University of Southampton

Effectiveness of an immune toolbox to determine the host response to infection and stress in two decapod species, *Homarus gammarus* (L.) and *Carcinus maenas* (L.).

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To my family

Abstract

Decapod crustaceans are ecologically and economically valuable, yet are vulnerable to a variety of stressors in both natural and farmed environments. Immune biomarkers have been commonly used to assess host transcriptional response to infection and disease in Decapoda. Here, the transcription of various immune genes was quantified to assess the host response of the European lobster, *Homarus gammarus* (L.) and the shore crab, *Carcinus maenas* (L.) to a number of stress scenarios. Selected immune genes included *carcinin*, (*C. maenas*) and *crustin* (*H. gammarus*), antimicrobial peptides, *peroxinectin*, a cell adhesion molecule and osponin and the zymogen *prophenoloxidase*. Prophenoloxidase cleaves to form active phenoloxidase which is involved in the melanisation of many invading pathogens. The immune gene transcription was quantified using real-time PCR. The efficacy of this suite of biomarkers was tested in the context of either pathogen history, as a predictor of viral or bacterial challenge in decapods or in response to non-pathogen related stress (high stocking density).

In *H. gammarus*, the predictive capacity of this 'toolbox' of immune genes was quantified in response to White Spot Syndrome Virus (WSSV) infection, a viral pathogen with a wide host range and known to cause mortality, most notably in penaeid species. For *C. maenas*, the transcription of the immune genes was quantified to assess the host impact of different pathogen burdens in two distinct, wild populations. Further, the predictive capacity of the biomarkers was quantified in *C. maenas* in response to two controlled infection studies with either a Gram positive (*Planococcus citreus*) or Gram negative (*Listonella anguillarum*) bacteria. Lastly, the wider applicability of this suite of biomarkers was assessed as a general indicator of stress in *C. maenas*.

Overall, the transcription of *carcinin, peroxinectin* and *prophenoloxidase* differed between *C. maenas* populations in response to pathogen assemblages and site-specific differences. In response to viral (*H. gammarus*) and bacterial (*C. maenas*) challenge and the non-pathogenic stress challenge (*C. maenas*), there was no significant change in *peroxinectin* and *prophenoloxidase* transcription. Interestingly, the transcription of *crustin* (*H. gammarus*) and *carcinin* (*C. maenas*) changed significantly in response to all of these challenges. This suggests a much wider role for these antimicrobial peptides as biomarkers in both viral and bacterial challenges and in response to stress associated with high stocking density. The efficacy of antimicrobial peptides in response to bacterial and viral pathogens and non-pathogen stressors should be assessed in other species to understand its applicability and limitations as a biomarker in the wider decapod community.

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Declaration of Authorship

I, Lauren Susan Hall, declare that the thesis entitled "Effectiveness of an immune toolbox to determine the host response to infection and stress in two decapod species, *Homarus gammarus* (L.) and *Carcinus maenas* (L.)." and the work presented in the thesis are both my own, and have been generated by me as the result of my own original research. I confirm that:

- This work was done wholly or mainly while in candidature for a research degree at this University.
- Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated.
- Where I have consulted the published work of others, this is always clearly attributed.
- Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work.
- I have acknowledged all main sources of help.
- Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself.
- None of this work has been published before submission.

Signed: .....
Date: .....

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List of abbreviations

Abbreviations	
CEFAS	Centre for Environment, Fisheries and Aquaculture Science
CNRQ	Calibrated Normalised Relative Quantity
DEPC	Diethyl pyrocarbonate
DHC	Differential Haemocyte Count
DNA	Deoxyribonucleic acid
E genes	Early genes that are expressed prior to viral DNA replication
EDTA	Ethylenediaminetetraacetic acid
EFSA	European Food Standard Authority
ERG	Endogenous reference gene
GOI	Gene of interest
GCs	Granular cells
IE genes	Immediate early genes that are expressed prior to viral DNA replication
IPTG	Isopropyl-β-D-thiogalactopyranoside
L genes	Late genes that are expressed post viral DNA replication
LPS	Lipopolysaccharide factor
OIE	Office International des Epizooties (World Organisation for Animal Health)
PAMP	Pathogen-Associated Molecular Pattern
PCR	Polymerase Chain Reaction
PGN	Peptidoglycan
PLIV	Post-larval stage IV
PRR	Pattern Recognition Receptor
ppA	proPO-activating enzyme
psu	practical salinity unit
qPCR	Quantitative Polymerase Chain Reaction
RNA	Ribonucleic acid
RQI	RNA Quality Indicator
SGCs	Semi-Granular Cells
SPF	Specific Pathogen Free
TAE Buffer	Tris-acetate-EDTA
TBE Buffer	Tris-borate-EDTA
THC	Total Haemocyte Count
WSD	White Spot Disease
WSSV	White Spot Syndrome Virus
X-Gal	substrate for β-galactosidase

1 Introduction

Decapods live in environments enriched with a variety of potentially harmful pathogens, such as viruses and bacteria (Jiravanichpaisal et al., 2006). As ectotherms, decapods interact intimately with their immediate environment and as such are vulnerable to an array of pathogens and stressors, such as toxins and extremes in temperature and salinity. The arsenal of host responses to any invading pathogen or indeed to any stress that threatens homeostasis, is provided by the innate immune system in decapods (discussed below). This complex system is required to identify and respond to anything that has the potential to cause the host harm. Over evolutionary time, the 'arms race' between the host and microorganisms has been dynamic leading to the efficient host immunity evident today (Cooper and Herrin, 2010; Bean et al., 2013). Nevertheless, some pathogens have developed strategies to evade this system. As susceptibility to various microorganisms is variable between species (Chang et al., 2011) there is no single model capable of describing host/pathogen interaction.

1.1 Relevance of host-pathogen interactions

1.1.1 Food security

As the estimated human population is projected to increase to 7.4-10.6 billion by 2050, with the average predicted at 8.9 billion (Anonymous, 2004), the associated demand for food will increase. Patterns in food consumption have changed over the past 50 years from pulses and roots towards vegetable oils, meat and sugar, along with an average increase of 400 kcal person^{-1} day^{-1}, termed a 'westernisation' of diet (reviewed in Stentiford et al., 2012). Concurrent with this change in food consumption was a growth in the global trade of food products. Within this trade one of the fastest growing food sectors has been aquaculture, which is predicted to contribute >50% of aquatic food products by 2015 (Bostock et al., 2010). Global aquaculture production of marine shrimp represent the most commercially valuable commodity at US$17.7bn, with crabs worth US$5.4bn and lobsters and crayfish valued at US$2.5bn (annual data for 2011; FAO, 2014).

Along with this increase in production, there has been an increase in disease prevalence which has been particularly evident in penaeid aquaculture. Disease in decapods is a growing concern in terms of impact on food security and the potential impact on susceptible wild fisheries. An increase in the international trade of commercially important decapods has increased the transfer of pathogenic agents to naive hosts (Walker and Mohan, 2009; Walker et al., 2011). Over a similar time period an increase in diseases has been reported (reviewed in Harvell, 1999; Peeler, 2012). In terms of penaeid aquaculture, disease has been associated with increased stress in relation to stocking density (Kautsky et al., 2000) and factors such as feeding on non-penaeid species, *e.g.* imported frozen crabs that may harbour novel penaeid diseases (Stentiford et al., 2012). This has been highlighted in the past by the increasing distribution of a significant viral pathogen, White Spot Syndrome Virus (WSSV), which has had a devastating effect on cultured penaeids, resulting in significant economic losses (Lightner, 1996). This

viral pathogen can infect a wide range of species, although susceptibility to this virus varies between host taxa (Chang et al., 2011). This example highlights the need for a more comprehensive understanding of susceptibility of the host to particular pathogens. In addition to disease in aquaculture, disease can occur in economically important decapods in natural populations (Dove et al., 2005; Glenn and Pugh, 2006) and can often be in relation to environmental stressors. These are discussed in more detail in the following section.

There is also a political motivation to monitor the disease state of important fisheries and ecologically significant species in the UK, to ensure that any listed parasites are not exported or imported to non-affected areas. This list of notifiable diseases is formulated by the World Organisation for Animal Health (OIE) (OIE, 2014). The OIE Aquatic Animal Health Code requires the 178 member countries to ensure appropriate surveillance is in place and report any evidence of notifiable diseases to their national official veterinary services. If evident, trading restrictions are enforced, with the overall aim to contain and prevent the distribution of pathogens, particularly where there are no effective treatment strategies (Stentiford et al., 2014). Any member country wishing to impose a greater level of protection than those imposed under the international trade agreements, must provide appropriate scientific justification under the World Trading Organisation (WTO) guidelines (Peeler, 2012).

1.1.2 Impact of environmental factors on host-pathogen interactions: Climate change and pollution

The environment heavily influences the interaction between the host and pathogen (Harvell et al., 2004). Pollutants and variables associated with climate change, *i.e.* changes in temperature, oxygen concentration in the marine environment and seawater pH, have the capacity to change the distribution of both the host and pathogen, affect host susceptibility and increase the frequency of epizootics (Karvonen et al., 2010).

1.1.2.1 Changes in the tolerance limits and biogeographical range of host species and pathogens

Future climate change scenarios are predicted to impact the physical, chemical and biological systems of the ocean (reviewed in Burge et al., 2014). Predictions for the northwest European continental shelf indicate warming between 1.5-4°C and a reduction in salinity of 0.2 psu by the end of the 21st century (Holt et al., 2010). Temperature, in particular, is known to have an impact on the biogeographical range of ectotherms in line with their thermal tolerance limits and also on the growth of pathogens (Harley et al., 2006; Karvonen et al., 2010). Increased sea-surface temperatures can also lead to other physical impacts such as increased stratification in the water column and changes in circulation patterns, such as modifications in local upwelling and alongshore advection patterns (Doney et al., 2012; Howard et al., 2013). These changes are likely to affect the distribution and therefore overall range of those species, such as *Homarus gammarus* and *Carcinus maenas*, with planktonic life stages (Gaylord and Gaines, 2000; Green et al., 2014). This may ultimately lead to novel host-pathogen interactions, although more positively, disease prevalence may decrease in some instances (Karvonen et al., 2010) particularly if the geographical overlap between the pathogen and host is reduced.

As well as physical changes in the environment, temperature also impacts the biology of the host and pathogens. In terms of pathogens, an increase in temperature facilitates growth (Karvonen et al., 2010). In terms of the host, the physiological responses to thermal stress are varied (reviewed in Somero,

2011), but include an increased metabolic demand that may ultimately result in hypoxia (Pörtner, 2008). Therefore, those species that are less efficient in acclimatising to changing environmental conditions or live closer to their thermal limits may be subject to changes in susceptibility to pathogens as a consequence of increased metabolic demands.

There are many examples in the literature that highlight changes in environmental variables impacting the susceptibility of host organisms to disease. For example, a thermal stress (23°C) experiment on the American lobster *Homarus americanus*, resulted in a decrease in haemolymph pH and 60% decrease in phagocytic activity (Dove et al., 2005). This would presumably affect immunocompetence. In line with this, increasing bottom water temperatures recorded off the north-east coast of the USA between 1999-2003 has been linked to epizootic shell disease in *H. americanus* (Glenn and Pugh, 2006). In another example, total haemolytic prophenoloxidase in the yellowleg shrimp, *Penaeus californiensis*, was found to increase with increasing salinity and significantly decrease with increasing temperature (Vargas-Albores et al., 1998). The authors suggested that these changes in the haemolytic prophenoloxidase activity, particularly in response to temperature, which was beyond the optimal range for this species, were likely to contribute to an increase in disease susceptibility.

Changes in the susceptibility of host species may also be compounded by changes in the biogeography of either the host species or the pathogen. A protozoan parasite *Perkinsus marinus*, (the cause of Dermo disease in the oyster *Crassostrea virginica*), was found to extend its geographical range in response to changing seawater temperatures (Cook et al., 1998). Previously, the range of this endoparasite extended from the Gulf of Mexico to lower Chesapeake bay on the east coast of the USA (Cook et al., 1998 citing Mackin et. al., 1950, Ray, 1954 and Andrews and Hewatt, 1957). An outbreak further north in lower Delaware Bay resulted in high prevalence (90-100%) in 1991, followed by further outbreaks in the subsequent years as far north as Maine on the east coast of the USA. This was beyond the previous range noted for *P. marinus*. Cook et al. (1998) determined that three warming periods (1974, 1984 and 1988-1991) all coincided with an increased prevalence of *P. marinus* and that an increase in winter seawater temperatures were key in facilitating the increased range of this parasite further north. This example highlights the potential for future changes in temperature to impact both the biogeography of pathogens and also the prevalence, presumably related to the increased temperature facilitating pathogen growth and transmission (Karvonen et al., 2010). Furthermore, a comparison of previous warmer periods suggested that more extreme short-term elevations in temperature were less likely to trigger *P. marinus* epizootics than less extreme but sustained temperature changes (Cook et al., 1998). Longer, milder, sustained temperature elevations would allow longer periods for the replication of pathogens and transmission to non-infected hosts (Karvonen et al., 2010).

Interactions between pathogens and naive hosts could occur as a result of changing environmental conditions. During 2006 and 2007 shellfish hatcheries from the North American Pacific coast suffered larval and juvenile mortalites as a result of *Vibrio tubiashii* infection in three previously undescribed bivalve species; the Pacific oyster *Crassostrea gigas*, the Kumamoto oyster *Crassostrea sikamea* and the geoduck clams *Panope abrupta* (Elston et al., 2008). This was determined to be associated with warmer sea-surface temperatures mixing with intermittent upwelling of nutrient-rich water containing *V. tubiashii*. This example highlights changing environmental conditions resulting in exposure of pathogens to novel hosts, the consequences of which would be difficult to predict. The effect of seawater acidification was tested on the early larval stages of *Homarus gammarus* (Arnold et al., 2009). The

authors exposed *H. gammarus* larvae to 1,200ppm CO_2 and observed a reduction in carapace mass. As the exoskeleton is the first line of defence for decapods, a reduction in carapace mass may have implications for increased disease susceptibility.

1.1.2.2 Effect of pollutants

Pollutants have been shown to effect susceptibility in a number of species. As an example, the effect of manganese was tested on the Norway lobster, *Nephrops norvegicus* (Hernroth et al., 2004). High levels of bioavailable manganese can be released from the soft sediments as a consequence of eutrophication. The authors noted a 60% reduction in total haemocyte count and a 75% reduction in the activity of granular haemocytes as a result of manganese exposure. In addition, a reduction in the gene coding for a RUNT-domain protein, known to be involved in maturing haemocytes, was evident. These changes in immune function would be likely to have a detrimental affect on the host's ability to deliver a competent immune response to invading pathogens. In another example, the freshwater giant prawn, *Macrobrachium rosenbergii* was exposed to *Lactococcus garvieae*, a Gram positive bacterium, in the presence of copper sulphate (Chen et al., 2001). This study observed both an increased virulence of *L. garvieae* and increased susceptibility of *M. rosenbergii* through a decrease in phenoloxidase activity. In this scenario, it could be argued that even without an effect on the host, the increased virulence could potentially effect the pathogenicity of the microorganism to the host.

All of these examples highlight the impact environmental change may have on the pathogens (changes in virulence and growth rates) and host susceptibility due to increased metabolic demand in order to compensate for the effect of environmental stressors. In addition, potential changes to the biogeoegraphical range of both host species and pathogens will likely change the interactions between them, the consequences of which are unknown.

1.2 Biomarkers

Concurrent with present and future changes in climate and concerns regarding food security is a need for appropriate biomarkers that are capable of identifying early evidence of host response to pathogens or to non-biotic stressors. Many techniques have been used to determine host response to infection and stress, from cellular (haemocyte counts) (Brown et al., 2004), sub cellular (Neutral Red Retention Assay) (Brown et al., 2004) to molecular assays (protein and gene expression) (Hernroth et al., 2004). Attempts to identify important host biomarkers to a range of different stressors is challenging and it may be that no one biomarker will be sufficient (Calfee et al., 2011; Damman et al., 2011).

The main objective of biomarkers is to provide a measurement of the host's response to a stressor. Therefore, biomarkers should be capable of measuring cellular, biochemical or molecular changes in biological fluids, cells or tissues (Hulka, 1990). In the context of human health Naylor (2003) extends this definition further to define biomarkers as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmaceutical responses to a therapeutic intervention". This could be transposed to crustacean biomarkers capable of assessing host response to disease and environmental stressors (pollutants, variations in temperature, salinity, seawater pH *etc.*). Biomarkers can be used in a number of ways that include identifying a dose-response relationship, identifying mechanisms that link exposure and disease/stress and

investigating the natural history of disease (Schulte, 1993).

Where possible, measurement of an external contaminant, (*e.g.* pollution or pathogen), in the immediate environment can inform the researcher of the level of exposure or dose of the stressor and thereby the relationship to the disease or stress process. However, it does not provide the researcher information regarding the effect of the stressor on the host organism. Choosing the appropriate biomarker to measure the response of the host to a stressor is important. The biomarker should be a quantifiable antecedent factor capable of modifying the risk of the host developing disease or detrimental effects from the stressor (Mayeux, 2004).

If selected correctly, the advantages of biomarkers include the ability to distinguish the effects of stressors between two or more groups. If validated properly, they can be sensitive, precise and reliable (Mayeux, 2004). However, care needs to be taken in interpreting the results. Biomarkers are not necessarily independent of other stressors and it can be difficult if the biomarker of choice is also responding to an unknown factor that may also be related to the stressor. This may produce variability that would need to be mitigated as much as possible either via preliminary studies or within the design of the experiment. Further variability can be a result of errors related to sample collection and laboratory processing. Again, this can be reduced by following established assay protocols and producing robust experimental designs (Mayeux, 2004). Furthermore, interpretation can be inhibited by the difficulty in establishing the normal range of a biomarker, particularly in a natural population where the synergistic or antagonistic effects of stressors may be complex and vary temporally. That said, endogenous host biomarkers provide a measure of response to a stressor that can inform researchers about the significance of a stressor in terms of host susceptibility, improve understanding regarding the mechanisms of host-pathogen or host-stressor interaction and inform decisions regarding treatment options (Mayeux, 2004) or policy decisions that can impact the health of the species of interest.

1.3 Rationale for research

The overall purpose of this research was to develop a 'toolbox' or suite of immune biomarkers in two economically and ecologically important decapod species, the European lobster *Homarus gammarus* (L.) and the shore crab *Carcinus maenas* (L.), to identify sub-lethal host response to both infection with pathogens and to environmental stress at the transcriptional level. Changes of immune gene expression at the transcriptional level do not necessarily infer synthesis of a functional protein (Frohlich et al., 2001), but they do provide very sensitive and early evidence of host response to a stressor. The three immune genes, each with varying functions, included the Type I antimicrobial peptide *crustin/carcinin*, the cell adhesive molecule and opsonin *peroxinectin* and the zymogen *prophenoloxidase*, converted to phenoloxidase for downstream melanisation (discussed below). The decapods were exposed to viral and bacterial challenges and non-biotic stress and also assessed in terms of exposure to natural pathogens in the wild. These varied challenges were designed to establish the significance, limitations and therefore practical application of these immune biomarkers (Clark et al., 2013a; Clark, 2014) in determining host response over a wide variety of stressors.

1.4 Immunity

There are two types of host immunity; innate (Medzhitov and Janeway Jr., 1997) and adaptive (Janeway Jr. et al., 2012). Adaptive immunity can be defined as the capacity for immunological memory in the form of appropriate immune cells that recognise a pathogen and therefore provide a targeted response. These specialised lymphocytic cells, are known as T and B cells.

T cells recognise a pathogen once small fragments of the pathogen have been processed and presented in combination with a 'self' receptor called a major histocompatibility complex (MHC) molecule. B cells have an antibody molecule on the cell surface that recognises the antigen on the pathogen without any prior need for processing (Storni et al., 2005). Specificity is derived from the antigen-specific receptors on the surfaces of T and B cells. These receptors are encoded by genes that result from somatic rearrangement of hundreds of germ-line gene fragments. The consequence of this is formation of millions of different antigen receptors, each with specificity for a different target antigen (Chaplin, 2010). This immunity is evident in vertebrates (Hirano et al., 2011).

Conversely, an innate immune response provides an immediate response to an invading pathogen, but does not have the appropriate cells to provide a tailored immune response to a specific pathogen. Innate immunity is also rapidly induced whereas the adaptive immune response is evident after antigen-specific T and B lymphocytes have undergone clonal expansion, which may take several days (Chaplin, 2010). Whilst different, these two types of immune systems work synergistically. Innate components can contribute to the activation of antigen-specific T and B cells and conversely, the antigen-specific T and B cells can recruit innate effector mechanisms thereby augmenting the innate immune response (Chaplin, 2010).

In invertebrates, the innate immune response, whilst lacking the antigen-specific T and B lymphocytes of the adaptive immune system, can provide the host organism with an efficient immune response despite lacking immunological memory. As this research focuses on host response in decapod crustaceans, the following details the innate immune effector processes with particular reference to the immune biomarkers (*crustin/carcinin, peroxinectin* and *prophenoloxidase*). As a footnote, whilst the description above provides a simple overview of the fundamental differences between these two immune systems, there is developing research regarding the capacity of the innate immune system in crustaceans to potentially produce targeted responses to pathogens. One hypothesis suggests the possibility of generating specific repertoires of pattern recognition receptors that may then control downstream activation (reviewed in Hauton et al., 2015). However, whilst an exciting prospect for future research in innate immunity, it is beyond the scope of this current research and therefore not discussed further in the following innate immunity overview.

1.4.1 Innate immunity in decapod crustaceans

The first line of defence against pathogens is the external cuticle, which acts as a physical barrier against invading pathogens. As the main site for invading pathogens is via the digestive tract, this is also, in part, lined with a chitinous membrane (Jiravanichpaisal et al., 2006). Beyond the physical protection of the cuticle, decapods rely on the innate immune system. Innate immunity can be traced back to the first metazoan organisms around 600 million years ago (Cooper and Herrin, 2010). As diverse multi-cellular organisms evolved, pathogens found new host opportunities, leading to evolution

of host defence systems (Cooper and Herrin, 2010).

1.4.2 Pattern recognition

Host defences all incorporate the use of germline-encoded pattern recognition receptors (PRRs). These soluble or cell surface host proteins recognise and respond to antigens on the surface of the pathogens through pathogen-associated molecular patterns (PAMPs) (Vazquez et al., 2009). The highly conserved molecular signatures are present in the cell walls of bacteria and fungi and have not been found in other multi-cellular organisms (Padhi and Verghese, 2008). In bacteria these are lipopolysaccharides (LPSs) and peptidoglycans (PGNs) and in fungi, β-1, 3-glucans (Vazquez et al., 2009). PRRs can recognise and bind to these cell surface molecules which in turn initiate cellular and humoral effector responses from the host that can inhibit or kill these invading pathogens (Cerenius et al., 2010). Cellular and humoral responses are discussed separately, however it is important to note that these systems respond concomitantly, so there is considerable overlap between the cellular and humoral host immune responses.

1.4.3 Cellular host defence

Haemocytes play a crucial role in the defence of the host against invading foreign particles and pathogens by participating in clotting functions, phagocytosis, haemocyte aggregation or nodule formation, and encapsulation. In decapod crustaceans, cellular defence involves activity from the three main haemocyte types; hyaline cells, semi-granular cells and granular cells. These cell types are distinguishable by morphological features and have been fully described in the freshwater crayfish, *Pacifastacus leniusculus* (D.) (Smith and Söderhäll, 1983), the freshwater crayfish *Astacus astacus* (Smith and Söderhäll, 1983b)(Smith and Söderhäll, 1983) and the shore crab *Carcinus maenas* (Söderhäll and Smith, 1983).

Hyaline cells contain very few or no granules and act to phagocytose biotic and non-biotic particles (Smith and Söderhäll, 1983). Semi-granular cells have numerous small granules, have a limited phagocytic role (Bachère et al., 1995; Itami et al., 1998; Jiravanichpaisal et al., 2006), but are predominantly involved in encapsulation processes (Jiravanichpaisal et al., 2006). Granular cells are not involved in phagocytosis, but have numerous large granules (Kobayashi et al., 1990). The granules in the semi-granular and granular haemocytes contain stores of molecules involved in humoral responses, such as *peroxinectin* and *prophenoloxidase* (Saha, 2011) which are released on exocytosis.

1.4.3.1 Haematopoeisis

Phagocytosis, nodule formation, encapsulation and clotting are all processes that recruit haemocytes for various functions in order to maintain homeostasis. Consequently, the titre of circulating haemocytes decreases. Maintaining an adequate population of circulating haemocytes is critical to protect against invading pathogens. To achieve this, production of haemocytes is regulated by haematopoeisis. This process synthesises and matures haemocytes which are then released into circulation (Chaga et al., 1995; Barreda and Belosevic, 2001). Haematopoeisis occurs continually, although the rate of production can vary according to homeostatic requirements. Factors influencing this can range from environmental stress, such as extremes in temperature and salinity to biological variables, such as invading pathogens and repeated sampling (Lightner et al., 1983; Jussila et al., 1997).

The location of the haematopoeitic tissue varies between species. Reports of a sheet-like tissue situated dorsally and dorsolaterally, particularly in the hollows by the cardiac and pyloric stomach have been reported in crayfish (Jiravanichpaisal et al., 2006). This tissue contains densely packed, small lobules comprising morphologically different cell types at various stages of maturation. This has not only been observed in *P. leniusculus*, but also in *Carcinus maenas* and the American lobster, *Homarus americanus* (reviewed in Johansson et al., 2000).

1.4.3.2 Phagocytosis

Phagocytosis is the primary mechanism for disposing of small non-biotic particles or biotic targets such as apoptotic cells, bacteria or protozoan cells. Phagocytosis is a form of endocytosis in which the target is engulfed, in this case, by hyaline cells. The phagocytic cell firstly recognises and then binds to the target. It is then transported into the cell via a vacuole known as a phagosome (Jiravanichpaisal et al., 2006). Lysosomes bind to the phagosome and release lysozymes and proteases to digest the target (Gordon et al., 1974).

Phagocyte activity appears to be species specific. For example, phagocytes have been observed to demonstrate activity against Gram negative bacteria such as *Pseudomonas* species and *Escherichia coli* in *Parachaeraps bicarinatus* (freshwater crayfish) and *Cherax extractor* (common yabby) (McKay and Jenkin, 1969). Whereas in *Callinectes sapidus*, phagocytes act against both Gram negative and Gram positive bacteria (Cassels et al., 1986). In the American lobster, *Homarus americanus*, variations in phagocytosis have been shown to vary in response to different Gram positive and Gram negative bacterial infections (Mori and Stewart, 2006). The authors demonstrated that immunogens from Gram negative bacteria induced a significant, but generally non-specific increase in phagocytosis that was dependent upon the immunogen concentration. In contrast, rather than a dose-dependent response, the increased resistance observed against Gram positive bacteria were likely to be as a result of modifications to the bacterial cell wall that increased the permeability of the bacterial cell which could then be agglutinated by the lobster haemolymph.

1.4.3.3 Nodule formation and encapsulation

Particles too large for phagocytosis can be contained through nodule formation and encapsulation. Nodules are aggregates of haemocytes, typically semi-granular and granular cells, that entrap pathogens such as bacteria (Lackie, 1988). Encapsulation occurs when compact layers of haemocytes, (between 5-30 layers), surround a non-self target (Vazquez et al., 2009). These processes restrict the movement and growth of the pathogen and are associated with melanisation, the end product of the prophenoloxidase activation cascade. Destruction of the target occurs by reduction of oxygen concentration, asphyxiation, activity of hydrolases and cytotoxic quinones and action of antibacterial peptides within the encapsulation (Söderhäll et al., 1994; Gillespie et al., 1997).

1.4.3.4 Clotting

Unlike mammals, crustaceans have semi-open circulatory systems. Damage to the cuticle and loss of appendages are not uncommon in decapods and can occur as a consequence of moulting or aggressive interaction with conspecifics or other species. Any resulting injury has the potential to lead to rapid haemolymph loss and allow pathogens and foreign particles entry into the body cavity. Clotting proteins are found in the haemolymph rather than haemocytes and are efficient at both preventing

haemolymph loss and preventing microorganisms from spreading throughout the haemolymph (Jiravanichpaisal et al., 2006; Vazquez et al., 2009).

Clotting has been studied in detail in a number of species including the freshwater crayfish. In this model, clotting is a result of cross-linking of a specific plasma clotting protein mediated by transglutaminase in the presence of calcium. This highly dense lipoprotein, evolutionarily related to vitellogenin, consists of two identical 210kDa subunits each containing lysine and glutamine sidechains. On detection of PAMPs in Gram negative bacteria by PRRs (Ariki et al., 2004), the haemocytes release transglutaminase through rapid exocytosis. Transglutaminase is activated by the calcium in the plasma and proceeds to cross-link the sidechains of the clotting proteins in the haemolymph resulting in large aggregates (Hall et al., 1999).

In other models, different clotting factors are used. In the phylogenetically ancestral horseshoe crab, *Tachypleus tridentatus* (L.), (class Merostomata), clotting is regulated by a proteolytic cascade. On detection of LPS and β-1, 3-glucans, components of the clotting system, stored in haemocyte granules, are released into the haemolymph (Iwanaga et al., 1998). These components, four serine proteinase zymogens, factors B, C, G and proclotting enzyme, trigger the conversion of soluble coagulogen into insoluble coagulant gel (Takaki et al., 2002). Factor C zymogen is highly sensitive to LPS and autocatalyses to it's active form (Iwanaga et al., 1992). Factor C activates factor B which in turn activates proclotting enzyme to clotting enzyme. Factor G zymogen autocatalyses to it's active form in the presence of β-1, 3-glucans and then directly activates the proclotting enzyme to clotting enzyme. This clotting enzyme then catalyses coagulogen to coagulant gel forming non-covalent bonds (Kawasaki et al., 2000). Interestingly, the amine-terminal portion of factor B and the proclotting enzyme contain a clip domain (an area of three di-sulphide bridges). The folding pattern of the di-sulphide bridges within the clip domain is identical to that of big defensin, an antimicrobial peptide identified in the haemocytes of *T. tridentatus*. Release of this clip domain during proteolytic cleavage may provide antimicrobial activity against invading pathogens during this clotting process (Iwanaga and Lee, 2005). This process is also observed in crayfish prophenoloxidase activating enzyme (ppA) (Wang et al., 2001) (discussed later).

1.4.4 Humoral host defence

Humoral responses include reactive oxygen intermediates (ROI), the production of anti-microbial peptides (AMPs) and the enzymatic cascade which regulates the production of melanin (Cerenius et al., 2010). This synopsis will focus on the function of the suite of genes used to assess host response to infection and stress in this research.

1.4.4.1 The prophenoloxidase cascade

The prophenoloxidase cascade is a fundamental part of innate immunity in many crustaceans as it is involved in many responses including melanisation, cell adhesion and cytotoxic reactions and aids phagocytosis and encapsulation reactions (Cerenius et al., 2008). There are species-specific variations in the details of the prophenoloxidase activating cascade between different invertebrates, so the following description is predominantly based on the system studied in the freshwater crayfish, *Pacifastacus leniusculus*. Ultimately, this cascade produces a terminal enzyme, phenoloxidase (PO), that initiates the biosynthesis of melanin, a brownish/black pigment known to provide pigmentation. In invertebrates

it also has three other major functions; cuticle sclerotisation, wound healing and immune defence such as inhibition of bacterial and fungal enzymes and encapsulation of foreign material including pathogens (Smith and Söderhäll, 1983; Söderhäll and Cerenius, 1998; Cerenius et al., 2008; Sugumaran, 2002). This complex system consists of pattern recognition receptors, proteinases and proteinase inhibitors (Söderhäll and Cerenius, 1998).

Prophenoloxidase, stored as a zymogen in the haemocyte granules (Cerenius et al., 2003), is released into the haemolymph by exocytosis upon recognition of very low concentrations of PAMPs by PRRs (Sritunyalucksana and Söderhäll, 2000). These PRRs include LPS and β-1, 3-glucans binding proteins (LGBP), β-1,3-glucans binding protein (βGBP) and peptidoglycan binding protein (PGBP) (Figure 1.1) (Cerenius and Söderhäll, 2004). Interestingly, peptidoglycans are thought to be recognised in *P. leniusculus*, not through the use of a specific recognition receptor, but through possible complex formation between novel serine proteinase homologues and LGBP (Liu et al., 2011).

Conversion of inactive prophenoloxidase to phenoloxidase is mediated by a trypsin-like serine proteinase, the prophenoloxidase activating enzyme, ppA (Aspan and Söderhäll, 1991). ppA is stored as a zymogen, proppA, and activated post infection or injury (Cerenius and Söderhäll, 2004). Arthropod ppAs commonly have clip domains. As previously discussed, these are areas containing di-sulphide bridges. This clip domain in crayfish has homologous amino acid sequences to both the antimicrobial peptide big defensin in the horseshoe crab *T. tridentatus* and also to mammalian β-defensin (Bensch et al., 1995). Cleavage of this domain in crayfish ppA has resulted in antimicrobial activity *in vivo* against Gram negative bacteria, such as *Escherichia coli* and also Gram positive bacteria such as *Micrococcus luteus* and *Bacillus megaterium*, suggesting a dual role for this serine protease (Wang et al., 2001). Phenoloxidase, also known as tyrosinase, is a copper-containing enzyme responsible for two main reactions in which tyrosine is converted to DOPA (dihydroxyphenylalanine) and then DOPA converted to DOPA-quinone, the precursor to melanin (Vazquez et al., 2009). This occurs through hydroxylation of monophenol to O-diphenol, followed by oxidation of O-diphenol to O-quinone. These intermediate products are highly toxic. Consequently, proteinase inhibitors play a role in ensuring ppA is not over-activated (Aspan et al., 1990). In freshwater crayfish this proteinase inhibitor is pacifastin. It encodes for a heavy chain consisting of three transferrin domains, including two that bind iron (Söderhäll and Cerenius, 1998). It also codes for a light chain domain containing a proteinase inhibitor with sequence similarity to proteinase inhibitors identified and cloned in other species such as the locust *Locusta migratoria* (Kromer et al., 1994). Alternatively, regulation of the prophenoloxidase activating system can be achieved by direct inhibition of phenoloxidase activity itself by the phenoloxidase inhibitor (POI) (Sugumaran and Nellaiappan, 2000).

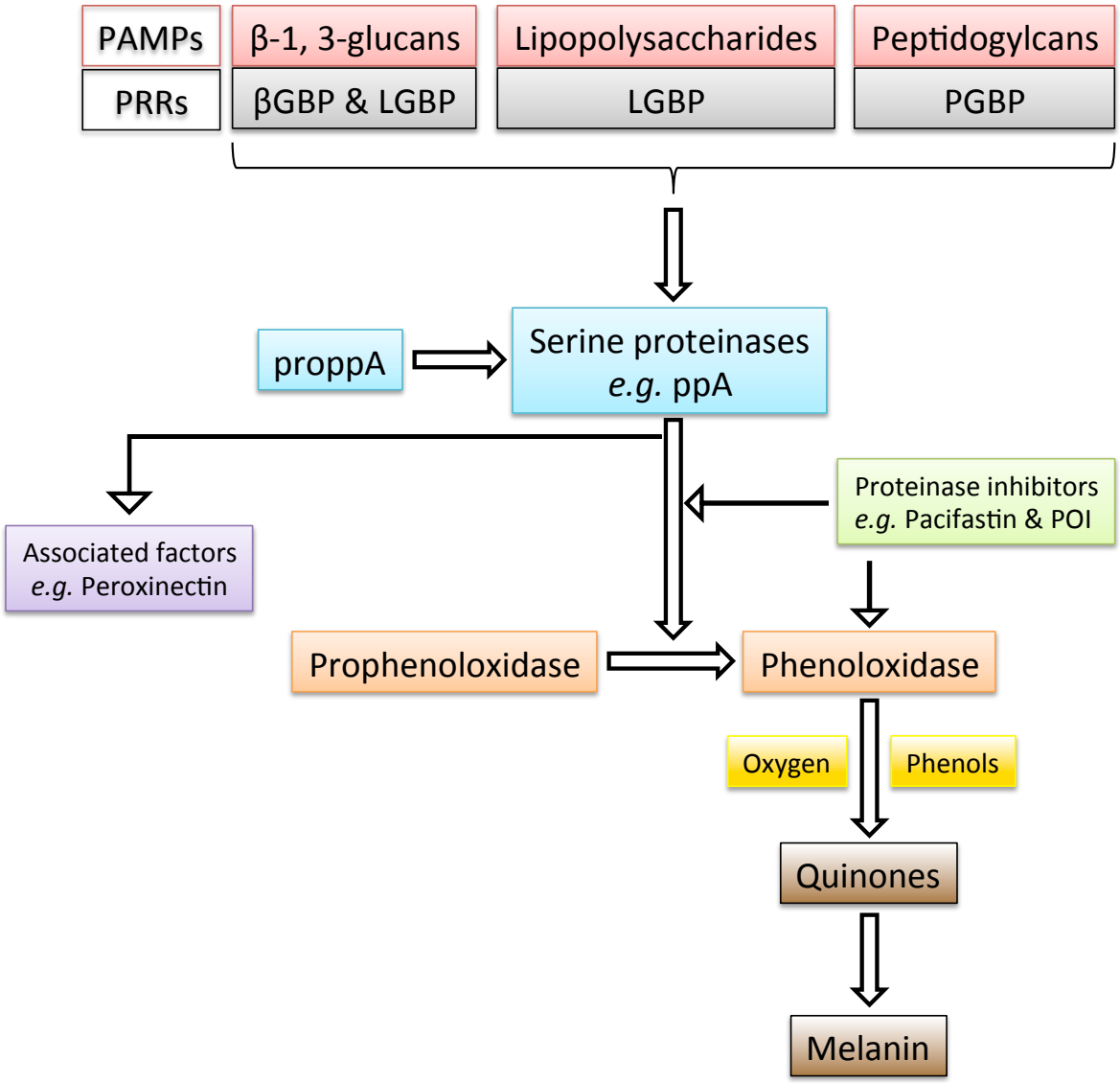


Figure 1.1: An overview of the prophenoloxidase activating system. Pathogen-associated molecular patterns (PAMPs) are recognised by pattern recognition receptors (PRRs), such as β-1, 3-glucans binding protein (βGBP), lipopolysaccharide and β-1, 3-glucans binding protein (LGBP) and peptidoglycan binding protein (PGBP). These PRRs induce conversion of prophenoloxidase to phenoloxidase through the action of serine proteinases, for example prophenoloxidase activating enzyme (ppA). ppA is stored in the pro-form as proPPA prior to activation post injury or infection. This process is regulated by proteinase inhibitors, pacifastin and prophenoloxidase inhibitor (POI), to prevent deleterious effects from over-production of toxic quinones. Phenoloxidase catalyses the hydroxylation of monophenol to O-diphenol and subsequent oxidation of O-diphenol to O-quinone ultimately resulting in the production of melanin. Associated factors, such as the cell-adhesive molecule, peroxinectin, is activated concomitantly with the prophenoloxidase activating cascade. Figure modified from Söderhäll and Cerenius (1998).

1.4.4.2 Peroxinectin

Peroxinectin, a 76kDa protein, was first isolated, purified (Johansson and Söderhäll, 1988) and cloned (Johansson et al., 1995) from the haemocytes of the crayfish *P. leniusculus*, but has since been isolated in a number of other decapod crustaceans (Thörnqvist et al., 1994; Sritunyalucksana et al., 2001; Liu et al., 2004). It has been observed to have a number of biological functions. These include cell adhesion (Johansson and Söderhäll, 1988), inducing degranulation (Johansson and Söderhäll, 1989), opsonic activity (Thörnqvist et al., 1994), peroxidase activity (Johansson et al., 1995) as well as promoting encapsulation (Kobayashi et al., 1990).

Peroxinectin in crayfish is synthesised in the haemocytes and stored in the granules of the cells until exocytosis upon stimulus. It is inactive when it is secreted from the cell but can be activated in the presence of microbial molecular signatures (PAMPs). Peroxinectin has a KGD (Lys-Gly-Arg) motif which binds to an integrin receptor (Johansson et al., 1999). In insects such as the tobacco hornworm *Manduca sexta* (L.), it has been suggested that haemocyte-specific integrin ligand-binding is significant in stimulating adhesion of plasmatocytes which leads to encapsulation (Levin et al., 2005).

In addition, peroxinectin also binds to another protein at the cell-surface. This protein shares amino acid sequences with the family of superoxide dismutases containing copper and zinc (CuZn-SOD) (Johansson et al., 1999). This interaction may serve to bring these two enzymes in close proximity. As peroxinectin functions as a peroxidase, this may facilitate the use of hydrogen peroxide (H_2O_2) as a substrate for peroxinectin to produce antimicrobial peptides (Sritunyalucksana and Söderhäll, 2000).

1.4.4.3 Antimicrobial peptides

Antimicrobial peptides (AMPs) are major components of the innate immune system. They have been classified into three main categories based on the amino acid sequences, secondary structure and functional properties; penaeidins, crustins and antilipolysaccharide factors (reviewed in Vazquez et al., 2009; Cerenius et al., 2010; Ghosh et al., 2011; Hauton, 2012).

Penaeidins

Penaeidins are small peptides (5-7kDa) of which there are four classes and several isoforms within each class (Destoumieux et al., 1997, 2000; Cuthbertson et al., 2002). They are stored within the granulocytes of penaeids and released into circulation upon microbial stimulus. Penaeidins contain both proline-rich (N-terminal) and cysteine-rich (C-terminal) domains, the latter containing three di-sulphide bridges (Destoumieux et al., 1997). Penaeidins have been reported to have both antifungal and antibacterial properties. As examples, Pen-2 and Pen-3a were reported to have broad antifungal activity and antibacterial activity predominantly against Gram positive bacteria (Destoumieux et al., 1999). Interestingly, Pen-5 from *Penaeus monodon* has been reported to have a possible role in antiviral immunity against White Spot Syndrome Virus (WSSV) (Woramongkolchai et al., 2011).

Crustins

Crustins, another category of antimicrobial peptides, have been reported in a wider range of taxa compared with penaeidins, including over 50 decapod species such as shrimp, crayfish, lobsters and crabs. In general, crustins are larger than penaidins (7-14 kDa) (Smith et al., 2008; Ghosh et al., 2011). The first crustin isolated was an 11.5 kDa cationic and hydrophobic protein from *Carcinus maenas*,

termed carcinin (Accession number AJ427538) (Relf et al., 1999). The term 'crustin' was later coined to describe subsequent isolation of cystein-rich genes with high homology to carcinin (Bartlett et al., 2002). Crustins have a non-conserved signal sequence at the N-terminal and a characteristic conserved cysteine-rich WAP (whey acid protein) domain at the C-terminal (Smith et al., 2008). Whey acid proteins are a family of proteins originally discovered from mammalian milk, that comprise 50 amino acids and two WAP domains (Ranganathan et al., 1999).

There are three crustin classifications based on structural differences; Type 1, Type 2 and Type 3 (Figure 1.2). Types 2 and 3 have a tightly packed core, consisting of eight cysteine residues stabilised by four di-sulphide bridges, known as the four di-sulphide core (4DSC) or WAP domain. Whereas, Type 1 crustins have a variable length cysteine-rich domain, but is rarely greater than six cysteine-rich residues (Smith et al., 2008).

Crustin Types I and II have a cysteine-rich domain between 46 and 52 amino acids in length, with a glycine-rich domain in Type II upstream of the cysteine-rich and WAP domains. It has been suggested that the hydrophobic glycine-rich domain may function as a trans-membrane domain (Bartlett et al., 2002). Type III crustin has a characteristic single WAP domain and short proline and arginine-rich domain upstream of the WAP domain (Smith et al., 2008). Type I crustin, (which includes carcinin), has been isolated in the shore crab *Carcinus maenas* (Brockton et al., 2007), signal crayfish *Pacifastacus leniusculus* (Jiravanichpaisal et al., 2007), homarid lobsters (Hauton et al., 2006; Christie et al., 2007) as well as some other crab species such as the spider crab *Hyas areneus* (Sperstad et al., 2009). Type II crustins are predominantly found in penaeid species such as *Litopenaeus vannamei*, *Litopenaeus setiferus* (Bartlett et al., 2002) *Fenneropenaeus chinensis* (Zhang et al., 2007) and *Penaeus monodon* (Amparyup et al., 2008), but has also been isolated in the red king crab *Paralithodes camtschaticus* (Sperstad et al., 2009). Lastly, Type III crustins are also found in penaeids, for example *Litopenaeus vannamei* (Jiménez-Vega et al., 2004) and *Penaeus monodon* (Chen et al., 2005).

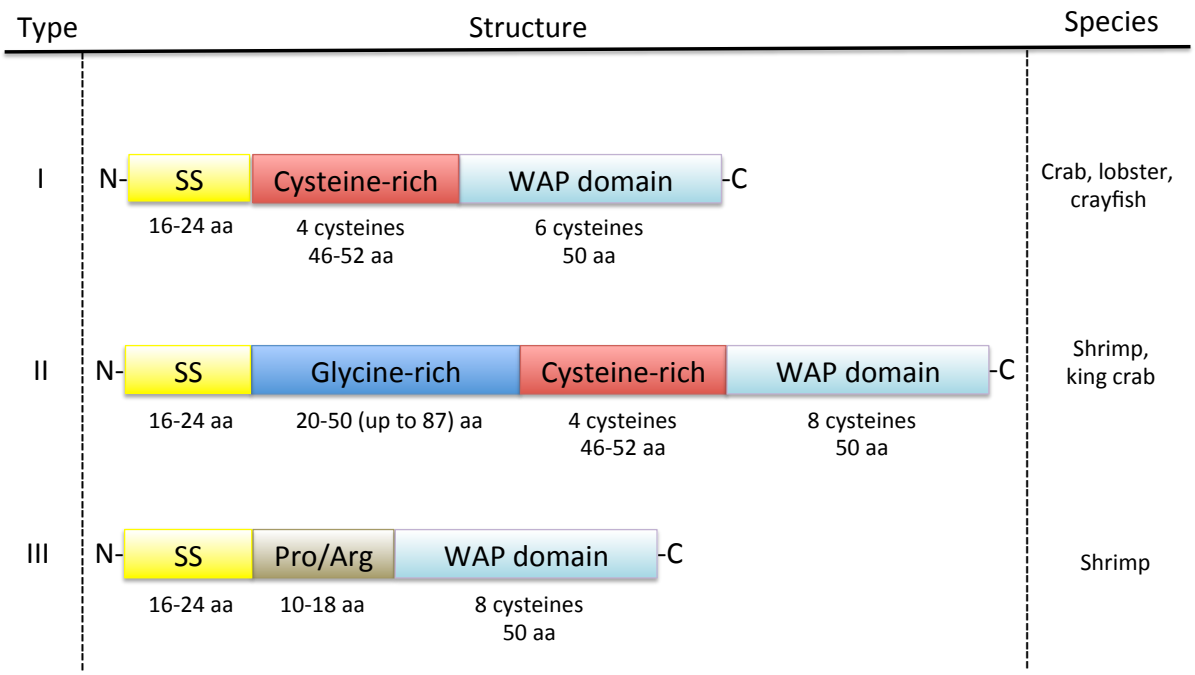


Figure 1.2: Crustin antimicrobial peptides, Types I, II and III. All types have an N-terminal signal sequence (SS) and a WAP (whey acid protein) domain C-terminal. Types I and II crustin have a cysteine-rich domain with 46–52 amino acids (aa), with an additional glycine-rich domain in Type II. Whereas Type III crustin consists of a number of proline and arginine residues. Examples of species types for each crustin type are given in the last column. Not to scale. Based on Smith et al. (2008) and Ghosh et al. (2011).

Crustins have been reported to have activity against a wide variety of microorganisms including Gram positive (Donpudsa et al., 2010; Mu et al., 2011) and Gram negative (Relf et al., 1999; Fall et al., 2010) bacteria, yeast (Antony et al., 2011a), and viruses (Prapavorarat et al., 2010), although it is important to note that many of these studies are based on transcriptional expression rather than the expression of functional proteins. Crustins are secreted from the haemocytes once they are produced, so remain in the haemolymph at a high titre (Hauton, 2012), but can be also be inducible upon microbial stimulation (Han-Ching Wang et al., 2010).

Antilipopolysaccharide factors

The final category of antimicrobial peptides are the antilipopolysaccharide factors (ALFs). ALF was first identified from the horseshoe crab, *Limulus polyphemus* (Tanaka et al., 1982). It has since been identified in other species such as *Carcinus maenas* (Towle and Smith, 2006), *Homarus americanus* (Beale et al., 2008; Clark et al., 2013a), *Pacifastacus leniusculus* (Liu et al., 2006), the mud crab *Scylla paramamosain* (Imjongjirak et al., 2007) and various penaeid species including *Fenneropenaeus chinensis* (Liu et al., 2005), *Marsupenaeus japonicus* (Nagoshi et al., 2006) and *Penaeus monodon* (Somboonwiwat et al., 2005).

ALFs are basic proteins approximately 100 amino acids in length, that can bind to LPS (Yedery and Reddy, 2009). These anitmicrobial proteins have been reported to show activity predominantly against Gram negative bacteria (Beale et al., 2008; Kadowaki et al., 2011), but also Gram positive bacteria (Li et al., 2014), fungi (Löfgren et al., 2009), protozoa (Löfgren et al., 2008) and also viruses (Prapavorarat

et al., 2010). For example, changes in transcriptional regulation were observed in *Pacifastacus leniusculus* and *Penaeus monodon* in response to WSSV infection (Liu et al., 2006; Tharntada et al., 2009) inferring potential antiviral activity in these host antimicrobial peptides.

1.4.4.4 Summary

To summarise, pathogens are recognised by the host via pattern recognition receptors which stimulate both cellular and humoral host responses. These involve phagocytosis, nodule formation, encapsulation and clotting. Processes such as haemocyte aggregation are facilitated by cell-adhesion which is mediated by peroxinectin. In addition, the prophenoloxidase cascade produces cytotoxic intermediates and melanin as an end product which functions to contain and kill the pathogens. The constitutive and induced production of antimicrobial peptides can directly kill pathogens by targeting the integrity of the bacterial cell membrane (Rosa and Barracco, 2010). Clearance of pathogens from the haemolymph often results in increased pathogen aggregations in the gill or hepatopancreas whereby they can be broken down (Hauton, 2012). Due to recruitment of haemocytes for the functions previously stated, there is often a decrease in total haemocyte count. These haemocytes are repleted via haematopoeisis.

As well as in response to pathogens, the expression of some antimicrobial peptides, *peroxinectin* and *prophenoloxidase* have also been observed to change in response to non-biotic environmental stimulation. A number of researchers have highlighted the response of these immune biomarkers to environmental stressors. Temperature is a significant abiotic factor that affects metabolism, oxygen consumption, growth, susceptibility to infection and survival (Hennig and Andreatta, 1998). *Prophenoloxidase* expression in the white-leg shrimp *Litopenaeus vannamei* was up-regulated in response to 30 minute heat-shock at 34°C-38°C (Loc et al., 2013). In response to hypoxic stress, the activity of phenoloxidase in the blue shrimp *Penaeus stylirostris* resulted in a significant increase in activity due to a decrease in phenoloxidase inhibitors regulating the prophenoloxidase cascade (Le Moullac et al., 1998). The expression of *peroxinectin* in the freshwater prawn *Macrobrachium rosenbergii* was found to be affected by moult cycle (variable expression), sodium alginate-containing diet (up-regulation of *peroxinectin*) and copper sulphate, benzalkonium chloride (surfactant), and trichlorfon (insecticide) (down-regulation of *peroxinectin*) (Liu et al., 2007a). In addition, microarray analysis in the black tiger shrimp *Penaeus monodon* observed a down-regulation of *crustin* in response to hypoxic stress (de la Vega et al., 2007). Clearly, these examples demonstrate that changes in environmental conditions have the ability to adversely influence immunocompetence in the host. Furthermore, these studies suggest *crustin*, *peroxinectin* and *prophenoloxidase* are credible as stress biomarkers in response to some environmental stressors.

1.5 Decapod species of interest

1.5.1 *Homarus gammarus* (L.)

Distribution

The European lobster, *Homarus gammarus* (Linnaeus 1758), is an economically significant decapod, valued as a high commodity product (Figure 1.3). It's range is confined to the north-eastern Atlantic Ocean from the Lofoten Islands in northern Norway extending further south to Sweden and Denmark, although not found in the Baltic Sea. This may be related to the lower salinity and temperature extremes in this region (Prodöhl et al., 2007). Their range extends around the coast of the United

Kingdom and Ireland and further south along the west coast of continental Europe to a southern limit around 30° latitude in the region of Morocco in Northern Africa. They are also found throughout the Mediterranean Sea, although at lower abundance (Jørstad et al., 2004; Prodöhl et al., 2007). There are known to be four major genetically distinct populations of *H. gammarus* based on allozyme, microsatellite and mitochondrial DNA analysis; a widespread Atlantic population and three others in northern Norway, the Netherlands and Mediterranean (Jørstad et al., 2004; Triantafyllidis et al., 2005).

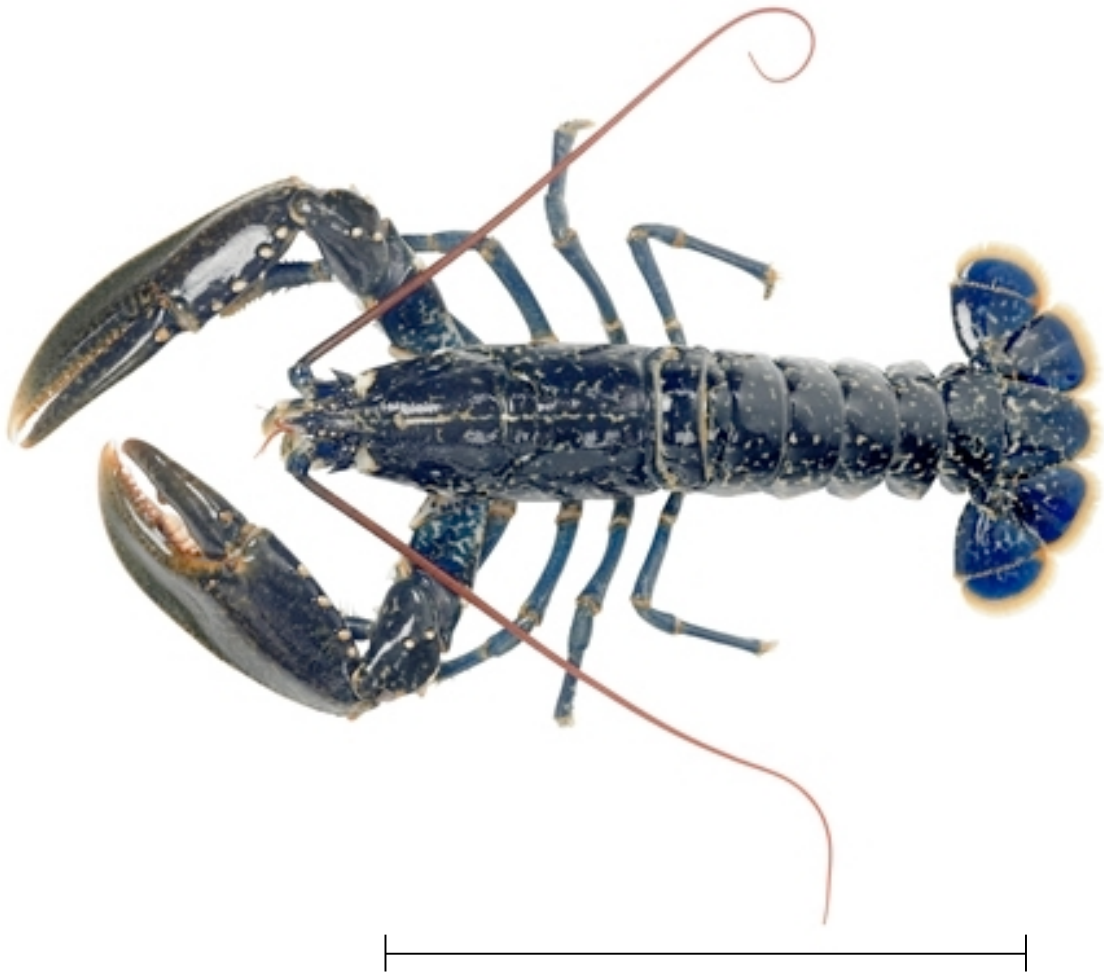


Figure 1.3: Adult *Homarus gammarus* (d'Or Bocuse, 2013). The European lobster is typically blue dorsally and lighter in colour ventrally. The two front appendages are specialised chelipeds for crushing and slicing prey. Scale bar represents 300mm.

Economic significance

Fishing catch has fluctuated over time (Figure 1.4). In the late 1950's and early 1960's global capture exceeded 3,000 tonnes per annum, although this reduced to less than 2,000 tonnes in the 1970's. The population in northern Norway experienced a collapse between 1960-1980 to less than 10% of pre-1960 annual catches. Subsequently, recruitment was reduced to this population (Agnalt et al., 2006). It has been placed on the International Union for Conservation of Nature (IUCN) Red List of Threatened Species (IUCN, 2013), although it is classified as 'least concern' due to the relatively wide distribution

and increased annual catch over the previous 30 years. More recently, annual recorded catches have been up to 5,651 tonnes. The fishing effort between 1950-2012 has been dominated by countries among the more northerly distributed range of *H. gammarus* such as the United Kingdom, followed by Ireland, France then Norway (FAO, 2014). *H. gammarus* can be worth up to three times as much as the American lobster, *Homarus americanus* as it is considered in general to have a better flavour (Barrento et al., 2009). Regulation of the European lobster fishery include an EU minimum landing size of 87mm carapace length (Cefas, 2011) which is broadly related to the mean size of sexual maturity (approximately 5-7 years) and V-notch schemes for berried females which legally protects them from fishing in order to increase total egg production to maintain and improve stocks in a particular area (Tully et al., 2000; Agnalt et al., 2006).

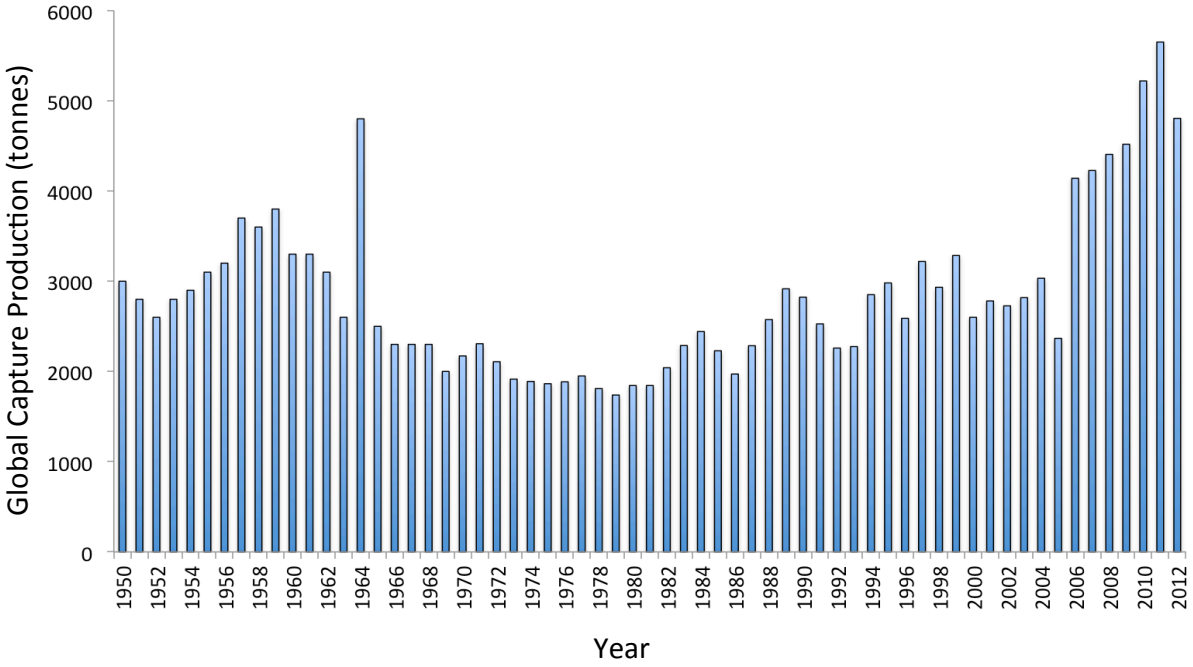


Figure 1.4: Global capture production (tonnes) of *Homarus gammarus* between 1950-2012 (FAO, 2014)

Life cycle and habitat

In terms of life cycle, *H. gammarus* undergoes a pelagic period post-hatching that usually lasts for 14-20 days depending on temperature. During this time, frequent ecdysis ensures rapid progression through Stages I to III until metamorphosis to Stage IV. At this stage *H. gammarus* settle on the benthos. Survival increases after this stage as they are less vulnerable to pelagic predation. Very little is known regarding the early benthic stages in *H. gammarus* as they are rarely seen at this ontogenetic stage in the wild. From laboratory investigations it is thought that they bury in the sediment for around two years post settlement feeding on benthic fauna (Howard and Bennett, 1979). They leave their burrows around 15mm carapace length and find crevices to inhabit as adults, typically in rocky and hard mud substrates. Depth range varies from the sublittoral fringe to 150m, although they are rarely found at depths beyond 50m (IUCN, 2013 citing Holthuis, 1991).

Disease susceptibility

Very few diseases are found in adult *H. gammarus*. However, *H. gammarus* is known to be susceptible to *Aerococcus viridans*, also known as Gaffkaemia (Gjerde, 1984). This Gram positive bacterium is known to infect the closely related *H. americanus*. It's thought to have occurred in *H. gammarus* due to the import of a number of *H. americanus* and then accidental release of live lobsters or discard of infected offal (Alderman, 1996). This pathogen survives well in holding facilities and can therefore be a significant disease in marketable lobsters. In the wild it can be found in benthic mud although incidence of infection in wild homarid lobsters is unclear (Cawthorn, 2011). Infection is likely to be as a result of damage to the cuticle as the bacteria does not survive gastric acid and cannot cross the integument (Cawthorn, 2011).

A parasitic copepod, *Nicothoë astaci*, referred to as lobster louse, is known to infect the gills of *H. gammarus* (Wootton et al., 2011). This parasite has been previously reported in Europe (Gotto, 1954; Sindermann and Rosenfield, 1967). A study around Lundy Island in the Bristol Channel observed *N. astaci* attached to the gill filaments of lobsters via maxillae, maxillipeds and an oral disc, through which it feeds on the lobster haemolymph.

H. gammarus is also vulnerable to shell disease which causes degradation of the cuticle (Davies et al., 2014). This is common in many crustaceans. There are two forms of shell disease; one mediated by chitinolytic bacteria and the other by non-chitinolytic bacteria (Vogan et al., 2008). Studies in shell disease in the edible crab *Cancer pagarus* and *H. americanus* have identified bacteria such as *Vibrio* spp. (Vogan et al., 2002), Flavobacteriaceae and *Pseudoalteromonas gracilis* associated with shell disease (Chistoserdov et al., 2005). A review of lobster health in populations around Lundy Island observed shell disease in *H. gammarus*, although these were mainly small melanised lesions that did not fully penetrate the cuticle (Wootton et al., 2012). This is in contrast to other species, such as *H. americanus*, whereby shell disease can penetrate a much more significant area of the exoskeleton (Shields et al., 2012). A pre-cursor or trigger for shell disease is thought to be as a result of injuries or abrasions (Quinn et al., 2012). Direct comparison of abrasion injuries between juvenile *H. gammarus* and *H. americanus* observed greater resistance to injury in *H. gammarus*. This was found to be related to a thicker cuticle and fewer number of pores in *H. gammarus*.

Homarus americanus, in particular, has previously been vulnerable to epizootic shell disease (ESD). A high prevalence of heavily eroded shell disease and wide-spread geographical distribution was reported from 1998 to 2004 along the north-east coast of the USA (Glenn and Pugh, 2006). Bacteria were observed on the leading edge of the lesions, which penetrated inward from the carapace surface (Glenn and Pugh, 2006). The high prevalence and wide-spread distribution of this bacterial infection of the cuticle was attributed to the interaction between water temperature, the sexual maturity of the lobsters and the intermoult duration (Glenn and Pugh, 2006). In particular, previously above average water temperatures between 1999 and 2003 was suggested as a driving factor that triggered the outbreak in ESD. The warmer water temperatures may not only encourage bacterial growth, but also influence the host in a number of ways. Sexual maturity has been observed to occur at smaller sizes in warmer waters (Aiken, 1980; Aiken and Waddy, 1986). After sexual maturity, the inter-moult duration increases considerably (Aiken, 1980), thereby increasing the length of exposure to the bacterial agent (Glenn and Pugh, 2006). These mechanisms may explain how water temperature could increase the prevalence and distribution of shell disease, thus exacerbating the impact of the infection.

Known occurrences of *H. americanus* in European waters, including one with observed symptoms of shell disease, highlights the potential for cross-infection and vulnerability to known and novel pathogens in *H. gammarus* (Davies et al., 2014). Similarly, *H. gammarus* is known to be vulnerable to a significant viral pathogen, WSSV (Bateman et al., 2012a,b), which has historically affected highly commercial penaeid species (Lightner, 1996). As *H. gammarus* is a commercially valuable species to the UK with hatcheries rearing larvae to ensure stocking densities are maintained (Hauton et al., 2007) and also vulnerable to WSSV, it was chosen as the experimental model with which to investigate host response to this commercially significant pathogen (Chapter 3).

1.5.2 *Carcinus maenas* (L.)

Distribution

The shore crab, *Carcinus maenas* (Linnaeus 1758), is indigenous to the British Isles (Figure 1.5). It's native range extends along the Atlantic European coast as far north as Norway and Iceland, as far east as the Baltic Sea and as far south as northern Africa to Mauritania (Darling et al., 2008). It is an exceptionally good species in establishing populations in non-native areas. These include Atlantic and Pacific North America (Grosholz and Ruiz, 1995; Carlton and Cohen, 2003), Australia, Japan and South Africa. Although there have been other appearances in South and Central America, Pakistan and Sri Lanka (Carlton and Cohen, 2003). It has been suggested that there have been three major periods where *C. maenas* has been translocated to new areas; the 1800s, 1850s-1870s and 1980s-1990s (reviewed in Carlton and Cohen, 2003). There are a number of different mechanisms by which *C. maenas* has been transported to non-indigenous areas over the years. Prior to ship hulls being replaced by steel and before the development of anti-fouling paints, boring by teredinid bivalves and the isopod genus *Limnoria*, provided dynamic habitats for fouling by opportunistic organisms such as *C. maenas*. Other transfer mechanisms included transport with solid ballast such as sand and rocks and via seawater pipes and sea chests of ocean-going vessels (Carlton, 1989). More recently, semisubmersible exploratory drilling platforms, ballast water, seaweed transported with commercial fisheries products, education and research and intentional release to create new fisheries have also contributed as methods to transport *C. maenas* to new areas (Carlton and Cohen, 2003).

Figure 1.5: Adult *Carcinus maenas* (Anonymous, 2011). Maximum carapace width is approximately 90mm. Scale bar represents 50mm

C. maenas has a carapace width up to 90mm and inhabits estuarine and marine areas as they are able to tolerate a wide salinity (Winkler et al., 1988) and temperature (Cohen et al., 1995) range as adults. *C. maenas* is omnivorous, feeding on molluscs (Ameyaw-Akumfi and Hughes, 1987), crustaceans (Elner and Hughes, 1978) and polychaetes (Calvez, 1987). As a voracious predator, *C. maenas* are often seen as a pest in non-native habitats due to the adverse impact on local flora and fauna (Glude, 1955; Morgan et al., 1980; Grosholz and Ruiz, 1995). Predation by *C. maenas* is an important factor in shaping the composition in marine benthic communities (Grosholz et al., 2000) and it is an important species in inter-tidal habitats. However, it has also adversely impacted ecosystems, for example, it has been implicated in the collapse of the soft-shell clam *Mya arenaria* in New England, USA (Glude, 1955) and reduced the population size of the scallop, *Argopecten irradians* in Connecticut, USA (Morgan et al., 1980). Yet, whilst there can be a negative impact regarding *C. maenas* as an invasive species associated with it's predatory effects on other species, more positively, *C. maenas* has been utilised as bait and is viable as a commodity product with a small fishery based Europe (Figure 1.6). Capture quantity between 1950-2012 was dominated in the main by Portugal, followed by Spain, France, UK and Ireland (FAO, 2014).

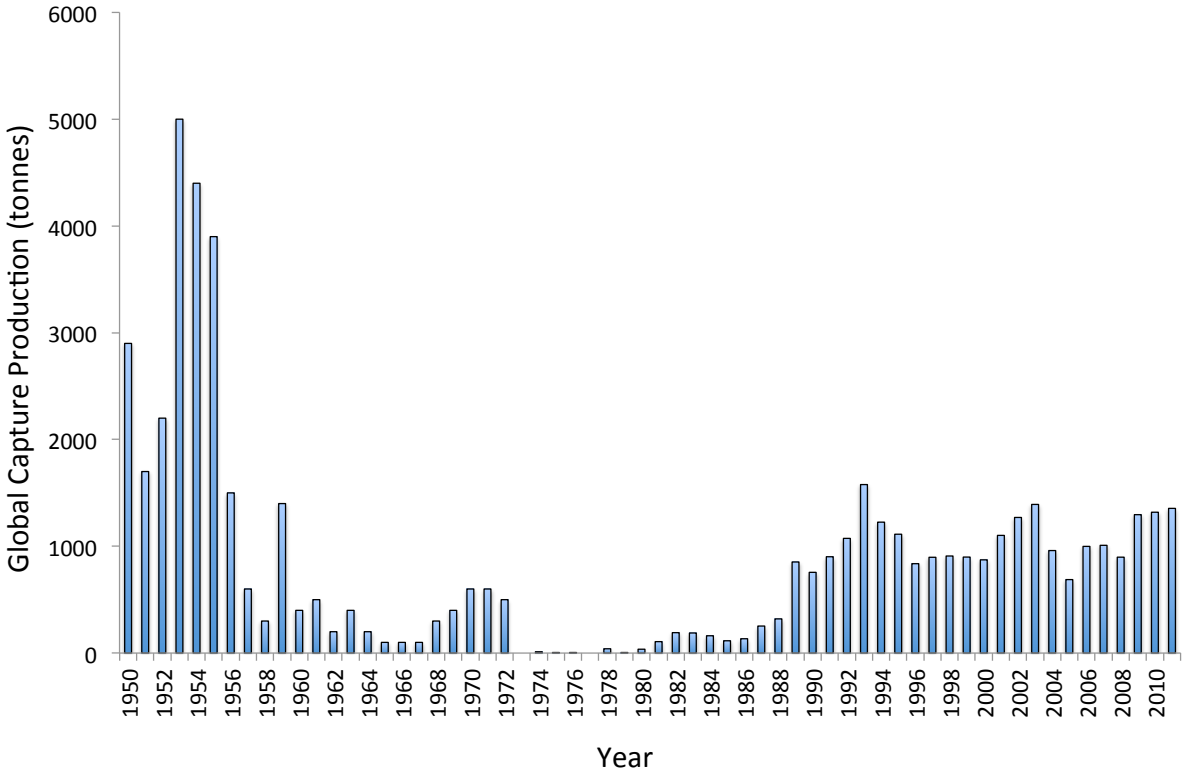


Figure 1.6: Global capture of *Carcinus maenas* between 1950-2012 (FAO, 2014).

