Comparative Study of two Australian Coxiella species: *Coxiella burnetii* and nov. sp. *Coxiella cheraxi* (TO-98)

Thesis submitted by:

Sarah E. Powell

September 2013

Submitted for the degree Masters of Science

In the School of Veterinary and Biomedical Sciences

James Cook University

Townsville, Australia

STATEMENT OF ACCESS

I, Sarah Powell, author of this work understand that James Cook University will make this thesis available for use within the University Library and via the Australian Digital Theses Network for use elsewhere.

I understand that, as an unpublished work, this thesis has significant protection under the Copyright Act and I do not wish to place further restriction of access to this work.

Signature: **Date:**

September 2013

DECLARATION OF ELECTRONIC COPY

I declare that the electronic copy of this thesis provided to James Cook University Library is, within the limits of technology available, an accurate copy of the thesis submitted**.**

Signature: **Date:**

September 2013

STATEMENT OF AUTHENTICITY

I, Sarah Powell, declare that this thesis is my own work and has not been submitted in any form for another degree or diploma at any other university or institution of tertiary education. Information derived from the published and unpublished work of others has been acknowledged in the text and an appropriate list of references given.

Signature: **Date:**

September 2013

DECLARATION OF ETHICS

The research presented and reported in this thesis was conducted within the guidelines for research ethics outlined in the *National Statement on Ethics Conduct in Research Involving Human* (1999), *the Joint NHMRC/AVCC Statement and Guidelines on Research Practice* (1997), the *James Cook University Policy on Experimentation Ethics, Standard Practices and Guidelines* (2001), and the *James Cook University Statement and Guidelines on Research Practice* (2001). The research methodology used in this thesis received clearance from the James Cook University Experimentation Ethics Review Committee (approval number A1680).

Signature: **Date:**

September 2013

ACKNOWLEDGEMENTS

This thesis is really the work of many people who have taken the time, energy and care to teach, supervise and help me conduct this research over the past few years. First and foremost, I'd like to thank my supervisors, particularly Leigh Owens for his guidance and patience with all that came with taking me on as an advisee. I'd also like to thank Dr. Ketheesan, Brenda Govan and Dr. Lynne Van Herwerden for their support and feedback throughout the research process. I'd like to acknowledge and thank Dr. Alanna Cooper, Dr. Jodie Morris and Jaqui Stevens, whose patience and training in the PC3 lab made the first half of this entire thesis possible. I owe the second portion of this thesis to the generosity of MARFU PhD student Rifiloe Thubake, Dr. Chaoshu Zeng and John Stevenson, Colin Valverde and John Jennings whose kind crayfish donations and accommodation made the research process significantly less stressful.

I'd also like to thank the School of Veterinary and Biomedical Sciences and their staff for taking me on as a researcher. Jenny Elliman for helping me get back on track with my molecular techniques, Dr. Jeff Warner for helping me file all that last minute paperwork, Laurie Reilly for his accommodation with the histology lab, Kathy and Ellen for answering all my seemingly never-ending questions about microbes and turtles. Emily, Louise and all of the technicians who helped ensure my orders and equipment were available and ready to go. I'd also like to offer particular thanks and gratitude to Rusaini, whose friendship, kindness and expertise made a lot of my late nights in lab incredibly productive and enjoyable.

Thanks also to the rest of the Aquapath and Postgraduate Cohort: Orachun, Daniel, Jenna, Anita, Ku Suphan and the gang for their contributions and advice regarding laboratory and experimental design as well as their wonderful food parties on the lawn.

A large portion of gratitude also goes to Christopher Ende at the GRS for his compassionate and unwavering support on the administrative and frisbee dramas that came along with my thesis writing process.

I want to thank the numerous physicians and nurses who helped me recover from my more serious bouts of illness and stay on track for a healthy finish to this project.

Lastly I'd like to thank my friends and family, without whose emotional support throughout a particularly difficult personal period of this project would have left me sans degree. Jennifer Newton, Brooke Dillon, Marilou Camera, Emily Tucker, Amy D Ho, Greenlakers Amy & Greg, Dr. Miin Chua, HMQ Anne Sloan, Jessica Nowicki, Darren Coker, Yan Xiang, Amelia Wegner, Evan Torres, Phill Hoying, Marisa Maglaty, Michael Kramer, Jane Davey, Jenny Orbaker, Chelsea D'Aprile and Tim Price.

Further Acknowledgements:

This study was funded by James Cook University School of Veterinary and Biomedical Sciences. Editorial advice was given only by supervisory committee. The author has no commercial conflicts of interest and no connections to the manufacturers of any of the disinfectants or products discussed in this paper.

ABSTRACT

In current literature, the genus *Coxiella* is monospecific with *Coxiella burnetii* as the only described member. *Coxiella burnetii* is the causative agent of Q-fever, a virulent disease found worldwide spread through inhalation, tick bites and occasionally through the ingestion of contaminated milk products (Center for Disease Control, 2010). Recent research suggests that there are emergent pathogenic members of *Coxiella* to be described. In 1990, heavy losses in the Australian redclaw crayfish industry led to the discovery of a potential disease pathogen closely related to *Coxiella* causing up to 80% mortality in crayfish. The previously unrecognized disease was described histologically, revealing an aetiological organism similar to Rickettsia known to cause disease (Owens et al. 1992; Ketterer et al. 1992). An additional genetic study via 16S rRNA analysis revealed that this new pathogen had a 95.6% similarity to *Coxiella burnetii*, effectively indicating the organisms are of the same genus (Tan and Owens 2000; Cooper et al. 2007). The bacterium was designated *Coxiella cheraxi* by Tan and Owens (2000) and due to its phylogenetic similarity to *Coxiella* we are proposing further research to understand it and its infection potential.

This study seeks to compare *Coxiella burnetii* and *Coxiella cheraxi* with respect to infectivity, pathogenicity, histopathology and subsequent implications for biosecurity and public health. This was achieved in two parts, the first of which illustrates a novel method and experiment whereby the first known aquatic invertebrate (*C. quadricarinatus*) was infected with three doses of *C. burnetii,* a Level 3 pathogen. Twenty crayfish in four replicate dose groups (control, 10^4,10^5, 10^6 cells/ml) of five animals each were infected and monitored for twenty one days and mortality, histopathology, PCR, qPCR and sequencing assessments were made post-trial. The laboratory design and ensuing experiment were the first of their kind and were successful in illustrating a novel, risk-averse laboratory design for monitoring and maintaining aquatic invertebrates within a Physical Containment Level 3 (PC3/BSL-3) facility. This design is a potential model for future experiments seeking alternatives to vertebrate hosts. The subsequent infection experiment successfully demonstrated the initial host potential of *C. quadricarinatus* for *C. burnetii*, with results indicating that crayfish may be less susceptible to *C. burnetii* exposure and act only as carriers of the pathogen in doses as low as 1 to 2 organisms. These results differed significantly from observed *Coxiella cheraxi* infections within Australian redclaw which are known to cause high mortality and morbidity.

The second part of this thesis explores the clinical manifestation and origin of a 2012 presumptive *C. cheraxi* outbreak in Australian redclaw crayfish at the Marine and Aquaculture Research Facilities Unit (MARFU) at James Cook University in Townsville, Australia. Cytoplasmic inclusion bodies, strong Gram negative bacteraemia and heavy mortalities observed in initial screening histopathology were strongly suggestive of previously observed *C. cheraxi* incidents (Owens et al. 1992, Ketterer et al. 1992). Resultant 16S rRNA sequencing indicated an 86% identity match to *C. cheraxi* and no other known pathogens thus tentatively suggesting the re-emergence of this pathogen within redclaw stock at MARFU and elsewhere. The work goes on to outline the consequent biosecurity response and diagnostic steps taken, and the successful tracking of the outbreak origin to one of three supplying commercial crayfish farms in northern Queensland. Finally the thesis illustrates the implications of the comparisons between *C. cheraxi* and *C. burnetii* as they relate to the fields of aquaculture, biosecurity and public health.

TABLE OF CONTENTS

Chapter Three

Novel system for maintenance and monitoring of aquatic invertebrates infected with a Level 3 organism

Chapter Four

Experimental Infection of *Coxiella burnetii* in Australian redclaw crayfish

Chapter Five

Evidence of RLO and *Coxiella cheraxi* infections in MARFU facility stock

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

16S rRNA	16S ribosomal RNA
ANOVA	Analysis of Variance
ATV	Antibiotics,Trypsin,Versene
Bp	Base Pair
BSC II	Biosafety Cabinet Level 2
BSL-3	Biosafety Level 3 (USA)
CDC	Center for Disease Control (USA)
Com1	Com1 protein PCR target
Ct	Cycle threshold value (qPCR)
DRIP	Phylogenetic clade in the Class Mesomycetozoea
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal Bovine Serum
H&E	Haematoxylin and Eosin Stain
Hep	Hepatopancreas
IUPAC	International Union on Pure and Applied Chemistry
LCV	Large cell variant
LPS	Lipopolysaccharide
MARFU	Marine and Aquaculture Research Facilities Unit (JCU)
MLSA	Multi-locus Sequence Analaysis
NIH	National Institute of Health (USA)
NHP	Necrotizing hepatopancreatitis
NMII/C4	Nine Mile II Clone 4 Strain
NTC	No-Template Controls (qPCR)
PBS	Phosphate buffered saline
PC3	Physical Containment Level 3 (Australia)
PCR	Polymerase Chain Reaction
PM	Pico-molar
PPE	Personal Protective Equipment
qPCR	Real-time Polymerase Chain Reaction
RLO	Rickettsia-like organism
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid

SCV	Small cell variant
SDC	Small dense cells (endospores)
SPD	Stained Prawn Disease
TE	Tris-EDTA
TEM	Transmission Electron Microscopy

GENERAL INTRODUCTION

Overview

Coxiella burnetii is the causative agent of Q-fever, a virulent disease found worldwide spread through inhalation, tick bites and occasionally through the ingestion of contaminated milk products. *C. burnetii* is global zoonoses with not only expansive reservoirs in mammals, but also birds and arthropods including ticks and crustaceans. As such its potential to infect other populations is significant given environmental opportunity (CDC, 2010).

In current literature the genus *Coxiella* is monospecific with *Coxiella burnetii* as the only described member. However, recent research suggests that there are more members of *Coxiella* to be described. Mass mortalities in the Australian redclaw crayfish industry in the 1990s led to the sequencing of one of the first such organisms revealing a potential new species of *Coxiella* bacteria, *Coxiella cheraxi* which is 95.6% homologous to *Coxiella burnetii* (Tan and Owens, 2000; Cooper et al. 2007). *C. burnetii* is classified as a Category B biological warfare agent and is known to cause virulent, debilitating disease in a wide-range of hosts including humans (Angelakis and Raoult 2010). This discovery opens doors to developing diagnostic and culturing techniques towards other potentially virulent RLO infections within both crustacean and human hosts and has the potential to act as a model towards improved prevention and vaccine development for Q-fever and related Rickettsiae infections.

In March of 2012, a presumptive outbreak of *Coxiella cheraxi* occurred at the Marine and Aquaculture Research Facility Unit (MARFU) at James Cook University, prompting the rapid screening and containment of approximately 200 redclaw crayfish. The diagnostic proceedings and general observations conducted during the investigation further contributed knowledge about *C. cheraxi* as a pathogen and provided a unique opportunity from which to trace the origin of the infection from local aquaculture farms. The outbreak also led to improved awareness about biosecurity and the risks associated with RLOs as well as other pathogenic organisms within an aquaculture environment.

This thesis seeks to illustrate a preliminary comparative study of the two Australian *Coxiella* species: *Coxiella burnetii* and *Coxiella cheraxi*. It observes how their similarities and differences in host preference, clinical manifestation and structure may contribute to the aquaculture and agriculture industries, biosecurity, defence and public health. Additionally we outline important gaps in research knowledge comparing emergent *Coxiella* species to better recognize the virulence, mechanisms of transmission and pathogenicity of these infectious agents.

Research Aims

The research aims for this project were based on two main components, the study of *Coxiella burnetii* infections within Australian redclaw and the study of a *Coxiella cheraxi* outbreak within Australian redclaw. The aims were as follows:

1) Determine the potential of *Coxiella burnetii*, a known pathogen in humans and livestock, to infect Australian redclaw crayfish *Cherax quadricarinatus* in order to identify whether Australian redclaw may also act as host to the human pathogen. If so, identify any clinical similarities between *Coxiella burnetii* and *C. cheraxi* infections in crayfish.

2) Detect, isolate and sequence presumptive samples of *Coxiella cheraxi* a closely related bacteria to *Coxiella burnetii,* within a suspected local outbreak of the pathogen at MARFU.

3) Determine a timeline and track infection origin of the MARFU outbreak of presumptive *Coxiella cheraxi* from supplying farms

4) Develop a preliminary comparative illustration of both *Coxiella* pathogens for future consideration.

CHAPTER 1

REVIEW OF THE LITERATURE

1.1 Introduction to Rickettsiae

Rickettsiae, described broadly, are Gram-negative obligate intracellular bacteria that cause fastidious and highly infectious diseases in humans worldwide including Rocky Mountain Spotted Fever, Typhus, Q-fever and African Tick Bite Fever (Scola and Raoult, 1997). Rickettsiae grow within the cytoplasm or nucleus of eukaryotic cells and multiply via host-cell destruction (Winkler 1990). Research over the past few decades shows Rickettsiae and Rickettsiae-like organisms (RLOs) as increasing pathogens with the aquaculture industry as well as within the field of public health.

The subfamily Rickettsiae currently contains 3 distinct genera: *Coxiella*, *Rickettsia* and *Rochalimaea*. While there has been some dispute over the actual placement of *Coxiella* within this group taxonomically, physiologically and clinically *Coxiella burnetii* is globally recognized as a Rickettsiae pathogen (American Society for Rickettsiology, 2013). Previous research has focused primarily on medically significant Rickettsiae and arthropod endosymbionts *Wolbachia* and *Cardinium*, with little focus on other arthropod Rickettsiae (Weinert et al. 2009). In recent years, however, more Rickettsiae infections have emerged and with the advent of 16S rRNA testing, been found to pose more serious potential economic and public health risks. In particular, emergent *Coxiella* bacterial forms were discovered in ticks and freshwater crayfish (Tan and Owens, 2000; Cooper et al. 2007; Jasinskas et al. 2007). As such, there has been a greater interest in studying and understanding more about these potentially pathogenic organisms. This is particularly true within aquatic environments where the effects of climate change in recent decades are of increasing importance with regards to rises in bacterial infections. Aquatic organisms are particularly vulnerable to elevated temperatures which often lead to a rise in microbial growth, reduction in oxygen levels and immunosuppression (Le Moullac and Haffner, 2000). To communities and markets dependent upon the health and reproductivity of potentially infected aquaculture species, further research to isolate pathogenic agents is imperative to maintaining successful populations.

1.2 *Coxiella burnetii*

A member of the subfamily Rickettsiae, *Coxiella burnetii* is a Gram-negative obligate intracellular cocillobacillus bacterium. Organisms range in size from 0.2-0.3µm by 0.7-1.0µm (Mattix et al. 2006). Phylogenetically they are most closely related to *Legionella* species. *C. burnetii* is distinguished from other Rickettsiae agents in that it contains endospores within infected host cells and undergoes complete lifecycle changes within the phagolysosome of the infected host cells (Mattix et al. 2006). *C. burnetii* occurs intracellularly in three morphologic forms: small cell variants (SCV), large cell variants (LCV) and small dense cells such as endospores (SDC). Each cell form has distinctive antigenic, morphological and metabolic differences as well as varying levels of resistance and virulence. Evidence suggests that SCV and SDCs, while metabolically inactive, are the bacteria within hosts that infect other host cells. LCVs express LPS on the cell surface and are metabolically active (Mattix et al. 2006; Angelakis and Raoult 2010). *Coxiella* strains are characterized by differences in lipopolysaccharide (LPS) biosynthesis and level of virulence which are separated into two antigenic states: phase I and phase II (Mattix et al. 2006). Chronic infection is marked by high levels of antibodies to phase I antigens while phase II antibodies are produced during acute infections. Asymptomatic cases are also marked by phase II antibodies (Knobel et al. 2013). Highly virulent phase I strains are most closely associated with a smooth LPS and phase II with a truncated or rough LPS surface. Phase II and phase I strains share an identical lipid A and some core sugars, however phase II lacks some essential biomarker sugars to *C. burnetii* including O-antigen sugars virenose and dihydrohidroxystreptose (Hoover et al. 2002).

As with most rickettsial pathogens, *Coxiella burnetii* is highly resistant to drying, heat and most disinfectants, making it particularly resilient and dangerous. It also has an unusually low infectious dose. In 1958 a formerly clandestine study by the United States Army, Operation Whitecoat, determined the infectious dose of *C. burnetii* required for acute infection in humans to be as few as 1-2 organisms (Pittman 2005; Jones et al. 2006). Due to this characteristic as well as the severity of individual infections, the United States government has classified *Coxiella burnetii* as a Class 3 category B biological terrorism agent for its threat potential in national and international bio-terrorism (Rotz et al. 2002; CDC, 2010). Research of *Coxiella* species and publication of new species has interdisciplinary relevance and is an important contribution towards global health research, biosecurity, aquaculture, agriculture and defence.

1.3 Q-fever

Coxiella burnetii is the known etiological agent of Q-fever, a virulent and often debilitating disease spread through inhalation, tick bites and occasionally through the ingestion of contaminated milk products. Historically, Q-fever manifests in a wide clinical spectrum of disease ranging from asymptomatic to seriously acute and chronic infections including endocarditis, vascular infection, and pneumonia (Knobel et al. 2013). Despite Q-fever's highly ubiquitous distribution world-wide, prior to epidemic outbreaks such as those observed in the Netherlands in 2007-2010 Q-fever was, and still is in some regions seldom considered a notifiable disease. As such, it is difficult to fully assess annual global incidence and morbidity of *Coxiella burnetii* infections. The Netherlands outbreak of over 3,500 recorded cases illustrates the severe and re-emergent nature of *C. burnetii* and highlights the necessity for improved control and surveillance methods. Epidemiological studies strongly suggest that Q-fever should be considered a current and emerging public health problem globally, particularly in Germany, France, Belgium, Switzerland, Italy, Spain, the United Kingdom, Greece, Israel, Kenya, Senegal, Canada and the United States as well as many other countries where Q-fever prevalence may be under reported or poorly surveyed (Scola and Raoult 1999; Waag 2007). In 2013, the CDC published a report that a new, more virulent strain of Q-fever has begun to circulate in French Guiana where the incidence rate has steadily been increasing since 1996 and at 150 cases/100,000 persons it is one of the highest recorded annual incidence rates in the world for Q-fever (Mahamat et al. 2013). This case particularly illustrates the emergent nature of *C. burnetii* infections and its relevance on a global scale.

1.4 Ubiquity and environmental occurrence of *Coxiella* and *Rickettsia*-like organisms (RLOs)

1.4.1 Hosts and Vectors

It is estimated that over two-thirds of the world's infectious diseases (approximately 1 billion worldwide) are caused by pathogens spread by wild and domestic animals (Karesh et al. 2012). *Coxiella burnetii* is a global zoonoses contributing to these estimates with expansive reservoirs in cattle, sheep, goats, canines, cats, squirrels, rats, mice as well as pigeons, chickens, ducks and other wild bird species (Mattix et al. 2006). Reptiles such as snakes, monitors and tortoises are also confirmed hosts (Yadav and Sethi 1979; Yadav and Sethi 1980). Marine mammals, including South American fur seals and South American sea lions have also exhibited infections indicating the versatility of *C.burnetii* in a range of

environments and habitats (Jurczynski and Flugger 2005). Arthropods are obligate primary hosts for all known Rickettsiae and current research indicates that most vertebrate Rickettsiae are first vectored by arthropods (Weinert et al. 2009). While most commonly found in ticks, Rickettsiae have also been detected in spiders and a number of crustaceans (Cordaux et al. 2007; Wang et al. 2011). As such its potential to infect other populations is significant given environmental opportunity (Mattix et al. 2006).

Geographically, *Coxiella* sp. have been detected worldwide on every continent except Antarctica and most countries with the exception of New Zealand. Cases resulting in infection and illness occur both in the developing and developed world with little apparent discrimination as most recently demonstrated by epidemic outbreaks in the Netherlands between 2007- 2010 where over 3,500 human cases were reported . Numerous smaller outbreaks have been reported almost annually in Europe, East Africa, the Middle East and North America (Kersh et al. 2010; Knobel et al. 2013; Van der Hoek et al. 2012).

Q fever remains a significant occupational hazard worldwide to abattoir workers, farmers, veterinarians and those in contact with livestock such as sheep, cattle and goats as well a number of other domestic and wild ruminants. Recent research also indicates that some endemic wildlife as well as domestic pets may also act as carriers to the disease (Cooper et al. 2011). Due to their apparent versatile nature in infecting a broad variety of hosts as well as a wide geographic distribution, further study of the environmental occurrence of Rickettsiae has potentially significant implications for agriculture, aquaculture and public health.

1.4.2 RLOs in Aquaculture Species

Economically, rickettsial pathogens affect aquaculture industries in both freshwater and marine environments. They are increasingly an instrument of the success or failure of these industries as some infecting agents have been known to cause up to 95% mortality with crippling consequences to those dependent upon them (Aranguren et al. 2006). In recent research Rickettsiae-like organisms have been described in a range of economically significant aquaculture species including oysters (Sun and Wu, 2004;), black abalone (Ben-Horin et al. 2013), the giant clam (Norton et al. 1993) scallops (Elston, 1986), mussels (Mialhe et al. 1987), Coho and Atlantic Salmon (Garces et al. 1991; Fryer et al. 1994) and a number of crustaceans (Wang 2011). Due to their ubiquitous range of hosts and virulence, a particularly rampant or infective RLO infection has the potential to devastate aquaculture industries worldwide.

1.5 RLOs in marine and freshwater Crustacea

The first Rickettsiae-like organism infection (RLO) in a crustacean was described in 1970 by Vago et al. in a terrestrial isopod *Armadillidium vulgare* from France. Subsequent RLO discoveries include infections in a wide range of crustacean hosts including amphipods, crabs, freshwater crayfish, shrimp and prawns (Federici et al. 1974; Nunan et al. 2003; Eddy et al. 2007). Rickettsial infections within Crustacea appear to occur in one of two types of infection as defined by tissue tropism (Tan and Owens, 2000). The first type of infection affects connective tissues throughout the body causing systemic infection while the second type of infection affects hepatopancreatic cells. The hepatopancreas serves as a digestive gland for crustaceans and is analogous to the liver in mammalian species. Interruption of this gland affects nutrient uptake and moulting strength (Owens et al. 1992).

1.5.1 Amphipods

The first Rickettsiae infection to be described in an aquatic species occurred in *Crangonyx floridanus* a freshwater amphipod from Florida in the United States. RLO infection exhibited intracytoplasmic saturation and lysis not unlike that seen in subsequent studies of crustacean infection with infection rates as high as 40% in selected populations (Federici et al. 1974; Nunan et al. 2003). There is no record of genetic analysis or additional classification for this RLO since the technology became available.

1.5.2 Crabs

RLO infections in freshwater and marine crabs are rare but significant as they show high mortality and virulence within affected populations. The first described pathogenic Rickettsiae infection in a marine crustacean was in the Mediterranean Shore crab *Carcinus mediterraneaus* by Bonami and Pappalardo in 1980. RLO infection in the Sète region of Southern France on the Mediterranean was believed responsible for mass mortalities among the Mediterranean shore crab *C. mediterraneaus* in the late 1970's (Wang 2011). Rickettsiae-like organisms were found in the cytoplasm of connective tissue between hepatopancreatic tubules as well as within the gut, gills and gonads of infected individuals. Additionally organisms were shown to multiply within intracytoplasmic vacuoles eventually causing cell lysis in infected host cells (Bonami and Pappalardo, 1980). Experimentally infected *C. mediterraneaus* died 15 days post-infection; however the method of inoculation did not necessarily mirror that of a natural infection. Further pathogenic classification and identification of this organism since the advent of 16S rRNA analysis has yet to take place.

The Blue King Crab *Paralithodes platypus* from St. Lawrence Island in the Eastern Bering Sea Alaska was also shown to exhibit at least one case of terminal rickettsial infection. The previously unknown RLO was found in a juvenile female. Infection appeared limited to hepatopancreatic tissues, causing partial to full necrotization of the tissue and cessation of normal function (Johnson 1984). The infection of hepatopancreatic epithelium in this scenario is consistent with other RLO infections found in crustaceans targeting primarily hepatopancreatic tissue (Owens et al. 1992; Wang 2011).

Other crab species with at least one or more known RLO infections include Golden king crabs, *Lithodes aequispina* and blue crabs *Callinectes sapidus* . Prevalence within aquacultural systems is relatively low, however and reported RLO prevalence was less than 3% within sampled shedding facilities (Messick and Kennedy, 1990). Additionally infected crabs from these populations exhibited little pathology (Messick, 1998). Further elucidation or investigation into these infectious organisms has yet to take place.

1.5.3 Prawns and Shrimp (Penaeids)

Studies of RLO infection within prawns and shrimp have shown significantly fewer mortalities than in other crustaceans however, understanding the pathogenicity and mechanisms of these infections are useful for future reference within the market. The spot prawn *Pandalus platyceros*, an economically important trap fishery species in British Columbia, was also found to be host to a previously undescribed RLO (Bower et al. 1994). Populations were exhibiting abnormal dark discoloration along the carapace and along the edges of body segments, a condition called stained prawn disease (SPD). When sampled and histologically examined, the disease was shown to be the result of a Gram-negative RLO. Affected prawns exhibited melanised haemocytic encapsulations below the cuticular epithelium as well as within adjacent connective tissues in advanced infection. Additionally melanotic nodules were found on the surface of the hepatopancreas and heart tissue of infected individuals. Phagocytes and hemocytes were the only observed infected cells containing granular micro colonies of Rickettsiae-like organisms with granular inclusion bodies up to 20 µm in diameter. During early stages of disease progression, experimentally infected prawns exhibited only hepatopancreatic infection via melanotic encapsulations. Ultrastructure of infected tissues indicated that the RLO causing SPD was monomorphic, with a spherical morphology and underwent reproduction via binary fission (Bower et al. 1994).

Necrotizing hepatopancreatitus (NHP) is a significant disease of Pacific white shrimp: *Penaeus vannamei* , an economically important species within the global prawn aquaculture industry (Krol et al. 1991). A novel Rickettsiae-like organism was isolated after severe NHP losses (between 20-95% mortality) caused difficulties for shrimp farming in Texas and Central and South America. Disease was characterized by infection within epithelial cells of the hepatopancreas where multiplication of 3 varying morphological forms of the same RLO was found within necrotized tissue (Frelier et al. 1992; Loy et al. 1996). While previously similar hepatopancreatic infections by RLOs have been described in penaeids *Penaeus marginatus* and *Penaeus stylirostris* (Brock et al. 1986) as well as *Penaeus merguiensis* (Lightner et al. 1985), descriptions of differing morphological forms of the same infection were not previously mentioned. Subsequent 16S rRNA analysis of infected cells from *P. vannamei* confirmed its phylogenetic relationship to Rickettsiae, although additional study and cultivation of these RLO pathogens for infectivity and further genetic placement has remained difficult. Recent advancements have identified the NHP causing RLO as a pleomorphic, Gram-negative, intracytoplasmic bacterium. It is a member of the α-subclass of proteobacteria but the organism remains to be further classified (Frelier et al., 1992; Lightner & Redman, 1994; Loy et al., 1996; Orales-Covarrubias 2010).

