm & Graf, 2012a; Nyholm & Graf, 2012b). In crustaceans, haemocytes play a vital role in the first order immune response to incursions of microbial pathogens (Fotedar et al., 2006). Consequently, haemocyte accumulation, which is associated with phagocytosis to alleviate bacterial infection, was commonly observed in the tail fans, especially the subepithelial connective tissues. The formation of pseudomembrane in American lobsters with epizootic shell disease was usually associated with a severely damaged endocuticle sometimes with suppuration, part of walling off the region of tissue damage when lesions penetrate through the cuticle (Shields et al., 2012). Bacteria were identified at the leading edges of different types of shell disease including TFN, but the various bacteria and their products which are responsible for the condition are difficult to identify by pathological methods alone, but have been previously characterised (Smolowitz et al., 2005; Zha et al., 2018b).

The pathology of TFN shows some distinct differences to other forms of shell disease. In American lobsters affected by epizootic shell disease, a melanised surface was found above the pseudomembrane (Smolowitz et al., 2005). In this study, melanin-enriched materials were observed within the pseudomembrane, suggesting that the formation of the pseudomembrane may be at an early stage of host melanisation in some sites (e.g., Fig. 6-2E). The distribution of TFN on lobsters appears to be mostly confined to the tail fan region, while other shell diseases found in lobsters are usually distributed more widely across the carapace and tail cuticle (Smolowitz et al., 2014). Other shell diseases, such as epizootic shell disease, frequently spread across the surface of the cuticle and are rarely reported to progress through the endocuticle (Smolowitz et al., 2005). By contrast, the spread of TFN was mostly confined to the tail fan region, however, the condition was typically found in both the cuticle layers and subepithelial connective tissues, suggesting that TFN is likely to progress through the endocuticle and even into the underlying tissues. The possible penetration into underlying connective tissues, and subsequent formation of granulomatous foci and caseous necrosis with melanisation and convoluted pseudomembranes are likely to disrupt the formation of a coherent layer of new carapace which enables the lobster to eliminate damaged tissue through moulting. This would contribute to the persistence of the condition in individual lobsters and may explain the ongoing persistence of the condition in the wild population of spiny lobsters (Freeman & MacDiarmid, 2009). Alternatively, disrupted carapace may create difficulties for moulting lobsters, possibly causing moult death syndrome, as reported in American lobsters with shell disease (Conklin et al., 1980).

Any form of injury to that disrupts the integrity of the external surface coating of the cuticle of the tail fan appears to be an essential precursor for the initiation of TFN in spiny lobsters (Musgrove et al., 2005), as has been found in other forms of shell disease (Quinn et al., 2012a; Tlusty & Metzler, 2012; Whitten et al., 2014). Signs of TFN in this study were found

to be associated with a variety of physical damage to the tail fan, including lacerations, puncture wounds, abrasion and severed tail fan elements (Musgrove et al., 2005; Vogan et al., 2008). Subsequent bacterial invasion through injuries, especially by bacteria with strong chitinolytic, proteolytic and lipolytic capabilities, are likely to result in continuing degradation of tissues radiating out from the initial injury site and into the inner cuticle and underlying tissue layers (Vogan et al., 2008; Zha et al., 2018a). A small number of injuries were observed on tail fans without any apparent TFN signs (e.g., Fig. 6-1A) and histological examination indicated damaged cuticle without melanosis or pathology. This suggests that injuries do not always lead to the development of TFN, perhaps as a result of an effective immune response or differences in pathogenicity of the suite of bacteria colonising the injured tail fan region.

The presence of TFN in some cuticle layers (i.e., exocuticle and endocuticle) is most commonly characterised by an inflammatory response resulting in indurated tissues with granulomatous foci and caseous necrosis accompanied by melanin production. Pathological changes associated with prevention of possible progression of TFN into subepithelial connective tissues include the formation of pseudomembranes, which can also involve the deposition of melanin to form a subsequent melanised layer (Shields et al., 2012; Smolowitz et al., 2005).

The recovery from shell diseases in lobsters is not frequently reported. American lobsters were found to moult away the cuticle affected by shell disease (Smolowitz et al., 1992; Tlusty & Metzler, 2012), but this situation is largely unknown in spiny lobsters and European lobster. The progression of shell disease was observed to be halted on lesions associated with cracked and punctured claws of European lobster during 8-weeks of rearing in laboratory, but the same recovery was not found for American lobsters with similar shell disease (Whitten et al., 2014). To our knowledge, there is no report of recovery of spiny lobsters from TFN.

Pathology was found in gills of two TFN-affected lobsters, which is consistent with another study reporting gill pathology in American lobsters with shell disease (Comeau & Benhalima, 2009). However, no pathology was found in hepatopancreases of the TFN-affected lobsters, which contrasted with the finding by Comeau & Benhalima, (2009), but consistent with the study reporting hepatopancreas pathology in American lobsters (Shields et al., 2012). Internal organ pathology was more frequently found in TFN-affected lobsters than unaffected lobsters in this study, suggesting that TFN is associated with internal organ pathology in *J. edwardsii*.

The culturable bacteria count in haemolymph was similar between lobsters with and without TFN, implying that culturable bacteria count was not associated with TFN. The presence of bacteria in the haemolymph of lobsters is common and not always associated with compromised health (Chistoserdov et al., 2005; Shields et al., 2012; Wang, 2011). Haemolymph bacteria count was lower in the affected lobsters with internal organ pathology than the other affected lobsters, suggesting a possible stimulation of an immune response resulting in enhanced elimination of bacteria in haemolymph or alternatively a variation in the species of bacteria involved.

6.5. Conclusion

In conclusion, TFN is likely to be initiated from sites of physical injury and appears to progress with an increasing variety of pathological changes mostly associated with the various immune defensive responses in lobsters. These diverse pathological signs included melanisation, caseous necrosis, pseudomembrane formation, detachment of epidermis or cuticle, haemolymph accumulation, and loss of epithelium and fibrosis. There was no evidence of recovery from TFN pathology among the samples examined either with or without TFN. The presence of TFN did not appear to be associated with elevated bacteraemia, but it was associated with internal organ pathology in nearly a third of affected

lobsters. While positive confirmation of the presence of bacteria in internal organs with pathology was not possible in this current study, the causes of the observed pathology and its significant association with TFN warrants closer examination.

7. Chapter Seven - Multiple opportunistic bacteria associated with tail fan necrosis in spiny lobster, *Jasus edwardsii*

7.1. Introduction

Tail fan necrosis (TFN) is a disease-like condition which involves the erosion of tail fan tissues found in wild and captive spiny lobsters (i.e., *Jasus edwardsii*, *Panulirus argus* and *P. elephas*) following initiation by physical damage to the tail fan cuticle (Mancuso et al., 2010; Musgrove et al., 2005; Porter et al., 2001; Zha et al., 2017). This condition is a subtype of shell disease (Shields, 2011; Vogan et al., 2008). Different etiologies of shell disease have been identified in the American lobster (*Homarus americanus*), including epizootic shell disease (ESD), enzootic shell disease (EnSD), impoundment shell disease (ISD), trauma-induced shell disease (TSD), diet-induced shell disease (DISD) and black spot shell disease (BSSD) (Castro & Somers, 2012; Chistoserdov et al., 2012; Meres et al., 2012; Quinn et al., 2013a). Shell disease was also found in some other species of lobsters, such as the European lobster (*Homarus gammarus*) and Norway lobster (*Nephrops norvegicus*) (Davies et al., 2014b; Ziino et al., 2002).

In Australasian waters, the spiny lobster species most commonly affected by TFN is *J. edwardsii* (Musgrove et al., 2005). For example, up to 17% of male *J. edwardsii* in north-eastern New Zealand had TFN over the course of a three year study (Freeman & MacDiarmid, 2009), while the prevalence of the condition was higher (33% - 43%) among captive *J. edwardsii* after around 30 weeks holding in sea-cages in South Australia (Bryars & Geddes, 2005). Lobsters affected with TFN commonly have impaired immune status and tail fan pathology, and a percentage of the TFN-affected lobsters were found to have affected internal organs (Zha et al., 2018d; Zha et al., 2017).

Bacteria have been isolated from the TFN-affected lesions of the affected spiny lobsters (Mancuso et al., 2010; Musgrove et al., 2005; Porter et al., 2001), and some of them have been used to induce TFN in experiments on captive lobsters (May, 2007). *Aeromonas* sp., *Pseudoalteromonas* sp., *Shewanella* sp. and *Vibrio* sp. were isolated from TFN-affected lesions of spiny lobsters (i.e., *J. edwardsii*, *P. argus* and *P. elephas*) (Mancuso et al., 2010; Musgrove et al., 2005; Porter et al., 2001). *Vibrio vulnificus*, *V. parahaemolyticus* and *V. alginolyticus* were used to inoculate artificially damaged tail fans and found to induce TFN in laboratory-held *J. edwardsii* in South Australia (May, 2007). In our previous studies, some specific characteristics, i.e. proteolytic, chitinolytic, lipolytic activity, biofilm formation, production of melanin and siderophones, were found to be associated with TFN in *J. edwardsii* (Zha et al., 2018a; Zha et al., 2018b). A group of representative bacterial isolates with all or most of these characteristics were identified and belong to several bacterial genera, i.e., *Aliivibrio* sp., *Paracoccus* sp., *Photobacterium* sp., *Pseudoalteromonas* sp., *Ruegeria* sp., *Shewanella* sp., *Stenotrophomonas* sp. and *Vibrio* sp. (Zha et al., 2018a; Zha et al., 2018b).

Several difficult issues are limiting the progression of the study of shell disease, including TFN. One of these issues is the ability to identify the status of shell disease lesions in lobsters, i.e., severity, stage, and the extent of progression of the condition. The severity (i.e., mild, moderate and severe) of shell disease in lobsters is usually defined according to the percentage of affected area in lobsters (Comeau & Benhalima, 2009; Smolowitz et al., 2005; Smolowitz et al., 2014), while there was also one study which defined the severity according to detailed histological changes (Smolowitz et al., 1992). The progression models of shell disease were proposed based on the bacterial communities present in different cuticle sites of captive American lobsters (i.e., affected spots and lesions, the nearby regions of cuticle) (Feinman et al., 2017), but the extent of the progression of shell disease within the affected lesions was seldom reported.

The objectives of this research were to examine the bacterial community in affected *J. edwardsii* to determine;

- the difference of bacterial communities in lesion of TFN affected lobsters versus those in unaffected cuticle of unaffected lobsters;

- whether there are a group of bacteria closely associated with TFN in *J. edwardsii*;

- whether TFN closely associated bacteria (if any) also exist in commercial fishers' gloves and pots;

7.2. Materials and methods

7.2.1. Sample collection

A total of 71 wild juvenile and adult *J. edwardsii* were collected over five days in May 2017 using commercial pots on the east coast of the North Island of New Zealand in commercial fished areas with high prevalence of TFN among lobsters (Freeman & MacDiarmid, 2009). The sample comprised 35 TFN-affected lobsters and 36 unaffected lobsters. A total of 92 samples were collected using dry sterile cotton swabs, from the tail fan lesions of 35 TFN-affected wild lobsters, as well as the corresponding tail fan regions of 36 unaffected lobsters (the margin of tail fan), as well as from 10 gloves used by commercial fishers whilst handing lobsters and 11 pots used for catching lobsters. Lobster and pot samples were collected immediately upon their recovery from the water and onto the fishing boat, while glove samples were collected at the end of each day after the harvesting activities were completed. Swab samples were stored in 1.5 ml Eppendorf tubes with filtered autoclaved seawater and transported in insulated boxes with icepacks and transferred to a -20 °C freezer.

7.2.2. Molecular methods

The cotton swabs and seawater in the Eppendorf tubes were transferred to the lysis buffer columns of MoBio PowerSoil DNA Isolation Kit (MoBio Laboratory Inc. USA), and DNA was extracted from individual samples according to the manufacturer's protocol, but using a TissueLyser II disruption system (QIAGEN, Germany) to agitate sample solutions (30 revolutions per s, 2 min, twice) (Wu et al., 2017).

DNA fragments were amplified with the modified primer pair 341F (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGN GGCWGCAG-3') and 785R (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC-3'), which targets the V3 and V4 region of bacterial 16S rDNA and is designed for optimal use on Illumina Miseq DNA sequencing platforms (Klindworth et al., 2013). DNA was amplified by using PCR conditions: (i) 95 °C for 3min; (ii) 30 cycles of 98 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s; (iii) 72°C for 5 min. 16S rRNA amplicons were single PCR reaction per sample. After PCR amplification, the products were individually purified using DNA Clean and Concentrator Kits (Zymo Research), per the instructions of the manufacturer (Hermans et al., 2017). The concentrations of purified PCR products were measured using a Qubit double-stranded DNA HS assay Kit (Life Technologies, USA) and the concentrations were normalized where required. The cleaned normalized PCR products were then submitted to New Zealand Genomics Ltd for sequencing on an Illumina Miseq sequencer (Illumina Inc. USA) using 2-by-300 bp chemistry. The sequencing provider attached a unique combination of Nextera XT dual indices (Illumina Inc. USA) to each DNA sample to allow multiplex sequencing before sequencing.

7.2.3. Bioinformatic analyses

Paired-end read DNA sequence data were merged and quality filtered using the Usearch sequence analysis tool (Edgar, 2013). After quality filtering, we de-replicated sequence data, removed 'singleton' sequences. Sequences were then chimera checked and clustered sequences into groups of operational taxonomic units (OTUs) based on sequence identity threshold ≥ 97% using the clustering pipeline UPARSE (cluster_otus command in usearch) (Edgar, 2013). Prokaryote phylotypes were then classified to corresponding taxonomy by implementing the RDP classifier routine in Quantitative Insights into Microbial Ecology, version 1.9 (QIIME v.1.9) against the Greengenes database (McDonald et al., 2012). Sequences identified as chloroplast and mitochondria were removed from the data set. Each sample was rarefied to 18300 randomly selected reads per sample, consistent with the most shallow sample.

7.2.4. Statistical analyses and comparison of bacterial communities

Permutation analysis of variance (PERMANOVA) was conducted in R using the 'adonis' command in the vegan package (Anderson, 2001). Alpha diversity metrics (i.e., the observed species, Shannon index) comparing lesion and unaffected cuticle were calculated in QIIME v.1.9 using the command alpha_diversity.py (Caporaso et al., 2010). Principal coordinates analysis (PCoA) of the Bray-Curtis dissimilarity metric from square-root transformed comparing bacterial composition was used to visualise the differences of bacterial communities in lesion and unaffected cuticle samples by using Primer 6 software (PRIMER-E Ltd, New Zealand).

Similarity percentage (SIMPER) analysis was performed to identify the 100 largest contributors to the dissimilarities between different groups (Luter et al., 2017). Among the top 100 contributors, the 30 OTUs with greater average abundance in affected lobsters than

unaffected lobsters were reported as TFN associated OTUs, and the remaining 70 were considered as OTUs associated with unaffected cuticle.

Linear Discriminant Analysis (LDA) Effect Size (LEfSe) was used to determine which OTUs differentiated diseased from healthy subjects in an program run by the Huttenhower Lab (Segata et al., 2011). The analysis was performed using Kruskal-Wallis sum-rank test (with $\alpha < 0.05$), followed by unpaired Wilcoxon rank-sum test (with $\alpha < 0.05$), and a one-against-all strategy for multi-class analysis (Wagner Mackenzie et al., 2017). In this study the LEfSe analysis was used to examine the OTUs differentiated TFN affected lobsters from those unaffected ones. Those OTUs with an LDA threshold above 3.0 and consistently significant across lesion were identified as TFN associated OTUs.

The TFN associated OTUs identified by both SIMPER and LEfSe analyses were defined as "TFN closely associated OTUs".

Partition around medoids (PAM) clustering algorithm was used for cluster analysis for the bacterial communities in all the tail fan samples, based on the Bray-Curtis dissimilarity metric. Before cluster analysis, the optimal cluster number was calculated by using average silhouette method (Rousseeuw, 1987).

7.3. Results

7.3.1. Sequence summary

Sequence analyses of the 92 swab samples yielded 6.27 million sequencing reads. After rarefying the data from each sample to 18300 reads, we identified 5669 unique operational taxonomic units (OTUs) defined at 97% sequence identity. These OTUs represented 47 bacterial phyla, 139 classes, 252 orders, 418 families and 663 genera. There were 4201 OTUs

identified among lesion, 4606 OTUs among unaffected cuticle, 2959 OTUs among gloves and 2297 OTUs among pots.

A total of 3559 OTUs were found in both lesion and unaffected cuticle, representing 99.2% of the total sequencing counts in the two types of samples; while 642 OTUs and 1047 OTUs only found in lesion and unaffected cuticle, respectively.

7.3.2. Taxonomic composition

OTUs affiliated with the phyla Proteobacteria and Bacteroidetes dominated both affected lesions and unaffected cuticle samples, while OTUs from Planctomycetes and Actinobacteria were secondary in abundance in lesions and unaffected cuticle samples (Fig. 7-1). Among the phyla, OTUs from Actinobacteria were over twice as abundant in lesion as in unaffected cuticle samples (7.4% versus 3.5%).