Life cycle

Berried females brood their eggs externally which then hatch into plankton as larvae. The larvae can undergo vertical migration in the water column to aid their transport to deeper coastal waters as larvae and return to the intertidal zone to metamorphose to the megalopa stage in which *C. maenas* settle on the benthos and develop as juvenile crabs. In the plankton they undergo four successive zoea stages through ecdysis, after a pre-zoea stage. Larvae of *C. maenas* were first successfully reared in the laboratory (Williams and Naylor, 1967) whereby the pre-zoea stage, the time between hatching and shedding of the embryonic cuticle, was found to be around 3-5 minutes. The following four zoea and megalopa stages lasts for in excess of fifty days at 12°C (Williams and Naylor, 1967). Overall, the life span of *C. maenas* varies between 4-7 years (Yamada and Hauck, 2001).

Disease susceptibility

The motivation for using *C. maenas* as a model species for this research is several-fold. They are ubiquitous and interact with many other species either via predation or as prey. Therefore, the potential for trophic transmission of disease is significant. They are also good indicators of ecosystem health and are known to harbour a broad range of pathogens at any one time, some of which can be pathogenic (Stentiford et al., 2012). These range from viruses, such as baculovirus and herpes-like virus to multicellular parasites such as *Microphallus primas* (helminth). Other examples include species such as the rhizocephalan barnacle *Sacculina carcini* which are known as parasitic castrators of *C. maenas* (Stentiford and Feist, 2005). This pathogen consists of a rootlet system that is present throughout the haemocoel and protrusion of an external egg sac on maturation. Bacterial infections include a rickettsia-like Gram negative α-proteobacteria. This infection is associated with a characteristic milky

appearance of the haemolymph and these bacteria appear to replicate in the fixed phagocytes of the hepatopancreas (Eddy et al., 2007). A wide range of other pathogens associated with *C. maenas* are discussed in Chapter 4.

1.6 Overall Project Aims

The overall aim of this research was to develop a suite of immune biomarkers that could be applied to assess various stress scenarios in two decapod species; *Homarus gammarus* (L.) and *Carcinus maenas* (L.).

Host response to infection

Controlled infection studies were performed to test the host response to a viral pathogen, (WSSV), in *H. gammarus* (Chapter 3) and Gram positive (*Planococcus citrus*) and Gram negative (*Listonella anguillarum*) bacteria in *C. maenas* (Chapter 5) to assess the predictive value of these biomarkers. Further, assessing baseline prevalence of disease in natural communities provides a foundation for assessing any changes in prevalence, (be they seasonal or otherwise), incidence and virulence of any disease outbreaks. In addition, geographically close populations of shore crabs were reported to have distinctly different pathogen assemblages (*pers. comm.* Kelly Bateman). Therefore, cumulative infection was comparatively assessed in two wild populations of *C. maenas* (Chapter 4) to determine the historical pathogen impact on the host using the suite of biomarkers.

Host response to non-biotic stress

Lastly, the toolbox was used to assess non-pathogen related stress in *C. maenas* (Chapter 6). Many decapods are subject to live commercial trading which can incorporate a number of stressors. However, overcrowding was taken as the main stressor under simulated transport and holding conditions to assess the wider application of the immune biomarkers.

2 Experimental Methods

2.1 Introduction

Investigations into *Homarus gammarus* and *Carcinus maenas* examined host response to pathogenic infection and stress. Specifically, immune-related gene transcription was analysed. These genes were chosen and isolated as immune biomarkers as they had the potential to have a wide application as a 'toolbox' to assess current and recent infection in these two species. However, changes in gene expression at a transcriptional level do not represent actual expression of immune proteins. Research to date has produced conflicting results with respect to correlating messenger RNA (mRNA) expression to protein expression. As examples in human health, drug resistant protein expression (MRP1, MRP2 and MRP3) in lung cancer was shown to correlate with the expression of mRNA (Young et al., 2001). Yet proteolytic enzymes (cathepsins) investigated in malignant melanomas did not show a consistent relationship between the transcription and protein expression levels (Frohlich et al., 2001). It may be that a correlation exists between gene transcription and protein expression under particular scenarios, but not under others, which is not unexpected as gene regulation occurs post-transcriptionally (Bustin and Nolan, 2004). At the transcriptional level of investigation no information is provided as to whether the mRNA is translated into a functional protein. Therefore, to provide a more holistic analysis of immune-related processes, other techniques were employed to investigate the host response at other levels of organisation; total haemocyte counts for *C. maenas* and histology and for both *C. maenas* and *H. gammarus*. All histology was processed and read by colleagues in CEFAS (Section 2.5).

A very well established assay, Polymerase Chain Reaction, was used to investigate the target gene transcription. Polymerase Chain Reaction, or PCR, is a method of exponentially amplifying a gene of interest via a thermal cycle with the use of a heat-stable DNA polymerase enzyme, (further explained in the next section). PCR was used in two parts. Firstly, conventional PCR was used to isolate and amplify the immune and endogenous reference target genes which were then sent for sequence confirmation using Sanger sequencing. Secondly, quantitative PCR was used to measure the transcription levels of the immune genes for each of the experimental investigations.

For each sample, numerous stages of processing were necessary before target genes could be isolated either for sequencing or to quantify gene transcription levels (Figure 2.1). As each stage had the potential to introduce error, strict protocols were adhered to. In particular, a set of standards known as the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) (Bustin et al., 2009) were used as a gold standard by which to process all samples.

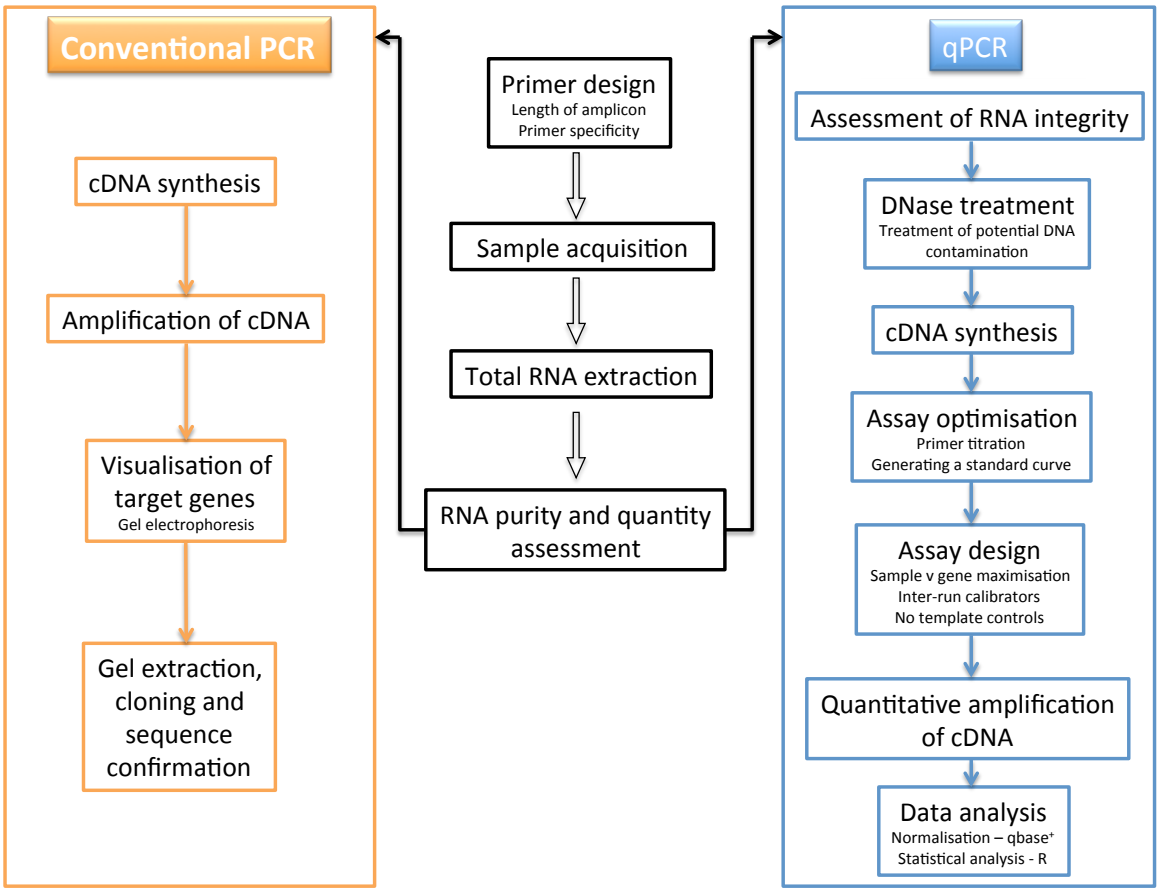


Figure 2.1: Illustration of workflow through conventional and quantitative PCR. All stages were processed according to standards highlighted in the MIQE guidelines (Bustin et al., 2009).

2.2 Background

2.2.1 Brief history

PCR has revolutionised molecular biology. It is a valuable tool in many disciplines ranging from food science (Meyer and Candrian, 1996), clinical diagnostics in both human (Reiss, 1991) and animal (Belak and Ballagi-Pordany, 1993) health, forensic science (Decorte and Cassiman, 1993) and environmental science (Braker et al., 1998). The original invention of PCR has been widely credited to Kary B. Mullis. Mullis discovered the concept of PCR in April 1983, the details of which were described in a short scientific report (Mullis, 1990). Whilst all the components that are required for PCR were available at the time, it was Mullis who realised the concept of using two short oligonucleotide primer sequences complimentary to the two opposing strands of DNA to isolate a given region of the genome and amplify the region between the primers in a repetitive heat cycle. Mullis was subsequently awarded 10,000 US$ from his employers, Cetus Corporation and the Nobel Prize for chemistry in 1993 (Bartlett and Stirling, 2003).

However, a documented earlier account also exists describing the process as ‘repair replication’. The aim of this work by Kleppe et al. (1971) was to develop techniques to synthesise DNA of specific

nucleotide sequences and then replicate these sequences enzymatically to produce a sufficient quantity of templates for future studies. Its likeness to PCR can be seen in the following description by Kleppe et al. (1971); "The DNA duplex would be denatured to form single strands. This denaturation would be carried out in the presence of a sufficiently large excess of the two appropriate primers. Upon cooling, one would hope to obtain two structures, each containing the full length of the template strand appropriately complexed with the primer. DNA polymerase will be added to complete the process of repair replication. Two molecules of the original duplex should result. The whole cycle could be repeated, there being added every time a fresh dose of the enzyme".

One of the early difficulties during this procedure was the need to add DNA polymerase during each cycle. This was a consequence of using DNA polymerase sourced from *Escherichia coli* bacterium. Heating the reagents during the denaturation step to separate the newly synthesised strands of DNA, unfortunately, also rendered the DNA polymerase inactive (Bartlett and Stirling, 2003). This repetitive addition of the enzyme was a very labour-intensive and error-prone process, inevitably adversely affecting the efficiency of the PCR. The timely isolation and purification of a stable DNA polymerase from the thermophilic bacterium *Thermus aquaticus*, or *Taq*, known to live in hot springs (Chien et al., 1976), eliminated the need for repeated addition of the enzyme during each cycle. This made the process receptive to automation. In addition, as this thermostable DNA polymerase was capable of functioning at incubation temperatures of 95°C, the overall performance of the reaction improved considerably in terms of specificity, sensitivity, yield and the length of target genes which could be amplified.

2.2.2 PCR process

The PCR process occurs as a heat cycle consisting of three main stages; denaturation, annealing and extension (Figure 2.2). Prior to commencement of cycling there is an initial five minute denaturation step at 95°C. This is an important step to ensure complete denaturation of the starting DNA template, which is significantly larger than the target of interest that is amplified in subsequent heat cycles and also to activate the DNA polymerase (Smits et al., 2009). This promotes efficient amplification of the target gene. After denaturation to separate the two DNA strands, short (∼20 nucleotides) priming sequences anneal to complimentary base pairs on each separated DNA strand in the 5' to 3' read direction. Hydrogen bonds form between the primers and DNA template and the DNA polymerase, *e.g. Taq*, then binds to this region and begins DNA synthesis using the deoxyribonucleotides that have been previously added to the reaction. This thermal cycle is repeated to achieve exponential amplification of the DNA template (providing there is sufficient quantities of reagents). A final elongation step ensures any remaining single strands of DNA are fully extended (Appendix, Chapter 2, Tables 8.1 and 8.2).

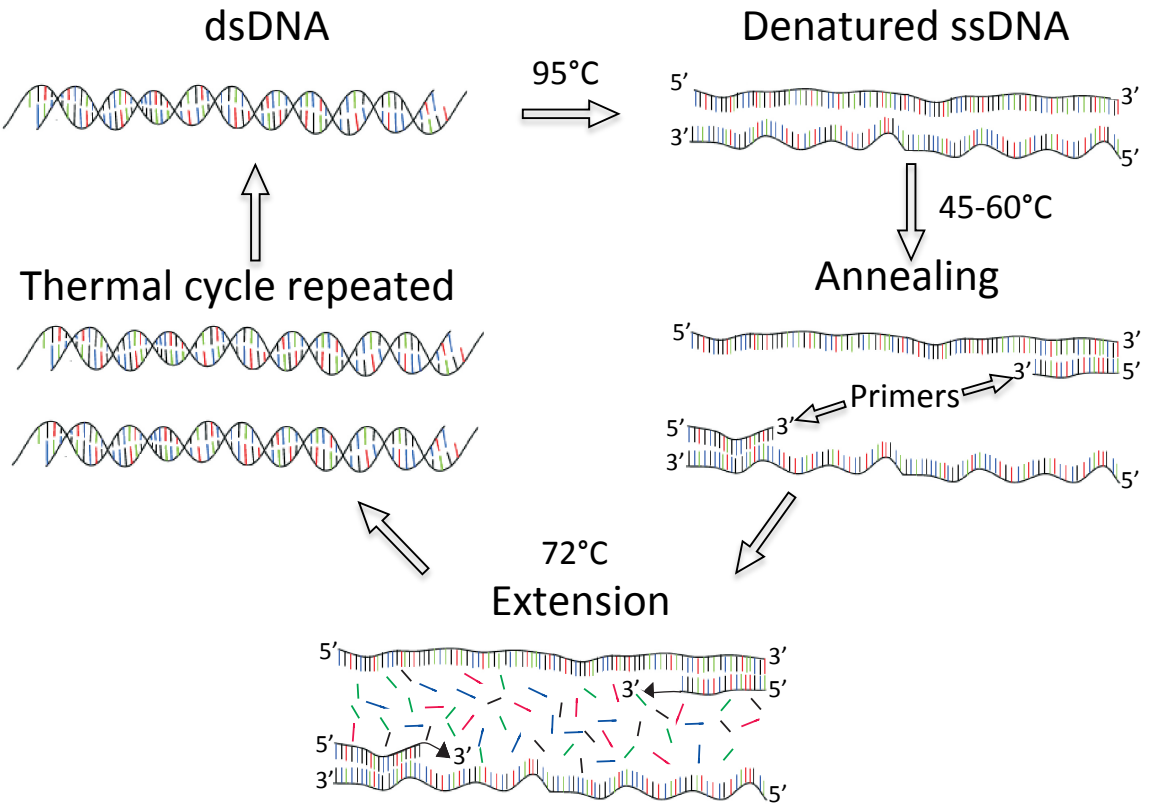


Figure 2.2: PCR schematic illustrating the three main stages of Polymerase Chain Reaction; denaturation of double stranded DNA (dsDNA) to form two single strands of DNA (ssDNA), annealing of the primers to bases on the template strand and extension to form the new DNA strand, resulting in an exponential amplification of the target gene of interest. Image adapted from Universiteit Gent (1999).

Only one strand of DNA exists at the beginning of the reaction when using complimentary DNA (cDNA) (reverse transcribed from mRNA) as a template. In this case, the first heat cycle produces the complimentary DNA strand using the primer sequences added to the reaction. Thereafter, two DNA strands serve as the template for the subsequent synthesis of new DNA strands.

2.2.3 Quantitative PCR

Fluorescence-based quantitative PCR (qPCR) follows the same principles as a conventional PCR assay, but has a much greater sensitivity with the capability to detect and measure very small quantities of nucleic acids (Bustin et al., 2009). Conventional PCR offers the user the opportunity to ask whether a gene transcript is being expressed or not, essentially requiring a 'yes' or 'no' response. Quantitative PCR allows the investigator to ask how much of a particular gene transcript is being expressed.

2.2.3.1 MIQE guidelines

All stages, from sample acquisition to data processing, follow a strict set of guidelines (Bustin et al., 2009) which stipulate the standards required to produce reliable and reproducible data. It is very easy to produce artifactual data if these guidelines are not adhered to, which can of course lead to

misinterpretation of results (Taylor et al., 2010). Therefore, correct preparation and processing of samples is fundamental in producing data in which the investigator can have confidence. Furthermore, these guidelines are designed to promote better experimental practice, consistency and transparency between different laboratories and therefore improve the integrity of the scientific literature (Bustin et al., 2009). That said, the main body of literature reporting data sourced from qPCR assays still does not include the minimum required information as stipulated by the MIQE guidelines. A survey of over 1,700 publications determined that whilst those authors citing the MIQE guidelines showed significant improvement in the technical details of experimental reporting, this remained vastly outnumbered by those publications in which the technical details were insufficient. In addition, reporting of RNA quality was very limited in papers reviewed between 2009-2011 and reporting of normalisation procedures was inadequate in papers reviewed between 2009-2013 (Bustin et al., 2013). This lack of transparency results in a lack of confidence in the results. Key factors highlighted in the MIQE guidelines, such as RNA integrity, normalisation and PCR efficiency of data, are discussed in the relevant sections later in the chapter.

2.2.3.2 Absolute and relative quantification

There are two methods by which quantification can be achieved; absolute or relative. Absolute quantification requires an external standard dilution curve to act as a calibrator by which to measure the gene transcription of unknown samples. This is a method often used to measure viral loads, for example. It requires identical amplification efficiencies of both the standard and unknown samples in both the reverse transcription and subsequent qPCR steps (Pfaffl, 2004) and therefore can be a more challenging assay to perform well. Relative quantification does not require comparison of unknown samples to a calibration curve, but instead compares the transcription of a target gene relative to an Endogenous Reference Gene (ERG). ERGs act as internal controls that are constitutively expressed regardless of experimental conditions (Bustin et al., 2010, 2009; Vandesompele et al., 2002). For the purposes of investigating gene transcription in both *Carcinus maenas* and *Homarus gammarus*, relative quantification was used and measured against the following reference genes; *actin, eef1a, gapdh and tubulin*. However, it has been shown that often reference genes can be differentially expressed (Vandesompele et al., 2002). As a result, the suitability of reference genes must be determined in order to achieve meaningful results.

2.2.3.3 Detection chemistries

There are a variety of fluorescent probes and dyes that can be used to quantify DNA amplification (Bustin and Nolan, 2004). The two main established methods are either sequence-specific DNA binding agents or fluorescent probes and non-sequence specific DNA binding agents (Pfaffl, 2004).

Sequence-specific binding

Sequence-specific fluorescent probes are designed against the target amplicon and many utilise fluorescent resonance energy transfer (FRET) or similar interactions between the fluorophore (which absorbs and emits photons) and quencher (which accepts the energy from a fluorophore) (Bustin and Nolan, 2004). A popular example of this are hydrolysis probes such as Taqman®. Hydrolysis probes contain reporter dyes at the 5' end and quenchers at the 3' end. Fluorescence remains low if the probe remains intact, which results as a consequence of no binding. However, upon complimentary binding, the activity of the *Taq* cleaves the probe at the 5' end and degrades it until the probe is denatured from

the amplicon. The fluorophore and quencher are released into solution during this process, spatially separating them, resulting in an increase in fluorescence.

Non sequence-specific binding

Non-specific detection chemistries, such as intercalating dyes, bind non-covalently to double-stranded DNA (dsDNA) and are stable over a wide range of temperatures (Bustin and Nolan, 2004). For the purpose of investigating gene transcription response in *H. gammarus* and *C. maenas*, an intercalating dye, SYBR® Green was used. During DNA amplification the fluorescent output increases relative to the number of dsDNA products (Figure 2.3). However, as intercalating dyes have the potential to bind to any dsDNA molecule, the specificity is determined entirely by the primers. Although this is no more specific than conventional PCR, melt curves can be used to assess the specificity of the target amplified. This is because the melting temperature of different dsDNA molecules varies according to factors such as GC content and amplicon length, therefore, different gene products can be differentiated according to the melting temperature (Bustin and Nolan, 2004).

In both methods there is a linear correlation between the fluorescent output and DNA concentration and thus the fluorescent signal can be used to calculate the amount of DNA template (Bustin and Nolan, 2004; Wittwer et al., 1997) using either relative or absolute quantification (Figures 2.3 and 2.4). The point at which a fluorescent signal is detected above a background level (threshold) is determined by the number of cycles, known as the C_q (quantification cycle) value and should occur during the exponential phase of the reaction (Figure 2.4).

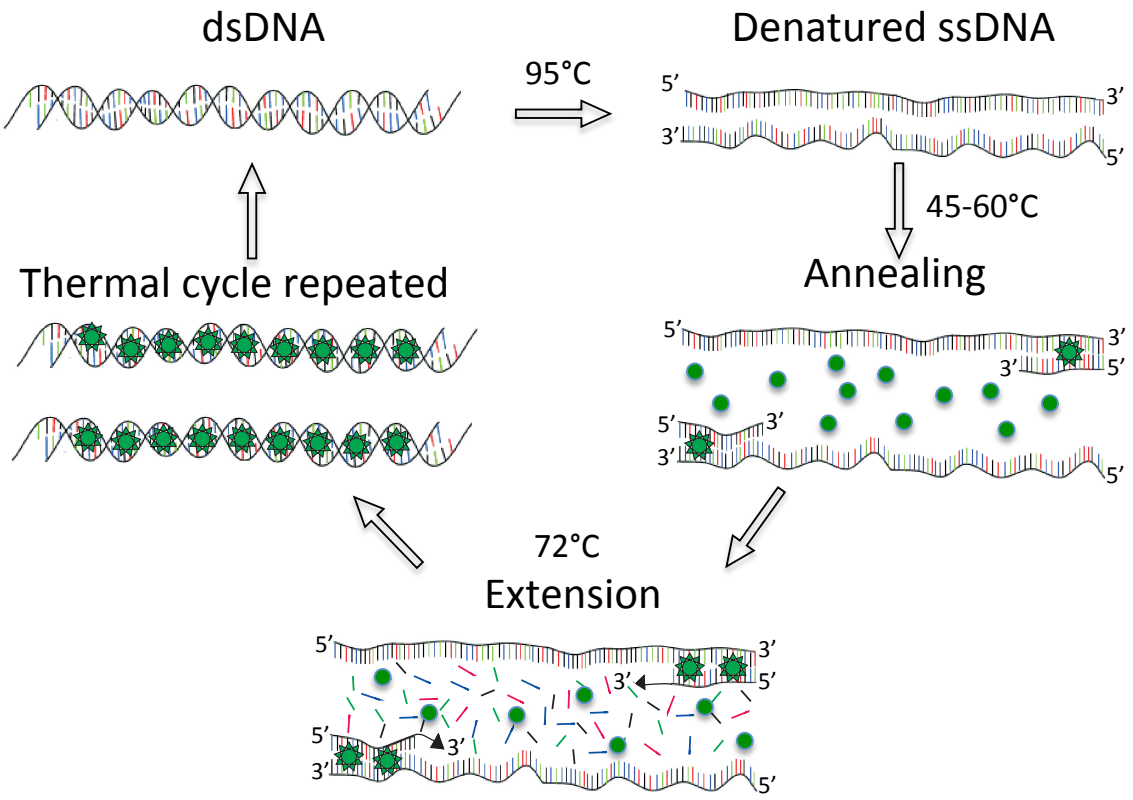


Figure 2.3: qPCR schematic illustrating the increase in fluorescent signal (represented by the green stars) as the primers anneal and extend complimentary to the dsDNA template. The fluorescent signal (green stars) is directly proportional to the quantity of amplified dsDNA above a background fluorescent signal and is also several orders of magnitude greater than the fluorescence emitted from the unbound dye (green circles) (Thermo Scientific, 2013). Image adapted from Universiteit Gent (1999).

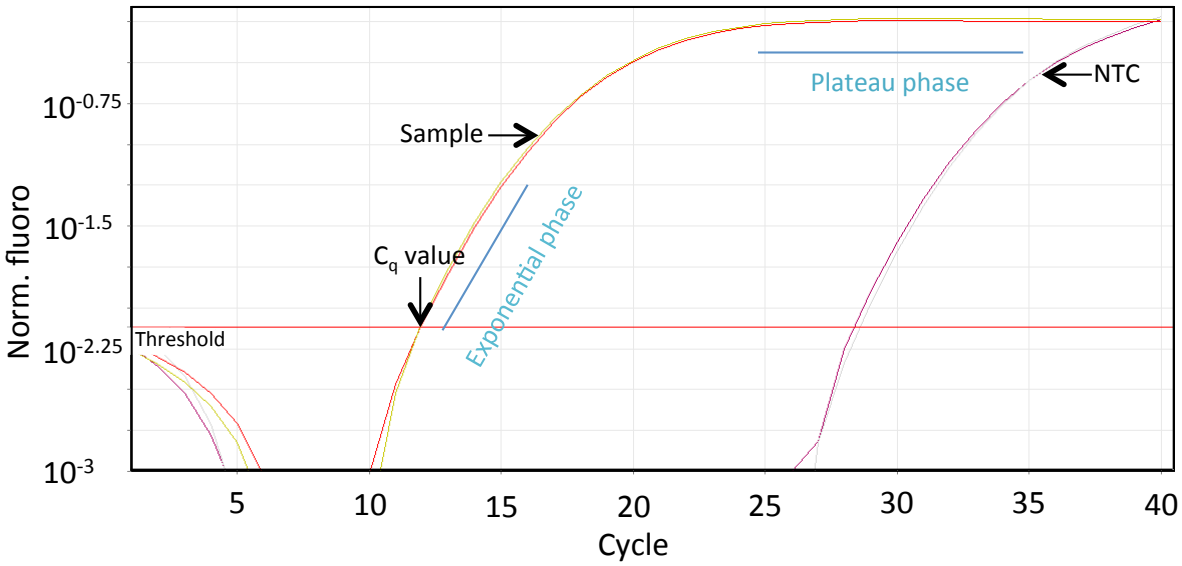


Figure 2.4: Amplification of *tubulin* gene transcript in *Carcinus maenas* determined by the fluorescence (Norm. fluoro.) against the number of qPCR cycles. The C_q value is taken at the point above which fluorescence can be determined above a background level. The reaction is exponential until reagents become limiting and the reaction finally plateaus. NTC represents the No Template Control.

2.2.3.4 Normalisation

Differences in gene transcription are caused either by true biological variation or non-biological variation. Normalisation serves to remove as much non-biological variation as possible. Non-biological variation can be introduced through various stages of sample processing. Some factors contributing to this variation can include different amounts of starting cDNA template, differences in the quality of the cDNA template and differences in reverse transcription and qPCR efficiencies (Vandesompele et al., 2009). It is therefore important to ensure each stage of processing is standardised, for example, ensuring the same concentration of RNA is DNase treated and reverse transcribed into cDNA.

The most common way to normalise data is to use ERGs as internal controls as they are the best markers to capture all of the non-biological variation (Vandesompele et al., 2009). The geometric mean, rather than the arithmetic mean, is calculated, as this better controls for abundance differences between genes as well as any potential outlying C_q values (Vandesompele et al., 2009). Investigations into the expression levels of 10 common reference genes by Vandesompele *et. al.* (2002) determined that using only a single reference gene for normalisation led to erroneous expression differences of between 3-fold in 25% of cases and 6-fold in 10% of cases. Therefore, it is recommended that multiple reference genes are used for normalisation (Bustin et al., 2009).

Reference gene stability

It is also necessary to determine the stability of the ERGs, as previously mentioned, which must be carried out anew prior to each experiment. This is achieved by calculating the ratio of two candidate reference genes. The expression ratio for ideal reference genes, represented by M in geNorm (Hellemans et al., 2007) (software available in qbase$^+$ designed to calculate pairwise comparison of reference gene

stability), would be constant across samples thus resulting in a low M value. An increasing variation of the ratio suggests one or both reference genes are not stably expressed. Alternatively, reference gene stability can also be determined using the Coefficient of Variation (CV) of the samples based on the Normalised Relative Quantities (D'Haene and Hellemans, 2010). Recommended M and CV values can vary from <0.2 and <10% respectively according to (D'Haene et al., 2010) or <0.5 and <25% respectively according to Hellemans *et. al* (2007) for relatively homogeneous samples (*e.g.* cell cultures from the same cell type). For more heterogeneous samples (*e.g.* comparison of cell types or biopsies) the M and CV values can increase to 1 and 50% respectively (Hellemans et al., 2007).

Pairwise variation can be determined for every candidate reference gene and then a Normalising Factor (NF) for the most suitable reference genes can be calculated, which is then used to convert Relative Quantities (RQs) into Normalised Relative Quantities (NRQs) (further described in section 2.4.5). In addition, the minimum number of reference genes that are required to calculate a reliable normalisation factor can be determined. This is achieved by analysing the pairwise variations between the normalisation factors after the inclusion of successive less stably expressed reference genes. A value V is produced, the lowest of which indicates the most stable number of candidate reference genes (Smits et al., 2009).

2.3 Isolation, amplification and sequencing of immune and reference genes using conventional PCR

2.3.1 Primer Design

Primers, short ($\sim$ 20 base pairs) nucleotide sequences used to synthesise the targeted DNA, were designed for the genes of interest or GOI (immune genes) and ERGs that were either not sequenced or whereby only EST (Expressed Sequence Tag) sequences were available (Table 2.1). Genes where the full sequence was known, *carcinin* (Accession number AJ427538) in *C. maenas*, *antimicrobial peptide* (*crustin*) (AJ786653) in *H. gammarus* and *prophenoloxidase* in both *Carcinus maenas* (DN202844) and *Homarus gammarus* (AJ581662), were identified using the EMBL-EBI (European Molecular Biology Laboratory-European Bioinformatics Institute) database and later used for the design of qPCR primers.

Degenerate primers for the remaining GOIs and ERGs were designed using the most conserved regions from alignments of nucleotide sequences from various phylogenetically similar species. Primer analysis, in terms of secondary structures, hairpins, palindromes, GC content and melting temperature, were analysed with both NetPrimer (PREMIER Biosoft, California, USA) and Eurofins MWG Operon (Ebersberg, Germany). NetPrimer was not suitable to analyse degenerate primers.

Table 2.1: *Carcinus maenas* and *Homarus gammarus* nucleotide sequences for the forward and reverse primers also showing the Primer Length (PL), melting temperatures of the primers (T_m) based on the Eurofins MWG Operon algorithm and the Sequence Amplicon Size (SAS). IUB nucleotide codes, used to design the degenerate primers, shown (Cornish-Bowden, 1985).

Carcinus maenas							
Genes	**F Primer**	**PL (bp)**	**T_m (°C)**	**R Primer**	**PL (bp)**	**T_m (°C)**	**SAS (bp)**
peroxinectin	GAGAACAACAACGAGCAGCATC	22	60.3	GCTGAATGTTGAGTGCCACCAG	22	59.4	655
actin	GTCGGYGAYGARGCWCARA	19	58.8	CRTCNGGNARYTCRTANGA	19	55.6	627
eef1a	CGTCGTTCGCCTTTGCCTT	19	58.8	GCTTGGTGGGCGTGAAGAACT	21	61.8	292
gapdh	CNGAYGCNCCNATGTTYGT	19	57.7	ACRTCRTCYTCNGTRTANCC	20	57.3	471
ppia	GAGACCTGCTATGTGACCTGACCTTC	26	66.4	GCTCAGAATCCAGTGAATCCAAGG	24	62.7	547
tubulin	GGNGCNGGNAAYAAYTGG	18	57.1	NGGRAANGGNACCATRTTNAC	21	57.9	495
ubiquitin	CHAATATHCARGAYAARGARG	21	54.3	VGAYTCYTTYTGRATRTTRTARTC	24	56.2	228
Homarus gammarus							
Genes	**F Primer**	**PL (bp)**	**T_m (°C)**	**R Primer**	**PL (bp)**	**T_m (°C)**	**SAS (bp)**
peroxinectin	TCCTCCGTMATGCAGTGGG	19	59.9	TCTGGAASAGATGRTTBGAC	20	55.6	812
actin	GTCGGYGAYGARGCWCARA	19	58.8	CRTCNGGNARYTCRTANGA	19	55.6	627
eef1a	CAGCACRGARCCCAAGTAYTC	21	60.8	AYACATCCTGSAGDGGRAGACG	22	62.7	292
gapdh	CNGAYGCNCCNATGTTYGT	19	57.7	ACRTCRTCYTCNGTRTANCC	20	57.3	471
ppia	ACTTYATGWKHCAGGGWGGMG	21	59.5	CRAADACHACRTGYTTBCCATC	22	58.1	220
tubulin	GGNGCNGGNAAYAAYTGG	18	57.1	NGGRAANGGNACCATRTTNAC	21	57.9	495
ubiquitin	CHAARATHCARGAYAARGARG	21	54.3	VGAYTCYTTYTGRATRTTRTARTC	24	56.2	228

IUB Nucleotide codes											
A ADENINE			C CYTOSINE			G GUANINE			T THYMINE		
Codes	N	V	B	H	D	K	S	W	M	Y	R
Bases	A, C, G, T	G, A, C	G, T, C	A, T, C	G, A, T	G, T	G, C	A, T	A, C	C, T	A, G

2.3.2 Haemolymph and tissue collection and preparation for gene analysis

2.3.2.1 Haemolymph

Haemolymph was aspirated from both *H. gammarus* and *C. maenas* to use for isolation and sequence confirmation of GOIs and ERGs and for analysis of gene transcription pre and post experiments.

The first articulation at the proximal end of the first periopod in an adult *H. gammarus* and the second periopod in *C. maenas* was first cleaned with 70% ethanol. A 1ml haemolymph sample was aspirated using a 25 gauge sterile needle, into a 2ml syringe pre-loaded with 1ml of species-specific anticoagulant (Table 2.2). Haemolymph samples were immediately placed on ice.

Table 2.2: Concentration of component anticoagulant reagents per 250ml of deionised RO (reverse osmosis) water used for *Carcinus maenas* (Söderhäll and Smith, 1983) and *Homarus gammarus* (Hauton and Smith, 2004)

	Carcinus maenas	***Homarus gammarus***
Sodium chloride	6.6g	8.0g
Glucose	4.5g	4.5g
Trisodium citrate	2.2g	2.2g
Citric acid	1.4g	1.4g
EDTA	0.7g	0.7g

Anticoagulant was made using pyrogen-free glassware and filter sterilized using a 0.2μm filter. Haemolymph samples were centrifuged at 3000*g* and 4°C for 7 minutes to form a pellet of cells. Samples not processed immediately were stored at -80°C in 1ml RNAlater® (Life Technologies™, Paisley, UK) after aspirating and discarding the anticoagulant. RNAlater® was added to those samples used only for isolation and sequencing of gene products. Haemolymph samples processed for analysis after experiments were stored in 1-1.5ml of TRI Reagent® (Sigma-Aldrich, Dorset, UK) at -80°C until further processing.

2.3.2.2 Tissue

Tissue was collected for gene transcription analysis only from *H. gammarus*. Whole tails were dissected from Stage IV juveniles, placed in o-ring sealed screw-cap cryovials and placed into liquid nitrogen before transfer to the -80°C freezer. This was to reduce the possibility of RNases degrading host tissue RNA. The experiment on *H. gammarus* involved working with a live virus, White Spot Syndrome Virus (WSSV). As this viral pathogen is listed by the Organization for Animal Health (OIE) as a notifiable disease (OIE, 2009), juvenile *H. gammarus* fed WSSV-positive tissue were processed to TRI-Reagent® in the biologically secure laboratory facilities within CEFAS, Weymouth.

2.3.3 Total RNA isolation and nucleic acid quantification

The extraction efficiency of total RNA is affected by a number of factors including the type and biomass of the sample, sufficient homogenisation of the sample, the physiological state of the animal and the genetic complexity (Bustin et al., 2009). Total RNA was isolated from *H. gammarus* tissue and *H. gammarus* and *C. maenas* haemolymph using theTRI Reagent® (Sigma-Aldrich, Dorset, UK) protocol (Appendix, Chapter 2). Any RNAlater® was aspirated from the microcentrifuge tube containing the haemolymph pellets and discarded prior to the addition of TRI Reagent®. The manufacturers protocol (Sigma-Aldrich, Technical Bulletin T9424) was then followed, adding chloroform and centrifuging to achieve phase separation of total RNA, DNA and proteins, allowing isolation of total RNA. Isopropanol precipitation followed by an ethanol wash resulted in a total RNA pellet for each sample. This was subsequently rehydrated by repeated pipetting in an appropriate volume of DEPC-(diethyl pyrocarbonate) treated Milli-Q® water (Appendix 1.9.1). Milli-Q® water, also known as Type 1 water, is ultrapure water that has been purified using an ion-exchange resin and deionisation.

The quantity and purity of RNA was analysed using Nanodrop (ND-1000 Spectrophotometer, Nanodrop®, Wilmington, USA). To assess the purity of the RNA, UV absorbance ratio's of 260nm and 280nm were assessed. For RNA, a 260:280 absorbance ratio of 2.0 is considered as pure. The other

measure of RNA purity used was the 260:230 ratio which was expected at 2.0-2.2 for RNA (NanoDrop, 2007). Ratio's <2.0 may represent contamination such as proteins, DNA or phenol which can alter the ratio (Bustin et al., 2009). For example, phenol and carbohydrates absorb near 230nm and TRI Reagent® absorbs at 230nm and approximately 270nm. Only those samples with acceptable purity values were processed for downstream analysis. Prior to use, Milli-Q® water was first used to calibrate the NanoDrop®, followed DEPC-treated Milli Q® water (the solution used to rehydrate RNA pellets) as a blank sample.

2.3.4 Determining RNA integrity (qPCR analysis only)

To produce high quality data, an assessment of the quality of RNA is essential. Degradation of RNA can be affected by many factors such as inadequate sample processing, exposure to heat or UV and cleavage by RNA enzymes (Vermeulen et al., 2011). Investigations into the effect of RNA degradation on reference gene stability from biopsy samples taken from ethmoidal and maxillary sinuses in humans, demonstrated that gene-specific variation was greater in degraded samples (Perez-Novo et al., 2005). As well as finding that the stability of reference genes varied in the different tissue types, emphasising the need to assess gene stability anew for each experiment, the authors noted that the reference genes that were highly stable in high quality samples were ranked amongst the most unstable in degraded samples. This inevitably effects data interpretation and highlights the need for proper quality control for all samples.

Although beyond the control of the investigator, it is worth being mindful that RNA of course will degrade *in vivo* in response to environmental stimuli in the process of natural regulation of mRNAs (Doma and Parker, 2007). However, optimising extraction protocols to maintain RNA integrity is something that can be controlled. The integrity of the RNA can then be assessed using an automated capillary electrophesis system. These systems generate a quantity and quality assessment and allow a visual inspection of the RNA integrity. An example of these is the Experion™ (Bio-Rad, Herts, UK) system.

Experion software generates RQI (RNA Quality Indicator) values that indicate the integrity of the RNA through assessment of 18S and 28S ribosomal RNA (Fleige and Pfaffl, 2006) (Figure 2.5). Intact RNA is indicated by a value of 10 and fully degraded RNA represented by a value of 1. Providing RNA integrity information is now a requirement of the MIQE guidelines (Bustin et al., 2009). High RNA integrity is key in obtaining meaningful and reproducible gene expression data from downstream qPCR processing (Fleige and Pfaffl, 2006; Vermeulen et al., 2011). It is also recommended that only samples of similar RQI values should be compared, *i.e.* comparing a sample with an RQI of 4 to a sample with an RQI of 9 would be inappropriate (Bustin et al., 2010). Only samples with high ($\geq$ 7.6) RQI values were compared for qPCR analysis.

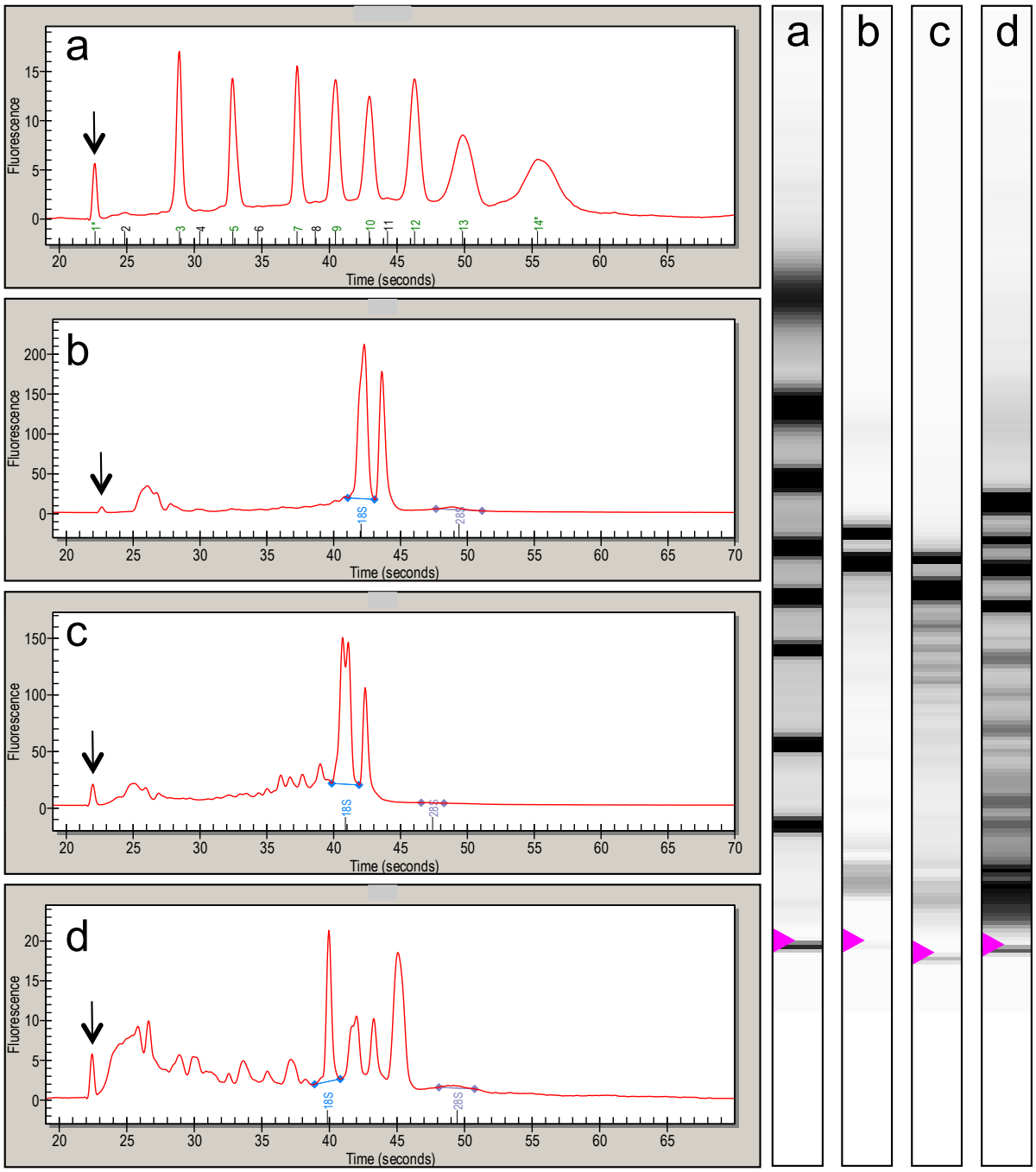


Figure 2.5: Virtual electropherogram (left) and gel (right) images of a) RNA ladder and b-d) RNA products extracted from *Carcinus maenas* and viewed via Experion™ software. The electropherogram shows the 18S (also seen as a dark band on the gel image) and 28S rRNA (ribosomal RNA) peaks. Electropherogram and gel images highlight various stages of RNA degradation; b) high quality (RQI 10), c) medium quality (RQI 7) and d) low quality (RQI 4,9). The pink triangles indicate the lower marker, (also indicated by the arrows on the electropherogram), which is evident at around 21 seconds, followed either by the ladder or sample profiles. The electropherogram profile 'b' was characteristic of all *Carcinus maenas* sampled.

2.3.5 Treatment of potential contaminants (qPCR analysis only)

No RNA extraction method can be described as being free of DNA contamination and so potential contamination is always a concern. It is preferred practice to treat any potential DNA contamination before reverse transcribing the RNA to cDNA. RNA samples for qPCR processing were therefore DNase treated using the TURBO DNA-free™ Kit (Life Technologies™, Paisley, UK) according to the manufacturers protocol (Appendix, Chapter 2). This removed any contaminating DNA through the enzymatic degradation of DNA in solution. This method was designed to remove trace to moderate quantities of DNA contaminants (less than 50μg DNA/ml RNA) to a level that is mathematically insignificant for qPCR (Ambion, 2012).

2.3.6 Complimentary DNA (cDNA) synthesis

Complimentary DNA (cDNA) was synthesized from total RNA using SuperScript II® Reverse Transcriptase for isolating genes for sequencing and SuperScript III® Reverse Transcriptase (Life Technologies™) for samples processed for qPCR, as per the manufacturers protocol (Appendix, Chapter 2). Samples were stored at -20°C until further processing.

2.3.7 Amplification of cDNA products using Polymerase Chain Reaction (PCR)

Reaction volumes of 25μl of the reagents were added to a sterile PCR tube on ice according to the manufacturers protocol (Taq PCR Handbook February 2008, Qiagen, Crawley, UK) (Appendix Chapter 2, Table **??**). Amplification of the cDNA products for both GOIs and ERGs were optimised (Appendix, Chapter 2, Tables 8.1 and 8.2). For *eef1a, gapdh, peroxinectin, ppia, tubulin* and *ubiquitin* genes between 0.2μl and 1.5μl of magnesium chloride 25mM was also added to the reaction mix (Appendix, Chapter 2, Table **??**) to improve the sensitivity and specificity of the PCR assay (Bustin and Nolan, 2004) and the volume of MilliQ® water adjusted accordingly to maintain a reaction volume of 25μl. For *eef1a* gene in *Carcinus maenas* and *eef1a, ppia* and *peroxinectin* in *Homarus gammarus*, 5x Q-solution, which changes the melting behaviour of DNA, was also added in view of previous difficulty in amplifying the gene product. Unfortunately, both *ubiquitin* and *ppia* were eliminated as candidate reference genes due to ongoing difficulty in successfully amplifying these gene products in both *C. maenas* and *H. gammarus*.

2.3.7.1 Visualising amplified cDNA products

PCR products were run at 76V on a 30ml 1% agarose gel made with 1 x TAE (Tris-acetate-EDTA) buffer containing 0.001mg/ml ethidium bromide. The PCR products were visualized using a Gel Doc™ UV transilluminator (Bio-Rad Laboratories, Inc., Hemel Hempstead, UK).

2.3.8 Confirming nucleotide sequences

2.3.8.1 Extraction and cloning of amplified gene products

PCR products of the correct amplicon size were extracted from agarose gel and purified using a QIAquick® Gel Extraction Kit (QIAGEN) according to the manufacturers protocol (Appendix, Chapter 2). Purified products were then inserted into a pGEM®-T Easy Vector consistent with the manufacturers protocol (Technical Manual TM042, Promega, Southampton, UK,). The ligated products were then incorporated into *Escherichia coli* bacterial strain JM109 High Efficiency Competent

Cells. After 1.5 hours of incubation at 37°C with shaking at 150rpm the cells were centrifuged at 1000*g* for 10 minutes forming a pellet at the distal end of the tube. The top 500μl were discarded and the cells resuspended in solution through repeated pipetting. The remaining 500μl solution was added to duplicate agar plates containing 100μg/ml of ampicillin with 100μl IPTG (100mM) and 20μl X-Gal (50mg/ml) added per plate. These cells were then incubated at 37°C for 16-24 hours. Combinations of blue (negative) and white (positive) colonies grew on the agar plates. The white colonies, which generally contained the inserts, were picked and confirmed to contain the correct size amplicon using PCR with M13 primers (Appendix, Chapter 2, Table 8.4). Colony PCR products were visualised on a 1% agarose gel to confirm the presence of the vector and insert.

2.3.8.2 Extraction and purification of plasmid DNA

Colonies were cultured overnight in 5ml Luria Broth containing 100μg/ml of ampicillin. Plasmids containing the insert were subsequently extracted from the bacterial cells using a QIAprep® Spin Miniprep Kit (QIAGEN, Ltd., Crawley, UK) following the manufacturers protocol (Appendix, Chapter 2). The purified plasmids were then sent for Sanger sequencing by Source BioScience LifeSciences Plc. (Oxford, UK).

2.3.8.3 Sequence analysis

Sanger sequencing (also referred to as dideoxy or chain termination sequencing) is a method used to identify the sequence of nucleotides in a DNA strand. This is achieved by amplifying the target sequence using the appropriate priming sequences, DNA polymerase, the normal nucleotides (dNTPs) and dideoxynucleotides (ddNTPs), but at comparably much lower concentrations (about one hundredth the concentration of the normal dNTPs). Dideoxynucleotides have been modified to contain a hydrogen group rather than a hydroxyl group on the 3' carbon. When incorporated into a sequence, this modification prevents further addition of nucleotides, thus terminating the chain. The DNA polymerase synthesises the DNA using the dNTPs, but on occasion a ddNTP will be integrated in the sequence. This results in a combination of different length amplicons which have been terminated randomly along the sequence depending on where the ddNTP was incorporated. By fluorescently labelling the four modified dideoxynucleotides, (ddATP, ddCTP, ddGTP and ddTTP), with a different coloured dye, the final product can be visualised in a single lane on a gel. As the different dyes fluoresce at different wavelengths it can be read by a laser to determine the different nucleotides and hence the sequence of the target gene.

Sequences were uploaded into Geneious Basic^{TM}5.3.4 (Biomatters Ltd., Auckland, New Zealand) and the plasmid sequences were identified and removed. Sequences were aligned and analysed using either Geneious Pro^{TM}5.3.4 (Biomatters Ltd., Auckland, New Zealand) or 4Peaks Version 1.7.1 (Mekentosj, Amsterdam, The Netherlands), then manually edited to omit or correct any errors in nucleotide bases between sequences. Nucleotide BLAST (Basic Local Alignment Search Tool) searches were conducted using the NCBI (National Centre for Biotechnology Information) server to confirm the correct order of the sequence (5' to 3' read direction) and to compare sequence identity against those of the same gene identified in other species.

2.4 Quantitative Polymerase Chain Reaction (qPCR)

2.4.1 Assay optimisation

Following the MIQE guidelines (Bustin et al., 2009, 2010) for qPCR standards, assay optimisation, including appropriate design of qPCR primers, validation of priming conditions and limits of detection, were carried out as follows.

2.4.1.1 qPCR primer design

Gene-specific primer pairs were designed for immune and reference genes for both *Homarus gammarus* and *Carcinus maenas* and viral genes for WSSV using Primer Express® software (Applied Biosystems, Life Technologies, California, USA) (Table 2.3). The recommended guidelines were followed;

1. The nucleotide length of each primer should be between 9 and 40 bases.
2. The annealing temperature of the primers should be between 58°C-60°C and the annealing temperature of the primer pairs should have less than a 2°C difference.
3. The amplicon length should be 50-150 nucleotides to prevent the formation of secondary structures and to produce efficient amplification.
4. GC content should be maintained within 30-80% with no more than two GC residues within the last five nucleotides at the 3′ end.

Only those primer pairs meeting the Applied Biosystems recommended guidelines for primer design were used.

Table 2.3: qPCR primer pairings with Primer Length (PL), annealing temperature (NetPrimer), and Sequence Amplicon Size (SAS) for all immune and endogenous reference genes for *Carcinus maenas* and *Homarus gammarus* and for all viral genes (WSSV).

Genes	Forward primer	PL (bp)	T_m°C	Reverse primer	PL (bp)	T_m°C	SAS (bp)
Carcinus maenas							
actin	TCACCAACTGGGACGACATG	20	59.36	GCCACACGGAGCTCATTGTAG	21	60.58	64
carcinin	TGAAGCACCACGTGTGCAA	19	59.51	CGGGTCTGCGATGTCTAATAATAA	24	60.78	65
eef1a	CTGGATGAGGCAACACAACAA	21	59.13	GTCTGACCACTGAGGGTTGATG	22	59.1	64
gapdh	TGCGGTGTGAATTTGGAGAA	20	59.63	GGTGCAGGAGGCATTGGAT	19	60.25	63
peroxinectin	CAACACAACCGTCTAGCCAAAG	22	60.11	AGAGGATCTCGTCGGTCCAA	20	59.19	65
prophenoloxidase	CTTGCCCGCTCCATTCC	17	59.29	GCGAGGCGATGACTCTTGAC	20	60.43	62
tubulin	CTCTGCTTCTTTGCGGATAACA	22	60.07	CACTACACGGAAGGTGCTGAAC	22	59.78	63
Homarus gammarus							
actin	ACTTGGCTGGACGTGACCTTA	21	59.66	GAAGGTGTAGCCACGCTCAGT	21	59.37	65
crustin	CACACCTGCAAGGGTCCAAT	20	60.35	GGGCAGCGGGTGGAA	15	58.14	62
eef1a	GGCTCGTTTTGAGGAAATCA	20	57.75	CAGGATTGTAACCAACCTTCTT	22	55.68	65
gapdh	GCTAAGGCTGTTGGCAAGGT	20	59.57	GGAAAGCCATGCCAGTAAGC	20	59.88	61
peroxinectin	CACTGAGGGTTGATGCTCTTGA	22	60.10	CTGACAGCCATACACACCATCTG	23	60.39	77
prophenoloxidase	TGAAGCTAGTCGAGGCATTGAC	22	59.74	CCAAATGGGTGAGGCGTAAT	20	59.86	65
tubulin	GCCCTGAAGACAATCGCATT	20	59.99	TGAACTTGTAGACTCCGTCCTTGA	24	60.78	67
White Spot Syndrome Virus (WSSV)							
ie1	GAGGCAGTCAGGAAGAGTGATCTAG	25	59.99	ATTCTTCGATGCCTCCATTGAG	22	60.88	94
dnapol	GTGGCTGACATGTTGGAAATACTT	24	60.22	TTCGAGCCGTAAAGAGTTTGC	21	60.04	69
vp28	GACATTCAAGGTGTGGAACAACAC	24	60.93	GCCAACTTCATCCTCATCAATAGA	24	60.30	65

Known viral gene sequences for *ie1* (HM778020 and from Wang et al., 2007), *dnapol* (AF365882) and *vp28* (HM484379) were used only for qPCR primer design.

2.4.1.2 Primer optimisation

To determine the minimum concentration of forward and reverse primers to achieve the optimum qPCR assay conditions, a variety of primer combinations (Table 2.4 and Figure 2.6) were tested with a suitable cDNA template (Nolan, 2004). This process was repeated for each immune, reference and viral gene (Appendix, Chapter 2, Figure 8.5). The most suitable combinations of primers were then used for further downstream processing. The final reaction volumes were 23μl.

Table 2.4: Primer combinations for titration. The most appropriate combinations of forward and reverse primers were chosen on a gene by gene basis.

Primers	**Primer concentration (nM)**								
Forward	50	300	900	50	300	900	50	300	900
Reverse	50	50	50	300	300	300	900	900	900

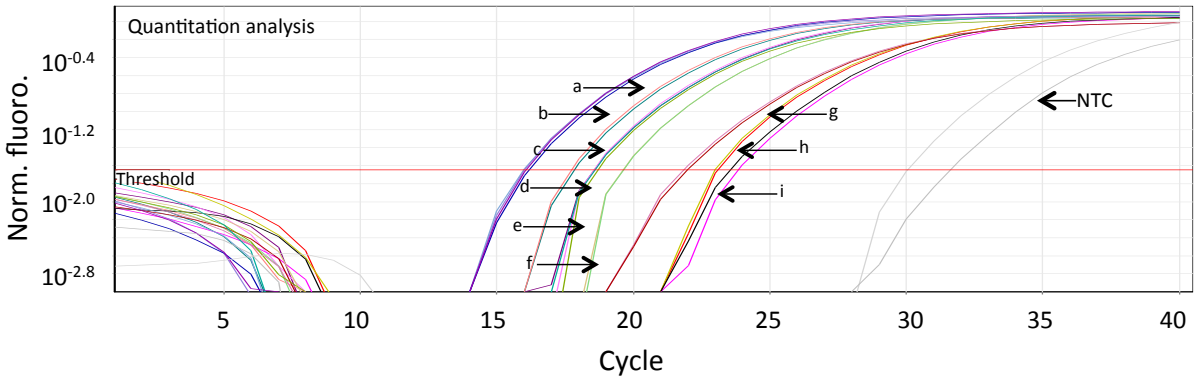


Figure 2.6: Example of primer titration of *gapdh* in *Homarus gammarus*. NTC represents the no template control sample. The following represent the forward and reverse primer concentrations respectively; a) 900nM, 900nM, b) 300nM, 900nM c) 50nM, 900nM d) 50nM, 300nM e) 900nM, 300nM f) 300nM, 300nM g) 900nM, 50nM h) 50nM 50nM i) 300nM, 50nM.

2.4.1.3 Generating a standard curve

In order to accurately quantify PCR efficiency, as well as define the limits of detection (the lowest concentration of cDNA template at which 95% of samples are detected (Bustin et al., 2009)), a calibration or standard curve was generated for each gene. A high PCR efficiency ($\geq$95%) is an essential contributing factor to the development of a robust and reproducible assay (Bustin et al., 2009). Typically, standard curves are created through a serial ten-fold dilution of cDNA. The replicate C_q values for each dilution are plotted against the log of the starting concentration and a best-fit linear regression line is fitted to the data (Lewis and Maughan, 2004). Three orders of magnitude should be used for the minimum standard curve, but ideally it should be over five or six dilutions (Bustin et al., 2009).

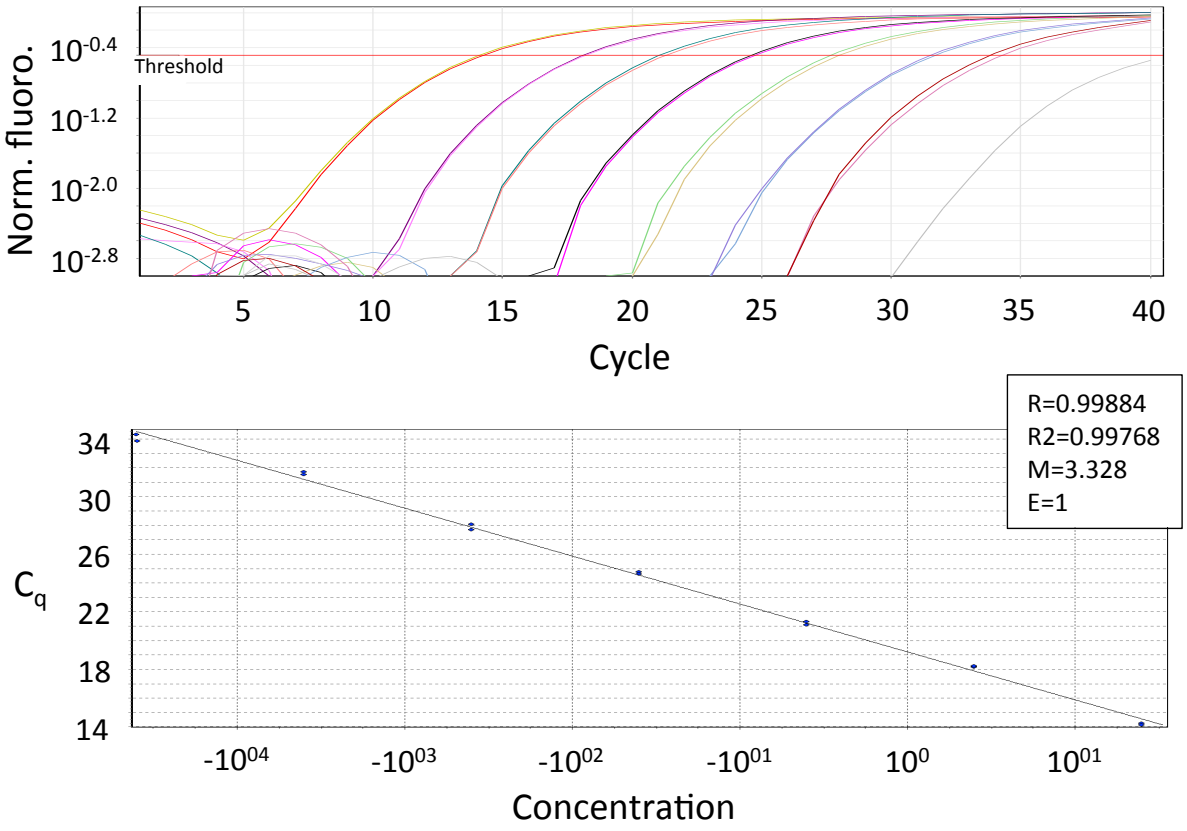


Figure 2.7: Example of a standard curve generated from a dilution series for *carcinin* (top image). Norm. Fluoro. respresents normalized fluorescence against the number of PCR cycles. A regression line is fitted to the C_q values (bottom image). The correlation coefficient, R, represents the goodness of fit of the regression line with the actual data, M represents the slope and E indicates the efficiency of the PCR reaction, i.e. 1. represents 100% efficiency.

Ideally, the dilution series should result in curves that are equally spaced at 3.3 cycles apart (Figure 2.7 top image). Inaccurate dilutions or contamination can result in anomalous dilution curves (Lewis and Maughan, 2004). A standard curve was generated for each gene and the slope of the standard curve was then used to determine the amplification efficiency using the following equation (Hellemans et al., 2007);

$$E = 10^{-1/slope} - 1 \tag{2.1}$$

The amplification efficiency (E) from each gene (Appendix, Chapter 2, Table 8.6) was later used to convert the logarithmic C_q values into Normalised Relative Quantities (NRQ).

2.4.2 qPCR assay design

There are two main experimental designs for relative quantification; sample maximisation or gene maximisation. Sample maximisation is designed to run as many samples as possible within a single run of the same gene. Therefore, different genes should be run in separate runs. Alternatively, gene maximisation analyses multiple genes within a single run, spreading samples across runs if necessary (Hellemans et al., 2007). As the focus for the work presented herein was to compare the gene transcription levels between samples, the sample maximisation approach was used for all investigations.

All reactions consisted of 25μl of Precision MasterMix containing SYBR® Green (Primerdesign Ltd., Southampton, UK), 4μl cDNA template, volumes of forward and reverse primers (Appendix, Chapter 2, Table 8.5) and Milli-Q water to a final volume of 50μl. A volume of 23μl was then pipetted into each duplicate strip tube. All qPCR runs were performed on the Corbett Research Rotor-Gene 3000^{TM} 72-well rotor using clear, 0.1ml strip tubes and caps (QIAGEN). This required multiple runs of the same gene, thus it was necessary to include Inter-Run Calibrators (IRCs). These were cDNA samples of the same template repeated in each run within a particular gene. This allowed for a calibration against the technical run-to-run differences between samples. All runs also included No Template Controls (NTCs) to assess for contamination and ascertain whether primer-diming had resulted in positive C_q values (Bustin et al., 2010). The qPCR thermal cycling is seen in Table 2.5 below.

Table 2.5: The denaturation, annealing and melt curve cycle for all samples processed for qPCR analysis.

Cycling	°C	Time	Number of cycles
Initial denaturation	95	10 mins	x1
Denaturation	95	10s	x40
Annealing (not acquiring SYBR® Green)	60	40s	
Annealing (acquiring SYBR® Green)	60	20s	
Melt curve	72	Ramp of 1°C every 5s to 95°C	

2.4.3 qPCR data analysis including qbase$^+$ workflow

2.4.3.1 Overview

The qbase$^+$ software was used to normalise and calibrate the raw C_q values into data ready for statistical analysis (Hellemans et al., 2007). Calculating changes in the transcription of target genes is based on the $\Delta\Delta C_q$ model (Livak and Schmittgen, 2001) which normalises with a single reference gene. The difference in C_q values between a target and reference gene is calculated and the differences in the ΔC_q values between samples are compared directly.

$$NRQ = 2^{\Delta\Delta C_q} \tag{2.2}$$

This model assumes 100% PCR efficiency, denoted by the 2 for the E of the exponential function in Equation 2.2, indicating that the PCR product doubles with each cycle (Bustin et al., 2009). To ensure accuracy using this method, the PCR efficiencies of both the genes must be comparable. This model has subsequently been adjusted to account for differences in the PCR efficiencies between the target and reference genes (Equation 2.3) (Pfaffl, 2001). (NRQ represents Normalised Relative Quantities, *goi* refers to gene of interest and *ref* refers to the Endogenous Reference Genes).

$$NRQ = \frac{E_{goi}^{\Delta C_q, goi}}{E_{ref}^{\Delta C_q, ref}} \tag{2.3}$$

Further development of this model (Equation 2.4) now accounts for the use of stably expressed multiple reference genes, as advocated by the MIQE guidelines, for improved normalisation (Hellemans et al., 2007) which forms the basis of the qbase$^+$ workflow. Note that this model uses the geometric mean rather than arithmetic mean to better account for abundance differences and outlying C_q values.

$$NRQ = \frac{E_{goi}^{\Delta C_q, goi}}{f\sqrt{\prod_0^f E_{ref_0}^{\Delta C_q, ref_0}}} \tag{2.4}$$

2.4.3.2 Workflow

The qbase$^+$ workflow (Hellemans et al., 2007), which was used to process raw C_q values into interpretable data, involved four major stages; calculation of the mean C_q values of the replicates (Equation 2.5), transformation of the logarithmic C_q values into a linear relative quantity using gene specific PCR efficiencies (Equation 2.6), normalisation (Equation 2.9) using a sample specific normalisation factor (Equation 2.7) and finally calibration (Equation 2.10) using qPCR run and gene specific calibration factors (Equation 2.8).

$$\overline{C_q}_{jkl} = \frac{\sum_{i=1}^{n} C_{q_{ijkl}}}{n} \tag{2.5}$$

The mean C_q values were calculated for replicates of a given sample and gene combination (jk) within a given run (l).

$$RQ_{jkl} = E_{jl}^{\Delta C_q}{}_{jkl} \tag{2.6}$$

The means were then transformed from the logarithmic C_q values into a linear scale or Relative Quantity (RQ) using the gene specific PCR efficiciency E_{jl}.

$$NF_k = f\sqrt{\prod_{p=1}^{f} RQ_{pk}} \tag{2.7}$$

The Normalising Factor (NF) was calculated for a given sample k based on the Relative Quantities (RQs) of the reference genes p.

$$CF_{jl} = c\sqrt{\prod_{m=1}^{c} NRQ_{jlm}} \tag{2.8}$$

The Calibration Factor (CF) for gene j in run l was calculated based upon the Normalised Relative Quantities (NRQs) of the Inter-Run Calibrators (IRCs) m.

$$NRQ_{jk} = \frac{RQ_{jk}}{NF_k} \tag{2.9}$$

Division of the Relative Quantities (RQ) by the Normalising Factor (NF) generated Normalised Relative Quantities (NRQ).

$$CNRQ_{jkl} = \frac{NRQ_{jkl}}{CF_{jl}} \tag{2.10}$$

Division of the Normalised Relative Quantities (NRQ) by the Calibration Factor (CF) generated the Calibrated Normalised Relative Quantities (CNRQ) which were then used for statistical analysis.

All calibrated data was exported from qbase$^+$ and statistical analysis was performed using R (RStudio©, Inc., Version 0.97.316, 2009-2012).

2.4.3.3 Quality control

Prior to processing any data to CNRQ values, it was important to visually assess the raw C_q values to determine whether they were of sufficient quality to incorporate in the analysis. This included assessment of reference gene stability (previously discussed in section 2.2.3), assessment of duplicate sample variability, no template controls, melt curves and dissimilar normalisation factors.

Duplicate samples

The use of duplicate samples serves to increase confidence in the results and the quality of the assay can be measured by assessing what proportion of duplicate samples fall within a defined limit (which can be adjusted by the user). Duplicate variation is determined by the ΔC_q or standard deviation. The standard deviation increases by 0.1 units for every 0.141 cycle difference between duplicates (D'Haene and Hellemans, 2010). The recommended threshold for duplicate variability is <0.5 C_q which correlates to a standard deviation of 0.35 (D'Haene and Hellemans, 2010; D'Haene et al., 2010).

No template controls

The no template controls should produce no C_q values. However, there are occasionally contamination or primer dimer issues with samples, as previously discussed. Providing there is a minimum difference of 5 C_q values between the No Template Control (NTC) and the closest sample, these issues can be ignored. This is because a value of 5 C_q represents a 32-fold or 3% difference between samples, which is considered to be below the technical error for replicates (D'Haene et al., 2010). The C_q value between the NTC and closest sample can be adjusted by the user in qbase$^+$. However, for all experiments, the limit was maintained at 5 C_q.

Melt curves

Melt curves, generated when using intercalating dyes, need to be assessed as a guide to determine whether the target sequence has been amplified. There are four main types of melt curves (Figure 2.8); a sharp peak is characteristic of a sequence specific reaction; non-specific reactions are often related to multiple peaks; broader, lower peaks at a lower temperature are often caused by primer diming and can be more pronounced with lower cDNA concentrations and finally a shoulder peak close to the main peak may be evident in longer amplicons with multiple melting domains of varying GC content (D'Haene and Hellemans, 2010). Assessment of melting curves for all samples was therefore essential to ensure only relevant samples were included for analysis.

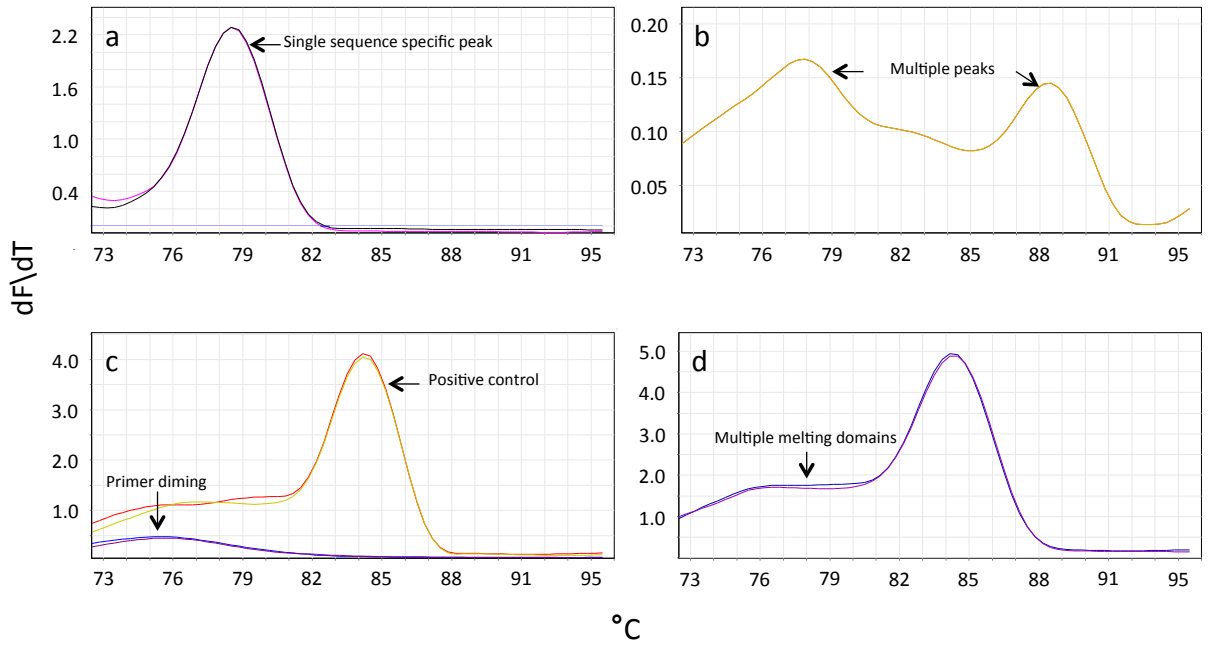


Figure 2.8: Illustration of variations in melt curves. a) Sequence specific (*eef1a* in *Homarus gammarus*), b) non-specific multiple peaks (*dnapol* in *Homarus gammarus*), c) primer diming (no template control) relative to a postive control (*vp28* in penaeid tissue) and d) multiple melting domains indicated by the shoulder peak preceding the dominant peak (*prophenoloxidase* in *Carcinus maenas*).