One of the few other sequenced examples of an RLO infecting shrimp species is from a study of farm-raised *Penaeus monodon* in Madagascar. Studies of severe mortalities in grow-out ponds yielded clinical signs of shell necrosis, white hepatopancreatic tissues and slowed clotting of haemolymph (Nunan et al. 2003). Histological examination showed more systemic tissue infection affecting connective tissues and cuticular epithelial cells rather than hepatopancreatic tissue infection. Detection methods used to detect and isolate specific RLO included PCR and *insitu* hybridization assays developed to recognize the 16S rRNA region of *Rickettsia* bacteria. Based upon these sequencing techniques as well as the organism's size, morphology, cytoplasmic locale and Gram-stain reaction the bacterium was characterized as a rickettsial agent (Nunan et al. 2003). However, further comparisons to 16S rRNA sequences from the same gene in other bacterial organisms are necessary before complete classification may take place.

Hepatopancreatic RLOs in penaeid populations have been reported globally in Hawaii and Mexico (Lightner 1996), Singapore (Chong and Loh, 1984), Brazil (Cohen and Isaar, 1990), the Mediterranean Sea (Vogt 1994) and Adriatic Sea (Vogt and Štrus, 1998). Systemic prawn RLOs have been reported in Malaysia (Anderson et al. 1987), Indonesia (Lightner et al. 1992) and Canada (Bower et al. 1996). RLO-infected *Fenneropenaeus chinensis* lymphoid

cell cultures were reported from China (Wang et al. 2011). RLOs have not been reported in wild or cultured prawns from Australia; however they have been isolated from farmed Australian freshwater redclaw crayfish, *Cherax quadricarinatus* (Owens et al. 1992; Ketterer et al. 1992; Tan and Owens 2000).

1.5.4 Freshwater Crayfish

Australian redclaw crayfish (*Cherax quadricarinatus*) are native to northern Australia and southern Papua New Guinea but they are farmed commercially worldwide, including countries such as Israel, China and Ecuador (Tan and Owens, 2000). Australian redclaw are ideal aquaculture species as they readily reproduce, are non-aggressive and are naturally adapted to tropical water temperatures (Jones 1990; Ketterer et al. 1992). Diseases affecting these animals are significant to the industry. In 1990, heavy losses in the Australian redclaw crayfish industry led to the discovery of a potential disease pathogen closely related to *Coxiella* causing up to 80% mortality in redclaw crayfish. Two variant species or strains of *C. cheraxi* have tentatively been described within the literature including a systemic and hepatopancreatic infecting forms. Systemic infection is characterized by dense basophilic microcolonies of Rickettsiae-like organisms within all organ systems, connective and epithelial vessels (Owens et al. 1992; Edgerton et al. 1995; Edgerton and Prior 1999). Infection within the hepatopancreas was characterized by inclusion bodies which post-replication lead to necrotization of hepatopancreatic tissue and eye stalks (Tan and Owens, 2000). The infection resembled that described by Bower et al. (1994) in prawns whereby infected individuals exhibited melanised nodules although these nodules were not as frequently noted in infected animals as sample sizes increased (Jiménez and Romero, 1997; Romero et al. 2000). The previously unrecognized disease in *C. quadricarinatus* was described histologically, revealing strong evidence of an etiological organism similar to *Rickettsia* bacteria known to cause disease. This organism was isolated and examined using transmission electron microscopy (TEM) in both Australia and Ecuador where it was believed to be of similar ancestry (Owens et al. 1992; Jiménez and Romero, 1997; Edgerton and Prior, 1999; Cooper et al. 2007). Additional genetic study via 16srRNA analysis revealed that this new pathogen exhibits a 95.6% homology to *Coxiella burnetii,* a known human pathogen. The bacteria was designated TO-98 and due to its phylogenetic similarity to *Coxiella* it is a suspected new species within the genus and has been tentatively named "*Coxiella cheraxi*" (Tan and Owens, 2000; Cooper et al. 2007).

1.6 Identification of *Coxiella cheraxi*

In current literature the genus *Coxiella* is monospecific with *Coxiella burnetii* as the only described member. However, as is evidence by the abovementioned research by Owens et al. (1992), Ketterer et al. (1992), Edgerton and Prior (1999), Tan and Owens (2000) and Cooper et al. (2007), there is significant evidence for the classification of an RLO infecting *Cherax quadricarinatus* closely resembling a new *Coxiella* species. Additionally, studies across many other arthropod groups have yielded a plethora of potential new *Coxiella* members. In a 2006 sampling of ticks from national parks in the United States, 53% of those sampled yielded bacterial species with 90% genetic homology or greater to *Coxiella* sp. using 16SrRNA methods (Jasinskas et al. 2007). *Coxiella*-like bacteria have been detected in hard ticks *Haemaphysalis longicornis, Rhipicephalus sanguineus* and soft tick *Ornithodoros moubata* (Jasinskas et al. 2007). At least one 16SrRNA study places *Rickettsia grylli* from crickets in close relation to the genus *Coxiella* (Cordaux et al. 2007). These findings highlight the current gap in classification and understanding we have for potential new pathogens and demonstrate the need for definitive classification of *Coxiella* species.

The potential discovery of a new *Coxiella* species has both economic and biomedical significance as the genus *Coxiella* is currently monospecific with the only described member an exceptionally virulent pathogen known to cause Q-fever. A new species within this genus provides a number of opportunities to further explore the significance of this finding. The remainder of this review will focus on this particular finding within *Cherax quadricarinatus* crayfish with *Coxiella* sp due to its close relation to vertebrate pathogens, complex development, pathogenicity and significance in both veterinary and public health studies.

1.7 *Coxiella cheraxi* as a Model for Infectivity and Vaccine Development

Coxiella cheraxi is one of the best categorized and most studied of any current RLO infections. Studies by Owens et al. (1992), Ketterer et al. (1992) Jimenez and Romero (1997) Edgerton and Prior (1999), Tan and Owens (2000) and Cooper et al. (2007) have all addressed the morphology, pathogenicity and infectivity of this novel RLO. Research has shown that *C. cheraxi* can be transmitted horizontally among redclaw populations, and it is believed that the gills may serve as a transmission site (Tan and Owens 2000). Despite frequent exposure to infected crayfish and worldwide translocation of Australian redclaw, no human infections from *C. cheraxi* have been reported and it is believed that slight

differences in organism morphology may account for this. Research by Cooper et al. (2007) suggests that *C. cheraxi* is morphologically a closer match to Phase II avirulent strains of *Coxiella burnetii,* as it lacks a smooth LPS within the cell wall. As such, it poses little risk for human infection but has a significant potential in providing further categorization and understanding for an improved vaccine which carries fewer risks than the current Q-Vax alternative. The close phylogenetic homology between *Coxiella burnetii* and *Coxiella cheraxi* (95.6%) as well as the 100% homology to the outer membrane protein com1 gene within *C. burnetii* indicates that *C. cheraxi* may elucidate a similar antigenic response within humans without manifestation of Q-fever infection, and therefore it may be a viable vaccine candidate (Cooper et al. 2007). Additional infectivity studies and definitive sequencing and classification will be necessary before such a conclusion can be made, however the prospects are promising.

While there are currently several vaccine alternatives being considered , as of 2013, there were only three existing vaccines available to protect against Q-fever infection and of these, only one has been accepted for safe and viable use in Australia (Angelakis and Raoult 2010; Peng et al. 2012). The whole-cell formalin inactivated Phase I vaccine Q-Vax ® is widely used throughout Australia and worldwide to protect commercial, abattoir and veterinary workers against infection. However, due to problems with hypersensitivity in individuals with previous *C. burnetii* exposure, the vaccine can only be given once and does not provide complete lifetime protection and immunity for some individuals (Chiu and Durrheim 2007; Angelakis and Raoult, 2010). Understanding the mechanisms of infection and isolating related *Coxiella* species from arthropods provides a potential opportunity for improved vaccine development. If live strains of *C. cheraxi* may be isolated from wild crayfish populations and subsequently used to induce antigenic response within mammals without clinical manifestation of disease then it may be possible to develop a safer, more effective commercial vaccine against Q-fever without the hypersensitivity risks associated with the current formalin inactivated Phase I form.

1.8 Research Trends

Most literature outlined within this review described the results of opportunistic findings, whereby a chance infection or mass mortality event initiated most research interest at the time rather than any global event or investigative trend. One particular area which may constitute a turning trend within the research would be within the past decade in

identifying the probability that *Coxiella* species are far more variant and abundant than was previously thought of this originally monospecific genus as represented by tick and crustacean samples. The increased interest in *Coxiella*-like bacteria in tick species, particularly in the US and in the isolated RLO from Australian redclaw crayfish are in turn generating elevated public health interests (Jasinskas et al. 2006; Cooper et al. 2007). While this research is still very much in its infancy, as no definitive species or complete sequences have yet to be published, this is a significant step towards a greater understanding of the pathogenic potential between human and arthropod hosts from the terrestrial, aquatic and marine environments.

1.9 Proposed Research Direction and Significance

The main constraint in the characterization and definitive classification of a majority of the above-named RLO infections within Crustacea is a lack of available organisms and data. Many of the above described infections occurred before adequate 16S rRNA technology was available or widely accepted making accurate phylogenetic molecular analysis ambiguous at best. Studies such as those by Vago et al. (1970), Bonami and Pappalardo (1980)and Brock (1986) rely primarily on physical description, TEM, light microscopy, fixed histological samples and comparison to known infecting agents for classification. It was not until the 1990s that 16S rRNA analysis emerged within these types of studies to properly sequence an RLO, allowing researchers to match infections to a specific family and genus on a molecular basis. Due to this fairly recent advancement, only a handful of the above described infections have been further elucidated via genetic analysis and none have been definitively classified (Table 1.1).

Of the selected studies, the critical issues were (i) the absence of followup investigations to determine prevalence or obtain isolated samples and (ii) the lack of cultured isolates from which to determine infectivity or sequence data. It is clear that there are significant research gaps in understanding the phylogenetic origin, pathogenicity and indeed, general abundance of Rickettsiae within the environment. The following thesis research intends to help breach this gap in understanding and data and while by no means comprehensive, offer some valuable contributions towards future RLO investigations.

Table 1.1:

Summary of Rickettsiae and Rickettsiae-like organism (RLO) infection in Crustacea

Host	Location/ Habitat	Tissue Infected	Tentative Identity Sequenced?	Source
Isopod *A. vulgare*	France Terrestrial	Connective and adipose tissue	*Rickettsiella grilli* Not sequenced*	Vago et al. (1970)
Amphipod *C. floridanus*	USA Freshwater	Hepatopancreas, epidermis, and free hemolymph	*Rickettsiella grilli* Not sequenced*	Frederici et al. (1974)
Crab *C. mediterraneus*	France Marine	Connective tissue of hepatopancreas, gut, gill and gonad	Rickettsiae group related to *Wolbachia* and *Rickettsiella* Not sequenced*	Bonami and Pappalardo (1980)
Crab *P. platypus*	USA/Alaska Marine	Hepatopancreatic epithelial cells	*Rickettsiella* Not sequenced*	Johnson (1984)
Shrimp *P. marginatus*	USA/Hawaii Marine	Hepatopancreatic epithelial cells	Family Rickettsiae Not sequenced*	Brock et al. (1986)
Shrimp *P. vannamei*	USA/Peru Marine	Hepatopancreatic epithelial cells	Family Rickettsiae No further ID 16S rRNA sequenced	Krol et al. (1991) Frelier et al.(1993) Loy et al. (1996)
Shrimp *P. monodon*	Madagascar Marine	Connective and epithelial tissues and free hemolymph	*Rickettsiella* sp. No further ID 16S rRNA sequenced	Bower et al. (1996) Nunan et al. (2003).
Crayfish *C. quadricarinatus*	Australia Freshwater	2 Types i)Connective tissues ii) Hepatopancreatic epithelial cells	Family Rickettsiae Genus *Coxiella* Species "*cheraxi*" **suggested** 16S rRNA sequenced	Owens et al. (1992) Cooper et al. (2007)

***** Adapted from Bower et al. 1996

1.10 Relevance to Current and Future Public Health

In March 2013, the CDC issued the first national reporting guidelines addressing Q-fever recognition, clinical diagnosis, treatment, management and care for healthcare professionals and occupational workers. The guidelines address treatment for both chronic and acute phases of the disease in all populations including pregnant women, children and occupational professionals at risk and it is eventually hoped a similar reporting model will

be adapted on a global scale (CDC 2013). Rickettsial infections remain a scourge to human populations worldwide and Q-fever is a very real problem for agricultural workers and those within the industry (veterinarians, abattoir workers) as well as agrarian populations worldwide. Its infecting agent, *C. burnetii*, is known to cause debilitating illness and is categorized by the US government as a bio warfare agent. As such it is also an increasing defence and public health concern (CDC, 2010).

Rickettsiae infections within commercially important species has implications not only for economic reasons when causing high mortality, but also for public health should any infections have transmission potential to human consumers as many Rickettsiae have shown versatility in transmission from arthropods to mammalian hosts (Mattix et al. 2006). *Cherax quadricarinatus* a commercially important stock in Australia and worldwide has proven a useful model from which to isolate a rickettsial agent and explore its genetic origin and infection potential. We therefore propose that further investigation and elucidation of the RLO infection isolated from Australian redclaw crayfish act as a launch point into definitive classification of RLO infection and uncovering its implications for both public health and the aquaculture industry.

By using genetic comparisons to known virulent Rickettsiae, we are better able to target known virulence genes that act as indicators of pathogenicity to identify possible epidemic threats. Additionally the culture and study of live samples of infected organisms is imperative if we are to better understand transmission and infectivity potential among aquaculture species as well as mammals for the purposes of vaccine development. It is clear from already published reports that further investigation and comparison of *C cheraxi* and *C. burnetii* is an ideal avenue from which to gain this knowledge.

While a number of potential new *Coxiella* and *Rickettsiella* species in arthropods have been identified via 16S rRNA sequencing in recent years, very little additional research has been done to establish the molecular taxonomic placement of many of these RLOs, particularly those infecting crustaceans (Jasinskas et al 2007; Cordaux et al 2007). Indeed, the most work to date on crustacean RLOs, according to available literature, has been studies by Tan and Owens (2000) Romero et al. (2000) and Cooper et al. (2007) in an attempt to definitively classify TO-98 as a new *Coxiella* species.

The absence of definitive and conclusive genetic information for any one of the abovementioned organisms, particularly *C. cheraxi* , further illustrates the necessity to investigate these organisms with more scrutiny and actively pursue environmental samples rather than leave discoveries to opportunistic happenstance as has been the historical trend. In the absence of information regarding diagnostic tests or long term follow up studies, it is thus far impossible to identify true prevalence of RLO infection within crustacean populations mentioned in this review. In a world of increasing public health risks, climate change induced microbial blooms and international instability, it is imperative that we gain a better understanding of the rickettsial organisms that cause(or are closely related to the bacteria causing) some of the most ubiquitous, virulent human diseases worldwide.

The research undertaken for this thesis seeks to create a more comprehensive comparative profile of *Coxiella burnetii* and *Coxiella cheraxi* emergent pathogens within Australian redclaw in the hopes of providing valuable contributions and appropriate admonishments to infectivity studies, laboratory design, diagnostic technique, biosecurity measures and analysis for future investigations.

CHAPTER 2

GENERAL MATERIALS AND METHODS

2.1 Biosafety

The James Cook University Biosafety Approval Number for this project is MI11-09**.**

2.2 Ethics Statement

This experiment was carried out in full compliance of institutional animal ethics (JCU A1680) and conducted in accordance with National Health and Medical Research Council, *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes 7th Edition, 2004*; and in compliance with the *Queensland Animal Care and Protection Act, 2001.*

2.3 Experimental Animals

Australian redclaw crayfish (*Cherax quadricarinatus*) were used as the primary experimental animal for this study (Fig 2.1). *C. quadricarinatus* are a useful laboratory species as they are non-aggressive, fast-growing, robust and can survive a range of water temperatures, pH and dissolved oxygen levels (Masser and Rouse, 1997). Redclaw were sourced from stocks at James Cook University Aquatic Pathology Holding Units, Marine Aquaculture Research Facilities Unit (MARFU) and commercial crayfish farms in northern Queensland.

Experimental redclaw were fed three times weekly with commercial chicken pellets (GoldenYolk®) and once monthly with vegetable greens to maintain health. Experimental animals for the PC3 laboratory experiment were initially held in large 1000 litre trough style tanks at the Aquatic Pathology Holding Unit at James Cook University (Fig 2.2). Tanks contained onion bag hides and PVC piping to reduce animal stress and each unit was fitted with a filtration system to maintain water quality. Water exchange and cleaning took place bi-weekly.

Figure 2.1: Australian redclaw crayfish (*Cherax quadricarinatus*)

Figure 2.2: Example of tank setup for housing Australian redclaw crayfish within the Aquatic Pathology Holding Unit at James Cook University.

2.4 Bacteriology

The bacterial isolate used as reference for this study was Nine Mile II clone four (NMII/C4) strain of *Coxiella burnetii* passaged by Dr. Alanna Cooper and sourced originally from the Australian Rickettsial Reference Laboratory, Geelong and maintained in cell culture.

2.5 Cell culture

Propagation of *C. burnetii* isolates took place in Vero cell culture. Vero cultures were performed in in 25 cm^2 cell culture flasks in DMEM (Invitrogen,Australia) with 2 mM L-glutamine, 4.5 g L^{-1} glucose, 110 mg L^{-1} sodium pyruvate (Invitrogen, Australia) and 5% FBS (JCU, Australia). Cells were incubated at 37°C with 5% CO_2 until cell budding was apparent.

Cells were passaged weekly. The monolayer was disrupted using ATV (JCU, Australia) and transferred to sterile falcon tubes and cells pelleted by centrifugation at 500**g* for 10 min at room temperature in sealable swing out buckets. Vero cells were suspended in fresh media and split into additional 0.2 µm filter capped 25 cm^2 cell culture flasks (adapted from Cooper 2011, unpublished thesis). Further detail of these methods may be found in Appendix 1 (sections 1.1 - 1.1.4).

2.6 Primer Design for Multi-Locus Sequence Analysis, Diagnostic PCR and 16S rRNA

Primers *Com1* and *IS1111*a for diagnostic assays and all additional MLSA primers were designed using Primer3 ® Software from reference sequence *Coxiella burnetii* RSA 493 Nine Mile Strain with a length of 1,995,281 nt consisting of 2143 genes coding for 2016 proteins (NCBI refseq: NC_002971). 16S rRNA primers 16s63F and 16s1387R were sourced from the literature (Tan and Owens,2000). All primers were synthesized and annealing temperatures calculated by Sigma-Aldrich (Sydney, Australia). Primers and annealing conditions are listed in Table 2.1.

2.7 Polymerase Chain Reaction (PCR) Identification of microbial isolates

Identification of *Coxiella sp*. was confirmed on standard PCR assay for the *com1* and IS111a genes of C*oxiella burnetii* (Table 2.1) and 16S rRNA universal primers 16s63F and 16s1387R (Tan and Owens, 2000). Amplifications were carried out on an Eppendorf ©Mastercycler. The thermocycler program for *com1* consisted of incubation at 94°C for 2 minutes followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing of primers at 63°C for 1 minute and 68°C at 1 minute. Finally an extension of 72°C for 5 minutes and a 12° holding temperature. This cycle was emulated for all primer sets with the appropriate adjustments only for annealing temperature.

Samples were setup in 25 µl reactions containing 12.5 µl GoTaq Green Hotstart Master Mix (Promega ©), 7-11µl nuclease-free water, 25-50 ng DNA template and 0.5 µl forward and reverse primers respectively. PCR products were visualized via UV on 1.8 % agarose gels stained with 1µ GelRed (Biotium ©) run at 150V for 45 minutes against known ladder standards (100bp by Invitrogen ®) to verify presence of amplicon.

2.8 Realtime PCR (qPCR)

2.8.1 Standard Curve

In order to quantify *C. burnetii* after cell harvest, a standard curve was generated using genome equivalents (GE) of *C. burnetii* Nine Mile II Clone 4 Strain. This strain was selected as the full genome size, sequence and GC content were previously determined (Beare et al. 2009). Genome equivalents were calculated using the formula listed in Appendix 1 (Section 1.2.3) (adapted from Cooper 2011, unpublished thesis).

2.8.2 Quantitation of com1

To quantify *com1* within the samples for dosing purposes, qPCR reactions of 20 µL volume were set up for a 36-well rotor in a RotorGene®3000 (Corbett Research, Australia). Specific qPCR primers targeting the *com1* marker were sourced from the literature (Marmion et al. 2005) and resuspended in Tris-EDTA buffer to a stock concentration of 100 pm μL^{-1} and stored at -20°C. Known 10^5 cells/ml replicate standards were included, as were positive and non-template controls (NTC). Cycling conditions for quantitative real time PCR consisted of initial denaturation for 5 min at 94°C, 40 cycles of denaturation for 10 sec at 94°C, annealing of primers for 10 sec at 62°C and extension for 20 sec at 72°C. A melt curve analysis was performed with an increase in temperature from 72°C to 95°C in 1°C increments (Cooper 2011, unpublished thesis). Based on this quantification cells were subsequently dosed into three infection groups of 10^4,10^5 and 10^6 cells/ml homogenized in phosphate buffered saline (PBS).

Table 2.1. List of amplification and sequencing primers used in MLSA sequencing

Gene product	Primer name	Primer Sequence (5'-3')	Annealing Temp (C°)	Reference
16S rRNA	16s63F	CAG GCC TAA CAC ATG CAA CTC	50-55	Tan and Owens 2000
	16s1387R	GGG CGG AGT GTA CAA GGC	45-50	Tan and Owens 2000
ank 2 (ankyrin repeat)	Ank190F	GCG CTG CAA CGA AGA GCT GC	61	Primer3-BLAST software*
190bp	Ank190R	TGA GAA AGT GAG CCG CGG CG	61	
hisS	HisS176F	CCT GCT GTT CCT TCC GGG CG	61	Primer3-BLAST software*
(histidyl-tRNA synthetase)				
176bp	HisS176R	TGC CGT TCG GTG GTG TCT GC	61	
com1	Com1F	TTC CAC GAC GCG CTG CTC AG	61	Primer3-BLAST software*
189bp	Com1R	GAC GAA CGT CGG GGT GCC TG	63	
IS111a (transposase)	Isa309F	ACA GAG CAT CCC GGG GGT GG	63	Primer3-BLAST software*
309bp	Isa309R	GCT AAC GCC ACA CAA GCG CG	61	
ATPase	ATP160F	CCA CAG CGC CGA ACT CCC AT	61	Primer3-BLAST software*
160bp	ATP160R	TGG CGA CGA TCT CTG CAC ACT	61	
dnRec	DnRec248F	TGC ATG CCG CCA CCA GTG AAA	61	Primer3-BLAST software*
(integrase/recombinase)	DnRec248R	GGC AAT CCC GCG CAT GGT TT	59	
248bp				
pshK	PshK119F	ACA ACC GCC CAA ATC GCG TCA	61	Primer3-BLAST software*
(polyketide biosyn)				
119bp	PshK119R	CAC CTG CAA TGA CCG CAC CCT	63	

rpoB	rpOB404F	TGC CAC CAA GCG GCT CGA AG	61	Primer3-BLAST software*
404bp	rpOB404R	GCT CTT TGG CGT CCG CAC CT	61	
luxR	LuxRFwd	TCG TGA AGC ACA ATG CGT CGC T	63	Primer3-BLAST software*
194bp	LuxRRev	AGC AGG TGG GTG GCA AGT AAG T	63	
recA	recAF481-504	ATC GCC CAT ATC ACC TTC AAT TTC	59	Primer3-BLAST software*
	recAR874-893	GAA AAA GCA GGC GCT TGG TA	55	

2.9 Sequencing

PCR products were purified using Wizard ® SV Gel and PCR clean-up system (Promega, Australia) and sent to Macrogen Corporation (Seoul, Korea) for sequencing. Resultant sequences were cleaned and aligned using Sequencher® and Geneious© Software (Biomatter ™). Resultant sequences were then compared with GenBank data by BLAST analysis (www.ncbi.nlm.nih.gov/blast) as well as the Ribosomal Database Project (Michigan State University, USA).

2.10 Histological preparation tissue dehydration

Crayfish were injected with approximately 0.5ml of Davidson's Fixative in their first and third abdominal segments and cut longitudinally for added preservation. They were then immersed in Davidson's fixative for approximately 48 hours and transferred to 95% ethanol for handling and preparation. The hepatopancreas, gills and muscles of all redclaw were placed in histology cassettes and dehydrated using a timed series of alcohols, xylene and finally embedded in paraffin wax (Appendix 2). Tissue sections were cut at 5µm using a rotary microtome into paired sections and stained with Mayer's haematoxylin and eosin (H&E) or Gram stain. Sections were screened under light microscopy (Olympus E © microscope) and images captured with a MicroPublisher 5.0 RTV camera (QImaging ®, Surrey Canada).

Graded ethanol was used to dehydrate tissues at concentrations of 70%, 80%, 90%, 95% and 100% respectively prior to xylene emersion. The procedure was run in a Shandon Elliot Processor® using a Tissue-Tek II embedding center, tissues were embedded with paraffin wax. A rotary microtome was used to cut sections at 5µm to be placed on slides. Tissue sections were dried in an oven at 60◦C for one hour.

2.11 Data analysis

Where appropriate, data was analyzed using univariate analysis of variance (ANOVA) on Statistica© program after meeting assumptions of normality as assessed by Q-Q plot. Differences between means were considered significant when $P < 0.05$.

CHAPTER 3

NOVEL SYSTEM FOR MAINTENANCE AND MONITORING OF AUSTRALIAN REDCLAW CRAYFISH INFECTED WITH A LEVEL-3 ORGANISM

3.1 Introduction

The significance and use of aquatic species in biomedical research has increased considerably in the past decade. The National Institute of Health (NIH) recognizes a number of aquatic organisms as viable comparative models that may reduce overall dependence on mammalian species for health-related research (Spitsbergen et al. 2009). Previously, aquatic invertebrates have been used as proxy for mammalian subjects to study a range of areas in immunology and pathogenic classification including antigen receptor diversification (Eason et al. 2004), allorecognition in immune response (Lightner et al. 2008) and innate immunity and immune defense (Millet and Ewbank, 2004). Others, such as the crustacean *Daphnia sp*. have been used as models for infectious disease and have shed light on RNA-interference based therapies that may be applied to human viral diseases (Ebert 2008; Krishnan et al. 2009).These models may also be applied more broadly to diseases that may either be vectored by or infect aquatic invertebrates with direct ties to public health.

The recent discovery of a novel *Coxiella* sp. in farmed populations of Australian redclaw crayfish (*C. quadricarinatus*) suggests that the human pathogen *Coxiella burnetii* , the causative agent of Q fever, may also have the potential to infect aquatic invertebrate hosts (Tan and Owens, 2000; Cooper et al. 2007). The ability to test infection potential within this host is vital towards future public health research and may pave the way for similar studies with other human pathogens found within the aquatic environment.