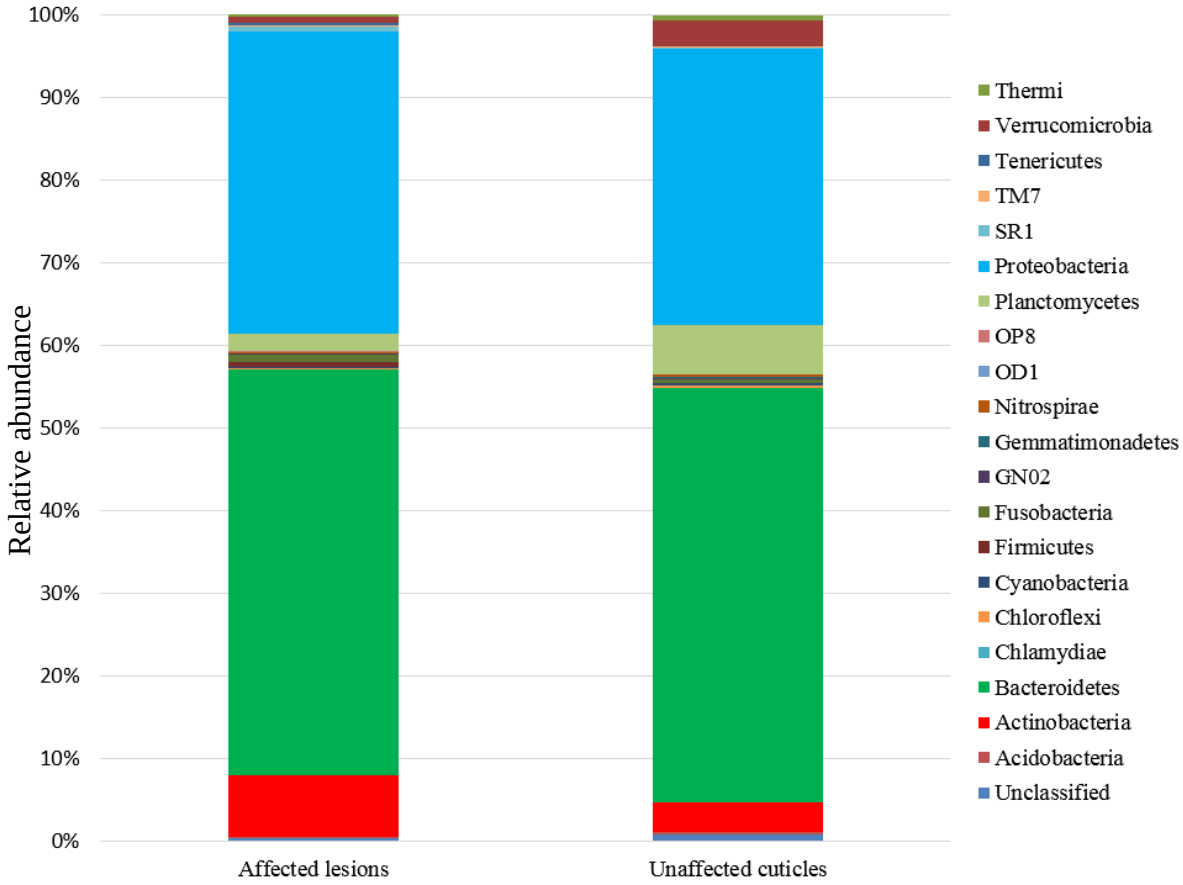


Fig. 7-1. Taxonomic composition of bacterial communities in affected lesions and unaffected cuticle samples, showing mean abundance at the phylum level. A few phyla with less than 0.1% of the samples were not included in this figure.

7.3.3. Difference of bacterial communities in lesion versus unaffected cuticle

PERMANOVA results showed the bacterial communities in lesion and unaffected cuticle were significantly different ($R^2 = 0.235$, $P = 0.001$). Alpha diversity revealed that the richness and diversity of bacterial communities in affected lesion were significantly less than those unaffected tail fan cuticle samples, across observed species (phylotypes) and Shannon index (Table 7-1).

Table 7-1 OTU richness (Observed species) and diversity (Shannon index) of bacterial communities in affected lesions versus unaffected cuticle samples.

Index	Affected (n = 35) (mean ± standard error)	Unaffected (n = 36) (mean ± standard error)
Observed species	836 ± 44 **	1308 ± 39 **
Shannon	5.56 ± 0.21 **	7.16 ± 0.23 **

Note: ** represents a significant difference with $P < 0.01$.

Bacterial communities in affected lesion and unaffected cuticle were distinct according to the Principal coordinates analysis (PCoA) based on Bray-Curtis dissimilarity metric (Fig. 7-2). Furthermore, the bacterial communities in affected lesions were more divergent than those in unaffected cuticle samples (Fig. 7-2).

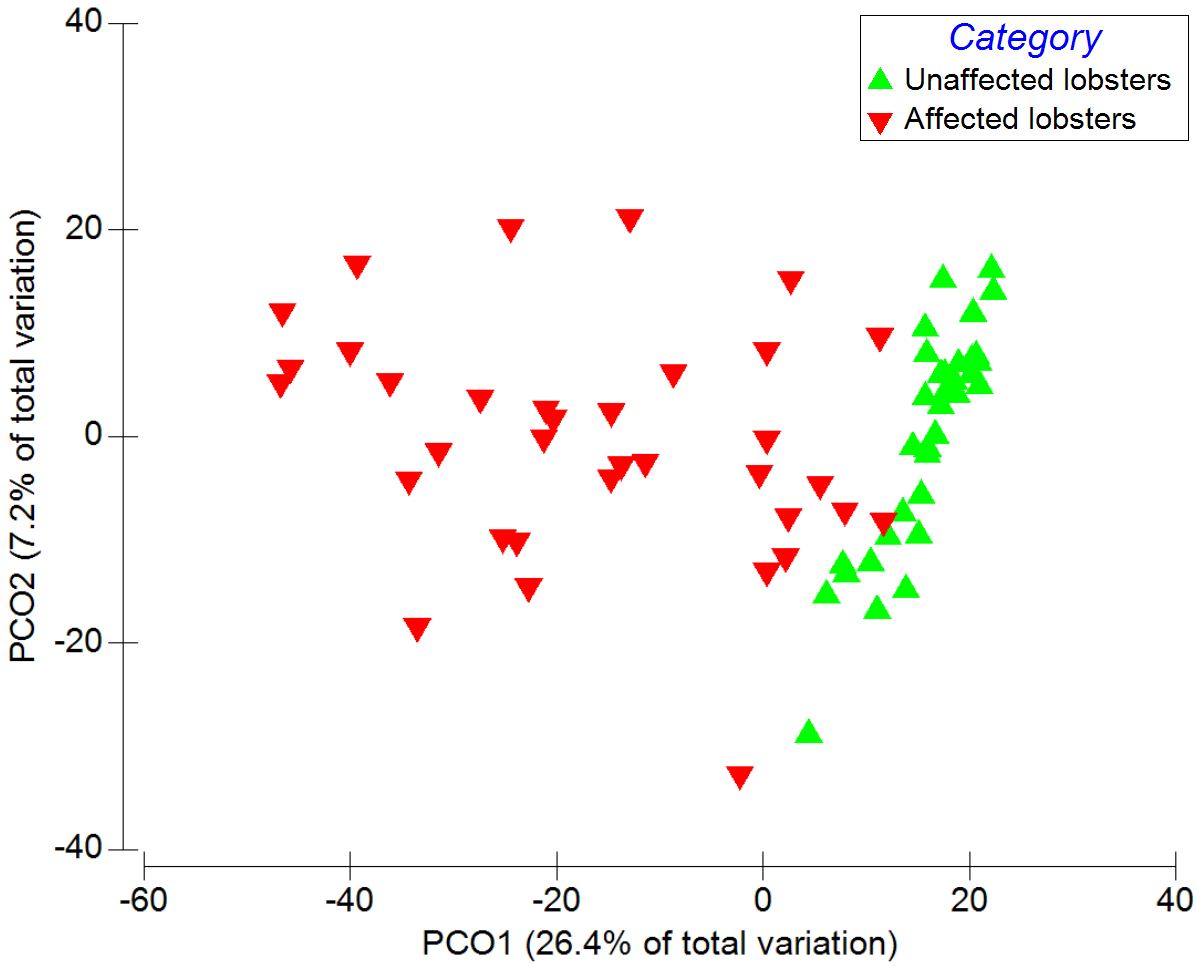


Fig. 7-2. Comparison of the bacterial community composition in affected lobsters (affected tail fan lesion) and unaffected lobsters (unaffected tail fan cuticle) using principal coordinates analysis based on the Bray-Curtis dissimilarity metric.

SIMPER analysis showed that similarities between affected and unaffected samples were generally low (SIMPER average dissimilarity = 60 %). The similarity within lesion (SIMPER average similarity = 40 %) was lower than within unaffected cuticles (SIMPER average similarity = 58 %). A total of 100 OTUs contributing most to the difference between bacterial communities in lesions versus unaffected cuticles were identified from SIMPER analysis (around 25% cumulative contribution). Among them, 30 OTUs were associated with lesion (Appendix B).

LEfSe analysis revealed that 23 OTUs with LDA score over 3.0 which were significantly more abundant in lesion than unaffected cuticle samples (Appendix C).

A total of 21 OTUs were identified to be associated with lesion from both SIMPER and LEfSe analyses (Fig. 7-3), among which six phylotypes ranked the top six in both analyses, i.e., *Aquimarina*, *Flavobacterium*, *Neptunomonas*, *Streptomyces*, *Flavobacteriaceae*, *Thiohalorhabdales* (Table 7-2). Apart from the six top OTUs, *Arcobacter*, *Neptunomonas*, *Streptomyces* and *Vibrio* were also closely associated with lesion, as well as the seven *Rhodobacteraceae* (such as *Loktanella*, *Octadecabacter* and *Ruegeria*) (Appendix D).

Table 7-2 Top Six TFN closely associated OTUs identified from both Similarity percentage (SIMPER) analysis and Linear Discriminant analysis Effect size (LEfSe) analysis

OTU ID	Taxonomy	Av.Abund (Unaffected)	Av.Abund (Lesion)	Contribution (%)	LDA score
2	*Aquimarina*	2.81	46.2	1.58	4.94
4	*Streptomyces*	0.32	27.86	0.94	4.47
5	Flavobacteriaceae	14.6	28.01	0.54	4.34
14	*Neptunomonas*	0.44	14.9	0.5	4.04
17	*Flavobacterium*	0.29	14.16	0.49	4.11
18	Thiohalorhabdales	1.95	15.63	0.47	4.02

Note: “LDA score” represented the score calculated by Linear Discriminant analysis.

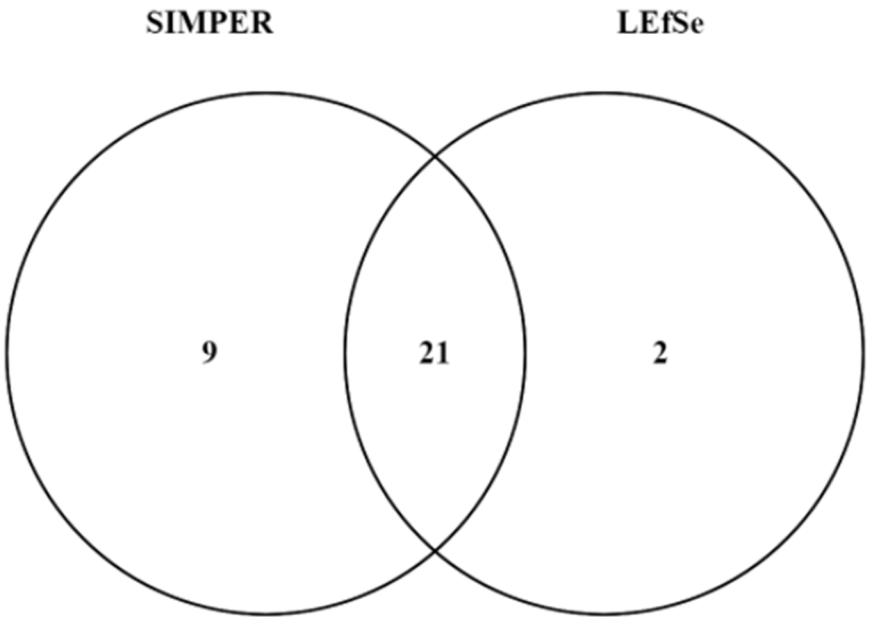


Fig. 7-3. The number of OTUs associated with lesion identified from SIMPER analysis and LEfSe analysis.

Silhouette analysis revealed that two clusters were the more optimal than the other numbers of clusters (Appendix E). In the two clusters, lesion communities of 13 affected lobsters were in the same cluster as the cuticle bacterial communities of unaffected lobsters (Cluster One), and the other 22 lesion communities were in another cluster (Cluster Two) (Appendix F).

7.3.4. TFN associated OTUs in glove and pot samples

Nine OTUs were commonly found in glove and pot, and eight OTUs were all found in gloves and pots, but with lower prevalence (Table 7-4). *Streptomyces* (OTU 4) was found in only one glove sample and three pot samples, while an unclassified Kilomiellaceae (OTU 106) was not found in any glove and pot.

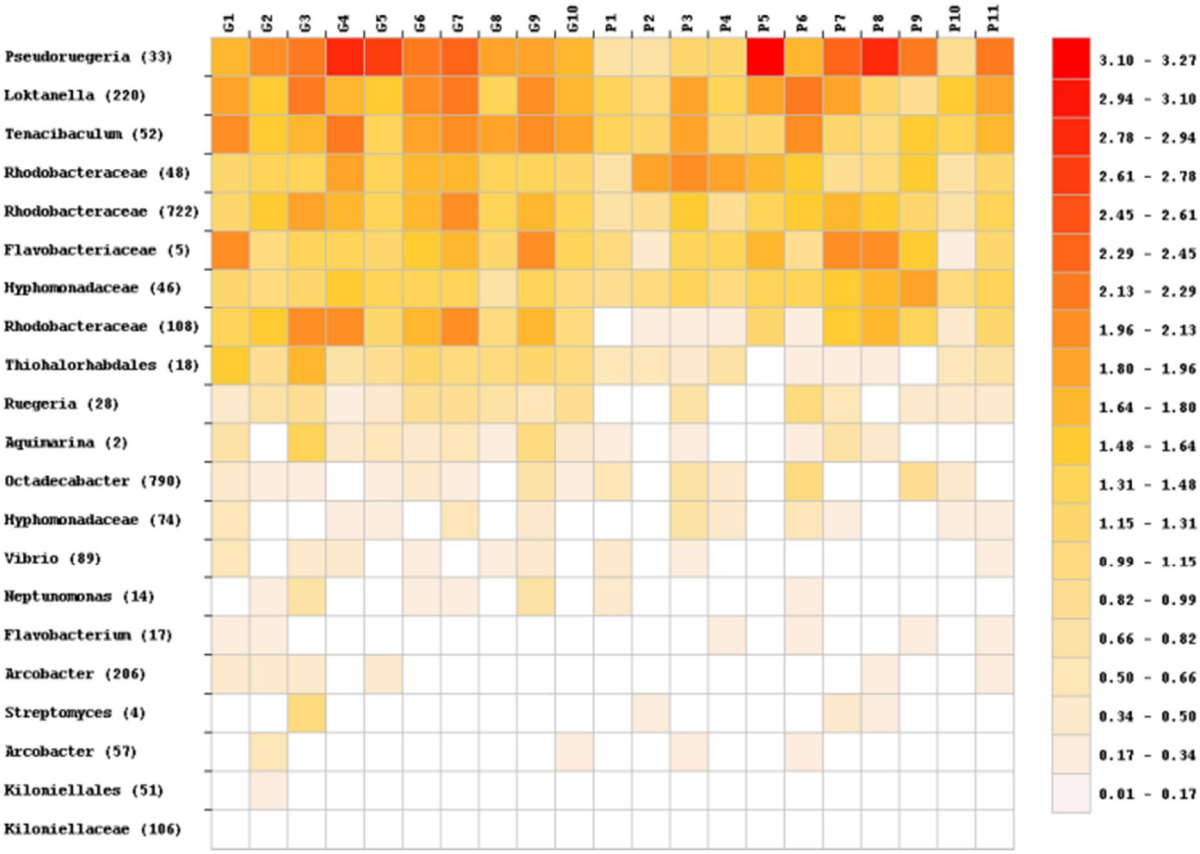


Fig. 7-4 Distribution of 21 TFN closely associated OTUs in glove and pot samples. Note: The data were lg(1+ raw read) transformed; The number right after the taxonomy were the corresponding OTU ID; “G” and “P” represented glove and pot samples respectively; white in block means absence.

7.4. Discussion

Bacterial communities in shell disease affected lesions have been reported in American lobster and European lobster (Chistoserdov et al., 2012; Feinman et al., 2017; Quinn et al., 2013a; Whitten et al., 2014), but seldom reported in affected spiny lobsters (i.e., TFN).

In this study, the bacterial communities in lesion were found to have lower richness and diversity than those in unaffected cuticle, and SIMPER analysis and PCoA analysis revealed that bacterial communities in lesion were more divergent within the affected lobster

individuals than those in the unaffected cuticle within unaffected lobster individuals. These findings that lesion bacterial communities with less diversity (than unaffected ones) but more divergent from each other were also reported in other diseases, such as bleaching disease in red seaweed (Kumar et al., 2016).

SIMPER and LEfSe analyses have been used for identifying the disease associated OTUs have been used in some other disease studies (Luter et al., 2017; Wagner Mackenzie et al., 2017). In this study, a group of 21 TFN closely associated OTUs were commonly identified from the two analyses, six of which were commonly identified as the top six important OTUs associated with TFN in both analyse, i.e., *Aquimarina*, *Flavobacterium*, *Neptunomonas*, *Streptomyces*, unclassified Flavobacteriaceae, unclassified Thiohalorhabdales. *Aquimarina* is reported to be associated with different types of shell disease in American lobsters (Chistoserdov et al., 2012; Quinn et al., 2013a; Quinn et al., 2012b; Whitten et al., 2014), but it has not been reported in spiny lobsters with TFN. Likewise, *Flavobacterium*, *Neptunomonas* and *Streptomyces* were seldom reported in the marine lobsters with shell disease (including TFN). Rhodobacteraceae (seven OTUs) were commonly identified from the 21 TFN closely associated phylotypes. These TFN closely associated OTUs may possibly played different roles in the lesion formation.

The TFN closely associated *Aquimarina* may possibly hydrolyse some types of lipid and protein (Whitman, 2015), which could be associated with the biofilm formation or possible degradation of cuticle in the marine lobsters (Zha et al., 2018a; Zha et al., 2018b). *Streptomyces* and *Neptunomonas* was found to produce various antibiotics and as potential probiotic bacteria (Kesarcodi-Watson et al., 2012; Niu et al., 2011). However, they were possibly associated with infection in aquatic organisms (Buller, 2014), such as spiny lobsters. For example, *Streptomyces* was reported to produce a variety of pigments including black and dark colours (Abdel-Haliem et al., 2013; Whitman, 2015), which may be associated with the

dark colour of the tail fan lesions. *Flavobacterium* have been identified in diverse biofilm structures (Basson et al., 2008) and found to be pathogenic to various aquatic organisms (Buller, 2014), which makes it possibly access the tail fan cuticle of spiny lobsters (Madsen et al., 2005). *Vibrio* and *Ruegeria* were found to be closely associated with TFN in this study, which is consistent with our previous studies demonstrating bacterial isolates from tail fan lesions belonging to some *Vibrio* and *Ruegeria* species commonly had all or majority of proteolytic, chitinolytic, lipolytic activities, biofilm formation, production of siderophore and melanin (Zha et al., 2018a; Zha et al., 2018b).