Normalisation factors

Ideally, the normalisation factors should be similar between samples. This was calculated from the geometric mean of the reference genes (Equation 2.7) and acts as an internal control to account for non-biological variation in gene transcription. A 2- to 3-fold difference is generally considered acceptable and within the experimental variation. Differences greater than this maybe due to differences in the quality of the sample, or possible differences in the amount of starting material, or stability of candidate ERGs.

2.5 Histopathology

All samples processed for histopathology were prepared and fixed according to CEFAS protocols (quality assurance ISO 9001). All *Carcinus maenas* were anaesthetised prior to dissection by placing on ice for a minimum of 30 minutes. In *Carcinus maenas*, muscle, gill, gonad, nerve, heart and hepatopancreatic tissue samples were dissected for histology. Hepatopancreas was also fixed in glutaraldehyde for Electro-Microscopy (EM) investigation if required. In *Homarus gammarus* the Stage IV juveniles were too small to excise specific tissue from. Therefore, the entire cephalothorax was dissected for histological processing by colleagues in CEFAS. All tissue samples were initially fixed in Davidsons Seawater fixative (Appendix, Chapter 2) for 24 hours and then transferred to 70% IMS (Industrial Methylated Spirit) for storage prior to further processing by CEFAS colleagues.

All histology was processed according to CEFAS standard operating procedures (Cefas, 2013). Fixed tissue samples were processed (dehydrated and infiltrated with paraffin wax) in a vacuum infiltration

processor. The tissue samples were then embedded in heated wax. Once cooled and hardened, wax blocks containing the fixed tissue samples were sectioned at 3-5μm thickness using a rotary microtome, floated onto glass slides and then dried on a hotplate to allow evaporation of water. The slides were deparaffinised, rehydrated and then stained with haematoxylin and eosin (H&E). Images were analysed by light microscopy (Nikon Eclipse E800) and captured using Lucia^{TM} Screen Measurement System (Nikon Instruments, United Kingdom). An example of normal histology, for reference, from a selection of *Carcinus maenas* tissue, is observed in the Appendix, Chapter 4, Figure 8.21.

2.6 Total Haemocyte Quantification

A quantitative analysis of total haemocytes pre- and post controlled infection studies was performed for *Carcinus maenas*. A volume of 0.1ml of haemolymph was aspirated from each crab into a 1ml sterile syringe pre-filled with 0.1ml of 20% seawater formalin (Appendix, Chapter 2).

The diluted haemolymph was mixed by gentle pipetting and a sub-sample placed onto a Neubauer haemocytometer taking care to completely cover the counting chamber under the cover slip and fill the 0.1mm chamber depth. The total number of haemocytes were counted in five of the large squares within the counting area.

Each of these squares measures a volume of 0.004mm^3 equating to 0.004μl. Total haemocytes were calculated per millilitre of haemolymph and adjusted for the 1:1 dilution using the following equation:

$$\left(\frac{\left(\frac{THC}{5}\right)\times 1000\mu l}{0.004\mu l}\right)\times 2 = THC ml^{-1} \tag{2.11}$$

2.7 Bacterial Load Quantification

The standard plate count method (Reynolds, 2013) was used to quantify the bacterial load of both Gram negative and Gram positive bacteria in *Carcinus maenas* as well as the background bacterial load in seawater. This method was used for quantifying live bacteria only. A dilution series was established using sterile marine saline (pH 7.4) (Appendix, Chapter 2) in order to accurately count the Colony Forming Units (CFU). A sterile 1ml pre-filled syringe of marine saline (0.1 ml) was used to aspirate 0.1ml of haemolymph from each crab. After gently mixing with a pipette, dilutions of haemolymph:marine saline were set up as follows: 1:1, 1:200, 1:2,000, 1:200,000, 1:2,000,000. A volume of 20μl of each dilutent was aseptically added to each duplicate plate, evenly spread and left for 48 hours at room temperature. Thereafter, the bacterial colonies were counted and the CFU ml^{-1} calculated.

3 Dissecting the response to White Spot Syndrome Virus in the European lobster, *Homarus gammarus* (L.)

Chapter Abstract

White spot syndrome virus (WSSV) causes high mortality in decapod crustaceans, notably in penaeid shrimps. Relatively free international movement of aquaculture animals and products has seen the range of WSSV extend globally from initial occurrences in Asia. WSSV is now listed as a notifiable disease by the World Organisation for Animal Health (OIE). Recently, European legislation (Directive 2006/88/EC) has deemed all decapod crustaceans to be potentially susceptible to infection. The wide host susceptibility range reported in the literature is supported when exposure to the virus occurs at European ambient conditions. However, work by CEFAS (Centre for Environment, Fisheries and Aquaculture Science) has demonstrated that the relative susceptibility to disease varies widely between host taxa. The immune genes, *crustin* (an antimicrobial peptide), *peroxinectin* (an opsonin and cell-adhesive molecule) and the zymogen *prophenoloxidase* (cleaved to phenoloxidase involved in the downstream production of melanin) were chosen as a 'toolbox' of biomarkers to investigate the host response to this significant viral pathogen in an economically valuable decapod crustacean, the European lobster *Homarus gammarus*. Concurrently, the progression of viral infection was quantified using the temporal expression of viral genes; *ie1, dnapol* and *vp28*. No viral gene expression was evident, indicating a lack of infection by WSSV. However, a host response in the form of an up-regulation of *crustin* transcription ($p<0.001$) was observed in the treatment group compared with the control group.

3.1 White Spot Syndrome Virus

Viruses occur throughout the biosphere and have been discovered to be the most abundant organisms in the oceans. Studies have revealed that a milliliter of seawater contains 10^4 to 10^8 virus-like particles (Wilcox and Fuhrman, 1994), with estimates of 10^{23} viral infections occurring in the oceans each second (Suttle, 2007). Therefore, with the ability to cause mortalities across a wide range of marine hosts, viruses have the capacity to not only influence the composition of marine communities, but adversely impact the sustainability of important fisheries.

3.1.1 Economic significance of White Spot Syndrome Virus

A major viral pathogen affecting a wide host range within decapod crustaceans is White Spot Syndrome Virus (WSSV). This viral pathogen was highlighted in the early 1990s as the causative agent

of high mortalities in aquaculture, most notably penaeid shrimps. Infection with the WSSV pathogen results in up to 100% mortality within 3-10 days in commercial shrimp farms (Lightner, 1996) from the onset of clinical signs (Lightner, 2011). This has resulted in huge economic losses to the shrimp farming industry over the previous two decades (Lightner, 1996).

3.1.1.1 Changes in the global food market

The global significance of WSSV relates to the growth in the trade of aquaculture products over the previous five decades. This has been heavily influenced by changes in the composition of the human diet, which is generally considered to be associated with the economical development of a nation. Global food consumption has increased by almost 400kcal per person per day between 1970 and 2000, which has been influenced by a shift towards an increase in processed foods during this time (Kearney, 2010). These changes in diet composition have been facilitated by changes to the infrastructure that supply the demand for a changing food market in terms of trading agreements between nations, supply chains and technology (Anderson, 2010).

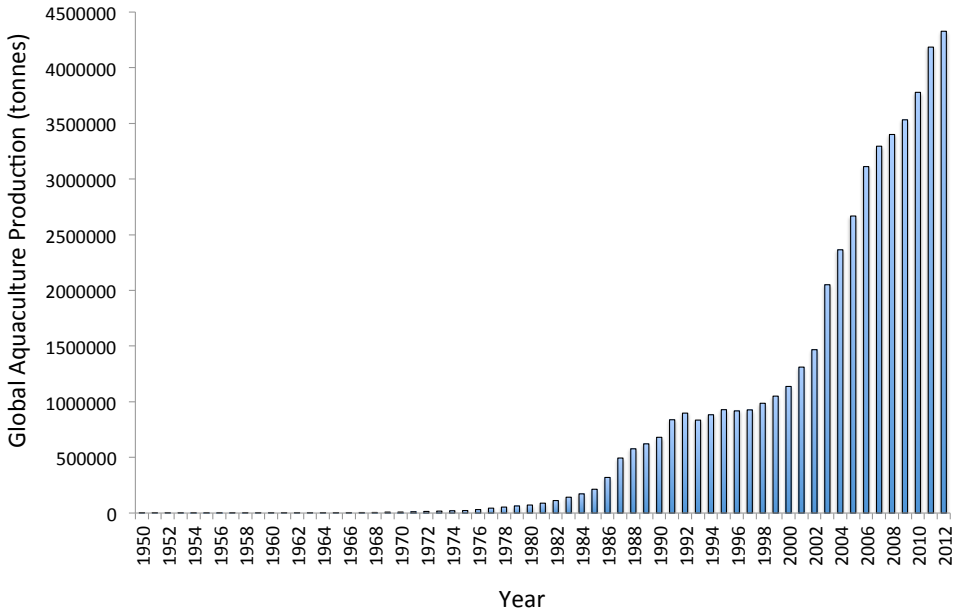


Figure 3.1: Global production of penaeid aquaculture from 1950-2012 (FAO, 2014)

The increase in demand for protein from live and frozen aquaculture products has seen a large increase in penaeid aquaculture production since the 1970's (Figure 3.1). In fact, aquaculture has been recognised as the fastest growing food sector over the previous 50 years contributing over 50 million tonnes, worth US$100 billion over this period (Bostock et al., 2010). Future estimates suggest over 50% of aquatic food products are to be supplied by aquaculture by 2015 (Bostock et al., 2010). Within the aquaculture sector, penaeids form a large proportion, contributing to in excess of 3 million tonnes, worth approximately US$12 billion (data for 2006; http://www.fao.org), the largest producers by volume of which are the Asian sub-continent, supplying 89% of global aquaculture (Bostock et al., 2010).

In 1992, at the beginning of the emergence of WSSV, farmed crustaceans, 90% of which were farmed shrimp, were reported to be valued around US$6.6 billion (Primavera, 1997). Economic losses have been estimated to be in the region of US$4-6 billion in Asia alone and more than US$1 billion in the US

between 1992 and 2001 (Rout et al., 2007). In fact, global cumulative losses have been estimated to exceed US$10 billion since it's emergence (OIE, 2009).

3.1.1.2 Contributing factors affecting the spread of WSSV

The international movement of live and frozen stock has primarily been responsible for the geographical spread of WSSV (Walker and Mohan, 2009). With Asia providing almost 90% of global penaeid aquaculture production, (penaeids historically being a major source of WSSV), it is widely recognised that there have been major deficiencies in the management of aquatic animal health (Walker and Mohan, 2009). Inadequate international and local quarantine inspection capabilities, illegal trading in unscreened stock as well as substitution of unscreened stock for Specific Pathogen Free (SPF) stock also presents an ongoing high risk for the international spread of WSSV (Walker et al., 2011). As well as the movement of products through international trade, the wide host range has also been implicated in contributing to the wide-spread geographical range of WSSV (Table 3.1) (Chang et al., 2011).

Table 3.1: Overview of species range of WSSV investigations in a variety of affected species groups.

Host group	Example reference
>18 Cultured and penaeid shrimp	Wongteerasupaya *et al.,* 1996 Durand *et al.,* 1997 Lu *et al.,* 1997 Chou *et al.,* 1998 Lightner *et al.,* 1998 Park *et al.,* 1998
8 Caridean species	Sahul-Hameed *et al.,* 2000 Shi *et al.,* 2000 Pramod-Kiran *et al.,* 2002
7 Lobster species	Chang *et al.,* 1998 Rajendron *et al.,* 1999
7 Crayfish species	Wang *et al.,* 1998 Corbel *et al.,* 2001 Jirvanichpaisal *et al.,* 2001 Edgerton, 2004 Jirvanichpaisal *et al.,* 2004
38 Crab species	Lo *et al.,* 1996 Kanchanaphum *et al.,* 1998 Sahul-Hameed *et al.,* 2001 Sahul-Hameed *et al.,* 2003
6 Non-decapod species	Supamattaya *et al.,* 1998 Otta *et al.,* 1999 Hossain *et al.,* 2001
Chaetognaths and Rotifera	Yan *et al.,* 2004 Ramirez-Douriet *et al.,* 2005 Yan *et al.,* 2007
Polychaete worms	Supak *et al.,* 2005 Vijayan *et al.,* 2005
Aquatic insect larvae	Lo *et al.,* 1996 Flegel, 1997 Ramirez-Douriet *et al.,* 2005

3.1.1.3 Legislative changes in response to WSSV emergence

The increasing geographical range of reported cases since the 1990s has resulted in WSD (White Spot Disease, caused by WSSV), being listed as a notifiable disease by the World Organization for Animal Health (OIE) (OIE, 2009). In addition, European legislation has listed all decapod crustaceans as potentially susceptible to WSSV (European Union, 2006). This amounts to in excess of 20,000 species, but with the current literature supporting 67 species as susceptible (Bateman et al., 2012b). All European member states are required to report occurrences of the disease and conduct specific health measures designed to limit, prevent and contain the spread of disease as well as ensuring sanitary conditions for the international trade of animals and their products (Walker and Mohan, 2009). Furthermore, WSD has been listed as a non-exotic disease in European legislation (EC Directive 2006/88) (European Union, 2006). This is based on anecdotal evidence of WSD in Southern Europe in the late 1990s (Stentiford and Lightner, 2011). The addition of WSD to European legislation recognises its significance in causing such large economic losses over nearly 30 years.

3.1.1.4 A European perspective

In terms of Europe, whilst there is a only a limited aquaculture industry for penaeids, there is a large coastal and off-shore crustacean fishery rivalling some fin-fish species in terms of value and production (Stentiford and Lightner, 2011). The EC Directive 2006/88 recognises the historical impact of WSSV in terms of broad host range and geographical distribution and the potential for WSSV to become established within either cultured or wild crustacean stocks within Europe.

Yet, whilst the potential for WSSV to adversely affect European crustacean fisheries has been recognised through this legislation, some high risk practices still remain. A small scale study of frozen shrimp, (imported for human consumption), within the UK supermarket industry confirmed the presence of WSSV via PCR (Bateman et al., 2012a). Prior investigations into the transmissiblilty of WSSV via frozen commodities has proved positive, resulting in 100% mortality of *Litopennaeus vannamei* after either injection of WSSV (Hasson et al., 2006; Durand et al., 2000) or *per os* administration of WSSV from imported infected shrimp (Durand et al., 2000). Anecdotal evidence indicates shrimp intended for human consumption are alternatively being used for angling bait (Oidtmann and Stentiford, 2011). This potentially provides a direct link between infected penaeids sourced via international trade, to wild crustacean populations within Europe and the UK (Oidtmann and Stentiford, 2011).

Recent work by CEFAS (Centre for Environment, Fisheries and Aquaculture Science) supports the wide range of potential host species when exposure to the virus occurs at European ambient conditions. However, the relative susceptibility to disease is highly variable between host taxa. Host susceptibility, as determined by EFSA (European Food Safety Authority), is based on four criteria (Algers et al., 2008);

A) Evidence of replication or growth of the organism.
B) Presence of a viable organism.
C) Presence of specific clinico-pathological changes.
D) Specific location of the pathogen within the host.

Based on these four criteria, there are three defined categories for susceptibility. Type 1 (high) which

includes peneaids, but also European freshwater species *Austropotamobius pallipes* (White claw crayfish) and *Pacifasticus leniusculus* (Signal crayfish) and one estuarine species *Eriocheir sinensis* (Chinese mitten crab), Type 2 (medium) including *Homarus gammarus* (European lobster), *Nephrops norvegicus* (Norway lobster) and *Cancer pagurus* (Edible crab) and Type 3 (low) including *Carcinus maenas* (shore crab) (Bateman et al., 2012b). The low-level susceptibility to disease reported for the European shore crab, *Carcinus maenas* (L.) is consistent with previous research that demonstrated a greater resistance to WSSV infection in *C. maenas* compared with other European decapod crustaceans (Table 3.2) (Corbel et al., 2001).

Table 3.2: Mortality of a range of European decapod crustaceans experimentally infected with WSSV via injection (Corbel et al., 2001).

Species	Mortality (%)	Time to mortality (days)
Liocarcinus depurator (L.)	100	8
Liocarcinus puber (L.)	100	9
Cancer pagarus (L.)	100	21
Carcinus maenas (L.)	10	4
Astacus leptodactylus (E.)	71	18
Orconectes limosus (R.)	91	20
Palaemon adspersus (R.)	100	7
Scyllarus arctus (L.)	91	14

Whilst the time to mortality was comparatively quicker in *C. maenas* (Table 3.2), overall mortality was markedly lower in relation to other decapod crustaceans. This curious finding has, as yet, not been fully explored.

The largest body of research to date has focused on infectivity studies within commercially valuable penaeid species. Whilst this research has provided a great deal of understanding regarding functional genomics in WSSV, the molecular mechanisms of infection remain relatively poorly understood. Furthermore, factors contributing to variability in the pathogenicity of this virus across different taxa have yet to be fully investigated. In particular, the European lobster, *Hommarus gammarus*, a commercially important species in terms of the UK and European fishery (Smith et al., 1998; Lizárraga-Cubedo et al., 2003; Triantafyllidis et al., 2005), is known to be moderately susceptible to infection with WSSV from feeding trials (Bateman et al., 2012b). WSSV was found to successfully infect and replicate in *H. gammarus* within 10 days post feeding of a single ration of WSSV-positive penaeid tissue. However, little is know regarding the host immune response of *H. gammarus* to infection with WSSV. Interestingly, no other viral pathogens have been described in this species or in homarid lobsters in general. Therefore, the susceptibility of *H. gammarus* to WSSV presented an opportunity to investigate, not only the viral transcription of WSSV, but also the immune competence of the host.

3.1.2 Pathogenesis of WSSV

3.1.2.1 Gross pathology in penaeid shrimps

Most of the described pathogenesis of WSSV infection has been based on penaeid species. Therefore, penaeids will be used as a model by which to describe the clinical signs of WSSV infection.

Infection is characterised by a rapid replication cycle and systemic infection. The replication cycle at 25°C is approximately 20 hours (EURL, 2008). The main clinical symptoms seen and from which the name of the virus is derived, are white spots on the inner surface of the cuticle (Wang et al., 1995) caused by the presence of calcium deposits approximately 0.5-2.0mm in diameter (Figure 3.2). In addition, the cuticle is often seen to be loose (Lightner, 2011).

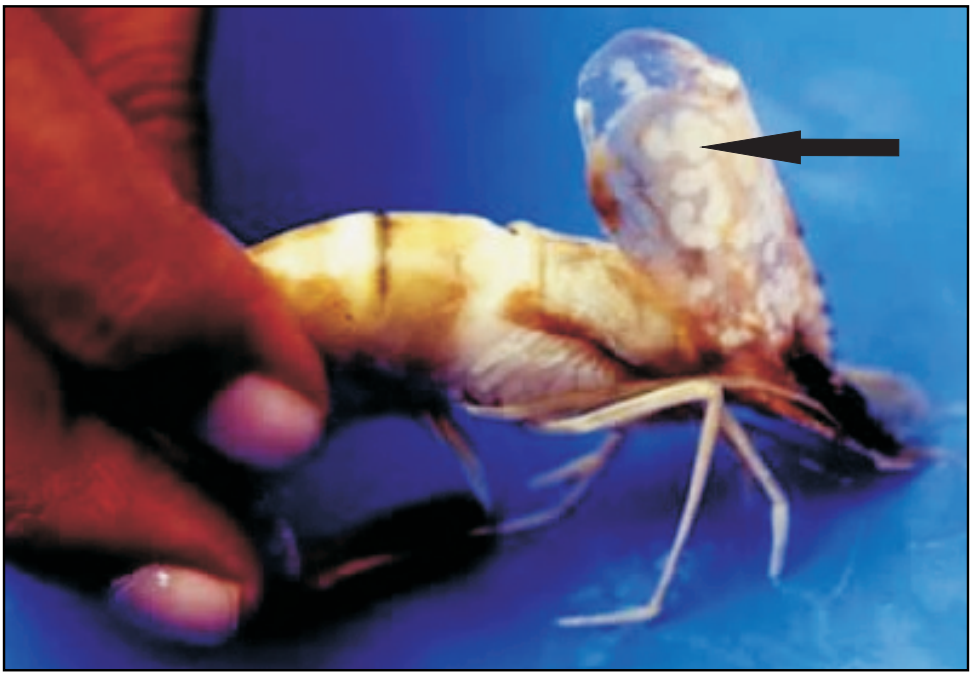


Figure 3.2: Gross pathology in penaeid shrimp include white calcium deposits (indicated by arrow) seen on the loose cuticle (Lotz, 2012).

There is also a high degree of colour variation, mainly a red/pink discoloration. Other general signs, although not definitively diagnostic, include an increase in lethargy, a rapid reduction in food intake and movement of moribund shrimp to the water surface and edges of the pond or tank in aquaculture farms, which commonly attract shrimp eating birds (Lightner, 2011).

Gross pathology in other crustacean species are less well documented, but are thought to include similar general symptoms seen in penaeid shrimp. However, observing white spots under the cuticle may be unlikely due to a thicker carapace in some of the comparatively larger crustacean species.

3.1.2.2 Histology

In penaeid shrimp, the connective and epithelial cells of the cuticle are commonly infected, but other cells, such as the hematopoietic tissues, antennal gland epithelium, lymphoid organ sheath and myocardial phagocytes can also become infected. Intranuclear inclusion bodies in hypertrophied nuclei are commonly identified in histological preparations during the acute phase of infection with WSSV (Lightner, 2011) (Figure 3.3).

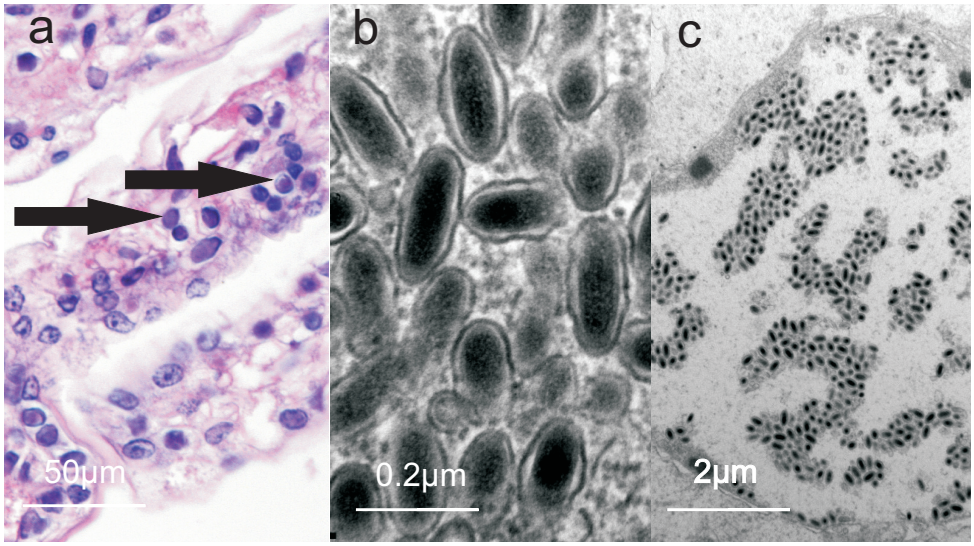


Figure 3.3: a). H&E stain of hypertrophied epithelial gill cells of WSSV infection in *Pennaeus vannamei* (arrows), (Scale 50μm). b). TEM image of longitudinal and cross section of WSSV virions in *P. vannamei* (Scale 0.2μm). c). TEM image of WSSV infected *P. vannamei* epithelial cell nucleus of the cuticle. (Scale 2μm) (EURL, 2008).

However, early developing intranuclear inclusion bodies (Figure 3.3c) may easily be confused with IHHNV (Infectious Hypodermal and Hematopoietic Necrosis Virus) infection due to similar resemblance. Differential diagnosis is easier during the late stage of infection as WSSV inclusion bodies are generally larger and more fully developed. Also, WSSV infected nuclei usually contain a single inclusion body. Nevertheless, a second diagnostic method, such as PCR (polymerase chain reaction) or ISH (insitu hybridization) with a DIG-labelled probe, is recommended to make a definitive diagnosis (Lightner, 2011).

3.1.2.3 Genetic description of WSSV

WSSV, once known as White Spot Bacilliform Virus, is a non-occluded, bacilliform, enveloped and circular double-stranded DNA virus (Lan et al., 2006) with an adenine/thymine content of 59% which is homogeneously distributed in the genome (Escobedo-Bonilla et al., 2008; van Hulten and Vlak, 2001). Around 300kbp in size (van Hulten et al., 2001a; Yang et al., 2001), WSSV was, until recently, considered one of the largest animal viruses (Filee and Chandler, 2008). Discoveries of seawater viruses with much larger genomes are now known; *Megavirus chilensis* (1.259 Mb) (Arslan et al., 2011), *Pandoravirus salinus* (>2.5 Mb) and *Pandoravirus dulcis* (>1.9 Mb) (Philippe et al., 2013).

WSSV has been completely sequenced and found to have three known isolates (van Hulten et al., 2001a; Chou et al., 1995; Chen et al., 2002). The genome size varies between these isolates; 305107bp (Yang et al., 2001) (GenBank Accession number AF332093), 292967bp (van Hulten et al., 2001a) (GenBank Accession number AF369029) and 307287bp (Chen et al., 2002) (GenBank Accession number AF440570), mostly due to several small insertions and one large deletion (Chen et al., 2002) in the regions between the DNA polymerase and the protein kinase genes (Walker and Mohan, 2009). The identity of nucleotide sequences between these isolates is 99.32% (Marks, 2005).

Originally, WSSV was commonly defined as an unassigned member of the *Baculoviridae* family (Francki et al., 1991) due to it's morphogenesis and nuclear replication (Durand et al., 1997). However, due to

its large size and lack of significant gene homology with baculoviruses and unique morphology this was subsequently reviewed. Genetic analysis has since defined WSSV as the only member within a new viral family (*Nimaviridae*) (Mayo, 2002) and genus (*Whispovirus*) (Mayo, 2002; Tsai et al., 2000) as approved by the International Committee on Taxonomy of Viruses (ICTV) (Mayo, 2002).

3.1.2.4 Morphological description of WSSV

Morphologically, WSSV has been described as ellipsoid in shape with a long tail-like structure at one extremity in the mature virion which can sometimes be seen in negatively stained electron micrographs (Durand et al., 1996, 1997; Wongteerasupaya et al., 1995). The size of the virion is variable (210-420nm x 70-167nm) (Figure 3.4) (Cesar et al., 1998; Chang et al., 1996) and has a tightly contained nucleocapsid (180-420nm x 54-85nm). These virions are protected through complex folding and assembly of macromolecules to ensure successful delivery of the viral genomes into the host cell. The nucleocapsid surface itself is comprised of fifteen distinct, vertical helices, with each helix composed of 13-15 capsomers around 8nm in diameter (Huang et al., 2001; Sanchez-Paz, 2010).

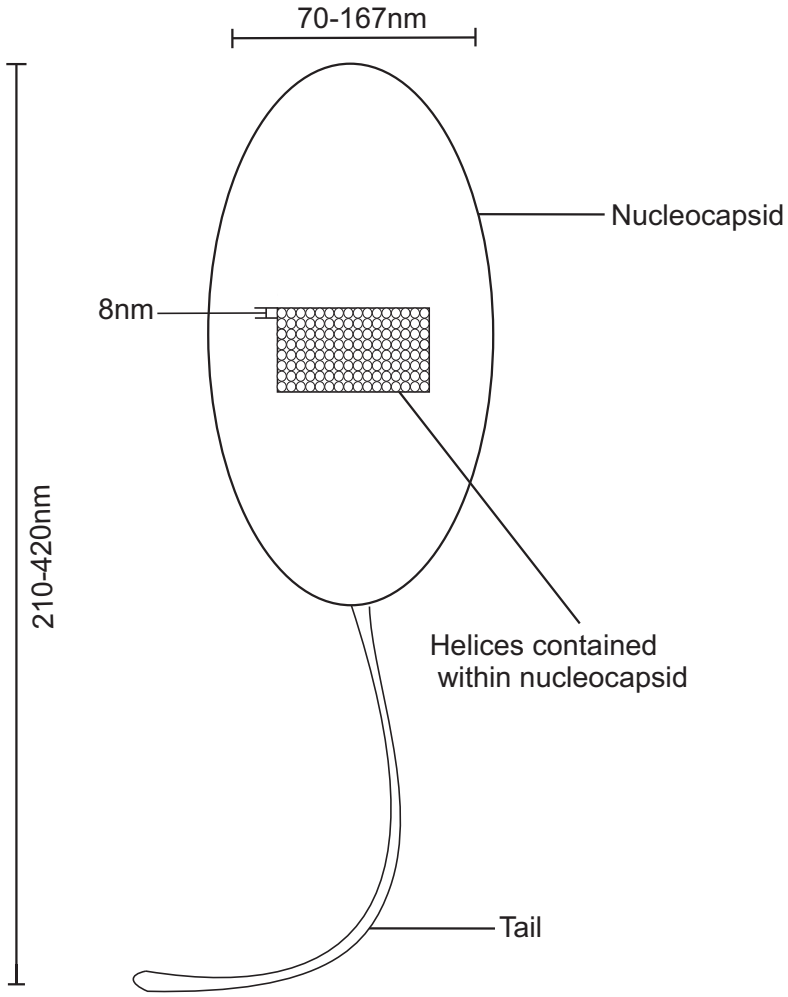


Figure 3.4: Schematic of a mature White Spot Syndrome Virus (WSSV) virion (from Sanchez-Paz, 2010)

3.2 Viral genes

Consistent with other dsDNA viruses, WSSV transcribes its genes in a temporal manner. Significant genes involved in important stages of the viral replication cycle were chosen and can be defined into three main temporal categories; immediate early (IE) and early (E) genes, which are expressed prior to viral DNA replication and late (L) genes which are expressed after viral DNA replication (Sanchez-Paz, 2010).

3.2.1 *ie1*

There are at least 16 identified immediately early genes (Li et al., 2009), however, *ie1* is the most highly expressed non-structural WSSV gene, suggesting it plays a crucial role in WSSV infection (Wang et al., 2008). Immediately early genes often encode for regulatory proteins involved in viral infection and an important function of these genes is to control host or viral gene expression (Li et al., 2009). The transcription of genes, such as *ie1* (also known as ICP11), is independent of *de novo* viral protein synthesis. This suggests that during viral infection, gene transcription is very likely driven only by host cell machinery and is insensitive to protein synthesis inhibitors (Lin et al., 2011; Liu et al., 2005). This is likely to be a very important contributing factor in determining host species range (Liu et al., 2005).

ie1 belongs to the zinc finger protein family and has been shown to have two distinguishable domains. The N-domain has specifically been shown to exhibit transactivation activity while the C-domain is responsible for DNA-binding activity (Liu et al., 2008). In terms of its DNA-binding activity, *ie1* has been reported to act as a DNA mimic by binding directly to the site of histone proteins (Wang et al., 2008). Histones are essential for packaging and ordering DNA into structural units called nucleosomes (Grunstein, 1990). *ie1* can bind to at least three core histone proteins; H2A, H2B and H3. This subsequently prevents host DNA from binding to these proteins. Specifically, *ie1* binds to the γhistone H2A.x that represents approximately 2-25% of total H2A histone expressed in cells. Histone H2A is required for maintaining genomic stability and once activated will accumulate in the nucleus at the site of double stranded breaks where it is involved in repair (Fillingham et al., 2006; Fernandez-Capetillo et al., 2004; Downs et al., 2000; Foster and Downs, 2005). Accumulation of γhistone H2A.x has been reported in the cytoplasm of WSSV-infected cells rather than the nucleus, which has been suggested to be the result of the DNA-binding action of *ie1* to this protein (Wang et al., 2008). Therefore, it seems probable that part of the function of *ie1* is to disrupt nucleosome assembly in the host cell.

3.2.2 *dnapol*

dnapol, defined as an early gene in terms of temporal transcription, is critical in determining the level of genomic replication. It is recognized through three highly conserved regions; Exo I, II and III and shows overall very low homology with other DNA polymerases (22-24%). Additionally, when compared with other viral DNA polymerases, *dnapol* from WSSV is much larger (7056 nucleotides compared with 2739-3732 nucleotides) (Chen, 2002).

The initiator region commonly starts with an adenine flanked either side by pyrimidines (Martins et al., 1994; Nikolov and Burley, 1997). The major transcriptional start point for *dnapol*, 27 nucleotides downstream from the TATA-box, is the A in CAGT motif (Chen, 2002), known to be one of the arthropod initiator motifs (Cherbas and Cherbas, 1993). In fact, 76.9% of the structural WSSV genes

also have this initiator motif (Tsai et al., 2004). Both the TATA-box and initiator sequence are key components of the RNA Polymerase II promotor, which recognizes the TATA-box, initiator sequence or a combination of both (Nikolov and Burley, 1997; Martins et al., 1994; Young, 1991). There is some suggestion that *dnapol* transcription could also be mediated by host RNA polymerase II, which occurs in insect baculoviruses (Chen, 2002).

3.2.3 *vp28*

The role of *vp28*, defined as a late gene, involves attachment to and penetration into the host cell (Yi et al., 2004). *vp28* is thought to be located on the surface of the virus particle as an integral membrane protein (Robalino et al., 2006) suggesting it plays a crucial role in systemic infection (van Hulten et al., 2001b). *vp28* contains hydrophobic regions that may function as an anchor to the envelope or possibly be involved in the formation of homo- or heterodimers (van Hulten et al., 2002). The multiple glycosylation sites on *vp28* are thought to contribute to recognition from the host cell surface receptor(s) (Sánchez-Martínez et al., 2007) and thus in turn contribute to WSSV infectivity.

Viral and immune genes were selected for their varied and critical functions. The viral genes have been selected due to their role in infectivity and the replication of the virus. The temporal expression of the viral genes also allows tracking of the viral replication process. Any inhibition of viral replication can be defined in a temporal manner and therefore identify potential processes or mechanisms that might be involved. Similarly, immune genes were selected based on their differential immune functions. Targeting genes with the capacity to interact with pathogens directly (*crustin*) or through immune processes that lead to cell adhesion (*peroxinectin*) and melanization (*prophenoloxidase*), (previously discussed in Chapter 1), provide a more holistic view of the host immune response and therefore potentially a greater understanding of the immune processes involved against this viral pathogen.

3.3 Research Aims

The primary objective of this study was to investigate the immune response, at a transcriptional level, of post-larval stage IV (PLIV) *Homarus gammarus* after exposure to WSSV through consumption of infected feed. This study was designed to provide a realistic assessment of the risk of contracting WSSV-infection in a temperate crustacean species via a natural entry route for this viral pathogen. The aim was to compare the host immune gene transcription of *Homarus gammarus* alongside the transcription of three candidate viral genes involved in various phases of viral replication. This study was also designed to assess the suitability of the immune genes as a predictive suite of biomarkers to investigate viral infection in a susceptible decapod crustacean.

3.3.1 Research Objectives

i. To determine whether changes in the transcription of candidate immune and viral genes were evident in *Homarus gammarus* PLIV during a 10-day trial post consumption of WSSV-infected feed.

ii. To determine viral load using the viral gene *vp28* to ascertain the comparative quantity and rate of viral replication in *Homarus gammarus* during the course of the infection trial.

iii. To establish how effectual the candidate host immune genes were as biomarkers in a commercially valuable species, *Homarus gammarus*, in response to infection with a significant viral pathogen.

3.4 Methods

3.4.1 Molecular methodology

Primer sequences were first designed, tested and used to isolate the target immune, reference and viral genes for confirmation of gene sequences and for qPCR (see Chapter 2 for details). All genes were optimised for primer concentrations and for template concentration and to acquire PCR efficiency values for calculation of relative gene expression (Appendix, Chapter 2). Processing samples for qPCR, including RNA isolation, analysis of RNA quantity, purity and quality, treatment of DNA contaminants, reverse transcription to complimentary DNA and qPCR assay and cycling conditions are detailed in Chapter 2 and Appendix, Chapter 2.

3.4.2 WSSV infection trial in *Homarus gammarus* PLIV

A 10-day WSSV-infection trial was conducted using post-larval stage IV (PLIV) *Homarus gammarus* juveniles. Stage IV juveniles were chosen as this is the first post-larval settlement stage and the small size of the lobsters allowed for increased replication which would be more difficult if using larger animals. A typical PLIV juvenile weighed approximately 130mg and was approximately 2cm in length (Figure 3.5). These juveniles were acquired from the National Lobster Hatchery, Padstow, UK and transported to CEFAS, Weymouth where they were acclimated to trial conditions for a period of 10 days.

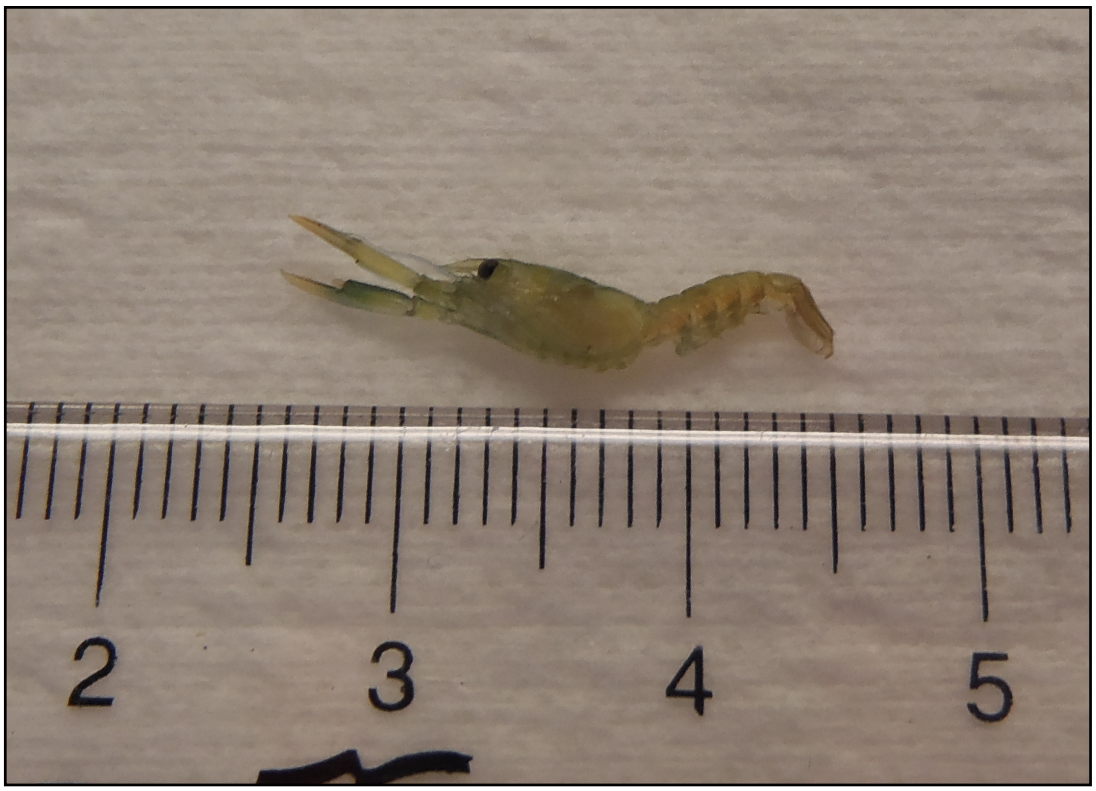


Figure 3.5: Illustration of the typical size of a post-larval stage IV juvenile *Homarus gammarus*. (Scale in centimeters).

3.4.3 Trial conditions

H. gammarus juveniles were kept in 30l flow-through tanks, each containing 20 juvenile lobsters within individual wells held within the upper water column. The tanks (Figure 3.6a) held UV treated, local, filtered seawater with a local salinity of ∼34 psu and a temperature of 20°C. This temperature was consistent with a WSSV inoculation study of *Homarus americanus*, also held at 20°C, that demonstrated successful infection and replication of the virus (Clark et al., 2013b). Salinity and temperature conditions were monitored and regulated via an in-house computer system. WSSV-fed lobsters were contained within the biosecure facilities in CEFAS, Weymouth, UK. Lobsters were monitored closely throughout the trial and any moribund or dead lobsters were removed and sampled immediately. Only lobsters alive at the time of sampling were included in the gene expression analysis.

At commencement of the trial, juvenile *H. gammarus* were fed 10% body weight, (after a 2-day period of starvation), of either WSSV positive tissue from the Chinese isolate UAZ 00-173B generated in *Penaeus chinensis* (Osbeck, 1765) in China in 1995 or WSSV negative tissue from *Penaeus vannamei*. The penaeid tissue was dissected into small blocks prior to feeding and then if not completely consumed was removed after a period of 12 hours. Thereafter, juveniles were fed on alternate days with crab cuisine pellets (West Dorset Aquatics, Weymouth, UK). A note was made of any food remaining in each individual well after this 12-hour period.

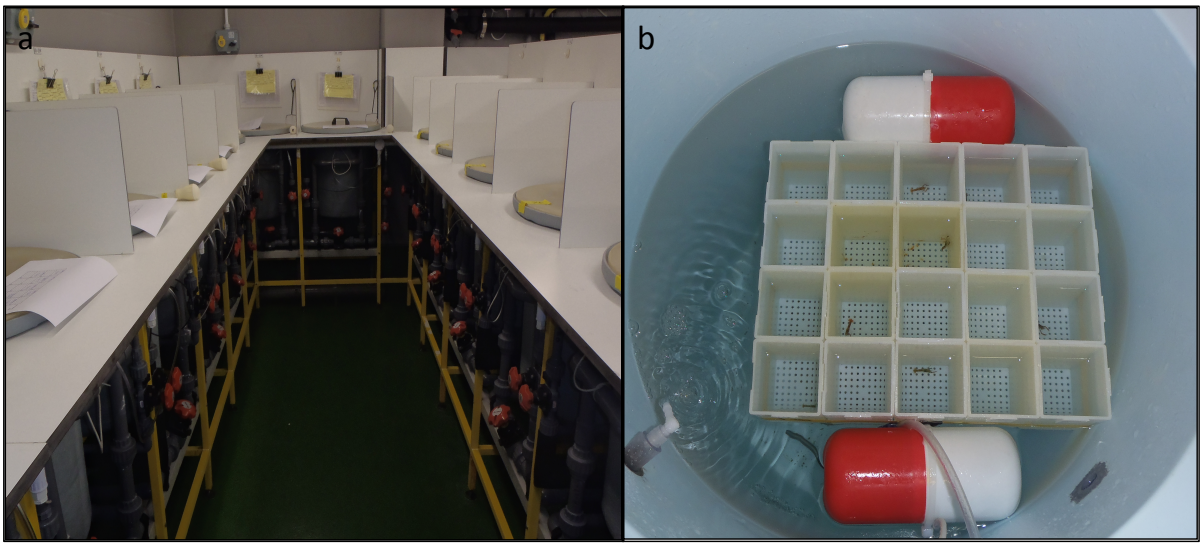


Figure 3.6: Experimental holding facilities showing (a) multiple tanks holding (b) 20 juvenile lobsters each within an individual compartment.

Water exchange occurred between feeding days by briefly lifting the wells out of the tank allowing full drainage of the water through the numerous perforations at the base of the wells (Figure 3.6b).

3.4.4 Sampling design

A total of 10 control animals and 15 experimental animals were sampled randomly at each of the following time intervals; 6h, 12h, 24h, 36h, 48h and daily thereafter until day 10. Five whole experimental animals from each time interval were immediately placed in a sterile tube, snap frozen in

liquid nitrogen and stored at -80°C for later viral load quantification. The remaining 10 animals in each group were first dissected, with the cephalothorax used for histology and electron microscopy by CEFAS colleagues and the tail placed in a sterile tube and immediately snap frozen and stored at -80°C for subsequent gene expression analysis.

3.4.5 Processing samples

Samples were processed in two stages. Firstly, samples were fully processed within the first 48 hours in order to observe early changes in gene transcription within the host and also observe the temporal progression of viral replication based on the 3 viral genes. Thereafter, preliminary analysis for days 3-10 was carried out on a subset of samples (data not shown), in order to isolate the key temporal stages that changes in host immune gene expression could be seen throughout the reminder of the trial. In addition, *per os* WSSV challenge in *Penaeus monodon* demonstrated later transcription of viral genes compared with inoculation of WSSV (Antony et al., 2011b). Therefore, in view of these findings, days 7 and 10 were fully processed in order to observe later changes in host immune transcription.

3.5 Results

3.5.1 RNA quality

RQI values are detailed in Appendix, Chapter 3, along with the raw $\log_{10}$CNRQ values. A total of 76.6% of samples had an RQI of 10 (Figure 3.7) and the remaining samples had an RQI above 7.1. Of those samples with an RQI $<$10, 11.6% were between 7.1-7.9, 19.2% were between 8.0-8.9 and 69.2% were between 9.0 and 9.9. Sample inclusion was based on RNA integrity $>$7.5, (previously discussed in Chapter 2, Section 2.3.4), which resulted in almost all of the samples being included.

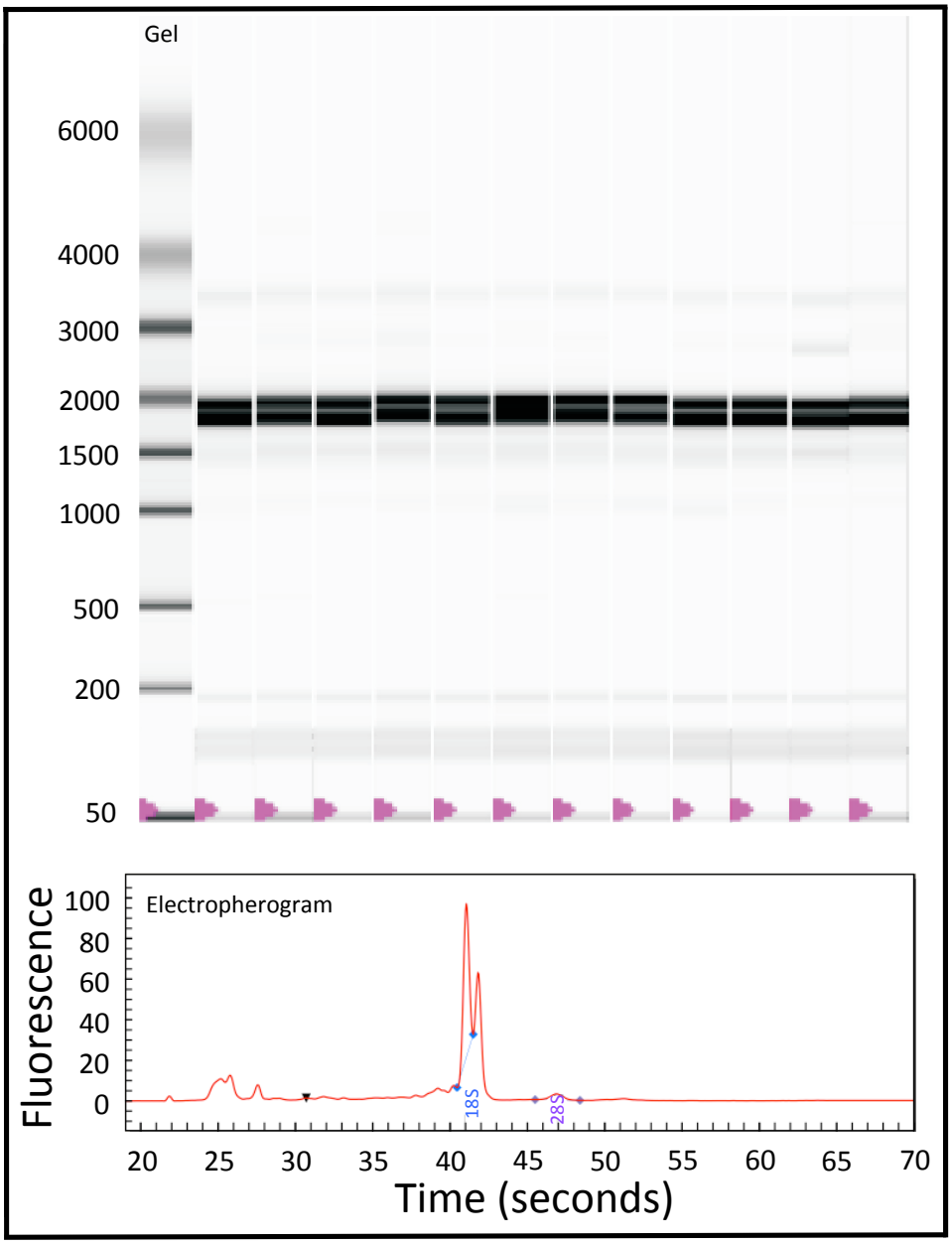


Figure 3.7: Virtual gel image of the RNA ladder in the first column (bp), (Product code 700-7255, Bio-Rad, UK) and RNA products extracted from WSSV-challenged *Homarus gammarus* in the subsequent columns, viewed via Experion™ software. All RQI™ (RNA Quality Indicator) values from the above image were 10, indicating high integrity of RNA (top image). Electropherogram indicating the 18S (also seen as a dark band on the gel mage) and 28S rRNA (ribosomal RNA) peaks (bottom image). The electropherogram profile above was characteristic of all *Homarus gammarus* sampled.

3.5.2 Reference gene stability

The reference gene stability was assessed using the geNorm algorithm in qbase$^+$ (Chapter 2). The average M and CV values for all reference genes were 2.182 and 3.748 respectively (Figure 3.8 and Table 3.3). This was the least stable combination, therefore *eef1a* was removed, improving both the M (1.740) and CV (0.880) values. However, the removal of *gapdh*, although causing a slight increase in the CV value to 0.979, did improve the M value to 1.496 and was subsequently considered the most stable combination of reference genes.

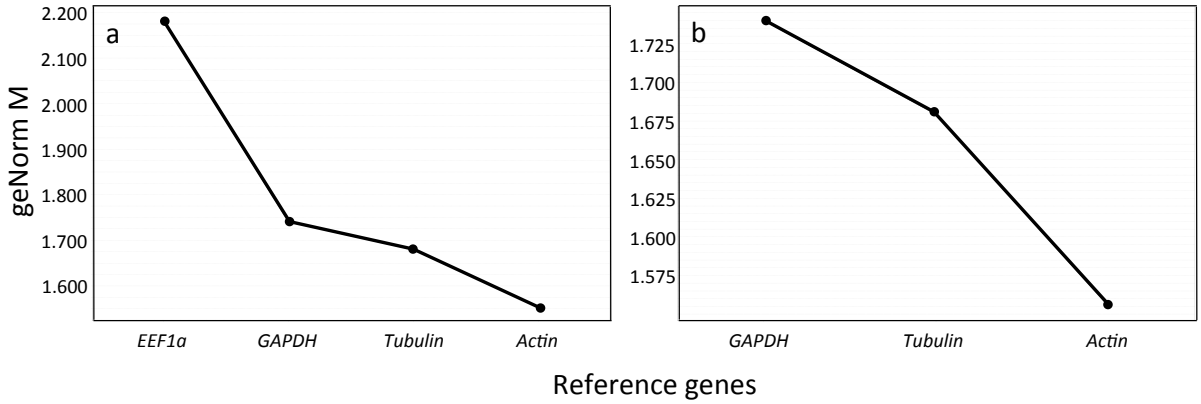


Figure 3.8: Reference gene stability values for a) all four reference genes and b) three reference genes after removal of the least stable reference gene (*eef1a*).

Table 3.3: The mean M and CV values for various reference genes. The most stable gene combination was *actin* and *tubulin*.

Endogenous reference genes	M	CV
actin, eef1a, gapdh, tubulin	2.182	3.748
actin, gapdh, tubulin	1.740	0.880
actin, tubulin	1.496	0.979

3.5.3 Feed consumption

At the 12-hour sampling interval all WSSV-infected and SPF *Penaeus vannamei* tissue was removed from each well. In the SPF-fed (control) group 70%, and in the WSSV-fed group 79% of lobsters had consumed the feed in its entirety.

3.5.4 Mortality

Mortality was low throughout the trial with one SPF-fed lobster mortality at 12 hours representing just 0.8% of the control lobsters and four WSSV-fed lobster mortalities at 12 hours (n=2), 48 hours (n=1) and at day 5 (n=1) representing 2% of the WSSV-fed lobsters.

3.5.5 Immune gene expression

3.5.5.1 Normal distribution and equal variance

Normal distribution (H_0) of data was assessed using the Shapiro-Wilks test for normality and the Bartlett test used to assess homogeneity of variance. As not all data were normality distributed (p <0.05) (Appendix, Chapter 3, Table 8.8) or equal in variance (p <0.05) (Appendix, Chapter 3, Table 8.9) statistical analysis was undertaken using the non-parametric Kruskal-Wallis test. This test is performed on ranked data, which does not make assumptions on normality or homogeneity of variance.

3.5.5.2 Results

All host immune genes showed a significant up-regulation in transcription (Table 3.4 and Figure 3.9) over the course of the 10 day trial. The effect of time on the change in gene transcription was significant at either p<0.001 for *crustin* SPF-fed and WSSV-fed and for *peroxinectin* WSSV-fed and significant at p<0.05 for *peroxinectin* SPF-fed and *prophenoloxidase* SPF-fed and WSSV-fed lobsters. In response to treatment, the gene transcription of both *peroxinectin* and *prophenolxidase* showed no statistical difference in expression between the lobsters that consumed WSSV-infected feed to those that consumed SPF-feed. However, the transcription of the anti-microbial peptide, *crustin*, increased (p<0.001) in those lobsters fed WSSV-infected tissue. This is seen in Figure 3.9 at 12 and 36 hours.

Table 3.4: Kruskal-Wallis statistical results for *crustin*, *peroxinectin* and *prophenoloxidase* over time in both the SPF-fed and WSSV-fed groups and between treatment groups. Statistically significant results are highlighted in red.

Crustin		
Time	SPF-fed	H = 27.01, d.f. = 6, p = <0.001
	WSSV-fed	H = 35.93, d.f. = 6, p = <0.001
Treatment		H = 13.50, d.f. = 1, p = <0.001
Peroxinectin		
TIme	SPF-fed	H = 13.79, d.f. = 6, p = <0.05
	WSSV-fed	H = 26.63, d.f. = 6, p = <0.001
Treatment		H = 0.007, d.f. = 1, p = >0.05
Prophenoloxidase		
TIme	SPF-fed	H = 16.00, d.f. = 6, p = <0.05
	WSSV-fed	H = 14.89, d.f. = 6, p = <0.05
Treatment		H = 2.86, d.f. = 1, p = >0.05

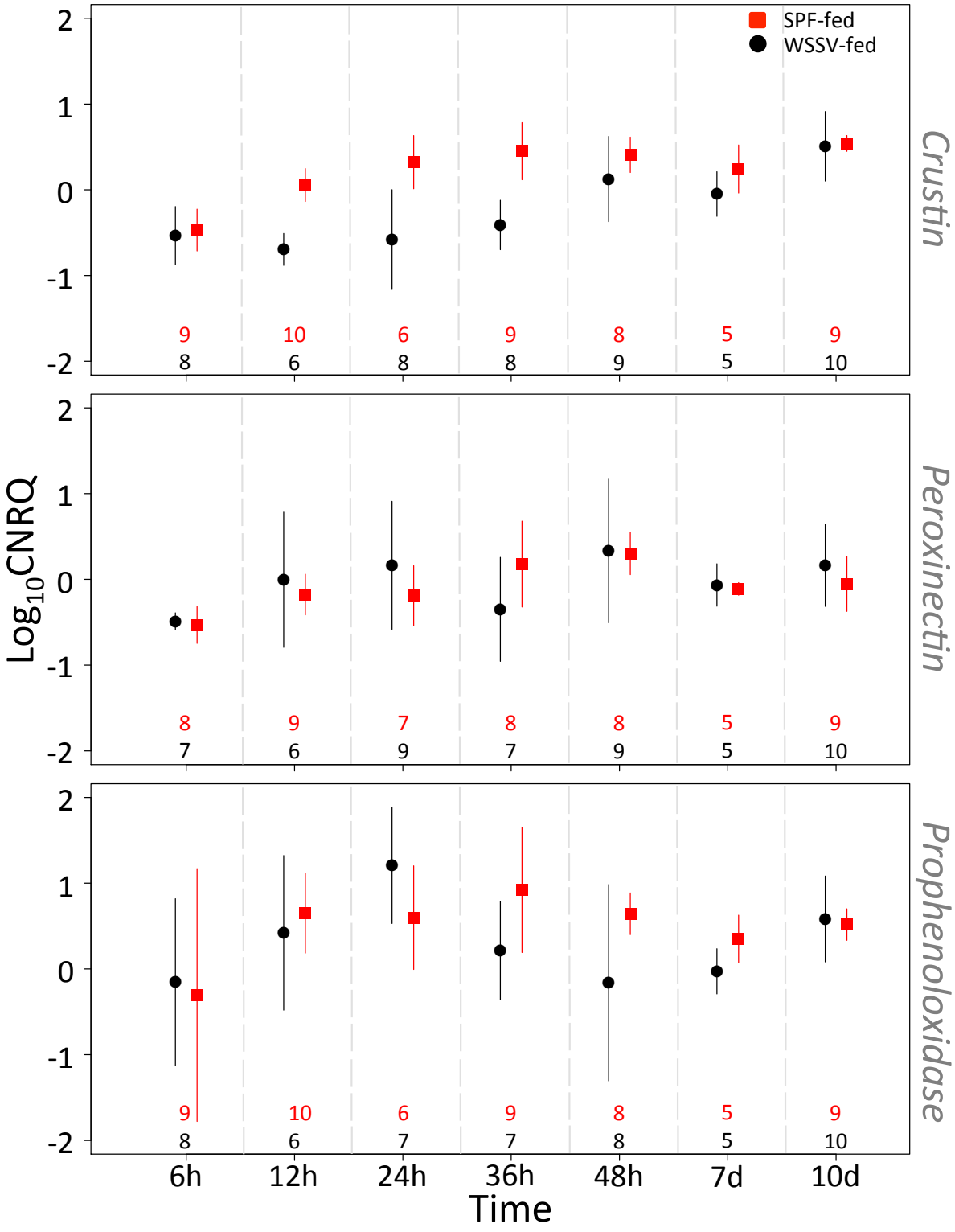


Figure 3.9: Transcription (Log_{10}CNRQ) of *crustin*, *peroxinectin* and *prophenoloxidase* in response to exposure to WSSV via feeding. Error bars are in standard deviation and the number of replicates are indicated for each immune gene at each time interval on the graph (WSSV-fed in red and SPF-fed in black).

3.5.6 Viral gene load

Viral gene load was analysed by colleagues in CEFAS, Weymouth (Kelly Bateman and Michelle Pond), after extraction of genomic DNA. No expression of *vp28* was evident either from nested PCR or qPCR analysis.

3.5.7 Viral gene transcription

Viral gene transcription of *ie1*, *dnapol* and *vp28* from the tails of PLIV *Homarus gammarus* were not transcribed during the 10-day trial. All qPCR runs included a positive control of WSSV-infected *Penaeus vannamei* tissue as well as a no-template control. The transcription of the positive control was expressed at approximately 12, 11 and 17 C_q values in *ie1*, *dnapol* and *vp28* respectively (Figures 3.10, 3.11, 3.12) confirming that the priming sequences and assay conditions successfully amplified the target genes.

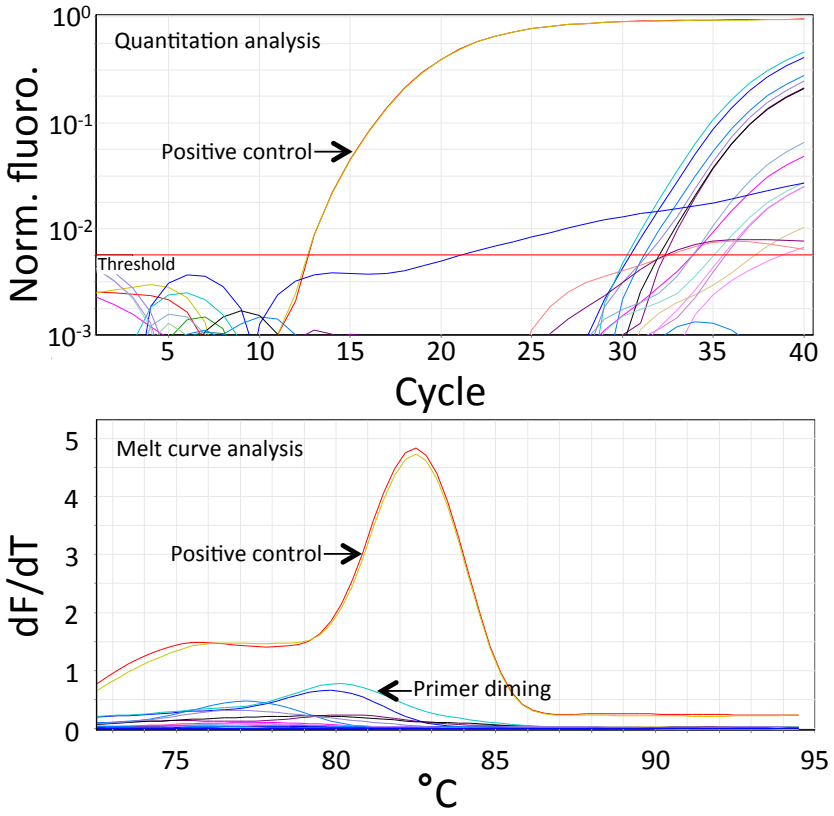


Figure 3.10: Raw qPCR normalised fluorescence data (quantitation analysis) and melt curves (dF/dT, change in fluorescence over time) of *ie1* transcription.

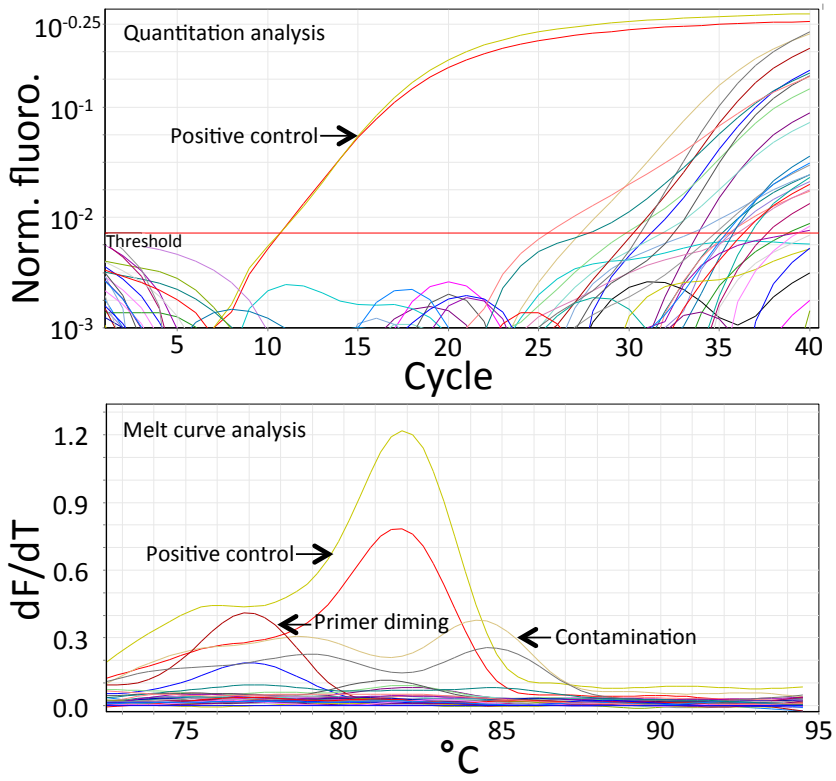


Figure 3.11: Raw qPCR normalised fluorescence data (quantitation analysis) and melt curves of *dnapol* transcription.

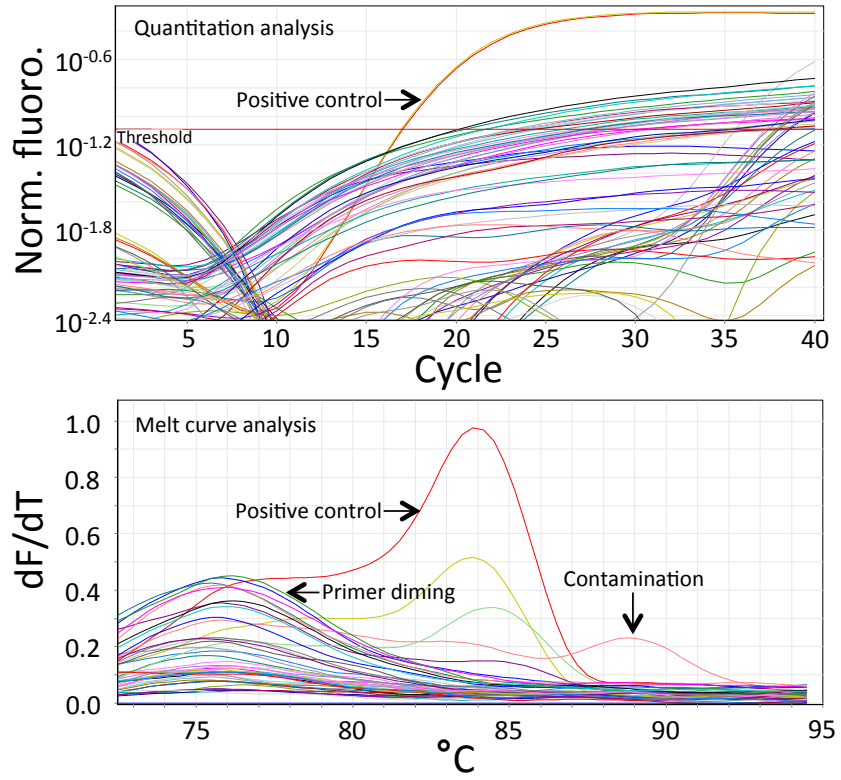


Figure 3.12: Raw qPCR normalised fluorescence data (quantitation analysis) and melt curves of *vp28* transcription.

3.6 Discussion

3.6.1 Immune and viral gene expression

The analysis of *crustin, peroxinectin* and *prophenoloxidase* showed definitive changes with an overall increase in expression with time in both the WSSV-fed and SPF-fed lobsters. Only the antimicrobial peptide, *crustin*, showed an up-regulation in expression in response to treatment. Curiously, during the 10-day trial no corresponding expression of the viral genes, *ie1*, *dnapol* or *vp28*, was evident.

To determine successful uptake of virions in the host, viral load (actual nucleic acid copy numbers per sample) was quantified by processing the genomic DNA (Valasek and Repa, 2005; Ginzinger, 2002). Genomic DNA was extracted from each of the five *Homarus gammarus* whole animal samples from each of the time intervals. As well as determining the presence of virions, the rate of production of the virions could also be established. However, there was no amplification of the *vp28* gene, used for quantifying viral load, suggesting the virus did not successfully infect and amplify within the host cells.

The overall results indicate a lack of WSSV infection in *Homarus gammarus*, but a definitive host response in terms of the increased *crustin* expression in the WSSV-fed lobsters compared with the SPF-fed lobsters. The following factors could have influenced successful viral infection and replication.

3.6.1.1 Assimilation of feed

Changes in the expression of viral genes are generally seen within hours of initial infection (Sanchez-Paz, 2010). However, in many studies the WSSV-infected material was delivered via injection (van Hulten et al., 2001b; Wu et al., 2005; Xu et al., 2009; Mejia-Ruiz et al., 2011). An important factor in this experiment was to determine the risk associated with a natural method of infection, *i.e.* ingestion of infected material, particularly as this is one of the major methods of transmission for WSSV.

The ingestion of WSSV-infected feed compared with inoculation of WSSV material is likely to result in a longer transition period before changes in viral gene expression are seen. A study investigating oral WSSV-infection challenge in *Penaeus monodon* reported that viral gene expression for the immediate early gene, *ribonucleotide reductase*, was not detected until day 2 post WSSV-oral challenge and the expression of *dnapol* and *vp28* were particularly low until day 5 post challenge (Antony et al., 2011b). In this example, a 5-day period was sufficiently long enough to observe transcriptional changes in immediate-early, early and late viral genes. However, it is important to note that shrimp in general are considered to be comparatively more susceptible to WSSV infection than *H. gammarus* (Bateman et al., 2012b) and therefore the virus may replicate more rapidly within the shrimp. That said, previous *per os* WSSV challenge in juvenile *H. gammarus* demonstrated viral replication within a day 10 period (Bateman et al., 2012a). Therefore the time period of 10 days for this study was likely to have been long enough to have seen viral replication providing there was adequate initial viral loading.

3.6.1.2 Adequate viral dosing?

It is important to point out that not all juvenile lobsters consumed the feed in its entirety. As previously stated 30% of the SPF-fed and 21% of WSSV-fed lobsters did not consume the entire feed. Nevertheless, in all cases at least some of the feed was consumed. This still introduces variability in terms of standardising the quantity of feed ingested. In addition, the feed administered was not

homogenised and was in fact blocks of tissue from an infected *Penaeus vannamei*. Consequently, it is possible that the viral load within each block was not comparable. In WSSV-fed juvenile *H. gammarus* from a previous feeding trial, low-dose fed lobsters did not exhibit the characteristic histology as seen in either high-dose fed juvenile lobsters or in penaeids, although mortality was higher (41%) compared with controls (17%) (Bateman et al., 2012a). It may be that the dose was not sufficient enough to establish pathogenic infection in the lobsters , but sufficient enough to produce a statistically significant host immune response in terms of the increased expression of *crustin* as seen in this study.

3.6.1.3 Viral latency

Whilst the 10 day time period for the feeding trial was deemed sufficient based on previous feeding studies (Bateman et al., 2012a; Antony et al., 2011b), several studies report a latent period in viral replication for WSSV. Latency is defined as the presence of the viral genome without the replication of infectious virions (He and Kwang, 2008) and is evident in other DNA viruses such as Epstein-Barr virus (Leight and Sugden, 2000) and cytolmegalovirus (Sinclair and Sissons, 2006). In terms of WSSV, inoculation of the mud crab, *Scylla paramamosain*, resulted in a latent period of 48 hours prior to viral replication (Du et al., 2013). Similarly, WSSV-inoculation of *Litopennaeus vannamei* saw the expression of *vp28* increase appreciably only after 48 hours. These are examples from inoculation studies whereby systemic infection is likely to have occurred rapidly. A feeding trial would require a longer time period to first passage through the gut before establishing systemic infection. Hypothetically this could facilitate an extended period of latency, particularly if this were compounded with low initial viral loading. Assuming this was the case in this juvenile *H. gammarus* feeding trial, then an extended trial period could have resulted in a transition from latent infection to the lytic stage of infection. Analysis of the expression of viral genes associated with latency in WSSV, such as WSSV403, believed to regulate latency via the ubiquitin ligase pathway in shrimp (He and Kwang, 2008), would be useful in future studies, to clarify the latency state of the virus.

3.6.2 Impact of temperature on immune gene expression

The expression of *crustin, peroxinectin* and *prophenoloxidase* were all significantly up-regulated over the course of the 10 day trial in both the WSSV-fed and SPF-fed lobsters (Table 3.4). Selecting 20°C as the holding temperature for the juvenile lobsters in the trial was a compromise between ensuring a temperature sufficient enough not to inhibit viral replication and one which is commonly seen in UK and European waters in the summer months, and recognising that this was towards the upper limit of the temperature range for *Homarus gammarus* (Tully et al., 2000; Smith et al., 1998). Despite acclimation of the juvenile lobsters to 20°C, the up-regulation of the immune genes within the SPF and WSSV-fed groups may be in response to thermal stress.