The Australian/New Zealand Safety Standard does not currently address aquatic containment facilities for PC3 based research (AS/NZS 2010). Developing laboratory standards by which these animals are monitored and maintained for virulent human diseases provides a valuable resource for research. Below we describe an inexpensive and easily managed protocol developed for the short term monitoring and disposal of *C. quadricarinatus* infected with *C. burnetii* within an approved Physical Containment Level 3

(PC3) Animal Facility (Biosafety Level -3 /BSL-3 USA). This chapter proposes a system as a novel, risk-averse approach to a broad range of disease research involving high risk pathogens using aquatic invertebrates.

3.2 Materials and Methods

3.2.1 Invertebrate host model

Australian redclaw crayfish (*Cherax quadricarinatus*) are native to northern Australia and southern Papua New Guinea but they are farmed commercially worldwide, including countries such as Israel, China and Ecuador (Tan and Owens, 2000). *C. quadricarinatus* were chosen as subjects of this experiment as part of a larger project exploring their potential as a host for a novel species of *Coxiella* (Chapter 4). Susceptibility of this organism to *Coxiella burnetii* and other virulent Rickettsiae is not known and presents a specific case whereby a standard operating procedure regarding a level 3 organism infection experiment in an aquatic invertebrate is necessary. *C. quadricarinatus* are ideal laboratory candidates for invertebrate infection experiments as they are robust, non-aggressive, fast-growing and can survive a range of water temperatures, pH and dissolved oxygen levels (Masser and Rouse,1997).

3.2.2 Infection Procedure

Healthy *C. quadricarinatus* (n=20) were sourced from stocks maintained at James Cook University Aquatic Pathology Holding Units. Animals were transported into the PC3 facility in dry, sealed autoclavable containers and were individually anesthetized immediately prior to infection using an ice slurry for approximately 45 seconds within a Class II biosafety cabinet (BSC II). Prior to anesthesia and infection, each crayfish was measured and weighed by turning the animals on their carapace rendering them temporarily immobile. *Cherax quadricarinatus* used in this study were between 7 - 10.7 cm in length from eyestalk to tail with an average mass of 28.9 grams (±8.5g) and all were chosen based on overall appearance of health and activity. Animals were randomly assigned into three experimental dose groups of passaged Nine Mile Clone 4 Phase II (9Mi/II/C4) strain of *Coxiella burnetii* supplied by James Cook University. Doses were determined using real-time PCR and animals were injected in equal 0.25mL increments with a syringe and 25

gauge needle on their 1st and 3rd abdominal segments (Fig 3.1). An additional control group was inoculated with 0.5 mL of PBS.

Experimental groups of C*. quadricarinatus* were individually added to pre-filled tanks using standard steel tongs within the BSC II taking care to minimize handling post-infection and to reduce potential for splashes. Crayfish were placed in the unit and tongs and cabinet surfaces were disinfected using a solution of 1:50 Trigene Advance® disinfectant within the BSC II. After placing animals in respective units, tanks were immediately sealed and an additional layer of Parafilm ® was added around the seal to minimize risk of leaks due to condensation. The entire unit was sprayed and wiped down using 1:50 Trigene Advance® disinfectant and placed on a single rolling table to transport it from the BSC II to its respective shelf location within the laboratory. Using a rolling table or trolley reduced potential for splash or spills while transporting water-filled tanks across the laboratory. Laboratory crayfish were maintained and monitored for twenty one days (Section 3.2.4). Tanks were transparent and their design and placement allowed observation of their activity and monitoring of filter functionality without moving units. Crayfish were observed twice daily (once in the morning and evening) for the first week post-infection to check for any deaths, behavioral changes or mechanical problems with units. Subsequently crayfish were monitored daily for the remainder of the experiment taking note of lethargy or changes in activity.

Figure3. 1: Inoculation of crayfish within a Class II Biosafety Cabinet

3.2.3 Containment

Three infection groups and one control group of five crayfish each were maintained in separately aerated, fully sealed and labeled 10 L plastic storage units transformed into tanks (dimensions: 37.5L x 24.5W x22H cm). Stocking density was one animal per tank for a total of twenty tanks. Prior to entering the PC3 facility, each tank was fitted with an independent self-adhering fish tank filter for animal health (Elite® Mini Underwater Filter by Hagen®) allowing adequate aeration throughout the experimental period and minimizing the need for frequent water contact and exchange. The filter model was chosen as it is easily accessible, functions for tanks up to 11 liters in volume and consists of removable parts able to withstand autoclave temperatures. These parts are separate from the filter's power units ensuring decontamination and potential for re-use. Filter units and power supplies were independently labeled corresponding to tanks and points where power cord exited each tank were sealed and fitted with waterproof tape to reduce the risk of leaks and contain any aerosolized water within the units. Tanks were filled with 5.5L single distilled H_20 which was sufficient to account for water exchanges and evaporation over the experiment but reduced the risk of spill and leaks during transportation. As there is no water source within the PC3 facility, all tanks were filled prior to entry and all additional water was brought into the laboratory in sealed 1L Schott bottles. Tanks were transported into the laboratory on a trolley shelving unit which remained in the laboratory for storage of tanks (Fig 3.2, Fig 3.3). Each tank was supplied with onion netting and pipe fittings as appropriate hides. Stocking density and size of containers ensured ample space, refuge and feeding while allowing clear observation and minimizing possibility of escape. Due to space constraints, tanks were designed to be stackable. A series of five labeled power strips were used to supply electricity to filter units. Wherever possible, power strips were placed above tanks to reduce any risk of water leakage into power source.

Figure 3.2: Containment and tank setup within approved PC3 laboratory facility

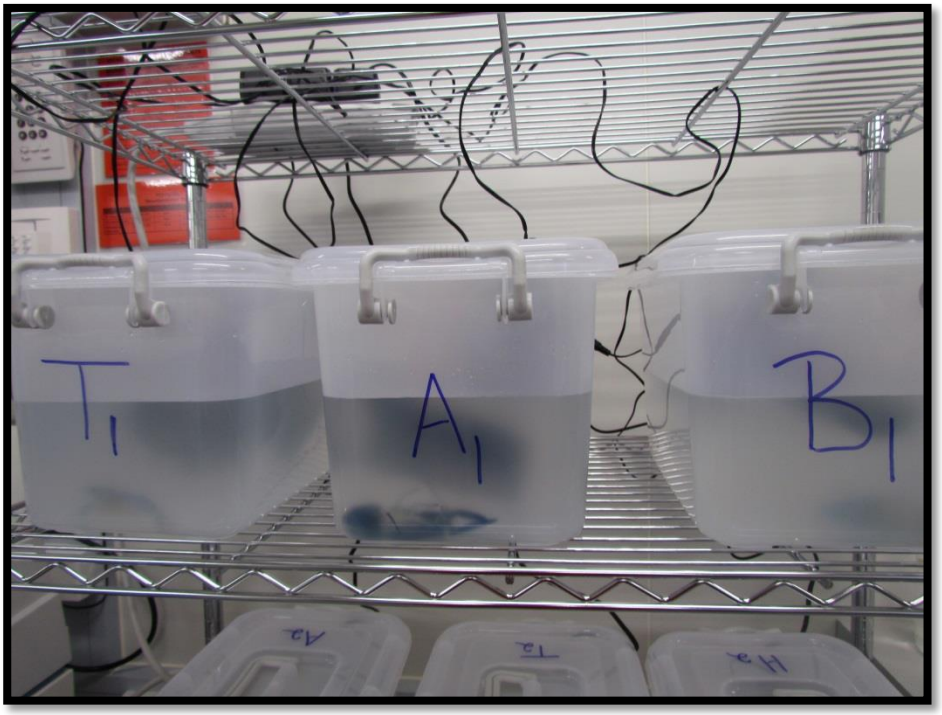

Figure 3.3: Tank labeling and randomized distribution within the PC3 facility.

3.2.4 Maintenance

Power supplies were run continuously and filters were checked daily to ensure they were functioning. Light-dark cycle was approximately 12:12 with a room and approximate water temperature of 25° C. Animals were fed commercial chicken pellets (Golden Yolk ®) three times weekly. For feeding, each tank was removed individually from its shelving unit, its filter power disconnected and moved via rolling table to a BSC II cabinet. Tanks were never carried to reduce splash potential. Each tank was only partially opened within the BSC II to minimize risk of aersolization and 3-5 pellets were placed into tanks. Tanks were immediately re-sealed and wiped down with Trigene Advance®.

Water exchange was performed only once for the duration of this twenty one day experiment due to the efficiency of filters, resistance to overfeeding and preference of *C. quadricarinatus* towards lower water quality. Water exchange was performed in the BSC II using Orcon ® Gasoline siphons which use a stop-valve and squeeze grip to control air pressure for removal of unwanted particulate matter in water and approximately 20-30% of water within tank with minimal aersolization. All controls underwent water exchange with separately labeled siphons prior to exchanging water for the infected population to prevent possible contamination. Infection groups underwent water exchanges separately from lowest to highest exposure groups with appropriately assigned siphons and equipment. When possible, a second BSC II was also used to separate manipulation between controls and infection groups. Contaminated water was exchanged with double-sealed siphons labeled according to dose group into 1L Schott bottles which were subsequently sealed, outer surface disinfected with Trigene Advance®, autoclaved and drained. Water was replaced in tanks using separately labeled siphons containing single distilled water in labeled 1L Schott bottles to minimize aersolization. After water-exchange, tanks were sealed, externally disinfected with Trigene Advance® and left within the BSC II for fifteen minutes for all potential aerosols to settle before continuing decontamination and removal of items from the cabinet. All surfaces and equipment were decontaminated with 1:50 Trigene Advance® disinfectant and siphons were cleaned and soaked in 1:50 Trigene Advance® disinfectant overnight, disassembled, rinsed with single distilled water and stored in sealed units which were also sprayed down before removing from the BSC II.

3.2.5 Disposal and Decontamination

At the completion of the experiment, crayfish were anesthetized via combination ice slurry and chilled water placed into each individual tank unit within the BSC II for approximately 3 minutes. Once immobile, crayfish were removed and their ventral nerve was promptly cut for euthanasia. A vertical cross-section of the crayfish was placed in a sealed container of Davidson's fixative for histological preparation while the remaining part of the animal was disposed of in a biohazard bag within the BSC II, sealed and wiped down with 1:50 Trigene Advance® then autoclaved. After 48 hours in Davidson's fixative, crayfish were transferred into 95% ethanol for storage prior to entire laboratory decontamination. All chemical reagents were contained within the PC3 facility until all experiments terminated and the laboratory underwent standard formaldehyde decontamination.

The PC3 facility is fitted with a 2-way autoclave whereby all waste (including liquid) exiting the facility must be autoclaved prior to removal (AS/NZS 2010). To comply with this standard, storage tanks and filter parts were previously tested to withstand autoclave sterilization temperatures and pressure. The autoclave conditions for a PC3 laboratory are approximately 121° C /103kPa for 45 minutes which is the approved standard with which to kill laboratory Rickettsiae (AS/NZS 2010). The ability to sterilize tanks and filter parts as sealed units minimized exposure of personnel to potentially contaminated tank water and facilitated easy disposal. Power section of filter units were removed, wiped down in 1:50 Trigene Advance ® and remaining filter parts were left sealed within tank units for autoclaving and disposal minimizing contact with potentially contaminated water. Post-sterilization, water and parts were removed from the autoclave for appropriate cleaning, disposal or re-use.

3.3 Results and Conclusions

This chapter reports a novel system specifically designed and developed to infect, monitor, maintain and dispose of tank units designed specifically to house aquatic invertebrates infected with a Level-3 human pathogen. This system is suggested as a baseline method for maintaining small populations of aquatic invertebrates within approved PC3 invertebrate facilities. It was demonstrated that with appropriate planning, it is possible to successfully implement an experimental design aiming to monitor the infection potential of a human pathogen in an aquatic invertebrate host within a controlled PC3 environment. There is a

growing need for standards addressing this vital gap in approved laboratory procedure. The findings here strongly suggest that the delineation and inclusion of a standardized baseline protocol for aquatic invertebrates within current PC3 facility standards is both possible and necessary.

CHAPTER 4

EXPERIMENTAL INFECTION OF AUSTRALIAN REDCLAW CRAYFISH (*CHERAX QUADRICARINATUS*) WITH *COXIELLA BURNETII*, THE AETIOLOGICAL AGENT OF Q-FEVER

4.1 Introduction

The discovery of a novel *Coxiella* sp. in farmed populations of Australian redclaw crayfish (*C. quadricarinatus*) suggests that the human pathogen *Coxiella burnetii* , the causative agent of Q fever, may also have the potential to infect aquatic invertebrate hosts (Tan and Owens, 2000 ; Cooper et al. 2007). The ability to test infection potential within this host is vital towards future public health research and may pave the way for similar studies with other human pathogens found within the aquatic environment. It also has significant potential within the field of aquaculture as a comparative model to *Coxiella cheraxi* and other emergent *Coxiella* species that may threaten production and incur substantial losses within the industry. This chapter outlines in detail the experiment undertaken within the design parameters described in Chapter 3. Due to the high level of genetic similarity between *C. burnetii* and TO-98 (*C. cheraxi*), as well as the known attenuation of *C. burnetii* within other arthropods such as ticks, Australian redclaw crayfish were infected in three dose groups with negative controls as a baseline for comparison.

The aim of this chapter was to investigate the infection potential of passaged isolates of *C. burnetii* within Australian redclaw crayfish. The intent was both independent determination of whether the crayfish may act as host to the human pathogen and a comparative immunological model of a crustacean to a RLO or *Coxiella* pathogens, specifically *Coxiella cheraxi*. Using *C. quadricarinatus* as a comparative model for cross-infection, we may also gain preliminary insight as to whether *C. cheraxi* may in turn have the potential to infect or generate antigenic response within mammals and therefore possibly provide a viable vaccine alternative for future research.

4.2 Materials and Methods

4.2.1 Crayfish preparation

Australian redclaw crayfish (*Cherax quadricarinatus*) were chosen as the subjects of this experiment due to its known potential as a host for a new species of *Coxiella* (see Tan and Owens, 2000). Crayfish were selected out of current experimental populations at James Cook University Aquatic Pathology Holding units. Animals were held at low stocking densities in tanks cleaned twice per month with overall high water quality. Animals were selected based upon size. Experimental subjects (N=20) were between 7 - 10.7 cm in length from eyestalk to tail with an average mass of 28.9 grams (±8.5g) and all were chosen based on overall appearance of good health and activity. Pre-trial, crayfish were fed on both green vegetables and commercial chicken pellets (Golden Yolk ®) three times per week for three weeks pre-infection to ensure maximal health and growth.

This experiment was carried out in full compliance of institutional animal ethics (JCU A1680) and conducted in accordance with National Health and Medical Research Council, *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes 7th Edition, 2004*; and in compliance with the Queensland Animal Care and Protection Act, 2001.

4.2.2 Laboratory Setup and Design

In the laboratory setup previously reported (Chapter 3), Australian redclaw crayfish were challenged with three doses of live Nine mile II Clone 4 Strain of *C. burnetii.* Twenty Australian redclaw crayfish were randomly distributed between four experimental treatments of control and inoculated crayfish with five replicates per treatment group.

4.2.3 Preparation of Inoculum

Vero cells, host of Nine mile II Clone 4 Strain of *C. burnetii* originally cultured by James Cook University in 2006 and stored in liquid nitrogen, were propagated and passaged for approximately three weeks using 10% FBS DMEM media (full protocol in Appendix 1). Cells were split 1:2 ratio approximately every 5-7 days depending on confluence.

4.2.4 *C. burnetii* Isolation from Vero Cells

Successfully infected cell-lines were pelleted in a PC3 laboratory BSC II Cabinet using antibiotics, trypsin, versene (ATV) and a cell scraper. Cells were placed into one 50ml falcon

tube and subsequently sonicated with a microtip sonicator (Misonix, USA) for 2x 40 second bursts at approximately 40 amps. Thirty second breaks were taken between bursts to reduce overheating. Cells were then left to sit within the BSC II for approximately 1 hour to allow for aerosols to settle. Cells were subsequently pelleted via centrifugation at 550**g* at 20˚C for 10 minutes. Bacterial supernatant was layered over 25% sucrose (2:3 ratio of supernatant to sucrose). Bacteria was then pelleted again at 2250**g* at 20^{o}C for 20 minutes. Supernatant was removed to less than 5 ml and a small aliquot of bacteria was removed for DNA extraction and quantification via qPCR (Appendix 1.1.3/1.1.4).

4.2.5 Dosing via qPCR (Adapted from Cooper 2011, unpublished thesis)

C. burnetii from vero cells underwent DNA extraction via Roche High Pure© PCR template with two modifications based on its resistance to lysozyme degradation: an additional 100µl lysozyme and 25 minute incubation step and a split elution step with a 2x 100µl buffer addition.

A standard curve was initially constructed for qPCR using genome equivalents (GE) of *C. burnetii* NMII/C4 (Figure 4.1b) (Cooper 2011 unpublished thesis). The full sequence of the genome of this strain and GC content were determined previously (Beare et al. 2009). Genome equivalents were previously calculated (Appendix 1.2.3).

To identify *C. burnetii* from vero cells and samples, a primer and probe sequence targeting the *com1* marker, a gene conserving for the outer membrane protein of *C. burnetii*, was sourced from the literature (Marmion et al.2005). The Taqman primers and MGB probe sequences were as follows: TP1f419 AAT CGC AAT ACG CTG CCA AA 438 (amplicon size 76bp), probe 447 FAM AGC AGC CGC TAA ACA TAMRA 461, TP1r494 AGC AGC GCG TCG TGG AA 478. Positions on first base of initiation codon = +1.

To quantify *com1* within the samples for dosing purposes, qPCR reactions of 20 µL were set up for a 36-well rotor in a RotorGene®3000 (Corbett Research, Australia). qPCR primers targeting the *com1* marker were resuspended in Tris-EDTA (TE) buffer to a stock concentration of 100 pm μL^{-1} and stored at -20°C. Standards of 10^5 cells/ml were included, as were positive and non-template controls (NTC). Cycling conditions for quantitative real time PCR consisted of initial denaturation for 5 min at 94°C, 40 cycles of denaturation for 10 sec at 94°C, annealing of primers for 10 sec at 62°C and extension for 20 sec at 72°C. A melt curve analysis was performed with an increase in temperature from 72°C to 95°C in 1°C increments (Cooper 2011, unpublished thesis). Based on this quantification (Fig 4.1a),

C. burnetii cells were diluted into three infection dose groups of 10^4,10^5 and 10^6 cells/ml homogenized in phosphate buffered saline (PBS).

A

B

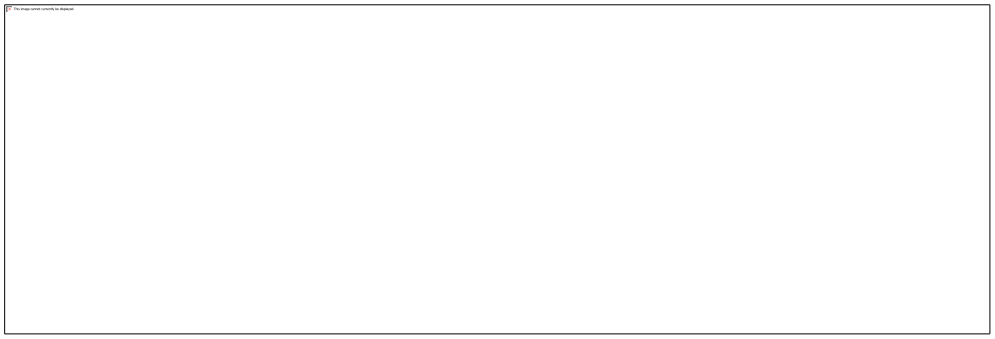

Figure 4.1: a) qPCR (realtime) quantitation report for dosing *C. burnetii* grown from a vero cell line – red: isolate C4 neat, green: standard isolate C4 at 10^5 cells/ml, blue: isolate C4 at 10^{-1} cells/ml, black: non-template control b) Standard curve for concentration values. Reaction efficiency 0.872(*=$10^{(-1/m)-1}$)

4.2.6 Infection challenge

Prior to inoculation, crayfish were sedated at approximately 12° C with chilled water. Doses were 10^4,10^5,10^6 cells/ml respectively, and were diluted into 0.5 mL aliquots of PBS. Crayfish were injected in equal 0.25mL increments with a syringe and 25 gauge needles into their 1st and 3rd abdominal segments. The control group was inoculated with 0.5 mL of PBS. The experimental period began on the day of injection (0) and concluded after 21 days.

Crayfish were observed twice daily (once in the morning and evening) for the first week post-infection to check for any deaths, behavioral changes or mechanical problems with holding units. Subsequently crayfish were monitored daily for the remainder of the experiment, taking note of lethargy or clinical signs. Dead crayfish were removed immediately and prepared for histology and standard PCR by splitting the cephalothorax in half, removing a tissue sample of hepatopancreas, two pleopods and extracting approximately 0.5 mL of haemolymph. One individual within the 10^4 cells/ml dose group was also found to be carrying eggs and upon termination of the experiment, one egg was also sampled for analysis. The remaining crayfish bodies were placed in Davidson's fixative for 48 hours before being transferred to 95% ethanol for histological preparation. Pleopods, muscle tissue and hepatopancreatic tissues were immediately placed in 95% ethanol for DNA extraction while haemolymph was collected in 1:1 ratio in citrate EDTA. At the end of the experiment all remaining crayfish were sacrificed and processed for histological and DNA screening.

4.2.7 Histology Preparation

Post-laboratory decontamination, all fixed crayfish tissues were removed in 95% ethanol from the PC3 facility for histological preparation. The hepatopancreas, pleopods and muscles of all redclaw were dehydrated using a timed series of alcohols, xylene and finally embedded in paraffin wax (Appendix 2). Tissue sections were cut at 5µm using a rotary microtome into paired sections and stained with Mayer's haematoxylin and eosin (H&E) as well as Gram Stain. Sections were screened under light microscopy (Olympus E © microscope) for the presence of inclusion bodies, inflammation and Gram negative bacteraemia. Digital photographs were taken with a MicroPublisher 5.0 ®RTV camera (QImaging ®, Surrey Canada).

4.2.8 DNA extraction and PCR for *C. burnetii* detection

Samples underwent DNA extraction under PC3 conditions. Extraction took place using Roche High Pure© Template Preparation Kit according to the manufacturer's instructions with the modifications previously described. DNA concentration was quantified using spectrophotometry (IMPLEN Nanospectophotometer ™)

DNA was tested first by standard PCR targeting a 189 base pair (bp) fragment of the *com1* gene, as described previously (Chapter 2). *Com1* primers were designed using Primer3 BLAST software (www.ncbi.nih.gov) and synthesized by Sigma-Aldrich (Sydney, Australia).

The forward and reverse primers for *com I* were Forward TTC CAC GAC GCG CTG CTC AG and Reverse GAC GAA CGT CGG GGT GCC TG respectively .

Samples were setup and visualized under conditions described in Chapter 2 (Section 2.7).

PCR products were purified using Wizard ® SV Gel and PCR clean-up system (Promega, Australia) and sent to Macrogen Corporation (Seoul, Korea) for sequencing. Sequences were cleaned against chromatograms, annotated and aligned using Sequencher® and Geneious© Software (Biomatters 2013). Resultant sequences were then compared with GenBank data by BLAST analysis (www.ncbi.nlm.nih.gov/blast) as well as the Ribosomal Database Project (Michigan State University, USA).

4.2.9 qPCR Preparation (Adapted from Cooper 2011)

Experimental samples were also quantified via qPCR using primers and Syto 9 fluorescence with reaction guidelines described in Section 4.2.5. The resulting data was compared to both the original standard curve and the pre-experimental dosing quantitation report to determine final bacterial concentrations.

4.2.10 Statistical Analysis

Statistical analyses were performed using Statistica® software. Mortalities between groups were investigated using a one-way univariate analysis of variance (ANOVA) after meeting assumptions of normality as assessed by q-q plots. Post-hoc analytical method was performed using the least significant difference to assess mean differences between independent variables. Differences between means were considered significant at $p < 0.05$

4.3 Results

4.3.1 Mortality and Size Data

After the 21 day challenge experiment, there was no significant statistical or clinical difference between mortality of *C. burnetii* inoculated groups and controls. The control group experienced the same mortality rate as the two lowest inoculation treatment groups at 20% (Table 4.3). The highest inoculation group with dose of 10^6 cells/ml experienced 0% mortality for the duration of the experiment. There was a variation in mass change between treatment groups. Inoculated crayfish had an average loss of body mass while the control group had an average gain when measured post-trial. The average change of weight of the control group was -0.36±1.59g (-0.27%) while for the 10^4,10^5,10^6 cells/ml infection

groups the mean change of mass was + 1.83±1.39g (+1.27%), +0.57±0.72g (+0.37%) and + 1.95±1.8g (+1.11%) respectively.

4.3.2 Histopathology

There was evidence of bacteraemia and inflammatory cells in all experimental groups including controls, however both pathologies were most pronounced within infection groups (Table 4.1). Forty percent of infected crayfish exhibited some form of systemic granuloma or inclusion bodies (Fig 4.2, Fig 4.3) and 80% of animals within inoculation groups demonstrated swollen or abnormal nuclei within the hepatopancreas and evidence of forced tubule separation (Fig 4.5). Histology also revealed that approximately 30% of the entire experimental population exhibited co-infection with presumptive reovirus. This included control, non-*Coxiella* exposed crayfish. The 10^6 cells/ml dose group of the infection population exhibited 100% changes expected for a *Coxiella* infection including facial plane inflammation in muscles, systemic inflammatory response, granulomas and generalized bacteraemia (Fig 4.3, Fig 4.4, Fig 4.5). Twenty percent of these samples presented evidence of inflammatory response within both the hepatopancreas and muscle tissue (Fig 4.4). All histological changes observed within experimental crayfish were considered clinically significant.

Table 4.1 Summary of histological findings for experimental groups of *Cherax quadricarinatus* exposed to *C. burnetii* + controls. Lesions were observed under light microscopy.