PAM clustering results implied the TFN lesions might experience different "states". It is difficult to determine the detailed "states" of TFN lesions, even if the actual appearances of TFN lesions were recorded (which was not conducted in this study). The lesion states could be the severity (mild and severe), progressing extent (less progressing and more progressing) or development stages (early and late stages), or a mixture.

Multiple hypotheses exist relating bacteria to lesion progression in shell disease, i.e., impaired host immune response, changed conditions caused by cuticle disruption, potential intraspecific suppression, dominance by pathogenic bacteria and potential bacterial virulent and enzymatic factors (Chistoserdov et al., 2012; Chistoserdov et al., 2005; Davies et al., 2014b; Feinman et al., 2017; Geddes et al., 2004; Meres et al., 2012; Musgrove et al., 2005; Quinn et al., 2013a; Quinn et al., 2012b; Shields, 2011; Shields, 2012; Shields et al., 2012; Smolowitz et al., 2014; Whitten et al., 2014; Zha et al., 2018a; Zha et al., 2018b; Zha et al., 2018d; Zha et al., 2017). We assumed the different hypotheses were possibly to exist in the lesion development, and the results in this study could provide some evidence for some of the bacterial hypotheses by conducting this study, such as intraspecific suppression, dominance by some opportunistic bacteria.

A group of nine TFN closely associated bacteria were commonly found in glove and pot samples, while the majority of other TFN closely associated ones were also found in both sample types. These facts suggested that glove and pot may be contaminated or possibly play a role in the transmission of TFN associated bacteria.

7.5. Conclusion

In conclusion, we found the bacterial communities in lesion were different from those in unaffected cuticle. A group of 21 OTUs were closely associated with TFN in *J. edwardsii*, six of which (i.e., *Aquimarina*, *Flavobacterium*, *Neptunomonas*, *Streptomyces*, unclassified Flavobacteriaceae, unclassified Thiohalorhabdales) were the most important ones associated with TFN. Gloves and pots used by commercial fishers are likely to be contaminated through the handling of affected lobsters and may also be a transmission agent of TFN associated bacteria among lobsters.

8. Chapter Eight - General Discussion

8.1. Overview

TFN significantly decreases the market value of spiny lobster (*Jasus edwardsii*) in New Zealand and Australia, and relates to the health of affected spiny lobsters. The condition may also spread more widely among the lobster population raising further concerns. Limited information has been available on this condition (Freeman & MacDiarmid, 2009; Musgrove et al., 2005), with the identity of the causative organisms and the potential influence of TFN on the immune status of lobsters being largely unknown. The overall aim of the research presented in this thesis was to improve our understanding of TFN in spiny lobsters, especially *Jasus edwardsii* in New Zealand. Addressing this research aim involved five specific research objectives which have been presented in individual chapters in this thesis, i.e.,

• Chapter Three mainly investigated the immune status of TFN-affected spiny lobsters (*J. edwardsii*).

• Chapter Four mainly examined whether bacterial proteolytic, chitinolytic and lipolytic activities are associated with TFN in *J. edwardsii*.

• Chapter Five mainly determined whether the virulence factors of bacteria were associated with TFN in *J. edwardsii*.

• Chapter Six investigated the pathology of affected tail fan and four selected internal organs in affected *J. edwardsii*.

• Chapter Seven investigated the TFN associated bacterial community and some abundant potential causative bacteria.

8.2. Key findings from this study

Six immune parameters were measured to assess the immune status of TFN-affected lobsters and unaffected ones, i.e., total haemocyte count (THC), haemocyte viability, total protein concentrations in plasma and haemocyte lysate supernatant (HLS), phenoloxidase (PO) activity in HLS and haemolymph bacterial count. The affected lobsters were found to have an impaired immunity, i.e., less THC and lower PO activity than unaffected lobsters.

Morphological features of affected lobsters were also recorded and analysed, and the affected lobsters were found to have more missing or damaged appendages (i.e., fewer antennae, pereiopods, pleopods). TFN was more extensive in endopod than exopod and telson, while the ventral area of tail fan affected with TFN was greater than the dorsal affected area. These results suggested that TFN either resulted in the compromised immune status of affected lobsters, or was facilitated by pre-existing reduced immunity.

Proteolytic, chitinolytic and lipolytic activities of bacteria from tail fan lesions and haemolymph of TFN-affected lobsters were assessed and compared with those from tail fan cuticle and haemolymph of unaffected lobsters (1678 bacteria successfully isolated in total). Chitinolytic bacterial isolates were more common on the tail fans and in the haemolymph of lobsters with TFN compared with those without TFN ($P < 0.001$), and the overall chitinolytic activity of bacteria present in lobsters with TFN was greater than in lobsters without TFN ($P < 0.001$). The bacterial isolates exhibiting all of proteolytic, lipolytic and chitinolytic activities were more prevalent on the tail fans and in the haemolymph of lobsters with TFN versus those in unaffected lobsters ($P < 0.001$). Some of the results, such as a similar overall enzymatic profile between tail fan bacteria and haemolymph bacteria of affected lobsters, and a significantly different enzymatic profile between tail fan bacteria and haemolymph bacteria of unaffected lobsters, suggest the possibility that tail fan bacteria may enter the haemolymph in the affected lobsters. Molecular methods identified 14 bacterial species from 35 isolates possessing this broad enzymatic profile associated with TFN, suggesting that not just one bacterial species is involved in TFN. Collectively these results suggest a group of bacteria with combined proteolytic, lipolytic and especially chitinolytic activity were associated with TFN in lobsters, possibly degrading the lobster cuticle or taking advantage of the cuticle nutrients for colonization.

In order to examine whether there are some more bacterial characteristics that are associated with TFN, a group of bacteria exhibiting proteolytic, chitinolytic and lipolytic activities (thereafter referred to PCL+ bacteria) were isolated from unaffected lobsters and identified. Additional characteristics of PCL+ bacteria from affected lobsters and unaffected lobsters were measured and compared. These characteristics included the morphology, biofilm formation, antimicrobial resistance, antimicrobial activity, production of siderophores, melanin and ammonia. Biofilm-formation was greater in PCL+ bacteria from both haemolymph and tail fan lesions of affected lobsters compared to those unaffected ones, while production of melanin and siderophores were both greater in the tail fan bacteria of

TFN-affected lobsters. By contrast, the other characteristics were similar in lobsters with and without TFN. The most common *V. crassostreae* PCL+ isolates from the affected lobsters had similar genomic patterns, while their genomic patterns were different in unaffected lobsters. These findings suggest that in addition to the bacterial enzymatic activities, some virulence factors of bacteria are also associated with TFN, i.e. biofilm formation, melanin and siderophore production.

The pathology of TFN-affected lobsters was investigated by gross observation and histopathology methods of tail fans, as well as four selected internal organs, i.e., mid-gut, hepatopancreas, heart and gill. TFN progression in the tail fan was characterised initially by caseous responses and haemocyte accumulation, followed by the spread of these responses together with melanisation. Additional pathological changes to the tail fans included pseudomembrane formation, detachment of epidermis or cuticle, clotted haemolymph and fibrosis. The establishment of the condition at points of injury is consistent with the penetration of TFN through the cuticle and tissue layers of the affected tail fans, which is rarely seen in other forms of shell disease. Among internal organs, pathological changes were found in a total of two mid-gut, four heart and two gill samples from eight lobsters with TFN, while no suspected changes were found in the organs of lobsters without TFN (Chi-square test, $P < 0.034$). These results suggest that TFN has different pathological features at different stages and the development of this condition is not limited to within the tail fan cuticle. A suite of bacteria may be associated with TFN, including some potentially invasive bacteria which may either activate the host immune response, or infect the lobsters further. These results suggest that TFN could induce different pathological changes in the affected tail fans, while pathological changes in the internal organs of affected lobsters also appeared to be associated with TFN.

The bacterial communities in tail fan lesions of TFN-affected lobsters, tail fan cuticle of unaffected lobsters, commercial fishers' gloves and lobster pots were investigated by 16S Illumina sequencing. Illumina sequencing of the 16S rDNA revealed that 3559 bacterial operational taxonomic units (OTUs) (99.2% abundance of the two types of samples) were found in all the affected lesion and unaffected cuticle samples. The tail fan lesions had a relatively distinct bacterial community and some abundant diseased core OTUs (i.e. *Aquimarina*) were part of the top largest contributors to the differences of bacterial communities in lesion versus unaffected cuticles. The bacterial community in TFN lesions had lower richness and diversity than that in equivalent samples from unaffected lobster

cuticle. Some of affected and unaffected bacterial communities were similar, suggesting a transition process in TFN affection, and different bacteria were found as potential indicators of different status of TFN progression. The TFN associated OTUs were also found in glove and pot samples, suggesting glove and pots may be contaminated by TFN-affected lobsters, or as potential transmission agents of the TFN-associated bacteria. The relevant results suggest that a group of bacteria are associated with TFN, and they were likely involved in different status of TFN in spiny lobsters.

8.3. Conclusion

The research results presented in this thesis demonstrated the impaired immune status and pathology associated with TFN in *Jasus edwardsii*, and also found the bacteria and bacterial characteristics associated with this condition. These results provide valuable information for further advancing the study of TFN and some new insights in the field of shell disease in crustaceans.

In the future, it is suggested that studies of shell disease could also focus on the bacterial activities. The results about the TFN-associated bacterial characteristics in this thesis were different from those of previous studies. For example, chitinolytic bacteria were found to be associated with shell disease (Mancuso et al., 2010; Tlusty et al., 2007; Vogan et al., 2002), however, the chitinolytic bacteria usually also have some other characteristics which we could not exclude for causing shell disease. We chose to characterise and compare the extent and presence of chitinolytic activity of bacteria isolated from affected versus unaffected tail fans, and found significant greater chitinolytic activity in the bacteria associated with TFN lesions. This strategy needs large amount of work and has seldom been used previously. Likewise, the other bacterial characteristics such as biofilm formation, production of melanin and siderophores have seldom been studied previously, but the relevant results could also provide some new sights for some further work in shell disease.

Understanding the initiation and progression of shell disease in crustaceans is challenging, because of the range of environmental variables that may be involved in influencing shell disease, and the diversity and variability in the bacterial flora associated with the cuticle surfaces of crustaceans. While methodologies for overcoming some of these complexities are improving, such as the recent emergence of advanced molecular genetic tools, it remains

challenging to describe the bacterial processes involved in shell disease. For this reason, the current study adopted two fundamental approaches to examining the role of bacteria and bacterial characteristics in TFN, i.e., culture-based methods and next-generation molecular sequencing of bacterial communities. Culture-based methods are limited by the culturability of bacteria, however, culture methods enable detailed characterisation of properties of individual bacteria which is difficult or impossible to achieve by other means. Take chitinolytic activity as an example, gene expression work on chitinolytic activity needs a comprehensive understanding of chitinolytic genes (Beier & Bertilsson, 2013). The current knowledge can provide some chitinase related genes, but not the genes relating to some other materials which could also use or degrade chitin (Bai et al., 2016). So in this case culture based methods are chosen for investigating the bacterial characteristics associated with TFN, and demonstrated the identities of some representative bacteria. *Vibrio crassostreae* was found to be a relatively common bacteria with proteolytic, chitinolytic and lipolytic activities, but it may not be the most abundant bacteria found in association with TFN, which is confirmed in the study by Illumina sequencing (i.e., *Aquimarina* sp.). It is reasonable to have different "key taxonomies" in culture -dependent versus -independent, as the TFN associated bacteria identified from culture-dependent study (Chapter Four) had the three enzymatic activities, while the TFN associated bacteria identified from culture-independent study (Chapter Seven) were those could distinguish the bacterial communities in lesion from unaffected cuticle. Future investigations should determine the roles of bacteria without the specific characteristics but identified to be associated with the lesion by Illumina sequencing.

8.4. Future research direction

8.4.1. Potential mechanisms of TFN

Some processes of TFN progression can be assumed based on the results. Bacteria associated with TFN in tail fan could take advantage of protein, chitin and lipid in the tail fan tissue; form biofilms to colonise in tail fan; produce siderophores and melanin to resist other normal bacteria for better incursion and form black materials in the lesions. However, some alternative processes in the progression and origins of the bacteria in lobster lesions still need to be further investigated.

The first hypothesis is some normal bacteria belonging to the normal bacterial flora on the tail fans of lobsters may become pathogenic after colonising new nutrient sources (i.e., exposed chitin, haemolymph clot and underlying tail fan tissue caused by damage to the cuticle). The second hypothesis is that TFN associated bacteria were attracted by the nutrient sources in the tail fan, and then progress to the development of TFN. The third hypothesis is that bacteria are selected by the host antibacterial activity (i.e., melanisation), during which some bacteria may be eliminated while some others may take advantage of the antibacterial materials (i.e., host induced melanin and antimicrobial peptides). The fourth hypothesis is that bacteria associated with TFN may be transferred from some transmission agents (i.e., glove and pot) (Zha et al., 2018c). Currently there is a lack of evidence sufficiently supporting these four hypotheses, but it is worthwhile to investigate them further.

8.4.2. Future research questions

The blackened appearance is always thought to be caused by host melanisation, but bacterial melanin production may possibly contribute to the black cuticle/lesion. It is worthwhile to further explore how some TFN associated bacteria possibly take advantage of tyrosine or relevant materials (which play important roles in host melanisation), or similar materials in the environment. Also, some bacteria themselves may become black/dark (not require tyrosine to form melanin). In our study we did not find many black bacteria (within 10 isolates) grown in the culture media during the large amount of bacteria isolation (over 2400 bacteria isolated initially, with 14 days for each of the three purifications). However, we did find some bacteria become brown/dark after culture for some time (typically after 14 days, which is consistent with the chronic TFN). These bacteria could be studied in future research.

8.4.3. Potential prevention and treatment of TFN

8.4.3.1. Prevention of injury

Injury is an important factor associated with shell disease, including TFN. Different fishing activities have been found to cause injuries to lobster cuticle, such as fishing by trap, trawl and freediving (Leland et al., 2013; Powrie & Tempero, 2009). Preventative measures for the alleviation of injury to lobster cuticle have been developed, such as bagging spiny lobster with fine-mesh nylon bags when handling and transporting (Musgrove et al., 2005). Improved capture methods that reduce injuries to lobsters need to be developed. For example,

more effective escape gaps that allow undersized lobsters to leave the pots so they are not repeatedly handled (Linnane et al., 2011), and hand collection of lobsters should be decreased (Leland et al., 2013).

8.4.3.2. Potential preventative measures for transmission

The severely affected spiny lobsters are less likely to recover by moult, and may possibly transfer the TFN associated bacteria to unaffected lobsters. Therefore, it is suggested to conduct a transmission experiment to confirm this situation. If the situation is true, then a removal of the severely affected lobsters may help alleviate the TFN in wild spiny lobster populations. It is also recommended to disinfect the fishing facilities (such as glove and pot), especially those have collected TFN-affected lobsters (Zha et al., 2018c).

8.4.3.3. Potential measures for treatment

TFN-affected lobsters were reported to be able to recover by moult (May, 2007), however, the large amount of melanisation is very likely to cause difficulty with the moult and subsequent death. Therefore, to cut the TFN-affected lesions and add Vaseline to the disinfected damaged cuticle could possibly alleviate the situation. But whether Vaseline has negative effect on lobster or strong attraction of pathogenic bacteria need to be examined first.

8.4.4. Connection of TFN with other shell diseases

Shell disease associated bacterial species could be variable in different crustacean species, and this may be true in different lobster species (Cook & Lofton, 1973; Hernandez-Divers et al., 2009; Huchette et al., 2006; Wang, 2011). It was found a group of TFN associated bacteria in TFN lesions by using next generation sequencing in this study, especially *Aquimarina*, which were usually regarded as the causative bacteria for shell disease in American lobsters (Meres et al., 2012; Quinn et al., 2012a; Quinn et al., 2012b; Whitten et al., 2014). While it is possible that some bacteria may be commonly associated with shell disease in some marine lobster species, variable bacterial communities are likely to play different roles in lobsters with different etiologies of shell disease (Chistoserdov et al., 2012; Feinman et al., 2017; Meres et al., 2012; Quinn et al., 2013a; Whitten et al., 2014; Zha et al., 2018c).