A response in a change in transcription levels of these immune genes are seen in other species. The antimicrobial expression of *carcinin* in *Carcinus maenas* in response to temperature was seen to increase in response to both extremes in low (5°C) and high (20°C) temperature (Brockton et al., 2007). In addition, the *crustinPm5* transcript in the tiger shrimp *Penaeus monodon* was inducible post heat shock from 28°C to 33°C for 2 hours followed by a 6 hour recovery period (Vatanavicharn et al., 2009). *Peroxinectin* was also up-regulated in response to heat stress in the oyster *Crassostrea gigas* (Lang et al., 2009). However, temperature stress has been reported to cause a decrease in the activity of prophenoloxidase as demonstrated in *Litopenaeus vannamei* that were exposed to their critical thermal

maxima (Diaz et al., 2013). The expression of prophenoloxidase in the SPF-fed lobsters is markedly more variable compared with that of the WSSV-fed lobsters and of both groups for the *crustin* and *peroxinectin* genes. In a WSSV-inocculation study in *H. americanus* an up-regulation of the redox protein thioredoxin was observed. This up-regulation could not be associated with the WSSV infection, but more likely corresponded to thermal stress (20°C) (Clark et al., 2013b). In *H. gammarus*, an overall up-regulation of all immune genes over 10 days was evident and may be in part related to a degree of thermal stress, although, other contributing factors such as small compartment sizes within the tank (Figure 3.6) and water exchanges on alternate days are also likely to contribute to a stress response. Alternatively, this may simply be a response to feeding. The SPF and WSSV penaeid tissue was not tested for other pathogen infections. The overall increase in the transcription of immune genes may therefore have resulted from a natural increase of immune defence with feeding and digestion.

3.6.3 Effectual immune gene response to a viral pathogen

The increase in *crustin* expression within the first 36 hours of the trial indicates a potential role for this antimicrobial peptide against WSSV. Antimicrobial peptides are produced in haemocyte granules, are readily available immediately after infection, can directly target pathogens, can be synthesised at a low metabolic cost and only very low concentrations are required (micromolar) to be effective against pathogens (Hancock, 2001). As previously stated, *crustin* has demonstrated increased expression in response to WSSV infection in *Penaeus monodon* (Antony et al., 2011b) and is therefore suggested to have antiviral properties. Unlike *peroxinectin* and *prophenoloxidase*, *crustin* is secreted from the haemocytes into the haemolymph immediately after synthesis (Hauton, 2012). Consequently, *crustin* is likely to respond to any pathogen more rapidly compared with *peroxinectin* and *prophenoloxidase*. It may be that the increased production of *crustin* was sufficient to prevent established WSSV infection in this case.

The lack of an established infection, potentially due to the initial increase in *crustin* expression in response to the virus, which may have been facilitated by insufficient viral loading and potential latency of the virus during the initial phase, limits the assessment of *peroxinectin* and *prophenoloxidase*. *Peroxinectin* acts as a cell adhesive molecule with opsonic functions (Johansson et al., 1999), capable of stimulating encapsulation (Kobayashi et al., 1990) and phagocytosis (Cerenius and Söderhäll, 2004). *Prophenoloxidase* exists as a zymogen in granular and to a lesser degree, semi-granular cells (Smith, 1996). In the presence of calcium, prophenoloxidase is converted to its active state of phenoloxidase through proteolytic cleavage by trypsin after its release to the haemocoel (Adachi et al., 2003). Phenoloxidase then catalyzes the *o*-hydroxylation of monophenols as well as the oxidation of diphenols to quinones. Quinones are then polymerized to melanin non-enzymatically (Smith, 1996; Cerenius and Söderhäll, 2004). Melanin, a brownish-black pigment, encapsulates pathogens in order to contain the infection by preventing or slowing its replication.

Certainly no evidence of a change in expression in *peroxinectin* and *prophenoloxidase* was demonstrated in response to WSSV-challenge throughout this trial. However, inoculation studies of WSSV in the mud crab *Scylla paramamosain* showed a four-fold up-regulation of *peroxinectin* within the first 72 hours post-infection followed by down-regulation at 96 hours (Du et al., 2013), suggesting antiviral properties of *peroxinectin* in this species, particularly during the early phase of infection. Similarly, antiviral

activity has been suggested for *prophenoloxidase* in response to WSSV-challenge. WSSV-injection in the red swamp crayfish, *Procambarus clarkii* demonstrated differential *prophenoloxidase* expression in nine different tissue types including intestine and haemocytes (Li et al., 2012). *Prophenoloxidase* increased in expression in the WSSV-challenged group in all nine tissue-types although not significantly in the intestinal tissue. Other studies have demonstrated differential tissue expression of *prophenoloxidase* in other species such as in the mud crab, *Scylla serrata*. In this case, *prophenoloxidase* was strongly expressed in the haemocytes, but not in the heart, gill, eyestalk, ovary, muscle, hepatopancreas, stomach or intestine (Ko et al., 2007). Therefore, an important consideration when investigating the host-pathogen interaction at the level of transcription is identifying the most appropriate immune gene to study for the species of interest according to the particular tissues sampled. In addition, due consideration should be given to the most appropriate tissues to sample.

In the case of the *H. gammarus* juveniles, it may be that both *peroxinectin* and *prophenoloxidase* expression would be unlikely to change in response to WSSV-challenge until a more systemic infection was evident. Therefore, it is not possible at this stage to invalidate these immune genes as biomarkers. Although ingestion of WSSV-infected tissue provides a more realistic assessment of transmission of WSSV to *H. gammarus* juveniles, stimulating a systemic infection via inoculation studies would provide a more comprehensive analysis of the roles *peroxinectin* and *prophenoloxidase* play in response to WSSV infection in this species. The difference in the host response of *crustin* transcription compared to that of *peroxinectin* and *prophenoloxidase* is also likely to be a reflection of the different mechanisms by which they are released. *Crustins* are made and secreted constantly whereas both *peroxinectin* and *prophenoloxidase* are released upon exocytosis when required.

3.6.4 Limitations with experimental design

Metabolic processes in *Homarus gammarus* are controlled by organs within the cephalothorax. Assuming any changes in gene expression would first occur within the cephalothorax, analysis of viral gene expression in the tails of *H. gammarus* would indicate a more established systemic infection. Although a change in the expression of *crustin* was evident within the first 36 hours within the tails, no amplification of the viral genes was seen. It is important to highlight only *vp28* was used as a biomarker to detect and determine viral load in the five whole lobsters sampled at each time interval. *vp28* is defined as a late gene in terms of the temporal succession of WSSV genes. Consequently, transcription of the immediate early (*ie1*) or early (*dnapol*) viral genes cannot be excluded.

The potential for low and variable viral load in the WSSV infected penaeid tissue could explain the lack of viral replication seen. Homogenisation and further analyses (PCR quantification) of viral load from the infected tissue would determine an accurate loading dose in all feed. Furthermore, extending the starvation period for a additional 24 hours may have ensured a greater uptake of feed from the juvenile *H. gammarus*. The WSSV inoculation trial in *H. americanus* found the greatest differential gene expression, based on micro-array analysis, occurred at 7 days (Clark et al., 2013b). Therefore, extending the time of this *H. gammarus* feeding trial for a longer period may have allowed development of WSSV infection, particularly if lower viral dose feed was consumed, compounded by the extended time required to assimilate the feed and a potential period of latency.

3.6.5 Concluding remarks

This is the first study to concurrently analyse the immune and viral gene transcription in *Homarus gammarus* to WSSV exposure. Susceptibility of *Homarus gammarus* to WSSV was confirmed from a previous high dose feeding trial whereby disease was evident within 10 days (Bateman et al., 2012a). The results presented here, however, demonstrate that infection of WSSV was not established in *Homarus gammarus* juveniles. The increase in *crustin* transcription during the early stages of the trial (<36 hours) suggest that the host was capable of mounting a response to WSSV, which may be why no systemic viral transcription was evident. This may have been facilitated by a potentially lower initial viral load. However, this is speculative, as the comparative viral load per unit of feed was not determined.

The increased transcription of *crustin* suggests this antimicrobial peptide has antiviral activity against WSSV in *H. gammarus*. Further investigation into the activity of *crustin* in response to WSSV could be explored using RNA inhibition technology to clarify the antiviral role of this immune gene against this viral pathogen.

The results in this study are in contrast to the prior feeding trial in *H. gammarus* whereby PLIV juveniles were fed high and low dose WSSV injected *Penaeus vannamei* tissue as well as commodity shrimp from a fish market and supermarket (Bateman et al., 2012a). Within the 10 day trial mortality of lobsters fed high dose WSSV reached 10% (15°C) and 55% (22°C). The low dose supermarket derived WSSV fed lobsters demonstrated mortality rates of 0% (imported from Honduras), 20% (imported from Ecuador) and 22% (imported from Vietnam) and tested 70%, 30% and 45% PCR positive respectively (Bateman et al., 2012a). The presence of WSSV virions were confirmed by histology, TEM, as well as nested PCR. Bateman *et. al.* (2012) demonstrated that successful infection and viral replication from WSSV-infected commodity shrimp is viable in *H. gammarus*. This also highlights the variation in the number of infected lobsters depending on the source of the infected material. Often smaller shrimp sold in supermarkets can have greater viral loads as there has been a tendency in the past to produce emergency harvests, whereby aquaculture stocks are sold at the onset of clinical signs (Durand et al., 2003).

The susceptibility of wild *H. gammarus* populations to infection with WSSV is likely to be determined by a variety of factors including viral dose (and therefore source of the contaminated tissue), water temperature and the effectiveness of the host immune response. In addition, future investigations should consider the role commensal gut biota play in host susceptibility in response to ingested WSSV-infected feed. Commensal biota have the potential to enhance host immunocompetence (Teixeira et al., 2008). Therefore, further investigations into the role of the gut in immunocompetence in decapod crustaceans is important in assessing the realistic expectation of WSSV becoming established within wild decapod crustacean populations within Europe. Furthermore, exploring the role of commensal bacteria in potentially providing a synergistic, efficient innate immune response would provide a more comprehensive picture as to the processes that contribute to immunocompetence within the gut in response to this viral pathogen (discussed further in Chapter 7: Final Discussion).

4 Assessment of immunocompetence in the shore crab, *Carcinus maenas* (L.), to natural exposure of pathogens

Chapter Abstract

UK populations of the shore crab *Carcinus maenas* host various pathogen assemblages. In particular, two geographically close populations in Weymouth, Harbour and Newton's Cove, on the south coast of the UK demonstrated remarkably different pathogen profiles as defined by histopathology. The immune biomarkers, *carcinin* (antimicrobial peptide), *peroxinectin* (cell adhesive molecule and opsonin) and the zymogen *prophenoloxidase* (cleaved to form active phenoloxidase, involved in the melanisation of many invading pathogens), were used to assess the immunocompetence of these populations in relation to their pathogen burden. Furthermore, RNA quality was assessed in context of the host pathogen burden. No change in gene transcription was observed within each location between *C. maenas* with pathogens and those without. However, there were significant changes in *carcinin* ($p<0.01$), *peroxinectin* ($p<0.001$) and *prophenoloxidase* ($p<0.01$) transcription in relation to location in those *C. maenas* with pathogens. Only *carcnin* ($p<0.001$) transcription changed in response to location in those crabs without pathogens.

4.1 Introduction

Pathogens have the ability to adversely impact natural and farmed fish and invertebrate stocks (Burkholder et al., 1995; Audemard et al., 2004; Nowak, 2007). Chapter 3 highlighted the example of WSSV (White Spot Syndrome Virus) infection in decapod crustaceans, particularly farmed penaeid species. Disease outbreaks within aquaculture are not entirely surprising considering the more highly stressed the environment in terms of the increased density of animals and therefore greater potential for compromised immune function (Sangamaheswaran and Jeyaseelan, 2001; Safeena et al., 2012). This can potentially allow proliferation of opportunistic pathogens. The effects of density on *Carcinus maenas* will be further explored in Chapter 6.

However, pathogens also have the ability to drive population dynamics in susceptible species in the wild (Dobson, 2004). Understanding natural prevalence of pathogens is critical for fisheries management and ecosystem health. The impact of these parasites on the host, in terms of their pathogenicity, can be very variable. This can be further complicated by the unknown pathogenicity of co-infections (Stentiford et al., 2003; Gismondi et al., 2012). It is important to assess the baseline prevalence of disease or infection in natural communities to provide a context for the frequency and virulence of disease outbreaks of either novel or known pathogens. Investigating host immune competence to

particular pathogens is a crucial part of understanding the variability in pathogen profiles evident in different populations.

4.1.1 Background

4.1.1.1 *Carcinus maenas*

As a well-studied decapod crustacean, the shore crab, *Carcinus maenas*, is a good model to investigate the impact of natural infection in the wild. *C. maenas*, a predominantly intertidal, eurythermal and euryhaline species, is widely distributed in North-West Europe (Hayward and Ryland, 1990 cited by Hebel et al., 1997) and has become an invasive species in areas such as the Atlantic and Pacific coasts of North America, South Africa, Australia and Japan (Carlton and Cohen, 2003; Hidalgo et al., 2005). An important species ecologically and economically in Europe, *C. maenas* is valued as bait for angling and as a commodity fishery, eaten in continental Europe (Eddy et al., 2007). *C. maenas* has also been recognised as an effective indicator species for ecosystem health (Hebel et al., 1997).

Importantly, *C. maenas* is susceptible to infection from an array of various pathogens and can harbour a number of pathogens at any given time. Infections can range from viral, fungal, protozoan and bacterial to larger multi-cellular parasites. Their ability to mount an immune response is crucial for sustaining a healthy fishery and arguably ecosystem. A UK study of *C. maenas* and their pathogen assemblage highlighted diverse profiles between populations in different locations (G. Stentiford, pers. comm., 2012). An example of two sites in Weymouth, UK, known to harbour different pathogen profiles, are Harbour, considered to be 'healthier' and Newton's Cove, considered to have a greater pathogen prevalence. This posed an interesting question in terms of whether the immunocompetence of *C. maenas* in these distinct, but geographically close populations differs?

4.1.1.2 Harbour versus Newton's Cove

The two survey locations are situated in close proximity to each other either side of a cliff peninsula, The Nothe, in Weymouth, UK. Despite this they remain distinct locations. Whilst it is important to establish the differences between these locations, the hydrodynamics of both sites were not investigated in this survey. Newton's Cove is a beach site approximately 0.5km in length, consisting of sand, shingle, large rocks and rock pools and is separated from the land by a sea wall overlooking Portland Harbour. Harbour is located on the other side of The Nothe peninsula. The collection site was situated toward the Harbour entrance at a crabbing site popular with local tourists. Harbour is host to a small fishing fleet and many small pleasure boats which results in a significant degree of boat traffic in the immediate area.

Figure 4.1: Sampling sites in Weymouth, UK (GoogleEarth, 2014) at latitude 50.606999, longitude -2.450820.

These locations present very different environments for *C. maenas*. Crabs in Harbour remain submerged throughout the entire tidal cycle. In addition, they are likely to have a different and supplemented dietary intake. Adult *C. maenas* generally feed on gastropods (*Littorina* spp.), mussels (*Mytilus edulis*), clams (*Mya arenaria*), algae and juvenile conspecifics (Crowthers, 1968 and Elner, 1977 cited by Sheehan et al., 2008). In Harbour this is supplemented by tourists crabbing who can use anything from fish to bacon as bait. Conversely, *C. maenas* at Newton's Cove are subject to different environmental stressors, such as aerial exposure during each tidal cycle. *C. maenas* are found to either bury into the sand or hide under large rocks or seaweed during this time. This period of aerial exposure also results in vulnerability to different prey, such as marine birds.

4.1.1.3 Research Aims

The objective of this survey was to determine and compare the pathogen profile between Harbour and Newton's Cove. In addition, the impact of natural infection on host immunocompetence at both locations was investigated.

4.1.2 Research Objectives

i. Identify the pathogen profiles at Harbour and Newton's Cove via histological investigations and compare these profiles.

ii. Determine how pathogenic infection affects the host immune transcription of *carcinin*, *peroxinectin* and *prophenoloxidase* in these natural populations of *Carcinus maenas*.

iii. Establish how effectual the candidate host immune genes were as biomarkers in natural populations of *Carcinus maenas*, in response to infection with a variety of pathogens.

Incidental finding - Variable RNA quality isolated from *Carcinus maenas* haemolymph.
iv. Determine how RNA quality affects the gene transcription of *carcinin*, *peroxinectin* and *prophenoloxidase*.

4.2 Methods

4.2.1 Preparation for molecular processing

Prior to sample collection, primers were designed to isolate the correct gene target using conventional PCR for those genes that were not sequenced or where only EST sequences were available, (*peroxinectin, actin, eef1a, gapdh* and *tubulin*). For *carcinin* (Accession number AJ427538) and *prophenoloxidase* (Accession number DN202844) full gene sequences were previously described. qPCR primers were designed for all immune and endogenous reference genes using Primer Express® software (Applied Biosystems, Life Technologies, California, USA) and then fully optimised for primer and cDNA template concentrations and qPCR efficiencies. (Full details in Chapter 2, Sections 2.4 and Appendix, Chapter 2, Tables 8.5 and 8.6).

4.2.2 Collection of *Carcinus maenas*

Carcinus maenas were collected from Harbour (latitude 50.606999, longitude -2.450820) and Newtons Cove (latitude 50.605365, longitude -2.448481), in Weymouth, Dorset (Figure 4.1) between June and September 2012. A total of 88 animals were collected from Harbour and 91 animals from Newtons Cove. All *C. maenas* were collected at low tide from Newton's Cove and throughout the tidal cycle in Harbour as they remained submerged at all times in this location. All animals were transported to the laboratory facilities in CEFAS (5-10 minutes walk from both sampling locations) and immediately anaesthetised by placing on ice for a minimum of 30 minutes prior to processing for gene expression analysis and histology. Ambient seawater temperature data from Weymouth was taken by Cefas and considered accurate ±1°C. For the sampling period of June, July, August and September 2012 the average temperatures were 15.2°C, 16.4°C, 18.0°C and 17.5°C respectively. Daily information on temperature data between March 2012 until October 2012 is available in Appendix, Chapter 4, Tables 8.17a and b (temperature data courtesy of Ian Tew, Cefas, Weymouth).

4.2.3 Sample processing

4.2.3.1 Molecular processing of haemolymph

From each animal, 1ml of haemolymph was aspirated using a pre-loaded syringe of 1ml of *Carcinus maenas*-specific anticoagulant (Chapter 2, Table 2.2). This was centrifuged (3000*g* and 4°C for 7 minutes) to form a pellet. After aspirating and discarding the anticoagulant, 1-1.5ml (depending on pellet size) of TRI Reagent® (Sigma-Aldrich, Dorset, UK) was added and the sample stored at -80°C until further processing. Sample processing for qPCR, including RNA isolation, quality, quantity and integrity analysis of the RNA, treatment of potential DNA carryover, reverse transcription to cDNA and qPCR cycling conditions, are detailed in Chapter 2, sections 2.3.3-2.3.6 and 2.4.2-2.4.3.

4.2.3.2 Histology

The tissues isolated and dissected for histology were heart, gill, muscle, gonad, nerve and hepatopancreas. The hepatopancreas was also isolated and fixed in glutaraldehyde for possible electron microscopy at a later date if required (Chapter 2, Section 2.5).

4.3 Results

4.3.1 Sex and size range of *Carcinus maenas*

The size range (carapace width) of *C. maenas* in Harbour was 42-77mm with a mean carapace width of 56.1mm and in Newton's Cove 21-60mm with a mean carapace width of 38mm. The details are described fully in Appendix, Chapter 4, Table 8.18. The male:female ratio was 1:1.4 and 1:1.05 in Harbour and Newton's Cove respectively. There was a significant ($p<0.0001$) difference in size between male and female *C. maenas* in Harbour with males being larger. There was also a significant difference in the size of both female and male *C. maenas* between locations with the larger crabs observed in Harbour (Table 4.1 and Figure 4.2).

Table 4.1: Statistical comparison of the size variation in *Carcinus maenas* between Harbour and Newton's Cove within each gender class and between males and females within each site.

Size v Location	Female	H = 48.29, d.f. = 1, $p = <0.01e^{-9}$
	Male	H = 44.39, d.f. = 1, $p = <0.01e^{-8}$
Size v Gender	Harbour	H = 14.98, d.f. = 1, $p = <0.01^{-2}$
	Newton's Cove	H = 0.01, d.f. = 1, $p = >0.05$

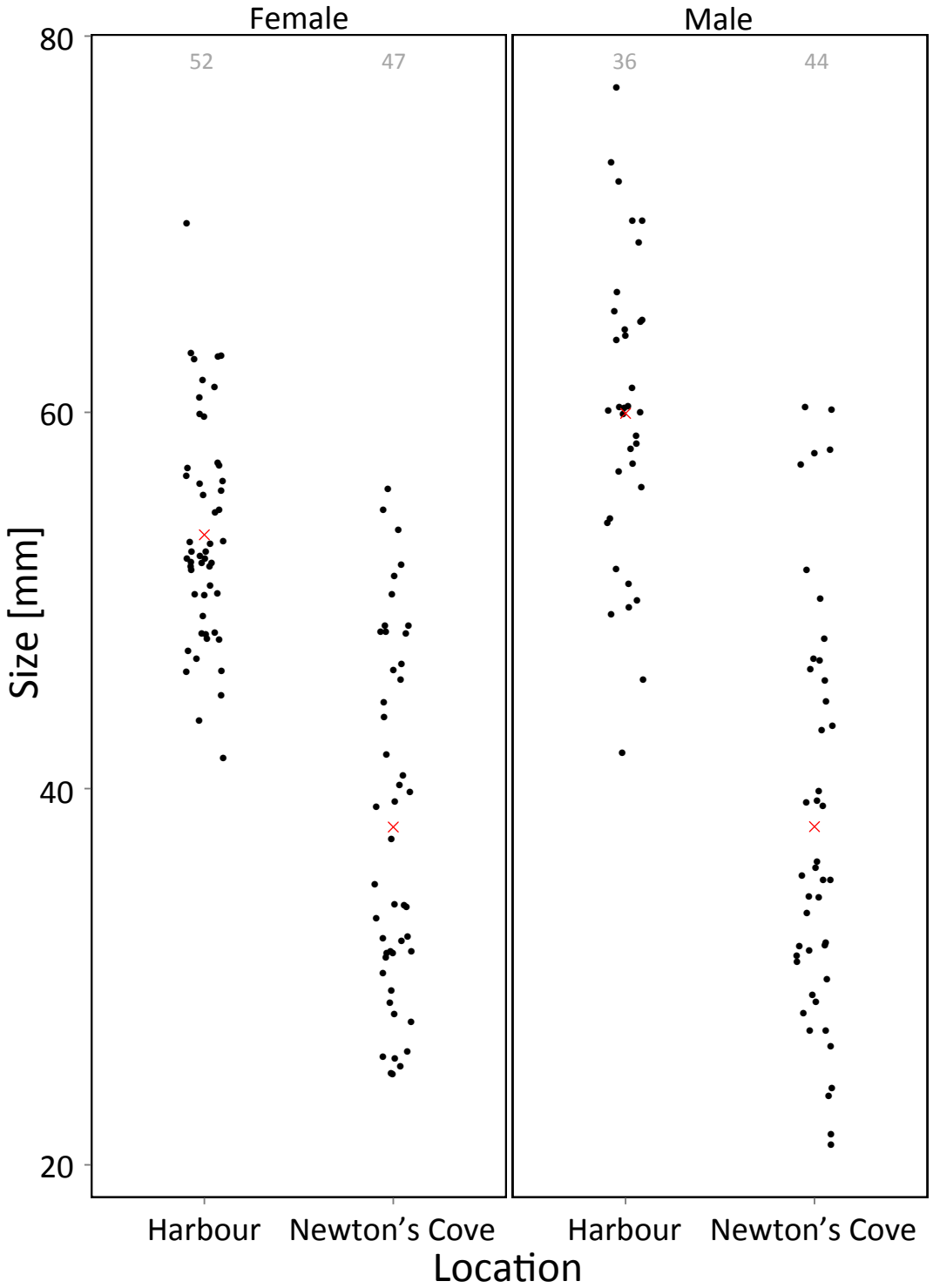


Figure 4.2: Size comparison of *Carcinus maenas* between gender and location. The number of replicates are indicated on the figure and the red 'X' indicates the mean size range in each category.

4.3.2 Identification of pathogens

Prevalence and pathogen identification were determined by histology. As well as identifiable pathogen groups, granulomas (an immune-related pathology), were included as an indication of pathological infection, despite no direct identification of a pathogen *per se*. The pathogen profiles of *C. maenas* from Harbour varied considerably to that of *C. maenas* from Newton's Cove (Table 4.2).

Table 4.2: Prevalence of pathogens from *Carcinus maenas* at Harbour and Newton's Cove including the comparison of pathogen-free crabs as determined by histology.

Pathogens	Harbour	Newton's Cove
Ameson sp. (Microsporidia)	0	4
CmBV (Virus)	0	4
Granuloma (Pathology)	12	23
Haplosporidium littoralis (Protist)	1	1
Hematodinium (Parasitic dinoflagellate)	3	0
HLV (Virus)	1	6
Microphallus sp. (Trematode)	5	82
Milky disease (Gram –ve bacterium)	1	5
Nematode	1	0
Sacculina sp. (Parasitic barnacle)	6	5
No pathogens	64	7
No. of *C. maenas* per site	88	91

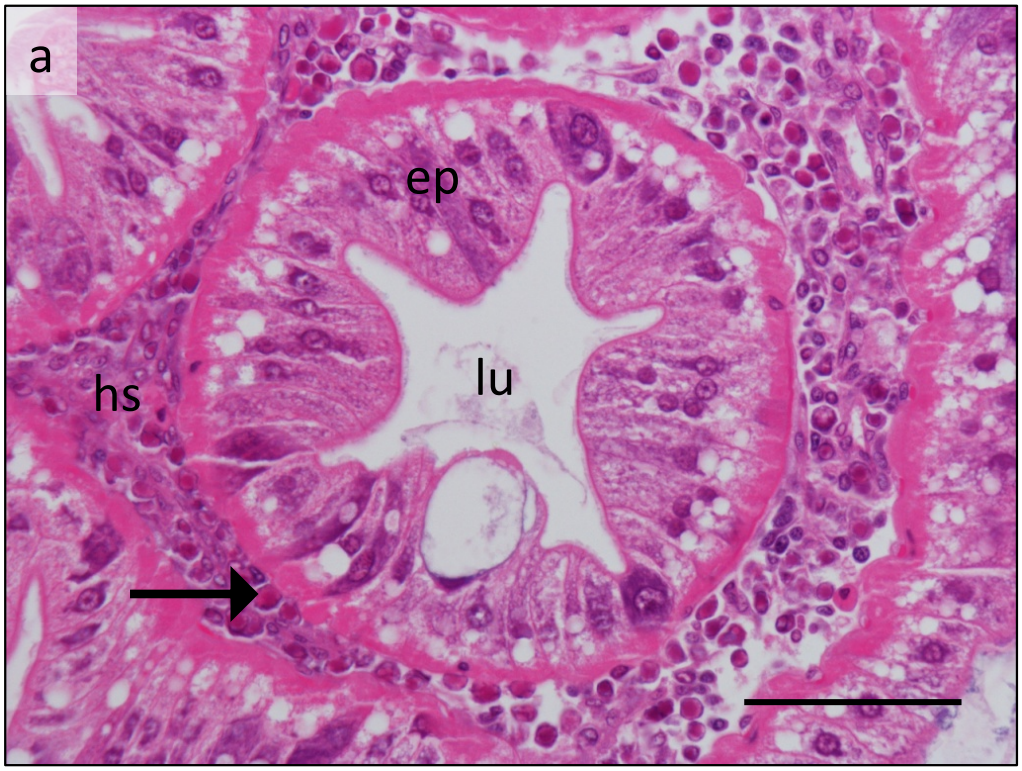
Most notable differences between the pathogen profile of crabs from these locations were the greater proportion of *C. maenas* at Harbour in which no pathogens were observed (73%) compared with Newton's Cove (5%) and the prevalence of *Microphallus* sp. in Newton's Cove (90%) compared with Harbour (6%).

4.3.3 *Carcinus maenas* histology

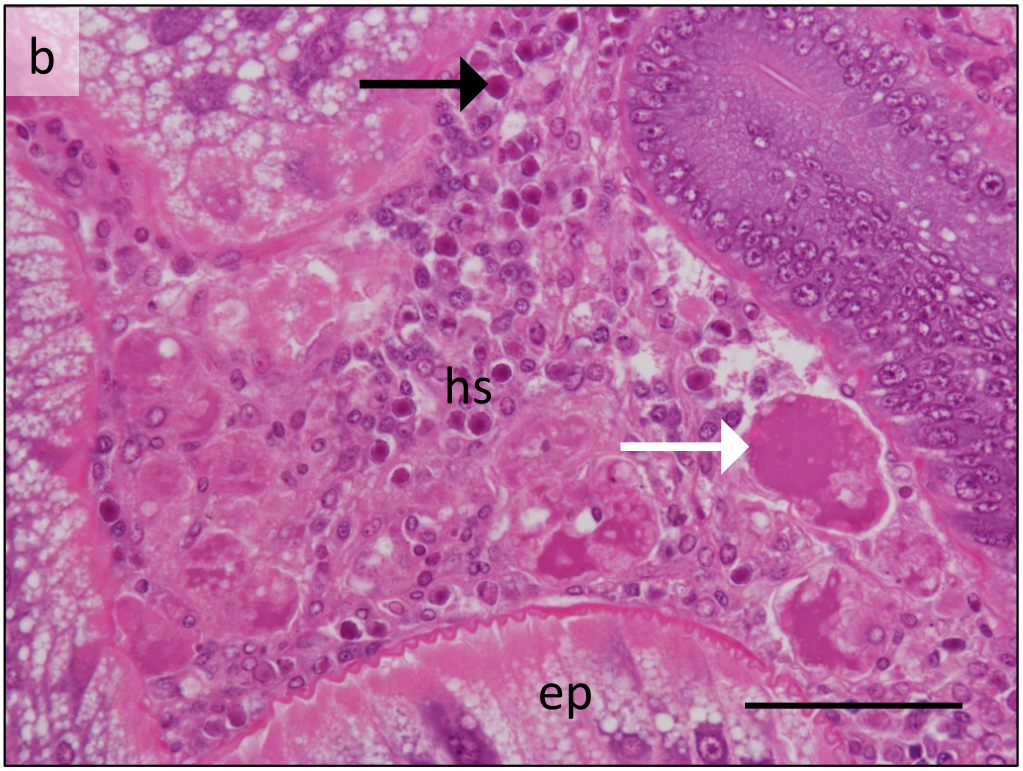
The variety of pathogens identified in *Carcinus maenas*, ranging from viral to helminth infections, are described below.

Viruses

A total of 10 crabs were observed to have viral infections (Figure 4.3). Herpes like virus (HLV) was observed in *C. maenas* at Harbour co-infected with Milky disease (n=1) (Figure 4.3b). In Newton's Cove, crabs observed to have HLV were also all co-infected with *Microphallus* sp. (n=6). A bacilliform virus in *Carcinus maenas*, termed *Cm*BV, was observed in crabs from Newton's Cove only (n=3). Two were co-infected with *Microphallus* sp. and one with *Microphallus* sp. along with non-specific pathology (granulomas).



(a)

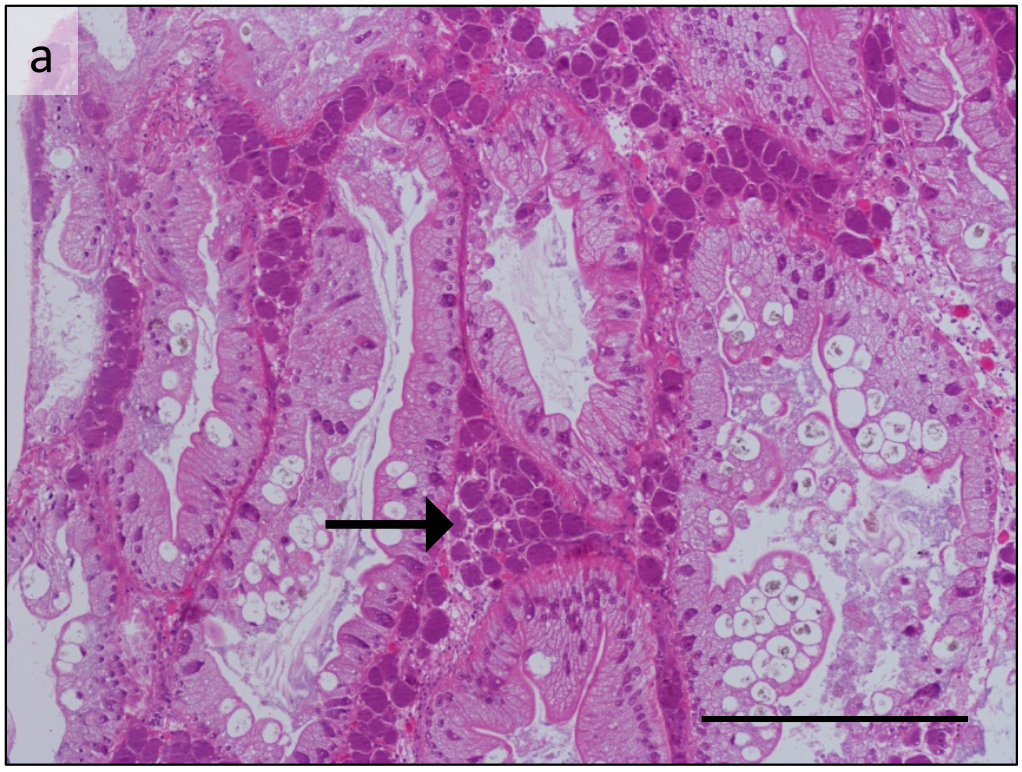


(b)

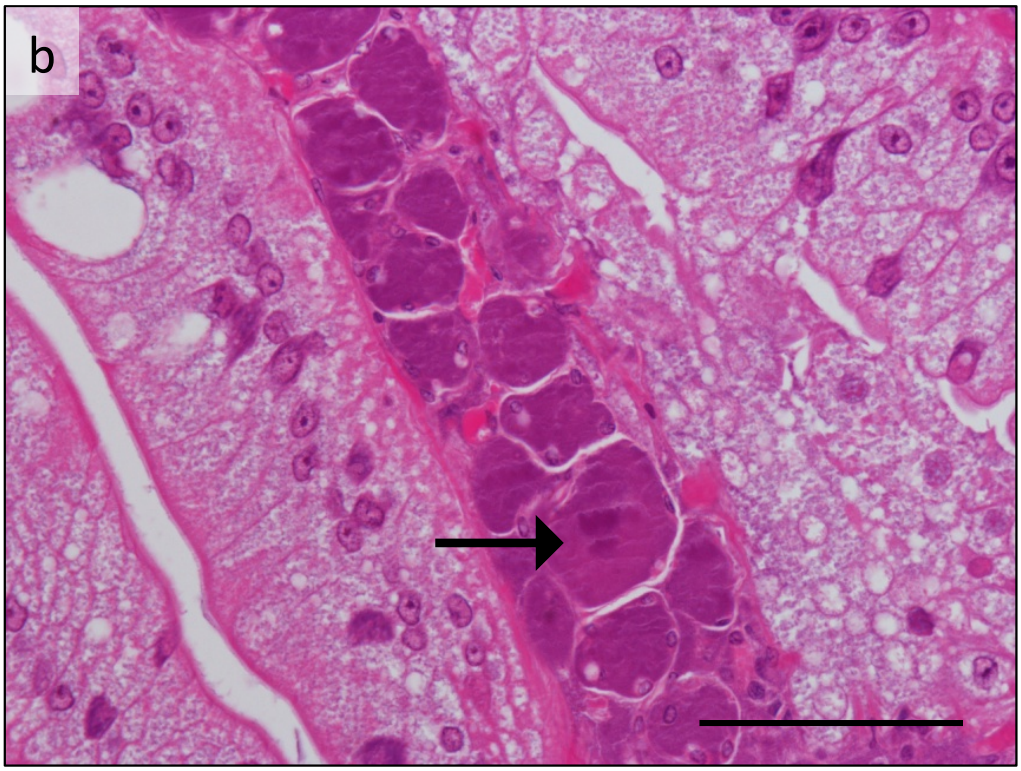
Figure 4.3: (a) Virus infected haemocytes (arrow) within the haemal space (hs) around the hepatopancreatic tubules, defined by the epithelial cells (ep) and lumen (lu), in *Carcinus maenas*. Scale bar 100μm. (b) Co-infection of viral infected haemocytes (black arrow) and intracellular bacteria within the fixed phagocytes (white arrow) in the haemal space around the hepatopancreatic tubules in *Carcinus maenas*. Scale bar 100μm. Both images H&E stained.

Bacteria

Ten bacterial infections were observed in *C. maenas* (Figure 4.4). Milky disease (α-proteobacteria) was identified in animals co-infected with herpes-like virus from Harbour (n=2) (Figure 4.3b) and in Newton's Cove co-infected with *Micropallus* sp. along with granulomas (n=8).



(a)

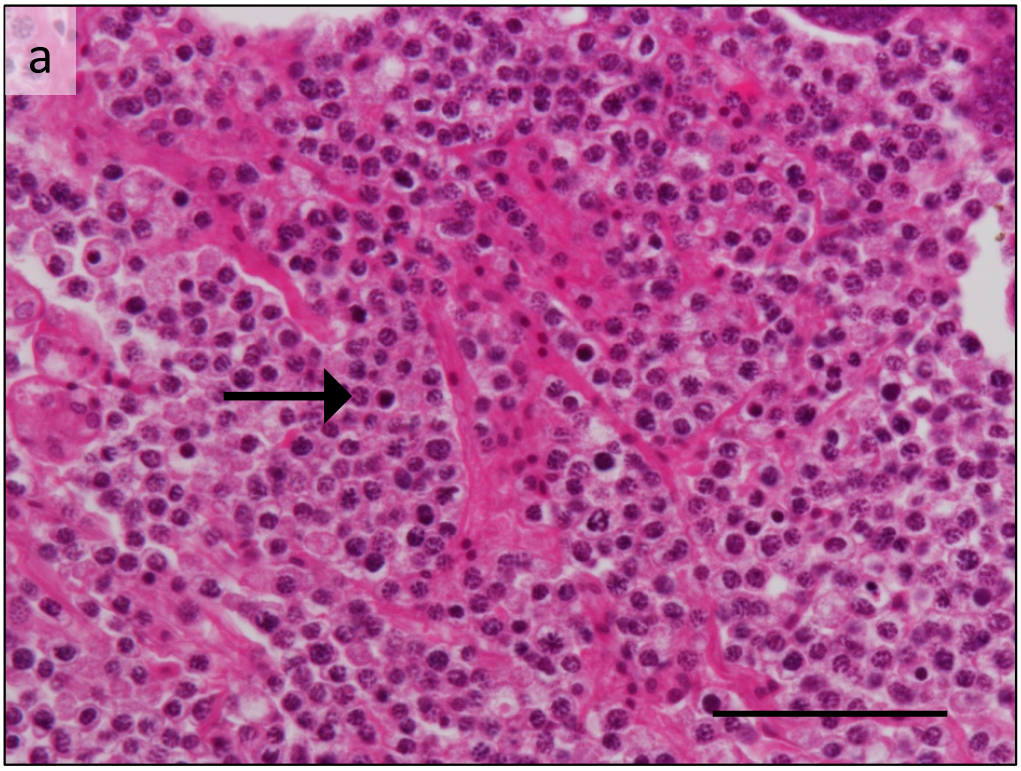


(b)

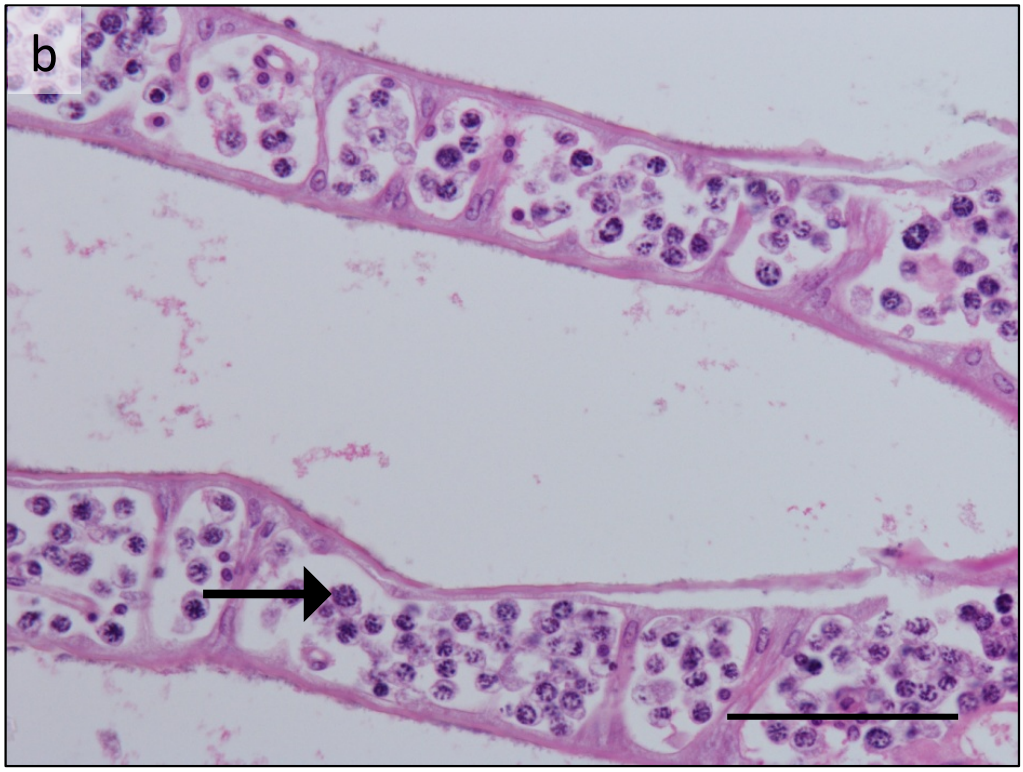
Figure 4.4: Histopathology of milky disease in *Carcinus maenas*. Intracellular bacteria within the fixed phagocytes (arrows) around the hepatopancreatic tubules. (Reviewed in Eddy et al., 2007). (a) Scale bar 200μm. (b) Scale bar 100μm. Both images H&E stained.

Dinoflagellate

The genus *Hematodinium* was identified only in *C. maenas* from Harbour (n=5). Two crabs were observed to host *Hematodinium* sp. as a single pathogen whilst two crabs were co-infected with *Hematodinium* sp. and *Sacculina* sp. and one with *Hematodinium* sp. and *Microphallus* sp. A significant *Hematodinium* sp. infection of the connective tissue of the heart, (showing significant decrease in the amount of expected connective tissue), and of the gill can be observed in Figure 4.5.



(a)



(b)

Figure 4.5: *Hematodinium* sp. infection in the (a) dilated haemal sinuses within the atrophied connective tissue and (b) within the gill of *Carcinus maenas*. Arrows indicate parasites containing the characteristic nuclear profiles, *i.e.* condensed chromosomes. Scale bar 100μm for both (a) and (b). Both images H&E stained.

Protists

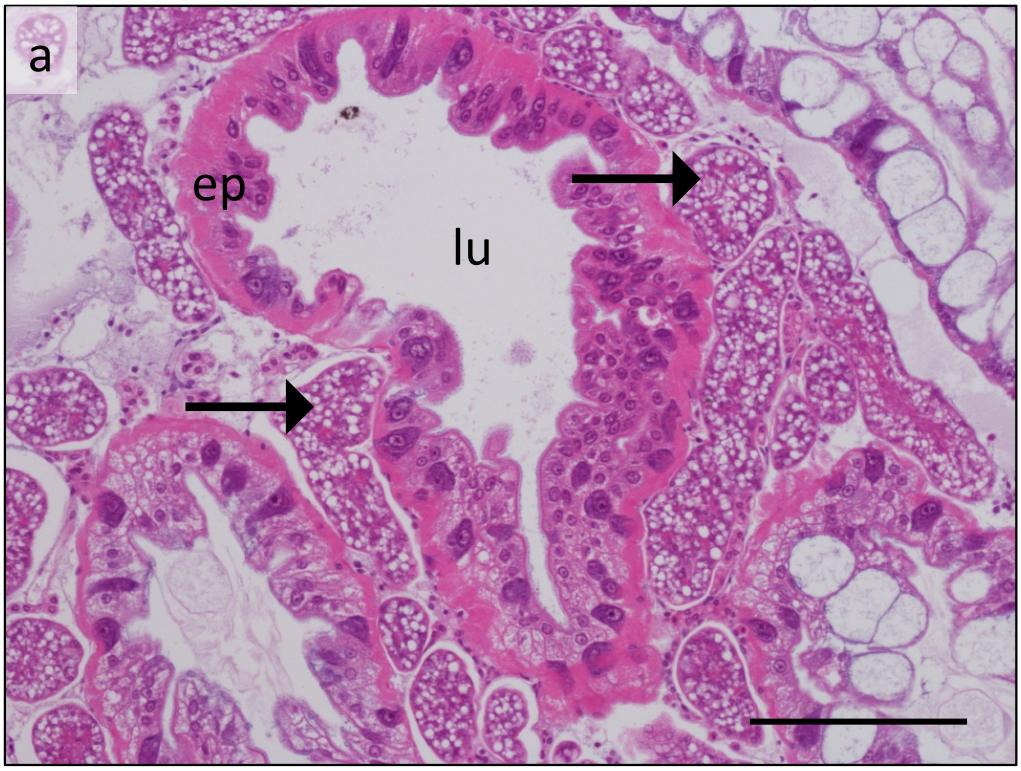
Haplosporidian-like pathogens were observed in two crabs, one from each location. *C. maenas* from Harbour was also found to have granulomas, whilst the crab from Newton's Cove was additionally infected with *Ameson* sp. and *Microphallus* sp..

Fungi

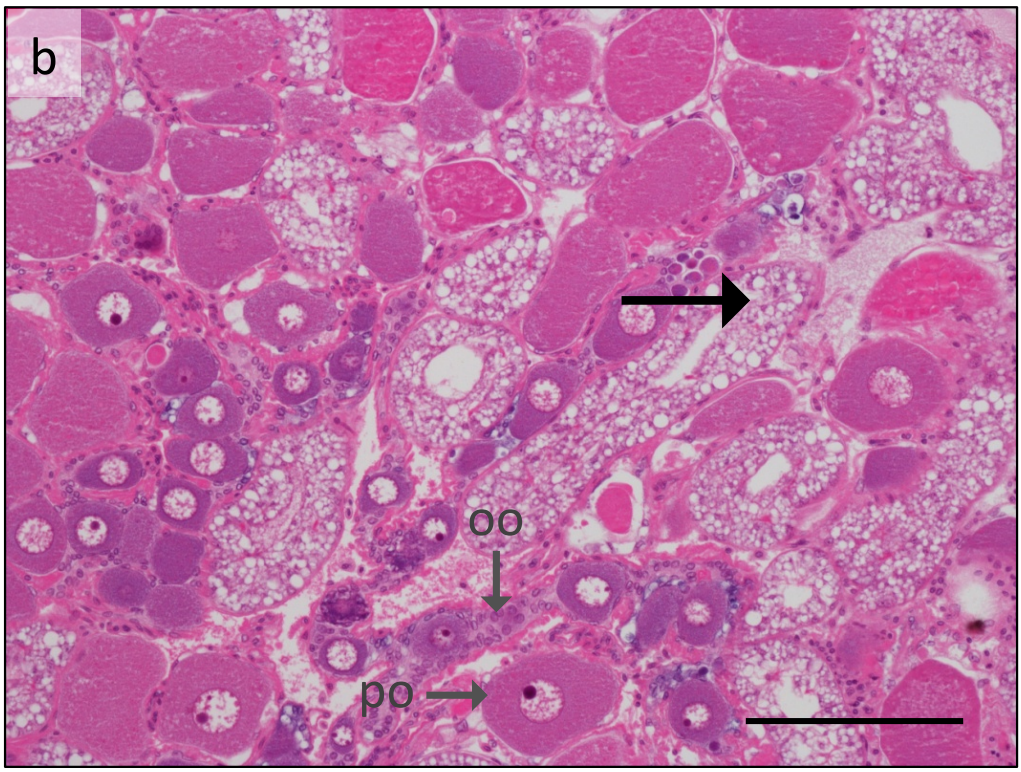
Ameson infection was evident only in Newton's Cove (n=4), co-infected with either *Microphallus* sp. (n=2), *Microphallus* sp. and *Haplosporidian* sp. (n=1) or *Sacculina* sp. (n=1).

Barnacle infection

Sacculina sp. was observed in both Harbour (n=5) and Newton's Cove (n=4). In Harbour, four crabs were infected solely with *Sacculina* sp. and one was co-infected with *Hematodinium* sp.. Whilst in Newton's Cove all *Sacculina* sp. positive crabs had additional infections; two crabs were identified with *Microphallus* sp., one crab was co-infected with *Ameson* sp. and one crab with Milky disease. Figure 4.6 shows *Sacculina* sp. infection in the haemal sinuses in the hepatopancreas (Figure 4.6a) and ovaries (Figure 4.6b).



(a)

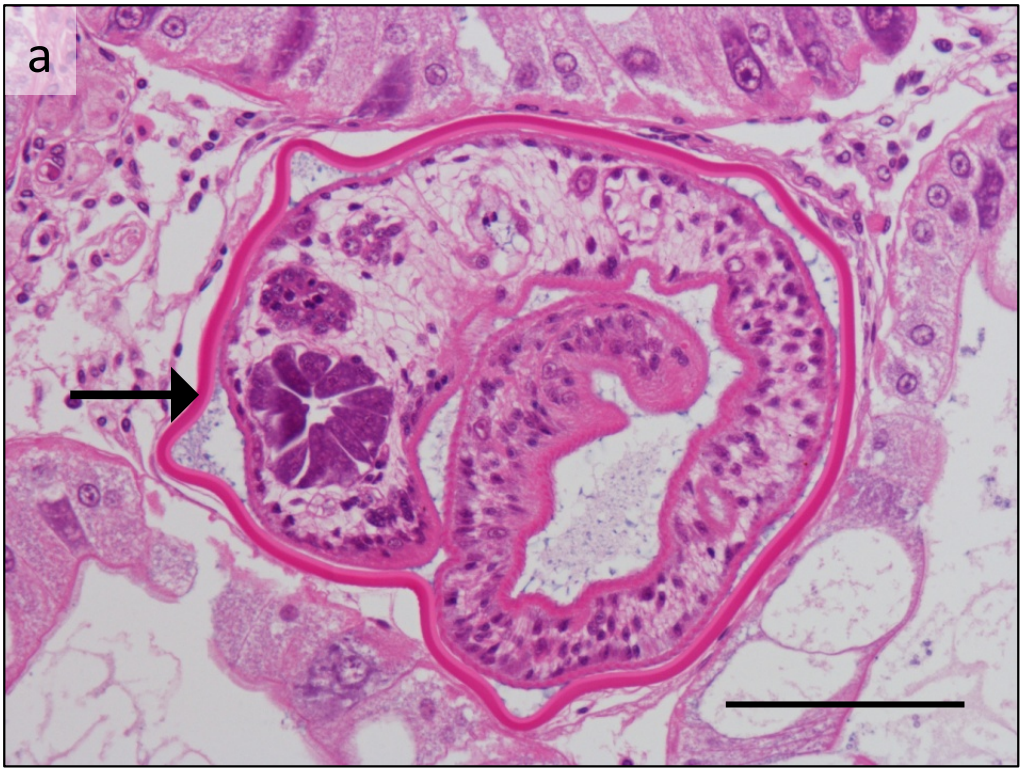


(b)

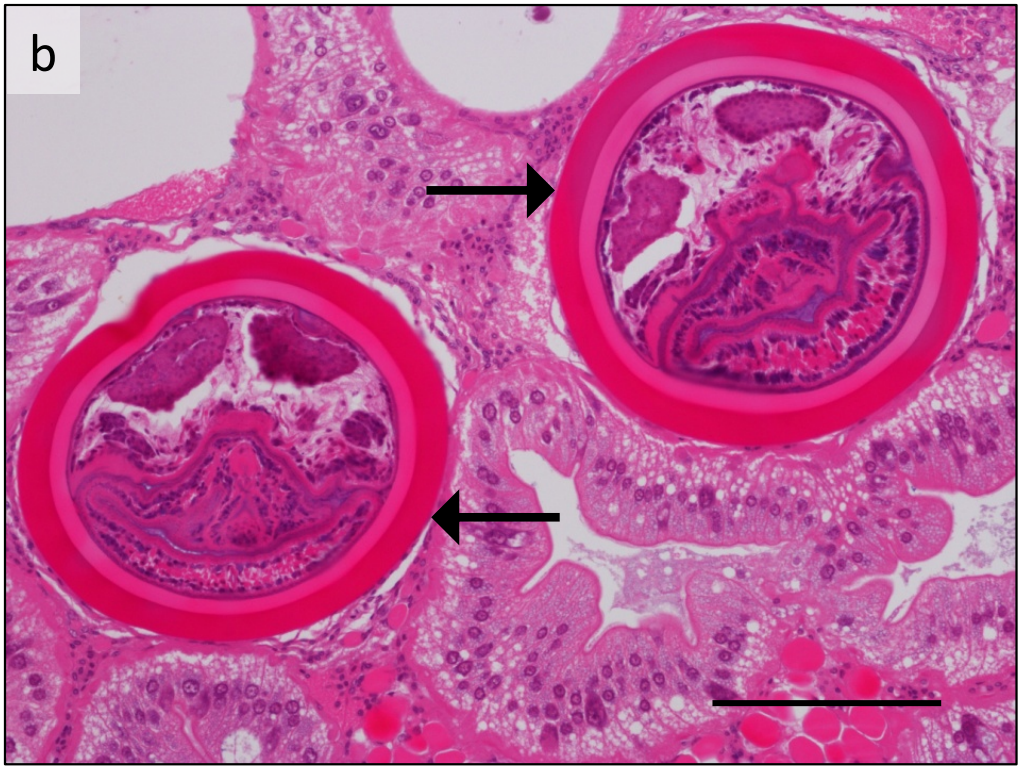
Figure 4.6: (a) Hepatopancreatic tubules identified by the epithelial cells (ep) and lumens (lu) and (b) ovaries of *Carcinus maenas* both surrounded by the rootlets of *Sacculina* sp. (large arrows). (b) The ovaries are at various stages of development evident from the different cells types; oogonias (oo) and primary oocytes (po), (small arrows). Scale bar 200μm for (a) and (b). Both images H&E stained.

Helminth infection

The digenean trematode *Microphallus* (Figure 4.7) was observed in high prevalence in Newton's Cove, (90%) and also in some crabs at Harbour, but at a much lower prevalence (6%). The high incidence of this pathogen at Newton's Cove results in a combination of co-infections with all other identified pathogen groups as well as single pathogen infection of this trematode.



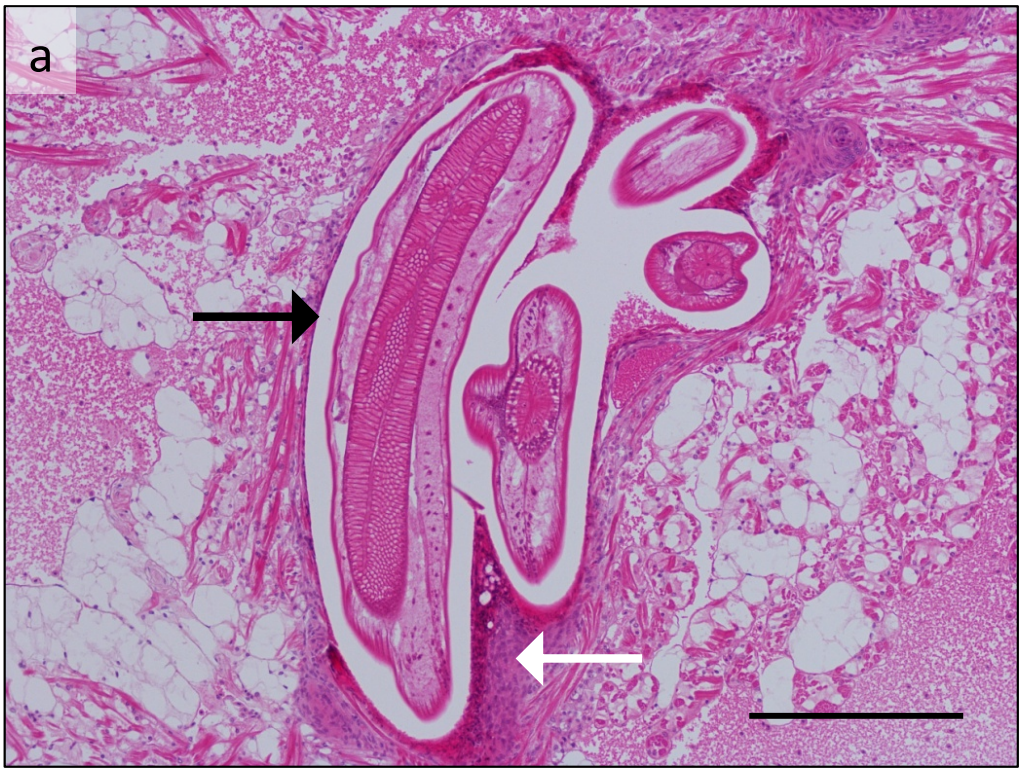
(a)



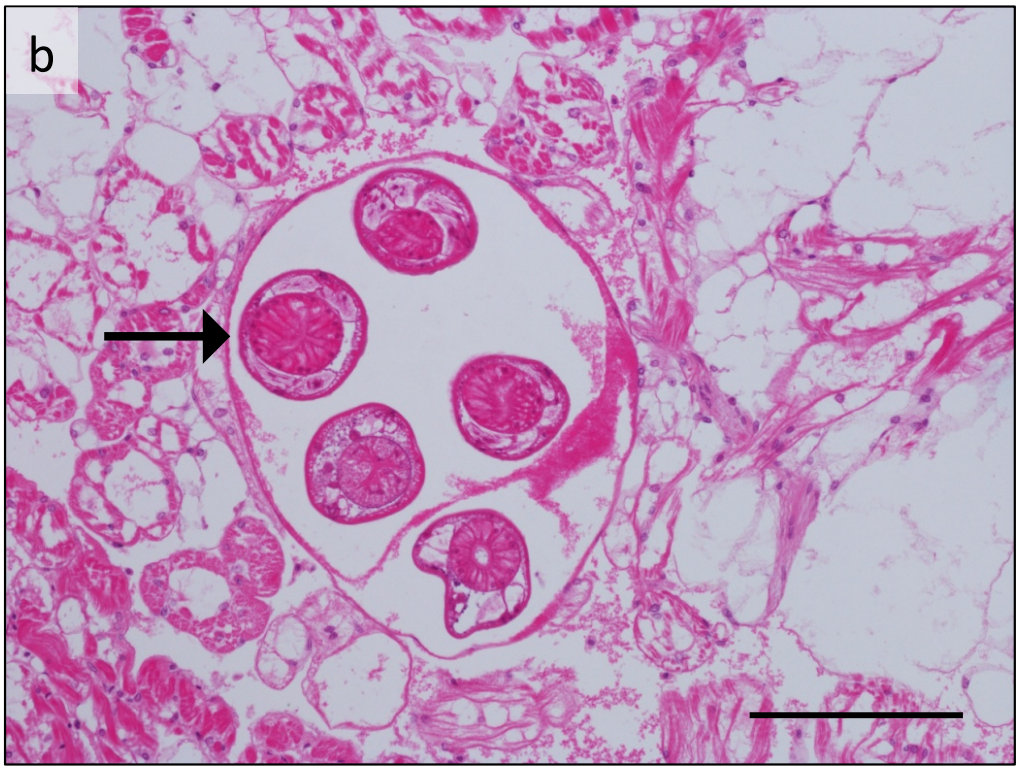
(b)

Figure 4.7: (a) Presumptive early and (b) late stage encapsulation of *Microphallus* sp. in the hepatopancreatic haemal space of *Carcinus maenas*. Scale bar (a) 100μm, (b) 200μm. Both images H&E stained.

A single nematode infection was identified in one *C. maenas* from Harbour. This crab was also observed to have granulomas. The nematode has not been identified to species, but can be observed to infect the heart sinus in Figure 4.8 with associated inflammatory responses indicated by the increased density of haemocytes around the nematode.



(a)



(b)

Figure 4.8: (a) Longitudinal and (b) cross section of a nematode pathogen in the heart sinus of *Carcinus maenas* (black arrows). (a) Inflammatory host response evident around the nematode (white arrow). Scale bar 500μm for (a) and (b). Both images H&E stained.

4.3.4 Analysis of similarity

Analysis of similarity (ANOSIM), a non-parametric analysis of the assemblage of pathogens that clusters similar profiles, (founded on permutation-based hypothesis testing), was carried out in PRIMER (Plymouth Routines In Multivariate Ecological Research) v6 software (PRIMER-E Ltd., 2006) (Clarke and Gorley, 2006). The non-metric multi-dimensional scaling (MDS) in Figure 4.9 represents the patterns in pathogen composition in low-dimensional space (2D) in rank order. Therefore, the relative distances between samples represents the similarity or dissimilarity between the animals in terms of their pathogen assemblage.

Multivariate analysis was based only on the presence or absence of pathogens. Pathogens were identified to either type (*e.g.* bacteria or virus) or to genus level if possible. Of those crabs with infection, many were infected with more than one pathogen, ('No pathogens' = 41.6%, 'Single pathogen' = 34.9%, 'Multiple pathogens' = 23.5% - data for both locations), the combinations of which can be seen in Figure 4.9c.

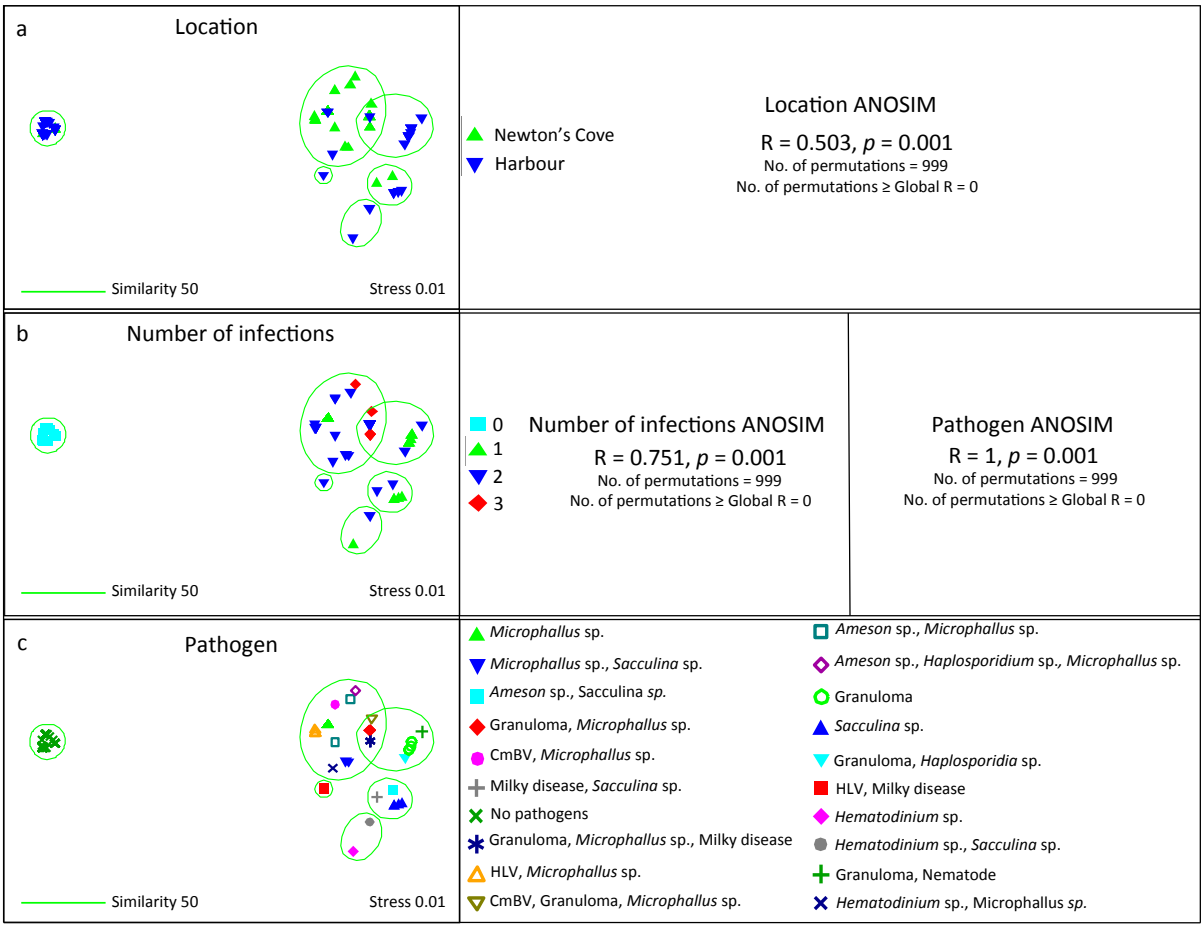


Figure 4.9: MDS of similarity/dissimilarity (at the 50% level) between groups highlighting various factors: a. location, b. the number of infections per crab and c. the various pathogen assemblages. Analysis of Similarity (ANOSIM) of pathogen profile for was calculated for location, the number of infections per crab and the pathogen profiles.

Similarity of the pathogen composition of the crabs was assessed in the context of location as a factor (Figure 4.9a) and repeated, factoring the number of infections per crab (Figure 4.9b) and the variation in pathogen profile (Figure 4.9c). As the mathematical model used for PRIMER does not guarantee the most optimal solution, the analysis requires a number of iterations. Therefore, the analyses were run for 50 random restarts resulting in a low stress of 0.01 indicating the 2D ordination highly represents the relationships amongst the data. As the stress of 0.01 occurred for all 50 restarts, sufficient iterations were considered to be performed.

The pathogen profiles in *Carcinus maenas* at Harbour and Newton's Cove were as similar as dissimilar ($R = 0.503$, $p = 0.001$) suggesting some overlap of pathogens, but also fundamental differences in pathogen assemblages between the locations. ANOSIM of pathogen profiles between crabs factoring the number of pathogen infections per crab revealed high dissimilarity ($R = 0.751$, $p = 0.001$) suggesting very varied combinations of co-infections between crabs. A more detailed look at the pathogen assemblages revealed 20 different pathogen profiles between crabs, highlighting a high level of dissimilarity ($R = 1$, $p = 0.001$). In each analysis, the number of permutations were equal or greater than the observed (global) $R = 0$ (Appendix, Chapter 4, Figure 8.22).

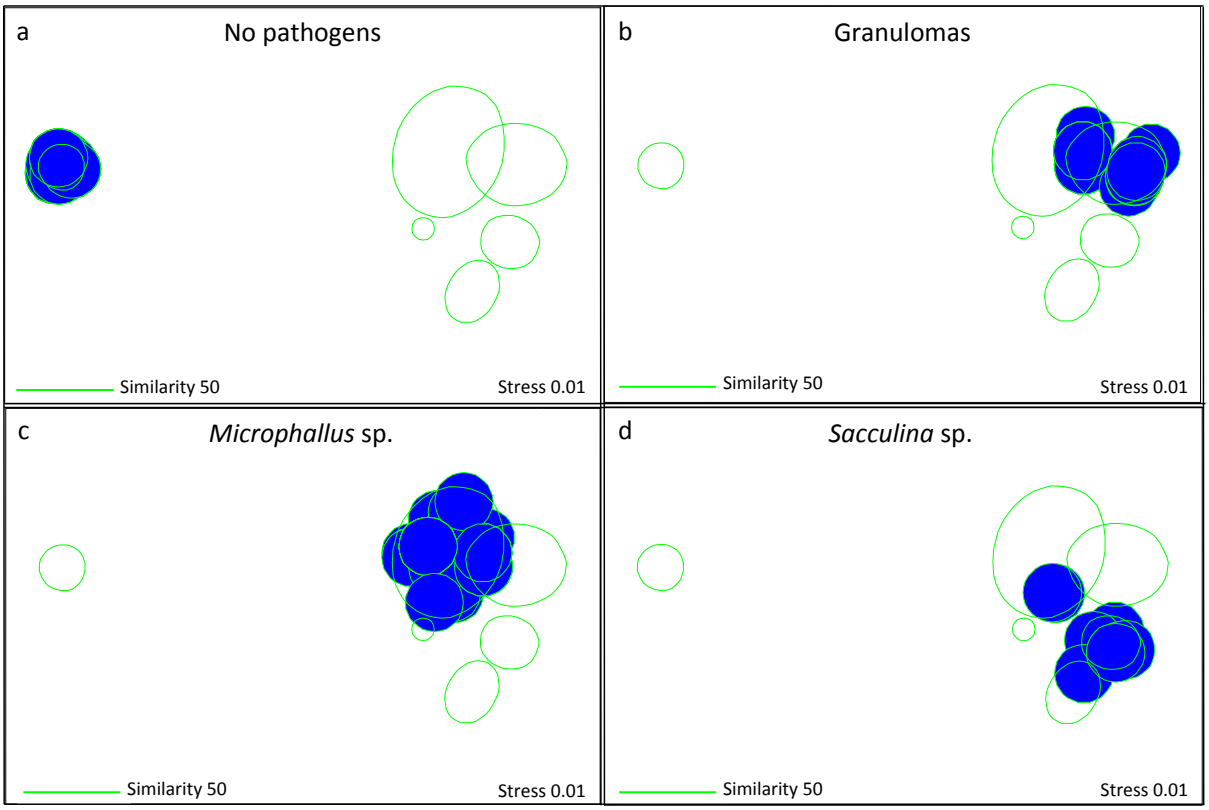


Figure 4.10: MDS of pathogen assemblages in *Carcinus maenas* highlighting the pathogen groups driving the similarity/dissimilarity at the 50% level.

The most notable dissimilarity between groups is that of *C. maenas* with no pathogens, (which dominated the profile in Harbour) and the remaining pathogen groups (Figure 4.10a). The other notable groups driving the relative (dis)similarities were granulomas, *Microphallus* sp. and *Sacculina* sp., (Figure 4.10b, c and d respectively).

4.3.5 Quality control for gene expression analysis

4.3.5.1 RNA quality

RNA quality, based on RQI values up to 10, was assessed using Experion™ software (full description in Chapter 2, Section 2.3.4) . Samples were categorised into four distinct groups and the percentage of incidence calculated; RQI 0.0-2.5 = 15.4%, RQI 2.6-5.0 = 8.7%, RQI 5.1-7.5 = 5.4% and RQI 7.6-10 = 70.5%.