Treatment	Myolysis (%)	Swollen nuclei %	Bacteraemia %	Reovirus or other co-infection %
Control PBS(C) n=5	20	40	40	20
Infection group 10^4cells/ml (Low) n=5	80	80	80	20
Infection group 10^5cells/ml (Medium) n=5	100	80	80	40
Infection group 10^6 cells/ml (High) n=5	100	100	100	40

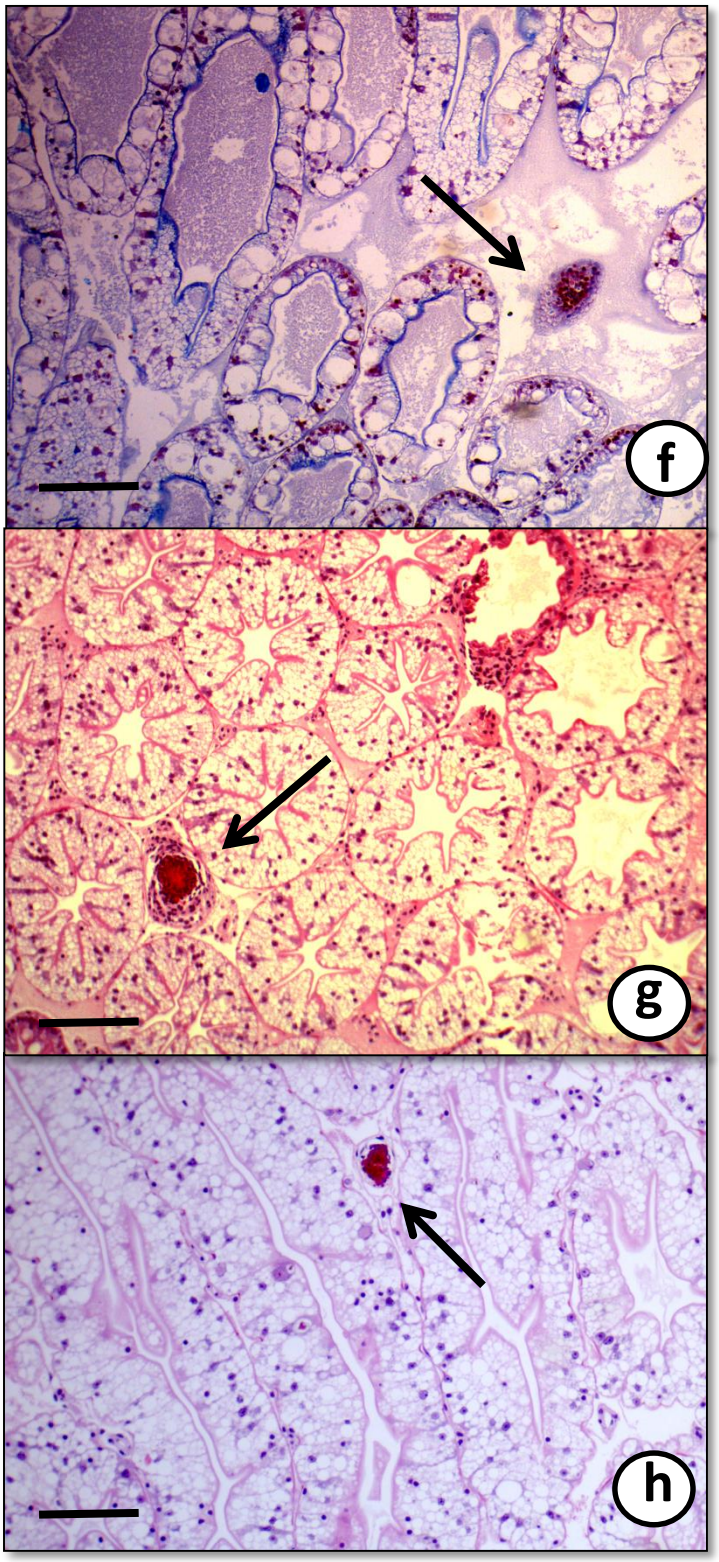

Figure 4.2: Light micrographs of haematoxylin and eosin (H&E) and Gram stained hepatopancreatic tissue from *Cherax quadricarinatus* infected with 10^4 and 10^5 cells/ml of *Coxiella burnetii.* (f) Tissue showing Gram negative bacterial masses (arrow) and separation of hepatopancreatic tubules in 10^4 cell/ml dosed crayfish. (g) HE stain of 10^4 infected crayfish with apparent tubule separation and granuloma formation (arrow). (h) HE stain of a 10^5 cell/ml dosed crayfish with an apparent granuloma formation (arrow) in its hepatopancreatic tissue. Scale bars 30µm.

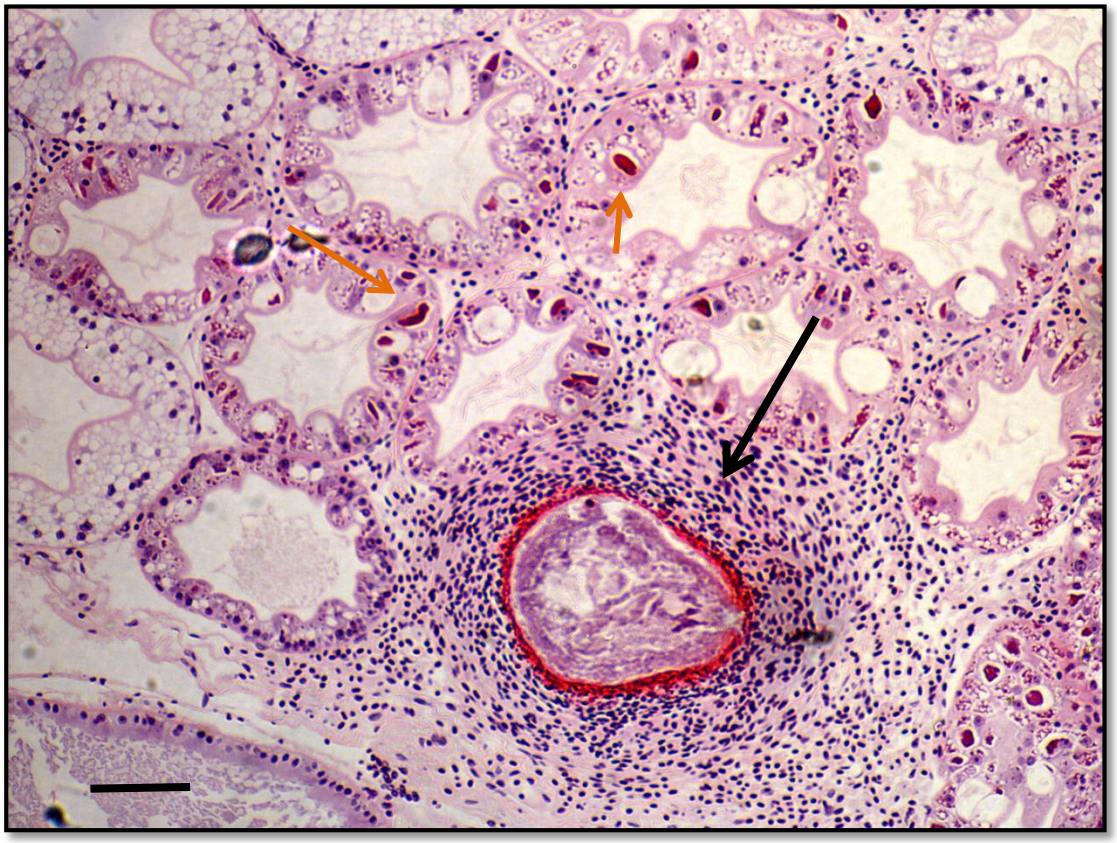

Figure 4.3: Light micrograph of HE stained hepatopancreatic tissue from *Cherax quadricarinatus.* Crayfish tissue illustrating a classic case of presumptive reovirus inclusions in tubule epithelial cells, as an apparent concurrent infection (orange arrows) as well as tubule encapsulation resulting in granuloma formation with haemocytic infiltration (black arrow) in a 10^6 cells/ml *Coxiella burnetii* dose infected animal. Scale bar 30µm.

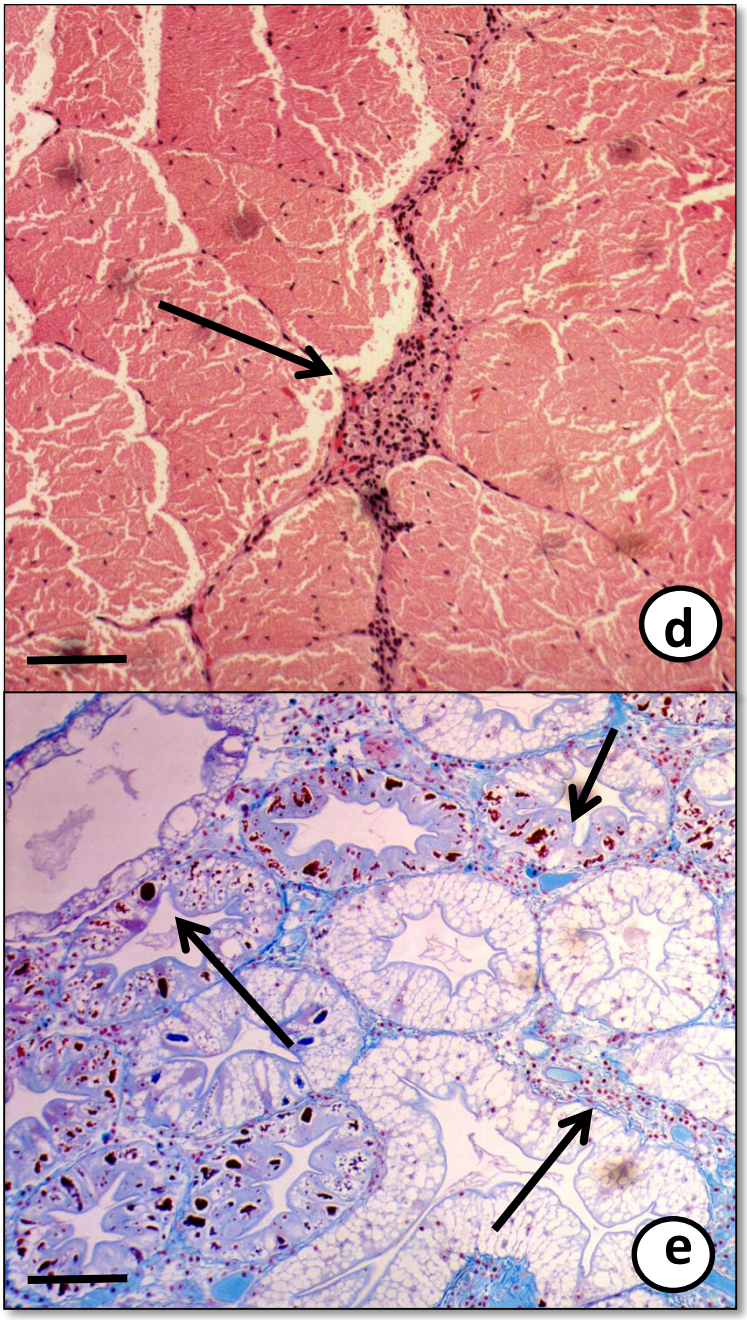

Figure 4.4: Light micrograph of H&E and Gram stained sections of muscle and hepatopancreatic tissue from *Cherax quadricarinatus* infected with 10^6 cells/ml dose of *Coxiella burnetii.* (d) Muscle tissue sample with apparent haemocytic infiltration (arrow) down the connective tissue fascial plain. (e) Sample of hepatopancreatic tissue with an apparent formation of Gram-negative haemocytic infiltration (bacteraemia) and reovirus-like inclusion bodies. Scale bars 30µm.

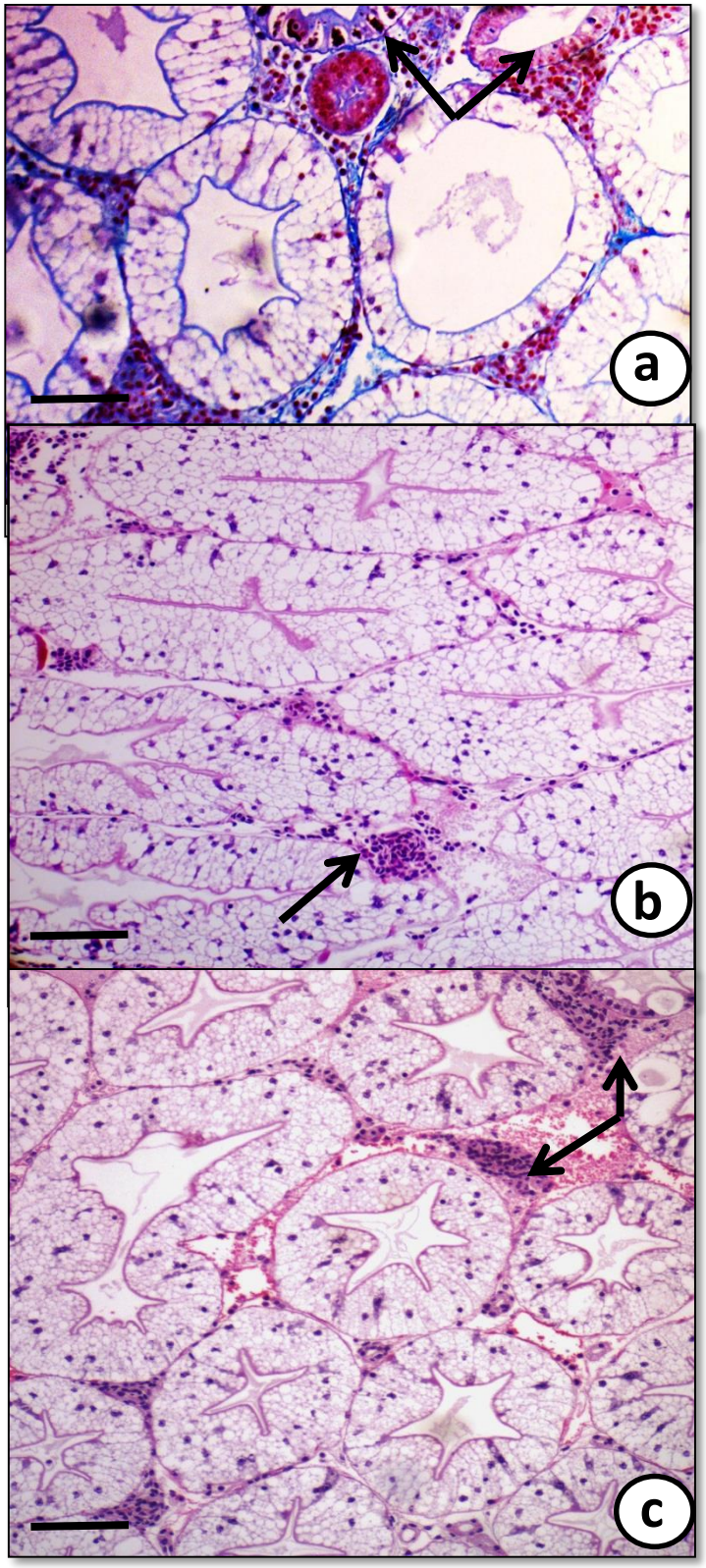

Figure 4.5: Light micrograph of the H&E and Gram stained hepatopancreatic tissue from *Cherax quadricarinatus* infected with 10^6 cells/ml of *Coxiella burnetii.* (a) Tissue showing Gram negative bacterial infection and granuloma formation (b/c) Sample H3 as HE stained tissue demonstrating haemocytic inflammation (arrows) and possible tubule separation invaded by haemocytes. Scale bars 30µm.

4.3.3 PCR results

Standard PCR testing for hepatopancreatic tissue and haemolymph of infected members indicated that 10 out of 15 inoculated samples (67%) were positive for *com1* when run next to positive and negative controls under the same thermocycling conditions. The 189 bp target amplicon was clearly observed from positive samples in both hepatopancreatic and haemolymph tissue samples and results were consistant for each crayfish. All controls were negative for the *com1* amplicon on standard PCR and some crayfish from every exposed experimental group tested positive (Table 4.3). The 10^6 cells/ml dose group was the only infection group to be 100% positive for the presence of *com1* on standard PCR (Figure 4.6).

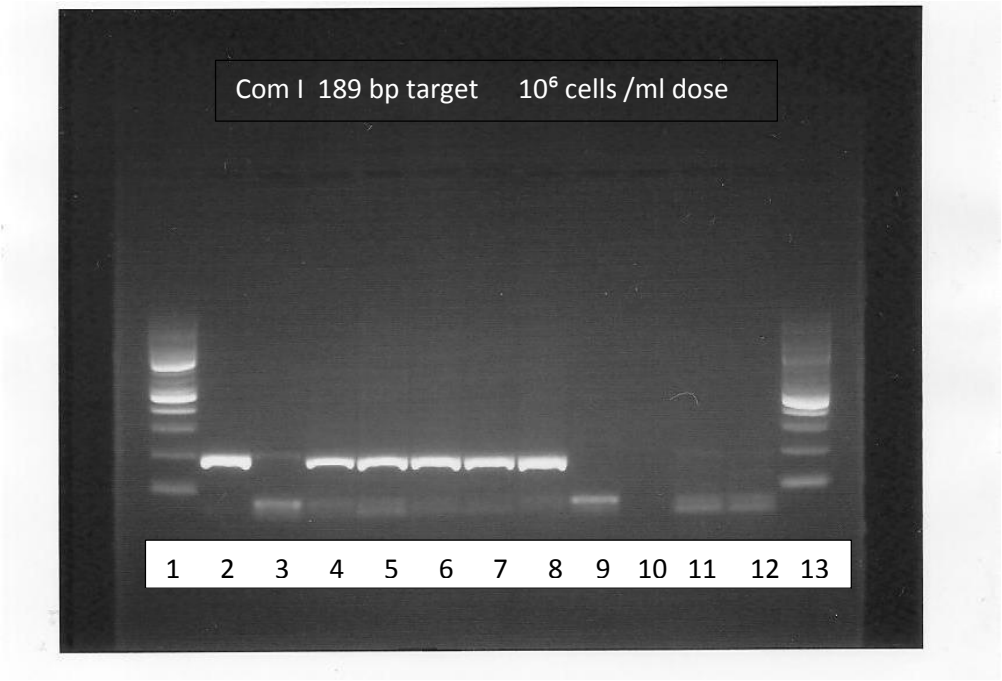

Figure 4.6: Standard PCR run to detect *com I* outer membrane protein at 189 base pairs within hepatopancreatic tissue (40-55ng/µl dilute concentration). 1.8% agarose gel (137v) for 10^6 cells/ml dosed crayfish infected with 9 mi/II/C4 strain of *Coxiella burnetii.* Lane 1)100bp Quickload ladder, 2) positive 9mi/II/C4 control from PC3, 3)positive control old stock DNA, 4)H1 hepatopancreas, 5) H2 hepatopancreas, 6) H3 hepatopancreas, 7)H4 hepatopancreas, 8) H5 hepatopancreas, 9)PBS negative control, 10)H_2O negative control, 11/12) positive control old stock, 13) 100bp Quickload ladder.

4.3.4 qPCR results

Primers used for the detection of *com1* within experimental samples were based on the marker used for the clinical detection of *C. burnetii* (see Marmion et al. 2005). The sequence of the expected amplicon for *com1* had a 100% identity match with the target sequence from *C. burnetii* in GenBank and did not have a significant match with any other organism listed. Results were compared against the previously acquired standard curve and both positive dose samples of *C. burnetii* at 10^4 cells/ml concentration and non-template controls were used. Eleven out of fifteen hepatopancreatic tissue samples (73%) from infected crayfish returned positive results in qPCR with varying bacterial loads from 38 to 0.65 copies. Cycle thresholds (Ct) varied from 32.65 to 38 (Table 4.2). The mean bacterial load and mean cycle thresholds for positive samples were 1.22×10^1 (±1.39x10) and 35.8 (±2.33) Ct respectively.

Table 4.2 qPCR quantitative report of bacterial copies and cycle time of *Coxiella burnetii* (NM4/II strain) for replicate assays in positively infected *Cherax quadricarinatus*

Infection Group	Copies	Ct	Mean copies	Mean Ct
10^4	6.56×10^{-1}	38.92		
10^4	2.05×10^0	37.60	1.35×10^0	38.26
10^5	2.41×10^0	37.91		
10^5	1.16×10^1	34.44		
10^5	3.87×10^0	36.86		
10^5	1.01×10^0	38.23	1.89×10^1	36.86
10^6	3.47×10^1	32.65		
10^6	1.02×10^1	34.77		
10^6	2.58×10^1	33.08		
10^6	4.60×10^0	36.63		
10^6	3.75×10^1	32.79	2.26×10^1	33.98
Overall Mean:	1.22×10^1±1.39x10	35.8 ±2.33		

In one sample, the egg membrane collected from a 10^4 cells/ml dose crayfish carrying eggs during the experiment was sampled and quantitative qPCR results revealed 1.10×10^0 copies per ml of *C. burnetii* DNA. Pleopods and haemolymph samples were assessed only by PCR.

Table 4.3 Summary of clinical and PCR data of Australian redclaw crayfish experimentally infected by *Coxiella burnetii*

Group	Start Weight (g)	End Weight(g)	Mortality	PCR (com1)	qPCR	Histology (General Bacteraemia)
T1	28.71	28.69	+ Day 5	-	-	NC
T2	28.79	29.28	-	-	-	+
T3	27.94	30.95	-	-	-	+
T4	26.56	25.55	-	-	-	-
T5	23.69	23.01	-	-	-	-
			20%	0%	0%	40%
A1	24.80	24.02	+ Day 3	NC	NC	NC
A2	27.98	26.76	-	-	-	+
A3	41.21	37.05	-	-	-	+
A3Egg			-	+	+	NC
A4	33.64	31.60	-	-	-	+
A5	16.04	15.07	-	+	+	+
			20%	33%	33%	80%
B1	15.92	14.27	-	-	+	+
B2	26.40	26.80	-	-	+	+
B3	26.17	25.57	-	+	+	+
B4	39.72	38.71	+ Day 16	+	+	+
B5	44.27	44.30	-	+	-	-
			20%	60%	80%	80%
H1	47.32	45.59	-	+	+	+
H2	46.40	46.37	-	+	+	+
H3	29.78	28.24	-	+	+	+
H4	28.14	22.40	-	+	+	+
H5	23.49	22.80	-	+	+	+
			0%	100%	100%	100%

Group Doses: T=Control, A=10^4, B=10^5,H=10^6 cells/ml

+ present/- absent NC - not collected due to decomposition

4.3.5 Sequencing

Samples from infected *C. quadricarinatus* hemolymph, hepatopancreas and pleopods were sent for 16S rRNA sequencing post-PCR testing for *com1*. Sequences were cleaned and aligned using Sequencher® and Geneious© Software (Biomatters 2013). Resultant sequences were then compared with GenBank data by BLAST analysis (www.ncbi.nlm.nih.gov/blast) as well as the Ribosomal Database Project (Michigan State University, USA). Sequences were between a 98-100% match for *C. burnetii com*1 or related gene marker.

Table 4.4: Sample 16S rRNA confirmation sequences from *C. quadricarinatus* experimentally infected with *Coxiella burnetii*

Sample	Length (bp)	Gene hit	Match %	E - value	Sequence
H1H8 (10^6 cells/ml) Hepato-pancreas	199	*Com1* (partial cds) *Coxiella burnetii* AC: HM804027.1	100	0.0	TTTTTCCNGGGCATTGCGCGACGCTAAATATACTAG GGTACCGTGTTGACATTAAATGATACTTTGGCAGC GTATTGCGATTAATTTAATTCTTTTTTGTGTTCTCCG TTTGCTACGGGTATCGCTCTTTTATGTGTGTGTTACT ACCGAAGGAGACGTTCTTTTACAATCGTTGGGTGC AAGATGACAACCTCCCCCC
H5H (10^6 cells/ml) Hepato-pancreas	202	Cbuk *Coxiella burnetii* Q154 AC: CP001020.1	100	1	CCGGGGCAGGACCAACGCAAGGGAGGACCAGTCT CTCGACACTCTGTCTGAACTGTGATTCTGGCCGCCG AGTGCCACTATCTCCGGACGCAGCCCCGTCTTCGAT CTCTTGCGTCGGCAAACCGACGAGGGTCCGCCCAG AATGCGGCATGAGCCACTCCCGAGAGCGTAGGGA ACAATAAATCAAGTCTGAGCATAGGCC
A3egg (10^4 cells/ml)	159	Coxiella burnetii strain Qi Yi 27kDa (com1) gene, partial cds AF317646.1	100	1e-76	GCATCCCAAGCATTGCAAAAAAAGACAGAAGCGCA ACAAGAAGAACACGCTCAACAAGCAATTAAAGAAA ATGCAAAGAAATTATTTAACGACCCTGCATCACCAG TGGCAGGCAATCCTCATGGCAATGTTACATTGGTTG AATTTTTCGATTATCAA

4.4 Discussion

This study was the first to report experimental infection of a PC3 level organism in an aquatic arthropod and the first to attempt a *C. burnetii* infection of Australian redclaw crayfish. While it is suspected that *C. quadricarinatus* is susceptible to novel *Coxiella sp.* as previously elucidated, no prior experiment has attempted to infect an aquatic species with the human pathogen for comparative immunity purposes (Tan and Owens, 2000; Cooper et al. 2007). In this study, infected redclaw demonstrated no apparent behavioural changes. Possibly a result of small experimental population size, there was no observed correlation between infection and mortality. However, post-mortem histological examination demonstrated infected crayfish displayed histopathology consistent with that of a general bacteraemia with Gram negative bacteria (Fig 4.5b). Lesions observed that were similar, but not identical to those observed in *Coxiella cheraxi* infections included granuloma formation, tissue separation and haemocytic infiltration (Fig 4.5c). In contrast to observed *C. burnetii* infections, there was no evidence of basophilic inclusion colonies and significant tissue tropism (Fig 4.7) that would be expected in a *C. cheraxi* infection based upon previous literature (Owens et al. 1992; Edgerton and Prior 1999).

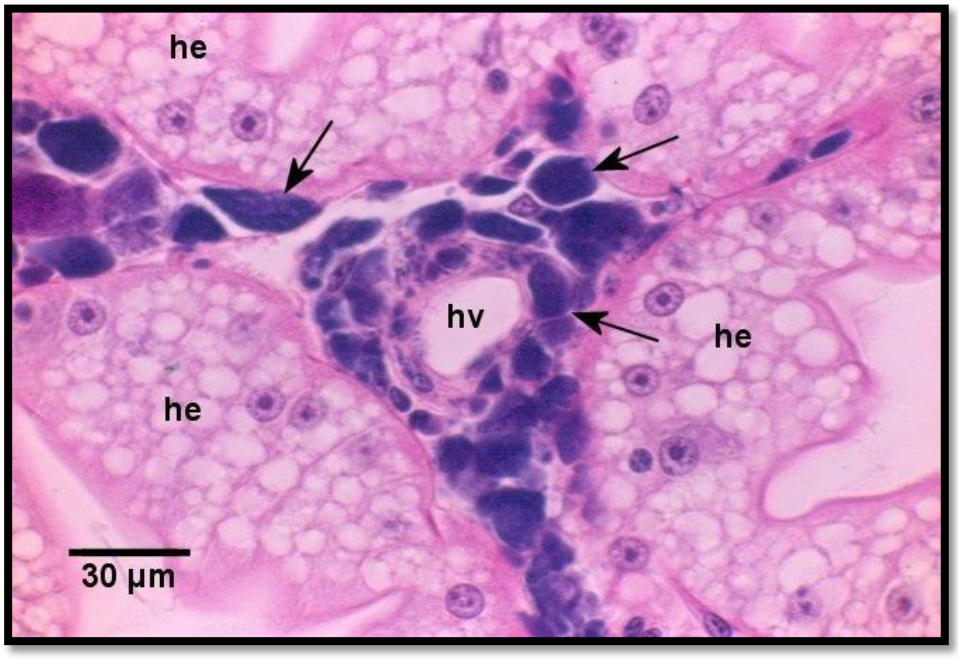

Figure 4.7 Example of *Coxiella cheraxi* infection illustrating strongly basophilic microcolonies, haemocytic infiltration and tubule separation (arrows). Hv=haemolymph vessel (Photo: Bower and Romero 2009).