Appendices

Appendix A

Detailed information of PCL+ (positive proteolytic, chitinolytic and lipolytic capabilities) bacterial isolates from spiny lobsters (*Jasus edwardsii*) with and without tail fan necrosis

Isolate	Species ID	Sou	Gra	Mot	Sha	Mel	Amm	Bio	Sid	Anti
PCL+ bacteria from unaffected lobsters										
H29T6	*Vibrio cyclitrophicus*	TF	-	+	rod	++	++	0.04 ± 0.001	-	P
H20B1	*Vibrio crassostreae*	Ham	-	+	curved rod	+	++	0.06 ± 0.01	++	E
H29T9	*Vibrio atlanticus*	TF	-	+	rod	++	++	0.07± 0.01	+	P
H17T17	*Vibrio crassostreae*	TF	-	+	curved rod	+	++	0.04 ± 0.003	++	P
H17T18	*Vibrio crassostreae*	TF	-	+	curved rod	+	++	0.05 ± 0.001	++	E
H29T4	*Vibrio crassostreae*	TF	-	+	curved rod	++	++	0.07 ± 0.01	-	E
H29B6	*Vibrio crassostreae*	Ham	-	+	curved rod	++	++	0.05 ± 0.002	-	P
H38T10	*Vibrio crassostreae*	TF	-	+	curved rod	+w	++	0.07 ± 0.01	++	P
H11T17	*Vibrio kanaloae*	TF	-	+	rod	+	++	0.14 ± 0.01	-	P
H36T3	*Photobacterium sanguinicacri*	TF	-	+	short rod	-	++	0.09 ± 0.01	+	E
H36B21	*Vibrio kanaloae*	Ham	-	+	rod	-	++	0.06 ± 0.004	+++	P
H36B23	*Vibrio crassostreae*	Ham	-	+	curved rod	++	++	0.08 ± 0.004	++	P
H38T5	*Vibrio crassostreae*	TF	-	+	curved rod	+	++	0.09 ± 0.003	++	P
H11T10	*Vibrio crassostreae*	TF	-	+	curved rod	+	++	0.07 ± 0.001	++	P
H17B2	*Vibrio crassostreae*	Ham	-	+	curved rod	+	++	0.07 ± 0.005	++	P

H38B16	*Vitellibacter vladivostokensis*	Ham	-	-	rod	++	++	0.16 ± 0.01	-	P
H36B15	*Pseudoalteromonas issachenkonii*	Ham	-	+	rod	+	++	0.29 ± 0.03	-	E
H11T16	*Vibrio toranzoniae*	TF	-	+	rod	++	+	0.29 ± 0.03	-	E
H38T15	*Vibrio atlanticus*	TF	-	+	rod	+++	++	0.07 ± 0.002	++	E
H18T20	*Photobacterium swingsii*	TF	-	+	short rod	-	+	0.07 ± 0.002	-	E
H18T19	*Vibrio splendidus*	TF	-	+	rod	++	++	0.15 ± 0.02	-	N
H11T2	*Vibrio atlanticus*	TF	-	+	rod	++	++	0.08 ± 0.01	++	E
H36T2	*Vibrio pomeroyi*	TF	-	+	rod	+	++	0.07 ± 0.01	-	P
H29T2	*Pseudoalteromonas haloplanktis*	TF	-	+	rod	-	++	0.08 ± 0.001	-	P
H18T6	*Vibrio tasmaniensis*	TF	-	+	rod	-	++	0.05 ± 0.002	-	P
H18T3	*Photobacterium sanguinicacri*	TF	-	+	short rod	-	+	0.17 ± 0.02	-	E
H28T8	*Photobacterium swingsii*	TF	-	+	short rod	-	++	0.10 ± 0.005	-	E
H28T7	*Pseudoalteromonas espejiana*	TF	-	+	rod	+w	++	0.35 ± 0.05	-	E
H0B2	*Vibrio tapetis*	Ham	-	+	rod	+w	+	0.08 ± 0.002	-	E
H0B3	*Vibrio tapetis*	Ham	-	+	rod	+w	+	0.12 ± 0.01	-	E
H0B4	*Vibrio tapetis*	Ham	-	+	rod	+w	+	0.08 ± 0.01	-	E
H19T6	*Paracoccus rhizosphaerae*	TF	-	+	cocci	-	+	0.06 ± 0.003	-	E
H19T12	*Sulfitobacter delicatus*	TF	-	-	rod	-	++	0.28 ± 0.03	-	E

H19T3	*Vitellibacter vladivostokensis*	TF	-	-	rod	-	++	0.04 ± 0.004	-	E
PCL+ bacteria from TFN-affected lobsters										
T24B11	*Vibrio splendidus*	Ham	-	+	rod	++	++	0.18 ± 0.01	-	P
T12B1	*Shewanella vesiculosa*	Ham	-	+	rod	+w	++	0.11 ± 0.01	++	E
H5DT3	*Vibrio crassostreae*	TF	-	+	curved rod	+w	++	0.18 ± 0.01	+++	P
H5DB5	*Vibrio crassostreae*	Ham	-	+	curved rod	++	++	0.28 ± 0.06	+++	P
T12B6	*Vibrio gigantis*	Ham	-	+	curved rod	+w	++	0.26 ± 0.02	+++	P
T22B7	*Vibrio gigantis*	Ham	-	+	curved rod	++	++	0.33 ± 0.03	+++	E
T9B13	*Pseudoalteromonas spiralis*	Ham	-	+	spiral	+	++	0.95 ± 0.03	+++	E
T24T3	*Vibrio atlanticus*	TF	-	+	rod	+++	++	0.15 ± 0.001	+++	E
T36T14	*Vibrio atlanticus*	TF	-	+	rod	++	++	0.17 ± 0.01	++	E
T36T15	*Photobacterium swingsii*	TF	-	+	cocci	-	+	0.12 ± 0.01	-	E
T22B17	*Vibrio gigantis*	Ham	-	+	curved rod	++	++	0.39 ± 0.01	++	P
T10T15	*Vibrio crassostreae*	TF	-	+	curved rod	+	++	0.22 ± 0.04	++	P
T24T4	*Vibrio atlanticus*	TF	-	+	rod	++	++	0.16 ± 0.01	++	E
T36T12	*Vibrio atlanticus*	TF	-	+	rod	++	++	0.14 ± 0.02	+++	E
T22T19	*Aliivibrio sifiae*	TF	-	+	coccoid-rod	-	+	0.14 ± 0.01	++	P
T12T8	*Vibrio crassostreae*	TF	-	+	curved rod	+++	++	0.21 ± 0.01	-	E
T12T7	*Vibrio crassostreae*	TF	-	+	curved rod	+++	++	0.16 ± 0.01	++	E

T12T12	*Vibrio crassostreae*	TF	-	+	curved rod	++	++	0.37 ± 0.003	++	P
T36T8	*Vibrio atlanticus*	TF	-	+	rod	++	+	0.18 ± 0.01	+	E
T22B21	*Vibrio crassostreae*	Ham	-	+	curved rod	++	++	0.10 ± 0.004	-	E
T9T15	*Vibrio crassostreae*	TF	-	+	curved rod	++	+	0.14 ± 0.02	+++	E
T12T10	*Vibrio crassostreae*	TF	-	+	curved rod	+	++	0.16 ± 0.02	+++	P
T24B22	*Vibrio crassostreae*	Ham	-	+	curved rod	+++	+	0.29 ± 0.03	+++	E
T9T14	*Vibrio atlanticus*	TF	-	+	rod	+	+	0.06 ± 0.002	-	E
T24B9	*Photobacterium swingsii*	Ham	-	+	cocci	-	++	0.11 ± 0.01	-	P
T36B4	*Vibrio crassostreae*	Ham	-	+	curved rod	+	++	0.42 ± 0.03	+++	P
T22B19	*Vibrio crassostreae*	Ham	-	+	curved rod	+	++	0.51 ± 0.03	+++	P
T22T20	*Vibrio cyclitrophicus*	TF	-	+	rod	++	++	0.90 ± 0.08	-	E
T9T5	*Vibrio crassostreae*	TF	-	+	curved rod	+	++	0.26 ± 0.03	-	P
T12B14	*Photobacterium lutimalis*	Ham	-	+	short rod	+	++	0.20 ± 0.02	-	P
T12B5	*Vibrio toranzoniae*	Ham	-	+	rod	+	++	0.10 ± 0.01	-	P
T12B22	*Stenotrophomonas rhizophila*	Ham	-	+	rod	++	++	0.12 ± 0.01	+	P
T9B5	*Photobacterium sanguinicancri*	Ham	-	+	cocci	-	++	0.32 ± 0.04	+	E
T24T14	*Vibrio cyclitrophicus*	TF	-	+	rod	++	++	0.25 ± 0.01	-	P
H5DT17	*Ruegeria pelagia*	TF	-	+	rod	+	++	0.41 ± 0.055	-	P

Note: Sou = source, Ham = haemolymph, TF = tail fan; Gra = gram reaction; Mot = motility; Sha = bacterial shape; Mel = ability to produce melanin, +w = weak positive (Rank = 0.5); Amm = ability to produce ammonia; Bio = ability to form biofilms; Sid = ability to produce siderophores; Anti = the ability to surmount antimicrobial activity in tail fan extract, “P” means bacterial growth were promoted by extract, “E” means bacteria grew at the similar rate with and without tail fan extract, “N” means bacterial growth was inhibited by extract.

Appendix B

Thirty TFN associated OTUs among the top 100 contributors to the difference of bacterial communities between lesion and unaffected cuticle identified by SIMPER analysis

OTUs	Contribution %	Taxonomy
2	1.58	k__Bacteria; p__Bacteroidetes; c__Flavobacteriia; o__Flavobacteriales; f__Flavobacteriaceae; g__Aquimarina; s__
4	0.94	k__Bacteria; p__Actinobacteria; c__Actinobacteria; o__Actinomycetales; f__Streptomycetaceae; g__Streptomyces
5	0.54	k_Bacteria; p_Bacteroidetes; c_Flavobacteriia; o_Flavobacteriales; f_Flavobacteriaceae
14	0.5	k_Bacteria; p_Proteobacteria; c_Gammaproteobacteria; o_Oceanospirillales; f_Oceanospirillaceae; g_Neptunomonas; s_
17	0.49	k_Bacteria; p_Bacteroidetes; c_Flavobacteriia; o_Flavobacteriales; f_Flavobacteriaceae; g_Flavobacterium
18	0.47	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Thiohalorhabdales; f__; g__; s__
15	0.24	k_Bacteria; p_Bacteroidetes
28	0.23	k_Bacteria; p_Proteobacteria; c_Alphaproteobacteria; o_Rhodobacterales; f_Rhodobacteraceae; g_Ruegeria
46	0.22	k_Bacteria; p_Proteobacteria; c_Alphaproteobacteria; o_Rhodobacterales; f_Hyphomonadaceae; g_; s_
206	0.22	k_Bacteria; p_Proteobacteria; c_Epsilonproteobacteria; o_Campylobacterales; f_Campylobacteraceae; g_Arcobacter; s_
33	0.2	k_Bacteria; p_Proteobacteria; c_Alphaproteobacteria; o_Rhodobacterales; f_Rhodobacteraceae; g_Pseudoruegeria; s_
51	0.2	k_Bacteria; p_Proteobacteria; c_Alphaproteobacteria; o_Kiloniellales; f_; g_; s_
722	0.19	k_Bacteria; p_Proteobacteria; c_Alphaproteobacteria; o_Rhodobacterales; f_Rhodobacteraceae
48	0.18	k_Bacteria; p_Proteobacteria; c_Alphaproteobacteria; o_Rhodobacterales; f_Rhodobacteraceae
106	0.18	k_Bacteria; p_Proteobacteria; c_Alphaproteobacteria; o_Kiloniellales; f_Kiloniellaceae; g_; s_
74	0.16	k_Bacteria; p_Proteobacteria; c_Alphaproteobacteria; o_Rhodobacterales; f_Hyphomonadaceae

89	0.16	k_Bacteria; p_Proteobacteria; c_Gammaproteobacteria; o_Vibrionales; f_Vibrionaceae; g_Vibrio
790	0.15	k__Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__Rhodobacterales; f__Rhodobacteraceae; g__Octadecabacter; s__
80	0.15	k_Bacteria; p_Bacteroidetes; c_Flavobacteriia; o_Flavobacteriales; f_Flavobacteriaceae; g_Maribacter; s_
108	0.15	k_Bacteria; p_Proteobacteria; c_Alphaproteobacteria; o_Rhodobacterales; f_Rhodobacteraceae
57	0.15	k_Bacteria; p_Proteobacteria; c_Epsilonproteobacteria; o_Campylobacterales; f_Campylobacteraceae; g_Arcobacter; s_
49	0.14	
360	0.14	k_Bacteria; p_Proteobacteria; c_Gammaproteobacteria; o_Thiohalorhabdales; f_; g_; s_
47	0.14	k_Bacteria; p_SR1; c_; o_; f_; g_; s_
1562	0.14	k_Bacteria; p_Bacteroidetes; c_Flavobacteriia; o_Flavobacteriales; f_Flavobacteriaceae; g_Tenacibaculum; s_
105	0.14	k_Bacteria; p_Proteobacteria; c_Epsilonproteobacteria; o_Campylobacterales; f_Campylobacteraceae; g_Arcobacter; s_
220	0.14	k_Bacteria; p_Proteobacteria; c_Alphaproteobacteria; o_Rhodobacterales; f_Rhodobacteraceae; g_Loktanella; s_
69	0.14	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Alteromonadales; f__Alteromonadaceae; g__Candidatus Endobugula; s__
125	0.13	k__Bacteria; p__Tenericutes; c__Mollicutes
52	0.13	k__Bacteria; p__Bacteroidetes; c__Flavobacteriia; o__Flavobacteriales; f__Flavobacteriaceae; g__Tenacibaculum

Appendix C

A group of 23 OTUs associated with lesion identified by LEfSe analysis

OTU ID	LDA score	Taxonomy
2	4.94	k__Bacteria; p__Bacteroidetes; c__Flavobacteriia; o__Flavobacteriales; f__Flavobacteriaceae; g__Aquimarina; s__
4	4.47	k__Bacteria; p__Actinobacteria; c__Actinobacteria; o__Actinomycetales; f__Streptomycetaceae; g__Streptomyces
5	4.34	k__Bacteria; p__Bacteroidetes; c__Flavobacteriia; o__Flavobacteriales; f__Flavobacteriaceae
17	4.11	k__Bacteria; p__Bacteroidetes; c__Flavobacteriia; o__Flavobacteriales; f__Flavobacteriaceae; g__Flavobacterium
14	4.04	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Oceanospirillales; f__Oceanospirillaceae; g__Neptunomonas; s__
18	4.02	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Thiohalorhabdales; f__; g__; s__
28	3.80	k__Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__Rhodobacterales; f__Rhodobacteraceae; g__Ruegeria
46	3.57	k__Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__Rhodobacterales; f__Hyphomonadaceae; g__; s__
48	3.56	k__Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__Rhodobacterales; f__Rhodobacteraceae
206	3.52	k__Bacteria; p__Proteobacteria; c__Epsilonproteobacteria; o__Campylobacterales; f__Campylobacteraceae; g__Arcobacter; s__
89	3.49	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Vibrionales; f__Vibrionaceae; g__Vibrio
33	3.45	k__Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__Rhodobacterales; f__Rhodobacteraceae; g__Pseudoruegeria; s__
51	3.41	k__Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__Kiloniellales; f__; g__; s__
74	3.36	k__Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__Rhodobacterales; f__Hyphomonadaceae
57	3.33	k__Bacteria; p__Proteobacteria; c__Epsilonproteobacteria; o__Campylobacterales; f__Campylobacteraceae; g__Arcobacter; s__
220	3.32	k__Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__Rhodobacterales; f__Rhodobacteraceae; g__Loktanella; s__
722	3.31	k__Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__Rhodobacterales; f__Rhodobacteraceae

106	3.16	k__Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__Kiloniellales; f__Kiloniellaceae; g__; s__
108	3.14	k__Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__Rhodobacterales; f__Rhodobacteraceae
52	3.12	k__Bacteria; p__Bacteroidetes; c__Flavobacteriia; o__Flavobacteriales; f__Flavobacteriaceae; g__Tenacibaculum
63	3.08	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Alteromonadales; f__Alteromonadaceae
790	3.04	k__Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__Rhodobacterales; f__Rhodobacteraceae; g__Octadecabacter; s__
87	3.03	k__Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__Rhodobacterales; f__Rhodobacteraceae

Appendix D

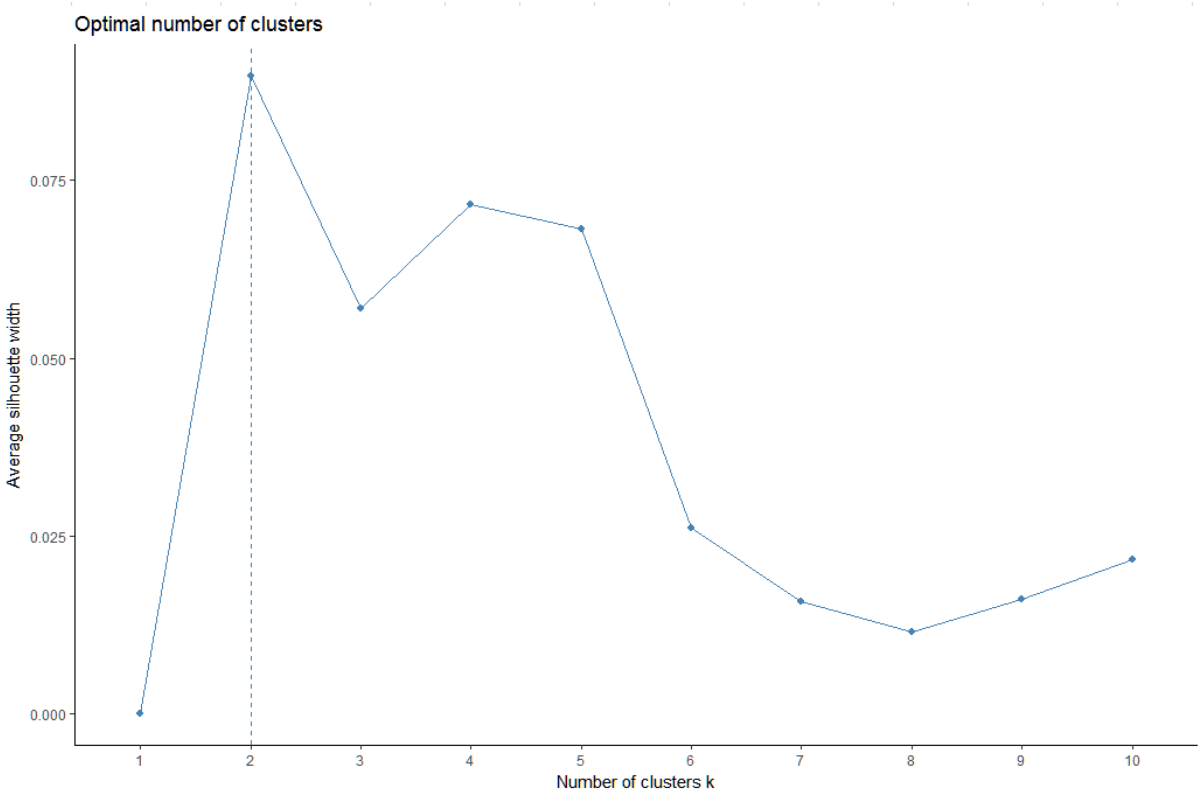
Taxonomy of 21 TFN closely associated OTUs identified by both SIMPER and LEfSe analyses