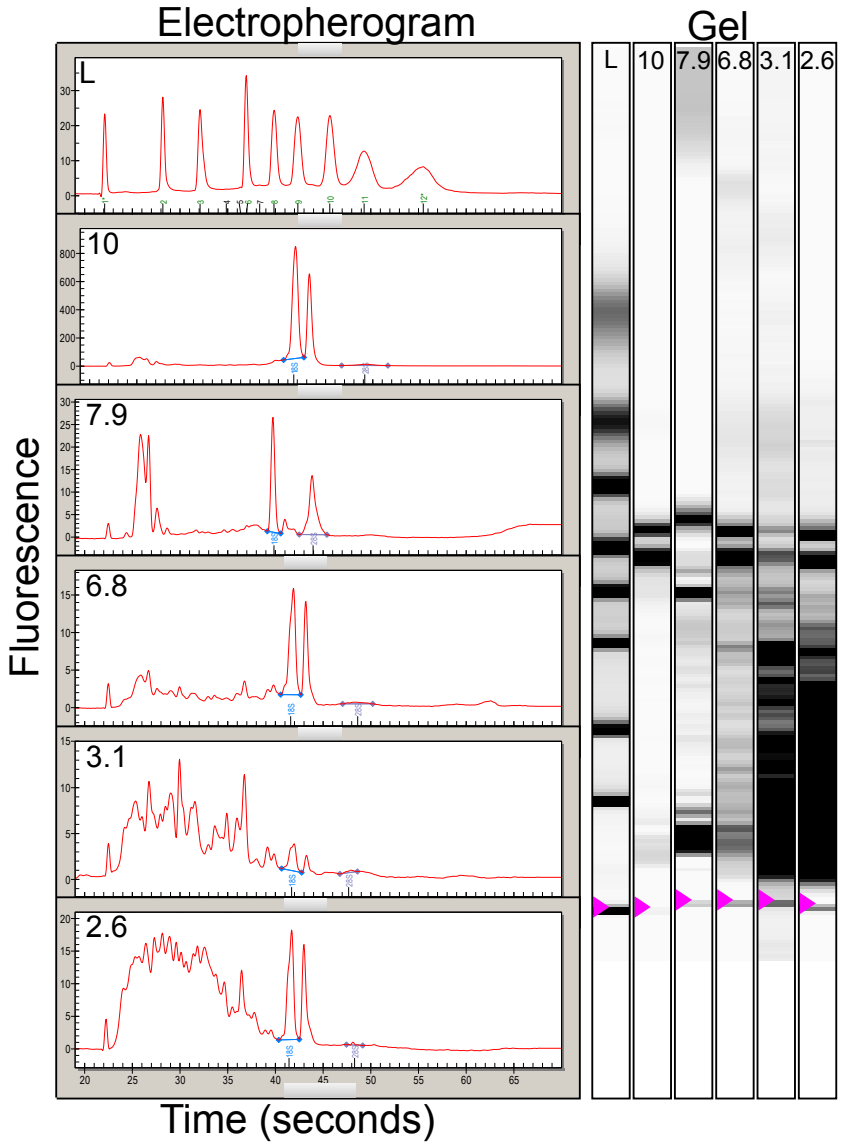


Figure 4.11: Electropherogram and gel analysis of RNA quality using Experion™ software. Illustrations include ladder (L) and highlight high quality RNA (RQI 10) to highly degraded RNA (RQI 2.6).

4.3.5.2 Reference gene stability

Endogenous reference gene stability was analysed using geNorm software within the qbase$^+$ programme (Hellemans et al., 2007) (further description in Chapter 2, Section 2.4.3). Average M values for all four ERG's were 1.466 with a CV of 0.572, *actin* being the most unstable of the ERG's (Figure 4.12 and

Table 4.3). M and CV values were further improved with the removal of *tubulin* as well. Therefore, the most stable combination of ERG's against which to quantify immune gene transcription was *eef1a* and *gapdh*.

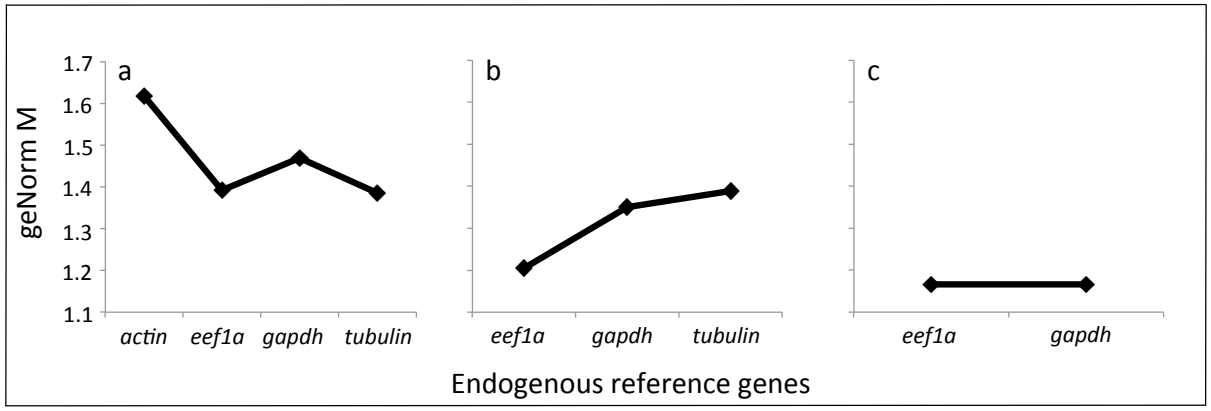


Figure 4.12: geNorm M analysis of ERG's from natural populations of *Carcinus maenas* in Weymouth, UK. The least stable ERG, *actin*, was removed, followed by removal of *tubulin* to leave the most stable ERGs, *eef1a* and *gapdh* with an average M value of 1.166.

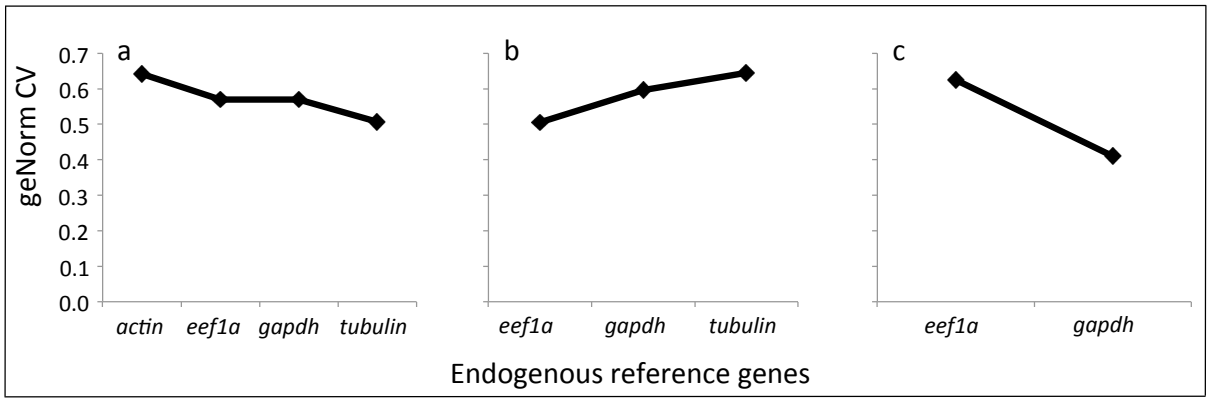


Figure 4.13: geNorm CV analysis of ERG's revealed an average CV of 0.572 using a) all four ERGs, b) 0.582 after removal of *actin* and c) 0.517 using only *eef1a* and *gapdh*.

Table 4.3: The average M and CV values for various endogenous reference genes. Removal of the most unstable gene, *actin*, improved the average M value from 1.466 to 1.314. The most stable gene combination was *eef1a* and *gapdh* with an average M value of 1.166.

Endogenous reference genes	M	CV
actin, eef1a, gapdh, tubulin	1.466	0.572
eef1a, gapdh, tubulin	1.314	0.582
eef1a, gapdh	1.166	0.517

4.3.5.3 Normal distribution and equal variation

Assessment of the normal distribution of the Log_{10}CNRQ data was based on the absence and presence of pathogens (determined by histology). Normal distribution and homogeneity of variance (H_0) was not evident for all groups (Appendix, Chapter 4). Therefore, the non-parametric Kruskal-Wallis H test performed on ranked data was used to analysis the statistical significance of immune gene expression differences between groups.

4.3.6 Analysis of gene transcription data in *carcinin*, *peroxinectin* and *prophenoloxidase*.

Gene expression varied considerably for all immune genes. This is seen in Figure 4.14 which shows the Log_{10}CNRQ values for *C. maenas* at both locations including all samples. Samples were categorised according to pathogen assemblage in each group. It was not possible to distinguish between what was a true reflection of the immune gene transcription relative to the ERGs in the animals in response to infection and that of artifact, potentially as a result of unreliable reverse transcription of cDNA from samples with degraded RNA. Therefore, statistical analysis of immune gene transcription was carried out only on those samples with an RQI $\geq$7.6.

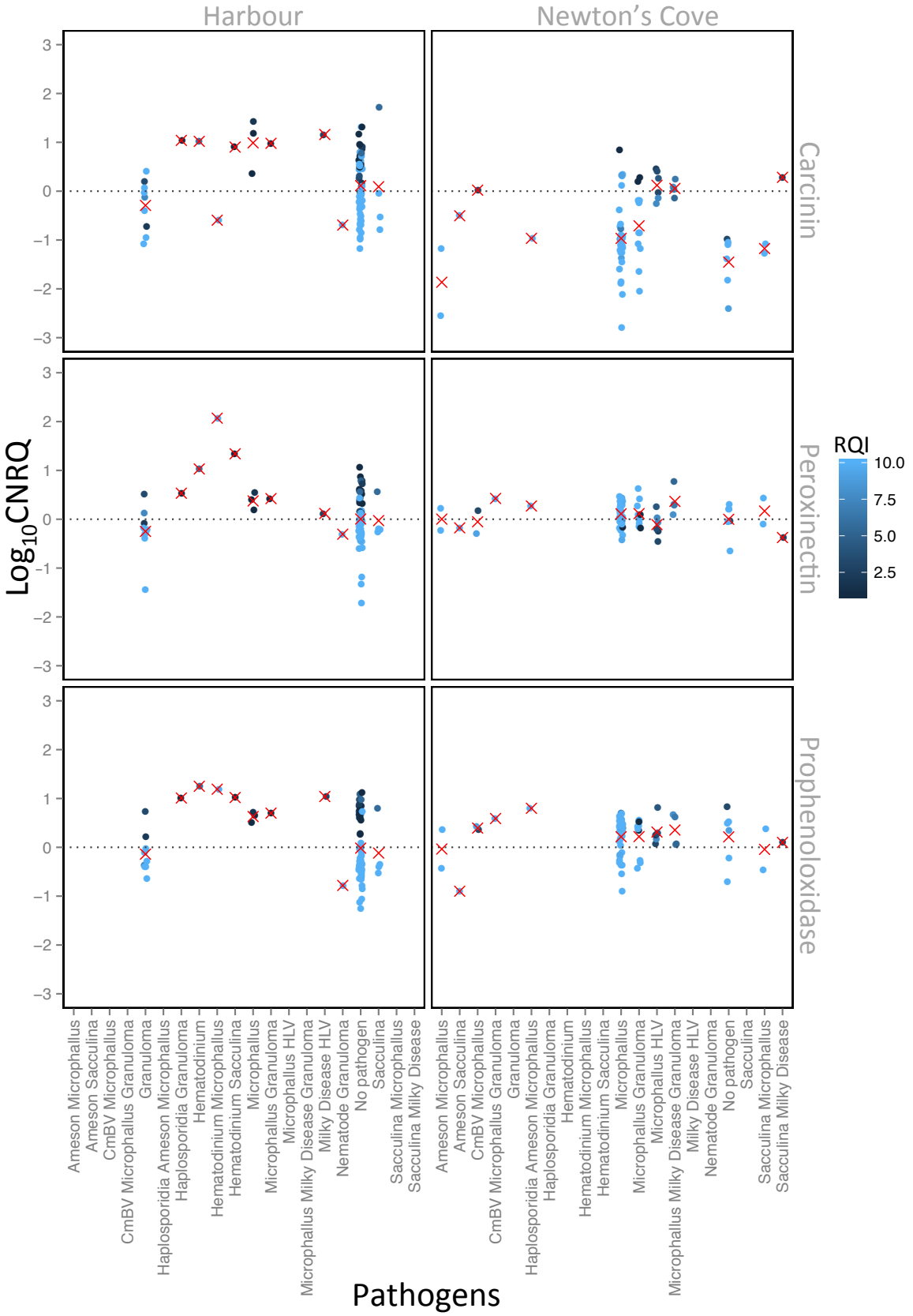


Figure 4.14: Overview of expression values (Log_{10}CNRQ) for all immune genes including all samples regardless of RNA quality. Red 'X' identifies the mean Log_{10}CNRQ. Lower quality RNA is indicated by progressively darker blue colour. Total n = 88 and 91 for Harbour and Newton's Cove respectively. The number of replicates varied from 0-56. See Appendix, Chapter 4, Table 8.20 for full details of the number of replicates per pathogen grouping.

4.3.6.1 Comparison of immune gene expression based on the absence or presence of pathogens within the host animals.

The following analysis was based on samples with RQI values >7.5. It was not possible to statistically analyse the gene transcription within the defined pathogen groups due to multiple infections of many *C. maenas*. This would have resulted in repeated statistical analysis of individual animals. Therefore, immune gene transcription was directly compared between those animals with and without infection within each location.

Within site analysis

Kruskal-Wallis non-parametric analysis did not reveal any significant statistical difference in the gene expression of *carcinin*, *peroxinectin* and *prophenoloxidase* ($p>0.05$) within each location (Table 4.4).

Table 4.4: Immune gene expression analysis based on the absence and presence of pathogens using the Kruskal-Wallis H test.

Gene transcription analysis between 'No Pathogen' and `Pathogen' groups within site		
Carcinin	Harbour	H = 1.93, d.f. = 1, p = >0.05
	Newton's Cove	H = 3.14, d.f. = 1, p = >0.05
Peroxinectin	Harbour	H = 0.44, d.f. = 1, p = >0.05
	Newton's Cove	H = 0.22, d.f. = 1, p = >0.05
Prophenoloxidase	Harbour	H = 0.08, d.f. = 1, p = >0.05
	Newton's Cove	H = 0.10, d.f. = 1, p = >0.05
Gene transcription analysis of 'No Pathogen' and 'Pathogen' groups between Harbour and Newton's Cove		
	No Pathogen	**Pathogen**
Carcinin	H = 12.41, d.f. = 1, p = <0.001	H = 8.12, d.f. = 1, p = <0.01
Peroxinectin	H = 2.41, d.f. = 1, p = >0.05	H = 12.91, d.f. = 1, p = <0.001
Prophenoloxidase	H = 3.17, d.f. = 1, p = >0.05	H = 8.24, d.f. = 1, p = <0.01

Between site analysis

Immune gene transcription was also analysed between Harbour and Newton's Cove within each defined group; 'No pathogen' and 'Pathogen'. *Carcinin* expression was significantly different between locations for both the 'No pathogen' ($p<0.001$) and 'Pathogen' ($p<0.01$) groups (Table 4.4), with comparatively lower transcription in *C. maenas* from Newton's Cove (Figure 4.15). Comparison of *peroxinectin* and *prophenoloxidase* expression in the 'No pathogen' group between locations did not show any significant difference ($p>0.05$). However, gene transcription of *peroxinectin* and *prophenoloxidase* in the 'Pathogen' group was significantly different between locations at $p<0.001$ and $p<0.01$ respectively, with higher gene expression in Newton's Cove compared with Harbour.

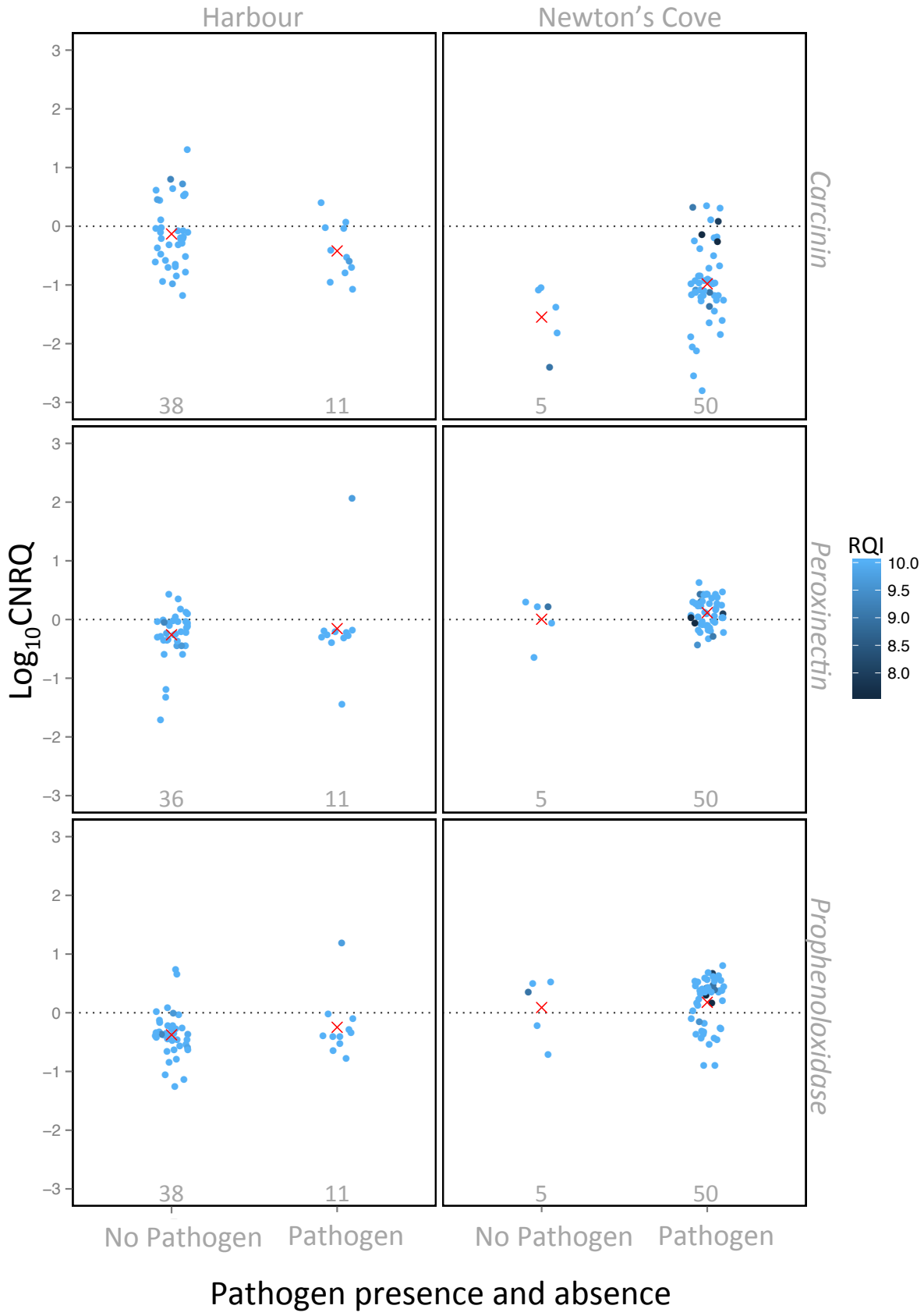
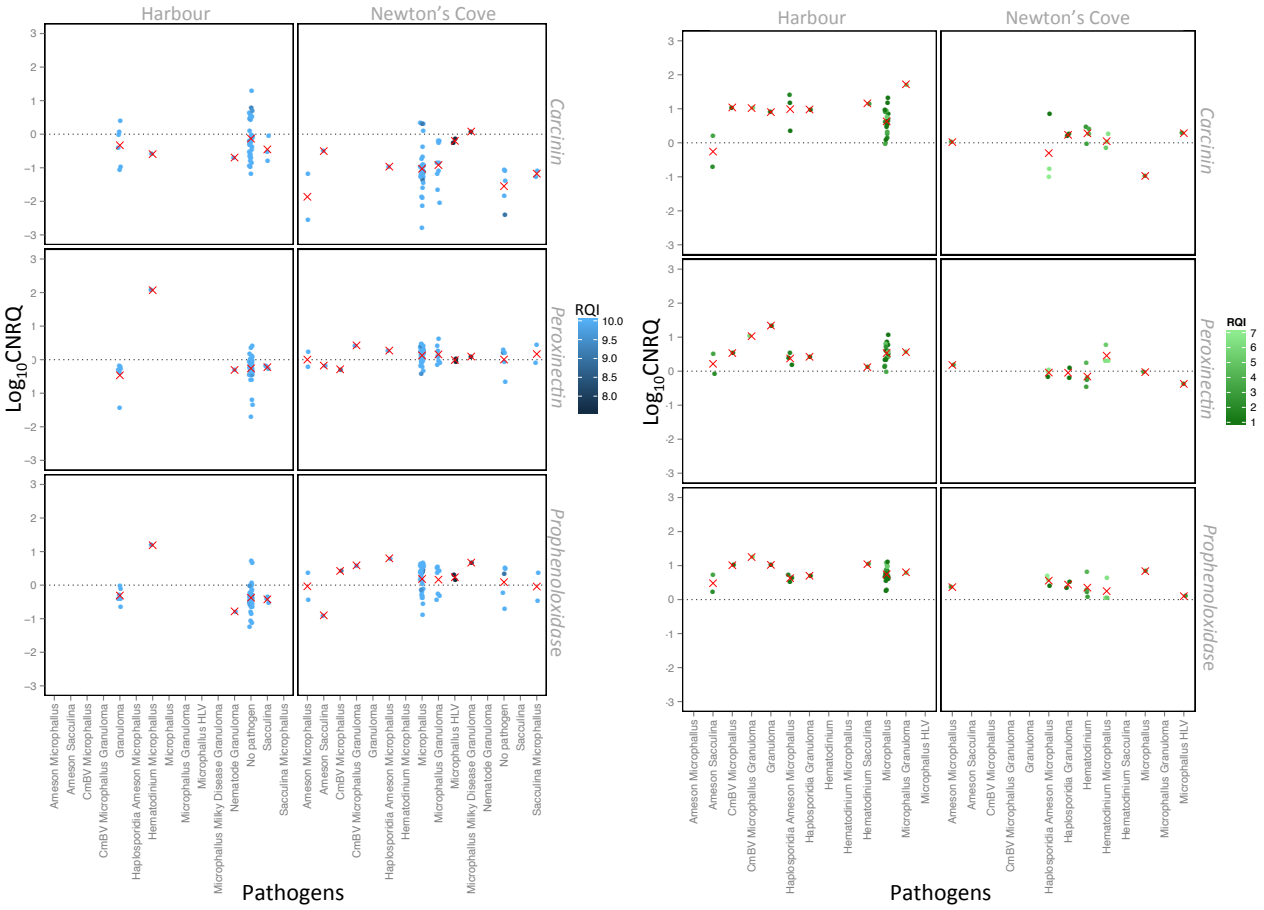


Figure 4.15: Analysis of absence and presence of pathogens on immune gene transcription, based on high quality data only. Red 'X' denotes mean Log_{10}CNRQ. The number of replicates are indicated under each category on the figure.

4.3.7 Comparison of immune gene transcription in relation to RNA quality.

Processing of all samples for gene expression also included assessment of RNA integrity. Fundamental differences were highlighted in the quality of some of the samples, such that it was not possible to confidently analyse the immune gene expression for samples with poor RNA integrity. For the purpose of maintaining high quality results as stipulated by the MIQE guidelines, only those samples with an RQI value ≥ 7.6 were included in any statistical analysis of gene expression. Figure 4.16 illustrates the differences in the transcription of immune genes between those with an RQI ≥ 7.6 and those with an RQI ≤ 7.5. As an overall qualitative assessment of gene transcription, many crabs identified as having low quality RNA (RQI ≤ 7.5) appeared to have greater transcription of immune genes in comparison to those crabs identified as having high quality RNA (Figure 4.16).



(a) High quality RNA only (≥ 7.6).

(b) Low quality RNA only (≤ 7.5).

Figure 4.16: Gene transcription for all immune genes at Harbour and Newton's Cove in relation to pathogen assemblage using both high and low RNA quality. a) Decreasing quality of RNA integrity is indicated by increasingly darker blue colouring (RQI 7.6-10). b) All high quality data has been removed leaving RQI values of 7.5 and below. Red 'X' indicates mean Log_{10}CNRQ. Total n = 88 and 91 for Harbour and Newton's Cove respectively. The number of replicates varied from 0-38 (high RQI) and 0-18 (low RQI). See Appendix, Chapter 4, Tables 8.21 and 8.22 for the full number of replicates per pathogen grouping for high and low RQI groups respectively.

Combinations of the different pathogen profiles related to the different categories of RNA quality are identified in Table 4.5. No single pathogen can be identified in relation to low RNA quality alone. All pathogens or pathology associated with low RNA quality are also identified either as a single infection or co-infection with other pathogens in samples from high quality RNA. Of all crabs sampled, 70% had RNA with RQI values ≥ 7.6 and 30% had variably degraded RNA (≤ 7.5). Comparison of high quality RNA found 48% of *C. maenas* were sourced from Harbour and the remaining 52% from Newton's Cove. The proportion of crabs with low quality RNA from Harbour and Newton's Cove were found to be 64% and 36% respectively. Interestingly, the dominant group driving the larger proportion of poor quality RNA at Harbour was the 'No pathogen' group, responsible for 40% of all low quality RNA samples (Table 4.5). The next largest group with low quality RNA were *C. maenas* with either *Microphallus* sp. or *Microphallus* sp. co-infected with herpes-like virus (HLV), each group consisting of 9% of the low RNA quality samples.

Table 4.5: Different combinations of pathogen assemblages in *Carcinus maenas* isolated from samples identified as having either variable RNA quality (high and low RQI) or high or low RQI only.

Pathogen assemblages	**Harbour**		**Newton's Cove**	
	High RQI (%)	**Low RQI (%)**	**High RQI (%)**	**Low RQI (%)**
CmBV, Microphallus sp.	0	0	2	2
Granuloma	7	4	0	0
Microphallus sp.	0	7	29	9
Microphallus sp., Granuloma	0	2	7	4
Microphallus sp., HLV	0	0	2	9
Microphallus sp., Milky disease, Granuloma	0	0	1	7
No pathogens	36	40	5	2
Sacculina sp.	3	2	0	0

Pathogen assemblages	**High RQI (%)**	
	Harbour	**Newton's Cove**
Ameson sp., *Microphallus* sp.	0	2
Ameson sp., *Sacculina* sp.	0	1
CmBV, Microphallus sp., Granuloma	0	1
Haplosporidia sp., *Ameson* sp., *Microphallus* sp.	0	1
Hematodinium sp., *Microphallus* sp.	1	0
Nematode, Granuloma	1	0
Sacculina sp., *Microphallus* sp.	0	2
Pathogen assemblages	**Low RQI (%)**	
	Harbour	**Newton's Cove**
Haplosporidia sp., Granuloma	2	0
Hematodinium sp.	2	0
Hematodinium sp., *Sacculina* sp.	2	0
Milky disease, HLV	2	0
Sacculina sp., Milky disease	0	2

4.4 Discussion

Understanding disease in the wild is made complicated by the influence of numerous biotic (*e.g.* host susceptibility to pathogens, trophic interactions) and abiotic factors (*e.g.* local hydrodynamic and ecotoxicology variables) that influence both the distribution of pathogens and host susceptibility to those pathogens. The focus of this study was to investigate the difference in host immunocompetence, in terms of transcription of important host immune genes (*carcinin, peroxinectin* and *prophenoloxidase*), in relation to the pathogen profile of two geographically close, but distinct wild populations of *Carcinus maenas*.

4.4.1 Size comparison of *Carinus maenas*

Initial comparison of the size difference of *Carcinus maenas* between locations revealed significantly larger crabs in Harbour (females $p<0.01^{-9}$, males $p<0.01^{-8}$) compared with Newton's Cove. In addition, there was no significant size difference in Newton's Cove, between male and female *C. maenas* ($p>0.05$), but males were larger than females in Harbour ($p<0.01^{-2}$) (Figure 4.2 and Table 4.1). This is likely to be a reflection of the increased exposure *C. maenas* at Newton's Cove were subject to each tidal cycle and the increase in food availability in Harbour. Particularly as Harbour is a popular crabbing site for tourists, whereby *C. maenas* frequently consumed bait. Furthermore, *C. maenas* have a greater opportunity to feed as they remain immersed throughout the entire tidal cycle. Conversely, *C. maenas* in Newton's Cove were emersed throughout part of the tidal cycle, reducing the opportunity to feed as their exposure to predators, such as birds, increased. The smaller size in Newton's Cove may facilitate concealment from predation in small rock crevices and under stones and boulders during emersion.

Reduced food intake has been shown to increase the intermoult period and reduce the moult increment in *C. maenas* (Breteler, 1975) as well as other species such as *Crangon crangon* (Oh and Hartnoll, 2000). In a study investigating the differential growth rates of different populations of adult male rock lobsters, *Jasus lalandii*, off the West coast of South Africa, availability of preferred prey was determined to be the driving factor responsible (Mayfield et al., 2000).

In addition, Trussell et al. (2006) argued that predation risk can strongly influence energy transfer and linked factors associated with predation to cause a 32% variation in growth efficiency, reducing growth by 44-76% in an intertidal rocky shore. This is consistent with the prediction that an increased predation risk reduces foraging activity, thus reducing individual growth (Lima and Dill, 1990; Werner and Anholt, 1993; Vadas et al., 1994). As well the potential for reduced food intake to limit growth in the *C. maenas* population at Newton's Cove, as the more exposed location, it is also possible that some of the energy available for growth was redistributed towards processes required for stress responses (Maltby, 1999). Ultimately, definitive experiments would be required in order to investigate the driving factors that were responsible for the significant differences in population size between *C. maenas* at Newton's Cove and those in Harbour.

4.4.2 Pathogen prevalence at Harbour and Newton's Cove

Pathogen identification revealed both similarities as well as fundamental differences in the pathogen profile of *C. maenas* populations between Harbour and Newton's Cove. Overall, there were 20 different

pathogen assemblages of either single or multiple pathogen infections of *C. maenas*, indicating considerable variation in the pathogen profiles. Analysis of similarity (ANOSIM) of pathogen assemblages between these *C. maenas* populations (R=0.503, p=0.001, Figure 4.9), suggests an overlap of pathogen taxa between locations, but also differences in pathogen profiles between these populations. These differences in pathogen profiles were predominantly driven by the number of crabs where no pathogens were observed (73% in Harbour and 5% in Newton's Cove). Futhermore, the dominant pathogen group overall was identified as *Microphallus* sp., representing 48% infection prevalence in total. The next most frequently observed pathogen was *Sacculina* sp., observed in 6.5% of all *C. maenas*. However, granulomas were evident in 19.5% of all crabs, although the source for this pathology could not be identified (Figure 4.10). In addition, there was considerable dissimilarity in the number of infections observed per crab (R=0.751, p=0.001) which also reflects the variety of pathogen assemblages observed in individual crabs.

Overview of pathogens in *Carcinus maenas*

Overall, there was a range of different phyla amongst the pathogens observed in *C. maenas*, including viruses, fungi, protists, bacteria and helminth infections.

Viruses

Two viral infections were observed; herpes-like virus (HLV) and an intranuclear bacilliform virus, (BV). HLV mainly infects the haemocytes (Johnson, 1978), as observed in *C. maenas*, although infection of the mesodermal cells of the male gonad in the mud crab *Rithropanopeus harrisii* (Sparks and Morado (1986) citing Payan and Bonami, 1979) and epithelial cells of the antennal gland of the blue king crab, *Parathithodes platypus* have been reported (Sparks and Morado, 1986). Bacilliform virus, termed *Cm*BV in *C. maenas*, is evident in other crustacea (Edgerton, 1996; Bateman and Stentiford, 2008). Severe infection can result in storage depletion in the reserve cells of the hepatopancreas, subsequently followed by necrosis and loss of the structural integrity of tissues (Stentiford and Feist, 2005).

Only 1% of crabs in Harbour compared with 10% in Newton's Cove were observed with viral infections. It is important to note that only presence and absence of pathogens was measured and prevalence does not necessarily relate to disease state. Interestingly, no immune response, in terms of apoptosis and tissue necrosis, was observed in the histological preparations in those crabs observed with viral infections. It may be that either some infections were not sufficiently systemic to result in a detectable host immune response, that the histopathology samples taken did not capture the immune response at a multi-cellular level or potentially the host immune system did not recognise the infection. In one *C. maenas*, co-infection of a viral pathogen and bacteria was observed (Figure 4.3b). It is not possible to know which pathogen was the primary infection, but it may be beneficial to the host to contract secondary infections that stimulate an immune response, (although not evident in this particular case). Hypothetically, the secondary infection may then result in an inadvertent host immune response against the primary pathogen. Conversely, it may be that primary infection results in immunosuppression in the host leading to secondary infections that may be too energetically demanding for the host to respond against (Slifka et al., 2003). Certainly in the case of the viral and bacterial co-infection, no pathology associated with immune response was detected. This in itself poses an interesting question as to how pathogens are able to avoid detection from the host?

Bacteria

Milky disease derives it name from the characteristic milky appearance of the haemolymph seen in infected crabs. The causative agent of the disease is a Gram negative bacterium, which is phylogenetically defined as a member of the $\alpha-proteobacteria$, based on the 16s rRNA gene, but with little similarity to *Rhodobacteriales* and distinct from other members of the *Rickettsiales* (Eddy et al., 2007). It is found to multiply in the fixed phagocytes situated within the extra-tubular haemal spaces of the hepatopancreas. Interestingly, in the histology samples observed in *C. maenas*, no evidence can be seen of any changes associated with a response from the circulating haemocytes to the presence of the bacteria (Figures 4.3b and 4.4), suggesting this pathogen was not recognised by the immune system of the host. Milky disease was evident in 1% of crabs in Harbour (female, 53mm carapace width) and 5% of crabs in Newton's Cove (male and females, size range 28-55mm carapace width). These crabs were sampled during June when seawater temperatures averaged 15.2°C. Eddy et al. (2007) noted a greater prevalence of Milky disease during the summer months in Swansea, Wales, UK where temperatures increased to 17-21°C in crabs maintained in aquarium conditions and also from a long-term survey of crabs surveyed from a pilot crab farm (Swansea, UK). In contrast, Eddy et al. (2007) also noted that prevalence of Milky disease in wild-caught *C. maenas* was <1% throughout the year. An annual survey of *C. maenas* for Milky disease in Harbour and Newton's Cove would be necessary to determine whether the comparatively greater prevalence in Milky disease observed in Newton's Cove correlated to increasing seawater temperature and to ascertain what other factors may be driving the difference in prevalence of Milky disease between these two locations.

Hematodinium

Hematodinium is a dinoflagellate infection considered to be highly pathogenic and therefore presents a significant mortality risk to the host (Stentiford and Shields, 2005). It was first described in 1931 (Chatton and Poisson, 1931 cited by Hamilton et al., 2009) as a rare parasite in the portunid crabs *Carcinus maenas* and *Liocarcinus depurator*, (swimming crab). It was not reported again until 2004, identified in one *C. maenas* from Southampton waters (Stentiford et al., 2004). *Hematodinium* has a wide host range (Stentiford and Shields, 2005; Morado et al., 2012; Small, 2012) and infection in the blue crab, *Callinectes sapidus*, suggests rapid proliferation of this parasite which may kill the host quickly (Shields and Squyars, 2000). This may explain why observations have been infrequent. In commercially important crustacean species such as *Chionoecetes bairdi*, (Tanner crabs), *Hematodinium* infection results in biochemical changes that render the tissue unpalatable for human consumption, referred to as Bitter Crab Disease, inevitably impacting the economic value of the fishery (Meyers et al., 1987; Taylor and Khan, 1995).

It typically infects the haemocoel in many of the internal organs of the host (gill, heart, hepatopancreas, muscle) (Meyers et al., 1987; Appleton and Vickerman, 1996). Figure 4.5a shows heavily dilated haemal sinuses within the connective tissue and heavy infection of the gill tissue (Figure 4.5b). *Hematodinium* can be observed with basophilic nuclei in the cells, a feature considered diagnostic (Stentiford and Feist, 2005). These multinucleate trophonts can be seen clearly in Figure 4.5. The tissues of infected hosts undergo dramatic alterations leading to respiratory dysfunction, lethargy and subsequent death (Small, 2012). The different isolates of *Hematodinium* bear few differential morphological characteristics to be able to identify to species level. Infection of *C. maenas* from the southern coast of the UK was putatively termed *Hematodinium perezi* (Stentiford and Feist, 2005), although the infection stage was too light to obtain sequence confirmation of species type (Small, 2012).

Interestingly, *Hematodinium* was not evident in the population of *C. maenas* at Newton's Cove, but were observed in 3% of the population at Harbour (male and female, size range 48-58mm carapace width). Previous outbreaks of *Hematodinium* in other species such as the velvet swimming crab *Necora puber* (Wilhelm and Mialhe, 1996) and the Norway lobster *Nephrops norvegicus* (Field and Appleton, 1995) have all been associated with either embayments, shallow sills or restricted coastal systems with entrained water masses. That said, outbreaks can occur in open water although depth may act to concentrate infection, such as in the case of the snow crab *Chionoecetes opilio* (Pestal et al., 2003). In this context it may be that the hydrodynamics of Harbour also play a role in facilitating some infections when they become evident, such as *Hematodinium*, through entrainment of water.

Haplosporidium

Haplosporidia are parasitic protists. The haplosporidian-like pathogen was first identified in *Carcinus maenas* in 2004 based on pathology and ultrastructure analysis (Stentiford et al., 2004). Later genomic DNA analysis confirmed the sequence as most closely related to the order Haplosporida, naming the pathogen *Haplosporida littoralis* (Stentiford et al., 2013b). Identifying *Haplosporidian* species is somewhat challenging as it is thought that there is significant variation in the morphology of the different life stages and potentially, some life stages may occur in other species. *Haplosporidium* infection in *C. maenas* is seen as uni- and mulitnucleate plasmodial life stage in haemocytes as well as the fixed phagocytes, connective tissue and myocytes and can cause significant modifications to the connective tissues and haemolymph (Stentiford et al., 2013b). Although, only two crabs could be seen to host *Haplosporidian* infection in this study, it is considered to be a mortality driver in populations with sufficient infection prevalence (Burreson et al., 2000). Previous prevalence of *H. littoralis* in *C. maenas* was somewhat higher than observed in this study. Other investigations revealed a 7.6% (Stentiford et al., 2013b) and 7% (Stentiford et al., 2004) prevalence at Newton's Cove, although the Harbour site was not studied. Nevertheless, considering *H. littoralis* adversely affects the integrity of connective tissue as well as causing haemolymph alterations, it is assumed that mortality results in those animals infected (Stentiford et al., 2013b). In view of the low prevalence of *H. littoralis* compared to previous investigations it would be interesting to analyse seasonal variability of this pathogen in this area.

Ameson

The fungi *Ameson* is an intracellular protozoan parasite, defined under the phylum Microsporidia. The species previously identified infecting *C. maenas*, *Ameson pulvis*, forms a clade with other microsporidians from marine crustaceans that demonstrate similar characteristics (Stentiford et al., 2013a). Morphological features of *Ameson* include microvilli extending from the spore surface and in general affect the skeletal musculature resulting in biomechanical as well as biochemical damage (Findley et al., 1981). However, there is significant plasticity amongst the morphological features of microsporidia making it challenging to identify. Commercially infected species such as penaeids lose their value due to the poor condition of the musculature (Lightner, 1996). *Ameson* sp. was identified only in 4% of *C. maenas* from Newton's Cove.

Sacculina

The genus *Sacculina* is a rhizocephalan barnacle infection of which there are many species. The infection associated with *C. maenas* is *Sacculina carcini*. The cyprid larvae of *S. carcini* will settle on the exoskeleton of the host, metamorphose into a developmental stage called kentrogon, which then

penetrates the carapace and injects the parasite into the haemocoelic fluid. After a period of a few months to around 3 years, the external reproductive body emerges from the abdomen of the host. This attracts male cyprids which fertilise the brood (Thresher et al., 2000). *S. carcini* is a parasitic castrator that can alter the physical, biochemical and behavioural traits of it's host (Werner, 2001). These changes can be extreme, such as modification of the male sexual characteristics leading to a female appearance (Veillet and Graf, 1958 cited by Stentiford and Feist, 2005; Høeg, 1995). The female bias at Harbour was 1:4 and at Newton's Cove 3:1. However, infection prevalence was relatively similar between Newton's Cove and Harbour at 6% and 7% respectively, therefore, as infection of *S. carcini* represents a relatively small proportion of both populations, the difference in sex ratio is unlikely to be pathogen related.

Crustaceans are particularly vulnerable to infection during ecdysis (Stentiford and Shields, 2005). The cyprid larvae of *S. carcini* have been demonstrated to settle preferentially on recently moulted compared with inter-moult *C. maenas* (Glenner and Werner, 1998). Potentially, younger age classes, *i.e.* those that moult more frequently, may therefore be more vulnerable to infection with *S. carcini*. Once infected, *S. carcini* can prevent any further moulting resulting in increased epi-fouling of the host (Thresher et al., 2000). The infections observed in *C. maenas* were widespread involving multiple tissue types, such a the hepatopancreas and ovaries (Figure 4.6).

Microphallus

Microphallus is a digenean trematode and the species associated with *C. maenas* is *Microphallus primas*. The life cycle of *M. primas* involves several stages associated with different host species. Larval stages are associated with molluscs such as *Hydrobia ulvae* (Saville and Irwin, 1991), whilst the adult parasites are found in the digestive tract of some marine birds (Dawes, 1968 and James et al., 1976 cited by Stentiford and Feist, 2005). It is the metacercarial cysts of the parasite that infect *C. maenas* and these were observed at different stages of infection in the hepatopancreas (Figure 4.7).

In general, pathogens that use *C. maenas* as an intermediary host are considered to have low pathogenicity (Kuris, 2005). Interestingly, the prevalence of *Microphallus* at Newton's Cove was significantly greater than that at Harbour (90% and 6% respectively). An important difference between the sites here are that *C. maenas* at Newton's Cove have a period of aerial exposure each tidal cycle which makes them more vulnerable to predation from marine birds. In addition, *Hydrobia ulvae* are ubiquitous around the UK shores, thereby providing *Microphallus* with the primary, secondary and tertiary hosts to complete its life cycle. *Microphallus* was observed in *C. maenas* in Harbour, but the prevalence was much lower. Whilst marine birds are still able to predate on *C. maenas* in Harbour, the complete submergence of *C. maenas* makes them less available as prey. The lower prevalence of *Microphallus* in Harbour may well be a reflection of the reduced availability of *C. maenas* as prey for marine birds and therefore less likelihood of *Microphallus* completing its life cycle.

Many infections have been described for *C. maenas* in the wild, but not all result in significant population losses. The analysis of pathogen genus or species was based on the presence and absence of pathogens and not severity of infection. Furthermore, the prevalence of some pathogens may vary with temperature and hence season, for example, Milky disease has been associated with greater prevalence during the summer months (Eddy et al., 2007). Ergo, this analysis represents just a snap-shot of pathogen assemblage in these two populations of *C. maenas*.

4.4.3 Immunocompetence of *Carcinus maenas* at Harbour and Newton's Cove

Immune gene transcription was also measured in relation to the presence and absence of pathogens based on the histology findings. No difference in the transcription of *carcinin*, *peroxinectin* or *prophenoloxidase* was evident between *C. maenas* identified either with or without pathogens at each location ($p>0.05$, Figure 4.15). Finer scale analysis of transcriptional changes between *C. maenas* hosting different pathogens might have revealed differences in the expression of immune genes. However, along with a lack of sufficient replication of some pathogens, no data was collected regarding the pathogen load of each crab, which also has the potential to affect immune gene transcription.

Interestingly, comparative analysis of immune gene transcription in *C. maenas* within each pathogen classification ('No pathogen' or 'Pathogen') revealed differences between the sites. In particular, the expression of *carcinin* was found to be lower in Newton's Cove for both the 'No Pathogen' ($p<0.001$) and 'Pathogen' ($p<0.01$) groups compared to *C. maenas* in Harbour. In contrast, the expression of *peroxinectin* and *prophenoloxidase* in those crabs observed with pathogens was greater at Newton's Cove compared with those at Harbour ($p<0.001$ and $p<0.01$ respectively). The difference in *carcinin* expression in the 'No Pathogen' group, in particular, suggests factors specifically associated with location play a role in determining the gene transcription at the population level. Furthermore, the comparatively greater *carcinin* transcription in Harbour may serve to maintain a lower pathogen burden in this population.

Carcinin, known to have activity against Gram positive bacteria, may also be involved in other cellular processes that relate to injury, stress and environmental factors (Brockton and Smith, 2008). In that case, the comparatively higher expression of *carcinin* in Harbour may suggest this site as the more stressful environment. As no abiotic variables were measured in this survey, it is not possible to attribute this increased gene expression of *carcinin* at Harbour to specific environmental stressors, so further investigations would be required. In contrast, there is also an argument for decreased expression of *carcinin* in Newton's Cove, possibly due to reallocation of energy in the hosts. This may be a result of wave action and potential dessication associated with aerial exposure, although interestingly this is not seen in *peroxinectin* or *prophenoloxidase*. Peroxinectin and prophenoloxidase are synthesised and stored in the granulocytes until exocytosis upon microbial stimulation (Johansson et al., 1999; Sritunyalucksana and Söderhäll, 2000), whereas *carcinin* is secreted into the haemolymph once synthesised (Hauton, 2012), so there is continual production. These different supply mechanisms may result in different immune gene expression profiles with energy expenditure. This may be reflected in the immune gene profiles observed between Harbour and Newton's Cove. However, further investigations that involve repeated haemolymph sampling of *C. maenas* under different environmental stressors, *e.g.* increasing emersion times, would shed light on whether increased energy demand differentially changes the immune gene expression profiles.

Although there was a marked difference in the overall expression of *carcinin* between the two populations of *C. maenas*, there remains high intra-group variability. This is particularly evident in the 'Pathogen' group in Newton's Cove. This may be explained by the increased pathogen prevalence at Newton's Cove which resulted in higher pathogen diversity and presumptive variation in infection load, which in turn may influence gene expression. The expression of *carcinin* has been demonstrated to be particularly variable in response to different pathogens, so this is consistent with that observation. As

an example, crustin (antimicrobial peptide) expression was shown to be down-regulated in *Homarus gammarus* in response to Gram-negative bacterial challenge after an initial up-regulation (Hauton et al., 2006), a pattern similarly demonstrated by the Type III crustin in *Litopennaeus vannamei* after exposure to *Vibrio alginolyticus* (Jiménez-Vega et al., 2004). Although, Type II crustin was seen to be down-regulated in response to *V. alginolyticus* challenge in *L. vannamei* (Vargas-Albores et al., 2004). This highlights much variation between species and the different isoforms of crustins and in response to pathogen type. Of course, the gene expression in *C. maenas* in this study has been analysed on a broader scale, so provides a snap-shot of immune gene transcription at a population level, rather than over time as described in the previous examples.

The up-regulation of *peroxinectin*, (an opsonin and phagocytic molecule) and *prophenoloxidase*, (a zymogen, converted to *phenoloxidase* which is involved in the melanisation of pathogens), in the 'Pathogen' group may be a reflection of the higher prevalence of infection in Newton's Cove, however, this is not significantly different in comparison to the 'No pathogen' group. It is important to note that the 'No pathogen' group in Newton's Cove consists of only 5% of *C. maenas* at this site, exposing greater variance in gene expression between samples. But as differential gene expression was also not seen in Harbour, it is more likely that infection *per se*, cannot fully explain this difference.

A possible explanation may be related to potential contamination effects in Harbour. Exposure to contaminated dredgings in the caridean shrimp, *Crangon crangon*, resulted in a reduction in total haemocyte count (THC) and subsequent decrease in *phenoloxidase* activity (Smith et al., 1995). THC was not measured in *C. maenas* in this study, but a reduction of haemocytes may explain the comparatively lower expression of both *peroxinectin* and *prophenoloxidase* in those crabs from Harbour in both the 'No pathogen' and 'Pathogen' groups, which would be consistent with the mechanism of their production (*i.e.* synthesised in the granulocytes and released on exocytosis). Although the difference in gene expression between sites was not statistically significant in the 'No Pathogen' group, the mean gene expression was greater in Newton's Cove compared with Harbour. A trend consistent with the gene expression from those crabs observed to be infected with pathogens (Figure 4.15). Based on this, it would be interesting to assess the difference in THC between these populations of *C. maenas* and if evident, ascertain the driving mechanisms that may influence haemocyte count. Of course, there was greater prevalence of pathogens at Newton's Cove compared with Harbour, so at a population level it would be expected that the expression of these genes (*peroxinectin* and *prophenoloxidase*) would be comparatively greater in order to encapsulate and kill the pathogens.

The snap-shot of gene expression presented here is likely to be attributed to many variables including the load and length of infection, co-infections and of course abiotic variables. Assessing the community level transcription of these two populations highlights fundamental differences in the overall level of immune gene transcription in these groups of *C. maenas* between locations. Finally, gene expression is, of course, dynamic and will vary depending on the length of exposure to a pathogen and the load in terms of it's physiological impact on the individual organism. Immune biomarkers, particularly a selection that encompass different functions, (as discussed in Chapter 1), respond to different pathogens and stressors. This provides more breadth and so can be a useful tool to assess immune transcription of animals in the wild. However, it is important to use established assays that measure host response at other levels of organisation (*e.g.* cellular or multi-cellular), such as histology. This would provide further context to the transcription of genes, particularly as factors affecting the individual can be

multi-variable and complex. Use of other assays that focus on cellular or multi-cellular levels of organisation, in concert with molecular tools, would provide an integrated and holistic response of an animal to pathogens to ascertain whether real immune response is evident (*i.e.* immune response can be seen at a cellular or multi-cellular level) or whether only transcriptional changes can be seen and what this then means for the population as a whole.

4.4.4 RNA quality

A considerable degree of time was dedicated to optimising extraction protocols for this study in order to ensure RNA integrity was preserved in all samples. Nevertheless, 30% of samples were observed to have RNA integrity with an RQI value ≤ 7.5. This begs the question as to what other factors may have contributed to RNA degradation?

Significance of RNA quality

Determining RNA quality is a fundamental part of processing samples for quantitative PCR. The MIQE (Minimum Information for publication of Quantitative real-time PCR Experiments) guidelines specifically stipulate that RNA quality must be reported (Bustin et al., 2009). Obtaining meaningful gene expression data can be limited by the quality of the initial RNA preparation. Quantitation of gene expression without validating RNA quality can, therefore, easily result in the misinterpretation of data (Taylor et al., 2011) and may also reduce the sensitivity of the qPCR assay to detect a low expression transcript. RNA degradation can occur naturally *in vivo* in response to environmental stimuli (Doma and Parker, 2007) such as nutrient and cytokine levels as well as hypoxia and tissue damage (Guhaniyogi and Brewer, 2001). Degradation may also occur as a result of poor storage and handling of samples (Perez-Novo et al., 2005), heat and UV exposure and cleavage by RNase enzymes (Vermeulen et al., 2011). However, in view of the the optimised extraction protocol used in this study, these variables have been ruled out as factors that may have affected RNA quality in *C. maenas*.

RNA degradation in relation to gene expression

The effect of RNA degradation on gene expression has been investigated by a number of authors (Perez-Novo et al., 2005; Copois et al., 2007). As an example, investigations into the effect of RNA degradation on the expression of endogenous reference genes has shown significantly differential expression of a tumour marker gene in two groups of neuroblastoma patients (Vermeulen et al., 2011). Normalisation of the endogenous reference genes is designed to remove variation between samples including differences in RNA quality (Vandesompele et al., 2002), although it is important not to compare samples with widely dissimilar RQI values (Bustin et al., 2010). Importantly, in this example, the process of normalisation did not mitigate the effect of degraded RNA according to the investigating authors. This results in a reduction in the sensitivity and accuracy of the qPCR assay as changes in the expression of the target genes are likely to be not only as a consequence of biology, but also artefact. Increased gene-specific variation has also been reported in other studies (Perez-Novo et al., 2005), (Fleige and Pfaffl, 2006) highlighting the necessity for efficient quality control of RNA.

There is still ongoing debate as to how much gene expression is effected by low quality RNA (Strand et al., 2007; Thompson et al., 2007; Popova et al., 2008) and this may well be influenced by different sensitivities of different genes to RNC_qA degradation. The complexity of the issue has lead to the recommendation of a study-specific cut-off for RNA quality (Vermeulen et al., 2011). However, this is

still arbitrary and set by the user. For the purpose of this study, data sourced from *C. maenas* samples with an RQI ≤ 7.5 was considered as low quality and therefore not included in the statistical analysis. This was based on a number of studies that demonstrated variable gene expression in samples with RQI values <7 (Taylor et al., 2011).

A qualitative assessment of the immune gene transcription of *C. maenas* between those with low (≤ 7.5) and high (≥ 7.6) quality RNA suggests that in many of the samples, apparent gene expression was greater in those crabs with degraded RNA (Figure 4.16). This is a curious finding as the expectation would be lower gene expression due to the lower number of starting transcripts of the target amplicons that would result from sheared RNA. Lower quality RNA generally results in high C_q values (Vermeulen et al., 2011) as demonstrated by Fleige and Pfaffl (2006). Hence, explaining this finding is somewhat difficult. However, this is presuming that the degradation occurs upstream of the target amplicon, which may not be the case if random primers are used. It is pertinent to note that the RQI value relates to the level of degraded total RNA, but not to where the mRNA is degraded. The priming strategy adopted for reverse transcription of mRNA to cDNA was to use oligo dTs. This is an important consideration in relation to RNA degradation. Oligo dTs are short deoxy-thymine nucleotides that bind to the poly-A tail at the 3' end of the strand and are widely used to convert mRNA to cDNA through reverse transcription. Consequently, where the amplified fragment of the gene is, based on the gene length and distance from the poly-A tail, may be important.

The curious finding of degraded RNA in the 'No Pathogen' *C. maenas* group at Harbour is unexpected. The 'No Pathogen' group represents 73% of *C. maenas* at Harbour, a third of which, therefore, have degraded RNA. Determining the cause of this is beyond the scope of this study, but it is important to understand the fundamental differences between these locations that might allude to factors that may compromise host RNA integrity.

4.4.5 Limitations

Overall, it is difficult to determine the true impact of pathogens on wild populations of *C. maenas* without longer studies that also incorporate seasonal variation. One of the challenges of this study was the lack of sufficient replication within the individual pathogen groups, in samples sourced from sufficiently high RNA quality, by which immune gene transcription could be measured. This was made more complex by the level of multiple pathogen infections observed in individual hosts, thus limiting the statistical tools available to appropriately assess the impact of a particular pathogen genus or species on the host. Host immune transcription was therefore measured purely on the presence or absence of pathogens within the host. In addition, immune gene transcription may change in response, not only to pathogen species, but to pathogen load. Assessment of pathogen load was not an objective of this study, therefore, interpretation of gene transcription needs to be in the context of pathogen presence or absence only.

Whilst histology was a useful method to identify the pathogens in *C. maenas*, it does not assess pathogen presence at the level of the whole organism. Therefore, it is possible that those crabs identified as having no pathogens may have had infections that were not isolated in the tissue samples dissected for histology. Furthermore, it is important to be mindful of other variables that have the potential to impact the host in terms of immune or stress response, such as local hydrodynamics, aerial

exposure and potential pollutants, the impact of which has not been explored in this study.

4.4.6 Key findings and ecological context

In summary, this study highlights fundamental differences in the pathogen profile of *Carcinus maenas* between these two geographically close populations. Pathogen prevalence in Newton's Cove was much greater compared with Harbour. However, there was no difference in the transcription of host immune genes within each location between those crabs observed to be infected with pathogens and those without. Yet, transcription differences of immune genes between sites were evident, with lower expression of *carcinin* in Newton's Cove compared to Harbour in both the 'No pathogen' and 'Pathogen' groups. The comparatively greater gene transcription of *carcinin* in Harbour may act to maintain a lower pathogen burden in this population. Conversely, comparatively greater transcription of *peroxinectin* and *prophenoloxidase* was evident in Newton's Cove in the 'Pathogen' group. Overall this suggests factors specific to location, rather than infection, were driving the host immune transcription differences between these populations of *C. maenas*. Furthermore, 30% of *C. maenas* were noted to have degraded RNA which was also likely to be associated with site-specific factors, but may potentially be compounded by parasitic infection.

It would be interesting to focus on potential site-specific drivers of RNA degradation for future studies. Major differences between these two environments, as previously stated, include aerial exposure of *C. maenas* each tidal cycle at Newton's Cove compared with complete submergence of *C. maenas* at Harbour. In addition, the high level of boat traffic in Harbour is likely to result in increased anthropogenic contaminants into the water column. Further investigations could determine the role of these contaminants on host RNA in *C. maenas*. Controlled infection studies on *C. maenas* of specific intracellular pathogens may also reveal an additional parasitic component affecting RNA integrity. Whilst an unexpected finding in this study, the importance of analysing RNA has been highlighted to ensure sensitive, robust and appropriate analysis is conducted on true biological gene-specific variation and thereby avoid any misinterpretation of gene expression data. Finally, the ability of these populations of *C. maenas* to compete both inter- and intra-specifically is dependent upon it's response to both biotic and abiotic factors that influence the fitness of the individual and in turn, the population. This study has highlighted the pathogen profiles between these populations and impact on immune function in the host, but also the potential for environmental factors to play a crucial role in terms of population fitness.

5 Testing the response of the immune toolbox with controlled infections in *Carcinus maenas* (L.)

Chapter Abstract

Carcinus maenas are susceptible to many invading pathogens including *Listonella anguillarum*, a Gram negative bacterium and *Planococcus citreus*, a Gram positive bacterium. These two bacteria were used to test the efficacy of the toolbox of immune biomarkers in *C. maenas* as indicators of recent infection to either a single Gram negative or Gram positive infection. *Listonella anguillarum* is globally distributed in the marine environment and *Planococcus citreus* is commonly found in intertidal sediments. *C. maenas* were inoculated with *L. anguillarum* or *P. citreus* and the transcription of *carcinin*, *peroxinectin* and *prophenoloxidase* were analysed along with histopathology, total haemocyte count and bacterial load. Overall, the transcription of *carcinin* was up-regulated in response to infection with *L. anguillarum* ($p<0.05$) and *P. citreus* ($p<0.05$). Unfortunately, bacterial infection was also evident within the control groups. The source of this secondary infection was not determined. Consequently, *carcinin* could not be endorsed as an appropriate biomarker in response to *L. anguillarum* and *P. citreus* infection in light of this secondary infection. Ultimately, repeat experiments would be required in order to validate the changes observed in *carcinin* transcription in response to *L. anguillarum* and *P. citreus* infection in *C. maenas*.

5.1 Introduction

5.1.1 Background

Outbreaks of disease caused by bacterial infections are known to have caused significant losses amongst commercially important decapod species (Lightner and Redman, 1994; Castro et al., 2012). Susceptibility to infection is influenced by numerous factors in the wild, such as environmental conditions (extremes in temperature, salinity, oxygen consumption), food availability and desiccation (Mente et al., 2003; Pearce and Balcom, 2005; Hardy et al., 2013). Disease outbreaks are likely to go unreported, however, in less commercially important species until the onset of an epizootic (Shields, 2012).

Low level infections are expected to occur naturally in wild populations and *Carcinus maenas* is a species known to be able to harbour a number of pathogens at any one time as illustrated in Chapter 4. The previous chapter investigated the immune response of two populations of *C. maenas* in response to

pathogen exposure in the wild. This approach necessitated a broader perspective whereby gene expression was assessed in terms of pathogen presence or absence only. Whilst this highlighted the diverse and complex pathogen profiles *C. maenas* were vulnerable to, it also prevented finer scale analysis of specific pathogen induced changes in gene transcription. This chapter focuses more specifically on controlled infection with either a Gram positive or Gram negative bacteria in adult *C. maenas* sourced from a wild population. Further, this study was designed to build on the population level survey from Chapter 4 and assess the capacity of the immune biomarkers as indicators of recent infection history, thereby testing the predictive ability of *carcinin, peroxinectin* and *prophenoloxidase* transcription post-infection challenge. In this case, single, controlled infection challenges were conducted in *C. maenas* against known pathogenic bacteria.

C. maenas are known to be susceptible to infection with *Listonella anguillarum* (Gram negative) (Hauton et al., 1997) and *Planococcus citreus* (Gram positive) (Relf et al., 1999) bacteria. In addition, the immune biomarkers selected, *carcinin, peroxinectin* and *prophenoloxidase*, have demonstrated activity against various bacterial challenges in crustaceans (Relf et al., 1999; Liu et al., 2007b; Zhang et al., 2007; Shockey et al., 2009; Burge et al., 2009; Du et al., 2013; Binggeli et al., 2014).

Listonella anguillarum

Listonella anguillarum (Canestrini, 1893), formerly *Vibrio anguillarum*, is a Gram negative, comma-shaped rod bacterium from the Family Vibrionaceae (Frans et al., 2011). It is flagellated, non-spore forming, halophilic and forms cream-coloured, round colonies (Figure 5.1) (Frans et al., 2011). *L. anguillarum* is widely distributed throughout the world (Rad and Shahsavani, 2008) and has been notable for epizootics in both fish and shellfish aquaculture (Bolinches et al., 1986; Lodeiros et al., 1987; Toranzo and Barja, 1990) resulting in significant economic losses (Frans et al., 2011). Infection studies in fish have revealed gross pathology such as necrosis, haemorrhagic lesions and boil-like lesions of the musculature (Tang et al., 2008). Infection occurs after attachment of the bacterium to mucosal surfaces such as the gut and gills (Rajan et al., 2013).

Planococcus citreus

Planococcus species are abundant in a variety of marine environments and are common bacteria in intertidal sediments (Relf et al., 1999; Engelhardt et al., 2001), a common habitat for *Carcinus maenas*. The genus *Planococcus* was created by Migula (1894) in order to differentiate motile cocci from micrococci. *Planococcus citreus* (Migula, 1894) is a Gram positive aerobic bacteria from the Family Planococcaceae. The cocci can be seen as single cells but also form pairs or tetrads, are actively motile and do not form spores (Farrow et al., 1994; Engelhardt et al., 2001). Colonies appear as yellow/orange in colour on most media (Figure 5.1) (Alvarez and Koburger, 1979).

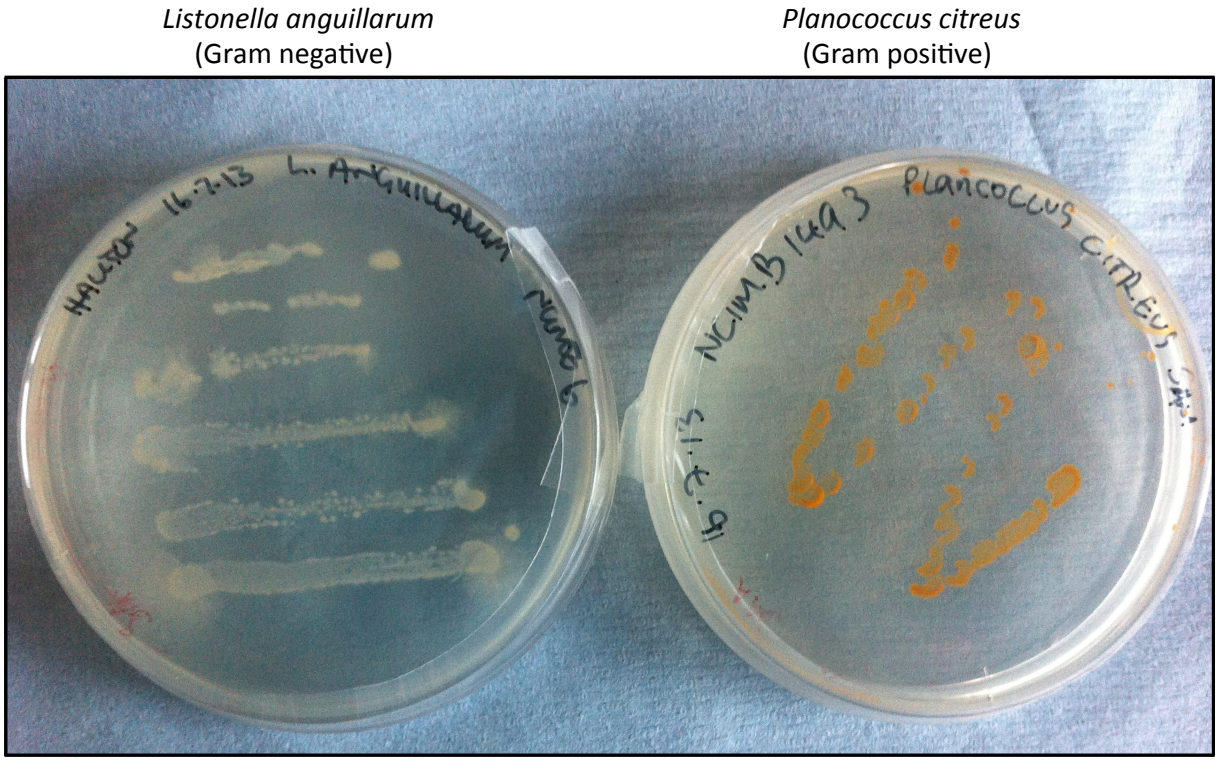


Figure 5.1: Bacterial colonies of *Listonella anguillarum* (cream) and *Planococcus citreus* (orange).

5.1.2 The role of *carcinin*, *peroxinectin* and *prophenoloxidase* in the host response to infection

Circulating haemocytes perform diverse immunological activities within the innate immune system of decapods. Functional attributes of haemocytes include the recognition of invading pathogens, aggregation of haemocytes, coagulation, adhesion, encapsulation, phagocytosis and the release of cytotoxic molecules through degranulation (Reviewed in Jiravanichpaisal et al. (2006) and Johansson et al. (2000)). Consequently, the number of circulating haemocytes are important. A decrease in the total number of haemocytes can be indicative of immune processes occurring through the host response to pathogens (Persson et al., 1987; Lorenzon et al., 1999), such as nodule formation (Martin et al., 1998) or due to haemocytic degranulation and release of peptides (Smith and Söderhäll, 1983; Johansson and Söderhäll, 1985). A deficient level of circulating haemocytes can present a threat to the survival of the individual animal unless haemocytes are replaced rapidly by the haematopoietic tissue. In addition, factors such as repeated sampling and infection can adversely influence the rate of haemocyte production (Saha, 2011).

It is generally accepted that there are three main morphologically distinct haemocyte types: hyaline, semi-granular and granular cells (Bachau, 1980) (Johansson et al., 2000). For the purpose of these investigations, total haemocyte count (THC) was monitored to determine the overall impact of bacterial infection on the host over the course of each infection trial. However, it is known that the immune genes are associated with a specific haemocyte type.

Peroxinectin, a cell adhesion molecule and opsonin is known to be stored in the granules of the

semi-granular and granular haemocytes (Johansson and Söderhäll, 1988). Upon microbial recognition, degranulation of haemocytes release *peroxinectin* from the storage granules (Barracco et al., 1991). *Peroxinectin* can then stimulate phagocytosis by hyaline cells (Johansson and Söderhäll, 1988) and encapsulation by semi-granular cells (Thörnqvist et al., 1994). *Prophenoloxidase* is stored as a zymogen also within the granules of semi-granular and granular haemocytes (Ashida and Söderhäll, 1984). *Prophenoloxidase* is cleaved to form active *phenoloxidase* after it is released through degranulation, which catalyses the O-hydroxylation of monophenols to diphenols and oxidises diphenols to quinones. Quinones can non-enzymatically polymerise to insoluble melanin, a brownish coloured pigment with a number of important functions in decapods. Melanin is involved in wound healing and sclerotisation (cuticle hardening) and importantly, through adhesion, encapsulates foreign particles including pathogens (Sugumaran, 2002). Both *peroxinectin* and *prophenoloxidase* are synthesised and then stored in the haemocytes until required.

Carcinin is an anti-microbial peptide (AMP), which generally targets the integrity of the bacterial cell membrane (Rosa and Barracco, 2010). AMPs are synthesised also within the granular haemocytes, but unlike *peroxinectin* and *prophenoloxidase* most AMPs are constitutively expressed (Relf et al., 1999; Jiménez-Vega et al., 2004; Brockton et al., 2007) and often at a high titre (Hauton et al., 2006).

5.1.3 Research Aims

The purpose of this study was to investigate the immune gene transcription in wild-caught *Carcinus maenas* in response to a sub-lethal, controlled dose of two known pathogenic agents; a Gram negative (*Listonella anguillarum*) and Gram positive (*Planococcus citreus*) bacteria post-infection.

5.1.3.1 Research Objectives

i. Determine the effect of controlled infection of both *Listonella anguillarum* and *Planococcus citreus* on the transcription of *carcinin*, *peroxinectin* and *prophenoloxidase* in *Carcinus maenas*.

ii. Measure the total haemocyte count intermittently during the infection and in relation to the gene transcription.

iii. Monitor the progression of infection through quantifying bacterial load.

iv. Establish how effectual the candidate host immune genes were as biomarkers post-controlled Gram positive and Gram negative infection in *Carcinus maenas*.

5.2 Experimental design

The infection trials were carried out as two individual experiments. The first population of *Carcinus maenas* were injected with either a placebo (n=15) or *Listonella anguillarum* bacteria (n=15). A second population of *C. maenas* were injected with a placebo (n=15) or *Planococcus citreus* bacteria (n=15).

5.2.1 Collection of *Carcinus maenas*

Carcinus maenas were collected from Marchwood, Southampton, UK (latitude 50.900242 and longitude -1.439896). Any crabs with obvious evidence of disease, such as shell disease or *Sacculina* were not sampled for this study. Local temperature and salinity were recorded as 14.5°C and 30.8 respectively at the site and time of collection. To eliminate variation in gene expression in relation to reproduction, only males were collected for this study. The size range varied from 36-57mm with a mean size in each category (*Listonella anguillarum* inoculated and controls and *Planococcus citreus* inoculated and controls) of 45mm, 46mm, 46mm and 45mm respectively.

Experimental set-up

Individual *C. maenas* were held in non flow-through 5l tanks, all with individual air lines. Seawater was autoclaved to eliminate pathogens that could infect *C. maenas* through the puncture wound caused by the needle used to administer the bacteria or placebo.

Seawater was measured daily for temperature, salinity and oxygen concentration (Appendix, Chapter 5, Table 8.35) to ensure stable acclimation and experimental conditions (temperature 12.9°C ±1.4, salinity 30.7 ±0.8 and oxygen concentration of 10.4mg l^{-1} ±0.5). All animals were acclimated for 14 days in the holding aquarium and a further 14 days in the experimental tanks. This was to allow for any pathogenic infections acquired in the wild to either eliminate those animals from the infection study or allow the crabs to adequately clear the infection. During both the acclimation and experiments animals were fed *ad libitum* on a mixed diet of either fish or *Crepidula fornicata*. All *C. maenas* were starved for 24 hours prior to sampling in order to standardise the energy input. Water changes (100%) took place during the interceding days.

Preparation of *Listonella anguillarum* or *Planococcus citreus* for inoculum

Bacteria colonies were grown from a master stock of either *Listonella anguillarum* (NCIMB 6) or *Planococcus citreus* (NCIMB 1493) for the inoculum. Colonies were scraped from a master plate and added to 30ml Marine Broth 2216 (Difco™), then left at room temperature at 200 revolutions/minute. Bacteria were removed during the exponential phase (9-12 hours) and re-plated on a new agar plate to maintain the stock. The remaining bacterial cells in the marine broth were transferred to a new 15ml falcon tube and centrifuged at 1000*g* at room temperature for 10 minutes. The marine broth was discarded and the pellet was washed in 1ml of marine saline three times (Appendix, Chapter 2, Section 7.5.3.4). The concentration of cells were titrated to achieve the target absorbance at 570nm and thus concentration for the inoculum (Table 5.1). The sub-lethal concentration of both *L. anguillarum* and *P. citreus* was determined empirically from controlled laboratory challenges (C. Hauton, 2014, pers. comm.) Each *Carcinus maenas* were inoculated with either 0.1ml of marine saline (placebo) or 0.1ml of bacteria.