While no statistical significance was observed, there was a slight reduction noted in weight of the inoculated crayfish post-trial, indicating a decline of health. Repeated PCR, qPCR and

sequencing were all consistent with the presence of a *Coxiella burnetii* infection with 73% of inoculated crayfish confirmed infected by the detection of the *com1* gene and subsequent 16S rRNA sequencing.

There was also evidence of possible vertical transmission of the infection. One crayfish in the 10^4 cells/ml infection bracket was carrying eggs at the time of infection, and subsequent sampling of hepatopancreatic tissue from this animal was negative for *C. burnetii* infection based on PCR and qPCR results (Table 4.3). However, egg membranes sampled from the same animal tested positive on PCR for *com1*. Further sequencing confirmed the presence of *C. burnetii* within the membrane and qPCR placed the approximate concentration of 1.10x10 copies per ml. This possibly supports previous reports of vertical transmission in both *C. burnetii* and *C. cheraxi* (Maurin and Raoult 1999; Tan and Owens, 2000).

Also of note, in this experimental population it appeared that approximately 30% of samples were concurrently infected with presumptive reovirus as diagnosed initially by histopathology. Reovirus infected individuals were found in all experimental groups and were sourced from the same population previously known to harbor the virus asymptomatically. It is unclear whether co-infection with this virus has a significant effect on gross pathology and infection of *C. burnetii* within experimental crayfish as a full survey for the virus was not performed on the experimental population. However, previous studies of RLO infections within prawns indicate that RLOs are frequently observed in association with multiple concurrent infections and therefore the possibility remains acknowledged (La Fauce and Owens 2007; Bower and Romero 2009). Histopathology was the most obvious pervasion of this pathogen whereby *Coxiella* infection manifestations may have been masked or exacerbated. For the purposes of this experiment, it was only noted that there was incidence of the virus in some individuals, as a more detailed survey and clinical diagnosis of the viral disease within the experimental population would be necessary to assess the significance of a co-infection.

This is the first reported evidence of successful infection and detection of *Coxiella burnetii* within Australian redclaw crayfish. While bacterial titres were not higher than $3.75x10^1$ copies, there was sufficient clinical evidence that *Cherax quadricarinatus* can carry *Coxiella burnetii* for a minimum of 21 days and that at least in one case, vertical transmission from adults to progeny potentially occurred. Due to a significant reduction in bacterial load from initial titres and the known presence of a co-infection of several animals, there is evidence

to indicate that *C. burnetii* replicates inefficiently within *C. quadricarinatus*. This suggests that *Cherax quadricarinatus* may have the ability to clear *Coxiella burnetii* as a feature of immunity or maintain infections at sub-clinical levels. In the case of *C. burnetii*, even low concentrations are epidemiologically significant due to its exceptional infectivity and the known ability of a dose as low as one organism to cause infection in an otherwise healthy human (CDC 2010). As such, further investigation into innate immunity and susceptibility on a larger scale is necessary to deduce the true host and carrier potential of Australian redclaw crayfish for *Coxiella burnetii.* This experiment illustrates an important research gap in comparative immunity and highlights some compelling questions about the physiological and biochemical differences between *Coxiella burnetii* and *Coxiella cheraxi* in arthropod and mammalian hosts. This information in turn, could provide valuable insight concerning both pathogens for the fields of aquaculture, public health and biomedicine.

CHAPTER 5

EVIDENCE OF RLO AND PRESUMPTIVE *COXIELLA CHERAXI* INFECTIONS IN MARFU FACILITY STOCK, JAMES COOK UNIVERSITY QUEENSLAND, AUSTRALIA

5.1 Introduction

In March of 2012, a graduate student working in the Marine and Aquaculture Research Facilities Unit (MARFU) at James Cook University noticed an abnormally high death rate in her recently acquired stock of juvenile Australian redclaw crayfish. Several screening samples were sent to the Aquapath Research Group at the School of Veterinary and Biomedical Sciences to determine cause of death. After initial histological screening, it was determined that there were abnormal Gram-negative cytoplasmic inclusion bodies within the connective tissues of the samples which closely resembled *Coxiella cheraxi* strain TO-98, an RLO previously described in *C. quadricarinatus*. Due to the known devastating affect these bacteria can have on aquaculture stock, immediate action for the definitive identification and containment of this pathogen was undertaken.

5.2 Materials and Methods

5.2.1 Site Survey

MARFU stock facilities are the primary facilities used to contain a range of marine and freshwater species under observation or experimental manipulation at James Cook University. While some basic biosafety measures are observed, at the time of investigation there were no comprehensive records of animal import or mandatory quarantine for farmed crustacean stock. It was also apparent that water sources were mixed and primary pipe sources and cleaning instruments were sometimes shared between tanks for different experiments without prior decontamination (personal observation).

Tanks housing the population of interest, Australian redclaw crayfish juveniles, were located outdoors with a permanent shade roof protecting tanks from rainwater and direct sunlight. Filtration units were also shared and contained activated charcoal. Average daily water temperature reading was 26.0°C and stocking density was approximately 100-150 juveniles per tank. Three tanks housed crayfish, with two of these sharing a water source (Fig. 5.1), while the third was located approximately 30 meters away on a separate block within the facility. All three tanks were experiencing abnormal mortality.

Figure 5.1: Tank setup at MARFU facility. N.B. two main tanks sharing pipes and water source (arrow).

5.2.2 Collection and Containment

Once identified as potentially infectious stock, approximately 200 live redclaw were transported from MARFU in moist, lightly stocked eskies to a set of three large quarantined tanks in a locked room at the School of Veterinary and Biomedical Sciences. As all tanks were located within close proximity to uninfected populations of *C. quadricarinatus* as well as a number of freshwater fish, biosafety measures were of significant concern. Tanks housing MARFU crayfish were filled with dechlorinated water via a designated water source and setup with separate filtration systems (non-charcoal). All waste water was decontaminated with chlorine (90 mg/L) prior to disposal into drainage system. Other biosafety measures included designated footwear and a Virkon® footbath (Dupont, USA), 95% ethanol disinfectant and dedicated separate food source. Additionally, all feeding and water exchanges were scheduled on opposite days to unaffected crayfish stock to further

reduce the risk of contact infection. Source tanks at MARFU were decontaminated and it was advised that all filters and pipe fittings were to be thoroughly cleaned with chlorine and decontaminated prior to disposal. Collected animals were observed for further signs of infection and mortality rates were recorded daily after a three day adjustment for transferred stock. Freshly dead crayfish were either frozen at -20°C or prepared for histology, DNA extraction and PCR screening to detect the presence of possible *Coxiella*.

5.2.3 DNA Extraction

DNA extraction was performed with Roche High Pure© PCR template using the same protocols described in Chapter 4 (Section 4.2.8).

5.2.4 Histology

Due to rapid post-mortem decomposition of tissues, only freshly dead or sacrificed crayfish were used for histological analysis. Twenty two crayfish of the 42 collected in total were used for histology sampling. Crayfish bodies were injected with approximately 0.5ml of Davidson's fixative in their first and third abdominal segments and cut longitudinally for added preservation. They were then immersed in the fixative for approximately 48 hours before being transferred to 95% ethanol for histological preparation. The hepatopancreas, gills and muscles of all redclaw were placed in histology cassettes and dehydrated using a timed series of alcohols, xylene and finally embedded in paraffin wax. Tissue sections were cut at 5µm using a rotary microtome into paired sections and stained with Mayer's haematoxylin and eosin (H&E) or Gram stain (Appendix 2). Sections were screened under light microscopy (Olympus E © microscope) and images captured with a MicroPublisher 5.0 RTV camera (QImaging ®, Surrey Canada).

5.2.5 PCR Diagnostics

Forty two crayfish as well as two water samples from MARFU were initially screened using standard PCR targeting the *com1* amplicon described previously (Chapter 4). Hepatopancreatic and muscle tissue were sampled from each crayfish. Samples were prepared and visualized under the same conditions described in Chapter 2 (Section 2.7).

In order to exclude the possibility of a *C. burnetii* infection and to more thoroughly characterize the MARFU infective agent, all samples were also screened using MLSA designed to detect known housekeeping genes of *C. burnetii* (Chapter 2, Fig 2.1). *Coxiella*

burnetii RSA 493 Nine Mile Strain was used as reference sequence. (refseq: NC_002971www.ncbi.nlm.gov/GenBank).

Primers for 16S rRNA were also used in the diagnostic process as was IS111a, a primer recognized for its clinical sensitivity to detect *C. burnetii* (see Vaidya et al. 2008). All primers were designed using Primer3 BLAST software (www.ncbi.nih.gov) and synthesized by Sigma-Aldrich (Sydney, Australia). Target sequences and thermocycling conditions are listed in Chapter 2. Positive controls were template 9mi/II/C4 strain from infected vero cells as well as known positively infected *C. burnetii* tissue samples from redclaw from the experiment described in Chapter 4. Negative controls were nuclease-free water and uninfected *C. quadricarinatus* hepatopancreatic tissue.

5.2.6 Sequencing

PCR products were purified using Wizard ® SV Gel and PCR clean-up system (Promega, Australia) and sent to Macrogen Corporation (Seoul, Korea) for confirmation of sequences. Resultant sequences were cleaned and aligned using Sequencher® and Geneious© Software (Biomatters 2013). Resultant sequences were then compared with GenBank data by BLAST analysis (www.ncbi.nlm.nih.gov/blast) as well as the Ribosomal Database Project (Michigan State University).

5.3 Results

5.3.1 Mortality Data

Collection of basic mortality data was attempted for quarantined animals for 32 days to detect any similarity to mortality rates observed in previous *Coxiella cheraxi* outbreaks. For an approximate sample size of 200 crayfish, 42% (83 animals) died in the 32 days after an initial three day allotted adjustment period. By the end of 60 days it was noted that an additional 26 crayfish died bringing the overall total to 109 crayfish (approximately 55% post-collection) and an average daily loss to 1.8 crayfish (± 1.2). Mortality rates and numbers from the same stock moved from MARFU were not formally reported prior to their removal from the facility. Estimates by MARFU for the three weeks leading up to collection period were between one to two casualties per day.

5.3.2 Histopathology

Histological analysis of the samples presented hepatopancreatic tissue with large, cytoplasmic inclusion bodies (Fig 5.2). Tubule separation was widespread and granuloma

formation was present and strongly basophilic based upon HE staining (Fig 5.3). Inclusion bodies were Gram-negative and found throughout the hepatopancreatic tissues of sampled crayfish (Fig 5.3). Muscle tissue appeared less commonly affected, however some connective tissue samples also presented with inclusion bodies consistent with an RLO infection (Fig 5.4). All twenty two crayfish samples collected from the MARFU stock exhibited gross histopathology (100% prevalence) within the hepatopancreas consistent with a *C. cheraxi* infection including Gram negative bacteraemia, cytoplasmic inclusion bodies and hypertrophy of endothelial cells (Owens et al. 1992; Ketterer et al. 1992).

Other notable pathologies included low level *Psorospermium c.f.* (Class Mesomycetozoea: Order Ichthyophonida) infections in most samples, a known spore of the parasitic DRIP clade of organisms from freshwater crayfish (Fig 5.4). No other concurrent infections were observed.

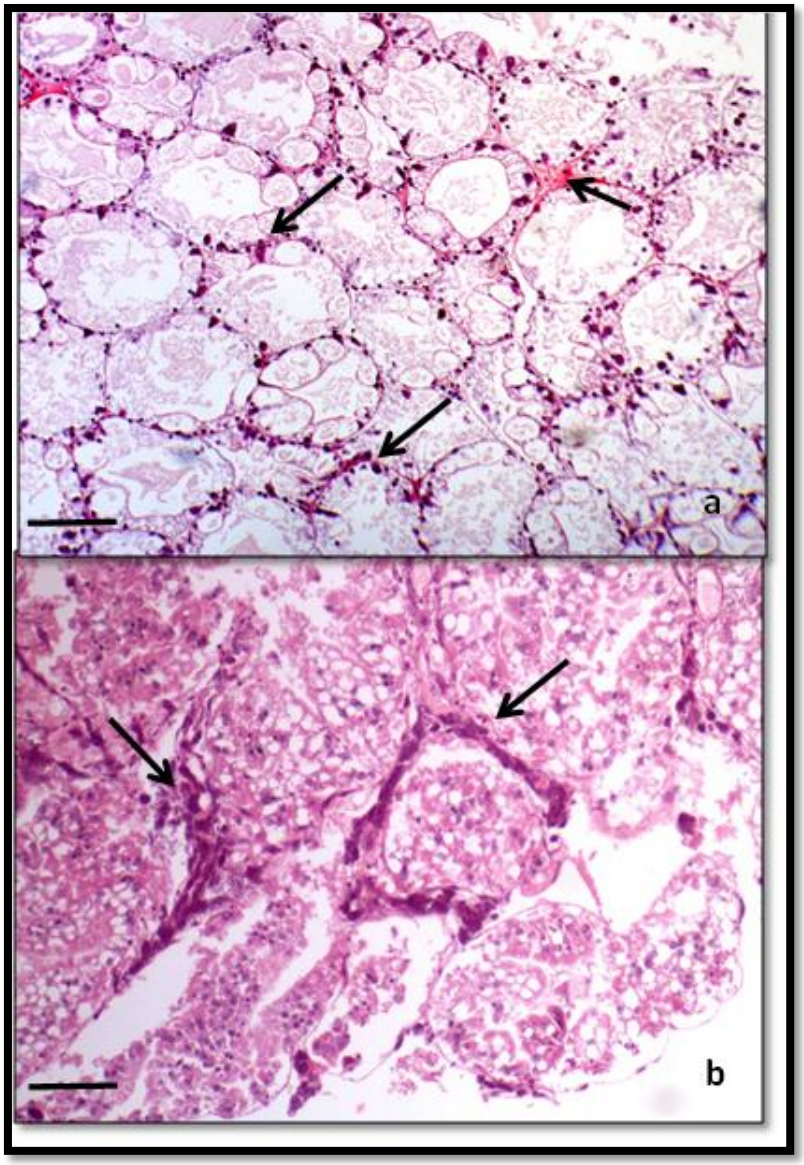

Figure 5.2: Light micrographs of haematoxylin and eosin (HE) stained hepatopancreatic tissue from *Cherax quadricarinatus* presumptively infected with *Coxiella cheraxi*. (a) A crayfish from MARFU showing widespread bacterial inclusions and mild separation of tubules (arrows). Some artefacts due to post-mortem autolysis present. Scale bar 100µm. (b) Crayfish from MARFU presenting progressed infection with strongly basophilic intracellular colonies (arrows). Scale bar 60µm.

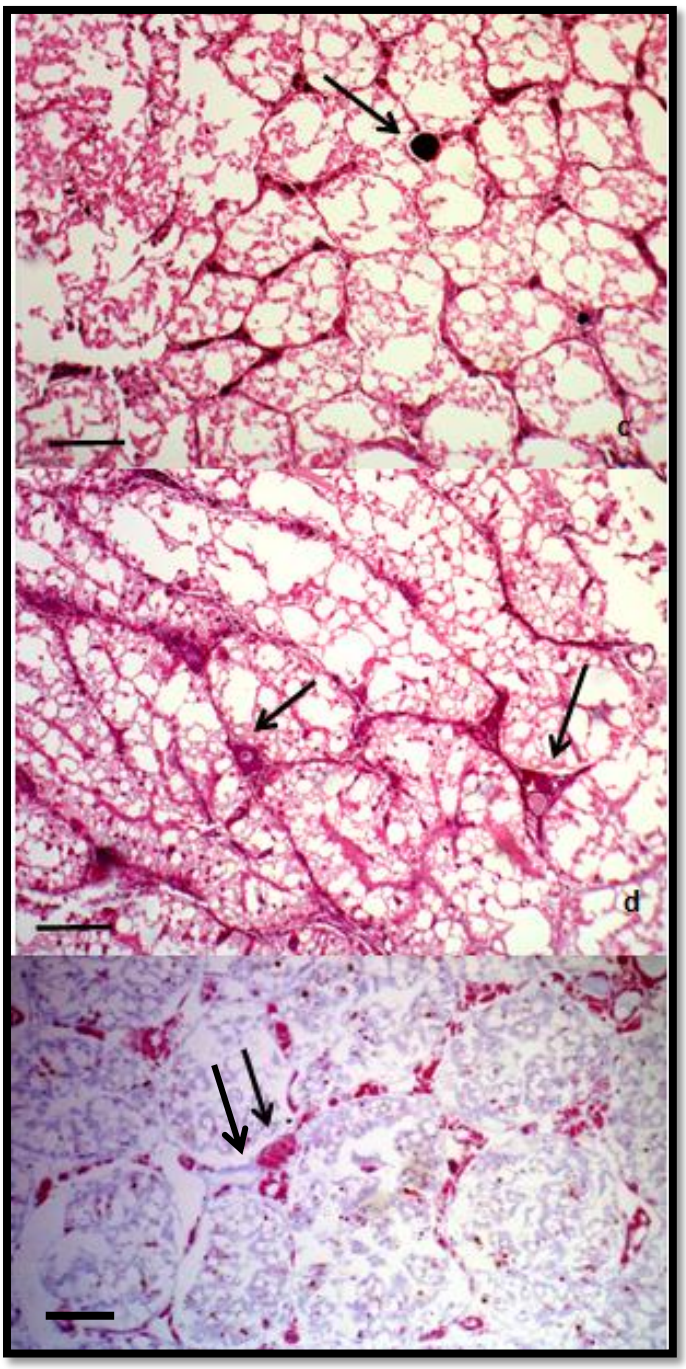

Figure 5.3: Light micrographs of hepatopancreatic tissue from *Cherax quadricarinatus* presumptively infected with *Coxiella cheraxi*. (c) Crayfish from MARFU showing widespread bacterial inclusions, separation of tubules and granuloma formation (arrows) H&E stain. (d) Crayfish from MARFU with progressed infection of strongly basophilic inclusion colonies (arrows) H&E stain. e) MARFU crayfish with Gram staining highlighting large Gram-negative colonies of presumptive *C. cheraxi* (arrows). Scale bar 30µm.

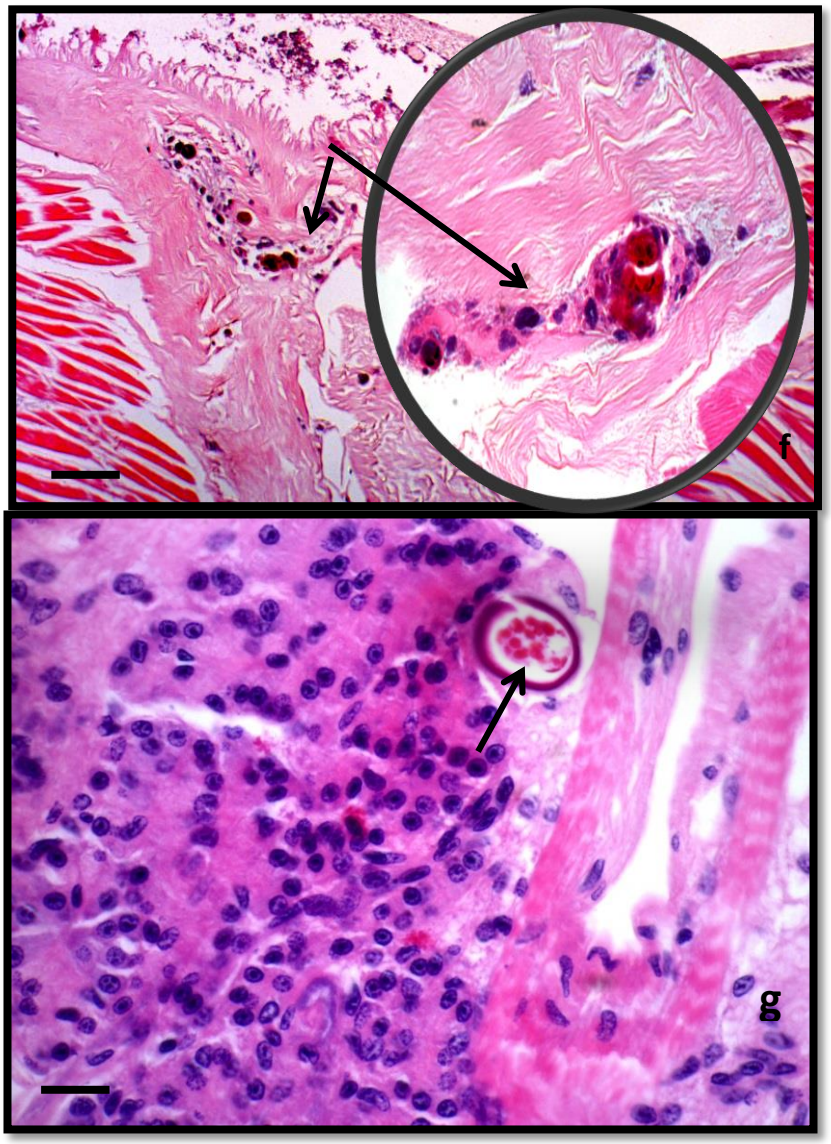

Figure 5.4: Light micrographs of haematoxylin and eosin (HE) stained hepatopancreatic and connective tissue from *Cherax quadricarinatus* presumptively infected with *Coxiella cheraxi*. (f) Connective tissue of crayfish from MARFU exhibiting large basophilic inclusion bodies (arrows) and containing possible *C. cheraxi* colonies.Scale bar 100µm/20µm. (g) Crayfish from MARFU with *Psorospermium* sp. co-infection. Scale bar 40µm.

5.3.3 PCR

Of the 42 crayfish tested by PCR, only six (14.3%) were positive for the *com1* marker, generating faint bands on standard PCR. Four of these were hepatopancreatic tissue samples while the other two were muscle tissue. Some samples exhibiting gross histopathology for an RLO infection generated false negatives by PCR. The two water samples collected were inconclusive. Some ambiguous secondary banding and by-products occurred for sample wells, while positive controls were clear and well defined in almost all cases. No samples tested conclusively positive on any other gene from the MLSA. All samples were also negative for amplicons of the IS111a virulence sequence, excluding the presence of *Coxiella burnetii*. Due to inconsistent results for *com1* positives, further 16S rRNA sequencing was undertaken on all MARFU samples to further examine the possibility of a *C. cheraxi* infection.

5.4.5 Sequencing

Of the 42 crayfish samples sequenced, there were two consensus 16S rRNA sequences developed based on quality and match post-clean-up. The first included 33 tissue samples from MARFU (generating 66 sequences) which was a contiguous set of 1237 base pairs with approximately 168 ambiguities. Samples consisted of both muscle and hepatopancreatic tissue. A BLASTn search indicated a 85% and 86% match respectively to an uncultured *Coxiella* bacterium clone partial sequence and *C. cheraxi* partial sequence. A consensus sequence developed with the reference sequence, *Coxiella cheraxi* confirmed this relationship with distance matrices of 86.5% between the MARFU consensus sequence alignment and *C. cheraxi* (refseq: EF413063.1 www.ncbi.nlm.gov/GenBank).

A second aligned consensus sequence for two additional hepatopancreatic samples was an 86% match with BLAST to a partial sequence of *C. cheraxi* with no other matches with known pathogenic organism within the database. The positive samples previously detected in standard PCR were included within the first consensus sequence.

Table 5.1: Consensus sequences from MARFU samples for *Coxiella sp.*

Sample	Length (bp)	Gene hit (Acc)	Match %	E - value	Consensus Sequence Refer to IUPAC ambiguity codes (Appendix 3)
Consensus sequence MARFU samples 1,2,3,4,7,8,9,10,11,12,14,15,16,17,18,20,21,23,24,25,26,27,28,29,30,31,32,33,34,35,37,39,41	1237	*Coxiella cheraxi Partial sequence* EF413063.1 Uncultured *Coxiella* bacterium clone EF09220.1	86	0.0	GGTGAGTAATGCSTRGGAAWYTACCTTATAGTGGGGGATAACCCGKG GAAACKMGGGCTAATACCGCATAATCTCKTMGGAGCAAAGCGGGG GATCTTCGGRCCTYGCGCTAMMRGATRAGCCYGCGTTGGATTAGCT WGTTGGTGRGGTAAAGGCYTACCAAGGCGACGATCCATAGCTGGTC TGAGAGGAYGATCAGCCACACTGGRACTGAGACACGGYCCAGACTCC TACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGSGMAASCCTG ATSCAGCMATGCCGCGTGTRTGAAGAAGGCCTTCGGGTTGTAAAGYA CTTTCAGTRGGGARGAARNTKSTTAAGATTAATACTCTTAAKCNKTTG ACGTTACCCACAGAASMAGCNCWGGCTRACTCTGNNTGCCAGCAGC CGCGGKANTACRGAGRGTGCAAGCGTTAATCGGAATTACTGGGCGTA AAGCGCRCSTAGKKRGWTMTKTAAGTMRGATGTGAAAKCCCTKGGC TWAASCTRGRAATTGCAYYYGATACTGRMTATCTAGAGTAYKGTAGA GGNNAGKGGWAKTTCCNNNGGTGTAGCGGTNMMMTGCGTAGAKA TCKGAARGAAYACCRGTGGCGANAGNGCGGCTATCTGGACMAATAC TGACACTSAGRTGCGAAAGCGTGGGGAGCAAACAGGATTAGAGACC CTGGTAGTCCACGCCGTMAACGATGWSAACNTRGMKGTTGKGASST WKACCTCKTRGNTWKCGRAGCYAACGCGTTARGTWSWCCGCCTGG GGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGGCCC GCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAAC CTTACCTACYCTTGACATCCWCAGAACTTRKCRGAAAYGACTKGNGTG CCTTCGGGAACTSWGTGACAGGTGCTGCATGGCTGTCGTCAGCTCGT GTCGTGAGATGTWGGGTTAAGTYCYGTAACGAGCGCAACCCTYRTCC TTAKKTGCCAGCRAGTAATGKYGGGAACTCYAAGGAGACTGCCGGTG ATNAAWYCSSAGGAANGGYGGGGAYGAYGTCAAGTCATCATGGCCC TTACGRGTAGGGCTACACACGTGCTACAATGNGCNGTAYAAAGNNG GYWGCSAWRCCGCGAGGTGGAGCKAATCCCAAAAAGNCTGYTSTTA NTCCSGATWGGAGTCTGCAACTCGACYCCWTG
MARFU samples 6 & 12	873	*Coxiella cheraxi Partial sequence* EF413063.1	86	0.0	ATCTCKTMGGAGCAAAGCGGGGGATCTTCGGRCCTYGCGCTAMMRG ATRAGCCYGCGTTGGATTAGCTWGTTGGTGRGGTAAAGGCYTACCAA GGCGACGATCCATAGCTGGTCTGAGAGGAYGATCAGCCACACTGGRA CTGAGACACGGYCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATT GGACAATGGGSGMAASCCTGATSCAGCMATGCCGCGTGTRTGAAGA AGGCCTTCGGGTTGTAAAGYACTTTCAGTRGGGARGAARNTKSTTAA GATTAATACTCTTAAKCNKTTGACGTTACCCACAGAASMAGCNCWGG CTRACTCTGNNTGCCAGCAGCCGCGGKANTACRGAGRGTGCAAGCGT TAATCGGAATTACTGGGCGTAAAGCGCRCSTAGKKRGWTMTKTAAG TMRGATGTGAAAKCCCTKGGCTWAASCTRGRAATTGCAYYYGATACT GRMTATCTAGAGTAYKGTAGAGGNNAGKGGWAKTTCCNNNGGTGT AGCGGTNMMMTGCGTAGAKATCKGAARGAAYACCRGTGGCGANAG NGCGGCTATCTGGACMAATACTGACACTSAGRTGCGAAAGCGTGGG GAGCAAACAGGATTAGAGACCCTGGTA GTCCACGCCGTMAACGATGWSAACNTRGMKGTTGKGASSTWKACCT CKTRGNTWKCGRAGCYAACGCGTTARGTWSWCCGCCTGGGGAGTAC GGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAG CGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCT ACYCTTGACATCCWCAGAACTTRKCRGAAAYGACTKGNGTGCCTTCG GGAACTSWGTG

5.5 Discussion

While initial *com 1* and MLSA PCRs were inconclusive, it was apparent from mortality, histopathology and the obtained 16S rRNA sequences that *C. cheraxi* or a closely related RLO was likely the agent of the MARFU outbreak. While mortality rates were not consistently recorded throughout the observed epizootic, the observations were comparable with previous incidences where the mortality pattern for infected crayfish was > 50% (Ketterer et al. 1992; Tan and Owens, 2000; Jimenez and Romero, 2002). This histological examination was largely consistent with observations from previous *Coxiella*

cheraxi and RLO infections, with large, Gram-negative basophilic inclusions and cytoplasmic bacterial colonies evident in infected crayfish (Fig 5.6)

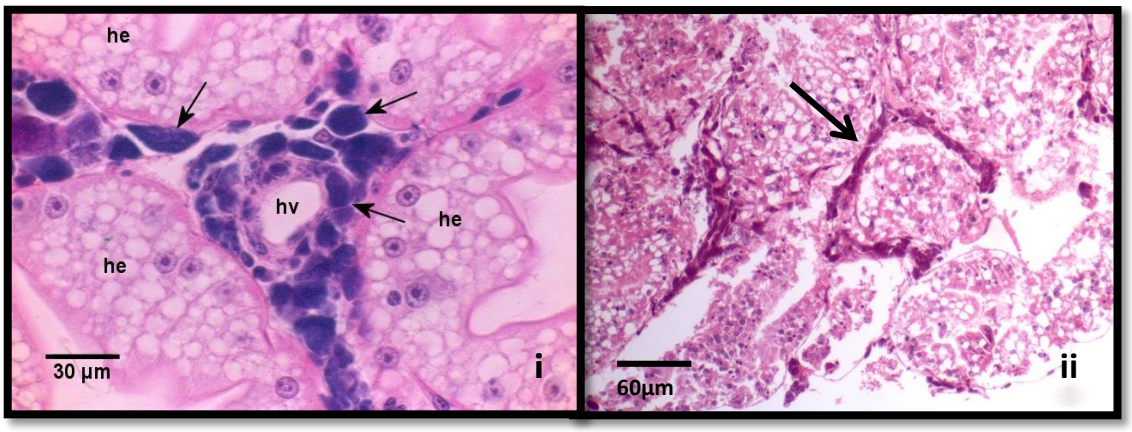

Figure 5.5: Comparison of suspected *C. cheraxi* infections from aquaculture samples of *C. quadricarinatus* under light micrograph and haematoxylin and eosin (HE) stain. (i) MARFU crayfish with suspected *C. cheraxi* infection collected in Canada in 2009 exhibiting large, strongly basophilic cytoplasmic inclusion bodies (arrows) (Bower and Romero, 2009). (ii) Sample collected from MARFU exhibiting similar pathology in early 2012. Both histology samples exhibit infected connective tissue and basophilic colonies (arrows). Note* Sample from 2012 also exhibits extensive artefact in form of post-mortem autolysis of hepatopancreas tubule epithelium possibly obscuring additional pathology.