OTU ID	Taxonomy
2	k__Bacteria; p__Bacteroidetes; c__Flavobacteriia; o__Flavobacteriales; f__Flavobacteriaceae; g__Aquimarina; s__
4	k__Bacteria; p__Actinobacteria; c__Actinobacteria; o__Actinomycetales; f__Streptomycetaceae; g__Streptomyces
5	k__Bacteria; p__Bacteroidetes; c__Flavobacteriia; o__Flavobacteriales; f__Flavobacteriaceae
17	k__Bacteria; p__Bacteroidetes; c__Flavobacteriia; o__Flavobacteriales; f__Flavobacteriaceae; g__Flavobacterium
52	k__Bacteria; p__Bacteroidetes; c__Flavobacteriia; o__Flavobacteriales; f__Flavobacteriaceae; g__Tenacibaculum
51	k__Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__Kiloniellales; f__; g__; s__
106	k__Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__Kiloniellales; f__Kiloniellaceae; g__; s__
74	k__Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__Rhodobacterales; f__Hyphomonadaceae
46	k__Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__Rhodobacterales; f__Hyphomonadaceae; g__; s__
722	k__Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__Rhodobacterales; f__Rhodobacteraceae
48	k__Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__Rhodobacterales; f__Rhodobacteraceae
108	k__Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__Rhodobacterales; f__Rhodobacteraceae
220	k__Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__Rhodobacterales; f__Rhodobacteraceae; g__Loktanella; s__
790	k__Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__Rhodobacterales; f__Rhodobacteraceae; g__Octadecabacter; s__
33	k__Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__Rhodobacterales; f__Rhodobacteraceae; g__Pseudoruegeria; s__
28	k__Bacteria; p__Proteobacteria; c__Alphaproteobacteria; o__Rhodobacterales; f__Rhodobacteraceae; g__Ruegeria

206	k__Bacteria; p__Proteobacteria; c__Epsilonproteobacteria; o__Campylobacterales; f__Campylobacteraceae; g__Arcobacter; s__
57	k__Bacteria; p__Proteobacteria; c__Epsilonproteobacteria; o__Campylobacterales; f__Campylobacteraceae; g__Arcobacter; s__
14	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Oceanospirillales; f__Oceanospirillaceae; g__Neptunomonas; s__
18	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Thiohalorhabdales; f__; g__; s__
89	k__Bacteria; p__Proteobacteria; c__Gammaproteobacteria; o__Vibrionales; f__Vibrionaceae; g__Vibrio

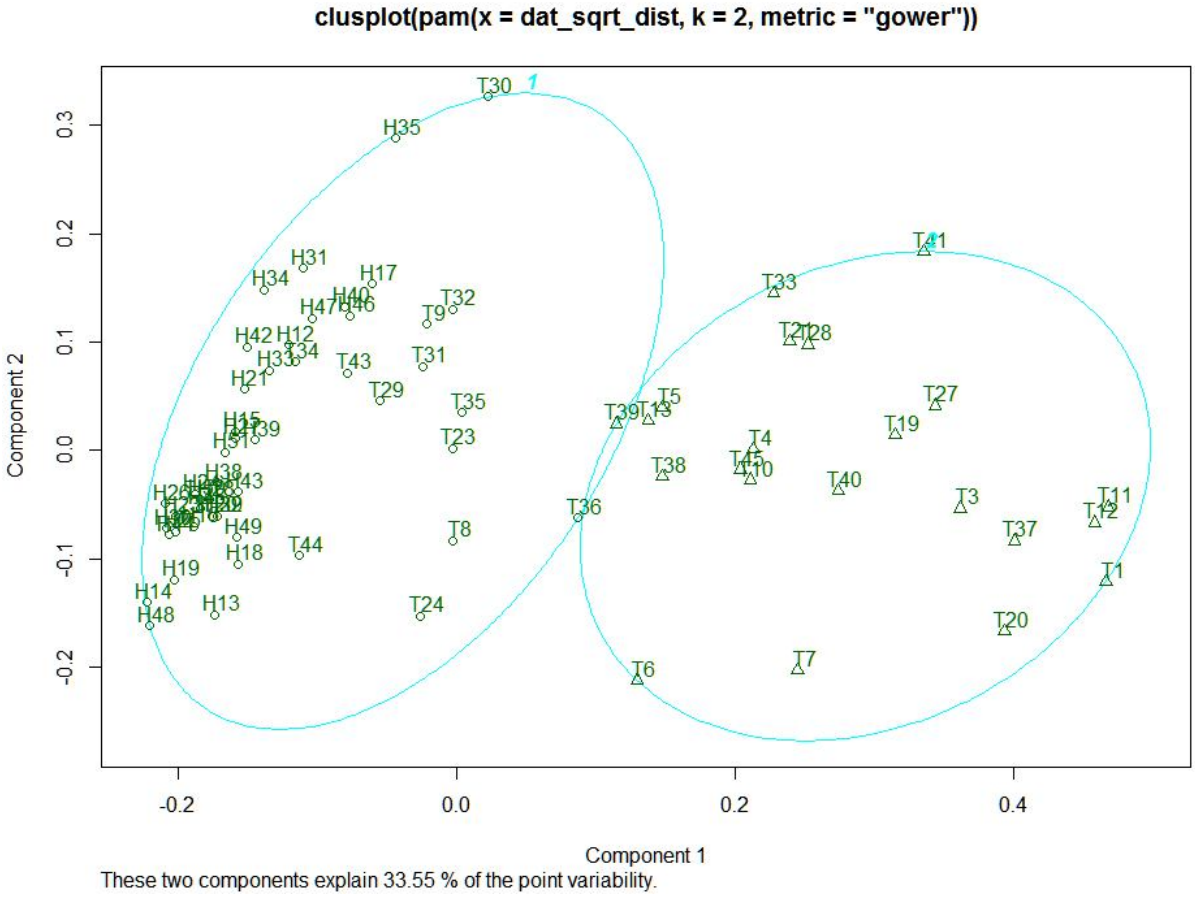
Appendix E

Silhouette analysis for identifying optimal cluster number of the bacterial communities in all the lobster tail fan samples



Appendix F

Partition around medoids (PAM) clustering algorithm for clustering the bacterial communities in all the tail fan samples based on the Bray-Curtis dissimilarity metric



References

Abdel-Haliem, M., Sakr, A., Ali, M., Ghaly, M. & Sohlenkamp, C. (2013). Characterization of *Streptomyces* isolates causing colour changes of mural paintings in ancient Egyptian tombs. *Microbiological Research, 168*, 428-437.

Ahmad, F., Ahmad, I. & Khan, M. (2008). Screening of free-living rhizospheric bacteria for their multiple plant growth promoting activities. *Microbiological Research, 163*, 173-181.

Allewell, N.M. (2016). Introduction to biofilms thematic minireview series. *Journal of Biological Chemistry, 291*, 12527-12528.

Amparyup, P., Charoensapsri, W. & Tassanakajon, A. (2013). Prophenoloxidase system and its role in shrimp immune responses against major pathogens. *Fish & Shellfish Immunology, 34*, 990-1001.

Arias-Barrau, E., Olivera, E.R., Luengo, J.M., Fernández, C., Galán, B., García, J.L., Díaz, E. & Minambres, B. (2004). The homogentisate pathway: a central catabolic pathway involved in the degradation of L-phenylalanine, L-tyrosine, and 3-hydroxyphenylacetate in *Pseudomonas putida*. *Journal of Bacteriology, 186*, 5062-5077.

Ayres, P. & Edwards, E. (1982). Notes on the distribution of “black spot” shell disease in crustacean fisheries. *Chemistry in Ecology, 1*, 125-130.

Bachman, M.A., Miller, V.L. & Weiser, J.N. (2009). Mucosal lipocalin 2 has pro-inflammatory and iron-sequestering effects in response to bacterial enterobactin. *PLoS Pathogens, 5*, e1000622.

Bai, Y., Eijsink, V.G., Kielak, A.M., Veen, J.A. & Boer, W. (2016). Genomic comparison of chitinolytic enzyme systems from terrestrial and aquatic bacteria. *Environmental Microbiology, 18*, 38-49.

Banerjee, A., Supakar, S. & Banerjee, R. (2014). Melanin from the nitrogen-fixing bacterium *Azotobacter chroococcum*: a spectroscopic characterization. *PloS ONE, 9*, e84574.

Baross, J.A., Tester, P.A. & Morita, R.Y. (1978). Incidence, microscopy, and etiology of exoskeleton lesions in the tanner crab, *Chionoecetes tanneri*. *Journal of the Fisheries Board of Canada, 35*, 1141-1149.

Bartlett, S., Wooster, G., Sokolowski, M., Dove, A. & Bowser, P. (2008). Naturally occurring bacteraemia in American lobsters, *Homarus americanus* Milne-Edwards, in Long Island Sound. *Journal of Fish Diseases, 31*, 19-25.

Basson, A., Flemming, L. & Chenia, H. (2008). Evaluation of adherence, hydrophobicity, aggregation, and biofilm development of *Flavobacterium johnsoniae*-like isolates. *Microbial Ecology, 55*, 1-14.

Battison, A., MacMillan, R., MacKenzie, A., Rose, P., Cawthorn, R. & Horney, B. (2000). Use of injectable potassium chloride for euthanasia of American lobsters (*Homarus americanus*). *Comparative Medicine, 50*, 545-550.

Becker, D.J., Streicker, D.G. & Altizer, S. (2015). Linking anthropogenic resources to wildlife-pathogen dynamics: a review and meta-analysis. *Ecology Letters, 18*, 483-495.

Behringer, D., Butler, M. & Shields, J. (2008). Effect of PaV1 infection on Caribbean spiny lobster (*Panulirus argus*) movement, condition, and survival. *Journal of Experimental Marine Biology and Ecology 359*, 26-33.

Behringer, D.C., Butler, M.J. & Shields, J.D. (2006). Ecology: avoidance of disease by social lobsters. *Nature, 441*, 421.

Behringer, D.C., Butler, M.J., Shields, J.D. & Moss, J. (2011). Review of *Panulirus argus* virus 1 - a decade after its discovery. *Diseases of Aquatic Organisms, 94*, 153-160.

Behringer, D.C., Butler, M.J. & Stentiford, G.D. (2012). Disease effects on lobster fisheries, ecology, and culture: overview of DAO Special 6. *Diseases of Aquatic Organisms, 100*, 89-93.

Beier, S. & Bertilsson, S. (2013). Bacterial chitin degradation - mechanisms and ecophysiological strategies. *Frontiers in Microbiology, 4*, 149.

Bensadoun, E.S., Burke, A.K., Hogg, J.C. & Roberts, C.R. (1996). Proteoglycan deposition in pulmonary fibrosis. *American Journal of Respiratory and Critical Care Medicine, 154*, 1819-1828.

Bethoney, N.D., Stokesbury, K.D., Stevens, B.G. & Altabet, M.A. (2011). Bait and the susceptibility of American lobsters *Homarus americanus* to epizootic shell disease. *Diseases of Aquatic Organisms, 95*, 1-8.

Bhadauria, V., MacLachlan, R., Pozniak, C. & Banniza, S. (2015). Candidate effectors contribute to race differentiation and virulence of the lentil anthracnose pathogen *Colletotrichum lentis*. *BMC Genomics, 16*, 628.

Bjornsdottir-Butler, K., McCarthy, S., Dunlap, P. & Benner, R. (2016). *Photobacterium angustum* and *Photobacterium kishitanii*, psychrotrophic high-level histamine-producing bacteria indigenous to tuna. *Applied and Environmental Microbiology, 82*, 2167-2176.

Blamey, L.K., Plagányi, É.E. & Branch, G.M. (2013). Modeling a regime shift in a kelp forest ecosystem caused by a lobster range expansion. *Bulletin of Marine Science, 89*, 347-375.

Breen, P.A., Starr, P.J. & Kim, S.W. 2005. A medium-term research plan for red rock lobsters (*Jasus edwardsii*). Ministry of Fisheries, Wellington, New Zealand.

Brisbin, M.M., McElroy, A.E., Espinosa, E.P. & Allam, B. (2015). Antimicrobial activity in the cuticle of the American lobster, *Homarus americanus*. *Fish & Shellfish Immunology, 44*, 542-546.

Brown, R.C. & Hopps, H.C. (1973). Staining of bacteria in tissue sections: a reliable Gram stain method. *American Journal of Clinical Pathology, 60,* 234-240.

Bryars, S.R. & Geddes, M.C. (2005). Effects of diet on the growth, survival, and condition of sea‐caged adult southern rock lobster, *Jasus edwardsii*. *New Zealand Journal of Marine and Freshwater Research, 39,* 251-262.

Buckling, A., Harrison, F., Vos, M., Brockhurst, M.A., Gardner, A., West, S.A. & Griffin, A. (2007). Siderophore-mediated cooperation and virulence in *Pseudomonas aeruginosa*. *FEMS Microbiology Ecology, 62,* 135-141.

Buller, N.B. (2014). Bacteria and fungi from fish and other aquatic animals: a practical identification manual, Cabi.

Butler, C.B. & Matthews, T.R. (2015). Effects of ghost fishing lobster traps in the Florida Keys. *ICES Journal of Marine Science, 72,* i185-i198.

Campos, A., Fonseca, P., Pilar-Fonseca, T., Leocádio, A.M. & Castro, M. (2015). Survival of trawl-caught Norway lobster (*Nephrops norvegicus* L.) after capture and release—Potential effect of codend mesh type on survival. *Fisheries Research, 172,* 415-422.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Pena, A.G., Goodrich, J.K. & Gordon, J.I. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nature Methods, 7,* 335.

Carvalho, P. & Phan, V. (1997). Oxygen consumption and ammonia excretion of *Xiphopenaeus kroyeri* Heller (Penaeidae) in relation to mass temperature and experimental procedures Shrimp oxygen uptake and ammonia excretion. *Journal of Experimental Marine Biology and Ecology, 209,* 143-156.

Castro, K., Angell, T., Somers, B., Tlusty, M., Halvorson, H., Smolowitz, R. & Sharma, U. (2005). Lobster shell disease in southern New England: monitoring and research. In: Michael Tlusty, Harlyn Halvorson, Roxanna Smolowitz & Usha Sharma (Eds.). *State of Lobster Science: shell disease workshop.* Aquatic Forum Series 05-1, pp. 165-172. The New England Aquarium, Boston, Massachusetts.

Castro, K.M., Cobb, J.S., Gomez-Chiarri, M. & Tlusty, M. (2012). Epizootic shell disease in American lobsters *Homarus americanus* in southern New England: past, present and future. *Diseases of Aquatic Organisms, 100,* 149-158.

Castro, K.M., Factor, J.R., Angell, T. & Landers, D.F. (2006). The conceptual approach to lobster shell disease revisited. *Journal of Crustacean Biology, 26,* 646-660.

Castro, K.M. & Somers, B.A. (2012). Observations of epizootic shell disease in American lobsters, *Homarus americanus,* in southern New England. *Journal of Shellfish Research, 31,* 423-430.

Cawthorn, R.J. (2011). Diseases of American lobsters (*Homarus americanus*): A review. *Journal of Invertebrate Pathology, 106,* 71-78.

Caza, M., Lépine, F. & Dozois, C.M. (2011). Secretion, but not overall synthesis, of catecholate siderophores contributes to virulence of extraintestinal pathogenic *Escherichia coli*. *Molecular Microbiology, 80*, 266-282.

Celi, M., Filiciotto, F., Vazzana, M., Arizza, V., Maccarrone, V., Ceraulo, M., Mazzola, S. & Buscaino, G. (2015). Shipping noise affecting immune responses of European spiny lobster (*Palinurus elephas*). *Canadian Journal of Zoology, 93*, 113-121.

Cerenius, L., Jiravanichpaisal, P., Liu, H.-p. & Soderhall, I. (2010a). Crustacean Immunity. *Advances in Experimental Medicine and Biology, 708*, 239-259.

Cerenius, L., Kawabata, S.-i., Lee, B.L., Nonaka, M. & Söderhäll, K. (2010b). Proteolytic cascades and their involvement in invertebrate immunity. *Trends in Biochemical Sciences, 35*, 575-583.

Cerenius, L., Lee, B.L. & Söderhäll, K. (2008). The proPO-system: pros and cons for its role in invertebrate immunity. *Trends in Immunology, 29*, 263-271.

Cerenius, L. & Söderhäll, K. (2004). The prophenoloxidase-activating system in invertebrates. *Immunological Reviews, 198*, 116-126.

Cerenius, L. & Söderhäll, K. (2012). Crustacean immune responses and their implications for disease control. In: Austin B (Ed.). Infectious disease in aquaculture: prevention and control, pp. 69-87. Woodhead Publishing, Cambridge.

Chaban, B., Hughes, H.V. & Beeby, M. (2015). The flagellum in bacterial pathogens: for motility and a whole lot more. *Seminars in Cell & Developmental Biology, 46*, 91-103.

Chatfield, C.H. & Cianciotto, N.P. (2007). The secreted pyomelanin pigment of *Legionella pneumophila* confers ferric reductase activity. *Infection and Immunity, 75*, 4062-4070.

Chistoserdov, A.Y., Quinn, R.A., Gubbala, S.L. & Smolowitz, R. (2012). Bacterial communities associated with lesions of shell disease in the American lobster, *Homarus americanus* Milne-Edwards. *Journal of Shellfish Research, 31*, 449-462.

Chistoserdov, A.Y., Smolowitz, R., Mirasol, F. & Hsu, A. (2005). Culture-dependent characterization of the microbial community associated with epizootic shell disease lesions in American lobster, *Homarus americanus*. *Journal of Shellfish Research, 24*, 741-747.

Chokesajjawatee, N., Zo, Y.-G. & Colwell, R.R. (2008). Determination of clonality and relatedness of *Vibrio cholerae* isolates by genomic fingerprinting, using long-range repetitive element sequence-based PCR. *Applied and Environmental Microbiology, 74*, 5392-5401.

Clark, K.F. (2014). Characterization and functional classification of American lobster (*Homarus americanus*) immune factor transcripts. *Fish & Shellfish Immunology, 41*, 12-26.

Comeau, M. & Benhalima, K. (2009). Internal organ pathology of wild American lobster (*Homarus americanus*) from eastern Canada affected with shell disease. *New Zealand Journal of Marine and Freshwater Research, 43*, 257-269.

Comely, C. & Ansell, A. (1989). The occurrence of black necrotic disease in crab species from the west of Scotland. *Ophelia, 30*, 95-112.

Conklin, D., D'Abramo, L., Bordner, C. & Baum, N. (1980). A successful purified diet for the culture of juvenile lobsters: the effect of lecithin. *Aquaculture, 21*, 243-249.

Cook, D.W. & Lofton, S.R. (1973). Chitinoclastic bacteria associated with shell disease in Penaeus shrimp and the blue crab (*Callinectes sapidus*). *Journal of Wildlife Diseases, 9*, 154-159.