Table 5.1: Bacterial target absorbance and concentration for *Listonella anguillarum* and *Planococcus citreus*.

Species	CFU/ml	Target absorbance
Listonella anguillarum (Gram -ve) NCIMB 6	$1x10^{8}$	0.3
Planococcus citreus (Gram +ve) NCIMB 1493	$4.5x10^{11}$	1

Gene expression preparation

Gene expression preparation for all immune and endogenous reference genes are fully described in Chapter 2. All genes were isolated with conventional PCR using primer sequences designed against either the full gene sequence or EST sequence (where target genes were not fully sequenced). All PCR amplicons were sent to Source BioScience LifeSciences Plc. (Oxford, UK) for sequence confirmation using conventional Sanger sequencing. qPCR primers isolated short ($\leq$ 150bp) amplicons for the genes of interest which were then optimised for primer and cDNA concentrations and PCR efficiencies.

5.2.2 Experiment

Immediately prior to inoculation, haemolymph was aspirated for baseline gene transcription, total haemocyte count and bacterial load (0 hours sample), (see below). *Carcinus maenas* were injected with either a placebo (marine saline) or bacterial (*Listonella anguillarum* or *Planococcus citreus*) dose into the unsclerotised articulation of the second periopod after cleaning the site with ethanol. Haemolymph samples were taken as per Table 5.2.

Table 5.2: Sampling of haemolymph for *Listonella anguillarum* and *Planococcus citreus* experiments.

Listonella anguillarum			
0 hours	**3 hours**		**72 hours**
Bacterial load	Bacterial load		Bacterial load
Gene expression	Total haemocyte count		Gene expression
Total haemocyte count			Total haemocyte count
INJECTION OF BACTERIA/SALINE			
Planococcus citreus			
0 hours	**3 hours**	**24 hours**	**48 hours**
Bacterial load	Bacterial load	Total haemocyte count	Bacterial load
Gene expression	Total haemocyte count		Gene expression
Total haemocyte count			Total haemocyte count
INJECTION OF BACTERIA/ SALINE			

The *L. anguillarum* infection trial continued for 3 days, whereas the *P. citreus* trial continued for 2 days, but with increased sampling frequency (Table 5.2). This second infection study was shortened after reviewing the preliminary results from the *L. anguillarum* study which showed later stage inflammatory processes in the histopathology and no classic decrease in THC post infection.

Gene expression

Haemolymph (1ml) was aspirated into a pre-loaded 2ml syringe containing 1ml of *Carcinus maenas*-specific anticoagulant (Chapter 2, Section 2.3.2, Table 2.2). This was immediately centrifuged at 3000*g* and 4°C for 7 minutes to form a pellet. The anticoagulant was aspirated and discarded and the pellet then mixed with 1-1.5ml of TRI-Reagent® depending on pellet size. After a short (days) storage at -80°C, RNA isolation, quality and quantity assessment, DNase treatment and reverse transcription to cDNA were all carried out according to the manufacturers protocols summarised in Chapter 2, Sections 2.3.3-2.3.6.

Total haemocyte counts

Haemolymph (0.1ml) was aspirated into a pre-loaded (0.1ml) syringe of 20% seawater formalin (Appendix, Chapter 2, Section 7.5.3.3) and measured as per Chapter 2, Section 2.6.

Bacterial load

Haemolymph (0.1ml) was aspirated into a pre-loaded (0.1ml) syringe of sterile marine saline (Appendix, Chapter 2, Section 7.5.3.4). Bacterial load was measured on duplicate marine agar plates (Appendix, Chapter 2, Section 7.5.3.1), summarised in Chapter 2, Section 2.7, for either a 5x (*L. anguillarum*) or 3x (adjusted for *P. citreus* after review) dilution series in order to enumerate the bacterial colonies. Autoclaved seawater, sourced from the aquarium, was also plated using the same method in order to assess bacterial load in the seawater.

Dissection

After the final aspiration of haemolymph, *C. maenas* were placed on ice for a minimum of 30 minutes prior to dissection in order to anaesthetise them. Dissection was carried out as per Chapter 2, Section 2.5. Tissue samples dissected for histology were heart, gill, nerve, gonad, muscle and hepatopancreas. Hepatopancreas was also dissected for electron microscopy should further investigations be required. Histology was processed and haematoxylin and eosin (H&E) stained by colleagues in CEFAS, Weymouth, UK according to Chapter 2, Section 2.5 and analysed by Dr. Grant Stentiford.

5.3 Results

5.3.1 Histology

Listonella anguillarum

Histology was taken at 72 hours post infection. Overall, general inflammatory processes were observed in the histology preparations of *C. maenas* inoculated with *L. anguillarum* although no bacterial inclusion bodies could be seen (Figures 5.2, 5.3, 5.4). Figure 5.2 shows a large granuloma with areas of melanisation and vacuoles within the connective tissues. In Figure 5.3 a granuloma with notable melanisation and vacuoles can be seen in the haemal space around the hepatopancreas. The heavy melanisation and lack of visible bacterial inclusion bodies are indicative of a later stage infection. Within the gill tissue, early nodule formation was observed. No bacteria were noted within the nodule.

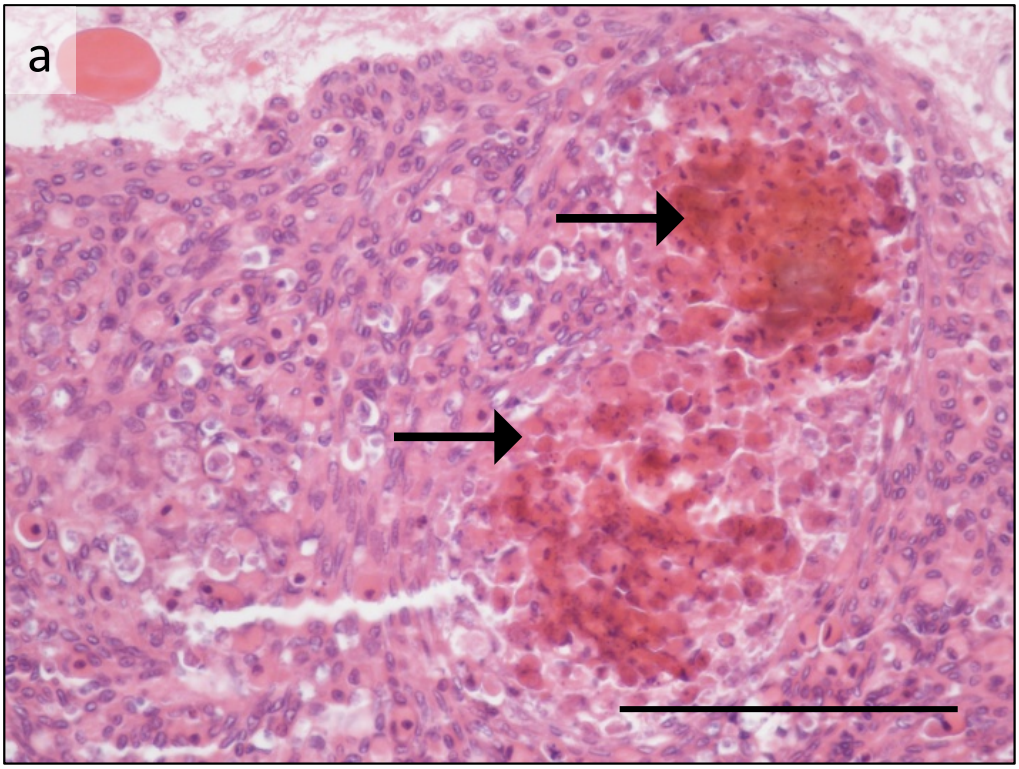


Figure 5.2: Late stage nodule formation (arrows) and encapsulation in the connective tissue of a *Listonella anguillarum* infected *Carcinus maenas*. Areas of melanisation can be seen suggesting later stage inflammatory processes. Scale bar 100μm. H&E stained

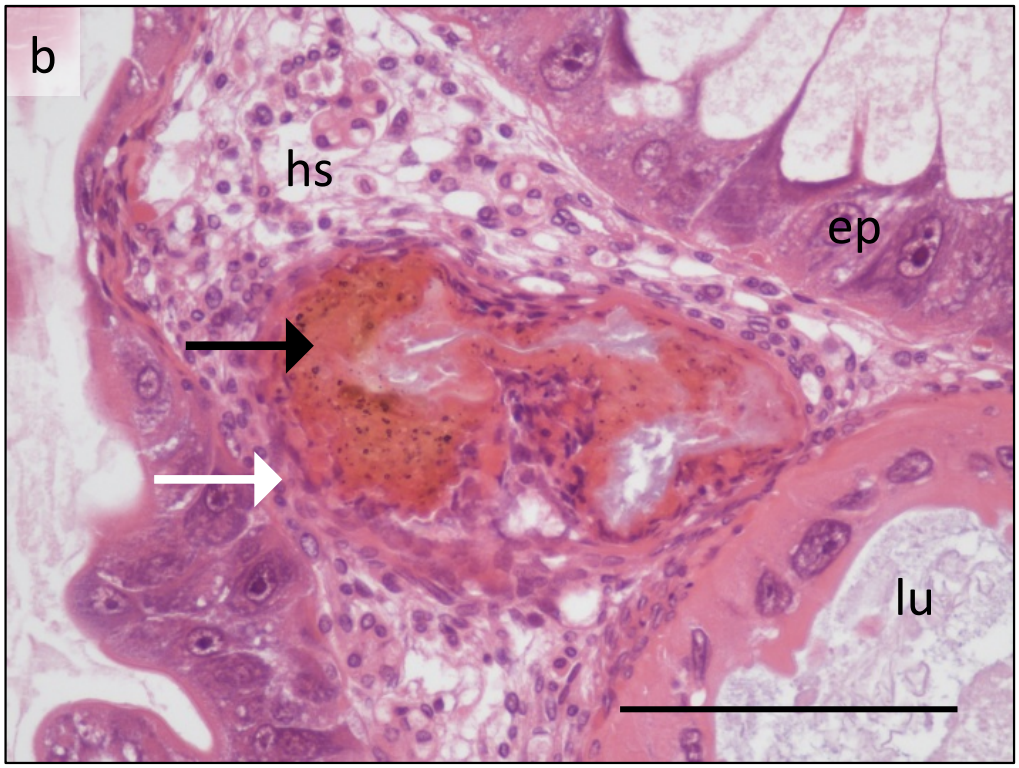


Figure 5.3: Heavily melanised granuloma in the haemal space (hs) of a *Listonella anguillarum* infected *Carcinus maenas*. The hepatopancreatic tubules are defined by the epithelial cells (ep) and lumen (lu). Scale bar 100μm. H&E stained.

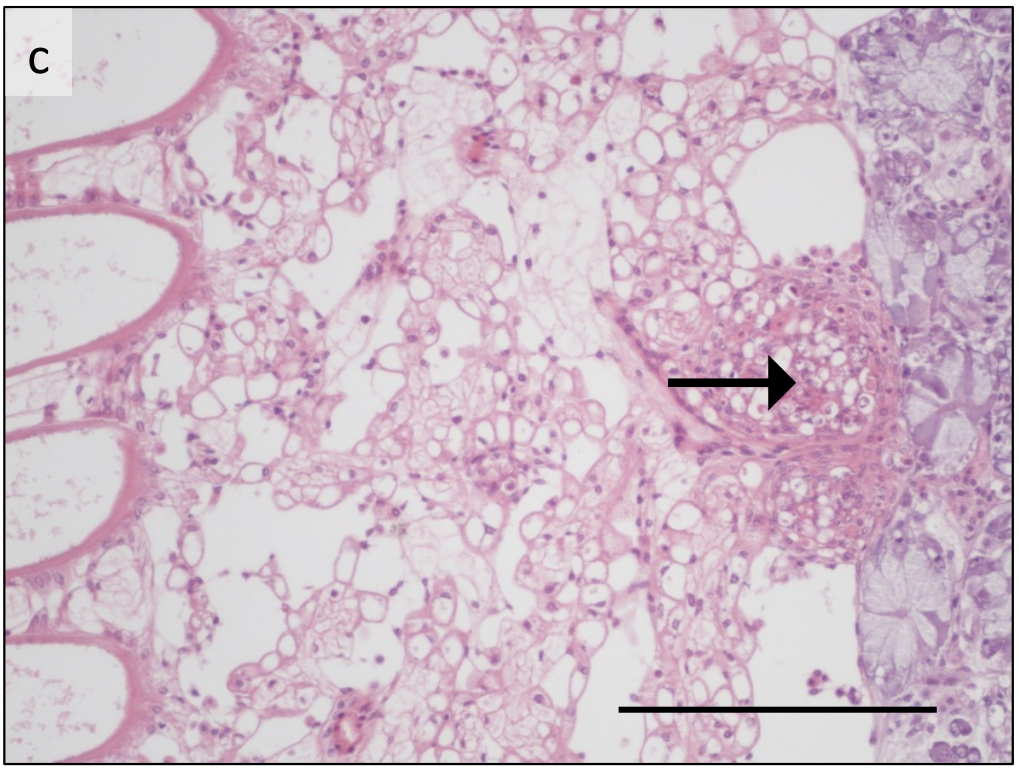


Figure 5.4: Early nodule formation observed within the gill stem in a *Listonella anguillarum* infected *Carcinus maenas*. Scale bar 200μm. H&E stained.

Planococcus citreus

Histology was taken at 48 hours post infection. Images show progression of infection from a single cell clearly containing bacteria (Figures 5.5 and 5.6), through to haemocyte recruitment (Figures 5.7, 5.8 and 5.9), aggregation (Figures 5.10 and 5.11) and melanisation (Figures 5.8-5.11). Progression of infection appears to overwhelm the host's immune responses (haemocyte aggregation and melanisation) in Figure 5.11. Bacteria were not evident in the control samples (data not shown).

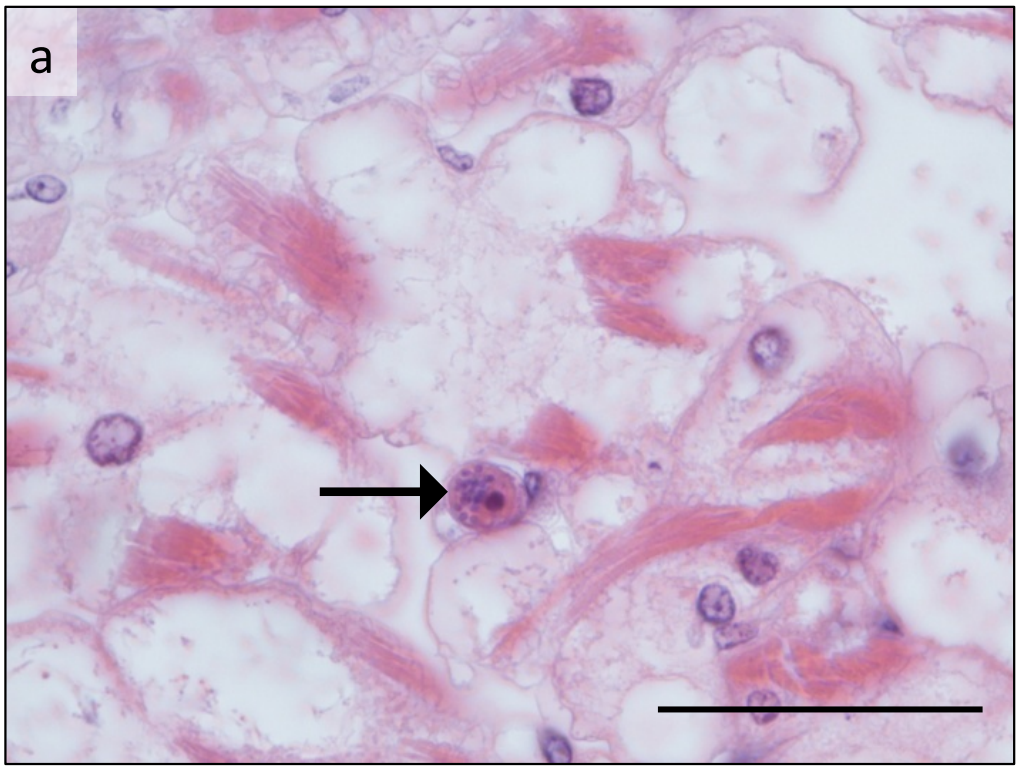


Figure 5.5: Bacteria visible (arrow) within a single cell in a *Planococcus citreus* infected *Carcinus maenas*. Histology image taken of the sinuses within the connective tissue from the heart. Scale bar 50μm. H&E stained.

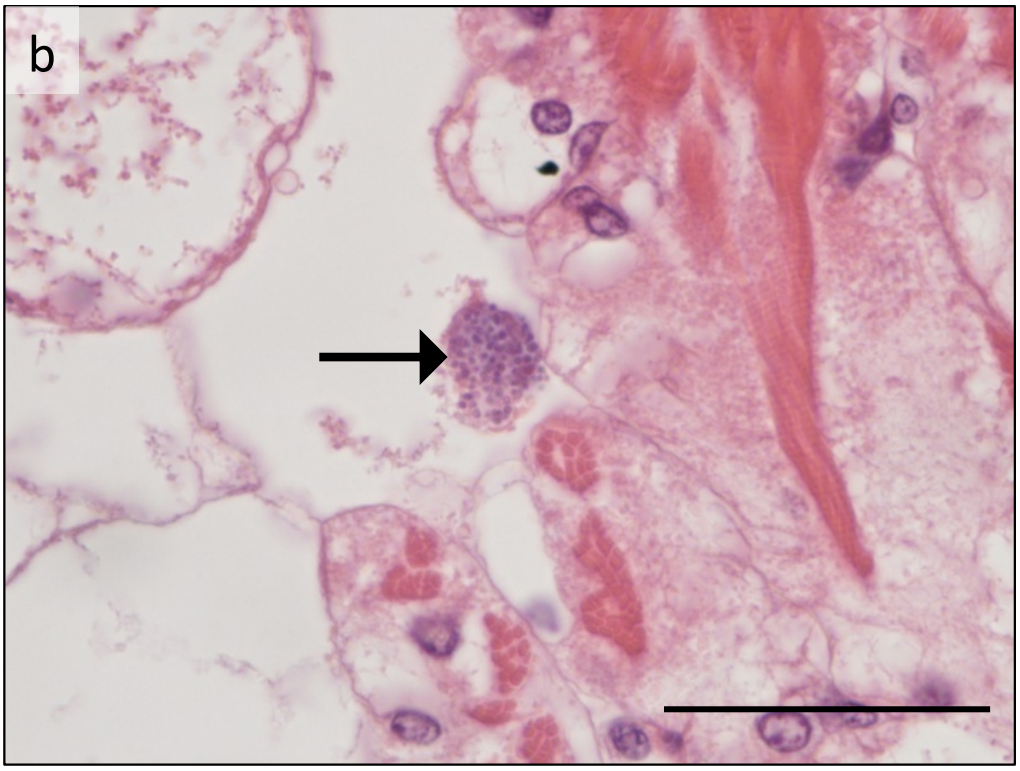


Figure 5.6: Micro-colony of bacterial cells (arrow) within the heart haemo-sinuses in a *Planococcus citreus* infected *Carcinus maenas*. Scale bar 50μm. H&E stained.

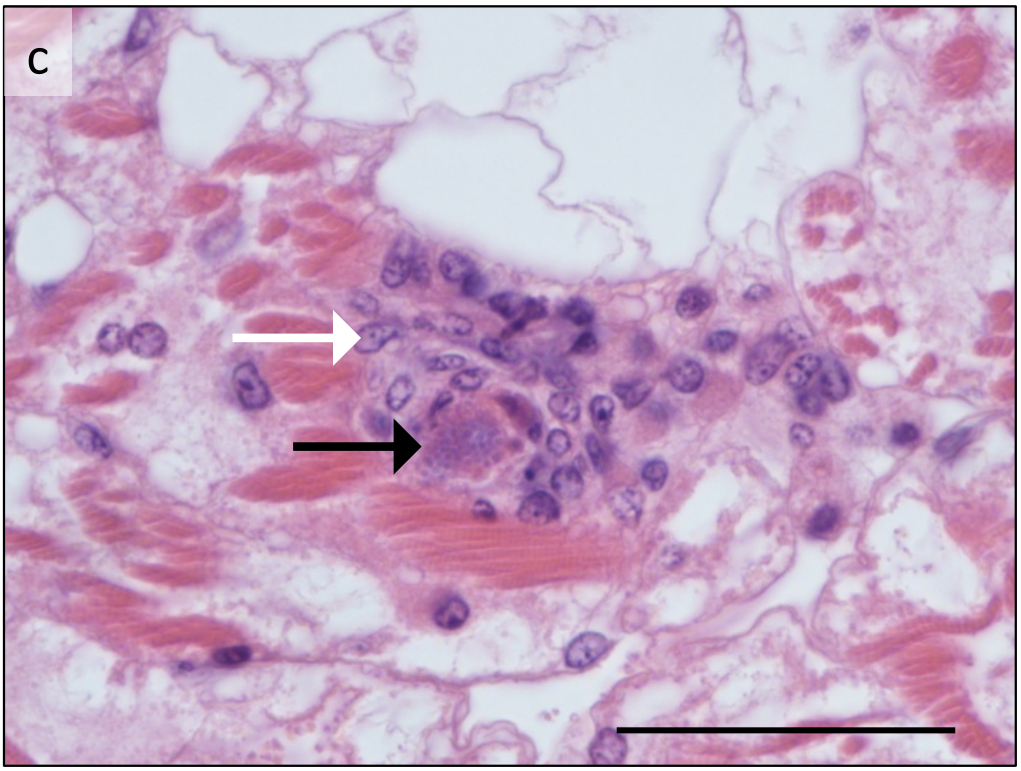


Figure 5.7: Early nodule with encapsulation of bacteria (black arrow) by haemocytes (white arrow) within the haemo-sinuses of the connective tissue of the heart in a *Planococcus citreus* infected *Carcinus maenas*. Scale bar 50μm. H&E stained.

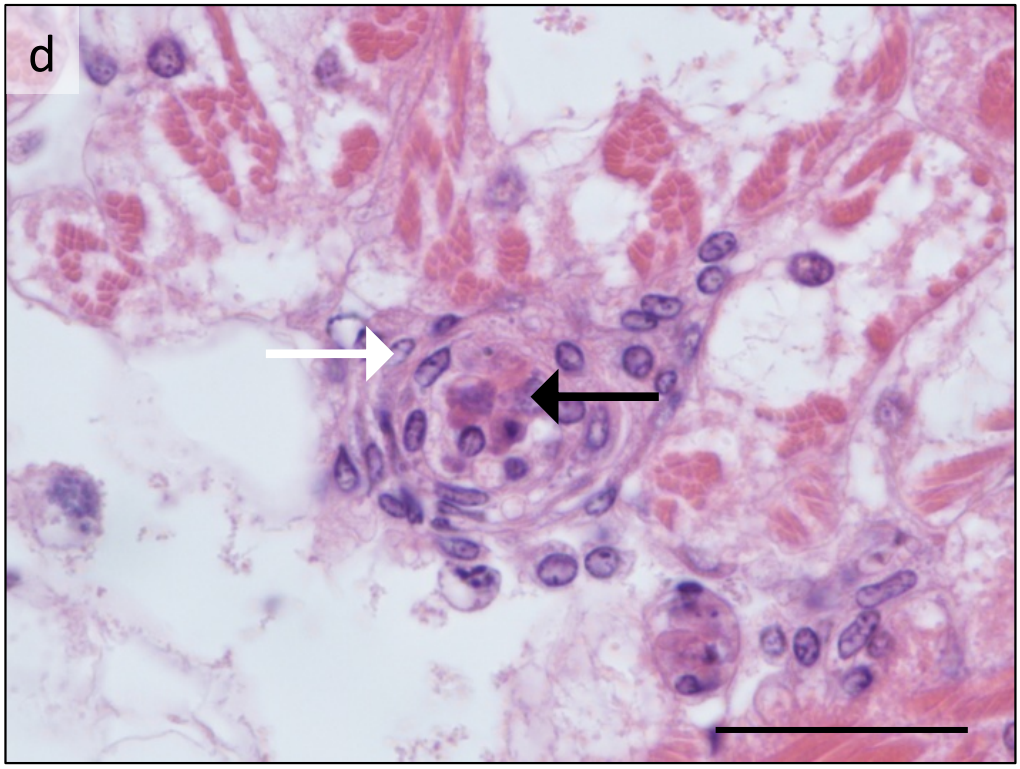


Figure 5.8: Histopathology image of the haemo-sinuses within the connective tissue of the heart in a *Planococcus citreus* infected *Carcinus maenas*. Progression of nodule formation and encapsulation by haemocytes (white arrow). Melanisation can be observed around the bacteria (black arrow) within the centre of the nodule. Scale bar 50μm. H&E stained.

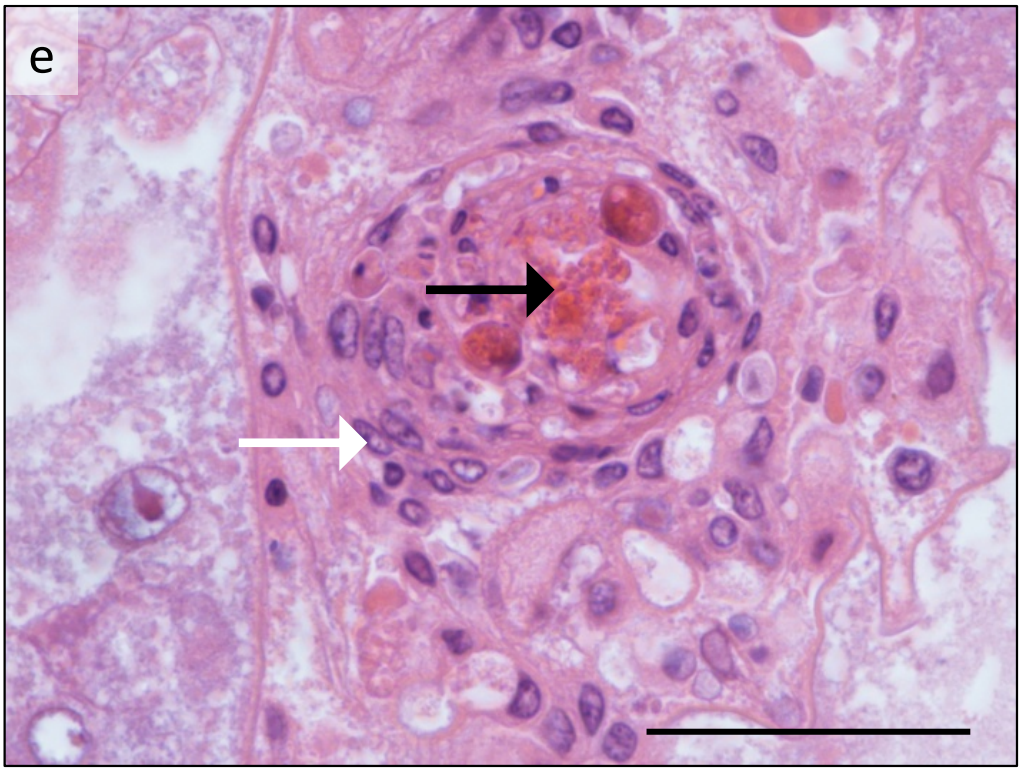


Figure 5.9: Histopathology image of the haemo-sinuses between the hepatopancreatic tubules in a *Planococcus citreus* infected *Carcinus maenas*. Progression of encapsulation by haemocytes (white arrow) and extensive melanisation within the centre of the nodule (black arrow). Scale bar 50μm. H&E stained.

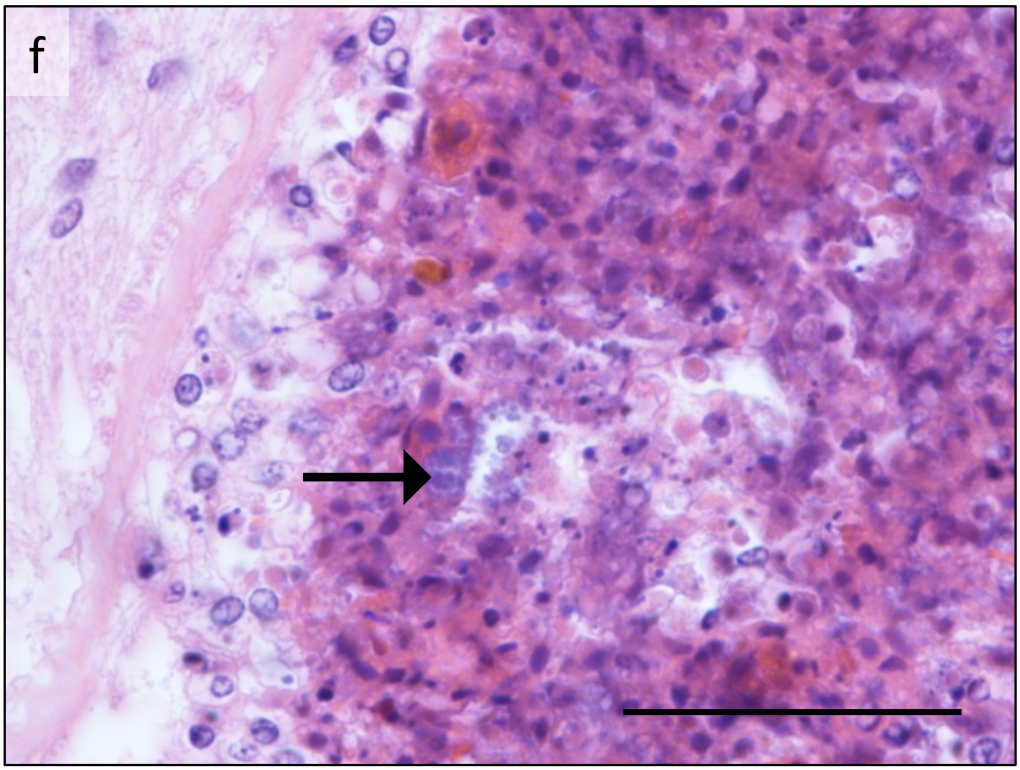


Figure 5.10: Necrotic nodule. Bacteria visible within the nodule (arrow) in a *Planococcus citreus* infected *Carcinus maenas*. Scale bar 50μm. H&E stained.

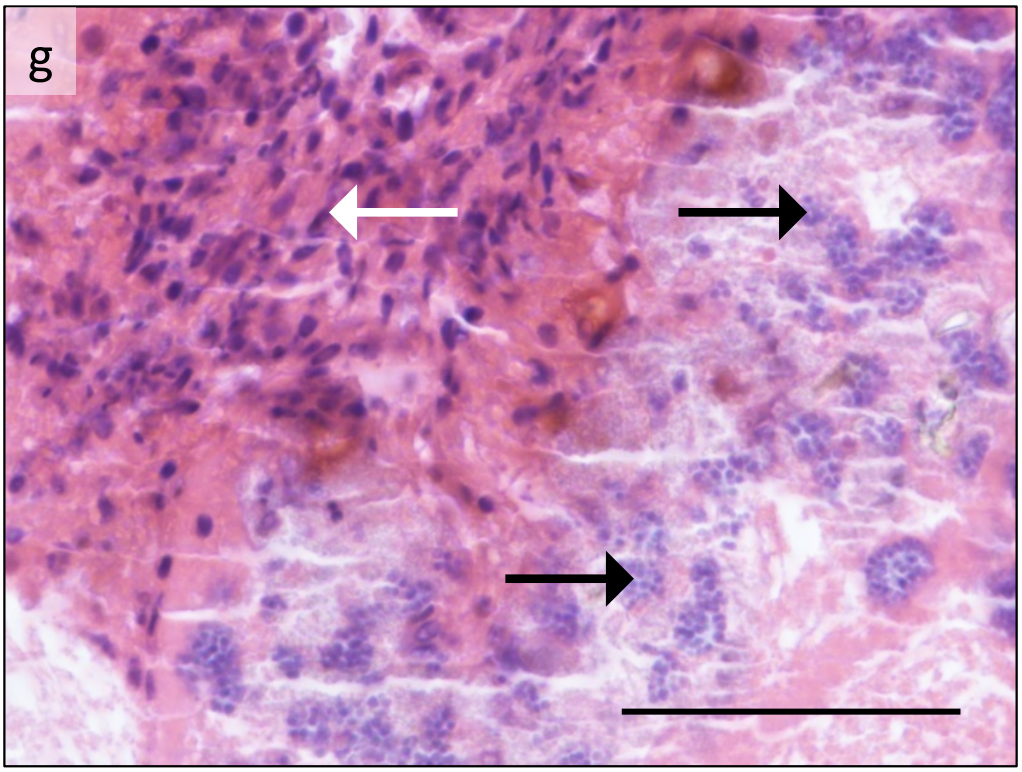


Figure 5.11: Multiple bacterial micro-colonies observed (black arrows) around necrotic tissue (white arrow) in a *Planococcus citreus* infected *Carcinus maenas*. Scale bar 50μm. H&E stained.

5.3.2 Gene expression analysis

5.3.2.1 RNA quality

The quality of the samples included in the final analysis were all within the previously defined criteria of ≥ 7.6 RQI (Chapter 4). However, a comparison of RNA quality was made between the samples from each experiment. The variation in RNA quality is highlighted in Table 5.3 and raw RQI as well as Log_{10}CNRQ values can be seen in Appendix, Chapter 5, Tables 8.23, 8.24, 8.25, 8.26. Notably, *C. maenas* infected with *P. citreus* had a comparably greater proportion of RNA <7 RQI, (no samples had RQI values between 7 and 8), than those crabs infected with *L. anguillarum*, 22.8% and 3.7% respectively (Table 5.3). These samples were analysed for qPCR to compare the gene expression profiles, but excluded from the final analysis (Figures 5.16 and 5.17 and Table 5.5) due to the difficulty in discriminating true biological variation from artefact as a result of degraded RNA (discussed in depth in Chapters 2 and 4).

Table 5.3: Variation in RNA quality from *Listonella anguillarum* and *Planococcus citreus* samples.

RQI	*Listonella anguillarum* (%)	*Planococcus citreus* (%)
10	70.4	54.4
>9	18.5	19.3
>8	7.4	3.5
<7	3.7	22.8

5.3.2.2 Reference gene stability evaluation

Average M and CV values for *Listonella anguillarum* and *Planococcus citreus.*

The most stable M and CV values for both the *L. anguillarum* and *P. citreus* infection trials were achieved using two endogenous reference genes.

Table 5.4: Assessment of the endogenous reference stability. The average M and CV values are highlighted for the different combinations of ERGs.

Planococcus citreus		
Endogenous reference genes	**M**	**CV**
actin, eef1a, gapdh, tubulin	1.136	0.485
actin, eef1a, tubulin	0.916	0.378
actin, eef1a	0.728	0.257
Listonella anguillarum		
Endogenous reference genes	**M**	**CV**
actin, eef1a, gapdh	0.710	0.289
eef1a, gapdh	0.570	0.199

Listonella anguillarum

GeNorm analysis defined *eef1a* and *gapdh* as the most stable combination of endogenous reference genes for *C. maenas* infected with *L. anguillarum* (Figures 5.12 and 5.13). The average M and CV values were 0.570 and 0.199 respectively after removal of *actin* (Table 5.4).

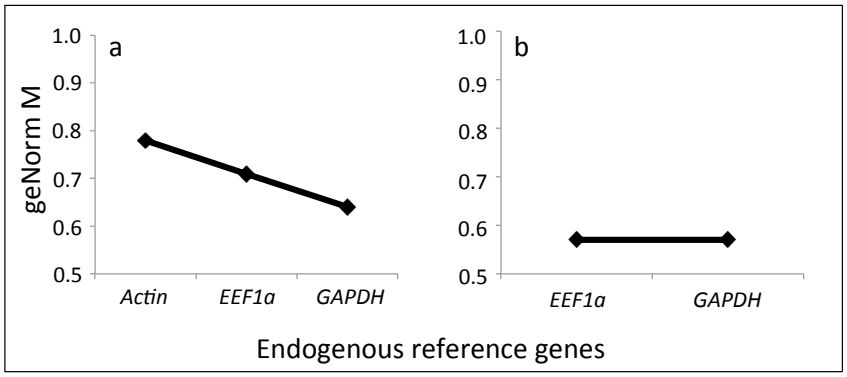


Figure 5.12: Endogenous reference gene stability assessment using geNorm M, for the *Listonella anguillarum* infection study. The removal of the most unstable ERG, *actin*, improved the average M value from 0.710 to 0.570.

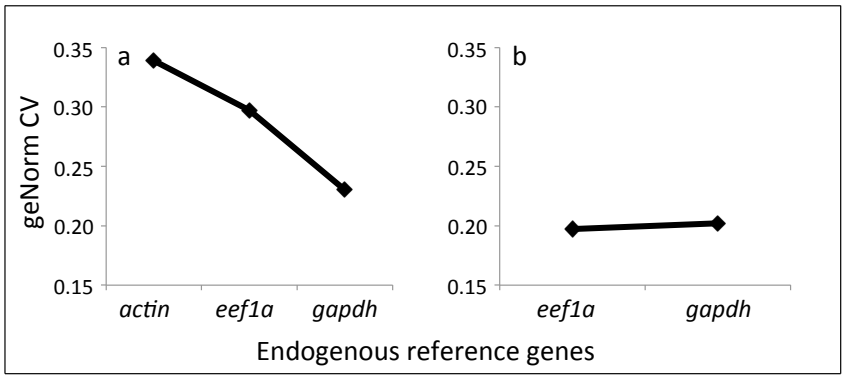


Figure 5.13: Endogenous reference gene stability assessment using geNorm CV, for the *Listonella anguillarum* infection study. Removal of *actin* decreased the average CV from 0.289 to 0.199.

<u>*Planococcus citreus*</u>

The most stable endogenous reference genes were defined as *actin* and *eef1a* with average M and CV values of 0.728 and 0.257 respectively (Figures 5.14 and 5.15 and Table 5.4).

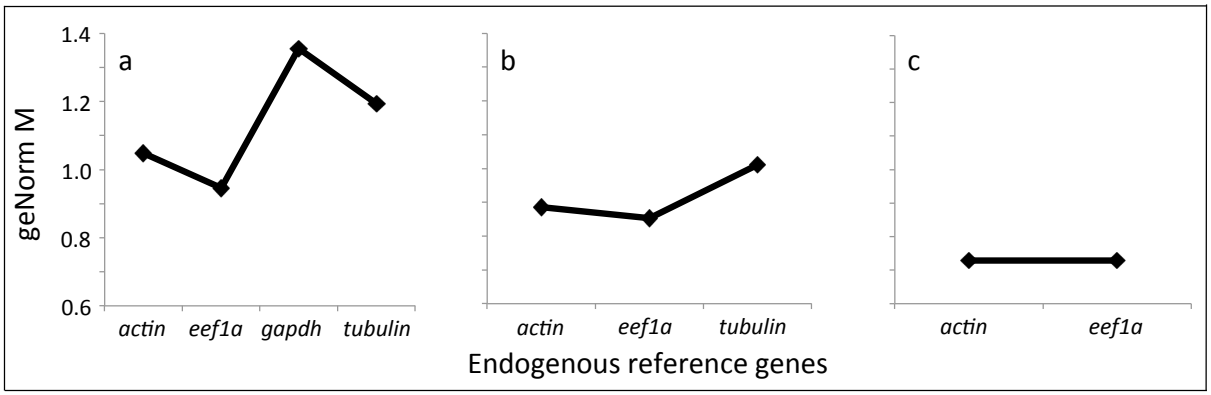


Figure 5.14: Endogenous reference gene stability assessment using geNorm M, for the *Planococcus citreus* infection study. The removal of the most unstable ERG, *gapdh*, improved the average M value from 1.136 to 0.916, but the most stable combination of ERGs was *actin* and *eef1a* resulting in an average M value of 0.728.

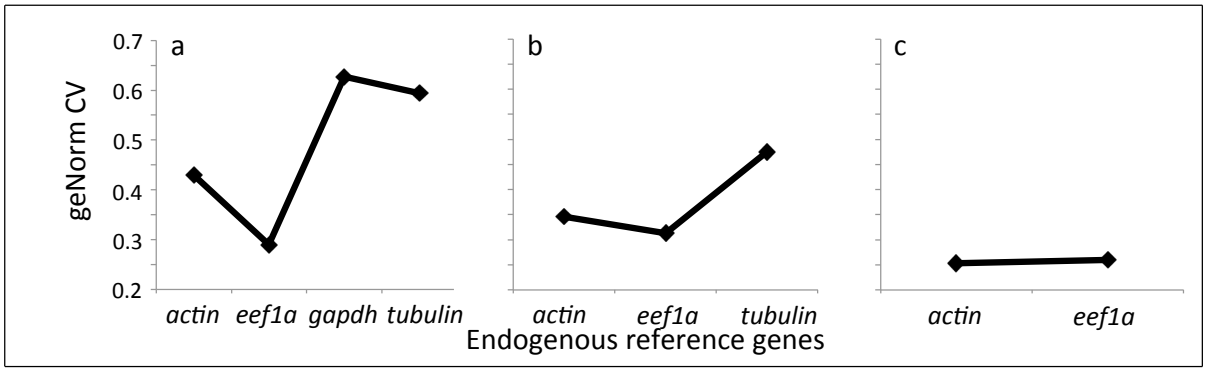


Figure 5.15: Assessment of the endogenous reference gene stability using the co-efficient of variation (CV) value for the *Planococcus citreus* infection trial. Removal of *gapdh* improved the CV from 0.485 to 0.378. The most stable combination of ERGs with a CV of 0.257 was *actin* and *eef1a*.

5.3.2.3 Gene transcription analysis

Listonella anguillarum

Gene transcription was assessed prior to inoculation at 0 hours and at 72 hours. Both *carcinin* and *prophenoloxidase* transcription increased over time in both the control and *L. anguillarum* infected crabs (Figure 5.16 and Table 5.5). Conversely, *peroxinectin* transcription decreased with time in both the control and treatment groups. *Carcinin* expression increased in the bacteria-infected *C. maenas* at 72 hours compared with the control crabs ($p < 0.05$).

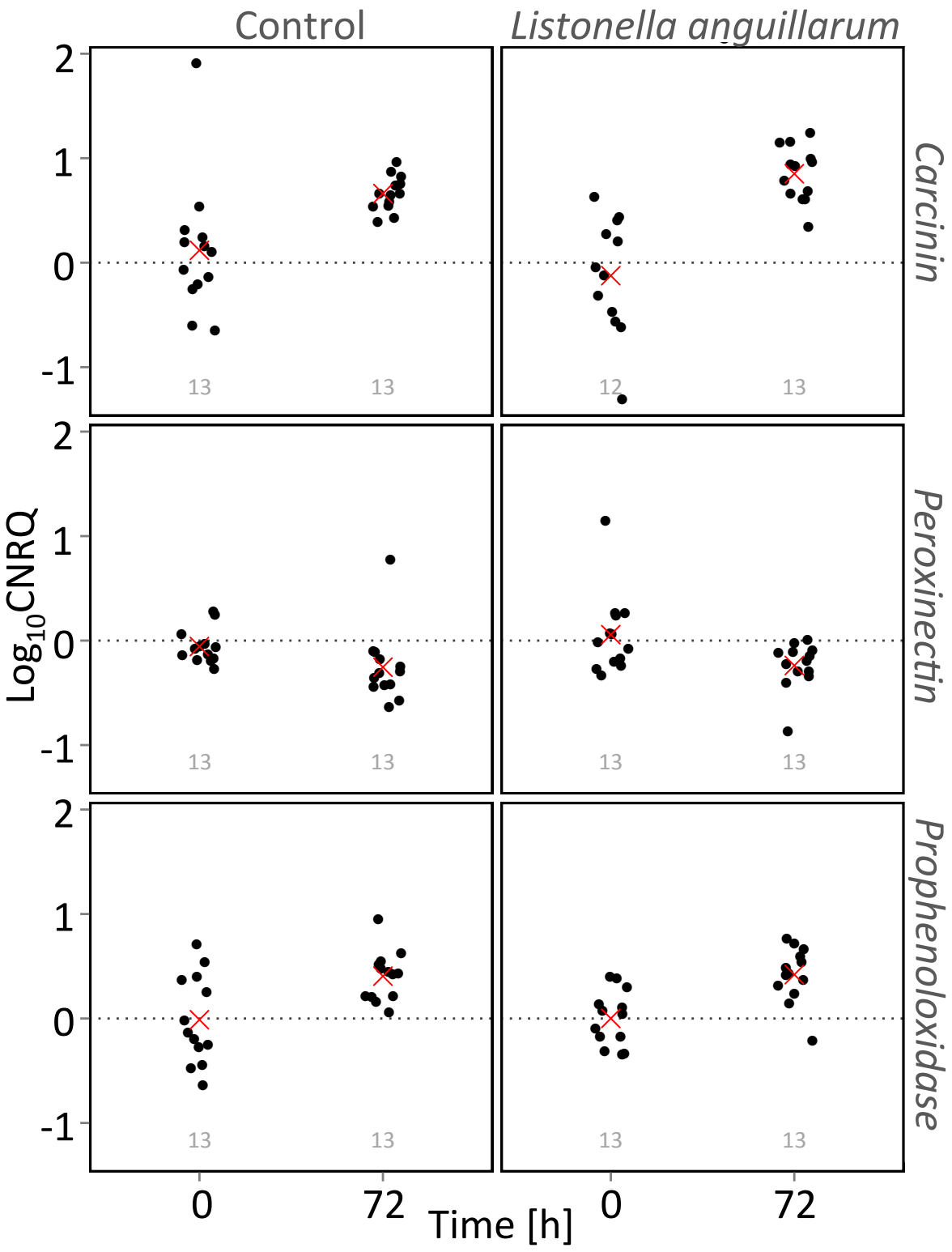


Figure 5.16: Gene transcription (Log_{10}CNRQ) in *Carcinus maenas* in response to marine saline (control) or *Listonella anguillarum* inoculation at 0 hours and 72 hours. The red 'X' marks the mean Log_{10}CNRQ. The number of replicates for each category are indicated on the figure.

Planococcus citreus

Gene transcription was measured at 0 hours prior to inoculation and at 48 hours. The *P. citreus* trial was reduced to 48 hours after preliminary analysis of the gene expression results from the *L. anguillarum* trial whereby no difference in gene expression was identified at 72 hours between treatment groups. Transcription of *carcinin* and *peroxinectin* was significantly up-regulated at 48 hours compared with 0 hours in the control group, although no significant change in transcription was evident in *prophenoloxidase* (Figure 5.17 and Table 5.5). In those crabs inoculated with *P. citreus* only *carcinin* transcription was significantly up-regulated over time. No differences in the transcription of *peroxinectin* or *prophenoloxidase* were observed between treatment groups at 48 hours. However, *C. maenas* inoculated with *P. citreus* were observed to have comparatively greater expression of *carcinin* than those *C. maenas* inoculated with marine saline ($p<0.05$).

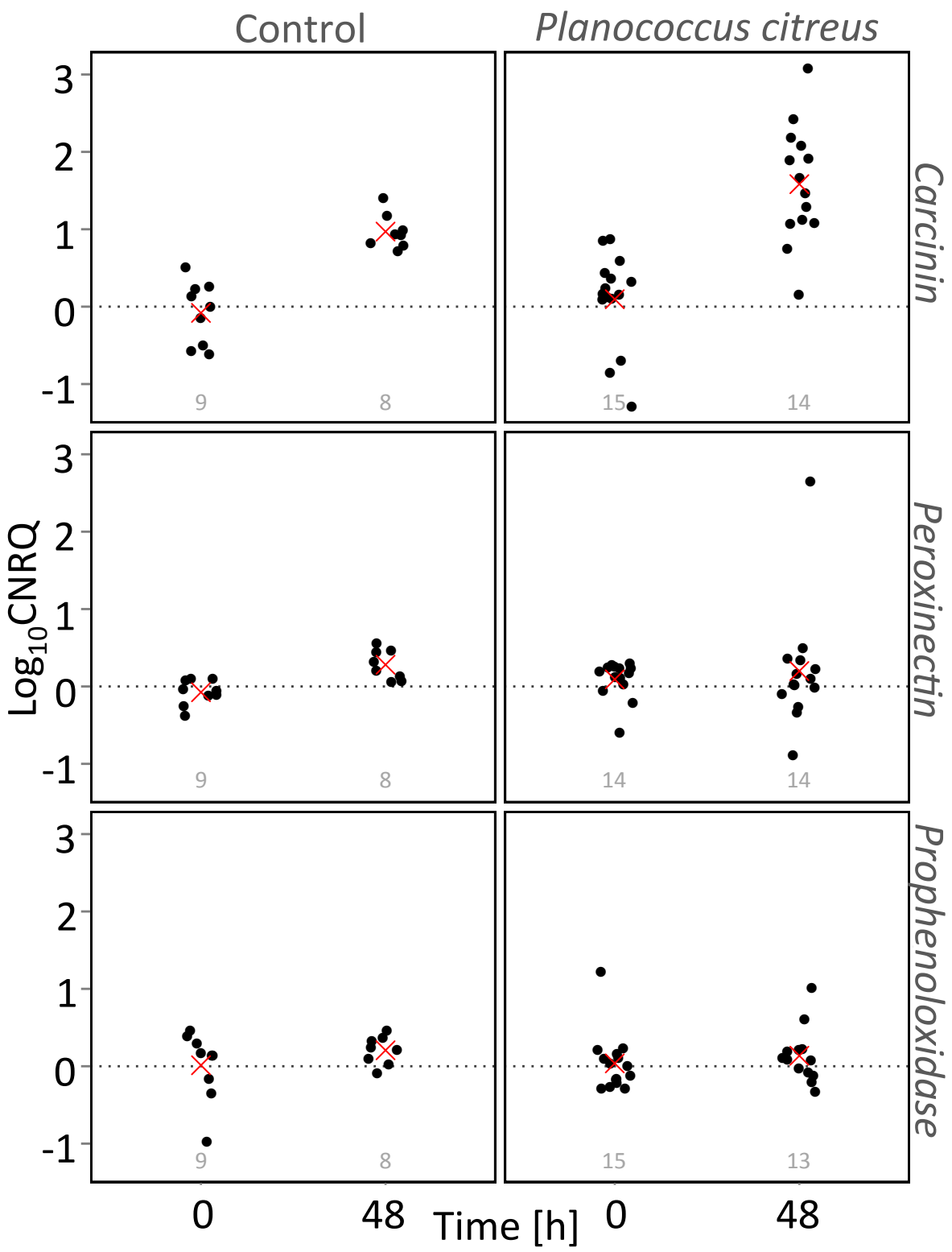


Figure 5.17: Gene transcription (Log_{10}CNRQ) in *Carcinus maenas* in response to marine saline (control) or *Planococcus citreus* inoculation at 0 hours and 48 hours. The red 'X' marks the mean Log_{10}CNRQ. The number of replicates for each category are indicated on the figure.

Table 5.5: Kruskal Wallis analysis for the *Listonella anguillarum* and *Planococcus citreus* infection trials. Statistically significant differences in gene expression are highlighted in red.

Listonella anguillarum			
Treatment	***Carcinin***	***Peroxinectin***	***Prophenoloxidase***
Control at 0 hours and 72 hours	H=12.3, d.f.=1, $p<0.001$	H=8.1, d.f.=1, $p<0.01$	H=5.9, d.f.=1, $p<0.05$
L. anguillarum inoculum at 0 hours and 72 hours	H=15.8, d.f.=1, $p<0.0001$	H=4.8, d.f.=1, $p<0.05$	H=10.9, d.f.=1, $p<0.001$
Control and *L. anguillarum* inoculum at 72 hours	H=3.4, d.f.=1, $p<0.05$	H=1.6, d.f.=1, $p>0.05$	H=0.19, d.f.=1, $p>0.05$
Planococcus citreus			
Treatment	***Carcinin***	***Peroxinectin***	***Prophenoloxidase***
Control at 0 hours and 48 hours	H=12, d.f.=1, $p<0.001$	H=8.3, d.f.=1, $p<0.01$	H=0.5, d.f.=1, $p>0.05$
P. citreus inoculum at 0 hours and 48 hours	H=17.4, d.f.=1, $p<0.0001$	H=0.2, d.f.=1, $p<0.05$	H=1.0, d.f.=1, $p>0.05$
Control and *P. citreus* inoculum at 48 hours	H=5.4, d.f.=1, $p<0.05$	H=2.6, d.f.=1, $p>0.05$	H=1.3, d.f.=1, $p>0.05$

Gene transcription in relation to total haemocyte count

Correlation between the THC and expression of each immune genes within each treatment group at each sampling time was analysed using the co-efficient of determination (R^2) (Table 5.6). The relationship between THC and immune gene expression is observed in Figures 5.18 and 5.19.

In the *L. anguillarum* infection trial, no correlation between the number of total haemocytes and the expression of *carcinin*, *peroxinectin* or *prophenoloxidase* could be observed ($p>0.05$) (Figure 5.18 and Table 5.6). Also, no correlation between the number of haemocytes and the expression of immune genes could be observed in all but one of the data groups in the *P. citreus* infection trial. For *prophenoloxidase* expression at 0 hours, 47% of the variation in the gene transcription could be explained by the number of total haemocytes ($F=12.7$, $R^2=0.47$, d.f.=1, 12, $p<0.01$) (Figure 5.19 and Table 5.6).

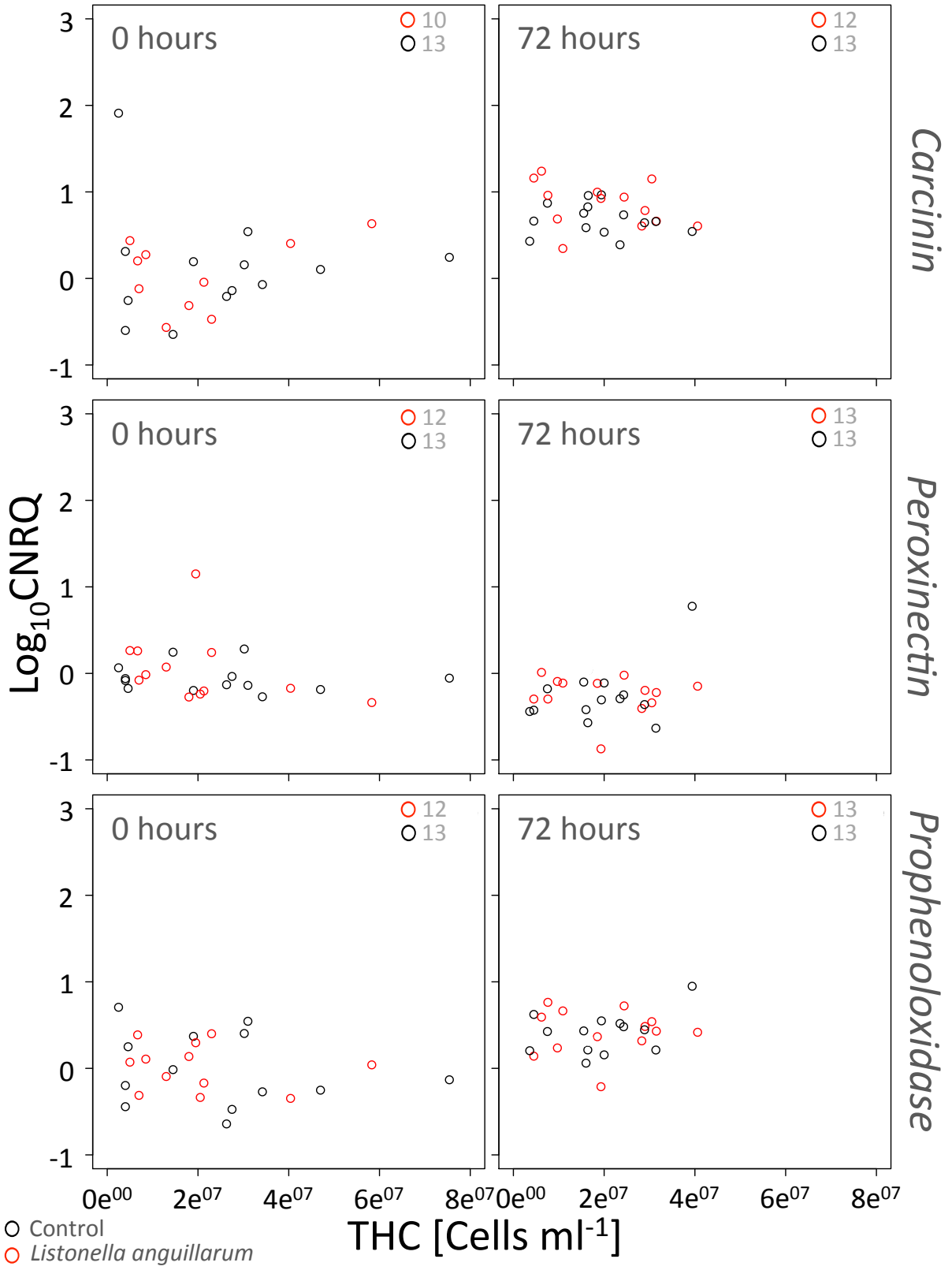


Figure 5.18: Immune gene transcription in relation to total haemocyte count in response to infection with *Listonella anguillarum*. Control crabs are highlighted in black and red were infected with *L. anguillarum*. No correlation between the immune genes and THCs was observed ($p>0.05$). The number of replicates for each category are indicated on the figure.

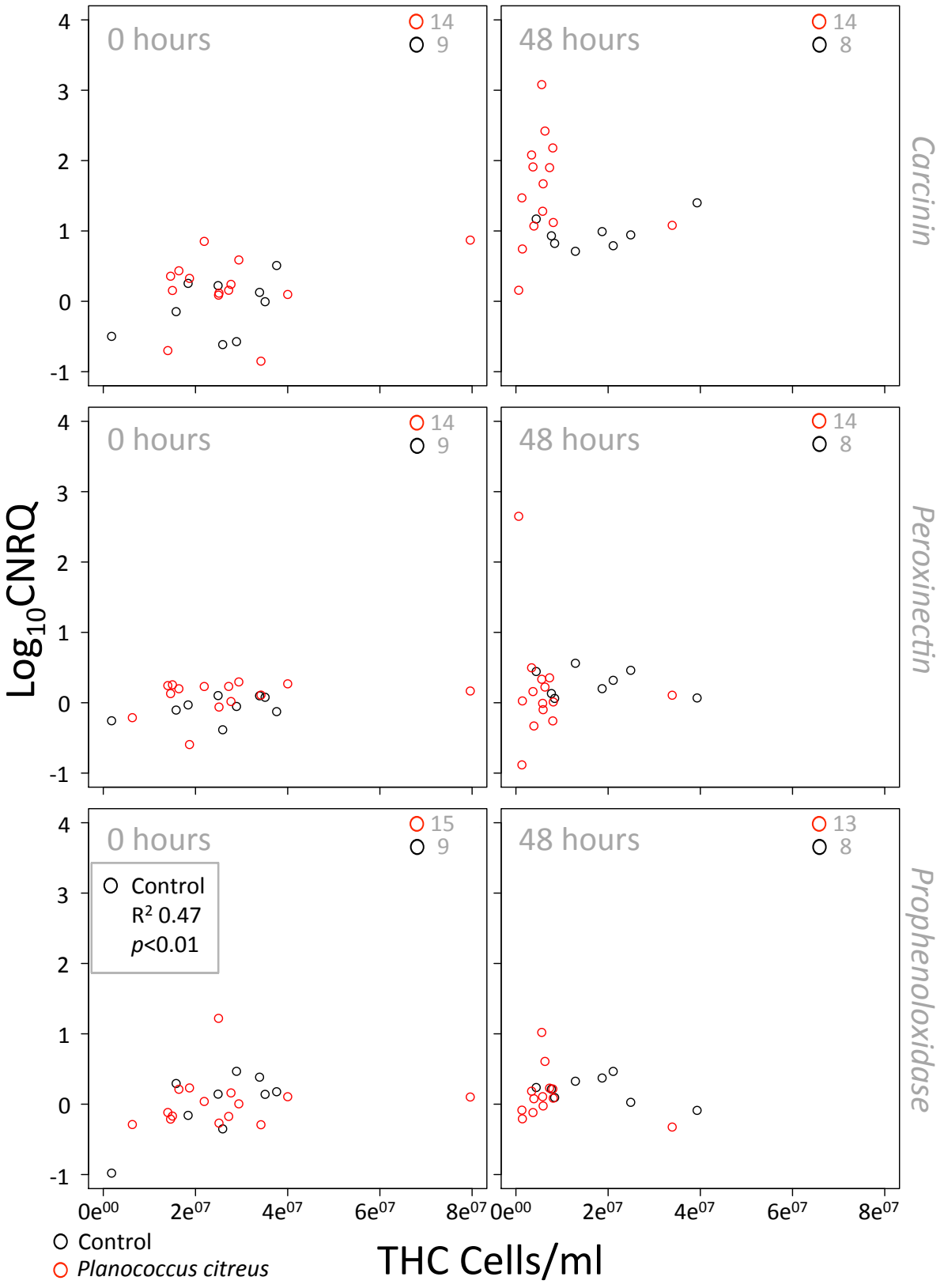


Figure 5.19: Immune gene transcription in relation to total haemocyte count in response to *Planococcus citreus*. Control crabs are highlighted in black and red were infected with *P. citreus*. For *prophenoloxidase*, 47% of the variation in gene transcription could be explained by THC (F=12.7, R^2-0.47, d.f.=1, p=<0.01). The number of replicates for each category are indicated on the figure.

Table 5.6: Correlation analysis between the THC and immune gene expression. *Listonella anguillarum* is highlighted in the shaded area and statistically significant correlations are highlighted in red.

Control		
	0 hours	**72 hours**
Carcinin	F = 0.03, R^2 = 0.09, d.f. = 1 , p >0.05	F = 0.29, R^2 = 0.06, d.f. = 1, p >0.05
Peroxinectin	F = 0.20, R^2 0.07= , d.f. = 1, p >0.05	F =2.81, R^2 = 0.12, d.f. = 1, p >0.05
Prophenoloxidase	F = 0.45, R^2 = 0.05, d.f. = 1, p >0.05	F = 1.90, R^2 = 0.06, d.f. = 1, p >0.05
Listonella anguillarum		
Carcinin	F = 0.05, R^2 = 0.09, d.f. = 1, p >0.05	F = 1.29, R^2 = 0.02, d.f. = 1, p >0.05
Peroxinectin	F = 0.43, R^2 = 0.05, d.f. = 1, p >0.05	F = 0.12, R^2 = 0.08, d.f. = 1, p >0.05
Prophenoloxidase	F = 0.79, R^2 = 0.00, d.f. = 1, p >0.05	F = 0.00, R^2 = 0.00, d.f. = 1, p >0.05
Control		
	0 hours	**48 hours**
Carcinin	F = 1.21, R^2 = 0.02, d.f. = 1, 11, p >0.05	F = 1.02, R^2 = 0.00, d.f. = 1, 10, p >0.05
Peroxinectin	F = 1.47, R^2 = 0.04, d.f. = 1, 12, p >0.05	F = 0.11, R^2 = 0.00, d.f. = 1, 10, p >0.05
Prophenoloxidase	F = 12.7, R^2 = 0.47, d.f. = 1, 12, p <0.01	F = 0.03, R^2 = 0.10, d.f. = 1, 10, p >0.05
	Planococcus citreus	
Carcinin	F = 2.79, R^2 = 0.11, d.f. = 1, 13, p >0.05	F = 4.00, R^2 = 0.20, d.f. = 1, 11, p >0.05
Peroxinectin	F = 0.66, R^2 = 0.00, d.f. = 1, 12, p >0.05	F = 1.22, R^2 = 0.02, d.f. = 1, 11, p >0.05
Prophenoloxidase	F = 0.20, R^2 = 0.00, d.f. = 1, 13, p >0.05	F = 2.10, R^2 = 0.09, d.f. = 1, 10, p >0.05

5.3.3 Total haemocyte count

The total haemocyte count was analysed at 0, 3 and 72 hours in *C. maenas* during the *L. anguillarum* infection trial. This was modified during the *P. citreus* trial to 0, 3, 24 and 48 hours (Figure 5.20).

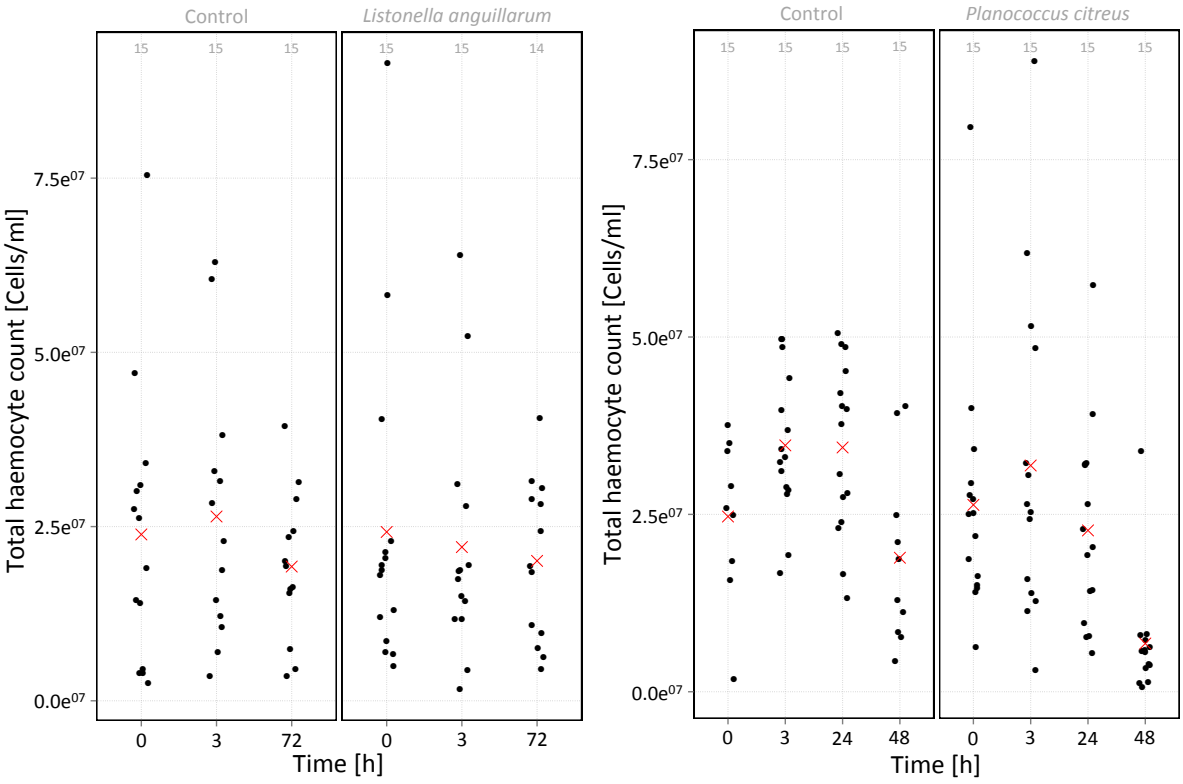


Figure 5.20: Total haemocyte count in *Carcinus maenas* in response to infection by *Listonella anguillarum* and *Planococcus citreus*. The red 'X' marks the mean THC within each group. Data represents all samples with RQI values $\geq$ 7.6. The number of replicates for each category are indicated on the figure.

No significant difference over time or between treatments at 0, 3 or 72 hours was observed during the *L. anguillarum* infection trial (Figure 5.20 and Table 5.7). Significant differences in THC during the *P. citreus* trial were observed over time in both the control and infected groups (p <0.001 and p <0.0001 respectively) and at 3 and 48 hours between the control and bacterially infected crabs in the *P. citreus* trial (p <0.05 and p <0.01 respectively).

Table 5.7: Kruskal Wallis analysis of total haemocyte counts (THC) within the *Listonella anguillarum* (shaded boxes) and *Planococcus citreus* infection trials. Statistically significant differences in THC are highlighted in red.

Control	0, 3, 72 hours	H = 0.50, d.f. = 2, p >0.05
Listonella anguillarum	0, 3, 72 hours	H = 0.03, d.f. = 2, p >0.05
Control v *Listonella anguillarum*	0 hours	H = 0.01, d.f. = 1, p >0.05
	3 hours	H = 0.46, d.f. = 1, p >0.05
	72 hours	H = 0.17, d.f. = 1, p >0.05
Control	0, 3, 24, 48 hours	H = 19.80, d.f. = 3, p <0.001
Planococcus citreus	0, 3, 24, 48 hours	H = 20.98, d.f. = 3, p <0.0001
Control v *Planococcus citreus*	0 hours	H = 0.07, d.f. = 1, p >0.05
	3 hours	H = 5.49, d.f. = 1, p <0.05
	24 hours	H = 1.83, d.f. = 1, p >0.05
	48 hours	H = 10.06, d.f. = 1, p <0.01

5.3.4 Bacterial load

Bacterial load was assessed at 0, 3 and 48 (*P. citreus*) or 72 hours (*L. anguillarum*). In both the *L. anguillarum* and *P. citreus* infection trials, bacterial load increased significantly over time ($p < 0.0001$). However, this was also evident in the control groups in both the Gram negative and Gram positive infection trials, $p < 0.01$ (*L. anguillarum*) and $p < 0.0001$ (*P. citreus*) (Figure 5.21 and Table 5.8).

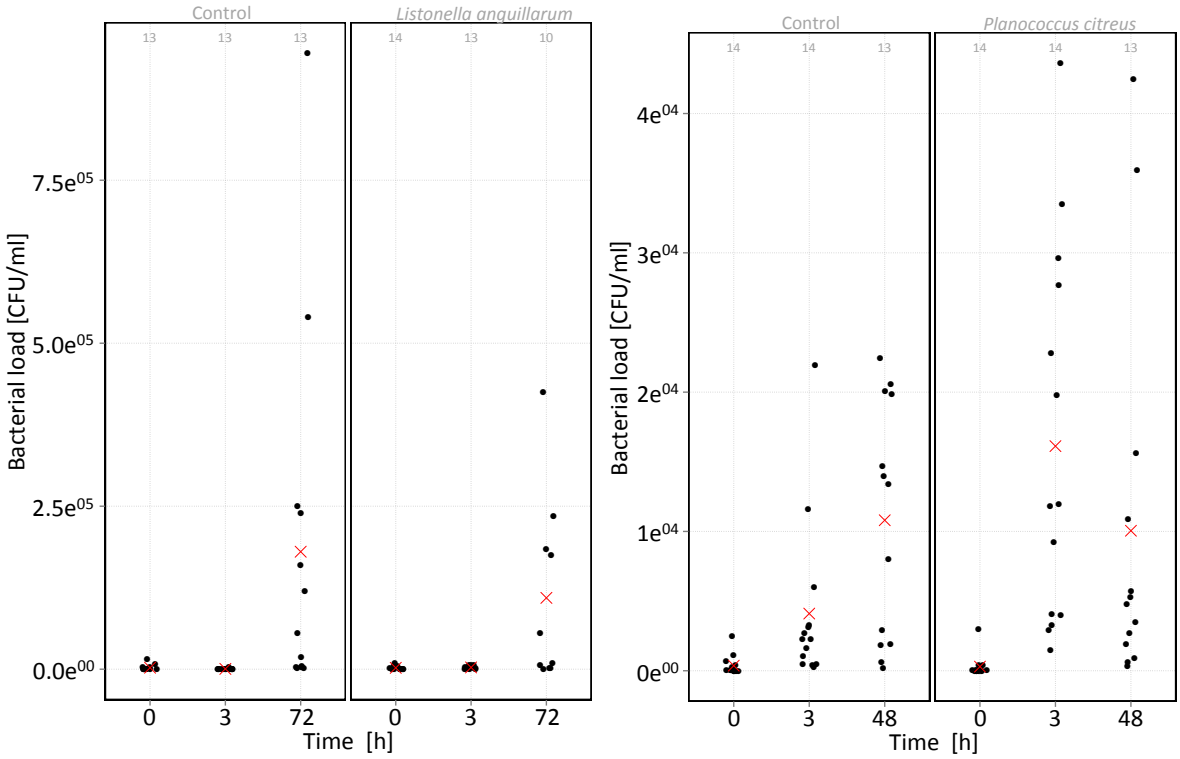


Figure 5.21: Bacterial load in *Carcinus maenas* during both the *Listonella anguillarum* and *Planococcus citreus* infection trials. Note the scales on the y axis are different. Data represents all samples with RQI values ≥ 7.6. The number of replicates for each category are indicated on the figure.