The presence of inclusion bodies in connective as well as hepatopancreatic tissue within the MARFU population is indicative of a systemic RLO, a description consistent with past *C. cheraxi* observations (Owens et al. 1992; Ketterer et al. 1992; Edgerton and Prior, 1999).

16S rRNA sequencing offered compelling confirmatory evidence that the infectious agent at MARFU was an RLO. Similar consensus sequences of 86-87% indicated that the infection was either a *Coxiella sp*. or a closely related RLO. As *C. cheraxi* has yet to be fully sequenced and submitted to the NCBI database, it is possible that the difference in matched sequence similarity is a data issue as BLAST treats searches and alignments with ambiguities as mis-matches, thereby possibly obscuring the full match identity of the organism.

Based on both histology and 16S rRNA sequencing results, the sensitivity of the previously designed diagnostic MLSA and PCR was called into question. Equipment troubles and likely a combination of inconsistency and inexperience in laboratory techniques may have obscured the initial screening and diagnosis of samples explaining the incongruency in PCR results versus those later presented in sequences. It is also possible that *com1* is less effective as a diagnostic marker for *C. cheraxi*. In order to increase screening sensitivity, it

would be necessary to fully isolate, culture and sequence the organism for a more specific diagnostic design.

The discovery of a potential outbreak of *C. cheraxi* at MARFU had a multitude of implications. Heavy mortalities experienced at MARFU had potential to spread to other crustacean stock, possibly other aquatic animals, or into local drainage areas where wild populations of redclaw and other susceptible animals may be exposed. As previous studies of *C. cheraxi* indicate mortality post-exposure could reach as high as 80%, it was imperative to not only investigate the organism in question, but also source and stem the infection (Ketterer et al. 1992). Upon further inquiry into MARFU's facilities, it was discovered that the infected crayfish had been sourced from three different local crayfish farms along a similar timeline, with no separation or initial quarantine between populations. As there was no evidence of previous outbreaks or issues with previous MARFU samples until the new crayfish were introduced, it was deduced that one or more of the supplying farms was the likely source of infection and that further investigation was necessary to assess this suspicion. This hypothesis is further investigated in Chapter 6.

CHAPTER 6

TRACKING PRESUMPTIVE *COXIELLA CHERAXI* RLO INFECTION FROM FARMED STOCKS IN NORTHERN QUEENSLAND, AUSTRALIA

6.1 Introduction

The discovery in March of 2012 of a suspected outbreak of *Coxiella cheraxi* in Australian redclaw crayfish at the MARFU at James Cook University led to a necessary investigation of the infection source. As prior outbreaks had not been observed at the MARFU facilities nor observed in any neighbouring tanks it was deduced that the infection was possibly sourced from one of the supplying crayfish farms. After investigating supply records it was apparent that between November 2011 and the outbreak in March of 2012, three different farms supplied juvenile Australian redclaw crayfish to MARFU for the same project. Populations were mixed in the same tanks with existing populations without quarantine or an isolation period prior to introduction. As the incubation period for *C. cheraxi* is not known and no farms were reporting abnormal mortalities, it could not be deduced from the timeline or geography alone which farm was the likely infection source. This chapter outlines the epidemiological investigation undertaken to try to trace the RLO infection source from MARFU and the steps necessary to prevent further infection.

6.2 Materials and Methods

6.2.1 Site Introductions

Three farms supplying crayfish were identified in northern Queensland (referred to as Farm A-C) as potential carriers for *C. cheraxi* into MARFU stock based on the timeline for delivery to the facility and the initial outbreak. Based on MARFU records and personal communication with local farmers, a basic timeline for stock delivery and mixing was established (Table 6.1). Farms were either researched remotely or surveyed in person depending on time and travel constraints.

Table 6.1 Approximate timeline for delivery of crayfish

Month of Delivery	Supplying Farm	Location
November 2011	Farm A	Townsville, Queensland
January 2012	Farm C	Mareeba,Queensland
February 2012	Farm A	Townsville, Queensland
March 2012	Farm B	Atherton, Queensland

Farm A

Farm A is a barramundi and redclaw crayfish farm located in Townsville, northern Queensland. It consists of ten semi-intensive grow-out ponds specific for growth and production of Australian redclaw (Fig 6.1). Biosafety measures were observed between front and back ponds with regards to feeding, monitoring and capture. Crayfish used for feeding, treatment or growth experiments were marked with latex tags for easy identification. Donated juveniles for MARFU were former experimental crayfish with latex tags. The farm reported no abnormal mortalities or reports of lowered production rates indicative of *C. cheraxi* infections prior to or during the supply period. *Coxiella cheraxi* was a suspected culprit of a mass die-off in a 2005 isolated incident at the farm, however no incidence of the disease had been observed on site since.

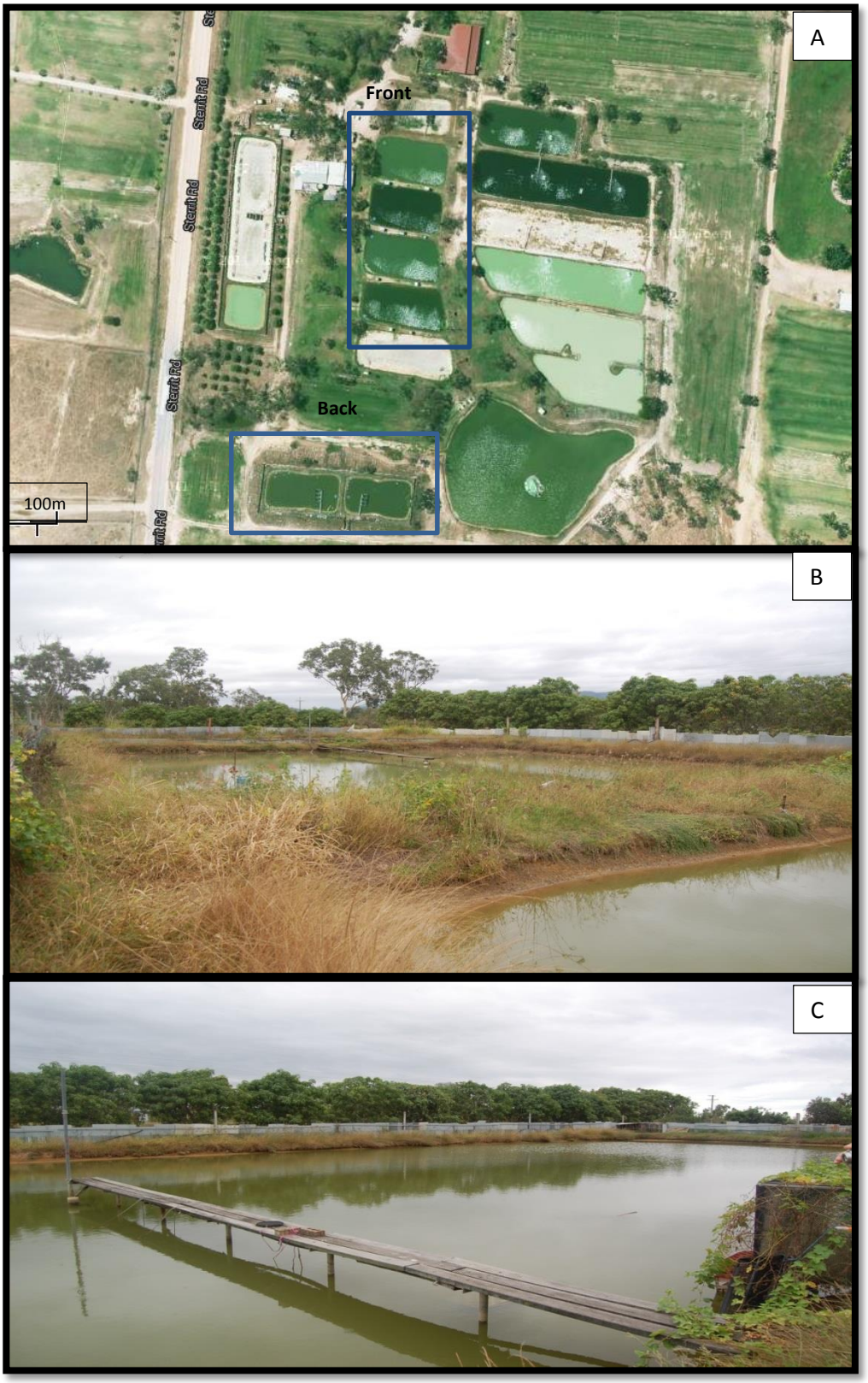

Figure 6.1: a) Aerial view of Farm A in Townsville, Queensland illustrating layout of ponds and general landscape features. Front and back ponds are highlighted in blue (GoogleEarth®) b/c) View of top two front ponds with catching dock, typical of redclaw farming facility.

Farm B

Farm B is an Australian redclaw farm based in Atherton, northern Queensland. The facility consists of 30 earthen based ponds of varying size and distribution on approximately 10 hectares of land (Fig 6.2). In the months leading up to the disease outbreak at MARFU, there were no reports of declining health or reduced production yield.

Figure 6.2: Aerial view of Farm B in Atherton, Queensland illustrating layout of ponds and general landscape features (GoogleEarth®)

Farm C

Farm C is an Australian redclaw crayfish farm based in Mareeba, northern Queensland. It consists of ten semi-intensive grow out ponds (Fig 6.3). Biosecurity information was not readily available; however in the months prior to the MARFU outbreak, the farm facility had been reporting an approximate 20% reduction in production load (personal communication with farmers). Additionally, crayfish from this farm delivered to MARFU in

late January of 2012 experienced a 90% mortality rate within 48 hours of their arrival. The surviving animals were mixed with replacement stocks from other farms.

Figure 6.3: Aerial view of Farm C in Mareeba, Queensland illustrating layout of ponds and general landscape features (GoogleEarth®)

6.2.2 Collection of Live Samples

In April and May of 2012, approximately 50 live *C. quadricarinatus* from Farm B and C were donated by supplying farms and delivered in transport coolers to JCU for further assessment and screening. In July 2012, twenty four crayfish from Farm A were collected in directly from two separate farm ponds in baited traps (Fig 6.4) and processed within one hour of collection. Prior to nucleic acid extraction and histological examination, all crayfish were submerged in an ice slurry for sedation. The cephalothorax was then cut midsagitally, whereby half was preserved in 95% ethanol for nucleic acid isolation and the other half fixed in Davidson's fixative for histological preparation.

Figure 6.4 Baited trap used at Farm A for collection of juvenile and adult *C. quadricarinatus* samples for analysis.

6.2.3 Histology Preparation

Twenty crayfish from both Farm B and Farm C were sacrificed for histology sampling while twenty four crayfish were surveyed from Farm A for a total of 64 animals. Crayfish bodies were prepared for histological analysis using the protocol described in Chapter 5.2.4.

6.2.4 DNA extraction and PCR for detection of *C. cheraxi*

Hepatopancreatic, pleopod and muscle tissue were sampled from each crayfish from farms A-C. Gills were also sampled from Farm A as part of a separate investigation. Extraction took place using Roche High Pure© Template Preparation Kit according to the manufacturer's instructions with the modifications previously described (Chapter 2). DNA concentration was quantified using spectrophotometry (IMPLEN Nanospectophotometer ™). Quantitative values were then observed and recorded for PCR.

Post-quantification, all samples were diluted into aliquots for PCR containing approximately 35 – 55ng of template DNA. PCR preparation took place in a biosafety cabinet under sterile conditions. All negative controls and samples were prepared and removed from the cabinet prior to positive sample preparation. Farms were screened and tested separately to avoid cross-contamination.

DNA from crayfish tissue samples were screened in a similar method to Chapter 5, initially with the *Coxiella* specific marker *com1* and IS111a as well as 16S rRNA primers. MLSA was not performed on these samples.

Samples were setup in 25µl reactions containing 12.5µl GoTaq Green Hotstart Master Mix (Promega ©), 7-11µl nuclease-free water, 25-50 ng DNA template and 0.5µl forward and reverse primers respectively. Annealing temperatures were calculated and optimized accordingly (Chapter 2). Samples were run on an Eppendorf ©Mastercycler. The thermocycler program for *com1* consisted of incubation at 94°C for 2 minutes followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing of primers at 63°C for 1 minute and 68°C at 1 minute. Finally an extension of 72°C for 5 minutes and a 12° holding temperature. Cycling conditions for IS111a and 16S rRNA primers are outlined in Chapter 2.

PCR products were visualized via UV on 1.8% agarose gels stained with 1µl GelRed (Biotium ©) run at 150V for 45 minutes against known ladder standards (100bp or 1kb by Invitrogen ®) to verify presence of relevant amplicons.

6.2.5 Sequencing

PCR products were purified and sent for sequencing using the protocol previously described in Chapter 5.2.5

6.3 Results

6.3.1 Histopathology

Farm A

Histological examination revealed a number of commensal flukes within gills of the redclaw, as well as other possible parasitic infections (Figure 6.5a). Melanisation and necrotic eosinophilic nodules and filaments indicative of opportunistic bacterial infection were observed in some crayfish. These pathological changes were associated with a number of hypertrophied nuclei observed in gills with a mild infiltration of haemocytes into infected regions suggestive of parvovirus (Figure 6.5b). Approximately 20.8% (5 out of 24) of redclaw developed hypertrophic nuclei in the gill epithelium. In the hepatopancreas, melanised nodules accompanied by haemocyte aggregations in the interstitial space between tubules (haemal sinuses) were observed (Figure 6.5c). Fibrous nodules were commonly found in the hepatopancreas. Eosinophilic reovirus-like inclusion body structures (Figure 6.5d) could be observed in the epithelium of the hepatopancreas of approximately 9 of 24 crayfish (37.5%).

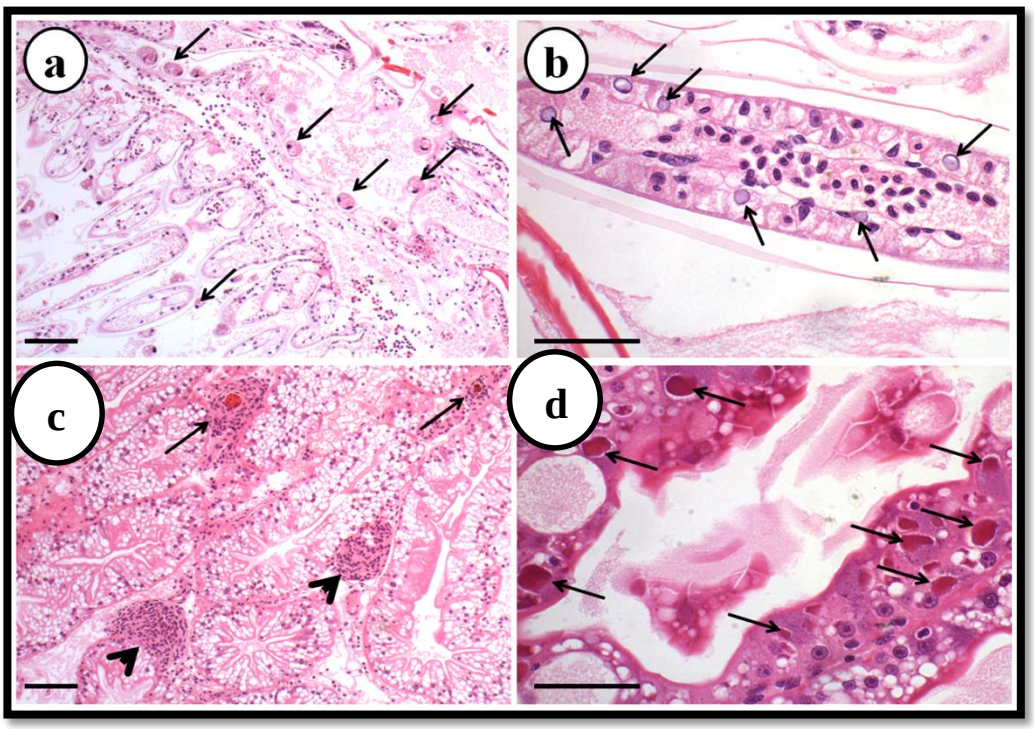

Figure 6.5. Light micrographs of haematoxylin and eosin (H&E) stained midsagittal section of the gills (a, b) and hepatopancreas (c, d) of *Cherax quadricarinatus*. (a) Numerous flukes (temnacephalids) in the gills (arrows) (b) Hypertrophied nuclei in the gill epithelium suggestive of parvovirus (c) Melanised nodules surrounded by multiple layer of haemocytes (arrow) and haemocyte aggregation initiating nodule formation (arrow head) in space between tubules (haemal sinuses) of hepatopancreas. d) Eosinophilic reovirus-like inclusion bodies were observed in the tubule epithelium of the hepatopancreas. Scale bars: 100 µm (a and c) and 50 µm (b and d). (Photo source b, d Rusaini James Cook University).

Farm B

Many crayfish observed from Farm B were clear of any obvious pathology however approximately 20% presented an unidentified bacteraemia within hepatopancreatic tissue and apparent eosinophilic granulomas with haemocyte infiltration (Fig 6.6b/Fig 6.6c). Approximately one quarter (25%) of samples exhibited a *Psoropermium* infection as well as another possible fungal infection (Fig 6.6a).

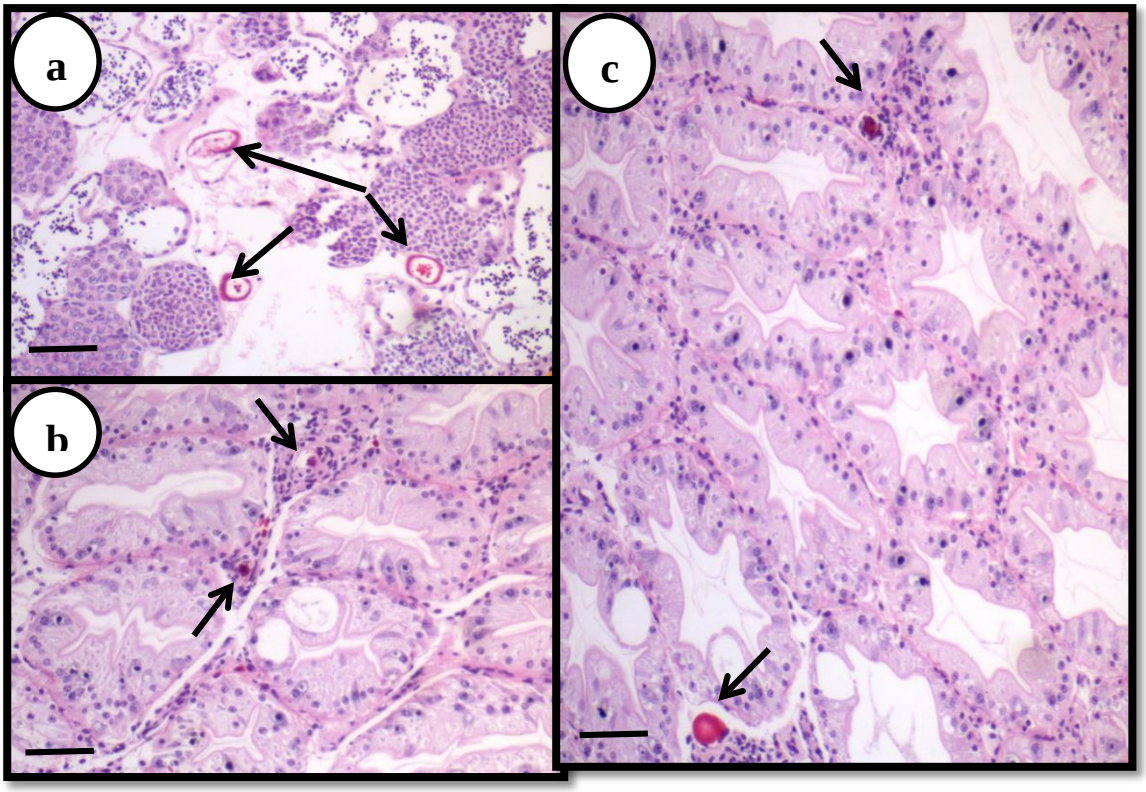

Figure 6.6. Light micrographs of the haematoxylin and eosin (H&E) stained tissue from *Cherax quadricarinatus* from Farm B (a) A crayfish demonstrating a *Psorospermium* infection with large multicellular clumping of spores (arrows) and possibly additional fungal infection. (b, c) Hepatopancreas with a minor bacteraemia, swollen nuclei with haemocytic infiltration around eosinophilic granulomas (arrows). Scale bars: 100 µm (a, b) 60µm (c).

Farm C

Histological examination revealed that approximately 20% of the sampled crayfish presented unidentified bacteraemia within the hepatopancreas, half of which (2/4) indicated the presence of unidentified Gram negative bacteria (Fig 6.7c/d).

Approximately 25% of samples demonstrated a mildly progressed unidentified fungal infection (Fig 6.7e). Another 20% of sampled crayfish also exhibited infections with cytoplasmic inclusion bodies, swollen nuclei and haemocytic infiltration similar to that observed in reovirus within the connective tissue (Fig 6.7a/c).

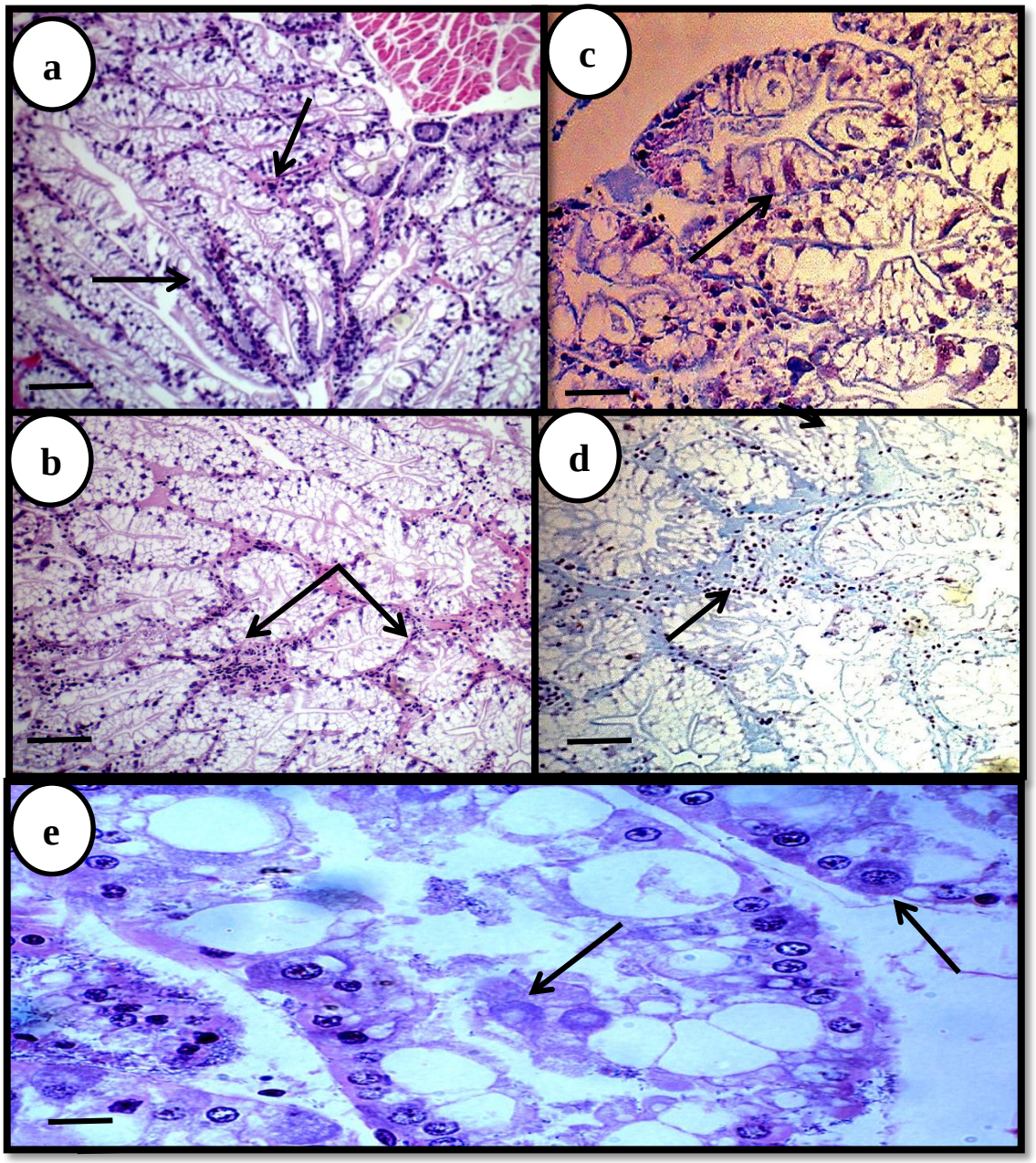

Figure 6.7 Light micrographs of the haematoxylin and eosin (H&E) and Gram stains of hepatopancreas from *Cherax quadricarinatus* from Farm C. (a) A crayfish from Farm C exhibiting haemocytic infiltration and possible reovirus –like infection (arrows). (b, c) Unidentified bacteraemia and inflammatory response (arrows), c) under Gram stain, autolysis present (d) shown to be a Gram negative infection. (e) Crayfish from Farm C exhibiting fungal infection within hepatopancreas tissues, possibly fungi or protozoan. Scale bars: 100µm (a-d); 40µm (e).