Cordero, O.X., Ventouras, L.-A., DeLong, E.F. & Polz, M.F. (2012). Public good dynamics drive evolution of iron acquisition strategies in natural bacterioplankton populations. *Proceedings of the National Academy of Sciences, 109*, 20059-20064.

Costa-Ramos, C. & Rowley, A.F. (2004). Effect of extracellular products of *Pseudoalteromonas atlantica* on the edible crab *Cancer pagurus*. *Applied and Environmental Microbiology, 70*, 729-735.

Costerton, J.W., Stewart, P.S. & Greenberg, E.P. (1999). Bacterial biofilms: a common cause of persistent infections. *Science, 284*, 1318-1322.

Coyne, V. & al-Harthi, L. (1992). Induction of melanin biosynthesis in *Vibrio cholerae*. *Applied and Environmental Microbiology, 58*, 2861-2865.

Crawford, J.M., Kontnik, R. & Clardy, J. (2010). Regulating alternative lifestyles in entomopathogenic bacteria. *Current Biology, 20*, 69-74.

Crear, B. & Forteath, G. (2002). Feeding has the largest effect on the ammonia excretion rate of the southern rock lobster, *Jasus edwardsii*, and the western rock lobster, *Panulirus cygnus*. *Aquacultural Engineering, 26*, 239-250.

D'alvise, P.W., Magdenoska, O., Melchiorsen, J., Nielsen, K.F. & Gram, L. (2014). Biofilm formation and antibiotic production in *Ruegeria mobilis* are influenced by intracellular concentrations of cyclic dimeric guanosinmonophosphate. *Environmental Microbiology, 16*, 1252-1266.

Davidson, G.W. & Hosking, W.W. (2004). Development of a method for alleviating leg loss during post-harvest handling of rock lobsters, Geraldton Fishermen's Co-operative, Geraldton.

Davies, C.E., Johnson, A.F., Wootton, E.C., Greenwood, S.J., Clark, K.F., Vogan, C.L. & Rowley, A.F. (2014a). Effects of population density and body size on disease ecology of the European lobster in a temperate marine conservation zone. *ICES Journal of Marine Science, 72*, i128-i138.

Davies, C.E., Whitten, M.M., Kim, A., Wootton, E.C., Maffeis, T.G., Tlusty, M., Vogan, C.L. & Rowley, A.F. (2014b). A comparison of the structure of American (*Homarus americanus*) and European (*Homarus gammarus*) lobster cuticle with particular reference to shell disease susceptibility. *Journal of Invertebrate Pathology, 117*, 33-41.

Deangelis, B.M., Cooper, R., Clancy, M., Cooper, C., Angell, T., Olszewski, S., Colburn, W. & Catena, J. (2012). Impacts of v-notching the American lobster. *Journal of Shellfish Research, 29*, 489-496.

Declercq, A., Chiers, K., Soetaert, M., Lasa, A., Romalde, J., Polet, H., Haesebrouck, F. & Decostere, A. (2015). *Vibrio tapetis* isolated from vesicular skin lesions in Dover sole *Solea solea*. *Diseases of Aquatic Organisms, 115*, 81-86.

Defer, D., Desriac, F., Henry, J., Bourgougnon, N., Baudy-Floc'H, M., Brillet, B., Le Chevalier, P. & Fleury, Y. (2013). Antimicrobial peptides in oyster hemolymph: the bacterial connection. *Fish & Shellfish Immunology, 34*, 1439-1447.

Destoumieux-Garzón, D., Rosa, R.D., Schmitt, P., Barreto, C., Vidal-Dupiol, J., Mitta, G., Gueguen, Y. & Bachère, E. (2016). Antimicrobial peptides in marine invertebrate health and disease. *Philosophical Transactions of the Royal Society B, 371*, 20150300.

Diggles, B., Hine, P., Handley, S. & Boustead, N. (2002). A handbook of diseases of importance to aquaculture in New Zealand. *NIWA Science and Technology Series, 49*, 200.

DiNardo, G.T., DeMartini, E.E. & Haight, W.R. (2002). Estimates of lobster-handling mortality associated with the Northwestern Hawaiian Islands lobster-trap fishery. *Fishery Bulletin, 100*, 128-133.

Drancourt, M., Bollet, C., Carlioz, A., Martelin, R., Gayral, J.-P. & Raoult, D. (2000). 16S ribosomal DNA sequence analysis of a large collection of environmental and clinical unidentifiable bacterial isolates. *Journal of Clinical Microbiology, 38*, 3623-3630.

Dubula, O., Groeneveld, J., Santos, J., Van Zyl, D., Brouwer, S., Van Den Heever, N. & McCue, S. (2005). Effects of tag-related injuries and timing of tagging on growth of rock lobster, *Jasus lalandii*. *Fisheries Research, 74*, 1-10.

Dyrynda, E. (1998). Shell disease in the common shrimp *Crangon crangon*: variations within an enclosed estuarine system. *Marine Biology, 132*, 445-452.

Edgar, R.C. (2013). UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nature Methods, 10*, 996-998.

Edgerton, B.F., Evans, L.H., Stephens, F.J. & Overstreet, R.M. (2002). Synopsis of freshwater crayfish diseases and commensal organisms. *Aquaculture, 206*, 57-135.

Emery, T.J., Hartmann, K., Green, B.S. & Gardner, C. (2016). Handled with care: Minimal impacts of appendage damage on the growth and productivity of the southern rock lobster (*Jasus edwardsii*). *Fisheries Research, 175*, 75-86.

Ernst, R.K., Ichikawa, J.K., Bangera, M.G., Selgrade, S., Burns, J.L., Hiatt, P., McCoy, K., Brittnacher, M., Kas, A. & Spencer, D.H. (2003). Genome mosaicism is conserved but not unique in *Pseudomonas aeruginosa* isolates from the airways of young children with cystic fibrosis. *Environmental Microbiology, 5*, 1341-1349.

Evans, L., Jones, J. & Brock, J. (2000). Diseases of spiny lobsters. In: Phillips Bf & Kittaka J (Eds.). *Spiny lobsters: fisheries and culture*, 2rd edition, pp. 586-600. Fishing News Books, Blackwell Science, Oxford.

Feinman, S.G., Martínez, A.U., Bowen, J.L. & Tlusty, M.F. (2017). Fine-scale transition to lower bacterial diversity and altered community composition precedes shell disease in laboratory-reared juvenile American lobster. *Diseases of Aquatic Organisms*, *124*, 41-54.

Fischbach, M.A., Lin, H., Zhou, L., Yu, Y., Abergel, R.J., Liu, D.R., Raymond, K.N., Wanner, B.L., Strong, R.K. & Walsh, C.T. (2006). The pathogen-associated *iroA* gene cluster mediates bacterial evasion of lipocalin 2. *Proceedings of the National Academy of Sciences*, *103*, 16502-16507.

Fisher, W.S., Nilson, E.H., Steenbergen, J.F. & Lightner, D.V. (1978). Microbial diseases of cultured lobsters: a review. *Aquaculture*, *14*, 115-140.

Floreto, E.A., Prince, D.L., Brown, P.B. & Bayer, R.C. (2000). The biochemical profiles of shell-diseased American lobsters, *Homarus americanus* Milne Edwards. *Aquaculture*, *188*, 247-262.

Fotedar, S., Evans, L. & Jones, B. (2006). Effect of holding duration on the immune system of western rock lobster, *Panulirus cygnus*. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *143*, 479-487.

Fotedar, S., Tsvetnenko, E. & Evans, L. (2001). Effect of air exposure on the immune system of the rock lobster *Panulirus cygnus*. *Marine and Freshwater Research*, *52*, 1351-1355.

Freeman, D. & MacDiarmid, A. (2009). Healthier lobsters in a marine reserve: effects of fishing on disease incidence in the spiny lobster, *Jasus edwardsii*. *Marine and Freshwater Research*, *60*, 140-145.

Frisch, A.J. & Hobbs, J.-P.A. (2011). Effects of autotomy on long-term survival and growth of painted spiny lobster (*Panulirus versicolor*) on the Great Barrier Reef, Australia. *Marine Biology*, *158*, 1645.

Garcia-Rivera, J. & Casadevall, A. (2001). Melanization of *Cryptococcus neoformans* reduces its susceptibility to the antimicrobial effects of silver nitrate. *Medical Mycology*, *39*, 353-357.

Gay, M., Berthe, F.C. & Le Roux, F. (2004). Screening of *Vibrio* isolates to develop an experimental infection model in the Pacific oyster *Crassostrea gigas*. *Diseases of Aquatic Organisms*, *59*, 49.

Geddes, M.C., Musgrove, R.J. & Thomas, C. 2004. Rock lobster enhancement and aquaculture subprogram: investigation of tail fan necrosis in live-held adult southern rock lobsters: final report. Fisheries Research and Development Corporation, Canberra.

Glenn, R.P. & Pugh, T.L. (2006). Epizootic shell disease in American lobster (*Homarus americanus*) in Massachusetts coastal waters: interactions of temperature, maturity, and intermolt duration. *Journal of Crustacean Biology*, *26*, 639-645.

Gomez-Chiarri, M. & Cobb, J.S. (2012). Shell disease in the American lobster, *Homarus americanus*: A synthesis of research from the New England lobster research initiative: lobster shell disease. *Journal of Shellfish Research, 31,* 583-590.

Gómez-León, J., Villamil, L., Lemos, M., Novoa, B. & Figueras, A. (2005). Isolation of *Vibrio alginolyticus* and *Vibrio splendidus* from aquacultured carpet shell clam (*Ruditapes decussatus*) larvae associated with mass mortalities. *Applied and Environmental Microbiology, 71,* 98-104.

Gopalan, U. & Young, J. (1975). Incidence of shell disease in shrimp in the New York Bight. *Marine Pollution Bulletin, 6,* 149-153.

Grandiosa, R., Mérien, F., Pillay, K. & Alfaro, A. (2016). Innovative application of classic and newer techniques for the characterization of haemocytes in the New Zealand black-footed abalone (*Haliotis iris*). *Fish & Shellfish Immunology, 48,* 175-184.

Hall-Stoodley, L., Costerton, J.W. & Stoodley, P. (2004). Bacterial biofilms: from the natural environment to infectious diseases. *Nature Reviews Microbiology, 2,* 95-108.

Haug, T., Kjuul, A.K., Stensvåg, K., Sandsdalen, E. & Styrvold, O.B. (2002). Antibacterial activity in four marine crustacean decapods. *Fish & Shellfish Immunology, 12,* 371-385.

Hermans, S.M., Buckley, H.L., Case, B.S., Curran-Cournane, F., Taylor, M. & Lear, G. (2017). Bacteria as emerging indicators of soil condition. *Applied and Environmental Microbiology, 83,* e02826-02816.

Hernandez-Divers, S.J., Hensel, P., Gladden, J., Hernandez-Divers, S.M., Buhlmann, K.A., Hagen, C., Sanchez, S., Latimer, K.S., Ard, M. & Camus, A.C. (2009). Investigation of shell disease in map turtles (*Graptemys* spp.). *Journal of Wildlife Diseases, 45,* 637-652.

Hernández-López, J., Gollas-Galván, T., Gómez-Jiménez, S., Portillo-Clark, G. & Vargas-Albores, F. (2003). In the spiny lobster (*Panulirus interruptus*) the prophenoloxidase is located in plasma not in haemocytes. *Fish & Shellfish Immunology, 14,* 105-114.

Hernández-López, J., Gollas-Galván, T. & Vargas-Albores, F. (1996). Activation of the prophenoloxidase system of the brown shrimp (*Penaeus californiensis* Holmes). *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology, 113,* 61-66.

Hernández-Romero, D., Solano, F. & Sanchez-Amat, A. (2005). Polyphenol oxidase activity expression in *Ralstonia solanacearum*. *Applied and Environmental Microbiology, 71,* 6808-6815.

Hernroth, B., Baden, S.P., Holm, K., André, T. & Söderhäll, I. (2004). Manganese induced immune suppression of the lobster, *Nephrops norvegicus*. *Aquatic Toxicology, 70,* 223-231.

Hernroth, B., Sköld, H.N., Wiklander, K., Jutfelt, F. & Baden, S. (2012). Simulated climate change causes immune suppression and protein damage in the crustacean *Nephrops norvegicus*. *Fish & Shellfish Immunology, 33,* 1095-1101.

Hoenig, J.M., Groner, M.L., Smith, M.W., Vogelbein, W.K., Taylor, D.M., Landers, D.F., Swenarton, J., Gauthier, D.T., Sadler, P. & Matsche, M. (2017). Impact of disease on the survival of three commercially fished species. *Ecological Applications, 29,* 2116-2127.

Holden, V.I. & Bachman, M.A. (2015). Diverging roles of bacterial siderophores during infection. *Metallomics, 7,* 986-995.

Homerding, M., Mcelroy, A., Taylor, G., Dove, A. & Allam, B. (2012). Investigation of epizootic shell disease in American lobsters (*Homarus americanus*) from Long Island Sound: II. Immune parameters in lobsters and relationships to the disease. *Journal of Shellfish Research, 31,* 495-504.

Hsu, A. & Smolowitz, R. (2003). Scanning electron microscopy investigation of epizootic lobster shell disease in *Homarus americanus*. *The Biological Bulletin, 205,* 228-230.

Huchette, S., Paillard, C., Clavier, J. & Day, R. (2006). Shell disease: abnormal conchiolin deposit in the abalone *Haliotis tuberculata*. *Diseases of Aquatic Organisms, 68,* 267-271.

Hwang, S.p. (2004). In vitro and in vivo antibacterial activity of shrimp chitosan against some intestinal bacteria. *Fisheries Science, 70,* 675-681.

Iversen, E. & Beardsley, G. (1976). Shell disease in crustaceans indigenous to South Florida. *The Progressive Fish-Culturist, 38,* 195-196.

Jacobs, M., Laufer, H., Stuart, J., Chen, M. & Pan, X. (2012). Endocrine-disrupting alkylphenols are widespread in the blood of lobsters from southern New England and adjacent offshore areas. *Journal of Shellfish Research, 31,* 563-571.

Jacobson, E.S. (2000). Pathogenic roles for fungal melanins. *Clinical Microbiology Reviews, 13*, 708-717.

Jaiswal, S., Sahoo, P.K., Ryan, D., Das, J.K., Chakraborty, E., Mohakud, N.K. & Suar, M. (2016). Altered virulence potential of *Salmonella* enteritidis cultured in different foods: A cumulative effect of differential gene expression and immunomodulation. *International Journal of Food Microbiology, 230,* 64-72.

Jayasree, L., Janakiram, P. & Madhavi, R. (2006). Characterization of *Vibrio* spp. associated with diseased shrimp from culture ponds of Andhra Pradesh (India). *Journal of the World Aquaculture Society, 37,* 523-532.

Jeffs, A. & Hooker, S. (2000). Economic feasibility of aquaculture of spiny lobsters *Jasus edwardsii* in temperate waters. *Journal of the World Aquaculture Society, 31,* 30-41.

Jeffs, A.G., Gardner, C. & Cockcroft, A. (2013). *Jasus* and *Sagmariasus* species. In: Phillips Bf (Ed.). *Lobsters: biology, management, aquaculture and fisheries,* 2rd edition, pp. 259-288. John Wiley and Sons, Oxford.

Jeffs, A.G. & James, P. (2001). Sea-cage culture of the spiny lobster *Jasus edwardsii* in New Zealand. *Marine and Freshwater Research, 52,* 1419-1424.

Jiménez, C.P., Huchin-Mian, J.P., Simões, N., Briones-Fourzán, P., Lozano-Álvarez, E., Arteaga, A.S., Pérez-Vega, J.A., Simá-Álvarez, R., Vazquez, C.R. & Rodríguez-Canul, R. (2012). Physiological and immunological characterization of Caribbean spiny lobsters *Panulirus argus* naturally infected with *Panulirus argus* Virus 1 (PaV1). *Diseases of Aquatic Organisms, 100*, 113-124.

Jiravanichpaisal, P., Lee, B.L. & Söderhäll, K. (2006). Cell-mediated immunity in arthropods: hematopoiesis, coagulation, melanization and opsonization. *Immunobiology*, *211*, 213-236.

Joint, I., Mühling, M. & Querellou, J. (2010). Culturing marine bacteria - an essential prerequisite for biodiscovery. *Microbial Biotechnology, 3*, 564-575.

Josenhans, C. & Suerbaum, S. (2002). The role of motility as a virulence factor in bacteria. *International Journal of Medical Microbiology*, *291*, 605-614.

Kaizu, A., Fagutao, F.F., Kondo, H., Aoki, T. & Hirono, I. (2011). Functional analysis of C-type lysozyme in penaeid shrimp. *Journal of Biological Chemistry, 286*, 44344-44349.

Kavanaugh, J.S. & Horswill, A.R. (2016). Impact of environmental cues on staphylococcal quorum sensing and biofilm development. *Journal of Biological Chemistry*, *291*, 12556-12564.

Kelly, S. (2001). Temporal variation in the movement of the spiny lobster *Jasus edwardsii*. *Marine and Freshwater Research, 52*, 323-331.

Kemp, J., Britz, P. & Cockcroft, A. (2009). Ammonia excretion dynamics in the east coast rock lobster *Panulirus homarus rubellus*. *Aquaculture, 286*, 296-300.

Kesarcodi-Watson, A., Miner, P., Nicolas, J.-L. & Robert, R. (2012). Protective effect of four potential probiotics against pathogen-challenge of the larvae of three bivalves: Pacific oyster (*Crassostrea gigas*), flat oyster (*Ostrea edulis*) and scallop (*Pecten maximus*). *Aquaculture*, *344*, 29-34.

King, N.G., Duncan, P.F., Kennington, K., Wootton, E.C. & Jenkins, S.R. (2014). Characterisation of shell disease syndrome in the brown crab, *Cancer pagurus*, in a discrete Irish Sea fishery. *Journal of Crustacean Biology, 34*, 40-46.

Kır, M., Kumlu, M. & Eroldoğan, O. (2004). Effects of temperature on acute toxicity of ammonia to *Penaeus semisulcatus* juveniles. *Aquaculture, 241*, 479-489.

Kjærgaard, K., Schembri, M.A., Hasman, H. & Klemm, P. (2000). Antigen 43 from *Escherichia coli* induces inter-and intraspecies cell aggregation and changes in colony morphology of *Pseudomonas fluorescens*. *Journal of Bacteriology, 182*, 4789-4796.