In both the *L. anguillarum* and *P. citreus* trials there was a significant difference in bacterial load between the control and bacterial inoculated *C. maenas* at 3 hours ($p < 0.001$ and $p < 0.01$ respectively). This is not evident in Figure 5.21 due to the scale of the graph, but can be seen in Appendix, Chapter 5, Figure 8.23.

Table 5.8: Kruskal Wallis analysis of bacterial load within the *Listonella anguillarum* (shaded boxes) and *Planococcus citreus* infection trials. Statistically significant differences in bacterial load are highlighted in red.

Control	0, 3, 72 hours	H = 20.45, d.f. = 2, p = <0.0001
Listonella anguillarum	0, 3, 72 hours	H = 9.35, d.f. = 2, p = <0.01
Control v *Listonella anguillarum*	0 hours	H = 1.45, d.f. = 1, p = >0.05
	3 hours	H = 12.73, d.f. = 1, p = <0.001
	72 hours	H = 0.22, d.f. = 1, p = >0.05
Control	0, 3, 48 hours	H = 21.45, d.f. = 2, p = <0.0001
Planococcus citreus	0, 3, 48 hours	H = 25.80, d.f. = 2, p = <0.00001
Control v *Planococcus citreus*	0 hours	H = 0.26, d.f. = 1, p = >0.05
	3 hours	H = 10.21, d.f. = 1, p = <0.01
	48 hours	H = 0.35, d.f. = 1, p = >0.05

Prior to the infection trials, autoclaved seawater plated immediately post autoclaving, grew no bacterial colony forming units (cfu) whereas $5e^{03}$ cfu grew from non-autoclaved seawater (both at 48 hours). Bacterial colonies were plated from the autoclaved seawater from both the *L. anguillarum* and *P. citreus* infection challenges (not routinely) and grew between $1.8e^{04}$ and $3.1e^{06}$. The data was based on less than 5 samples across treatments (control and infected) and therefore lacks sufficient replication.

5.4 Discussion

The overall aim of this study was to investigate the host transcriptional response of *Carcinus maenas* specifically to past exposure (at either 48 hours or 72 hours) to two known pathogenic agents; the Gram negative bacteria *Listonella anguillarum* and Gram positive bacteria *Planococcus citreus*. Immune gene transcription along with total haemocyte count, histology and bacterial load were investigated to provide a comprehensive analysis of host response. It is important to note that the population of *C. maenas* collected for these infection studies were sourced from the wild. Care was taken to ensure any *C. maenas* with obvious external pathology of infection, such as those with necrotic shell disease or external evidence of *Sacculina* (*i.e.* evidence of externa), were eliminated from this study at the time of collection. Also, *C. maenas* were acclimated for a total of 28 days to ensure any animals with significant disease were eliminated from this study. However, low level infections within any of these animals could not be discounted. That said, it was important to use wild-caught *C. maenas* to better represent the host immune gene transcription to infection that may be evident in natural populations, rather than specific pathogen-free animals.

5.4.1 Histology

Listonella anguilarum

Histology, analysed at the end of each infection trial, was particularly revealing. It was clear from the *L. anguillarum* infection trial that inflammatory responses were evident. Granulomas could be seen in the connective tissue (Figure 5.2), hepatopancreatic haemal space (Figure 5.3) and within the gill (Figure 5.4). Some of the features within these histology preparations, such as established melanisation, indicate later stage inflammatory processes. Furthermore, the absence of visible bacterial inclusion bodies suggests the infection within these tissue preparations had been successfully cleared.

Planococcus citreus
Preliminary assessment confirmed a lack of host transcriptional change in *peroxinectin* and *prophenoloxidase* expression in the *L. anguillarum* challenge (discussed later) with late stage inflammatory processes observed in the histology images. Therefore, the sampling of *C. maenas* for the *Planococcus citreus* infection trial was shortened by 24 hours with the aim of observing transcriptional changes in all biomarkers along with inflammatory responses evident in the histology.

The histology identified animals at different stages of infection, illustrated in Figures 5.5 to 5.11. These images record the progress of infection from a single cell to an established bacterial infection. Figure 5.5 shows an intact cell membrane, whereas the integrity of the cell membrane in Figure 5.6 appears compromised. Progression of infection is evident in Figures 5.7 to 5.9 with bacteria surrounded by haemocytes, some of which appear to be undergoing degranulation and encapsulation along with evidence of melanisation (Figures 5.8 and 5.9), suggesting that the host is responding specifically to the bacteria. Figure 5.10 shows a more established infection with aggregation of haemocytes and melanisation around visible bacteria. Finally, Figure 5.11 clearly shows significant immune response from the host (haemocyte aggregation and melanisation) to significant bacterial infection (numerous bacterial cells unchecked by the haemocytes). Subsequent histological sampling may have shed light on whether *C. maenas* were effective in responding to the established infection with *P. citreus*.

5.4.2 Immune gene transcription and bacterial load

Transcription of immune genes was assessed prior to infection and at the end of each infection trial.

Listonella anguillarum
The transcription of *carcinin* and *prophenoloxidase* in both the control and bacteria-infected crabs increased significantly over the course of the three day trial (Table 5.5). Conversely, the expression of *peroxinectin* decreased over time in both the control and *L. anguillarum* infected animals. There was a concurrent increase in the bacterial load in the bacteria-infected crabs and unexpectedly in the control crabs. This is likely to explain the increase in immune transcription over time in both treatment groups (control and infected). In addition, there was a significant increase in the bacterial load in the *L. anguillarum* infected crabs at 3 hours compared with the control animals ($p<0.001$).

The seawater was autoclaved in order to prevent inadvertent contamination of animals, via the inoculation site, from bacterial agents within the seawater. Whilst the autoclaving itself was effective, there was subsequent contamination of the autoclaved seawater (data not shown). The colonies plated from the control animals and autoclaved seawater were of a similar colour (cream) so were not distinguishable from *L. anguillarum* colonies and as a consequence were included in the overall quantification of bacterial load. It is therefore probable that the seawater was contaminated post-autoclaving, potentially from bacteria associated with the animals themselves. Another possible cause of the observed increase in bacterial load in the control crabs was contamination of marine saline (not determined). However, as this was used across both treatment groups any effect on gene expression would have been consistent in control and inoculated crabs.

Interestingly, the transcription of *peroxinectin* and *prophenoloxidase* between treatment groups at 72 hours was not statistically significant. Yet, *carcinin* transcription was significantly up-regulated at 72

hours in the bacteria-infected crabs compared to the control crabs. This is in contrast with the early description by Relf *et. al.* (1999) describing *carcinin* activity only against Gram positive marine or salt-tolerant bacteria, although some antimicrobial peptides (6.5 kDa and 14 kDa) from *Carcinus maenas* are known to have activity against both Gram negative and Gram positive bacteria (Schnapp et al., 1996). In general, antimicrobial peptides such as crustins, *carcinin* being classified as a Type I crustin (Smith et al., 2008), have demonstrated varied expression profiles in response to infection. Crustin expression in the European lobster, *Homarus gammarus*, was down-regulated from 3 hours post inoculation in response to infection with *L. anguillarum* (Hauton et al., 2006). In contrast, crustin expression in the granular cells of the spider crab, *Hyas araneus*, was found to have a small, but significant up-regulation in response to *L. anguillarum* challenge (Sperstad et al., 2010). Crustins in other species, such as *Penaeus monodon*, can show activity against both Gram positive (*A. viridans*) and Gram negative (*Escherichia coli* 363 and *Vibrio harveyi*) bacteria although this was considered unusual (Amparyup et al., 2008) as other *P. monodon* crustins have only demonstrated activity against Gram positive bacteria such as *Staphylococcus aureus* and *Streptococcus iniae* (Supungul et al., 2008). Based on this information, it would appear there is considerable variety within this antimicrobial group in terms of activity profile.

A key footnote to these variations in *crustin* transcription in the literature is that activity profiles may also be a function of whether the study was *in vivo* or *in vitro*. For example, assessment of crustin peptides in *Litopenaeus vannamei* demonstrated *in vitro* activity against Gram positive bacteria, whereas *in vivo* they demonstrated activity against *Vibrio penaeicida*, a Gram negative bacteria (Shockey et al., 2009). This complexity makes it challenging to fully assess the range of activity of different crustins across different species. As suggested by Shockey et al. (2009), *in vivo* crustin activity may require the action of other molecules or optimal conditions in order to function against certain microbes which may explain differences in crustin activity between *in vivo* and *in vitro* methods.

In addition to the different activity profiles, the expression profile of different crustin isoforms within a single species can also vary. A Type II crustin in *Penaeus vannamei* up-regulated expression in response to infection with *Vibrio alginolyticus* at 12-24 hours post inoculation (Vargas-Albores et al., 2004), but Type III crustin was initially up-regulated post inoculation (3 and 6 hours) and then down-regulated at 24 hours (Jiménez-Vega et al., 2004). However, care needs to be taken when comparing across the literature as often, the scale of experiments are varied and the dose of bacteria differs.

The immune expression at 72 hours highlights changes in transcription only in *carcinin*. In view of the bacterial load data from 3 hours and the histology highlighting immune processes (haemocyte aggregation and melanisation), it is likely that if transcriptional changes in *peroxinectin* and *prophenoloxidase* occurred between treatment groups, it would be evident earlier during the infection. Mixed *L. anguillarum* (Gram negative) and *S. aureus* (Gram positive) infection in the Chinese shrimp *Fenneropenaeus chinensis* resulted in earlier expression of *peroxinectin* at 6 and 12 hours post injection (Dong et al., 2009). This was also evident in *Vibrio alginolyticus* (Gram negative) infection in *Penaeus vannamei* at 6, 12 and 24 hours post infection (Liu et al., 2005). Expression of *prophenoloxidase* post injection of *L. anguillarum* in *Fenneropenaeus chinensis*, has also shown early transcriptional changes whereby an initial down-regulation (6 hours) was followed by an up-regulation (12 hours) (Gao et al., 2009). That said, there was also evidence of transcriptional changes in *prophenoloxidase* at 72 hours (Gao et al., 2009), suggesting, in this species at least, *prophenoloxidase* could be relevant during later *L.*

anguillarum infection. With the exception of this example, it is likely that *peroxinectin* and *prophenoloxidase* are expressed by the host early during the infection process in order to eliminate the pathogen.

Planococcus citreus

Transcription varied over time (0-48 hours) between the different immune markers. In the control animals the transcription of *carcinin* ($p<0.001$) and *peroxinectin* increased over time ($p<0.01$) and in the *P. citreus* infected animals only *carcinin* transcription increased significantly over time ($p<0.0001$) (Figure 5.17 and Table 5.5). No transcriptional changes were evident in the expression of *prophenoloxidase* over time or between treatment groups. However, there was a significant up-regulation of *carcinin* between the control and *P. citreus* inoculated *C. maenas* ($p<0.05$) at 48 hours.

Bacterial load increased throughout the trial in both the control and *P. citreus* infected animals (Figure 5.21 and Table 5.8). Again, the increase in bacteria in the control animals could potentially have been a result of secondary infection via the inoculation site as a consequence of contaminated seawater. There is also the possibility that the increased bacterial load in the controls was a direct result via contamination of marine saline as previously discussed. However, these bacterial colonies were distinguishable by colour (cream) with the colonies plated from *P. citreus* infected *C. maenas* (yellow). Those animals infected with *P. citreus* yielded a combination of the two differently coloured bacterial colonies, although most colonies were yellow. The cream coloured colonies, were consistent with those observed in the control animals and from the autoclaved seawater. To be consistent with the methodology, all bacterial colonies were included in the quantification of bacterial load. However, there was a significant ($p<0.01$) increase in the bacterial load between those *C. maenas* infected with *P. citreus* compared with the control crabs at 3 hours post-inoculation.

Interestingly, there was an increase in the transcription of *peroxinectin* only in the control group and not in the *P. citreus* infected group. Therefore, it could be that *peroxinectin* was responding to the secondary infection acquired as a consequence of contaminated seawater. The lack of a change in *peroxinectin* transcription over time in the *P. citreus* crabs and between treatment groups suggests a lack of phaogytic activity from the host cells. This is consistent with the appearance of bacterial cells unchecked by the haemocytes as evident in Figure 5.11. Consistent with this is a significant decrease in THC at 48 hours in the *P. citeus* infected *C. maenas* compared with the control crabs, which may explain the lack of *peroxinectin* transcription in the infected crabs.

Curiously, whilst there was evidence of melanisation in the *P. citreus* infected crabs, there was no discernible change in the transcription of *prophenoloxidase* over time or between treatment groups. Although it is known that transcription of genes does not automatically infer expression of functional proteins (Chen, 2007), taking the increase in *carcinin* transcription between treatment groups at 48 hours together with the histological observations of bacterial cells, there is an argument for an immune response from *C. maenas* as a consequence of infection with *P. citreus*. Activity of *carcinin* has been well established against Gram positive bacteria and the increase in *carcinin* expression in this study is consistent with previous reports demonstrating activity against *P. citreus*. (Relf et al., 1999). However, a change in transcription was not observed in *peroxinectin* or *prophenoloxidase* (*P. citreus* infected group only), although there was histological evidence of haemocyte aggregation and melanisation. In view of the established inflammatory responses observed in the histology, changes in *peroxinectin* and

prophenoloxidase transcription would most probably have been evident earlier in the trial. A significant ($p<0.01$) reduction in THC at 48 hours in the *P. citreus* infected group, (discussed later), may have contributed to a lack of transcription of these genes, particularly as *peroxinectin* and *prophenoloxidase* are synthesised and then stored in the granules of the haemocytes and then released upon microbial stimulation. As a consequence, a reduction of haemocytes could potentially reduce the immune efficiency of *C. maenas* (Hauton et al., 1997). In view of this and the histology indicating significant bacterial infection (Figure 5.11), it would have been interesting to determine later expression changes in *peroxinectin* and *prophenoloxidase* after recovery of the haemocyte population.

5.4.3 Total haemocyte count

Total haemocyte count was an important biomarker to indicate the impact of infection on host immunocompetence due to the multi-functional role of the haemocytes (discussed in Chapter 1).

Listonella anguillarum

There was relatively similar haemocyte counts in both the control and *L. anguillarum* infected crabs (Figure 5.20 and Table 5.7) throughout the Gram negative infection trial. This was not the expected infection response. Classically, a reduction in THC occurs as haemocytes are recruited for functions such as aggregation (Figure 5.2), nodule formation and encapsulation (Hauton et al., 1997). This population is then subsequently replaced through haematopoeisis. THC response to bacterial infection in the blue crab *Callinectes sapidus* was tested using several Gram negative bacterial and one Gram positive bacterial species. These included three γ-proteobacteria (*Vibrio harveyi, Psychrobacter* sp., and *Serratia marcescens*), an α-proteobacteria (Ruegeria pomeroyi), type strains of *Serratia marcescens* and *Escherichia coli* and the Gram positive *Bacillus coral* (Johnson et al., 2011). Haemocytopenia resulted as a consequence of infection with *B. coral* and all Gram negative bacterial infections bar *Serratia marcescens*. Significanty, the reduction in THC was evident shortly post injection (0.5 hours). Furthermore, as lipopolysaccharide (LPS) a is component of bacterial cell walls and induces pattern recognition receptors (PRRs) to stimulate host cellular and humoral pathways, purified LPS was bound to polystyrene beads and injected into *C. sapidus* (Cerenius et al., 2010). The LPS beads also induced haemocytopenia comparable to that induced by *V. campbelli*, a Gram negative γ-proteobacteria known to cause rapid haemocytopenia (Holman et al., 2004). This suggests LPS plays a key role in the response of the haemocytes to bacterial infection (Johnson et al., 2011). This rapid decrease in circulating haemocytes is in contrast with the findings in this study.

In view of the haemocyte aggregation and melanisation observed in Figures 5.2 and 5.3, processes that require cell adhesion and induction of the prophenoloxidase cascade, an increased transcription of *peroxinectin* and *prophenoloxidase* would be expected. As these genes are stored in the haemocyte granules, they would be released on exocytosis. The lack of discernible change in THC may explain the lack of any changes in *peroxinectin* and *prophenoloxidase* transcription between the control and infected samples during this infection challenge (Figure 5.16). In view of the lack of differential *peroxinectin* and *prophenoloxidase* transcription between treatment groups at 72 hours and significant increase in bacterial load at 3 hours in the *L. anguillarum* animals, the resolution of THC sampling was modified for the *P. citreus* infection trial.

Planococcus citreus

Conversely, the earlier sampling and shorter time intervals (Table 5.2) revealed significant differences in THC during the *P. citreus* infection trial. Over time, THC first increased from 0 hours to 3 hours and then eventually decreased to much lower haemocyte counts at 48 hours in both the control ($p<0.001$) and *P. citreus* ($p<0.0001$) infected crabs. THC decreased significantly in the *P. citreus* infected crabs at 3 hours ($p<0.05$) and 48 hours ($p<0.01$) compared with the controls (Figure 5.20 and Table 5.7). It may be that the freely circulating haemocytes involved in immune processes, such as the aggregation of haemocytes observed in Figures 5.2, 5.3 and 5.4, had not been fully replaced within the sampling time periods.

Summary

Whilst the overall purpose of the infection study was to test the efficacy of the immune 'toolbox' during the recovery phase, THC was used as a marker to follow the infection progress. No evidence of haemocytopenia was evident in the *L. anguillarum* challenge and THC decreased only at 48 hours during the *P. citreus* challenge. This difference in THC profile evident in this study could be explained if the sub-lethal dose administered was not sufficient enough to induce rapid haemocytopenia, in which case a lag in THC would be reasonable to expect. However, in view of the late stage inflammatory processes evident in many of the histology images, particularly that of *L. anguillarum*, it would be more likely that the sampling timing did not correlate with the reduction in THC. Only further investigations could determine this.

5.4.4 Relationship between THC and gene transcription

Carcinin, peroxinectin and *prophenoloxidase* are all stored in the granules of the haemocytes, with only *carcinin* being constitutively expressed (Ashida and Söderhäll, 1984; Johansson and Söderhäll, 1988; Relf et al., 1999; Jiménez-Vega et al., 2004; Brockton et al., 2007). In view of this, the relationship between the immune biomarkers and THC was investigated to ascertain whether a correlation existed.

However, no correlation was evident between the expression of immune genes and the total haemocyte count in the *L. anguillarum* infection trial (Figure 5.18 and Table 5.6). This was also the case in the *P. citreus* trial bar one result: the transcription of *prophenoloxidase* at 0 hours in the control crabs ($p<0.01$). The correlation between *prophenoloxidase* expression and THC revealed an R^2 of 0.47 (p 0.01), meaning that 47% of the variation in *prophenoloxidase* transcription could be explained by the total haemocyte count. Although statistically significant, this was a weak correlation and was not observed in any other treatment groups. As the proportion of granular to non-granular haemocytes were not defined, differential haemocyte counts may be a more informative method of defining the relationship between these immune genes and THC.

5.4.5 Limitations

Secondary infection

The most significant limitation during both the *Listonella anguillarum* and *Planococcus citreus* infection trials was the secondary infection. A potential source could have been via accidental contamination of the marine saline which was used as a placebo injection for the control crabs. The marine saline was filter sterilised and all equipment associated with its preparation was also sterilised. Whilst care was taken when handling and aspirating the marine saline, there was a minimal risk of

contamination. This may explain the increased bacterial load in the control *Carcinus maenas* groups in both the Gram positive and Gram negative bacterial infection experiments (Figure 5.21).

In addition, preliminary infection studies would have been useful to determine whether autoclaving of seawater would have been a better option compared with non-autoclaving. It is possible that the secondary infection, if sourced from contamination to the autoclaved seawater, was able to flourish as there were no other microorganisms to outcompete. Another option would have been to autoclave equipment such as tanks. However, this ultimately would have made no difference if the secondary infection was sourced from the animals themselves. Overall, there was no practical way to eliminate the risk of secondary infections and ultimately it was not the aim of the study to create conditions that were not transferable to the natural environment. *C. maenas* are vulnerable to an array of microorganisms and their susceptibility to a known dose of a known pathogen in the context of their life history, potential background infections and environmental conditions presented a more representative natural host immune response.

RNA quality

An interesting observation revealed the difference in RNA quality between the samples taken from the animals in the *L. anguillarum* infection trial compared to those crabs in the *P. citreus* infection trial. Notably, RNA quality from *C. maenas* from the *L. anguillarum* infection trial, overall, were of high quality with only 3.7% with an RQI <7. Whereas in the *P. citreus* infection trial 22.8% of samples had an RQI <7. Consistent with previous experiments (Chapters 3 and 4), only samples with an RQI $\geq$ 7.6 were included in data analysis. To maintain consistency and comparison of all data, the THC and bacterial load data were also restricted to those samples with an RQI $\geq$ 7.6. It is difficult to determine whether the *P. citreus* bacteria were the key factor affecting the quality of host RNA in this case. It is known that pathogens are able to adversely affect the quality of RNA (Dunoyer and Voinnet, 2005). The earlier sampling time during the *P. citreus* infection trial (48 hours) may have ensured that more haemocytes with lower RNA quality were aspirated for gene expression analysis (Figure 5.20). It may be that later sampling during the *L. anguillarum* infection trial (72 hours) allowed sufficient time to generate a new population of haemocytes to replenish those that were involved in immune processes. In which case, those circulating haemocytes would presumably be of better quality. To determine this would have required further sampling at earlier (*L. anguillarum*) and later (*P. citreus*) times, which would potentially compromise the health of the animals through excessive haemolymph aspiration. This is really a separate question from this study, asking what impact these pathogens have on the quality of the host RNA at various stages during the infection process. To best answer this question would require a dedicated, separate experiment which is beyond the scope of this study, but is worth future investigation.

5.4.6 Key findings

The overall aim of this study was to determine whether the 'toolbox' of immune biomarkers was effective at indicating recent infection history in recovered or late stage recovering *C. maenas* to Gram positive or Gram negative bacterial infection. Overall, both the *L. anguillarum* and *P. citreus* infection trials revealed transcription changes in *carcinin* in response to infection. Histology confirmed immune processes during both experiments as well as the presence of bacterial cells during the *P. citreus*

infection. Changes in total haemocyte count were only evident during the *P. citreus* challenge. A secondary infection was evident, although this was consistent across both treatment groups in each trial. As a consequence it was not considered to obscure any potential changes in immune gene transcription or total haemocyte count between treatment groups that would be related to the controlled Gram positive or Gram negative infection.

Carcinin was a useful biomarker in observing the host response to these infection. *Peroxinectin* and *prophenoloxidase* however, did not change significantly in transcription between treatments. The overall impression was that the haemocyte populations in the *Listonella anguillarum* challenge had recovered from the initial infection, which was reflected by the lack of *peroxinectin* and *prophenoloxidase* transcription observed in this infection. This was also consistent with the later stage inflammatory processes evident in the histology. Whilst the haemocyte population were not fully recovered from the *Planococcus citreus* infection challenge and bacterial inclusion bodies were observed in the histology, *peroxinectin* and *prophenoloxidase* transcription changes were not evident in response to infection. This suggests that *carcinin*, which is secreted continuously into the haemolymph (Hauton, 2012) and up-regulated as necessary, is an appropriate biomarker at recording host response to previous infection over a longer time-scale (days), whereas *peroxinectin* and *prophenoloxidase* are more likely to be relevant over the shorter time-scales (hours).

Unfortunately, the contamination issue consistent across both the Gram positive and Gram negative infection trials has undermined the interpretation of these results. Ultimately, these experiments would need to be repeated in order to validate the data.

6 The role of *carcinin, peroxinectin* and *prophenoloxidase* in *Carcinus maenas* (L.) as early stress biomarkers in response to increased stocking density

Chapter Abstract

The application of *carcinin, peroxinectin* and *prophenoloxidase* has commonly been used to assess the host response to infection and disease. To date, there has been no known research into their role in response to stress in decapods. Stress is commonly experienced by marketable decapods during the capture, grading, shipment and holding phases prior to sale. The application of this suite of biomarkers was assessed in response to crowding stress in *Carcinus maenas*, commonly experienced during the shipment and holding of decapods, to test the efficacy of these genes as potential early biomarkers of stress. *Carcinus maenas* were subject to short (11 hours) and longer (7 days) periods of crowding to simulate transport and holding of animals. In addition, THC and histology were analysed to assess an established index of stress (THC) alongside that of gene transcription and assess the pathogen burden (histology) of *Carcinus maenas*. Only the transcription of *carcinin* was significantly down-regulated ($p<0.01$) in response to crowding stress. This occurred at 11 hours suggesting possible recovery after initial stress.

6.1 Introduction

Previous chapters have focused on the application of the immune biomarkers *carcinin, peroxinectin* and *prophenoloxidase* as predictors of viral challenge (Chapter 3), indicators of immune status in the context of the pathogen history between two populations of *Carcinus maenas* (Chapter 4) or as indicators of recent pathogen history in response to bacterial challenge (Chapter 5) in *C. maenas*. This study aims to ascertain the wider applicability of this suite of biomarkers as a general indicator of stress in decapod crustaceans.

Stress can be described as a threat to homeostasis (Chrousos, 1998). A stress response can be defined in physiological terms as an adaptive mechanism that allows the organism to maintain homeostasis in response to a stress or stressors (Barton, 2002). Disturbance to homeostasis results in physiological adjustments, such as changes in ion levels and also metabolic functioning and the release of stress proteins. These physiological effects of stress are designed to provide a fitness advantage (Adamo, 2012). However, homeostatic instability may lead to compromised immune function resulting in increased susceptibility to infection and disease.

6.1.1 Impact of stress on immune function

The purpose of an acute stress response is to rapidly access energy stores to heighten responsiveness in order to react to the stressor (Adamo, 2012). Stress at the individual level is mediated by neuroendocrine factors (Charmandari et al., 2005). Generally, in invertebrates, the acute stress response is initiated by the release of a biogenic amine (Adamo, 2008). In crustaceans these include adrenaline, noradrenaline (Aparicio-Simòn et al., 2010) and serotonin (Lorenzon et al., 2005). This is followed by the release of a second neuroendocrine factor, which mobilise energy reserves that can be used for a fight or flight response (Adamo, 2012). The impact of this response on the host can vary between species, but one of the associated disadvantages can be a transient depression in immunocompetence.

An intriguing study in vertebrates investigated the trade-off between the increased energy demands associated with reproductive effort and the effect on the immune response in birds. Deerenberg et al. (1997) investigated the reproductive effort in the zebra finch, *Taeniopygia guttata*, by quantifying the antibody response to injected sheep red blood cells. In all non-breeding birds, antibodies to the sheep red blood cells were produced, whereas in breeding *T. guttata* only 47% of finches produced antibodies. Furthermore, there was also a decrease in antibody response with an increase in brood size, suggesting increased reproductive effort in this species resulted in reduction in immunocompetence (Deerenberg et al., 1997). In invertebrates, the relationship between stress and immunocompetence is not clear in many species, but several hypotheses have been suggested (summarised by Adamo, 2008).

The 'energy crisis' hypothesis describes the reallocation of a greater proportion of energy to the stress response. This is thought to be an evolutionary adaptation that diverts energy from immune processes to the more immediate requirements imposed by the stressor (Dhabhar, 2002). However, some mechanisms involved in immunosuppresion, such as apoptosis, may result in increased energy expenditure (Dhabhar, 2002). How much energy would therefore be saved from immunosupression is unclear.

The 'resource crunch' theory, diverts energy from the immune response as described previously, but in this case, rather than a generalised shift in energy allocation, specific reallocation of energy occurs in relation to very specific physiological changes. An interesting example in the field cricket *Gryllus texensis*, suggests competing demand for apolipophorin III (apoLpIII) protein between the stress response and immune surveillance (Adamo et al., 2008). Release of the neuroendocrine octopamine in *G. texensis* post exertion (flying or fighting), induces the mobilisation of lipids from the fat body. This is achieve by a conformational change in apoLpIII to bind with high-density lipoproteins to form low-density lipoproteins in order to increase the lipid carrying capacity. However, in the previous conformational form, apoLpIII acts in immune surveillance. This reduction in the original conformation of apoLpIII, reduces the immune surveillance capacity of the organism, which may lead to an increase in disease susceptibility (Adamo et al., 2008).

The 'over-excitation' hypothesis is a response designed to avoid immune hyperactivation. This could protect the animal from the adverse effects of the immune inflammatory response as a result of the physiological response to the stressor (Råberg et al., 1998). Increased physical demands on the animal can lead to damage to tissues that may increase inflammatory responses. Immunosuppression, in this case, would avoid inappropriate or unnecessary energy expenditure on inflammatory responses (Råberg

et al., 1998). In this scenario, the risk of infection is outweighed by the level of damage associated with the hyperactivation of the immune system.

In addition, the stress response is likely to be influenced by factors including duration of the stressor, dose/intensity and type of stressor (Adamo, 2012). The biochemical stress responses are dynamic and availability of energy to respond effectively to the stressor, will be a trade-off between other systems competing for energy, such as the immune system.

6.1.2 *Carcinus maenas* and stress

In terms of *C. maenas*, stressors can be many and varied, but include adverse water quality conditions (fluctuations in temperature, salinity, dissolved O_2 and pH and contamination of the water), physical variables (emersion, physical disturbance through handling or interactions with other conspecifics and injury) and nutrient availability, which can all impact on metabolism, growth, immunity and survival (Le Moullac and Haffner, 2000). Many of these stressors can occur from the live trade of decapod crustaceans as a commercial commodity. Penaeid species, in particular, are a common example of decapods sold in consumer food markets. The survival and quality of the product is an essential consideration for both holding and live transport of decapods (Neil, 2012).

6.1.3 Commercial implications

Previous researchers have investigated some of the drivers of stress associated with the trade of decapods and the effect on the host. Understanding sub-lethal changes in the host, may have implications for modifying holding and transport conditions that would optimise the physiological condition of marketable decapod crustaceans. This could prevent or suppress proliferation of opportunistic pathogens, improve the quality of the product and prevent economic losses. Understanding the impact of handling, transport and holding conditions on live crustaceans can inform the fishing community, processors and wholesalers in order to optimise conditions throughout the whole process and promote good practice (Neil, 2012).

Codes of Practice offer guidelines for both the transportation and holding phases of commercially important species (Jacklin and Combes, 2007). These recommendations include procedures for reducing stress prior to and during transport and holding. As an example, live crabs should be kept in tanks, wet wells or floating cages and generally at lower temperatures (2-10°C). This reduces the metabolism of the crabs which ensures survival, particularly for more sensitive species (APEC Fisheries Working Group, 1999 cited by Fotedar and Evans, 2011). These recommendations may be adapted to account for variation in stress tolerance between species. More susceptible species to stress include lobsters, which are considered to be sensitive to changes in temperature, dissolved oxygen concentrations and overcrowding, whereas freshwater crayfish are considered to be relatively tolerant to the stressors associated with live transport (Fotedar and Evans, 2011).

The length of the transportation and holding stages vary according to the end market, *e.g.* local (UK) or long (UK to continental Europe). In addition, some live markets are known to maintain animals for several days (Paterson and Spanoghe, 1997) in response to market demand (Bernasconi and Uglow, 2008) and in some cases, such as high commodity species like *Homarus americanus*, this holding period

can last for up to six months. For some species, holding environments can result in physical disturbance and excessive crowding with no hiding areas for the crustaceans (Fotedar and Evans, 2011). For example, crabs are commonly transported at high densities which result in crabs at the bottom of the tanks being compressed by those crabs above them as well as by the handlers (Barrento et al., 2010). Increased stocking densities in *Penaeus monodon* have been found to increase mortalities and decrease growth (Nga et al., 2005). Nga et al. (2005) concluded that the decrease in growth was related to physical factors such as competition for resources, whereas the likely driver of mortality was ammonium toxicity. In view of these examples, stocking density was taken as the primary stressor to investigate the response of the biomarkers in *C. maenas*.

Many studies have investigated the effect of live transport on economically valuable species such as the American lobster *Homarus americanus* (Chang et al., 998b; Lorenzon et al., 2007), spiny lobster *Panulirus cygnus* (Jussila et al., 1999, 2001; Fotedar et al., 2006), the edible crab *Cancer pagarus* (Webster, 1996; Barrento et al., 2010) and the Norway lobster *Nephrops norvegicus* (Bernasconi and Uglow, 2008) using a variety of indices by which to measure stress. Theses indices include plasma glucose (Dall, 1974; Lorenzon et al., 2008), lactate (Whiteley and Taylor, 1992; Lorenzon et al., 2008; Barrento et al., 2009), haemolymph pH (Lorenzon et al., 2008; Barrento et al., 2009) and haemolymph oxygen concentration (Taylor and Whiteley, 1989), phenoloxidase activity (Sritunyalucksana et al., 1999; Sanchez et al., 2001), phagocytic index (Cheng et al., 2003) and reactive oxygen intermediates (ROS) (Muñoz et al., 2000). However, there is no literature on the transcription of *carcinin, peroxinectin* and *prophenoloxidase* in response to crowding of decapods. These genes are often investigated in the context of infection, but not more generally in relation to specific stressors. This is interesting considering the previously discussed effect of stress on immunosuppression.

Total haemocyte count (THC), known to be an important indices of the health status of decapods (Lorenzon et al., 2007), has been used as a biomarker in species such as *Cherax tenuimanus* (hairy marron) (Jussila et al., 1999) and *Panulirus cygnus* (spiny lobster) (Jussila et al., 2001) specifically in response to transportation stress and handling. In view of the established use of THC to indicate health status in decapods (Truscott and White, 1990; Le Moullac et al., 1998; Vargas-Albores et al., 1998; Jussila et al., 1999, 2001; Celi et al., 2013), it was also quantified in *C. maenas* during this experiment. Furthermore, the ability of an animal to overcome stress is, in part, dependent upon the existing health status at the time of exposure. Consequently, histology was taken to determine the background health of this population of *C. maenas* and to provide context for any changes in gene transcription observed, particularly as this population was sourced from the natural environment and therefore would be exposed to naturally occurring pathogens in the wild.

For this study, two scenarios were investigated. The first was 11 hours to simulate transport of live decapod crustaceans and 7 days, to represent holding of decapods at live markets. The limited literature with respect to *carcinin, peroxinectin* and *prophenoloxidase* transcription in response to stocking density in decapods, presented an opportunity to assess the validity of these genes as stress biomarkers. Transcriptional analysis of these genes was performed alongside THCs, as an established indicator of stress in decapods, as well as the background histology. This could potentially widen the application of this suite of biomarkers beyond assessment of pathogen infections and disease, to assessment of immunocompetence in relation to stress associated with stocking densities.

6.1.4 Research Aims

The aim of this study was to assess the affect of crowding in *Carcinus maenas* under simulated transport (11 hours) and holding (7 days) scenarios using the suite of biomarkers, developed for the pathogen infection studies (Chapters 2 and 5) and disease survey (Chapter 4), that may indicate sublethal changes that could impact the health of the animals.

6.1.5 Research Objectives

i. Establish how effectual the candidate host immune genes were as stress biomarkers in *Carcinus maenas*, in response to two different crowding scenarios.

ii. Compare the total haemocyte counts as an established stress biomarker at 11 hours (simulating transportation) and 7 days (simulating holding conditions).

iii. Assess the histopathology to determine the background infection status of *Carcinus maenas*.

6.2 Experimental design

6.2.1 Preparation for gene expression analysis

Preparation of immune and endogenous reference genes are detailed in Chapter 2. To first isolate the genes, short priming sequences were designed against the full gene sequence or EST sequence (where target genes were not fully sequenced). Each target gene was amplified using conventional PCR. These target amplicons were then sent for sequence confirmation to Source BioScience LifeSciences Plc. (Oxford, UK) using conventional Sanger sequencing. After sequence confirmation, qPCR primers were designed to isolate short ($\leq$ 150 base pairs) amplicons for quantification of gene transcription using the intercalating dye, SYBR® Green. Prior to quantification, all immune genes and ERGs were optimised for primer and cDNA concentrations and PCR efficiencies (Appendix, Chapter 2, Tables 8.5 and 8.6).

6.2.2 Collection of *Carcinus maenas*

All adult *Carcinus maenas* were collected from Marchwood, Southampton (latitude 50.900242 and longitude -1.439896). To eliminate variability in gene expression in relation to reproduction, only males were collected for this study. Any animals with external signs of disease, (*e.g. Sacculina* externa or necrotic shell disease), were not collected for this study. The experiment was conducted in four batches; control at 11 hours, crowded at 11 hours, control at 7 days and crowded at 7 days with 9 or 10 crabs in each group. Prior to acclimation to study conditions, animals were first acclimated to aquarium conditions at an ambient temperature of 18°C for two weeks to ensure any animals with overt existing pathological disease were eliminated from this study.

6.2.3 Acclimation and experimental set-up

C. maenas were each held in individual 5l non-sealed, non-flow-through tanks with air lines, to acclimate to study conditions (12°C, salinity 31, and O_2 10mg/ml with a 12 hour light/dark cycle) for a period of 7 days. Although, 12°C is slightly above some of the generic recommended guidelines (APEC Fisheries Working Group, 1999 cited by Fotedar and Evans, 2011), this temperature is consistent with

that used to transport species such as *Cancer pagurus* from the UK to live markets in Portugal (Barrento et al., 2009) or Italy (Lorenzon et al., 2008) in which refrigerated vehicle transport systems range between 10-13°C. *C. maenas* were first placed in the 5l tanks at ambient temperatures (18°C) and then tanks were relocated to a temperature-controlled room to chill slowly to 12°C. Decreasing the water temperature can increase survival (Coyle et al., 2006), but rapid chilling has been known to cause limb loss in some penaeid species (Fotedar and Evans, 2011). Crabs were fed *ad libitum* on a diet of either fish or *Crepidula fornicata*. Water changes (100%) were conducted on alternate days after first acclimating the water to study conditions (see above).

During the experiment (11 hours or seven days), *C. maenas* were held either as individual animals in a single 5l tank (nine crabs in total) or as a group of 10 crabs in a single 5l tank. Seawater was analysed daily for temperature, salinity and oxygen saturation, during the acclimation and experimental periods, to ensure stable conditions throughout. *C. maenas* were fed the day prior to the 11 hour trial and *ad libitum* throughout the 7 day trial, but starved for 24 hours prior to haemolymph aspiration for gene transcription analysis. The feeding strategy was founded on previous work that determined feeding of fresh-water prawns, *Macrobrachium rosenbergii*, during holding, resulted in increased survival compared to those prawns that were not fed (Kubaryk and Harper, 2001). This strategy was used rather than starvation, to assess the sub-lethal effects of high stocking stress without introducing variables that may act as additional mortality drivers.

6.2.4 Haemolymph sampling

Samples for total haemocyte count and gene expression were taken at either 11 hours or 7 days in the control and crowded groups. After cleaning the first articulation of the second periopod with ethanol for each crab, 0.1ml of haemolymph was aspirated into a 1ml syringe preloaded with 0.1ml of 20% seawater formalin (Appendix, Chapter 2, Section 7.5.3.3) and enumerated as per Chapter 2, Section 2.6. Using the same needle puncture site, 1ml of haemolymph was aspirated into a 2ml syringe preloaded with 1ml of *Carcinus maenas*-specific anticoagulant (Chapter 2, Section 2.3.2, Table 2.2). This anticoagulated haemolymph was immediately centrifuged at 3000*g* and 4°C for 7 minutes to form a pellet of cells. The anticoagulant was aspirated and discarded and the haemolymph cells were mixed with 1-1.5ml (depending on pellet size) of TRI Reagent® and stored at -80°C until further processing. After a short (days) storage time, RNA isolation, quality and quantity analysis, DNase treatment and reverse transcription to cDNA were carried out according to the manufacturer's protocols summarised in Chapter 2, Sections 2.3.3-2.3.6 respectively. Consistent with the previous chapters, only samples with RQI values ≥ 7.6 were included in the data analysis.

6.2.5 Dissection

C. maenas were placed on ice for a minimum period of 30 minutes after haemolymph aspiration prior to dissection to anaesthetise them. Details of the dissection protocol are described in Chapter 2, Section 2.5. Histological samples were taken for heart, gill, nerve, gonad, muscle and hepatopancreas.

6.3 Results

6.3.1 Size range of *Carcinus maenas*

The carapace width was recorded for each *C. maenas*. The size range is detailed in Table 6.1.

Table 6.1: Size range detailing the carapace width (mm) of *Carcinus maenas* in the control and crowded groups at 11 hours and 7 days. The last row shows the mean size range in each group with standard deviation in parentheses.

Control		**Crowding**	
Carapace width (mm)			
11 hours	**7 days**	**11 hours**	**7 days**
53	54	53	60
55	51	54	52
60	49	57	53
48	54	62	57
50	51	57	59
59	53	56	56
60	51	51	50
55	52	55	50
46	55	48	53
52			48
53.8 (4.9)	52.2 (1.9)	54.8 (4.0)	53.8 (4.0)

6.3.2 Assessment of water quality

The water quality was maintained within acceptable and stable conditions throughout the acclimation and experimental periods (temperaure 12°C ±0.5, salinity 31 ±0.8 and O_2 10mg/ml ±0.9).

6.3.3 Assessment of endogenous reference genes

Endogenous reference gene (ERG) stability was assessed in qBase$^+$ using geNorm. The most stable combination of ERGs were *actin, eef1a* and *tubulin* with an average M value of 0.451 and CV value of 0.186 (Figures 6.1 and 6.2 and Table 6.2).

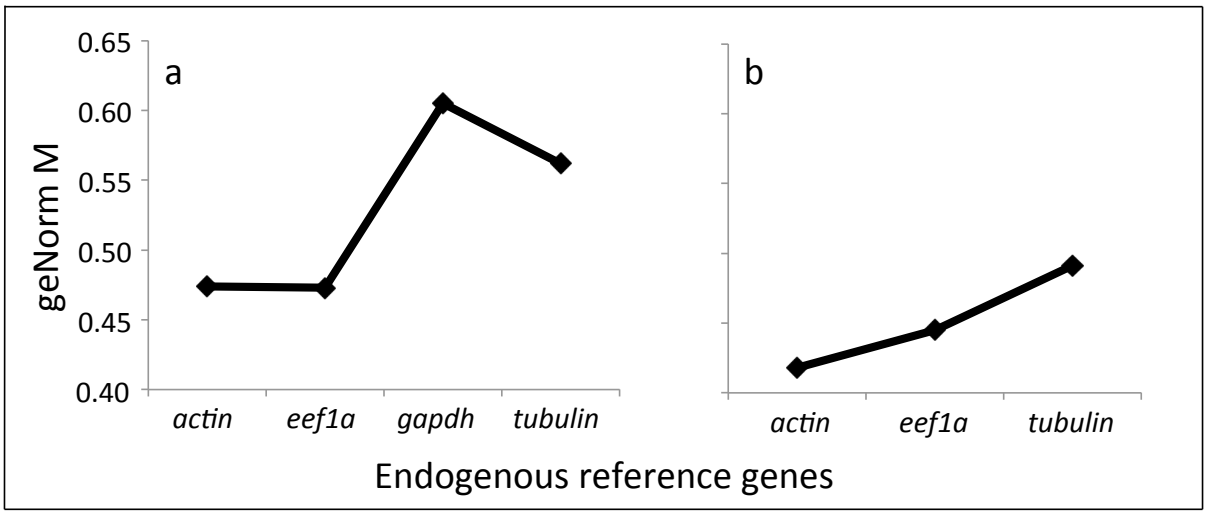


Figure 6.1: Endogenous reference gene stability assessment using geNorm M. Removal of *gapdh* improved the average M from 0.528 to 0.451.

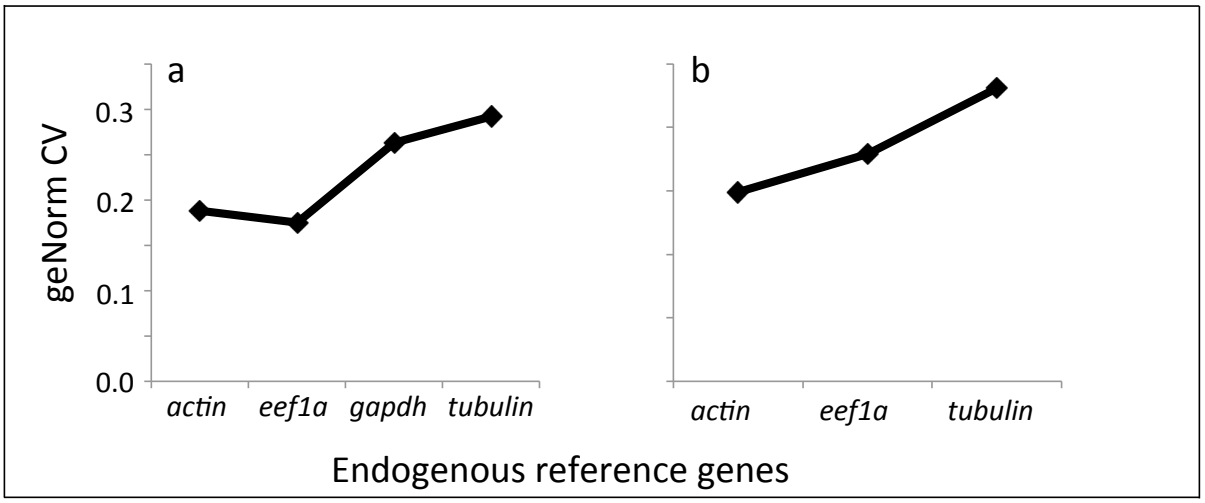


Figure 6.2: Endogenous reference gene stability assessment using geNorm CV. Removal of *gapdh* improved the CV from 0.230 to 0.186.

The least stable ERG in terms of CV was *tubulin* (0.292) rather than *gapdh* (0.263). However, *gapdh* was removed in view of the improved M value. (M value for *gapdh* was 0.605 compared with 0.562 for *tubulin*.)

Table 6.2: The average M and CV values are highlighted for the different combinations of ERGs.

Endogenous reference genes	M	CV
actin, eef1a, gapdh, tubulin	0.529	0.230
actin, eef1a, tubulin	0.451	0.186

6.3.4 Gene transcription

Gene transcription was measured at 11 hours and 7 days. There was a significant difference (p <0.01) in the transcription of *carcinin* at 11 hours, with comparatively lower transcription in the crowded group, but no other significant changes in expression were evident. Transcription of *peroxinectin* was lower at 7 days compared with 11 hours (p<0.05) in the control animals whereas *prophenoloxidase* was comparatively greater in both the control (p <0.01) and crowded (p <0.05) *C. maenas* over time (Figure 6.3 and Table 6.3).

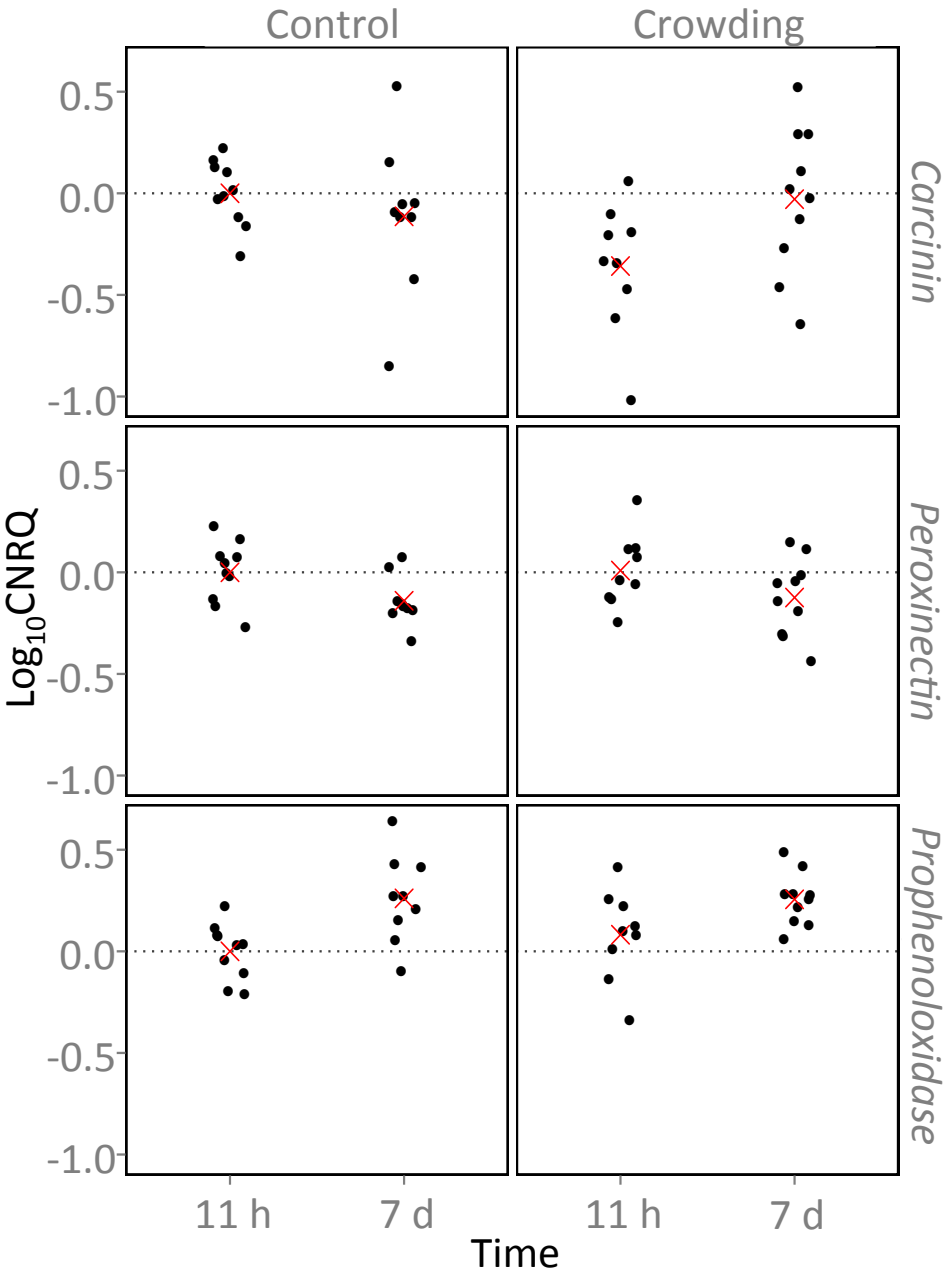


Figure 6.3: Immune gene transcription between the control and crowded animals at 11 hours and 7 days. The red 'X' denotes the mean Log_{10}CNRQ values for each group. n=9 for 11 hours in the crowded group and 7 days in the control group. n=10 for 11 hours in the control group and 7 days in the crowded group.

Table 6.3: Statistical evaluation of gene transcription between control and crowded *Carcinus maenas* at 11 hours and 7 days using Kruskal-Wallis. Significant differences are highlighted in red.

Immune genes	**Treatment**		**Kruskal - Wallis**
Carcinin	11 hours v 7 days	Control	H = 1.22, d.f. = 1, p >0.05
		Crowding	H = 3.52, d.f. = 1, p >0.05
	Control v Crowding	11 hours	H = 7.71, d.f. = 1, p <0.01
		7 days	H = 0.24, d.f. = 1, p >0.05
Peroxinectin	11 hours v 7 days	Control	H = 3.84, d.f. = 1, p =0.05
		Crowding	H = 1.71, d.f. = 1, p >0.05
	Control v Crowding	11 hours	H = 0.01, d.f. = 1, p >0.05
		7 days	H = 0.17, d.f. = 1, p >0.05
Prophenoloxidase	11 hours v 7 days	Control	H = 6.83, d.f. = 1, p <0.01
		Crowding	H = 4.51, d.f. = 1, p <0.05
	Control v Crowding	11 hours	H = 1.40, d.f. = 1, p >0.05
		7 days	H = 0.03, d.f. = 1, p >0.05

6.3.5 Total haemocyte count

The total haemocyte counts were widely variable between animals, particularly in the control animals at 11 hours. No significant differences in THC were evident between the groups (Figure 6.4 and Table 6.4).

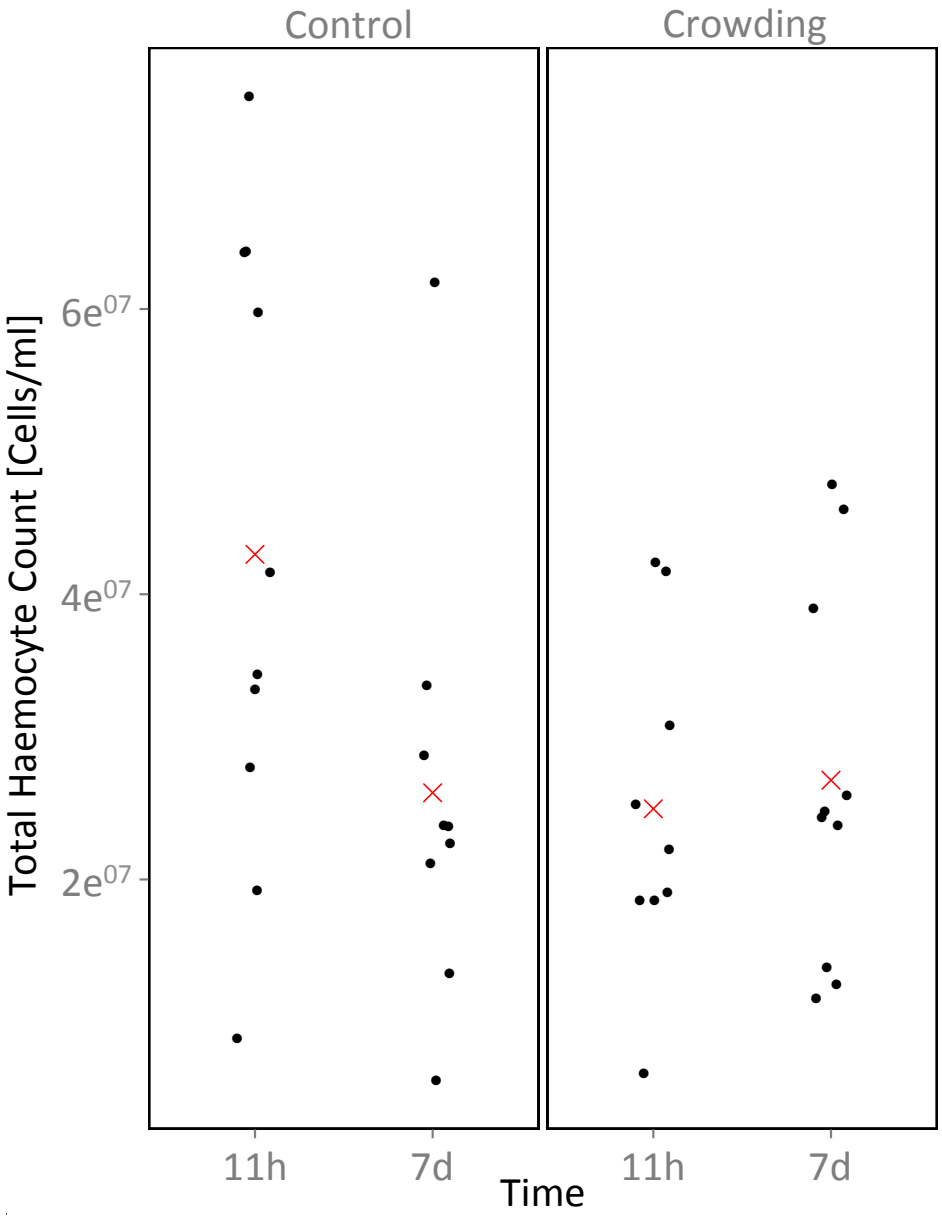


Figure 6.4: Comparison of total haemocyte count between control and crowded *Carcinus maenas* at 11 hours and 7 days. The red 'X' represents the mean total haemocyte count [cells/ml]. n=9 for 11 hours in the crowded group and 7 days in the control group. n=10 for 11 hours in the control group and 7 days in the crowded group.

Table 6.4: Statistical evaluation of total haemocyte count between control and crowded *Carcinus maenas* at 11 hours and 7 days using Kruskal-Wallis. No significant differences were evident.

	Control	**Crowding**
11 hours v 7 days	H = 3.23, d.f. = 1, p >0.05	H = 0.11, d.f. = 1, p >0.05
	11 hours	**7 days**
Control v Crowding	H = 3.53, d.f. = 1, p >0.05	H = 0.28, d.f. = 1, p >0.05

6.3.6 Histology

The pathogen burden included *Microphallus* and *Sacculina* species, *Cm*BV and microsporidian infections. In addition, some animals had non-specific haemocyte aggregations or granulomas in which infection could not be identified. No *C. maenas* was identified as having a particularly significant or overwhelming infection based on the histology images. The proportions of infections identified are highlighted in Table 6.5. (Some examples can be seen in the Appendix, Chapter 6, Figure 8.24).

Table 6.5: Pathogen burden based on the histology from each treatment group. (NAD is nothing abnormal detected). The total numbers of *Carcinus maenas* per treatment are noted in the final column.

Trial conditions		***Microphallus* sp. (%)**	***Sacculina* sp. (%)**	**Microsporidia (%)**	***Cm*BV (%)**	**Haemocyte aggregates/granulomas (%)**	**NAD (%)**	**Total numbers**
11 hours	**Control**	40	20	10	0	50	30	**10**
	Crowding	66	11	0	0	44	11	**9**
7 days	**Control**	22	0	11	0	33	44	**9**
	Crowding	30	20	40	10	30	20	**10**

6.4 Discussion

The main objective of this study was to determine the viability of these biomarkers as indices of stress, specifically in relation to stocking density in *C. maenas*. Early identification of stress associated with increased stocking densities would allow modification of practices, such as adjustment of holding times to allow sufficient recovery from stress prior to onward transport. This may serve to reduce mortality and may also improve the quality of the commodity species being traded. As *C. maenas* were sourced from a wild population, the background infection status was also assessed in terms of histology alongside THC as an established biomarker of stress in decapods.

6.4.1 Assessment of *carcinin, peroxinectin* and *prophenoloxidase* transcription

Carcinin

The response of the biomarkers was varied. Only *carcinin* transcription was significantly different with increased stocking density. This difference in transcription occurred only at 11 hours and was not observed at seven days. The fact that no difference in *carcinin* expression could be seen at seven days between the control and crowded groups, may be a reflection of recovery from an initial acute stress response over the longer time period.

In theory, the comparatively lower *carcinin* transcription at 11 hours in the crowded group could potentially have been a consequence of the reallocation of energy as described in either the 'energy crisis' hypothesis, indicating general reallocation of energy to stress pathways, or the 'resource crunch' theory, which describes very specific changes in energy allocation or function of specific proteins (Adamo, 2008). The lower transcription of *carcinin* would likely render *C. maenas* more susceptible to opportunistic pathogens. This could then result in proliferation of low-level infections, that could explain the increased transcription of *prophenoloxidase* at seven days (although this doesn't explain the greater *prophenoloxidase* transcription evident in the control crabs over time, or the lack of comparatively greater *peroxinectin* transcription in the crowded group at seven days).

Differences in antibacterial activity in the western rock lobster, *Panulirus cygnus*, were evident after simulated six hour shipment under submerged, sprayed and emersed scenarios (Tsvetnenko et al., 2001). After seven days, the greatest antibacterial activity was found in sprayed *P. cygnus* whilst the lowest was in those submerged. The authors concluded that handling stress was the dominant factor affecting the increased antibacterial activity. Considering this, the fact that *C. maenas* were submerged throughout the acclimation and experiment, could also mean reduced handling, (comparative to sprayed and emersed conditions), and therefore lower antibacterial activity. Furthermore, no increased inflammatory responses (increased haemocyte aggregations and melanisation), indicative of pathogenic infection, were observed in the histology images at seven days that differed from 11 hours (Table 6.5 and Appendix, Chapter 6, Figure 8.24), that would warrant increased antibacterial activity. This indicated no apparent increase in infection in the high stocking density group, that might have been expected due to the close proximity of crabs which would facilitate the transfer of pathogens.

Overall, it is likely that the comparatively lower *carcinin* transcription was a result of reallocation of energy to stress responses during the acute stress phase (short-term). The lack of any developing infections, which *C. maenas* would have been more susceptible to as a consequence of reduced transcription of *carcinin*, may have been facilitated by the fact they were submerged throughout the acclimation and experimental periods and therefore would have been subject to less handling. Of course, this is speculative and further experiments would need to validate this.

Peroxinectin

The transcription of *peroxinectin* was significantly different only in the control crabs over time (p=0.05). *Peroxinectin* is involved in mediating phagocytosis (Thörnqvist et al., 1994). In decapods exposed to other stressors such as temperature (Dove et al., 2005), starvation (Pascual et al., 2006) and hypoxia (Holman et al., 2004), levels of phagocytosis have been known to decrease. This is consistent with the lower *peroxinectin* transcription observed in the control *C. maenas* at seven days compared with 11 hours. It is difficult to determine, based on this finding alone, whether the comparatively lower *peroxinectin* transcription at seven days represents a stress response. However, previous research indicates animals subject to high stocking densities, such as farmed penaeids, that can often reach densities of 150m^{-2} and up to 400m^{-2} in controlled recirculated tank culture (FAO, 2014), are considered to be stressed. This often results in an increase in disease prevalence (Kautsky et al., 2000). As the change in *peroxinectin* transcription was not seen in crowded *C. maenas* or between treatments, it is more probable that the reduction of *peroxinectin* is a reflection of a lack of release of peroxinectin through degranulation of haemocytes and exocytosis, thereby indicating a non-stressed state. As the change in *peroxinectin* transcription was not a function of the increased stocking density, this gene is

not considered appropriate as a biomarker for this stressor in *C. maenas*.

Prophenoloxidase

The transcription of *prophenoloxidase* was significantly different over time in both the control and crowded groups of *C. maenas*. As previously discussed, transference of contaminants and pathogens is likely to increase during close contact with conspecifics, through interactions that may lead to injury or wound development, leaving *C. maenas* more susceptible to invading pathogens. Consequently, increased *prophenoloxidase* transcription, which is cleaved to form *phenoloxidase*, would be required for downstream melanisation processes. Taken in isolation, this could potentially explain the comparatively greater transcription in the crowded group of *C. maenas* at seven days. However, if this response were to be explained by an increase in opportunistic pathogens, then an increase in the transcription of *peroxinectin* would also be expected in the crowded group over time. *Peroxinectin* is known to be released concomitantly with the prophenoloxidase cascade and is involved in several important immune functions including cell adhesion (Johansson and Söderhäll, 1988), opsonic activity (Thörnqvist et al., 1994), inducing degranulation (Johansson and Söderhäll, 1989), peroxidase activity (Johansson et al., 1995) and promoting encapsulation (Kobayashi et al., 1990), all highly important during the process of containing and killing pathogens. However, an up-regulation of *peroxinectin* transcription was not evident.

Alternatively, the increase in *prophenoloxidase* transcription in the control and crowded groups, could be a consequence of the disturbance and handling associated with the water changes that occurred on alternate days throughout the seven day experiment. Investigations into the effect of repeated handling on the Pacific whiteleg shrimp, *Penaeus vannamei*, found a decrease in total protein in the haemolymph (Mercier et al., 2006). The authors suggested various reasons for this including use of proteins for immune processes such as recognition of invading pathogens, clotting and involvement in the phenoloxidase system. Similarly, total protein was found to decrease in *Homarus americanus* in response to transport stress when held at both 6°C and 15°C (Lorenzon et al., 2007). Studies into the stress response of *phenoloxidase* found changes in activity in response to temperature (Gomez-Jimenez et al., 2000; Cheng et al., 005b) and salinity (Vargas-Albores et al., 2008), but interestingly, also as a result of captivity (Sanchez et al., 2001).

Investigations into the immune response of *Litopennaeus setiferus* as a result of seven days acclimation at 27°C, found a decrease in haemolymph protein and THC and an increase in phenoloxidase activity in the acclimated group compared to those that were freshly caught (Sanchez et al., 2001). This pattern is consistent with the increase in *prophenoloxidase* transcription observed ($p<0.01$) and lower mean THC (not significant) in the control *C. maenas*. The authors suggested that these results could be explained by a change in the regulatory mechanism of the *prophenoloxidase* system as hypothesised by Le Moullac (1998). Le Moullac (1998) subjected the blue shrimp, *Penaeus stylirostris*, to hypoxic stress and found a decrease in THC concurrent with an increase in *phenoloxidase* activity. The *prophenoloxidase*-activating system is activated by an enzyme, the *prophenoloxidase* activating enzyme, or ppA (Cerenius et al., 2010). This is in turn regulated by inhibitors, such as α_2-macroglobulin (Hergenhahn et al., 1988) and pacifastin (Hergenhahn et al., 1987), which inhibits the proteinase trypsin. α_2-macroglobulin does not bind to the catalytic site of the proteinase, but instead undergoes a conformational change that traps the proteinase. This results in inhibition of hydrolysis of only high molecular weight substrates. Low molecular weight substrates are still able to access the active site of

the trapped proteinase (Hergenhahn et al., 1988). The proteinases trapped by α_2-macroglobulin would not be available to pacifastin-like inhibitors, which are thought to be the main inhibitory factor in crayfish (Aspan et al., 1990). Although Le Moullac (1998) found an increase in the α_2-macroglobulin activity in the hypoxic plasma of *P. stylirostris*, the total anti-trypsin activity was comparatively lower in hypoxic plasma compared with sufficiently oxygenated plasma. Consequently, there would have been less antiprotease activity in the hypoxic plasma to regulate the ppA which could explain why there was a lower THC concurrent with increased phenoloxidase activity. This scenario is consistent with the *prophenoloxidase* profile evident in this study in *C. maenas*. This mechanism could explain the greater transcription in *prophenoloxidase* and no difference (crowding group) or a comparatively lower *peroxinectin* transcription (control group). Further experiments designed to measure the transcription of these biomarkers alongside quantification of inhibitory proteinases would be necessary to determine whether this mechanism could explain the different *peroxinectin* and *prophenoloxidase* profiles observed.

Importantly, there was no transcriptional difference in *prophenoloxidase* in response to stocking density. Therefore, consistent with *peroxinectin*, *prophenoloxidase* cannot be endorsed as a relevant biomarker by which to assess the host response to acute stress associated with stocking density.

6.4.2 Relevance of *carcinin*, *peroxinectin* and *prophenoloxidase* as biomarkers of stress in response to high stocking density in *Carcinus maenas*

Acute stress associated with high stocking density is likely to result in reallocation of energy away from immunocompetence to the more immediate requirements imposed by crowding stress. *Carcinin* appears to be a relevant biomarker of crowding stress in the short-term as indicated by the comparatively lower transcription in the crowded group (11 hours). *Peroxinectin* transcription decreased in the control group over time, but this was not likely to reflect a stress response.

Despite the potential increased susceptibility that would occur as a consequence of lower *carcinin* transcription, the comparatively greater *prophenoloxidase* transcription over time is unlikely to be due to an increase in pathogen prevalence, as this is not supported by the *peroxinectin* transcription profile and the histology. More likely, this may be associated with handling and/or captivity in view of the findings by Sanchez et al. (2001); Tsvetnenko et al. (2001) and Mercier et al. (2006). It would be interesting to definitively determine whether mechanisms such as those proposed by Le Moullac et al. (1998) were fundamental in the response of *prophenoloxidase* in this case.

Whilst there may be other stressors associated with the experiment that may have resulted in changes to transcription in *peroxinectin* and *prophenoloxidase*, under these treatment conditions, these two genes cannot be endorsed as appropriate stress indicators in response to stocking density alone in *C. maenas*. Some biomarkers may be useful under some scenarios, but not under others. As an example, haemolymph glucose in *Penaeus monodon* was found to be elevated after a reduction of dissolved oxygen and increase in dissolved carbon dioxide, but did not indicate adverse effects of crowding or water pH between 5.0 and 8.3 (Hall, 1998). This difference in response to stressors appears to be reflected in the different responses of *carcinin*, *peroxinectin* and *prophenoloxidase* to stress associated with stocking density.

6.4.3 Total haemocyte counts

Changes in THC are dependent upon the nature and impact of the stressor itself. A decrease in THC is often reported in the literature in response to stressors such as hypoxia (Le Moullac et al., 1998), infection, ecdysis and the presence of wounds (Hose et al., 1992). Handling, which has also been a factor during this stocking density experiment due to necessary water changes, has been reported to lead to a chronic decrease in THC (Lorenzon et al., 1999). Others report an increase in THC as a consequence of emersion, post-harvest handling, transportation (Jussila et al., 2001) and on arrival to processing facilities (Jussila et al., 1997). This was thought to be a consequence of increased haematopoeisis compensating for the loss of haemocytes recruited for other purposes.

THC has been used as an index of health in the transportation of species such as *Cherax tenuimanus* (Jussila et al., 1999) and *Panulirus cygnus* (Jussila et al., 2001), causing an increase in THC in response to short-term physical disturbance and handling. A consequence of increased THC is potentially increased immunocompetence in these decapods. *P. cygnus* sampled over a period of ten days to assess the effect of holding duration on immune parameters (clotting time, differential haemocyte counts, THC and protein concentration), found an initial recovery after day 1 post capture and handling, with THCs returning to baseline levels within 16-48 hours following transfer to holding tanks (Fotedar et al., 2006) suggesting the lobsters had recovered from the acute handling phase. However, further adjustments in immune parameters were also observed on day four, suggesting the possibility of delayed recovery or alternatively an unidentified stressor (Fotedar et al., 2006).

Contrary to these findings, no statistically significant changes in THC were observed between stocking densities in *C. maenas* at either 11 hours or seven days. There was considerable intragroup variation in THC, particularly in the control crabs at 11 hours (Figure 6.4). Undetected wounds, particularly within the crowded *C. maenas* groups through interaction with conspecifics, could have decreased THC by removing haemocytes from the circulating haemolymph for clotting functions, contributing to the intragroup variation. However, there was no significant difference in the histology from the crowded groups compared to the control groups that would corroborate this.

The mean THC at 11 hours in the control group ($4.28e^{07}$) was apparently greater compared with the 11 hour crowded *C. maenas* group ($2.50e^{07}$), although this was not statistically significant (Figure 6.4). There was a lower mean THC at seven days ($2.70e^{07}$) compared with 11 hours in the control crabs, again not statistically significant, but was consistent with the lower transcription of *peroxinectin* (p=0.05) over time. As previously noted, this was in contrast to the profile of *prophenoloxidase* (discussed above). However, THC was not significantly different over time or between treatments, so does not indicate a stress response in the high stocking density *C. maenas* group. Ideally, in view of the intragroup variation, future investigations should increase sample size to validate that no changes in THC occur between the control and high stocking density groups.

6.4.4 Assessment of histology

Opportunistic infections are known to occur rapidly under increased homeostatic disturbance such as capture and transport (Johnson, 1976). This can result through mechanisms such as injury (Bergmann et al., 2000). This may then allow low-level infections to proliferate. The comparatively lower *carcinin* transcription in the crowded group at 11 hours could, over time, lead to proliferation of opportunistic

pathogens as a result of compromised immune function. One notable difference in the histology was the number of microsporidian infections in the crowded group at seven days (n=4) compared to the crowded group at 11 hours (n=0) (Table 6.5 and Appendix, Chapter 6, Figure 8.24c). However, as this was based on a small data set, more replication would be required to determine whether there was a correlation between the comparatively lower *carcinin* transcription at 11 hours and the increased prevalence of microsporidia. No other differences between treatment groups or time were noted. With the possible exception of microsporidia, the lack of significant differences in the THC, *peroxinectin* and *prophenoloxidase* transcription between treatment groups and lack of mortalities throughout the experimental period, the observed infections noted from the histology were likely to represent the pre-existing pathogen burden of *C. maenas*.