6.3.2 PCR

Farm A

PCR analysis of twenty four hepatopancreas and muscle samples (48 total tissue samples) yielded no hits on the *com1* or 16S rRNA markers for *C. cheraxi.* However, the known positive controls for *C. burnetii* and tissue from *C. quadricarinatus* infected with *C. burnetii*

presented as bright, positive amplicons with the expected lengths of 189 base pairs and 1324 base pairs respectively.

Farm B

Approximately twenty one hepatopancreas and muscle samples (42 total tissue samples) yielded 0% hits on the *com1* or 16S rRNA markers for *C. cheraxi.* However the known positive controls presented expected amplicons as above.

Farm C

Of twenty hepatopancreas and muscle tissue samples (40 total), there were four possible hits for *com1* in hepatopancreatic tissue whereby two samples presented clear 189bp amplicons. Clear amplicons were also present for both positive *C. burnetii* controls (Figure 6.8). Repeated PCR with original aliquots and again with initial source DNA excluded the possibility of contamination within the samples. There were no positive amplicons for the IS111a clinical marker, excluding the presence of *Coxiella burnetii*. 16S rRNA PCR produced ambiguous bands which were excised at the expected amplicon lengths, cleaned and sent for additional sequencing.

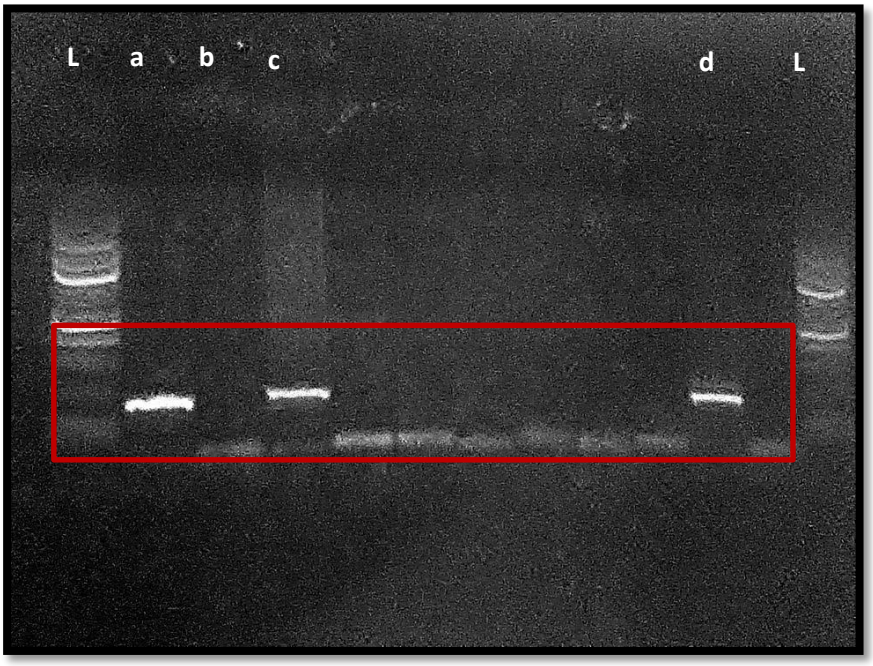

Figure 6.8. PCR amplification with *com1* primer showing 189bp amplicon for both control samples and sample from Farm C. amplicon. a) Positive control 9mi/II/C4 isolate template. b) Negative control H_2O. c) Positive control *C. quadricarinatus* hepatopancreatic tissue infected with *C. burnetii* from PC3 stock (Chapter 4). d) Farm C hepatopancreatic tissue sample. PCR products are electrophoresed on a 1.8% agarose/GelRed. L: 100 bp DNA marker (Quickload Invitrogen ®)

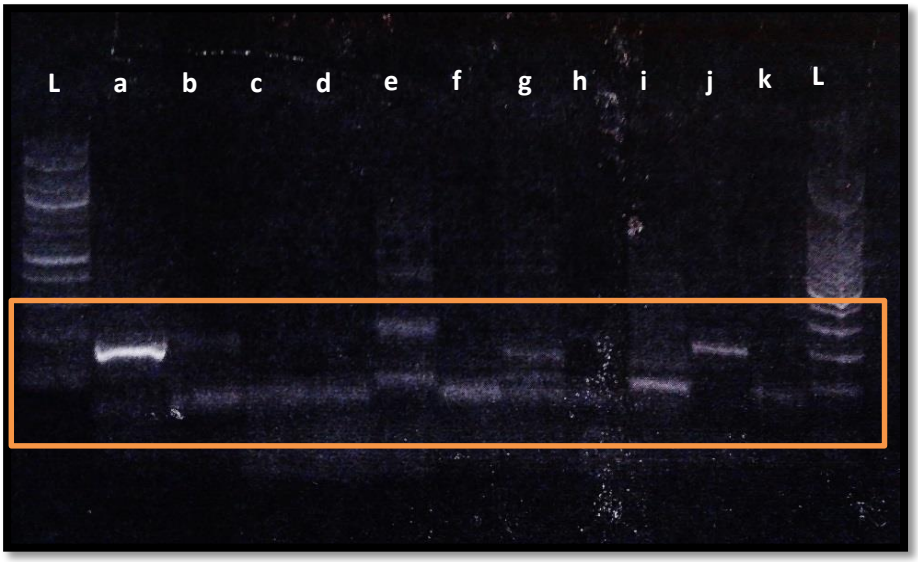

Figure 6.9. PCR amplification with *com1* primer showing 189bp amplicon for both control samples and sample from Farm C. Possible amplicons. a)Positive control 9mi/II/C4 isolate template. b) Lower template amount of positive control (5µl). (c) Negative control H_2O. (d) Sample from Farm C with no amplicon. (e) Farm C hepatopancreatic tissue with possible positive amplicon with secondary banding. (f) Farm C hepatopancreatic tissue sample with no amplicon. (g) Farm C hepatopancreatic tissue with possible amplicon and secondary banding. (h) Farm C hepatopancreatic tissue with no amplicon. (i) Farm C hepatopancreatic tissue with possible banding for different product or larger primer dimer. (j) Farm C hepatopancreatic tissue with positive 189bp amplicon. (k) Farm C muscle tissue with no apparent amplicon. PCR products were electrophoresed on a 1.8% agarose/GelRed. L: 100 bp DNA marker (Quickload Invitrogen®).

6.3.3 Sequencing

Post-PCR, 16S rRNA sequencing was performed on all farm samples regardless of whether or not they amplified for *com1*. The products from Farm C that did amplify for *com1* were excised and also sent for 16S rRNA sequencing. When positive results from Farm C were aligned and developed as a consensus sequence , they yielded an 85% identity match to *Coxiella cheraxi* partial sequence. When these individual results were aligned with MARFU consensus sequences (Chapter 5) there was a contiguous 83% identity match of the two samples to *Coxiella cheraxi* partial sequence (Table 6.2). When sequences from all farms were cleaned and aligned, no consensus sequence between farms was viable. When aligned as mutually exclusive groups, sequences from Farm A, B and C also did not individually yield any results positive for *Coxiella* sp. Only the abovementioned individual sequences from Farm C indicated the presence of *C. cheraxi*. Other potential bacterial components found during sequencing were only known symbionts and non-pathogenic bacteria (Appendix 4).

Table 6.2: Consensus sequences from MARFU samples and Farm C for *Coxiella sp.*

Sample	Length (bp)	Gene hit (Acc)	Match %	E-value	Consensus Sequence Refer to IUPAC ambiguity codes (Appendix 3)
Farm C Samples #16 and 12	1351	*Coxiella cheraxi Partial sequence* EF413063.1	85	0.0	CYMYCSTCTNNTASAYSTCTNNCCWRGAGNNNNNNGARTMCTGNNTK NATKYCCSRKGAANNNNNCSGSARGTNNNCYTGGGKYTWCNCCCGGCG TAGGAWTCWACYTTWTAGWGGGGGWTAACCCGGGGAARCTMGRGC TAWNMCSGCWNMAYCYYWTSGGANNAAASCGGGGGATYTTCSGRCC TYGCGNTRAAAGATRASCCNNCGKTGGATTAGCTTGTTGGKGGGGTWA AGGCYYACCMAGGCGACGAYCCATAGCTGGTCTGAGAGGAC--- GATCAGCCNCACTGGNGACTGAGACNCGGCCCAGAC-NTCCT---- ACGGGAGGNAGCAGTGGGGAATANTGGACAATGGGG- GAAACCCTGATSCAGCNAATGCCGCGTGTGTGAAGAAGGCCTTCGGGT TGTAAAGCACTT-----TCAGYGGN-- GGAAGAAATTCTTAAGATKAWTRCTMWTAANNGCN--GT- TGACGTTACYCACAGAAGAAGCACYGGCTAACTCYGTGCCAGCAGCCGC GGTAATACRGAGRGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGC GCRCGYAGGYGGWTRKWTAAGTYRGATGTGAAAGCCCYGGGCTYAACC TGGGAATTGCAYYYRAWACTGKMYAKCTAGAGTMTKGTAGAGGRKRGT RGAATTYCMGGTGTAGCGGTGAAATGCGTAGAKATCKGRARGAAYACC RGTGGCGAAGGCGGCYMYCTGGACMAAKACTGACRCTSAGGTGCGAA AGCGTGGGGAGCAAACAGGATTAGAKACCCTGGTAGTCCACGCCGTMA ACGATGNMGAWYTRGMKGYTGKGWSSTTKASMYSTKRSTWSCGRAGC YAACGCGTTAARTYSWCCGCCTGGGGAGTACGGYCGCAAGRTTAAAACT CAAAKGAATTGACGGGGGCCCGCACAA----GCGGTGG---AGCA TGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACCCTTGA-CATC- ---NNCTCRGAACTTGNCAGAAATGAN------- NCTKGNGTGCCTTCGGGAACNNTRAGTSACAGG- TGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGT-- TAAGTC-N-----CCGYA---ACGAGCGCAACCCTYG-- TCCTTWGTTGCCAGCR---NAGTAA---TGTCG- GGAACTCTARGRAGACTGCCGGTGATANACCGGAGGANGGYGGGGAT- -GANTGTCAAGNCATCATGGCCCTTACGGST--- NAGGGSTACMCACGTGCTACNATGGGCAGT----ACAAA- GGGTTGCCAAG-- NCCGCGANNTGNAGCNAATCCCNAAAAGCTGCTCTTANTCCGGANAG GARNCTGCAACYCGACYCCNNNNNNYTGGAATCGSMTSYTANGAWYS MATN
Consensus sequence MARFU samples 1,2,3,4,7,8,9,10,11,12,14,15,16,17,18,20,21,23,24,25,26,27,28,29,30,31,32,33,34,35,37,39,41 and Farm samples 16 and 12	455	*Coxiella cheraxi Partial sequence* EF413063.1	83	0.0	AAAGCGGGGGATCTTCGGRCCTYGCGCTAMMRGATRAGCCYGCGTTG GATTAGCTWGTTGGTGRGGTAAAGGCYTACCAAGGCGACGATCCATAG CTGGTCTGAGAGGAYGATCAGCCACACTGGRACTGAGACACGGYCCAG ACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGSGMAAS CCTGATSCAGCMATGCCGCGTGTRTGAAGAAGGCCTTCGGGTTGTAAA GYACTTTCAGTRGGGARGAARNTKSTTAAGATTAATACTCTTAAKCNKTT GACGTTACCCACAGAASMAGCNCWGGCTRACTCTGNNTGCCAGCAGC CGCGGKANTACRGAGRGTGCAAGCGTTAATCGGAATTACTGGGCGTAA AGCGCRCSTAGKKRGWTMTKTAAGTMRGATGTGAAAKCCCTKGGCTW AASCTRGRAATTGCAYYYGATACTGRMTATCTAGAGTAYKGTAGAGGNN AGKGGWAKTTCCNNNGGTGTAGCGGTNMMMTGCGTAGAKATCKGAA RGAAYACCRGTGGCGANAGNGCGGCTATCTGGACMAATACTGACACTS AGRTGCGAAAGCGTGGGGAGCAAACAGGATTAGAGACCCTGGTAGTCC ACGCCGTMAACGATGWSAACNTRGMKGTTGKGASSTWKACCTCKTRG NTWKCGRAGCYAACGCGTTARGTWSWCCGCCTGGGGAGTACGGTCGC AAGATTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGA GCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACYCTTGACA TCCWCAGAACTTRKCRGAAAYGACTKGNGTGCCTTCGGGAACTSWGTG ACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTWGGTAA GTYCYGTAACGAGCGCAACCCTYRTCCTTAKKTGCCAGCRAGTAATGKYG GGAACTCYAAGGAGACTGCCGGTGATNAAWYCSSAGGAANGGYGGGG AYGAYGTCAAGTCATCATGGCCCTTACGRGTAGGGCTACACACGTGCTA CAATGNGCNGTAYAAAGNNGGYWGCSAWRCCGCGAGGTGGAGCKAA TCCCAAAAAGNCTGYTSTTANTCCSGATWGGAGTCTGCA

6.4 Discussion

While PCR and histopathology gave insightful clues to the source of presumptive *C. cheraxi* in the MARFU outbreak, final sequencing left plenty of questions and ambiguities about the prevalence and possible identity of the pathogen. The positive detection of *com1* in PCR and suspicious histological analysis suggested that Farm C was the likely source of infection at MARFU. Sequencing further supported this hypothesis, however only a small number of samples from Farm C showed evidence of a *Coxiella* infection.

Further screening and investigation would be necessary to confirm the prevalence of *C. cheraxi* within Farm C samples as only 10% (2/20) hepatopancreatic tissues were positive for infection via 16S rRNA sequencing, while 0% of muscle samples tested positive. Most PCR screening appeared clear of an RLO based infection with the exception of four possible *com1* positive markers. Possible flaws in technique, difficulty with imaging equipment and several incidents of broken thermocyclers may have contributed to inconsistency in data. Histopathology indicated possible fungal infections as well as Gram negative bacteria within connective tissues, the latter of which is consistent with *C. cheraxi* infection. Previous reports of *C. cheraxi* infection found a possible correlation between ectoparasitic commensals such as *Lagenophrys* sp. and the presence of an RLO (Tan and Owens, 2000). Unidentified ectoparasites were observed on the gills of some sacrificed crayfish from Farm C during histological preparation, however further analysis was not undertaken. Farm C also had a history of an approximate 20% decline in productivity for the year prior to investigation, prompting the suspicion that while *Coxiella cheraxi* may not be the primary disease agent affecting the population mortality rates, there was an apparent overall decline in health within the farm stock. The only other indication that *C. cheraxi* may have affected mortality within the Farm C population is a 90% loss experienced upon arrival to MARFU in January of 2012, which is an expected mortality rate for a *C. cheraxi* outbreak (Ketterer et al. 1992). Sequencing data, while compelling, still only yielded a small percentage of positive samples and of these, only an 83% and 85% identity match respectively to *Coxiella cheraxi* within GenBank. While aligned sequences demonstrated relatively good quality for positive sequences, the lower identity scores or absence for some products may be a result of poor quality DNA or sequencing technique, which in part could be ameliorated by cloning samples prior to sequencing. Due to time, financial and personal constraints this was not a feasible option at the time of analysis, but is readily acknowledged here and is suggested as a method to be included in future investigations

These data may indicate a number of possible explanations. The first possibility is that the *C. cheraxi* outbreak at MARFU was an isolated incident, whereby crayfish from Farm C did carry *C. cheraxi* in a reduced capacity as a subclinical carrier with perhaps only a few infected cells. After a year or so of production loss as a result of possible exposure to *C. cheraxi* prior to their translocation to MARFU, surviving animals may have developed an immunity to *C. cheraxi* and therefore its clinical presentation and mortality rates would not be overly apparent within the farmed population until mixed with a non-resilient population(s) such as those found at MARFU. Evidence from Chapter 4 whereby crayfish may clear *Coxiella burnetii* or retain it at a very small bacterial load indicates that this is a possibility in terms of the crayfish immune response. Mortality rates in the past during *C. cheraxi* epizootics have reached 80-90% but as such, there is still a small survival rate which may be an indication of resilience to the pathogen. Another possible explanation would be the likelihood of a different, previously unidentified but closely related RLO infection whereby the current screening and diagnostic techniques are not sufficient for specific detection. *Aquicella* sp., a closely related RLO generally believed to be non-pathogenic were detected in some of the same sequence samples from MARFU at 85% and 80% respectively when undergoing BLAST (Table 5.1) (data not shown). These were not detected in any farm stock and could be a result of activated charcoal used in filtration systems within MARFU (Chapter 5). All of these scenarios indicate the need for further investigation and a more rigorous and consistent diagnostic technique to confirm the source of the MARFU outbreak.

6.5 Conclusions

In attempting to trace a presumptive *Coxiella cheraxi* infection from three Queensland farms, it was readily apparent that all farms within the study contributed some biosecurity risk to MARFU either through parasitic introduction of flukes or *Psorospermium* sp, unidentified bacteria, or virus. In the case of Farm A, evidence of a parvovirus were prevalent in histology (Fig 6.5b) (Rusaini et al. 2013) with the presence of hypertrophic nuclei with rarefied chromatin without intranuclear inclusion bodies in the gills (Evans and Edgerton, 2002). Based on subsequent sequencing results by another graduate student studying the pathogen, the presence of parvovirus was confirmed in Farm A (Rusaini et al. 2013). In Farm B, the presence of *Psorospermium* sp. was apparent (Fig 6.6a) and as these were not observed in any other farm stock, it was likely the source of the parasitic infection observed in the screened MARFU crayfish (Chapter 5). All farms presented unidentified

bacteraemia in some capacity and thus likely also carried infections of varying degrees of risk and severity to previously unexposed crayfish. The detection of *Coxiella cheraxi* within several Farm C samples, while not conclusive as an outbreak source, further illustrated the risk of mixing stock and the imperative need for MARFU and similar facilities to impose stricter import and quarantine guidelines as a basic measure of biosafety and stock protection.

CHAPTER 7

GENERAL DISCUSSION

Over two thirds of human infectious diseases worldwide are reportedly caused by pathogens shared with domestic and wild animals. Zoonoses are a growing threat to global health and security, with over one billion estimated cases annually (Karesh et al. 2012). As such, the study of zoonotic pathogens such as *Coxiella* species can provide valuable perspectives on emergent pathogen ecology and transmission and can also inform disease control programs for livestock, aquaculture stock and humans. By comparing infection potential of a relatively well known and well-studied pathogen, *C. burnetii* within Australian redclaw, we were able to gain better understanding into possible host mechanism and infectivity of a devastating emergent disease in aquaculture caused by the crayfish pathogen *C. cheraxi.*

In Chapter 3, a risk-averse laboratory design for monitoring and maintaining aquatic invertebrates infected with the Level 3 organism *Coxiella burnetii* was described. With some improvements and modifications it is a functional, financially viable and secure means by which researchers may gain better understanding of Level 3 organisms and in turn, contribute towards future disease research and biomedicine. Furthermore, the demonstrated use of an aquatic invertebrate to study a virulent human pathogen is a valuable method for consideration with potential for future use in the fields of aquaculture, biomedicine, public health and defence. Some suggested changes to the basic setup described in this thesis include more transparent tanks able to withstand autoclave temperatures for improved observation. Additionally, further precautions may be the addition of transparent secondary containers which would contain units for transport to and from the BSC and add further preventative measures against leaks, cracks and aerosol release within the laboratory.

Chapter 4 was the first reported experiment of a Level 3 organism infecting an aquatic invertebrate. It was also the first reported study of an experimental infection of *Coxiella burnetii* in Australian redclaw crayfish. While qPCR quantitation of hepatopancreatic tissue samples indicated that the bacteria were not replicating optimally in the crayfish, there was evidence to suggest they can carry the organism at low infective doses. If this response is related to the crayfish immune response alluded to in observed *Coxiella cheraxi* infections

(Chapter 6), it could be problematic from a biosecurity standpoint as sub-clinically infected carriers may harbor infective pathogens asymptomatically. As Australian redclaw are native to northern Queensland and globally are a widely farmed and also widely invasive species within lakes, rivers and waterways, the susceptibility on any level of these animals to carry or host *Coxiella burnetii* is potentially a threat to global health (Snovsky and Galil, 2011). Furthermore the proximity of many waterways to farmland and agricultural runoff whereby livestock most inclined to carry and expel infective doses of *C. burnetii* is another reason for concern and increased awareness.

Further refinements to the technical basis of this study would be the need for a larger sample population screened for co-infection prior to sampling, more comprehensive qPCR throughout the infection period and a longer experimental timeline as the incubation period, susceptibility and overall infectivity of *C. burnetii* within crayfish is not known. A longer sample period may also allow for more conclusive histology and qPCR as well as a more accurate portrait of morbidity. Of particular note, was that *C. burnetii* infection in crayfish appeared to present as a response to general bacterial infection attacked extracellularly, unlike the intracellular immune response expected based upon observations with *C. cheraxi* infections. As such, the results outlined in this thesis, while intriguing, remain very preliminary and fertile ground for future investigation.

The discovery of a presumptive *C. cheraxi* outbreak at MARFU in 2012 (Chapter 5) was a unique opportunity to compare *Coxiella* pathogen infections in morbidity, infectivity and genetics. While the experiment in Chapter 4 enabled a controlled *Coxiella burnetii* infection, the histopathology and morbidity results were not as was expected or observed in known *Coxiella cheraxi* infections in Australia redclaw. Infection in muscle tissue and the lack of large cytoplasmic inclusion bodies and basophilic colonies within connective tissues were apparent, as was the lack of overall morbidity within most animals. If these features are a true reflection of the disease exposure in crayfish, they may indicate a feature of virulence or pathogenicity in crayfish lacking in *Coxiella burnetii* and present in *Coxiella cheraxi* that may account for the high mortality rates and gross histopathology only when infected with the latter. This may be further associated with the lack of homologous genes required for LPS synthesis, a known virulence feature of phase I *C. burnetii* strains (Cooper et al. 2007). The histology observed in the presumptive *C. cheraxi* outbreak at MARFU was consistent with previous observations. Due to highly suggestive histopathology and mortality rates, diagnostic sequencing was initially targeted at a known homologous gene

to *C. burnetii*, *com1.* While precautions were taken to ensure DNA quality and purity, the resultant ambiguities in results for both PCR and subsequent sequencing may be a result of inexperienced sampling technique, equipment inconsistencies and problems frequent throughout the analysis (three thermocyclers were necessary during this phase of research due to breaks and power failures) , and the lack of cloning for most samples. While positive DNA was fairly high quality post-sequencing, the lack of more positive samples or higher similarity between sequences may be the result of one of the abovementioned issues. Due to these ambiguities it is also possible that a closely related but genetically distinct emergent RLO may be to blame, however the presence of *com1* as a clinical diagnostic marker is extremely suggestive that *Coxiella cheraxi* is in fact the culprit pathogen and relevant for the comparisons outlined within this work. The researcher acknowledges that a more specific diagnostic technique as well as consistent cloning would be necessary to gain more conclusive results regarding this pathogen at both MARFU and in tracking farm stock infections. Due to time, financial and personal constraints this was not undertaken. However for the intents and purposes of this study, there was sufficient evidence from the obtained data that *Coxiella cheraxi* or a very closely related RLO was the presumptive agent in the MARFU outbreak and at Farm C and that as such, is an excellent representative case study on transmission and biosecurity risks associated with emergent *Coxiella* and other RLOs when the appropriate preventative measures are not observed during the import of aquaculture stock.

A theme that was evident throughout the epidemiological study tracing the originating farm of the MARFU outbreak was an overall lack of biosecurity (Chapter 6). While preliminary results and circumstantial evidence suggested the most likely origin of the MARFU *C. cheraxi* outbreak as Farm C, the lack of quarantine, immediate mixing of stock (even those with high mortality rates) and a lack of basic preventative measures at MARFU were likely the biggest offenders in fuelling the outbreak observed in 2012. A feature evident in stock from all farms was the presence of a bacteria, virus or parasite unique to each farm whereby upon their immediate stock introduction to MARFU posed an unnecessary risk to other animals housed within the facility. The discovery of *Psorospermium* infections in Farm B and their subsequent discovery in MARFU histological samples was another example of likely transmission between farms. The confirmed presence of a presumptive parvovirus in Farm A, a known source for MARFU stock, was another cause for concern and may have been a contributing factor to the overall decline in health of MARFU animals infected with *C. cheraxi.* As the traceback experiment in

Chapter 6 illustrates, regardless of the pathogen involved, the overall theme is clear whereby the investigation into the outbreak of *Coxiella cheraxi* at MARFU is a valuable cautionary tale for future stock maintenance and import in a world where emerging RLO pathogens are on the rise ,posing risks to aquaculture stocks and possibly the human populations that consume them.

The breadth of this thesis only begins to cover some of the important and emerging questions surrounding the comparison between two emergent *Coxiella* species as they relate to Australian redclaw. However, in establishing a baseline of laboratory design and methodology as well as admonishments for improved biosecurity against both *Coxiella* species and other emerging RLO-like pathogens within aquaculture, we have made some valuable preliminary progress on the study of these pathogens. In the future, and with further improvement and understanding, we hope to contribute towards continued prevention, treatment and understanding of these and similar emergent pathogens.

LITERATURE CITED

American Society of Rickettsiology (2013) Q-fever and *Coxiella burnetii.* Available from: <http://www.cas.umt.edu/rickettsiology/>. [5 July 2013]

Anderson IG, Shariff M, Nash G, Nash M (1987) Mortalities of juvenile shrimp, *Penaeus monodon*, associated with *Penaeus monodon* baculovirus, cytoplasmic reo-like virus, and rickettsial and bacterial infections, from Malaysian brackishwater ponds. Asian Fish Sci 1:47—64.