Klindworth, A., Pruesse, E., Schweer, T., Peplies, J., Quast, C., Horn, M. & Glöckner, F.O. (2013). Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Research, 41*, e1.

Klompmaker, A.A., Chistoserdov, A.Y. & Felder, D.L. (2016). Possible shell disease in 100 million-year-old crabs. *Diseases of Aquatic Organisms, 119*, 91-99.

Kough, A.S., Paris, C.B. & Butler IV, M.J. (2013). Larval connectivity and the international management of fisheries. *PloS ONE, 8*, e64970.

Kristensen, E. (2008). Mangrove crabs as ecosystem engineers; with emphasis on sediment processes. *Journal of Sea Research, 59*, 30-43.

Kumar, V., Zozaya‐Valdes, E., Kjelleberg, S., Thomas, T. & Egan, S. (2016). Multiple opportunistic pathogens can cause a bleaching disease in the red seaweed *Delisea pulchra*. *Environmental Microbiology, 18*, 3962-3975.

Kunkel, J.G., Nagel, W. & Jercinovic, M.J. (2012). Mineral fine structure of the American lobster cuticle. *Journal of Shellfish Research, 31*, 515-526.

Landers, D., Tlusty, M., Halvorson, H., Smolowitz, R. & Sharma, U. (2005). Prevalence and severity of shell disease in American lobster Homarus americanus from eastern Long Island Sound, Connecticut. In: Michael Tlusty, Harlyn Halvorson, Roxanna Smolowitz & Usha Sharma (Eds.). *State of Lobster Science: shell disease workshop.* Aquatic Forum Series 05-1, pp. 94-97. The New England Aquarium, Boston, Massachusetts.

Laufer, H., Chen, M., Johnson, M., Demir, N. & Bobbitt, J.M. (2012). The effect of alkylphenols on lobster shell hardening. *Journal of Shellfish Research, 31*, 555-562.

Le Roux, F., Zouine, M., Chakroun, N., Binesse, J., Saulnier, D., Bouchier, C., Zidane, N., Ma, L., Rusniok, C. & Lajus, A. (2009). Genome sequence of *Vibrio splendidus*: an abundant planktonic marine species with a large genotypic diversity. *Environmental Microbiology, 11*, 1959-1970.

Lear, G., Niyogi, D., Harding, J., Dong, Y. & Lewis, G. (2009). Biofilm bacterial community structure in streams affected by acid mine drainage. *Applied and Environmental Microbiology, 75*, 3455-3460.

Leland, J.C., Butcher, P.A., Broadhurst, M.K., Paterson, B.D. & Mayer, D.G. (2013). Damage and physiological stress to juvenile eastern rock lobster (*Sagmariasus verreauxi*) discarded after trapping and hand collection. *Fisheries Research, 137*, 63-70.

Lemire, A., Goudenège, D., Versigny, T., Petton, B., Calteau, A., Labreuche, Y. & Le Roux, F. (2015). Populations, not clones, are the unit of vibrio pathogenesis in naturally infected oysters. *The ISME Journal, 9*, 1523-1531.

Li, X. & Roseman, S. (2004). The chitinolytic cascade in Vibrios is regulated by chitin oligosaccharides and a two-component chitin catabolic sensor/kinase. *Proceedings of the National Academy of Sciences of the United States of America, 101*, 627-631.

Lin, J., Yan, X.J., Zheng, L., Ma, H.H. & Chen, H.M. (2005). Cytotoxicity and apoptosis induction of some selected marine bacteria metabolites. *Journal of Applied Microbiology, 99*, 1373-1382.

Linnane, A., Penny, S., Hawthorne, P. & Hoare, M. (2015). Residency and movement dynamics of southern rock lobster (*Jasus edwardsii*) after a translocation event. *Marine and Freshwater Research, 66*, 623-630.

Linnane, A., Penny, S., Hoare, M. & Hawthorne, P. (2011). Assessing the effectiveness of size limits and escape gaps as management tools in a commercial rock lobster (*Jasus edwardsii*) fishery. *Fisheries Research, 111*, 1-7.

Lorkin, M., Geddes, M., Bryars, S., Leech, M., Musgrove, R., Reuter, R. & Clarke, S. 1999. Seabased live holding of the southern rock lobster, *Jasus edwardsii*: a pilot study on long term holding and feeding. SARDI & South Australian Research and Development Institute, Adelaide.

Louden, B.C., Haarmann, D. & Lynne, A.M. (2011). Use of blue agar CAS assay for siderophore detection. *Journal of Microbiology & Biology Education, 12*, 51.

Luján, A.M., Gómez, P. & Buckling, A. (2015). Siderophore cooperation of the bacterium *Pseudomonas fluorescens* in soil. *Biology Letters, 11*, 20140934.

Luter, H.M., Bannister, R.J., Whalan, S., Kutti, T., Pineda, M.-C. & Webster, N.S. (2017). Microbiome analysis of a disease affecting the deep-sea sponge *Geodia barretti*. *FEMS Microbiology Ecology, 93*.

Madsen, L., Møller, J.D. & Dalsgaard, I. (2005). *Flavobacterium psychrophilum* in rainbow trout, *Oncorhynchus mykiss* (Walbaum), hatcheries: studies on broodstock, eggs, fry and environment. *Journal of Fish Diseases, 28*, 39-47.

Mancuso, M., Costanzo, M., Maricchiolo, G., Gristina, M., Zaccone, R., Cuccu, D. & Genovese, L. (2010). Characterization of chitinolytic bacteria and histological aspects of shell disease syndrome in European spiny lobsters (*Palinurus elephas*) (Fabricius 1787). *Journal of Invertebrate Pathology, 104*, 242-244.

Matthews, T.R. (2001). Trap-induced mortality of the spiny lobster, *Panulirus argus*, in Florida, USA. *Marine and Freshwater Research, 52*, 1509-1516.

May, D.G. 2002. Identification and characterization of bacteria associated with tail fan necrosis of southern rock lobsters (*Jasus edwardsii*) BSc (Hons) thesis. The University of Adelaide, Adelaide, Australia.

May, D.G. 2007. Investigation of tail fan necrosis of live-held southern rock lobsters. PhD thesis. The University of Adelaide, Adelaide, Australia.

Maynard, J., Van Hooidonk, R., Harvell, C.D., Eakin, C.M., Liu, G., Willis, B.L., Williams, G.J., Groner, M.L., Dobson, A. & Heron, S.F. (2016). Improving marine disease surveillance through sea temperature monitoring, outlooks and projections. *Philosophical Transactions of the Royal Society B: Biological Sciences, 371*, 20150208.

McDonald, D., Price, M.N., Goodrich, J., Nawrocki, E.P., DeSantis, T.Z., Probst, A., Andersen, G.L., Knight, R. & Hugenholtz, P. (2012). An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea. *The ISME Journal, 6*, 610.

Meng, Q., Li, W., Liang, T., Jiang, X., Gu, W. & Wang, W. (2010). Identification of adhesin-like protein *ALP41* from *Spiroplasma eriocheiris* and induction immune response of *Eriocheir sinensis*. *Fish & Shellfish Immunology, 29*, 587-593.

Merella, P., Alemany, F., Carbonell, A. & Quetglas, A. (1998). Fishery and biology of Norway lobster *Nephrops norvegicus* (Decapoda: Nephropidae) in Mallorca (western Mediterranean). *Journal of Natural History, 32*, 1631-1640.

Meres, N.J., Ajuzie, C.C., Sikaroodi, M., Vemulapalli, M., Shields, J.D. & Gillevet, P.M. (2012). Dysbiosis in epizootic shell disease of the American lobster (*Homarus americanus*). *Journal of Shellfish Research, 31*, 463-472.

Miller, M.R., White, A. & Boots, M. (2006). The evolution of parasites in response to tolerance in their hosts: the good, the bad, and apparent commensalism. *Evolution, 60*, 945-956.

Milligan, R., Albalat, A., Atkinson, R. & Neil, D. (2009). The effects of trawling on the physical condition of the Norway lobster *Nephrops norvegicus* in relation to seasonal cycles in the Clyde Sea area. *ICES Journal of Marine Science, 66*, 488-494.

Moeller, R., Horneck, G., Facius, R. & Stackebrandt, E. (2005). Role of pigmentation in protecting *Bacillus* sp. endospores against environmental UV radiation. *FEMS Microbiology Ecology, 51*, 231-236.

Musgrove, R.J. (2000). Molt staging in the southern rock lobster *Jasus edwardsii*. *Journal of Crustacean Biology*, *20*, 44-53.

Musgrove, R.J., Geddes, M.C. & Thomas, C. (2005). Causes of tail fan necrosis in the southern rock lobster, *Jasus edwardsii*. *New Zealand Journal of Marine and Freshwater Research*, *39*, 293-304.

Myers, A. & Tlusty, M.F. (2009). A long‐term assessment of the physiological effects of herring (*Clupea harengus*) as a dietary component of the American lobster (*Homarus americanus*). *New Zealand Journal of Marine and Freshwater Research, 43*, 173-183.

Ngamwongsatit, P., Buasri, W., Pianariyanon, P., Pulsrikarn, C., Ohba, M., Assavanig, A. & Panbangred, W. (2008). Broad distribution of enterotoxin genes (*hblCDA*, *nheABC*, *cytK*, and *entFM*) among *Bacillus thuringiensis* and *Bacillus cereus* as shown by novel primers. *International Journal of Food Microbiology*, *121*, 352-356.

Nguyen, V.D., Pham, T.T., Nguyen, T.H.T., Nguyen, T.T.X. & Hoj, L. (2014). Screening of marine bacteria with bacteriocin-like activities and probiotic potential for ornate spiny lobster (*Panulirus ornatus*) juveniles. *Fish & Shellfish Immunology, 40*, 49-60.

Niu, S., Li, S., Chen, Y., Tian, X., Zhang, H., Zhang, G., Zhang, W., Yang, X., Zhang, S. & Ju, J. (2011). Lobophorins E and F, new spirotetronate antibiotics from a South China Sea-derived *Streptomyces* sp. SCSIO 01127. *The Journal of Antibiotics*, *64*, 711.

Nosanchuk, J.D. & Casadevall, A. (2006). Impact of melanin on microbial virulence and clinical resistance to antimicrobial compounds. *Antimicrobial Agents and Chemotherapy*, *50*, 3519-3528.

Nunan, L.M., Poulos, B.T., Navarro, S., Redman, R.M. & Lightner, D.V. (2010). Milky hemolymph syndrome (MHS) in spiny lobsters, penaeid shrimp and crabs. *Diseases of Aquatic Organisms, 91*, 105-112.

Nyholm, S.V. & Graf, J. (2012a). Knowing your friends: invertebrate innate immunity fosters beneficial bacterial symbioses. *Nature Reviews: Microbiology, 10*, 815-827.

Nyholm, S.V. & Graf, J. (2012b). Knowing your friends: invertebrate innate immunity fosters beneficial bacterial symbioses. *Nature Reviews Microbiology*, *10*, 815-827.

Oliver, M. & MacDiarmid, A. (2001). Blood refractive index and ratio of weight to carapace length as indices of nutritional condition in juvenile rock lobsters (*Jasus edwardsii*). *Marine and Freshwater Research, 52*, 1395-1400.

Ooi, M.C. 2014. Detection and quantification of bacteria in cultured juvenile spiny lobsters (*Panulirus ornatus*). MSc thesis. University of Tasmania, Tasmania.

Parsons, D.M. & Eggleston, D.B. (2005). Indirect effects of recreational fishing on behavior of the spiny lobster *Panulirus argus*. *Marine Ecology Progress Series, 303*, 235-244.

Patel, R. (2005). Biofilms and antimicrobial resistance. *Clinical Orthopaedics and Related Research*, *437*, 41-47.

Perdomo-Morales, R., Montero-Alejo, V., Perera, E., Pardo-Ruiz, Z. & Alonso-Jiménez, E. (2007). Phenoloxidase activity in the hemolymph of the spiny lobster *Panulirus argus*. *Fish & Shellfish Immunology, 23*, 1187-1195.

Pettersen, V.K., Mosevoll, K.A., Lindemann, P.C. & Wiker, H.G. (2016). Coordination of metabolism and virulence factors expression of extraintestinal pathogenic *Escherichia coli* purified from blood cultures of patients with sepsis. *Molecular & Cellular Proteomics*, *15*, 2890-2907.

Plonka, P.M. & Grabacka, M. (2006). Melanin synthesis in microorganisms-biotechnological and medical aspects. *Acta Biochimica Polonica*, *53*, 429-443.

Pollock, D.E. (1986). Review of the fishery for and biology of the Cape rock lobster *Jasus lalandii* with notes on larval recruitment. *Canadian Journal of Fisheries and Aquatic Sciences*, *43*, 2107-2117.

Ponprateep, S., Tharntada, S., Somboonwiwat, K. & Tassanakajon, A. (2012). Gene silencing reveals a crucial role for anti-lipopolysaccharide factors from *Penaeus monodon* in the protection against microbial infections. *Fish & Shellfish Immunology*, *32*, 26-34.

Porter, L., Butler, M. & Reeves, R.H. (2001). Normal bacterial flora of the spiny lobster *Panulirus argus* and its possible role in shell disease. *Marine and Freshwater Research, 52*, 1401-1405.

Powell, A. & Rowley, A.F. (2005). Unchanged prevalence of shell disease in the edible crab *Cancer pagurus* four years after decommissioning of a sewage outfall at Langland Bay, UK. *Diseases of Aquatic Organisms, 68*, 83-87.

Powrie, W. & Tempero, G.W. (2009). Comparison of injuries to New Zealand rock lobsters (*Jasus edwardsii*) caused by hand versus snare collection. *New Zealand Journal of Zoology*, *36*, 83-87.

Prince, D.L. 1997. Studies on the etiology and pathogenesis of shell disease in the American lobster, *Homarus americanus*. PhD thesis. University of Maine, Maine, USA.

Prince, D.L. & Bayer, R.C. (2005). Are all lobsters created equal? Understanding the role of host susceptibility in the development of shell disease in *Homarus americanus*. In: Michael Tlusty, Harlyn Halvorson, Roxanna Smolowitz & Usha Sharma (Eds.). *State of lobster science: shell disease workshop.* Aquatic Forum Series 05-1, pp. 58-67. New England Aquarium, Boston, Massachusetts.

Quinn, R.A., Cawthorn, R.J., Summerfield, R.L., Smolowitz, R. & Chistoserdov, A.Y. (2013a). Bacterial communities associated with lesions of two forms of shell disease in the American lobster (*Homarus americanus*, Milne Edwards) from Atlantic Canada. *Canadian Journal of Microbiology, 59*, 380-390.

Quinn, R.A., Hazra, S., Smolowitz, R. & Chistoserdov, A.Y. (2017). Real-time PCR assay for *Aquimarina macrocephali* subsp. *homaria* and its distribution in shell disease lesions of *Homarus americanus*, Milne-Edwards, 1837, and environmental samples. *Journal of Microbiological Methods, 139*, 61-67.

Quinn, R.A., Metzler, A., Smolowitz, R.M., Tlusty, M. & Chistoserdov, A.Y. (2012a). Exposures of *Homarus americanus* shell to three bacteria isolated from naturally occurring epizootic shell disease lesions. *Journal of Shellfish Research, 31*, 485-493.

Quinn, R.A., Metzler, A., Tlusty, M., Smolowitz, R.M., Leberg, P. & Chistoserdov, A.Y. (2012b). Lesion bacterial communities in American lobsters with diet-induced shell disease. *Diseases of Aquatic Organisms, 98*, 221-233.

Quinn, R.A., Smolowitz, R. & Chistoserdov, A. (2009). Eukaryotic communities in epizootic shell disease lesions of the American lobster (*Homarus americanus*, H. Milne Edwards). *Journal of Shellfish Research, 28*, 913-922.

Quinn, R.A., Smolowitz, R. & Chistoserdov, A.Y. (2013b). Culture-independent analysis of bacterial communities in hemolymph of American lobsters with epizootic shell disease. *Diseases of Aquatic Organisms, 103*, 141-148.

Randall, D. & Tsui, T. (2002). Ammonia toxicity in fish. *Marine Pollution Bulletin, 45*, 17-23.

Reichenbach, H. (1992). The Genus *Lysobacter*. In: Albert Balows, Hans G. Trüper, Martin Dworkin, Wim Harder & Karl-Heinz Schleifer (Eds.). *The Prokaryotes*, pp. 3256-3275. Springer, New York.

Ridgway, I., Taylor, A., Atkinson, R., Chang, E. & Neil, D. (2006). Impact of capture method and trawl duration on the health status of the Norway lobster, *Nephrops norvegicus*. *Journal of Experimental Marine Biology and Ecology, 339*, 135-147.

Roch, P. (1999). Defense mechanisms and disease prevention in farmed marine invertebrates. *Aquaculture, 172*, 125-145.

Rogers, H.A., Taylor, S.S., Hawke, J.P. & Lively, J.A.A. (2015a). Variations in prevalence of viral, bacterial, and rhizocephalan diseases and parasites of the blue crab (*Callinectes sapidus*). *Journal of Invertebrate Pathology, 127*, 54-62.

Rogers, H.A., Taylor, S.S., Hawke, J.P., Schott, E.J. & Lively, J.A.A. (2015b). Disease, parasite, and commensal prevalences for blue crab *Callinectes sapidus* at shedding facilities in Louisiana, USA. *Diseases of Aquatic Organisms, 112*, 207-217.

Rosas, Á.L. & Casadevall, A. (1997). Melanization affects susceptibility of *Cryptococcus neoformans* to heat and cold. *FEMS Microbiology Letters, 153*, 265-272.

Rosen, B. (1967). Shell disease of the blue crab, *Callinectes sapidus*. *Journal of Invertebrate Pathology, 9*, 348-353.

Rousseeuw, P.J. (1987). Silhouettes: a graphical aid to the interpretation and validation of cluster analysis. *Journal of Computational and Applied Mathematics, 20*, 53-65.

Ruiz-Rodríguez, M., Valdivia, E., Martín-Vivaldi, M., Martín-Platero, A.M., Martínez-Bueno, M., Méndez, M., Peralta-Sánchez, J.M. & Soler, J.J. (2012). Antimicrobial activity and genetic profile of enteroccoci isolated from hoopoes uropygial gland. *PloS ONE, 7*, e41843.