Angelakis E, Raoult, D (2010) Q fever. *Veterinary Microbiology* 140:297-309.

Aranguren LF, Brinez B, Aragon L, Platz C, Caraballo X, Suarez A, Salazar M (2006) Necrotizing hepatopancreatitis (NHP) infected *Penaeus vannamei* female broodstock: Effect on reproductive parameters, nauplii and larvae quality. *Aquaculture* 258:337-343.

Australia/New Zealand Standards (2010) Safety in laboratories. Part 3: microbiological aspects and containment facilities. Sydney and Wellington: Standards Australia International Ltd. AS/NZS 2243.3:2010.

Beare PA, Howe D, Cockrell DC, Omsland A, Hansen B, Heinzen RA (2009) Characterization of a *Coxiella burnetii* ftsZ mutant generated by Himar1 transposon mutagenesis. *Journal of Bacteriol*ogy 191:1369–1381.

Ben-Horin T, Lenihan HS, Lafferty KD (2013) Variable intertidal temperature explains why disease endangers black abalone. Ecology 94:161–168

Bonami JR, Pappalardo R (1980) Rickettsial infection in marine Crustacea. *Experientia* 36:180–181.

Bower SM, McGladdery SE, Price IM (1994) Synopsis of Infectious Diseases and Parasites of Commercially Exploited Shellfish: Rickettsia and Chlamydia of Crabs. *Ann. Rev. Fish Dis* 4:1-199.

Bower SM,Meyer GR, Boutillier JA (1996) Stained prawn disease (SPD) of Pandalus platyceros in British Columbia, Canada caused by Rickettsial infection. *Dis Aquat Org* 24:41-54.

Bower SM, Romero X (2009) Synopsis of Infectious Diseases and Parasites of Commercially Exploited Shellfish: Rickettsia of Crayfish. Available from Fisheries and Oceans Canada: <http://dev-public.rhq.pac.dfo-mpo.gc.ca/science/species-especes/shellfish-coquillages/diseases-maladies/pages/ricketcy-eng.htm> [April 2013].

Brock JA, Nakagawa LK, Hayashi T, Teruya S, Campen HV (1986) Hepatopancreatic rickettsial infection of the penaeid shrimp, *Penaeus marginatus* (Randall), from Hawaii. *Journal of Fish Diseases* **9**: 73–77.

Center for Disease Control (2010) Viral and Rickettsial Zoonoses Branch: Q-Fever. National Center for Health Statistics, USA. Available from <www.cdc.gov/ncidod/qfever> [September 23, 2013]

Center for Disease Control (2013) Diagnosis and Management of Q Fever - United States, 2013: Recommendations from CDC and the Q Fever Working Group. *MMWR Recomm Rep*.:62:1-30.

Chiu CK and Durrheim DN (2007) A review of the efficacy of human Q fever vaccine registered in Australia. *Public Health Bulletin* 18:133-136.

Chong YC, Loh H (1984) Hepatopancreas chlamydial and parvoviral infections of farmed and marine prawns in Singapore. *Singapore Veterinary Journal* 9: 51-56.

Cohen D, Isaar G (1990) Rickettsial disease of Macrobrachium rosenbergii larvae: gross signs, diagnosis and treatment. In: World Aquaculture vol III, 3rd edn. Blackwell Science, Oxford pp. 75.

Cooper A, Layton R, Owens L, Ketheesan N, Govan, B (2007) Evidence for the classification of a crayfish pathogen as a member of the genus *Coxiella*. *Letters in App Microbiol* 45:558-563.

Cooper A, Hedlefs R, Ketheesan N, Govan, B (2011) Serological evidence of *Coxiella burnetii* infection in dogs in a regional centre. *Australian Veterinary Journal* 89: 385–387.

Cooper A (2011) Unpublished PhD research thesis. School of Veterinary and Biomedical Sciences, James Cook University Townsville, Australia.

Cordaux R, Paces-Fessy M, Raimond M, Michel-Salzat A, Zimme M, Bouchon D (2007) Molecular Characterization and Evolution of Arthropod-Pathogenic *Rickettsiella* Bacteria. *Appl Environ Microbiol* 73: 5045-5047.

Eason DD, Cannon JP, Haire RN, Rast JP, Ostrov DA, Litman GW (2004)Mechanisms of antigen receptor evolution. *Semin Immunol* 16:215-226.

Ebert D (2008) Host-parasite coevolution: Insights from the *Daphnia*-parasite model system. *Curr Opin Microbiol* 11:290-301.

Eddy F, Powell A, Gregory S, Nunan LM, Lightner DV, Dyson PJ, Rowley AF, Shields RJ (2007) A novel bacterial disease of the European shore crab, *Carcinus maenas*–molecular pathology and epidemiology. *Microbiology* 153:2839–2849.

Edgerton B, Owens L, Harris L, Thomas A, Wingfield M (1995) A health survey of farmed redclaw crayfish, *Cherax quadricarinatus* (von Martens), in tropical Australia. *Freshw Crayfish*. 10:322-338.

Edgerton BF, Prior HC (1999) Description of a hepatopancreatic rickettsia-like organism in the redclaw crayfish *Cherax quadricarinatus*. *Dis Aquat Org* 18: 63-69.

Elston R (1986) Occurrence of branchial rickettsia-like infections in two molluscs, *Tapes japonica* and *Patinocteu yessoensis* with comments on their significance. *J Fish Dis* 9:69–71.

Evans LH, Edgerton BF (2002) Chapter 10: Pathogens, Parasites and Commensals. In Biology of Freshwater Crayfish, Vol DM Holdich ed. Blackwell Science, Oxford, pp. 377-438.

Frederici BA, Hazard EI, and Anthony DW (1974) Rickettsia-like organism causing disease in a crangonid amphipod from Florida. *Appl. Microbiol.* **28** :885–886.

Frelier PF, Sis RF, Bell TA, Lewis DH (1992). Microscopic and ultrastructural studies of necrotizing hepatopancreatitis in Pacific white shrimp (*Penaeus vannamei*) cultured in Texas. *Vet. Pathol.* 29, 269–277.

Frelier PF, Loy JK, Kruppenbach B (1993) Transmission of necrotizing hepatopancreatitis in *Penaeus vannamei. J Invertebr Pathol* 61:44–48.

Fryer JL, Lannan CN (1994) Rickettsial and chlamydial infections of freshwater and marine fishes, bivalves and crustaceans. *Zool Stud* 33:95–107.

Garces LH, Larenas JJ, Smith PA, Sandino S, Lannan CN, Fryer JL (1991) Infectivity of a rickettsia isolated from Coho salmon Oncorhynchus kisutch . *Dis Aqu Org* 11: 93-97.

Hayakijkosol, Orachun, and Owens, Leigh (2011) Investigation into the pathogenicity of reovirus to juvenile Cherax quadricarinatus. *Aquaculture* 316:1-4

Hoover TA, Culp DW, Vodkin MH, Williams JC, Thompson HA (2002) Chromosomal DNA deletions explain phenotypic characteristics of two antigenic variants, phase II and RSA 5149 (crazy), of the *Coxiella burnetii* Nine Mile strain. *Infect. Immun* 70:6726-6733.

Jasinskas A, Zhong J and Barbour AG (2007) Highly prevalent *Coxiella* sp. bacterium in the tick vector *Amblyomma americanum*. *Appl Environ Microbiol* 73:334–336.

Jimenez R, Romero X (1997) Infection by intracellular bacterium in red claw crayfish, *Cherax quadricarinatus* (von Martens) in Ecuador. *Aquacult Res* 28:923-929.

Jones CM (1990) The biology and aquaculture potential of the tropical freshwater crayfish *Cherax quadricarinatus*. Queensland Department of Primary Industries, Information Series Q 190028, Brisbane.

Jones RM, Nicas M, Hubbard A, Reingold A (2006) The infectious dose of *Coxiella burnetii* (Q Fever). *Applied Biosafety* 11:32-41.

Johnson PT (1984) A rickettsia of the blue king crab, *Paralithodes platypus*. *J. Invert. Pathol.* 44: 112–113.

Jurczynski K, Flugger M (2005). A Q-fever infection in marine mammals at Tierpark Hagenbeck in Hamburg: case report. *Verh.ber. Erkrg. Zootiere* 42.

Karesh W B, Dobson A, Lloyd-Smith J O, Lubroth J, Dixon M A, Bennett M, Heymann D L (2012) Ecology of zoonoses: natural and unnatural histories. *The Lancet*, *380*(9857), 1936-1945.

Ketterer PJ, Taylor DJ, Prior HC (1992) Systemic rickettsia-like infection in farmed freshwater crayfish, *Cherax quadricarinatus*. In *Diseases in Asian Aquaculture*, I. Asian Fisheries Society, Fish Health Section, Manila, Philippines pp: 173–179.

Kersh GJ, Wolfe TM, Fitzpatrick KA, Candee AJ, Oliver LD, Patterson NE, Self JS (2010) Presence of *Coxiella burnetii* DNA in the Environment of the United States, 2006 to 2008. *Applied and Environmental Microbiology* 76: 4469-4475.

Krishnan P, Gireesh-Babu P, Rajendran KV, Chaudhari A (2009) RNA interference-based therapeutics for shrimp viral diseases. *Dis Aquat Organ* 86:263-272

Krol RM, Hawkins WE, Overstreet RM (1991) Rickettsial and mollicute infections in hepatopancreatic cells, of cultured Pacific white shrimp (*Penaeus vannamei)*. *J. Invert. Pathol.* **57**:362–370.

Knobel DL, Maina AN, Cutler SJ, Ogola E, Feikin DR, Junghae M, Halliday JE, Richards AL, Breiman RF, Cleaveland S, Njenga MK (2013) *Coxiella burnetii* in humans, domestic ruminants, and ticks in rural western Kenya. *Am J Trop Med Hyg*. 3:513-8.

La Fauce K & Owens L (2007) Investigation into the pathogenicity of *Penaeus merguiensis* densovirus (*Pmerg*DNV) to juvenile *Cherax quadricarinatus*. *Aquaculture* 27:31-38

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

Lightner DV, Redman RM, Williams RR, Mohoney LL (1985) Resent advances in penaeid virus disease investigations. *J World Maricult Soc* 16:267–274.

Lightner DV, Redman RM (1994) An epizootic of necrotizing hepatopancreatitis in cultured penaeid shrimp (Crustacea: Decapoda) in northwestern Peru. *Aquaculture,* 122, 9–18.

Lightner DV (1996) Epizootiology, distribution and the impact on international trade of two penaeid shrimp viruses in the Americas. Revue Scientifique et Technique Office *International des Epizooties* 15(2): 579-601.

Lightner A, Schust DJ, Chen YB, Barrier BF (2008) The fetal allograft revisited: Does the study of an ancient invertebrate species shed light on the role of natural killer cells at the maternal-fetal interface? Clin Dev Immunol 2008: 631920

Loy JK, Dewhirst FE, Weber W, Frelier PF, Garbar TL, Serban IT, Templeton JW (1996) Molecular Phylogeny and *In Situ* Detection of the Etiologic Agent of Necrotizing Hepatopancreatitis in Shrimp. *Appl. Environ. Microbiol* 9:3439-3445.

Mahamat A, Edouard S, Demar M, Abboud P, Patrice J-Y, La Scola B (2013) Unique clone of *Coxiella burnetii* causing severe Q fever, French Guiana. *Emerg Infect Dis* July 2013.

Marmion BP, Storm PA, Ayres JG (2005) Long-term persistence of *Coxiella burnetii* after acute primary Q fever. *Monthly Journal of the Association of Physicians* 98:7-20.

Masser MP, Rouse DB (1997) Australian red claw crayfish. Southern Regional Aquaculture Center (SRAC) Publication No. 244. Southern Regional Aquaculture Center, Stoneville, MS. 8 pp

Mattix M, Zeman DH, Moeller R, Jackson C, Larsen T (2006) Clinicopathologic Aspects of Animal and Zoonotic Diseases of Bioterrorism. *Clini Lab Med* 26:445-489.

Maurin M, Raoult D (1999) Q fever. *Clin. Microbiol. Rev*. 12**:**518-553.

Messick GA (1998) Diseases, parasites, and symbionts of blue crabs (*Callinectes sapidus*) dredged from Chesapeake Bay *J. Crustacean Biol* **18:** 533–548.

Messick GA, Kennedy VS (1990) Putative bacterial and viral infections in blue crab *Callinectes sapidus* Rathbun, 1896 held in a flow-through or recirculation system. *J. Shellfish Res* **9**:33–40.

Mialhe E, Chagot D, Boulo V, Comps M, Ruano F, Grizel H (1987) An infection of *Ruditapes decussatus* (Bivalvia) by Rickettsia. *Aquaculture* 67:258–259.

Millet AC, Ewbank JJ (2004) Immunity in *Caenorhabditis elegans*. *Curr Opin Immunol* 16:4-9.

Norton JH, Shepherd MA, Perkins FP, Prior HC (1993) Perkinsus-like infection in farmed golden-lipped pearl oyster *Pinctada maxima* from the Torres Strait, Australia. J lnvertebr Pathol 62:105-10

Nunan LM, Poulos BT, Redman RM, LeGromellec M, Lightner DV (2003) Molecular detection methods developed for a systemic rickettsia-like bacterium (RLB) in *Penaeus monodon* (Decapoda: Crustacea) *Dis. Aquat. Organ* 53: 15-23.

Orales-Covarrubias MS, Lozano-OlveraRY, Hernandez-Silva (2010) Necrotizing hepatopancreatitis in cultured shrimp caused by extracellular and intracellular bacteria. *Tilapia & Camarones* 5: 33–39.

Owens L, Muir P, Sutton D, Wingfield, M (1992) The pathology of microbial diseases in tropical Australian Crustacea. In: Shariff M, Subasinghe RP, Arthur JR (eds) Diseases in Asian aquaculture 1. Fish Health Section, Asian Fisheries Society, Manila, Philippines, pp. 165-172.

Peng Y, Zhang Y, Mitchell WJ, Zhang G (2012) Development of a lipopolysaccharide-targeted peptide mimic vaccine against Q fever. *J Immunol.* 189(10):4909-20

Pittman PR (2005) An assessment of health status among medical research volunteers who served in the Project Whitecoat program at Fort Detrick, Maryland *Mil Med* 170(3):183-7

Romero X, Turnbull JF, Jiménez R (2000) Ultrastructure and cytopathology of a Rickettsia-like organism causing systemic infection in the redclaw crayfish, *Cherax quadricarinatus* (Crustacea: Decapoda), in Ecuador. *J Invertebr Pathol* 76:95–104

Rotz LD (2002) Public health assessment of potential biological terrorism agent Q fever. *Clinical Microbial. Rev*. 12:518-55

Rusaini, La Fauce KA, Elliman J, Bowater RO, Owens L (2013) Endogenous Brevidensovirus-like elements in Cherax quadricarinatus: friend or foe? *Aquaculture* 399: 136-145.

Scola B, Raoult D (1997) Minireview: Laboratory diagnosis of Rickettsioses: Current approaches to diagnosis of old and new Rickettsial diseases. *Journal of Clinical Microbiology* 35: 2715-2727

Snovsky G, Galil BS (2011) The Australian redclaw crayfish Cherax quadricarinatus (von Martens, 1868) (Crustacea: Decapoda: Parastactidae) in the Sea of Galilee, Israel. Aquatic Invasions, 6:S29-S31.

Spitsbergen JM, Blazer VS, Bowser PR, Cheng KC, Cooper KR, Cooper TK, Frasca S, Jr, Groman DB, Harper CM, Law JM, Marty GD, Smolowitz RM, St Leger J, Wolf DC, Wolf JC (2009) Finfish and aquatic invertebrate pathology resources for now and the future. *Comp Biochem Physiol C. Tox Pharm*. 149:249-57

Sun JF, Wu X (2004) Histology, ultrastructure, and morphogenesis of a rickettsia-like organism causing disease in the oyster, *Crassostrea ariakensis* Gould. *Journal of Invertebrate Pathology* 86:77-86

Tan CK, Owens L (2000) Infectivity, transmission and 16S rRNA sequencing of a Rickettsia, *Coxiella cheraxi sp. nov.*, from the freshwater crayfish *Cherax quadricarinatus. Dis Aquat Org 41 :*115—122

Vago C, Meynadier G, Juchault P, Legrand J, Amargier A, Duthoit J (1970) Une maladie rickettsienne chez les Crustaces Isopodes. *CR Acad Sci Paris* 271: 2061–2063.

Vaidya (2008) Comparison of PCR, Immunofluorescence Assay, and Pathogen Isolation for Diagnosis of Q Fever in Humans with Spontaneous Abortions. *J. Clin. Microbiol*. 46: 2038-2044

Van der Hoek W, Hogema BM, Dijkstra F, Rietveld A, Wijkmans CJ, Schneeberger PM, Zaaijer HJ (2009) Relation between Q fever notifications and *Coxiella burnetii* infections during the 2009 outbreak in the Netherlands. *EuroSurveill*. 17(3)

Vogt G (1994) Double-infection of the hepatopancreas of the shrimp *Palaemon elegans* with rickettsia and a reo virus. In: International Symposium on Aquatic Animal Health: program and abstracts. Seattle, United States.

Vogt G, Štrus J (1998) Diseases of the shrimp *Palaemon elegans* (Crustacea: Decapoda) in the Bay of Piran, Adriatic Sea. *Journal of Natural History* 32(10-11): 1795-1806

Waag D (2007) *Coxiella burnetii*: Host and bacterial responses to infection. *Vaccine*. 25:7288-7295

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

Weinert LA, Weren JH, Aebi A, Stone GN, Jiggins FM (2009) Evolution and diversity of Rickettsia bacteria. *BMC Biol.* 7: 6.

Winkler HH (1990) Rickettsia species: As organisms. *Annu. Rev. Microbiol* 44:131-153.

Yadav MP, Sethi MS (1979) Poikilotherms as reservoirs of Q-fever (*Coxiella burnetii*) in Uttar Pradesh. *J Wildl Dis.* 15:15-7

Yadav MP, Sethi MS (1980) A study on the reservoir status of Q fever in avifauna, wild mammals and poikilotherms in Uttar Pradesh (India). *Int J Zoonoses.* 7:85-89

APPENDIX 1

1.1 Preparation, Culturing and Harvesting of Vero Cell Lines

1.1.1 Vero culture media

DMEM
10% HI-FBS
2mM L-Glutamine
4.5g/L Glucose*
110 mg/L Sodium pyruvate*

*Glucose and sodium pyruvate dissolved in a small amount of DMEM/water and filtered into media through 0.2um filter

1.1.2 Culturing Vero cells (Protocol adapted from Cooper 2007/Stevens 2010)

1. Pre-warm media to 37°C
2. Pre-warm beaker of water
3. Thaw cells in beaker of warm water for 2 mins

   Do not immerse cryovial at or below lid line to prevent contamination

   Do not thaw cells longer than 2 mins (DMSO is toxic at room temperature)
4. Wash cells

   8-10ml DMEM, 15ml falcon tube

   Centrifuge 500 x g, 5 min, 20°C
5. Decant and resuspend cells in a small amount of media by pipetting up and down
6. Add cells to culture flask(s) and top up flask(s) with media

   a) 3x small flasks (75 cm^2), 5ml/flask OR large flask (175 cm^2), 20ml
   b) Incubate cells in 37°C CO_2 incubator and passage after 1 week

7. Check cells at day 5/6 and when confluent, split 1:2

1.1.3 Isolating *Coxiella burnetii* from Vero cells

***IMPORTANT-Undertaken post-training and certification within a PC3 facility with appropriate PPE ***

1. Harvest infected Vero cells
    - Depending on how many flasks being harvested, cells may need to be pelleted and resuspended in 20-30ml media to fit in one 50ml pot or falcon tube for sonication
2. Sonicate cells with the microtip sonicator for 2 X 40 sec bursts at 40 amps
    - Allow a 30 second break between sonications to prevent cell overheating
    - Wear ear muffs as important PPE
    - Change gloves after removing hands from the cabinet due to aerosol production. Wait ~20-30 mins for aerosols to settle if isolating another strain to avoid cross-contamination.
3. Pellet Vero cell debris by centrifugation at 550 x g at 20°C for 10 mins
4. Layer bacterial supernatant over 25% sucrose at a 2:3 ratio of supernatant to sucrose
    - Use a pasteur pipette and add supernatant slowly at first and by running the supernatant down the tube wall
5. Pellet bacteria by centrifuging at 2, 250 x g at 20°C for 20 mins
6. Take off the supernatant and leave behind ≤5ml sucrose for the bacteria to sit
7. Take a small aliquot of bacteria to do a DNA extraction for subsequent bacterial quantification by qPCR

1.1.4 Extracting *C. burnetii* DNA

Follow the protocol for bacterial DNA extraction as laid out in the Roche High Pure PCR Template Preparation kit ® manual with the following 2 modifications:

1) Use 100ul of lysozyme and incubate for 25 mins, instead of 5ul for 15 mins due to *C. burnetii* resistance to lysozyme degradation
2) Do 2 X 100ul elution steps instead of eluting with 200ul straight up, to ensure you are getting all DNA.

Reduce the final volume afterwards if low yield suspected. Similarly, the kit calls for 200ul of sample, may use less or more, but take dilutions into account when calculating bacterial concentration. Note: Binding Buffer contains 6M Guanidine-HCL,10Mm urea, 10Mm Tris-HCl, 20% Triton-X-100 (v/v)

1.2 qPCR Preparation and Quantitation*

1.2.1 qPCR reagents for Rotorgene® 3000

Reagent	Working volume
ddH_20	**to 720 µL**
SYTO-9 mix (Invitrogen)	**1×**
Forward primer (Sigma Genosys)	**300 nM**
Reverse primer (Sigma Genosys)	**300 nM**
Template DNA	**10-30 ng**

1.2.2 Mastermix Recipe for *com1*

	Stock	Final
ImmoMix	2X	1X
Primers (com1)	10µM	0.3µM
Syto 9	5mM	0.01mM
Water	-	-

Total Volume: *19µl master mix + 1µl template DNA (20µl total rxn)*

1.2.3 qPCR Genome equivalents formula calculation

Quantitation of NMII/C4 DNA in fmol:

$$\frac{\text{X ng NMII/C4 DNA } \mu L^{-1}}{0.66 \text{ ng fmol}^{-1} \text{ bp}^{-1} \times 2.016\times10^{3} \text{ bp}}$$

Conversion to genome equivalents:

$$\frac{\text{X fmol } \mu L^{-1} \times 1 \text{ mol} \times 6.02\times10^{23} \text{ copies mol}^{-1}}{1\times10^{12} \text{ fmol}}$$

Source (Alanna Cooper-Extraction Methods *Coxiella burnetti*- 9 Mile II/ C4 strain 2011).

APPENDIX 2

2.1 Histological Preparation: Fixative, Tissue Embedding and Staining

2.1.1 Davidson's fixative

Acetic Acid	**115 mL**
Absolute Ethanol	313mL
Formaldehyde	220 mL
Distilled Water	352 mL

2.1.2 Haematoxylin and eosin (H & E) stain

Mayer's haematoxylin

Haematoxylin	**2.0 g**
Sodium iodate	0.4 g
Aluminum ammonium sulphate	100.0 g
Citric Acid	2.0 g
Chloral hydrate	100.0 g
Distilled Water	2.0 L

2.1.3 Scott's tap water

$NaHCO_3$

8.75 g

$MgSO_4.7H_2O$

50.00 g

Distilled Water

2.50 L

2.1.4 Young's Eosin

Eosin

15.0 g

Erythrosin

5.0 g

Calcium Chloride

5.0 g

Distilled Water

2.0 L

2.1.5 Gram Stain

2% Crystal Violet

Crystal Violet

2.0 g

Ammonium Oxalate

0.8 g

95% Alcohol

20.0 mL

Distilled Water

8.0 mL

2.1.6 Lugol's Iodine

Iodine Crystal 1.0 g Postassium Iodide 2.0 g Distilled Water 100.0 mL

2.1.7 Twort Stain

0.2% Alcohol neutral red 100.0 mL 0.2% Alcohol fast green 11.3 mL

1M Phosphate buffered saline (PBS)

NaCl	**8.0 g**
KCl	0.20 g
$Na2HPO_4$	1.44 g
$KH2PO_4$	0.24 g
Distilled Water	1.0L

Haematoxylin and Eosin Staining Applied to dried tissues
1.Xylene 2 minutes
2.Xylene 2 minutes
3.Ethanol 2 minutes
4. Ethanol 1 minute
5. Ethanol 1 minute
6. Tap water 1 minute
7. Mayer's Hemotoxylin* 8 minutes
8.tap water 1 minute
9. Scott's tapwater substitute* 30 seconds
10.Young's Eosin * 4 minutes
11. tap water 1 minute
12.Ethanol 10 dips
13.Ethanol 1 minute
14.Xylene 2 minutes
15. Xylene 1 minute
16. Mounted on glass slides in DPX (dibuthylphthalate-polystyrene-xylene)
17. Slides were dried overnight in an incubator at 37◦C.

APPENDIX 3

3.1: IUPAC Code for Sequence Alignment Ambiguities

Nucleotide Code: Base:

---------- -----

A.....Adenine

C.....Cytosine

G.....Guanine

T (or U).....Thymine (or Uracil)

R.....A or G

Y.....C or T

S.....G or C

W.....A or T

K.....G or T

M.....A or C

B.....C or G or T

D.....A or G or T

H.....A or C or T

V.....A or C or G

N.....any base

. or -.....gap

APPENDIX 4

4.1: Table of sequence alignments obtained for farm stock (Chapter 6) other than *C. cheraxi*

Samples	Identity	Score	Identity
Farm C_Group_2_SP44_63F.ab1	0.191	2237	*Synechococcus sp*. CCAP 1479/10; HE975006
Farm C_Group_2_SP45_1387R.ab1	0.955	355	uncultured bacterium; 116.F12; EU356999
Farm C_Group_2_SP46_1387R.ab1	1	391	*Aeromonas sp*. 001; AF295658
Farm C_Group_2_SP47_63F.ab1	0.191	2237	*Synechococcus sp*. CCAP 1479/10; HE975006
Farm C_Group_2_SP49_1387R.ab1	0.216	148	uncultured *Azoarcus sp*.; 071; GU556288
Farm B_Group_2_SP50_63F.ab1	0.183	2237	*Synechococcus sp*. CCAP 1479/10; HE975006
Farm B_Group_2_SP52_63F.ab1	0.182	2237	*Synechococcus sp*. CCAP 1479/10; HE975006
Farm B_Group_2_SP53_63F.ab1	0.189	1813	uncultured bacterium; FW111; AF523982
Farm C_Group_2_SP54_63F.ab1	1	391	*Aeromonas sp*. 001; AF295658
Farm C_Group_2_SP55_1387R.ab1	1	391	*Aeromonas sp*. 001; AF295658
Farm C_Group_2_SP56_63F.ab1	1	362	uncultured *Aeromonas sp*.; SSCP12; EU704142
Farm C_Group_2_SP57_63F.ab1	1	362	uncultured *Aeromonas sp*.; SSCP12; EU704142
Farm C_Group_2_SP58_1387R.ab1	0.946	736	uncultured bacterium; GammaPro04; JF261656
Farm B_Group_2_SP59_63F.ab1	0.186	1987	uncultured *Psychromonas sp*.; F1C69; AY794084