Russo, T.A., Shon, A.S., Beanan, J.M., Olson, R., MacDonald, U., Pomakov, A.O. & Visitacion, M.P. (2011). Hypervirulent *K. pneumoniae* secretes more and more active iron-acquisition molecules than "classical" *K. pneumoniae* thereby enhancing its virulence. *PLoS ONE, 6*, e26734.

Rycroft, N., Radcliffe, K., McDougal, E., Halverson, J., Gerlach, G., Deppermann, J. & Atema, J. (2012). No olfactory recognition of shell disease in American lobsters, *Homarus americanus*. *Journal of Shellfish Research, 31*, 527-532.

Safari, O., Shahsavani, D., Paolucci, M. & Mehraban Sang Atash, M. (2015). The effects of dietary nucleotide content on the growth performance, digestibility and immune responses of juvenile narrow clawed crayfish, *Astacus leptodactylus leptodactylus* Eschscholtz, 1823. *Aquaculture Research, 46*, 2685-2697.

Sant'Ana, A.S., Barbosa, M.S., Destro, M.T., Landgraf, M. & Franco, B.D. (2012). Growth potential of *Salmonella* spp. and *Listeria monocytogenes* in nine types of ready-to-eat vegetables stored at variable temperature conditions during shelf-life. *International Journal of Food Microbiology, 157*, 52-58.

Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W.S. & Huttenhower, C. (2011). Metagenomic biomarker discovery and explanation. *Genome Biology, 12*, R60.

Shields, J.D. (2011). Diseases of spiny lobsters: a review. *Journal of Invertebrate Pathology, 106*, 79-91.

Shields, J.D. (2012). The impact of pathogens on exploited populations of decapod crustaceans. *Journal of Invertebrate Pathology, 110*, 211-224.

Shields, J.D. (2013). Complex etiologies of emerging diseases in lobsters (*Homarus americanus*) from Long Island Sound 1. *Canadian Journal of Fisheries and Aquatic Sciences, 70*, 1576-1587.

Shields, J.D., Wheeler, K.N. & Moss, J.A. (2012). Histological assessment of the lobster (*Homarus americanus*) in the "100 lobsters" project. *Journal of Shellfish Research, 31,* 439-447.

Shivaji, S., Begum, Z., Rao, S.S.S.N., Reddy, P.V.V.V., Manasa, P., Sailaja, B., Prathiba, M.S., Thamban, M., Krishnan, K.P. & Singh, S.M. (2013). Antarctic ice core samples: culturable bacterial diversity. *Research in Microbiology, 164,* 70-82.

Sindermann, C. (1989). Shell disease in marine crustaceans - a conceptual approach. *Journal of Shellfish Research, 8,* 461.

Skaar, E.P. (2010). The battle for iron between bacterial pathogens and their vertebrate hosts. *PLoS Pathogens, 6,* e1000949.

Slifkin, M. (2000). Tween 80 opacity test responses of various Candida species. *Journal of Clinical Microbiology, 38,* 4626-4628.

Smaoui, S., Elleuch, L., Bejar, W., Karray-Rebai, I., Ayadi, I., Jaouadi, B., Mathieu, F., Chouayekh, H., Bejar, S. & Mellouli, L. (2010). Inhibition of fungi and gram-negative bacteria by bacteriocin BacTN635 produced by *Lactobacillus plantarum* sp. TN635. *Applied Biochemistry and Biotechnology, 162,* 1132-1146.

Smith, V.J., Brown, J.H. & Hauton, C. (2003). Immunostimulation in crustaceans: does it really protect against infection? *Fish & Shellfish Immunology, 15,* 71-90.

Smolowitz, R., Chistoserdov, A.Y. & Hsu, A. (2005). A description of the pathology of epizootic shell disease in the American lobster, *Homarus americanus*, H. Milne Edwards 1837. *Journal of Shellfish Research, 24,* 749-756.

Smolowitz, R., Quinn, R., Cawthorn, R., Summerfield, R. & Chistoserdov, A. (2014). Pathology of two forms of shell disease of the American lobster *Homarus americanus* Milne Edwards in Atlantic Canada. *Journal of Fish Diseases, 37,* 577-581.

Smolowitz, R.M., Bullis, R.A. & Abt, D.A. (1992). Pathologic cuticular changes of winter impoundment shell disease preceding and during intermolt in the American lobster, *Homarus americanus*. *The Biological Bulletin, 183,* 99-112.

Söderhäll, K. & Cerenius, L. (1992). Crustacean immunity. *Annual Review of Fish Diseases, 2,* 3-23.

Söderhäll, K. & Sritunyalucksana, K. (2002). The prophenoloxidase activating system in invertebrates: a short review. *A New Model for Analyzing Antimicrobial Peptides with Biomedical Applications,* 131-142.

Sokal, R.R. & Rohlf, F.J. (2012). Biometry: the principles and practice of statistics in biological research 4th edition, W. H. Freeman, San Francisco, CA.

Starr, P.J., Haist, V. & Breen, P.A. 2013. Data for the 2014 stock assessment of red rock lobsters (*Jasus edwardsii*) in CRA 3. Ministry for Primary Industries, Wellington.

Steneck, R., Hughes, T., Cinner, J., Adger, W., Arnold, S., Berkes, F., Boudreau, S., Brown, K., Folke, C. & Gunderson, L. (2011). Creation of a gilded trap by the high economic value of the Maine lobster fishery. *Conservation Biology, 25*, 904-912.

Stentiford, G., Neil, D., Peeler, E., Shields, J., Small, H., Flegel, T., Vlak, J., Jones, B., Morado, F. & Moss, S. (2012). Disease will limit future food supply from the global crustacean fishery and aquaculture sectors. *Journal of Invertebrate Pathology, 110*, 141-157.

Stentiford, G.D. & Neil, D.M. (2011). Diseases of *Nephrops* and *Metanephrops*: a review. *Journal of Invertebrate Pathology, 106*, 92-109.

Stevens, B.G. (2009). Effects of epizootic shell disease in American lobster Homarus americanus determined using a quantitative disease index. *Diseases of Aquatic Organisms, 88*, 25-34.

Stewart, E.J. (2012). Growing unculturable bacteria. *Journal of Bacteriology, 194*, 4151-4160.

Suginta, W. (2007). Identification of chitin binding proteins and characterization of two chitinase isoforms from *Vibrio alginolyticus* 283. *Enzyme and Microbial Technology, 41*, 212-220.

Sykes, D. (2017). Counting the beasts - Research programmes in New Zealand lobster fisheries. *The Lobster Newsletter, 30*, 5-6.

Tada, M., Kohno, M. & Niwano, Y. (2010). Scavenging or quenching effect of melanin on superoxide anion and singlet oxygen. *Journal of Clinical Biochemistry and Nutrition, 46*, 224-228.

Tanaka, K.R., Belknap, S.L., Homola, J.J. & Chen, Y. (2017). A statistical model for monitoring shell disease in inshore lobster fisheries: A case study in Long Island Sound. *PloS ONE, 12*, e0172123.

Taylor, C.C. (1948). Shell disease as a mortality factor in the lobster (*Homarus americanus*). *Department of Sea and Shore Fisheries, Augusta, Maine, Fisheries Circular, , 4*, 1-8.

Theopold, U., Schmidt, O., Söderhäll, K. & Dushay, M.S. (2004). Coagulation in arthropods: defence, wound closure and healing. *Trends in Immunology, 25*, 289-294.

Theriault, M., Vanleeuwen, J., Morrison, M. & Cawthorn, R. (2008). Risk factors for the development of shell disease in impounded populations of the American lobster, *Homarus americanus*. *Journal of Shellfish Research, 27*, 1239-1245.

Tlusty, M.F. & Metzler, A. (2012). Relationship between temperature and shell disease in laboratory populations of juvenile American lobsters (*Homarus americanus*). *Journal of Shellfish Research, 31*, 533-541.

Tlusty, M.F., Myers, A. & Metzler, A. (2008). Short- and long-term dietary effects on disease and mortality in American lobster *Homarus americanus*. *Diseases of Aquatic Organisms, 78*, 249-253.

Tlusty, M.F., Smolowitz, R.M., Halvorson, H.O. & DeVito, S.E. (2007). Host susceptibility hypothesis for shell disease in American lobsters. *Journal of Aquatic Animal Health, 19*, 215-225.

Tsai, G.-J. & Hwang, S.-P. (2004). In vitro and in vivo antibacterial activity of shrimp chitosan against some intestinal bacteria. *Fisheries Science*, *70*, 675-681.

Vafopoulou, X., Laufer, H. & Steel, C.G. (2007). Spatial and temporal distribution of the ecdysteroid receptor (EcR) in haemocytes and epidermal cells during wound healing in the crayfish, *Procambarus clarkii*. *General and Comparative Endocrinology*, *152*, 359-370.

Valeru, S.P., Rompikuntal, P.K., Ishikawa, T., Vaitkevicius, K., Sjöling, Å., Dolganov, N., Zhu, J., Schoolnik, G. & Wai, S.N. (2009). Role of melanin pigment in expression of *Vibrio cholerae* virulence factors. *Infection and Immunity*, *77*, 935-942.

Vega, E., Barclay, L., Gregoricus, N., Williams, K., Lee, D. & Vinjé, J. (2011). Novel surveillance network for Norovirus gastroenteritis outbreaks, United States. *Emerging Infectious Diseases*, *17*.

Verghese, B., Radhakrishnan, E. & Padhi, A. (2007). Effect of environmental parameters on immune response of the Indian spiny lobster, *Panulirus homarus* (Linnaeus, 1758). *Fish & Shellfish Immunology*, *23*, 928-936.

Versalovic, J., Koeuth, T. & Lupski, R. (1991). Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucleic Acids Research*, *19*, 6823-6831.

Vogan, C.L., Costa-Ramos, C. & Rowley, A.F. (2001). A histological study of shell disease syndrome in the edible crab *Cancer pagurus*. *Diseases of Aquatic Organisms*, *47*, 209-217.

Vogan, C.L., Costa-Ramos, C. & Rowley, A.F. (2002). Shell disease syndrome in the edible crab, *Cancer pagurus* - isolation, characterization and pathogenicity of chitinolytic bacteria. *Microbiology*, *148*, 743-754.

Vogan, C.L., Llewellyn, P.J. & Rowley, A.F. (1999). Epidemiology and dynamics of shell disease in the edible crab *Cancer pagurus*: a preliminary study of Langland Bay, Swansea, UK. *Diseases of Aquatic Organisms*, *35*, 81-87.

Vogan, C.L., Powell, A. & Rowley, A.F. (2008). Shell disease in crustaceans - just chitin recycling gone wrong? *Environmental Microbiology*, *10*, 826-835.

Vogan, C.L. & Rowley, A.F. (2002). Dynamics of shell disease in the edible crab *Cancer pagurus*: a comparative study between two sites on the Gower Peninsula, South Wales, UK. *Diseases of Aquatic Organisms*, *52*, 151-157.

Vogt, G. (2012). Ageing and longevity in the Decapoda (Crustacea): a review. *Zoologischer Anzeiger*, *251*, 1-25.

Wagner Mackenzie, B., Waite, D.W., Hoggard, M., Douglas, R.G., Taylor, M.W. & Biswas, K. (2017). Bacterial community collapse: a meta‐analysis of the sinonasal microbiota in chronic rhinosinusitis. *Environmental Microbiology, 19*, 381-392.

Wahle, R.A., Dellinger, L., Olszewski, S. & Jekielek, P. (2015). American lobster nurseries of southern New England receding in the face of climate change. *ICES Journal of Marine Science, 72*, i69-i78.

Wahle, R.A., Gibson, M. & Fogarty, M. (2009). Distinguishing disease impacts from larval supply effects in a lobster fishery collapse. *Marine Ecology Progress Series, 376*, 185-192.

Wang, H. & Breuil, C. (2002). A second reductase gene involved in melanin biosynthesis in the sap-staining fungus *Ophiostoma floccosum*. *Molecular Genetics and Genomics, 267*, 557-563.

Wang, L., Kounatidis, I. & Ligoxygakis, P. (2014a). *Drosophila* as a model to study the role of blood cells in inflammation, innate immunity and cancer. *Frontiers in Cellular & Infection Microbiology, 3*, 113.

Wang, W. (2011). Bacterial diseases of crabs: a review. *Journal of Invertebrate Pathology, 106*, 18-26.

Wang, X.W. & Wang, J.X. (2015). Crustacean hemolymph microbiota: Endemic, tightly controlled, and utilization expectable. *Molecular Immunology, 68*, 404-411.

Wang, X.W., Xu, J.D., Zhao, X.F., Vasta, G.R. & Wang, J.X. (2014b). A shrimp C-type lectin inhibits proliferation of the hemolymph microbiota by maintaining the expression of antimicrobial peptides. *Journal of Biological Chemistry, 289*, 11779-11790.

Weng, Y., Chen, F., Liu, Y., Zhao, Q., Chen, R., Pan, X., Liu, C., Cheng, Z., Jin, S. & Jin, Y. (2016). *Pseudomonas aeruginosa* enolase influences bacterial tolerance to oxidative stresses and virulence. *Frontiers in Microbiology, 7*, 1999.

Whitman, W.B. (2015). Bergey's manual of systematics of Archaea and Bacteria, Wiley Online Library.

Whitten, M., Davies, C.E., Kim, A., Tlusty, M., Wootton, E.C., Chistoserdov, A. & Rowley, A.F. (2014). Cuticles of European and American lobsters harbor diverse bacterial species and differ in disease susceptibility. *MicrobiologyOpen, 3*, 395-409.

Wlnstanley, R. (1976). Marking and tagging of the southern rock lobster *Jasus novaehollandiae* Holthius off Tasmania. *New Zealand Journal of Marine and Freshwater Research, 10*, 355-362.

Wu, J., Anderson, B.J., Buckley, H.L., Lewis, G. & Lear, G. (2017). Aspect has a greater impact on alpine soil bacterial community structure than elevation. *FEMS Microbiology Ecology, 93*.

Wu, Y., Wen, J., Ma, Y., Ma, X. & Chen, Y. (2014). Epidemiology of foodborne disease outbreaks caused by *Vibrio parahaemolyticus*, China, 2003-2008. *Food Control, 46*, 197-202.

Young, J.S. & Pearce, J.B. (1975). Shell disease in crabs and lobsters from New York Bight. *Marine Pollution Bulletin, 6*, 101-105.

Zeng, X., Xia, W., Wang, J., Jiang, Q., Xu, Y., Qiu, Y. & Wang, H. (2014). Technological properties of *Lactobacillus plantarum* strains isolated from Chinese traditional low salt fermented whole fish. *Food Control, 40*, 351-358.

Zha, H., Jeffs, A., Dong, Y. & Lewis, G. (2018a). Characteristics of culturable bacteria associated with tail fan necrosis in the spiny lobster, *Jasus edwardsii*. *Bulletin of Marine Science (Accepted, in press)*.

Zha, H., Jeffs, A., Dong, Y. & Lewis, G. (2018b). Potential virulence factors of tail fan necrosis associated bacteria in spiny lobster, *Jasus edwardsii*. *Journal of Fish Diseases, 41*, 817-828.

Zha, H., Jones, B., Lewis, G., Dong, Y. & Jeffs, A. (2018c). Multiple opportunistic bacteria associated with tail fan necrosis in spiny lobster, *Jasus edwardsii Submitted for publication*.

Zha, H., Jones, B., Lewis, G., Dong, Y. & Jeffs, A. (2018d). Pathology of tail fan necrosis in the spiny lobster, *Jasus edwardsii*. *Journal of Invertebrate Pathology, 154*, 5-11.

Zha, H., Lewis, G., Alfaro, A., Wang, S., Dong, Y., Grandiosa, R. & Jeffs, A. (2017). Immune status of the spiny lobster *Jasus edwardsii* with tail fan necrosis. *Diseases of Aquatic Organisms, 126*, 229-238.

Zhang, J., Liu, Y., Tian, L., Yang, H., Liang, G. & Xu, D. (2012). Effects of dietary mannan oligosaccharide on growth performance, gut morphology and stress tolerance of juvenile Pacific white shrimp, *Litopenaeus vannamei*. *Fish & Shellfish Immunology, 33*, 1027-1032.

Zhang, L., Wang, Y., Liang, J., Song, Q. & Zhang, X.-H. (2016a). Degradation properties of various macromolecules of cultivable psychrophilic bacteria from the deep-sea water of the South Pacific Gyre. *Extremophiles, 20*, 663-671.

Zhang, S., Pan, J., Lu, W., Yan, Y., Wang, H., Wiegel, J. & Zhao, B. (2016b). *Halomonas urumqiensis* sp. nov., a moderately halophilic bacterium isolated from a saline-alkaline lake. *International Journal of Systematic and Evolutionary Microbiology, 66*, 1962-1969.

Zhang, X., Zhu, Y.-T., Li, X.-J., Wang, S.-C., Li, D., Li, W.-W. & Wang, Q. (2016c). Lipopolysaccharide and beta-1, 3-glucan binding protein (LGBP) stimulates prophenoloxidase activating system in Chinese mitten crab (*Eriocheir sinensis*). *Developmental & Comparative Immunology, 61*, 70-79.

Zhang, Y. & Chen, Y. (2007). Modeling and evaluating ecosystem in 1980s and 1990s for American lobster (*Homarus americanus*) in the Gulf of Maine. *Ecological Modelling, 203*, 475-489.

Zheng, G., Chen, Z.-G., Jiang, R.-J. & Yang, Z.-J. (2015). *Feifantangia zhejiangensis* gen. nov., sp. nov., a marine bacterium isolated from seawater of the East China Sea. *Antonie van Leeuwenhoek, 108*, 1441-1447.

Zhi, S., Banting, G., Li, Q., Edge, T.A., Topp, E., Sokurenko, M., Scott, C., Braithwaite, S., Ruecker, N.J. & Yasui, Y. (2016). Evidence of naturalized stress-tolerant strains of Escherichia coli in municipal wastewater treatment plants. *Applied and Environmental Microbiology*, *82*, 5505-5518.

Ziino, G., Giuffrida, A., Stancanelli, A. & Panebianco, A. (2002). Shell disease in *Nephrops norvegicus* from the Mediterranean Sea. Morphological and hygienic remarks. *Archiv für Lebensmittelhygiene, 53*, 134-136.