6.4.5 Limitations

Increasing the resolution of data, in terms of sampling frequency, may have allowed finer scale changes in THC or gene transcription to be observed between treatments or over time. There is always a balance between sufficient sampling frequency to identify changes in immune status and not inadvertently compromising the health status of the animals due to excessive haemolymph aspiration. Furthermore, excessive haemolymph aspiration, may have resulted in more difficult interpretation of the actual impact of stocking density and associated stressors on *C. maenas* due to the induced lower haemocyte counts caused by the sampling itself. Frequent haemolymph sampling in larger species such as *Cancer pagurus*, *Panulirus cygnus* or homarid lobsters is likely to be tolerated more readily compared to smaller species such as *C. maenas*, due to the difference in avalilable haemolymph volume between species. That said, baseline gene expression and THC assessed prior to the experiment in each treatment group would have allowed more informative interpretation of the changes observed in these biomarkers.

6.4.6 Key findings and future recommendations

As codes of practice are providing guidelines to encourage the optimal practices to reduce stress and also to ensure the greatest economic value for the commodity being traded, mortality is becoming less appropriate as a means to assess stress. Therefore, earlier warning indices of stress, such as plasma lactate, glucose and others, as previously described, are being used. Identifying relevant biomarkers at the transcriptional stage would provide even earlier indications of homeostatic disturbance in response to particular stressors, potentially further reducing the loss of valuable commodity stock either through mortality or poor quality.

In terms of *C. maenas*, it is unlikely that this whole suite of biomarkers, *carcinin, peroxinectin* and *prophenoloxidase*, would be sufficient to identify stress specifically caused by high stocking density. Only the antimicrobial peptide, *carcinin*, demonstrated significant transcriptional differences in response to stocking density. This was only observed over a short time-scale (11 hours), suggesting possible recovery after initial stress. The transcription of the other two biomarkers, *peroxinectin* and *prophenoloxidase*, were significantly different between 11 hours and seven days. However, this was as a result of unidentified stressors that may be associated with transport and holding, but was not in response to stocking density.

Consequently, only *carcinin* can be endorsed as a relevant biomarker to assess stress associated with increased stocking density in *C. maenas*. A key difference in the suitability of these genes as biomarkers

may lie in the mechanism of delivery. *Carcinin* is secreted continuously and is also inducible, whereas both *peroxinectin* and *prophenoloxidase* are synthesised and then stored in the granulocytes until required. Therefore, any changes in immunocompetence as a result of stressors, such as a shift in the energy available for immune responses, will more likely be reflected in a peptide which is continuously secreted, *i.e.* carcinin. The capacity of *carcinin* or *crustins* to reflect the host response to stress at the transcriptional level, should be investigated in other species, to understand it's applicability and limitations in the wider decapod community. Recovery of different species to handling and increased stocking densities will vary and the resolution of haemolymph sampling frequency should be modified accordingly, ensuring the sampling itself does not compromise the animals.

Only a short part of the whole marketing process has been addressed in this study. Assessment of stress during the capture and grading procedures as well as during transport and holding would provide a profile of stress and recovery throughout the entire marketing chain which may identify practices that could be modified to reduce stress in some species. In addition, it would be useful to investigate the impact of length of storage time on the risk of decapods developing pathogenic infections. This would provide an opportunity to assess the applicability of *carcinin* in terms of assessing cumulative stress in decapods over longer time periods.

7 Final discussion

7.1 Overview

The overall aim of this study was to develop a 'toolbox' of immune markers in two decapod crustaceans to answer questions on the host response to various biotic (infection) and non-biotic stressors. These challenges consisted of a viral infection (*Homarus gammarus*), assessment of natural pathogen burden in two wild populations of *Carcinus maenas*, recent pathogen history (recovery) against Gram negative and Gram positive bacterial challenges (*C. maenas*) and stocking density stress (*C. maenas*). The toolbox consisted of three genes (*crustin/carcinin, peroxinectin* and *prophenoloxidase*) involved in various immune-related functions. The antimicrobial peptide *crustin/carcinin* is known to be secreted continuously from the haemocytes once they are synthesised (Hauton, 2012), but transcription can be up-regulated in response to microbial stimulus (Han-Ching Wang et al., 2010). *Peroxinectin* is a cell-adhesive molecule and opsonin and is activated in the presence of pathogen associate molecular patterns (PAMPs), concommittently with the prophenoloxidase cascade (Sritunyalucksana et al., 2001). *Prophenoloxidase*, stored as a zymogen in the granules of the haemocytes (Cerenius et al., 2003), is cleaved to form phenoloxidase through the action of serine proteinases and then non-enzymatically converted to melanin (Vazquez et al., 2009). Unlike the antimicrobial peptides, both *peroxinectin* and *prophenoloxidase* are synthesised and stored in the haemocytes until release on exocytosis.

Selection of biomarkers can be challenging as the capacity to measure the response to a wide range of stressors may not be possible using just one biomarker (Calfee et al., 2011; Damman et al., 2011). Therefore, the motivation for the selection of these immune genes was their involvement in varied downstream immune responses. The mechanisms of crustin/carcinin are not fully understood, but some researchers suggest they act directly on destabilising the integrity of the pathogen membrane (Rosa and Barracco, 2010) or possible disruption of DNA synthesis or cell metabolism (Devine and Hancock, 2002) after translocation into the cytoplasm of the pathogen (discussed later) (Rosa and Barracco, 2010). Peroxinectin has a number of biological functions including cell adhesion (Johansson and Söderhäll, 1988), degranulation (Johansson and Söderhäll, 1989), opsonic activity (Thörnqvist et al., 1994) and it also promotes encapsulation (Kobayashi et al., 1990). Melanin, produced as the downstream end product of the prophenoloxidase cascade, functions in cuticle sclerotisation, wound healing and encapsulation of foreign material including pathogens (Smith and Söderhäll, 1983; Söderhäll and Cerenius, 1998; Cerenius et al., 2008; Sugumaran, 2002). The selection of this suite of biomarkers allowed quantification of a wide repertoire of host immune responses anticipated as a consequence of the various immune and stress challenges. The overall aim was to determine the scope and validity of this toolbox in two economically and ecologically important decapods, *Homarus gammarus* and *Carcinus maenas*.

Relevant biomarkers that are able to quantify change in host response to disease and stress are important. The increased pressure to provide sufficient food for a growing human population

(Anonymous, 2004) has lead to the growth of aquaculture in shellfisheries (FAO, 2014), particularly that of penaeids (FAO, 2014) and also development of facilities that can maintain wild-caught species, such as *Homarus americanus*, in order to supply market demand. Stocking densities are commonly high in aquaculture ponds in the penaeid industry (Moriarty, 1998) which can be stressful to the host, leading to a compromised immune response (Kautsky et al., 2000) and therefore increased susceptibility to pathogens. High stocking densities also facilitate rapid transmission of opportunistic pathogens that can have a devastating effect on the population (*e.g.* WSSV). Disease outbreaks (bumper car disease, *Anophryoides haemophila*) have also been associated with wild-caught lobsters (*H. americanus*) in holding facilities in Nova Scotia, Canada (Greenwood et al., 2005). These examples effect food security and have the capacity to affect the trade of these highly valuable commodities.

The World Organisation for Animal Health (OIE) recognise the potential for the distribution of pathogens through the trade of commercially important species. Member countries of the OIE are required to provide information on listed and emerging diseases. Furthermore, member countries that are classified as disease-free from particular pathogens, must enforce early monitoring systems that could detect emergence of disease (OIE, 2014). Biomarkers, once validated, are a useful tool not only in assessing the host response to stress and disease, but also as a surveillance mechanism that can provide early detection of disease.

Similarly, biomarkers can be useful indicators of host response to changing conditions associated with environmental variables. This will become increasingly important with the predicted climate changes anticipated (Holt et al., 2010). It could impact the susceptibility of the host (Dove et al., 2005), virulence of microorganisms (Chen et al., 2001) and result in biogeographical changes in the distribution of host species and pathogens (Cook et al., 1998) that could occur as a consequence of changes to hydrodynamics (Doney et al., 2012; Howard et al., 2013) and seawater temperature (Glenn and Pugh, 2006).

7.2 Summary of key findings

7.2.1 Dissecting the response to White Spot Syndrome Virus (WSSV) in the European lobster, *Homarus gammarus* (L.)

The European lobster, *Homarus gammarus*, was chosen as the model species by which to test the role of the biomarkers in WSSV (White Spot Syndrome Virus) infection due to the economical significance of the this species (FAO, 2014) and it's susceptibility to this pathogen (Bateman et al., 2012b). WSSV has a large host range (see Chapter 3, Table 3.1) and has had a devastating impact on penaeid aquaculture in the past (Lightner, 1996). WSSV has also been inadvertently imported into the UK via contaminated penaeid products (Bateman et al., 2012a), so there remains potential for transmission of this viral pathogen to susceptible species around the UK (Oidtmann and Stentiford, 2011).

Whilst the transcription of *crustin, peroxinectin* and *prophenoloxidase* all increased over the 10 day WSSV-challenge, only the antimicrobial peptide, *crustin*, up-regulated transcription in response to treatment. Interestingly, no viral gene transcription was evident, inferring no established viral infection. However, as this was a feeding trial, the gut of the post-larval stage IV (PLIV) *H. gammarus* may have played a significant role in impeding viral replication. In which case, two intriguing factors may have

contributed to inhibition or prevention of viral infection; elevated expression of antimicrobial peptides in the gut (Soonthornchai et al., 2010) and the role of commensal microbiota, which could have augmented the host innate immune response in the gut (Abt et al., 2012).

7.2.1.1 Gut immunity? A hypothesis

Potential for localised immune response?

Ingestion of pathogens often mean passage through the gut before systemic infection can be established. Potentially viral gene expression could be inhibited or delayed due to processes in the gut impeding viral replication. The crustacean gut is protected almost entirely by a chitinous membrane protecting the rest of the anatomy from invading pathogens. It is only the midgut that lacks the cuticle layer and possesses just an epithelial and basal layer (Leonardo et al., 2005). Additionally, the acidic environment of the gut is able to inactivate and digest many invading pathogens including viruses (Jiravanichpaisal et al., 2006). Host specificity can therefore be determined by the ability of WSSV to cross the basal membrane of the digestive tract. For example, differential susceptibility in two genera of shrimp, *Marsupenaeus* sp. and *Palaemon* sp. from ingestion of WSSV-infected feed has been reported (Leonardo et al., 2005). The WSSV virions were unable to cross the basal membrane in the *Palaemon* sp. whereas infection via the midgut (analogous to the intestine) in *Marsupenaeus* sp. was successful. This was denoted by the presence of virions in the nucleus of circulating haemocytes at different stages of morphogenesis, suggesting successful viral replication.

Expression of immune genes such as antimicrobial peptides can also be relatively elevated in the gut and then further expressed upon exposure to pathogens (Soonthornchai et al., 2010). Exposure to the Gram-negative bacterium *Vibrio harveyi* in *Penaeus monodon* showed that three anti-microbial peptides, (*anti-lipopolysaccharide factor isoform 3*, *crustin* and *penaeidin*) were all expressed at relatively high levels in the anterior midgut suggesting a local immune response to this pathogen (Soonthornchai et al., 2010). Whether this increase in expression translated to the protein level was not determined by the authors in this study.

Crustins are known to show activity against both Gram-positive and Gram-negative bacteria (Smith et al., 2008). Although lobsters contain mainly Type I crustins (Brockton et al., 2007; Hauton et al., 2006; Christie et al., 2007) (predominantly exhibiting activity against Gram-positive bacteria (Smith, 2011)), a type III crustin (crustin-3) was found to be over-expressed in WSSV-challenged *Penaeus monodon* suggesting it may have antiviral properties (Antony et al., 2011b). The antibacterial/antiviral mechanism of crustins are not fully understood. However, potential mechanisms do include destabilizing the integrity of the outer membrane of the pathogen (Rosa and Barracco, 2010; Hancock, 2001; Huang, 2000), or possible disruption of cell metabolism or DNA synthesis (Devine and Hancock, 2002) after translocation into the cytoplasm of the pathogen (Rosa and Barracco, 2010).

The role of commensal gastrointestinal bacteria on the innate immune response

To explore the hypothesis of gut immunity further, it is crucial to understand how commensal bacteria can contribute towards a competent host immune response. Microbes are known to colonise mucosal surfaces within the gut of multicellular organisms. These microbes are considered to be an integral part of the response of the host against ingestion of pathogenic bacteria or viruses (Ganal et al., 2012). However, the role of the microbiota in relation to innate immune function is not fully understood. Yet,

there are numerous examples in the literature of how commensal bacteria can influence or stimulate the innate immune system in response to other invading pathogens. The majority of these studies are in regard to the mammalian intestinal systems (Kinnebrew and Pamer, 2012), although an increasing body of work has also investigated the role of gastrointestinal commensal microbes in insects such as *Drosophila* species (Ryu et al., 2010), the gypsy moth *Lymantria dispar* (Broderick et al., 2010), the migratory locust *Locusta migratoria manilensis* (Zheng and Xia, 2012), the bumble bee *Bombus terrestris* (Riddell et al., 2011) and mosquitoes *Aedes aegypti* (Ramirez et al., 2012). These examples highlight how important the interaction between the innate immune receptors and microbial flora are in developing a healthy intestinal immune system.

Intestinal epithelium is a semi-permeable membrane that is colonised by commensal microbial communities, yet, is able to restrict microbes and microbial products from crossing this membrane and infecting the host (Kinnebrew and Pamer, 2012). Microbial molecules are sensed by host pattern recognition receptors which then activate signalling cascades to regulate specific immune-related proteins designed to eliminate pathogens (Kinnebrew and Pamer, 2012; Lee and Söderhäll, 2001). These signals from commensal bacteria within the gut have the ability to either augment innate immune responses after pathogenic infection, or conversely, exacerbate pathogenic infection (Abt et al., 2012).

The bacterially stimulated induction of signalling pathways can also up-regulate the transcription of antiviral genes. In *Drosophila melanogaster*, infection with the intra-cellular bacteria *Wolbachia* sp. resulted in increased resistance to three RNA viral infections (*Drosphila* C virus, Nora virus and Flock House virus) (Teixeira et al., 2008). In response to influenza A viral infection of the respiratory tract in humans, commensal bacteria were found to provide signals which lead to the expression of interleukin (produced by macrophages of the innate immune system), which in turn produced inflammatory responses, fever and sepsis. This suggests commensal bacteria are able to influence this innate signalling pathway in response to viral infection (Ichinohe et al., 2011). Furthermore, deliberate manipulation of the commensal bacterial community in the steady state in a mouse model, adversely affected the hosts' ability to produce a competent antiviral immune response. This suggests the commensal bacteria calibrated the activation threshold of the innate immune responses from the host (Abt et al., 2012).

Whilst the picture is currently incomplete regarding WSSV infection in *Homarus gammarus*, it is important to consider the role the gut and commensal bacteria play in potentially impeding or preventing infection of ingested pathogens. An increase in the transcription of *crustin* relative to the control group, without concurrent viral replication was evident in this study. This poses an interesting question as to whether this increase in *crustin* expression was related or initiated by a local immune response within the gut? Unfortunately, isolating the gastro-intestinal tract for separate analysis of gene expression or histology was not possible due to the small size of the post-larval stage IV *H. gammarus*. Repeat infection studies on larger *H. gammarus* would facilitate dissection of the digestive tract to allow separate analysis of *crustin* expression and determine the role the gut plays in the immunocompetence of the host.

There is a suggestion of antiviral activity of *crustin* in *H. gammarus* in response to consumption of WSSV-feed, although this cannot be definitely proven without directly investigating the antiviral properties of *crustin* in this species. For future studies, RNAi techniques can be used to inhibit or silence *crustin*, followed by WSSV challenge, which would provide important information in determining

the antiviral role of this gene in *H. gammarus*. This would establish the significance of *crustin* in the host immune response to WSSV and determine whether it was a key contributing factor in preventing successful viral infection. Similarly, identifying and altering the gut microbiota followed by repeat WSSV-challenge may provide insights into the role that commensal microbiota play in this species in response to infection. Particularly as adult *H. gammarus* are know to be susceptible only to a few diseases (Gjerde, 1984; Davies et al., 2014). Whilst there is a growing body of research investigating the role of gut immunity in insects (Eleftherianos et al., 2008; Glittenberg et al., 2011; Riddell et al., 2011; Garcia-Garcia et al., 2013; Lee and Brey, 2013) and penaeids (Rengpipat et al., 2000; Li et al., 2007; Soonthornchai et al., 2010), there is scope to extend this research more broadly to decapods in general. This may lead to much more information regarding the differential susceptibility of decapods to various pathogens, particularly as ingestion is a major route of transmission for microorganisms.

7.2.2 Assessment of immunocompetence in the shore crab, *Carcinus maenas* (L.), to natural exposure of pathogens

Carcnius maenas was chosen for it's accessibility and the fact that shore crabs are known to harbour a number of pathogens at any given time (Stentiford et al., 2012). The immune biomarkers, *carcinin, peroxinectin* and *prophenoloxidase*, have been used to measure host immune response often to single infections (Brockton and Smith, 2008; Du et al., 2013; Roux et al., 2002). This study was aimed at using the biomarkers to assess the comparative host immune response between populations of *C. maenas* with multiple and very different pathogen profiles.

Whilst there was an overlap in the genus and species of pathogens present in *C. maenas* at both Harbour and Newton's Cove, there were also fundamental differences resulting in very different pathogen profiles. Similar to the WSSV study, only the antimicrobial peptide *carcinin* had significantly ($p<0.001$ = 'No Pathogen' group, $p<0.01$ = 'Pathogen' group) different transcription profiles at the population level between sites. This was attributed to the presence or absence of pathogens, (*C. maenas* in Harbour were 73% pathogen-free, determined by histology, compared with only 5% pathogen-free in Newton's Cove), and also to non-identified factors associated with the locations themselves. The comparatively greater expression of *carcinin* in Harbour may also explain the lower incidence of infection evident at this site. The cause of the greater *carcinin* transcription was not explored further during this study. However, it would be interesting to identify factors between these sites that may have contributed to the difference observed in *carcinin* transcription. The drivers of the comparatively higher *carcinin* transcription at Harbour have the potential to decrease host susceptibility to pathogens and therefore to influence the health of *C. maenas* at the population level.

In addition, 30% of haemolymph samples from *C. maenas* were noted to be of lower quality RNA (RQI $\leq$7.6). In terms of the locations, the Harbour site was likely to be vulnerable to contaminants as a result of the high degree of boat traffic in the immediate area. An interesting direction for future research would be to investigate the impact of contaminants on host RNA, through exposure experiments and on the expression of the immune biomarkers, in particular, *carcinin*. One method would be to transplant *C. maenas* from one location to the other (*e.g.* from Newton's Cove to Harbour), or from both sites to a neutral location. Subsequent gene expression and RNA quality analysis could determine the influence of the environment on the host. Furthermore, information on water quality (particularly pollutants) at both sites would potentially reveal the key differences between

sites that could be linked to the host's gene transcription response and haemolymph RNA quality.

Modes of pathogen transmission are variable, such as predation, close contact with conspecifics and other species, but also include exposure to free pathogens in the water column. In order to more fully understand the drivers that result in varied pathogen profiles between these populations, it would be informative to compare the type and load of pathogens from the water inhabited by *C. maenas*. New and exciting techniques, such as Next Generation Sequencing (NGS), would facilitate analysis of environmental DNA, by using species-specific genomic regions (DNA barcodes) to identify samples (Shokralla et al., 2012). Identified pathogens could then be correlated to those found in the host to shed further light on the range of susceptibility in this host decapod and the difference in pathogen exposure between populations. Of course, functional annotation is likely to restrict identification of all pathogens, particularly the more cryptic and novel species. However, with improving annotation, this would be an interesting area to pursue, that would likely drive future investigations into host-pathogen relationships and determining reasons that may drive susceptibility and resistance in the host. Other factors, including identification of prey species and analysis of local hydrodynamics that may effect the distribution of pathogens, would also provide a more comprehensive assessment to determine the drivers that contribute to the variation in pathogen type between populations.

7.2.3 Testing the response of the immune toolbox with controlled infections in *Carcinus maenas (L.)*

Many studies to date have quantified the transcription of *carcinin, peroxinectin* and *prophenoloxidase* in response to early stages of bacterial infection (Brockton and Smith, 2008; Burge et al., 2009; Li et al., 2012). As a continuation from the survey of host immune transcription in response to the natural pathogen burden in two wild populations of *C. maenas* (Chapter 4), the biomarkers were tested in their capacity to indicate recent infection to a single Gram negative (*Listonella anguillarum*) or Gram positive (*Planococcus citrus*) bacterial infection. *C. maenas* are susceptible to infection with both of these pathogens. *L. anguillarum* is distributed globally (Rad and Shahsavani, 2008) and has been identified as the causal agent in fish and shellfish epizootics in aquaculture (Bolinches et al., 1986; Lodeiros et al., 1987; Toranzo and Barja, 1990). *P. citreus* is commonly found in intertidal sediments (Relf et al., 1999; Engelhardt et al., 2001) in areas also inhabited by *C. maenas*.

In both infection challenges, histological evidence of haemocyte aggregation and melanisation, suggested prior expression of *peroxinectin* and *prophenoloxidase*, key genes involved in these processes. However, changes in the transcription of these genes were not evident at 48 hours (*P. citrus*) and 72 hours (*L. anguillarum*). The presence of bacteria in the histology images from the *P. citreus* infected *C. maenas* suggested earlier stages of infection compared with the *L. anguillarum* histology, in which no bacteria were evident. The THC profiles also differed between bacterial challenges with no discernible change in THC during the *L. anguillarum* infection and a significant decrease ($p<0.01$) at 48 hours in the *P. citreus* infected *C. maenas* compared with the controls. These THC profiles did not fit the classic haemocyte response expected (*i.e.* rapid haemocytopenia post infection). This lack (*L. anguillarum*) or delay (*P. citreus*) in haemocytopenia may have been due to an inadequate infection dose or as a consequence of the timing of haemocyte sampling. Sampling of haemolymph for THC and bacterial load analysis was aimed at following the progression of infection, whereas haemolymph sampling for gene expression was aimed at assessing the recovered or recovering phase of *C. maenas* in response to

the Gram negative and Gram positive infection.

Similar to the previous investigation, the transcription of *carcinin* did change in response to treatment. In fact, *carcinin* transcription was up-regulated in response to both *L. anguillarum* and *P. citreus* challenges. This suggests *carcinin* was a useful biomarker at recording the host response to these bacterial infections over longer time periods (days). Conversely, *peroxinectin* and *prophenoloxidase* were more likely to have been appropriate biomarkers over shorter time-scales (hours). Similar to the WSSV infection trial in *H. gammarus*, these two bacterial infection challenges could be repeated post *carcinin* silencing to test the role of this antimicrobial peptide to Gram positive and Gram negative bacterial infection in *C. maenas*. Unfortunately, the contamination observed during both the *L. anguillarum* and *P. citreus* infection trials inevitably requires these experiments to be repeated in order to validate the data.

7.2.4 The role of *carcinin, peroxinectin* and *prophenoloxidase* in *Carcinus maenas* (L.) as early stress biomarkers in response to increased stocking density

Examples of host *carcinin, peroxinectin* and *prophenoloxidase* expression in response to microbial infection in a variety of different decapod species has been well documented (Liu et al., 2005; Wang and Chen, 2005; Hauton et al., 2006; Burge et al., 2009; Gao et al., 2009; Sperstad et al., 2010). The wider applicability of this suite of biomarkers was tested against the non-biotic stressor of high stocking density. No known evidence was presented in the literature to date of host stress response as a consequence of crowding stress using this suite of biomarkers, although the biomarkers have been used to assess host response against environmental variables in previous studies (Le Moullac et al., 1998; Liu et al., 2007a; de la Vega et al., 2007; Loc et al., 2013).

Consistent with the previous investigations, only *carcinin* transcription significantly ($p<0.01$ at 11 hours) decreased in response to increased stocking density. This was only evident during the shorter simulation. No change in *carcinin* transcription was evident at seven days. This suggests probable recovery from the initial increase in stocking density. The transcription of *peroxinectin* was lower in the control group at seven days compared with 11 hours ($p=0.05$) and *prophenoloxidase* was also comparatively greater at seven days in both the control ($p<0.01$) and high stocking density ($p<0.05$) groups. However, transcription of *peroxinectin* and *prophenoloxidase* was not significantly different in response to stocking density. Under these treatment conditions, these two biomarkers cannot be endorsed as appropriate stress indicators in *C. maenas* in response to only stocking density.

The mechanism of how *carcinin* is expressed (continuously and inducible upon stimulation) compared with *peroxinectin* and *prophenoloxidase* (synthesised and then stored in the haemocyte granules until required), is probably key in determining the suitability of these genes as biomarkers of non-biotic stress. Increased stress is likely to require redistribution of energy from immune responses to factors associated with a stress response. A biomarker involved in immune responses, that is secreted continuously, such as *carcinin*, is more likely to reflect a redistribution in energy, such as a down-regulation in transcription, as reflected in the *carcinin* expression at 11 hours. The capacity of *carcinin* or other *crustins* as biomarkers of non-biotic stress should be investigated in the wider decapod community to determine the sensitivity and limitations of use.

In terms of further work in relation to the transport and holding of decapods for live market, *carcinin* could be used to assess a profile of stress throughout the entire capture and processing chain. In particular, the capture and grading processes prior to holding and transport, are considered to be particularly stressful (reviewed in Neil, 2012). A stress profile could determine suitable conditions to optimise recovery post capture and grading to increase survival and product quality (Neil, 2012).

7.3 Summary

The outstanding feature throughout this project was the significant change in the transcription of the antimicrobial peptide *crustin/carcinin* in the host response throughout each of the challenges. This research has demonstrated it's use as a biomarker of short-term host response to viral challenge (WSSV in *Homarus gammarus*) and indicator of later host response to Gram negative and Gram positive bacterial challenge in *C. maenas* during the recovery phase of infection. In addition, *carcinin* has potential in determining the host response to pathogen burden at a population level, as evident from the analysis of the two populations of *C. maenas* in Weymouth, UK. Furthermore, *carcinin* transcription was evident in *C. maenas* in response to short-term high stocking density stress.

7.3.1 What do we know about antimicrobial peptides?

7.3.1.1 General structure of antimicrobial peptides

Antimicrobial peptides (AMPs) are evolutionarily ancient (Zasloff, 2002) and are known to consist of a high diversity of variants (Rosa and Barracco, 2010). In crustaceans there are known to be 15 families of AMPs, 14 of which are known in decapods. They are found in the haemolymph and in epithelial surfaces most exposed to microbes (Rosa and Barracco, 2010). The physicochemical properties of AMPs are thought to be related to their broad-spectrum activity (Hancock, 2001; Pushpanathan et al., 2013). Structurally, AMPs contain discrete regions (*e.g.* Chapter 1, Figure 1.2) consisting of cationic, hydrophilic and hydrophobic amino acids arranged in an amphipathic structure (Maloy and Kari, 1995). Overall they have a net positive charge due to the number of cationic amino acids within the hydrophobic region (Pushpanathan et al., 2013).

7.3.1.2 Mechanisms of function

The action of antimicrobial peptides on microbes involve compromising the integrity of the microbial cell wall. Electrostatic interaction between the cationic, positively charged portion of the AMP and the negatively charged microbial cell surface (as a consequence of the presence of *e.g.* lipopolysaccharides), is followed by possible translocation into the cytoplasm of the microbial cell through the hydrophobic region of the AMP (Rosa and Barracco, 2010; Pushpanathan et al., 2013). Pathogens can be destroyed either as a result of disruption of the cell membrane or action of the AMP on specific intracellular targets that inhibit processes such as cell-wall synthesis, cytoplasmic membrane formation, nucleic acid and protein synthesis, and enzymatic activity (Brogden, 2005). AMPs that act on permeabilising the microbial cell membrane are cationic and this category includes crustins.

Mechanisms that lead to membrane damage of the microbial membrane include thinning of the membrane, pore formation or lipid bilayer disruption (Lohner and Prenner, 1999). A number of models describe different mechanisms that require aggregation and binding of the AMPs to the microbial cell

membrane to permeate the microbial cell wall. These include the barrel-shaped stave, toroidal and carpet models (described by Brogden (2005); Sengupta et al. (2008) and Pálffy et al. (2009)). In brief, in the barrel-stave pore model, multiple AMPs aggregate on the microbial cell surface and cross the cell membrane so that the hydrophilic region faces the lumen of the pore and the hydrophobic region faces the lipid bilayer creating barrel-shaped staves (Pálffy et al., 2009). Similar to the barrel-shaped mechanism, in the toroidal model, the AMPs aggregate and penetrate the lipid bilayer, but form a monolayer connecting the inner and outer lipid bilayers (Mor and Nicolas, 1994). In the carpet model, the AMPs cover the microbial membrane which it then penetrates once a threshold has been reached, first causing thinning and expansion of the outer membrane layer, followed by the formation of numerous micelle-like units (Zasloff, 2002; Pálffy et al., 2009). Each of these mechanisms result in leakage of the cellular contents and therefore death of the microbe. The mechanisms of entry highlighted are generic and the specific entry mechanism utilised by crustins in decapods is not clear.

Some microbes are able to circumvent the effects of AMPs through mechanisms that secrete digestive enzymes that destroy the AMPs or simply as a consequence of a lower density of acidic lipids in the microbial outer membrane (Zasloff, 2002). However, in general, targeting the microbial cell membrane has proved to be a very effective mechanism used by AMPs in response to many microbial infections. One of the features of antimicrobial peptides is the lacking of any unique epitopes that can be recognised by pathogens and acted upon, *e.g.* by proteases (Zasloff, 2002). Another key aspect of their success is that they are constitutively expressed at a high titre (Hauton, 2012) and inducible on stimulation, delivery is rapid, which is aided by their small size (5-14kDa) and only small quantities are required to be effective (Cuthbertson et al., 2002; Zasloff, 2002; Smith et al., 2008; Ghosh et al., 2011).

Some AMPs are also thought to be multi-functional. Whether this is as a result of their multiple domains or generally due to the overall cationic, amphipathic conformtion is not clear (Bachère et al., 2004; Rosa and Barracco, 2010). In crustins for example, protease inhibitory functions are thought to be associated with the 4DSC region (Ranganathan et al., 1999), which may be involved in inhibiting microbial proteases (Cerenius and Söderhäll, 2004) or possibly regulating the host prophenoloxidase cascade (Rosa and Barracco, 2010). Antiprotease properties have been reported only in Type III crustins, so it is not clear whether this function is true for all crustins (Amparyup et al., 2008). Furthermore, in some taxa, such as insects, a repertoire of different isoforms of AMPs with similar structural features, can be expressed in response to different microbial challenges, providing the host with a comprehensive defence against a wide range of microbes (Bulet et al., 1999) which therefore makes it more difficult for microbes to circumvent or destroy.

7.4 Key challenges and concluding remarks

7.4.1 Determining crustin function

Whilst there is a large body of research on AMPs in general, there is still relatively little known regarding the specific function of crustins in decapods. Studies involving gene-silencing, (*e.g.* using techniques such as RNAi), would determine the role of crustins in decapods and provide important information regarding *in vivo* activity. This information could also expand on the findings in this research in determining, as a biomarker, the scope of disease or stress response from the host that crustins could reflect.

Interestingly, Shockey et al. (2009) noted differences in the activity of crustins between *in vivo* and *in vitro* studies. The authors suggested this could have been a result of the different methodologies, but also may have been related to synergistic effects *in vivo* with other molecules or from optimum conditions. This point highlights the requirement for appropriate experiments that provide the most relevant information. Furthermore, defining synergistic effects with other immune effectors would be a critical step forward in determining the overall function of crustin and is another important avenue to explore. In order to achieve this, an increase in the amount of functionally annotated genomic sequence data is required for economically and ecologically important decapods. A lack of functional annotation is one of the major obstacles that restricts the speed at which progress is currently made (Hauton, 2012). A fully transcribed host genome in *H. gammarus* and *C. maenas* with functional annotation is fundamental in gaining further insights into the mechanisms employed by either the host or microorganism and in determining the role crustins play.

7.4.2 Improving transparency in the scientific literature

One of the over-riding elements of this research was to provide data that had been scrupulously assessed in terms of validity and quality. As highlighted in Chapter 2, interpretation of data can be significantly affected by the quality of the data analysed. The gene transcription data presented throughout each study, was rigorously assessed and based on samples with high quality RNA. The low quality RNA was noted and discussed in the relevant chapters.

Guidelines exist to help researchers process samples correctly from sample acquisition to data analysis. In view of the technique used to assess host immune gene expression (qPCR), the MIQE guidelines (Bustin et al., 2009) were used as a gold standard by which to process samples and data. These guidelines also stipulate the essential and desirable information that should be submitted in support of the research undertaken. As highlighted by Bustin et al. (2013), much of the current literature, whilst improving, still needs to provide sufficient technical details to inform the scientific community of the quality and therefore relevance of the data presented. In addition, it is also fundamental to provide sufficient details in order for other groups to corroborate the research findings and to be able to compare infection or non-biotic stress challenges between researchers. As recognised by Hauton et al. (2015), provision of metadata is necessary to make more significant advances in the field of crustacean immunology. Particularly as the classic conception of the innate immune system has more recently been challenged, with increasing knowledge of more complex interactions and a growing impetus to focus on potential specificity in innate immunity (reviewed in Hauton et al., 2015). These advances in our understanding of arthropod immunity have been facilitated by the technological development, particularly in NGS and RNA-Sequencing, especially within Insecta. To progress more efficiently in our understanding of immune response in Decapoda and improve the integrity of the published scientific data, researchers should routinely include the minimum required information stipulated in the relevant guidelines.

Gaining a comprehensive understanding of host-pathogen interactions requires detailed knowledge of how the genetic profile of a population may effect susceptibility to pathogens, details of the compounding and mitigating influence of co-infections, the influence of environmental factors (water quality, temperature, salinity and hydrodynamics), trophic interactions, nutritional supply (Bean et al.,

2013) and more detailed investigations regarding the role of the gut microbiome on the host immunity. This research highlights the capacity for the wide application of *crustin/carcinin* as a biomarker in *Homarus gammarus* and *Carcinus maenas*. It would interesting to test the capacity of crustin as a biomarker in other decapods species. For the future there are many exciting, new and developing tools that will help answer questions relating to host-pathogen interactions.

8 Appendices

8.1 Chapter 2

Protocols for molecular processing of samples

8.1.0.1 Preparation of DEPC-treated Milli-Q water

The following solution was prepared in a fume cupboard. DEPC stock was stored in the fridge.

1. Open the DEPC stock in the fume cupboard to allow the fumes to vent.
2. Prepare a 0.1% solution of DEPC using Milli-Q water and leave in the fume cupboard overnight.
3. Autoclave the solution for 15 minutes at 121°C.

8.1.0.2 Liquid nitrogen-based homogenisation of crustacean tissue and haemolymph

1. As required, collect small volumes of liquid nitrogen (<2 litres) in a hand-held dewar using thermal protective gloves.
2. Remove samples to be homogenised from the -80°C and place immediately in the liquid nitrogen. Ensure only to use appropriate screw-top tubes for use in the liquid nitrogen.
3. Using tongs, remove the sample from the liquid nitrogen and homogenise using a hammer and grinder.
4. Place into TRI-Reagent® immediately in a 2ml sterile microcentrifuge tube (TRI-Reagent® to be used in the fume-hood only) and further homogenise with an electric homogeniser.
5. Ventilate any excess liquid nitrogen in the fume-hood overnight until dewar is empty.

8.1.0.3 Extraction of total RNA from biological tissues using TRI Reagent®

TRI Reagent®, Sigma-Aldrich.

1. Homogenise the sample in TRI Reagent® (1ml per 50-100mg) of tissue in a 1.5-2ml microcentrifuge tube.
   The volume of the tissue should not exceed 10%.
2. The sample may be stored at -80°C at this point if necessary.
3. Allow to stand for 5 minutes at room temperature to ensure complete dissociation of the nucleoprotein complexes.
4. Add 0.2ml of chlororform (molecular grade) per 1ml of TRI-Reagent® used. Cover the sample tightly and shake vigorously for 15 seconds, then allow to stand at room temperature for 15 minutes. (Dispose of any tips in the 'halogenated' waste).

5. Centrifuge at 12,000g for 15 minutes at 4°C to separate the phases (protein, DNA and RNA).
6. Aspirate the top RNA phase and transfμer to a new sterile microcentrifuge tube. Dispose of the organic (protein) and interphase (DNA) into the halogenated waste container.
7. Add 0.5 ml isopropanol (2-propanol) per ml of TRI Reagent® and mix gently by tipping upside down and back.
8. Allow the sample to stand for 5-10 minutes on ice. To increase the yield of RNA, the sample can be left at -20°C.
9. Centrifuge at 12,000g for 10 minutes at 4°C. The RNA precipitate will form a pellet on the side and bottom of the tube.
10. Remove the supernatant, briefly centrifuge again and syringe out the final volume of isopropanol from the tube.
11. Wash the RNA pellet by adding 1ml (minimum) of 75%-80% ethanol per ml of TRI reagent®.
12. Discard remaining ethanol, vortex the sample and then centrifuge at 7,500g for 5 minutes at 4°C (centrifugation is slower to prevent shearing of the RNA).
13. Remove the ethanol by pipetting and briefly air-dry the RNA pellet for 5-10 minutes on ice. Do not let the RNA pellet dry completely, as this will greatly decrease the solubility of the pellet.
14. Add an appropriate volume of DEPC-treated deionised water.

8.1.0.4 Assessment of quantity and purity using NanoDrop® software

NanoDrop® 1000 Spectrophotometer, Thermo Scientific.
General operation

1. With the sampling arm open, pipette 1μl of the sample onto the lower measurement pedestal.
2. Close the sampling arm and initiate a spectral measurement using the operating software on the PC. The sample column is automatically drawn between the upper and lower measurement pedestals and the spectral measurement made.
3. When the measurement is complete, open the sampling arm and wipe the sample from both the upper and lower pedestals using a soft laboratory wipe. Simple wiping prevents sample carryover in successive measurements for samples varying by more than 1000-fold in concentration.

8.1.0.5 RNA quantity and integrity analysis using Experion™ software

Experion™ RNA StdSens Analysis Kit, Bio-Rad.
Clean electrodes before run.

1. Fill a cleaning chip with 800μl Experion™ electrode cleaner. Check to make sure there are no air bubbles trapped in the reservoir. Gently tap the side of the cleaning chip to dispel any bubbles. Label this cleaning chip as the electrode cleaner chip.
2. Open the lid of the electrophoresis station and place the chip on the platform.
3. Close the lid and leave the chip in the instrument for 2 minutes.

4. Fill a separate cleaning chip with 800μl DEPC-treated water. Label this cleaning chip as the DEPC water chip.
   If this cleaning chip is being used for the first time, treat the chip with the Experion^{TM} electrode cleaner to remove any RNase contamination prior to use. To do this, completely fill a new cleaning chip with Experion^{TM} electrode cleaner, let it sit for 5 minutes, discard the solution, and then thoroughly rinse (4-5 times) the chip with DEPC-treated water.
5. Open the lid and remove the cleaning chip containing the electrode cleaner; replace it with the chip containing the DEPC-treated water.
6. Close the lid and leave the chip in the instrument for 5 minutes to rinse the electrodes.
7. Replace the DEPC-treated water in the DEPC water chip and repeat the rinse step for 60 seconds.
8. Open the lid and remove the DEPC water chip.
9. Leave the lid open and wait about 60 seconds for any water remaining on the electrodes to evaporate.
10. When finished for the day, remove liquid from the cleaning chips.

Equilibrate reagents to room temperature.

1. Remove the RNA Stain (blue cap), RNA loading buffer (yellow cap), and 1 tube of RNA gel (green cap) from storage and equilibrate to room temperature for 15-20 minutes. Keep the stain covered at all times to avoid exposure to light.
   If filtered gel was previously prepared, remove it from storage and equilibrate as detailed above.
2. Vortex the contents of each tube and briefly centrifuge to bring the solutions to the bottom of the tubes. Make sure the DMSO in the stain is completely thawed before proceeding.

Filter gel and prepare the gel stain.

1. Pipet 600μl RNA gel (green cap) into a spin filter tube.
2. Centrifuge the gel at 1,500g for 10 minutes. Confirm that all of the gel has passed through the filter and then discard the filter.
   Use the filtered gel within 4 weeks of preparation. After 4 weeks, the filtered gel should be re-filtered and can be reused.
3. Pipet 65μl filtered gel into an RNase-free microcentrifuge tube. Add 1μl of RNA stain to the tube. Briefly vortex the solution. Keep the gel-stain (GS) solution protected from light.
   This is enough gel-stain solution for 3 chips. Increase the amount of gel stain, if required, by using a 65:1 ratio of gel and stain.
4. Cap the RNA stain tightly, since DMSO is highly hygroscopic, and store it in the dark.

Preparing the RNA ladder and samples.

1. Remove the RNA ladder (red cap) from storage and allow it to thaw on ice.
2. Determine the amount of RNA ladder required. A total of 1μl RNA ladder is required for each chip, plus an extra 1μl RNA ladder to account for variations in pipeting. As examples, when running 1 chip, 2μl of RNA ladder will be required; for 3 chips, 4μl of RNA ladder should be used.

3. Pipet the required amount of RNA ladder into an RNase-free microcentrifuge tube.
4. Prepare all samples by pipetting 2-3μl sample into RNase-free microcentrifuge tubes. RNA samples should be dissolved in RNase-free water or TE Buffer, as higher ionic strength buffers will effect sensitivity and quantitation accuracy.
5. Denature the ladder and samples for 2 minutes at 70°C.
6. Cool the denatured ladder and samples by immediately placing the tubes on ice for 5 minutes.
7. Spin down the ladder and samples in a microcentrifuge for 3-5 seconds. Store on ice until used.

Prime chip with gel-stain solution.

1. Open the Experion^{TM} priming station by pressing down on the front lever.
2. Remove an Experion^{TM} RNA StdSens chip out of its packaging and place it on the chip platform, matching the arrow on the chip with the alignment arrow on the chip platform. A post on the chip prevents insertion in the wrong position. Do not force the chip into position.
3. Pipet 9μl of filtered gel-stain solution into the well labeled GS (gel priming well). Insert the tip of the pipet vertically and to the bottom of the well when dispensing. Do not expel air at the end of the pipetting step.
   Placing the pipet tip at the edge of the well or allowing the gel to slide down the wall of the well may lead to bubble formation at the bottom of the well. It is acceptable to allow 1-2 small bubbles at the surface. Dislodge bubbles at the bottom with a clean pipet tip, or remove the gel-stain and refill the well.
4. Carefully close the priming station by gently pressing down on lid. The lid should snap completely closed.
5. Set the pressure setting to B and the time setting to 1, as specified by the alphanumeric code on the chip.
6. Press the Start button. The 'Priming' message will illuminate on the LCD screen, the priming station will pressurise, and the timer will count down. Complete priming requires approximately 30 seconds. Do not open the priming station during the count-down.
7. An audible signal indicates that priming is complete, and a 'Ready' message will be displayed. Open the priming station by pressing down on the release lever.
8. Turn the chip over and inspect the micro-channels for bubbles or evidence of incomplete priming. The glass chip will appear opaque and the micro-channels will be difficult to see if they are primed properly. If you detect a problem, such as a bubble or incomplete priming, prime a new chip.
9. Place the chip on a clean surface for loading samples.

Load the samples and RNA ladder and vortex the chip.

1. Pipet 9μl of the gel-stain solution into the other well labeled GS.
2. Pipet 9μl of filtered gel into the well labeled G.

3. Pipet 5μl of the loading buffer (yellow cap) in each sample well (1-12) and the ladder well, labeled L.
   Use a new pipet tip for each delivery to prevent contamination of the loading buffer stock. Alternatively, remove 70μl of loading buffer into an RNase-free tube and pipet 5μl to each well from this volume.
   Make sure the pipet tip is centered and positioned vertically all the way to the bottom of the well. Avoid introducing bubbles into the bottom of the wells. It is acceptable to allow 1-2 small bubbles at the surface.
   All wells should be filled with the loading buffer, even when fewer than 12 samples are run. The chip will not run properly unless all wells are filled.

4. Pipet 1μl of denatured RNA ladder into the well labeled L.
   Every chip must have the RNA ladder loaded into the ladder well for accurate quantitation of samples and for sample alignment.

5. Pipet 1μl sample into each of the 12 sample wells.

6. If running fewer than 12 samples, add 1μl loading buffer, TE buffer, or DEPC-treated water to the unused sample well(s).

7. Place the chip in the Experion^{TM} vortex station.

8. Turn on the vortexer, which will operate for 60 seconds and then automatically shut off. Remove the chip when the vortexer stops.

9. Start the run immediately (within 5 minutes) to prevent excessive evaporation and poor results or a chip performance error.

Run RNA StdSens analysis protocol on the Experion electrophoresis station.

1. Turn on the power to the Experion^{TM} electrophoresis station by pushing the green button in the centre of the front panel. The steady green LED above the button indicates that the unit is on.

2. Launch the Experion^{TM} software.

3. Open the lid of the electrophoresis station. Place the primed chip, which has been loaded with samples, on the chip platform. Ensure that the chip is seated properly and then carefully close the lid.

4. Select New Run. Select the RNA StdSens protocol (Eukaryotic total RNA, Prokaryotic total RNA, mRNA).

5. Select the number of samples to run. Click the Start button to begin the chip run.

6. After a run has started, the green LED in the centre of the front panel on the electrophoresis station will begin blinking.

7. When the chip run is complete, a 'Run complete' message will be displayed. Remove the chip from the electrophoresis station and dispose of it in the appropriate waste. To prevent contamination of the electrodes, do not leave the chip in the electrophoresis station for an extended period of time. Also, it is good practice to immediately insert the DEPC water chip (see below) as soon as the RNA chip is removed to prevent samples and/or buffers from drying on the electrodes.

Clean instrument electrodes after the run.

1. Fill the cleaning chip labeled DEPC water with 800μl DEPC-treated water. Gently tap the side of the cleaning chip to remove any trapped bubbles from the wells.
2. Open the lid of the electrophoresis station and place the cleaning chip on the chip platform.
3. Close the lid and leave it closed for about 60 seconds.
4. Open the lid and remove the DEPC water chip.
5. Allow the electrodes to dry for 30-60 seconds.
6. Close the lid.
7. Replace the water in the cleaning chip after use to avoid contamination. For storage, remove the water from the cleaning chip and store the chip in a clean location.

8.1.0.6 DNase treatment of RNA samples

TURBO DNA-free™ Kit, ambion® by Life Technologies™, Cat. no. AM 1907

1. Add 0.1 volume of 10x TURBO DNase Buffer and 1μl of TURBO DNase to the RNA and mix gently.
2. Incubate the mixture at 37°C for 20-30 minutes.
3. Add resuspended DNase Inactivation Reagent (typically 0.1 volume or 2μl for routine DNase treatment, whichever is the greater) and mix well.
4. Incubate for 5 minutes at room temperature, mixing occasionally. Gently flick the tube 2 or 3 times during the 5 minutes to redisperse the DNase Inactivation Reagent. (NB. If the room temperature cools below 22-26°C then move the tubes to a heat block).
5. Centrifuge at 10,000 x *g* for 1.5 minutes and transfer the RNA to a new tube.

8.1.0.7 Production of cDNA from mRNA using reverse transcriptase II

Superscript™ II Reverse Transcriptase, Invitrogen™.
(Used for reverse transcribing samples for confirmation of nucleotide sequences).

1. Add the following components to a nuclease-free microcentrifuge tube:
   1μl of Oligo(dT)$_{12\text{-}18}$, or 50-250ng random primers or 2pmole gene-specific primer (GSP).
   1μl of 1ng - 5μg total RNA or 1-500ng mRNA.
   1μl of dNTP mix (10mM each).
   Sterile distilled water to 12μl.
2. Heat the mixture to 65°C for 5 minutes and quick chill.
3. Collect the contents of the tube by brief centrifugation and add:
   4μl of 5x First-Strand Buffer
   2μl of 0.1M DTT
   1μl of RNaseOUT™ (40 units/μl). RNaseOUT™ (Cat. No. 10777-019) is required if using <50ng starting RNA.

4. Mix contents of the tube gently. If you are using oligo$(dT)_{12\text{-}18}$ or GSP, incubate at 42°C for 2 minutes. If you are using random primers, incubate at 25°C for 2 minutes.

5. Addμl (200 units) of SuperScript^{TM} II RT and mix by pipetting gently up and down. If you are using less than 1ng of RNA, reduce the amount of SuperScript^{TM} II RT to 0.25μl (50 units) and add sterile, distilled water to a 20μl final volume. If you are using random primers, incubate tube at 25°C for 10 minutes.

6. Incubate at 42°C for 50 minutes.

7. Inactivate the reaction by heating at 70°C for 15 minutes.

8.1.0.8 Production of cDNA from mRNA using reverse transcriptase III

Superscript^{TM} III Reverse Transcriptase, Invitrogen^{TM}.
(Used for reverse transcribing samples for downstream qPCR).
The following 20μl reaction volume can be used for 10pg-5μg of total RNA or 10pg-500ng of mRNA.

1. Add the following components to a nuclease-free microcentrifuge tube:
   1μl of oligo $(dT)_{20}$ (50μM; or 200-500ng of oligo $(dT)_{12\text{-}18}$), or 50-250ng of random primers, or 2pmol of gene-specific primers
   10pg-5μg total RNA or 10pg-500ng mRNA
   1μl of 10mM dNTP mix (10mM each dATP, dGTP, dCTP and dDTP at neutral pH)
   Sterile distilled water to 13μl

2. Heat the mixture to 65°C for 5 minutes and incubate on ice for a minimum of 1 minute.

3. Collect the contents of the tube by brief centrifugation and add:
   4μl of 5x First-Strand Buffer
   1μl of 0.1 M DTT
   1μl of RNaseOUT^{TM} Recombinant RNase Inhibitor (Cat. no. 10777-019, 40 units/μl). NB: The use of RNaseOUT^{TM} is essential when using less than 50ng of starting RNA
   1μl of SuperScript^{TM} III RT (200units/μl). NB: If generating cDNA longer than 5kb at temperatures exceeding 50°C using a gene-specific primer or $oligo(dT)_{20}$, the amount of SuperScript^{TM} III RT may be increased to 400U (2μl) to increase the yield.

4. Mix the solution by pipetting up and down gently. If using random primers, incubate at 25°C for 5 minutes.

5. Incubate at 50°C for 30-60 minutes. Increase the reaction temperature to 55°C for gene-specific primers. Reaction temperatures may also be increased to 55°C for difficult templates or templates with high secondary structures.

6. Inactivate the reaction by heating at 70°C for 15 minutes.

8.1.0.9 PCR reagents and thermal cycling

Table 8.1: Quantity of PCR reagents used for gene isolation in (a) *Carcinus maenas* and (b) *Homarus gammarus*.

Carcinus maenas	***actin***	***eef1a***	***gapdh***	***tubulin***	***ubiquitin***	***peroxinectin***
10x Taq Reaction Buffer (contains Tris.Cl, KCl,$(NH_4)_2SO_5$, 15mM $MgCl_2$; pH 8.7 at 20°C)	2.5	2.5	2.5	2.5	2.5	2.5
Magnesium Chloride 25mM	-	1.5	1.5	0.2	0.2	-
Taq® 5units/µl	0.2	0.2	0.2	0.2	0.2	0.2
dNTP mix 100mM	1	1	1	1	1	1
Forward primer	2	2	2	2	2	2
Reverse primer	2	2	2	2	2	2
5x Q	-	1	-	-	-	-
cDNA	1	1	1	1	2	1
MilliQ®	16.3	13.8	14.8	16.1	15.1	16.3

(a)

Homarus gammarus	***actin***	***eef1a***	***gapdh***	***ppia***	***tubulin***	***peroxinectin***
10x Taq Reaction Buffer (contains Tris.Cl, KCl,$(NH_4)_2SO_5$, 15mM $MgCl_2$; pH 8.7 at 20°C)	2.5	2.5	2.5	2.5	2.5	2.5
Magnesium Chloride 25mM	-	1.5	1.5	1.0	0.2	1.0
Taq® 5units/µl	0.2	0.2	0.2	0.2	0.2	0.2
dNTP mix 100mM	1	1	1	1	1	1
Forward primer	2	2	2	2	2	2
Reverse primer	2	2	2	2	2	2
5x Q	-	1	-	1	-	1
cDNA	1	1	1	1	1	1
MilliQ®	16.3	13.8	14.8	14.3	16.1	14.3

(b)

Table 8.2: PCR cycling for genes in *Carcinus maenas*.

	Carcinus maenas		
actin	**Temperature**	**Time**	**No. of cycles**
Initial denaturation	95	5 mins	x1
Denaturation	95	30 s	x32
Annealing	54	30 s	
Extension	72	60 s	
Final extension	72	5 mins	x1
eef1a	**Temperature**	**Time**	**No. of cycles**
Initial denaturation	95	5 mins	x1
Denaturation	95	30 s	x35
Annealing	54	30 s	
Extension	72	30 s	
Final extension	72	5 mins	x1
gapdh	**Temperature**	**Time**	**No. of cycles**
Initial denaturation	95	5 mins	x1
Denaturation	95	30 s	x32
Annealing	52	30 s	
Extension	72	60 s	
Final extension	72	5 mins	x1
tubulin	**Temperature**	**Time**	**No. of cycles**
Initial denaturation	95	5 mins	x1
Denaturation	95	30 s	x30
Annealing	50	30 s	
Extension	72	90 s	
Final extension	72	5 mins	x1
ubiquitin	**Temperature**	**Time**	**No. of cycles**
Initial denaturation	95	5 mins	x1
Denaturation	95	30 s	x32
Annealing	50	30 s	
Extension	72	15 s	
Final extension	72	5 mins	x1
peroxinectin	**Temperature**	**Time**	**No. of cycles**
Initial denaturation	95	5 mins	x1
Denaturation	95	30 s	x32
Annealing	55	30 s	
Extension	72	60 s	
Final extension	72	5 mins	x1

Table 8.3: PCR cycling for genes in *Homarus gammarus*.

	Homarus gammarus		
actin	**Temperature**	**Time**	**No. of cycles**
Initial denaturation	95	5 mins	x1
Denaturation	95	30 s	x32
Annealing	54	30 s	
Extension	72	60 s	
Final extension	72	5 mins	x1
eef1a	**Temperature**	**Time**	**No. of cycles**
Initial denaturation	95	5 mins	x1
Denaturation	95	30 s	x35
Annealing	54	30 s	
Extension	72	30 s	
Final extension	72	5 mins	x1
gapdh	**Temperature**	**Time**	**No. of cycles**
Initial denaturation	95	5 mins	x1
Denaturation	95	30 s	x32
Annealing	52	30 s	
Extension	72	60 s	
Final extension	72	5 mins	x1
ppia	**Temperature**	**Time**	**No. of cycles**
Initial denaturation	95	5 mins	x1
Denaturation	95	30 s	x35
Annealing	52	40 s	
Extension	72	30 s	
Final extension	72	5 mins	x1
tubulin	**Temperature**	**Time**	**No. of cycles**
Initial denaturation	95	5 mins	x1
Denaturation	95	30 s	x30
Annealing	50	30 s	
Extension	72	90 s	
Final extension	72	5 mins	x1
peroxinectin	**Temperature**	**Time**	**No. of cycles**
Initial denaturation	95	5 mins	x1
Denaturation	95	30 s	x40
Annealing	56	30 s	
Extension	72	60 s	
Final extension	72	5 mins	x1

8.1.0.10 DNA gel electrophoresis

1. First rinse a gel kit (gel tray and combs) with deionised water.
2. Prepare a 1% agarose gel using 1xTBE (Tris-borate-EDTA) buffer and heat in the microwave ensuring complete dissolution of the agarose. (Use thermally insulated gloves to remove from heat).
3. 1μl of stock ethidium bromide (500μg ml^{-1}) was added per 10ml of the cooled agarose gel in a fume cupboard.
4. Set up the gel kit, pour the cooled agarose into the gel tray and place a well-comb at one end.
5. Remove any bubbles using one end of a pipette tip.
6. Leave the gel to cool, indicated by turning opaque in colour.
7. Once set, remove the well-comb and place the gel tray into 1x TBE solution.
8. Load the appropriate DNA ladder and DNA samples with loading buffer.
9. Run the gel at 76V for 30-60 minutes (depending on the size of the gel).
10. Visualise the PCR products using a UV transluminator.

8.1.0.11 Nucleic acid extraction from agarose gel using the Qiagen QIAQuick gel extraction kit

QIAprep® Miniprep, QIAGEN.

1. Excise the DNA fragment from the agarose gel with a clean, sharp scalpel.
   Minimize the size of the gel slice by removing extra agarose.
2. Weigh the gel slice in a colourless tube. Add 3 volumes of Buffer QG to 1 volume of gel (100mg 100μl).
   For example, add 300μl of Buffer QG to each 100mg of gel. For >2% agarose gels, add 6 volumes of Buffer QG. The maximum amount of gel slice per QIAquick column is 400mg; for gel slices >400mg use more than one QIAquick column.
3. Incubate at 50°C for 10 minutes (or until the gel slice has completely dissolved). To help dissolve the gel, mix by vortexing the tube every 2 - 3 minutes during the incubation.
   Important: Solubilise the agarose completely. For >2% gels, increase incubation time.
4. After the gel slice has dissolved completely, check that the colour of the mixture is yellow (similar to Buffer QG without dissolved agarose).
   If the colour of the mixture is orange or violet, add 10μl of 3M sodium acetate, pH 5.0, and mix. The colour of the mixture will turn to yellow.
   The adsorption of DNA to the QIAquick membrane is efficient only at pH $\geq$7.5. Buffer QG contains a pH indicator which is yellow at pH $\geq$7.5 and orange or violet at higher pH, allowing easy determination of the optimal pH for DNA binding.
5. Add 1 gel volume of isopropanol to the sample and mix.
   For example, if the agarose gel slice is 100mg, add 100μl isopropanol. This step increases the yield of DNA fragments <500bp and >4kb. For DNA fragments between 500bp and 4kb, addition of isopropanol has no effect on yield. Do not centrifuge the sample at this stage.

6. Place a QIAquick spin column in a provided 2ml collection tube.

7. To bind DNA, apply the sample to the QIAquick column and centrifuge for 1 minute.
   The maximum volume of the column reservoir is 800μl. For sample volumes of more than 800μl, simply load and spin again.

8. Discard flow-through and place the QIAquick column back in the same collection tube.
   Collection tubes are reused to reduce plastic waste.

9. Recommended: Add 0.5ml of Buffer QG to QIAquick column and centrifuge for 1 minute.
   This step will remove all traces of agarose. It is only required when the DNA will subsequently be used for direct sequencing, *in vitro* transcription, or microinjection.

10. To wash, add 0.75ml of Buffer PE to QIAquick column and centrifuge for 1 minute.
    NB: If the DNA will be used for salt-sensitive applications, such as blunt-end ligation and direct sequencing, let the column stand 2-5 minutes after addition of Buffer PE, before centrifuging.

11. Discard the flow-through and centrifuge the QIAquick column for an additional 1 minute at 17,900 x*g* (13,000rpm).
    Important: Residual ethanol from Buffer PE will not be completely removed unless the flow-through is discarded before this additional centrifugation.

12. Place QIAquick column into a clean 1.5ml microcentrifuge tube.

13. To elute DNA, add 50μl of Buffer EB (10mM Tris-HCL, pH 8.5) or water (pH 7.0−8.5) to the center of the QIAquick membrane and centrifuge the column for 1 minute. Alternatively, for increased DNA concentration, add 30μl elution buffer to the center of the QIAquick membrane, let the column stand for 1 minute and then centrifuge for 1 minute.
    Important: Ensure that the elution buffer is dispensed directly onto the QIAquick membrane for complete elution of bound DNA. The average eluate volume is 48μl from 50μl elution buffer volume, and 28μl from 30μl.
    Elution efficiency is dependent on pH. The maximum elution efficiency is achieved between pH 7.0 and 8.5. When using water, make sure that the pH value is within this range, and store DNA at -20°C as DNA may degrade in the absence of a buffering agent. The purified DNA can also be eluted in TE (10mM Tris-HCL, 1mM EDTA, pH 8.0), but the EDTA may inhibit subsequent enzymatic reactions.

14. If the purified DNA is to be analysed on a gel, add 1 volume of Loading Dye to 5 volumes of purified DNA. Mix the solution by pipetting up and down before loading the gel.
    Loading dye contains 3 marker dyes (bromophenol blue, xylene cyanol, and orange G) that facilitate estimation of DNA migration distance and optimisation of agarose gel run time.

8.1.0.12 Preparation of agar plates

(Step 1)

1. Add 8g of Luria Broth and mix with 200ml of Milli-Q water in a flask.

2. Cover the top with tin foil and autoclave solution.

3. When the solution has cooled to 50°C, add 0.02g of ampicillin to make a final concentration of 100μg/ml. (Keep swirling the solution).
4. Once the ampicillin is added pour the solution onto the plates ensuring the neck of the flask is passed through a flam prior to pouring.
5. Allow plates to set in the laminar flow cupboard.
6. Plates can be stored in the fridge prior to use and must be used within one month.

Step 2

1. Place the plates in the oven at 37°C for a short period if removed from the fridge.
2. Add IPTG (isopropylthio-β-galactoside) 100μl of 100mM and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) 20μl of 50mg/ml and spread equally over the plate.
3. Place the plates at 37°C for a minimum of 30 minutes to allow the IPTG and X-Gal to absorb into the agar.

8.1.0.13 Cloning of DNA fragments using the Invitrogen TOPO TA vector technology and chemically competent *Esherichia coli* cells

pGEM®-T and pGEM®-Easy Vector Systems, Promega
Protocol for ligation

1. Briefly centrifuge the pGEM®-T or pGEM®-Easy Vector and Control Insert DNA tubes to collect the contents at the bottom of the tubes.
2. Set up ligation reactions as below. (NB. Protocol optimised to increase yield of positive colonies).

Reaction Component	Standard Reaction
2x Rapid Ligation Buffer, T4 DNA Ligase	5 µl
pGEM®-T or pGEM®-T Easy Vector (50ng)	1 µl
PCR product	2 µl
T4 DNA Ligase (3 Weiss units/µl)	1 µl
Nuclease-free water to a final volume of	10 µl

3. Mix the reactions by pipetting and incubate overnight at 4°C (optimised).

Protocol for transformation

1. Use 2 Luria Broth/ampicillin/IPTG/X-Gal plates for each ligation reaction. Equilibrate the plates to room temperature.
2. Centrifuge the tubes containing the ligation reaction to collect the contents at the bottom. Add 2μl of each ligation reaction to a sterile 1.5ml microcentrifuge tube on ice.
3. Remove the tube(s) of frozen JM109 High Efficiency Competent Cells from storage and place on ice until just thawed (about 5 minutes). Mix the cells by gently flicking the tube. Avoid excess pipetting as the cells are extremely fragile.

4. Carefully transfer 50μl of cells into each tube prepared in Step 2.
5. Gently flick the tubes and place them on ice for 20 minutes.
6. Heat-shock the cells for 45-50 seconds in a water bath at exactly 42°C (do not shake).
7. Immediately return the tubes to ice for 2 minutes.
8. Add 950μl of room-temperature SOC medium to the tubes containing the cells transformed with the ligation reactions.
9. Incubate for 1.5 hours at 37°C with shaking (∼150rpm).
10. To increase the yield of colonies plated, centrifuge at 1,000 x*g* for 10 minutes.
11. Remove the top 500μl of solution, re-suspend the pellet by gently pipetting, plate 250μl of the remaining solution to each duplicate plate (adapted from protocol).

Table 8.5: Thermal cycling for colony PCR

Cycling	Temperature (°C)	Time	No. of cycles
Initial denaturation	95	5 mins	x1
Denaturation	95	30 s	x30
Annealing	55	30 s	
Extension	72	60 s	

8.1.0.14 Plasmid extraction from transformed *Escherichia coli* using the Qiagen QIAprep kit

QIAprep® Miniprep, QIAGEN.
Growth of bacterial cultures prior to purification

1. Pick a single colony from a freshly streaked selective plate and inoculate a culture of 1-5ml LB medium containing ampicillin. Incubate for 12-16 hours at 37°C with vigorous shaking (∼150rpm).
   Growth for more than 16 hours is not recommended since cells begin to lyse and plasmid yields may be reduced. Use a tube with a volume of at least 4 times the volume of the culture.
2. Harvest the bacterial cells by centrifugation at >8000rpm (6800 x*g*) in a conventional, table-top microcentrifuge for 3 minutes at room temperature (15-25°C). The bacterial cells can also be harvested in 15ml centrifuge tubes at 5400 x*g* for 10 minutes at 4°C. Remove all traces of supernatant by inverting the open centrifuge tube until all medium has been drained.

Plasmid DNA purification using the QIAprep Spin Miniprep kit

1. Resuspend pelleted bacteria cells in 250μl of Buffer P1 and transfer to a microcentrifuge tube. Ensure that RNase A has been added to Buffer P1. No cell clumps should be visible after resuspension of the pellet.
   If LyseBlue reagent has been added to Buffer P1, vigorously shake the buffer bottle to ensure LyseBlue particles are completely dissolved. The bacteria should be resuspended completely by vortexing or pipetting up and down until no cell clumps remain.

2. Add 250μl of Buffer P2 and mix thoroughly by inverting the tube 4-6 times.
   Mix by gently inverting the tube. Do not vortex, as this will result in shearing of genomic DNA. If necessary, continue inverting the tube until the solution becomes viscous and slightly clear. Do not allow the lysis reaction to proceed for more than 5 minutes.
   If LyseBlue has been added to Buffer P1 the cell suspension will turn blue after addition of Buffer P2. Mixing should result in a homogeneously coloured suspension. If the suspension contains localised colourless regions or if brownish cell clumps are still visible, continue mixing the solution until a homogeneously colourless suspension is achieved.

3. Add 350μl of Buffer N3 and mix immediately and thoroughly by inverting the tube 4-6 times.
   To avoid localised precipitation, mix the solution thoroughly, immediately after addition of Buffer N3. Large culture volumes ($\geq$5ml) may require inverting up to 10 times. The solution should become cloudy.
   If LyseBlue reagent has been used, the suspension should be mixed until all trace of blue has gone and the suspension is colourless. A homogeneous colourless suspension indicates that the SDS (Sodium Dodecyl Sulphate) has been effectively precipitated.

4. Centrifuge for 10 minutes at 13,000 rpm ($\sim$17,000 xg) in a table-top microcentrifuge.
   A compact white pellet will form. These are destroyed *E. coli* cells.

5. Apply the supernatants from Step 4 to the QIAprep spin column by decanting or pipetting.

6. Centrifuge for 30-60 seconds. Discard the flow-through.

7. Recommended: Wash the QIAprep spin column by adding 0.5ml Buffer PB and centrifuging for 30-60 seconds. Discard the flow-through.
   This step is necessary to remove trace nuclease activity when using *endA*$^+$ strains such as the JM series, HB101 and its derivatives, or any wild-type strain, which have high levels of nuclease activity or high carbohydrate content. Host strains such as XL-1 Blue and DH5α^{TM} do not require this additional wash step.

8. Wash QIAprep spin column by adding 0.75ml of Buffer PE and centrifuging for 30-60 seconds.

9. Discard the flow-through and centrifuge for an additional 1 minute to remove residual wash buffer.
   Important: Residual wash buffer will not be completely removed unless the flow-through is discarded before this additional centrifugation. Residual ethanol from Buffer PE may inhibit subsequent enzymatic reactions.

10. Place the QIAprep column in clean 1.5ml microcentrifuge tube. To elute DNA, add 30μl (adjusted from protocol) of Buffer EB (10mM Tris-HCL, pH 8.5) or water to the centre of each QIAprep spin column, let stand for 1 minute and centrifuge for 1 minute.

8.1.1 qPCR optimising results and gene sequences

8.1.1.1 Primer concentrations for qPCR

Table 8.6: .
Primer concentration for qPCR optimising in *Carcinus maenas* and *Homarus gammarus*.

	Carcinus maenas			***Homarus gammarus***		
Gene	**Forward primer (nM)**	**Reverse primer (nM)**	**C_q**	**Forward primer (nM)**	**Reverse primer (nM)**	**C_q**
actin	50	50	10.75, 10.85	50	50	9.10, 8.97
carcinin/crustin	50	50	8.40, 8.32	300	50	8.06, 8.15
eeF1a	50	300	11.85, 11.94	50	50	9.04, 9.17
gapdh	50	50	13.41, 13.38	300	900	15.98, 16.01
peroxinectin	50	50	16.18, 16.15	50	50	20.81, 21.00
prophenoloxidase	300	50	11.01, 10.95	50	50	12.89, 13.18
tubulin	50	50	14.31, 14.28	300	300	11.30, 11.28

8.1.1.2 qPCR efficiencies

Table 8.7: qPCR efficiencies and standard errors for both *Carcinus maenas* and *Homarus gammarus* based on the standard dilution curves.

	Carcinus maenas		*Homarus gammarus*	
Gene	**PCR efficiency**	**SE**	**PCR efficiency**	**SE**
actin	1.967	0.043	2.081	0.041
carcinin/crustin	1.995	0.021	2.216	0.016
eef1a	1.985	0.029	2.011	0.032
gapdh	2.035	0.046	2.047	0.036
peroxinectin	2.003	0.027	2.094	0.042
prophenoloxidase	2.156	0.148	1.995	0.078
tubulin	2.021	0.073	1.998	0.068

8.1.1.3 Target gene sequences isolated for qPCR

Table 8.8: .
qPCR gene sequences for *Carcinus maenas* and *Homarus gammarus* in the 5' to 3' read direction. (SAS represents Sequence Amplicon Size in base pairs).

Gene	***Carcinus maenas***	SAS
actin	5' TCACCAACTGGGACGACATGGAGAAGATCTGGCATCACACTTTCTACAATGAGCTCCGTGTGGC 3'	64
carcinin	5' ACCTGCCTGAAGCACCACGTGTGCAAGACTGCCGAATATCCTTATTATTAGACATCGCAGACCCG 3'	65
eef1a	5' CTGGATGAGGCAACACAACAAGGTGGCCTTCGAGCTACAGAGCATCAACCCTCAGTGGTCAGAC 3'	64
gapdh	5' TGCGGTGTGAATTTGGAGAAGTACTCTAAGGACATGAAAGTGGTATCCAATGCCTCCTGCACC 3'	63
peroxinectin	5' CAACACAACCGTCTAGCCAAAGACCTTGCCCACTTCAACCCATCTTGGACCGACGAGATCCTCTT 3'	65
prophenoloxidase	5'CTTGCCCGCTCCATTCCCAGAGGCACGGTTTTCTCCATCTTCGTCAAGAGTCATCGCCTCGC 3'	62
tubulin	5' CTCTGCTTCTTTGCGGATAACATCGAGGACAGAGTCGACAAGTTCAGCACCTTCCGTGTAGTG 3'	63
	Homarus gammarus	
actin	5' ACTTGGCTGGACGTGACCTTACTGACTACCTGATGAAGATCCTGACTGAGCGTGGCTACACCTTC 3'	65
crustin	5' CACACCTGCAAGGGTCCAATTTTTTAACTCCTTGCGCAAGATCAGTGTTCCACCCGCTGCCC 3'	62
eef1a	5' GGCTCGTTTTGAGGAAATCAAAAAGGAACTGAATGCCTACGTAAAGAAGGTTGGTTACAATCCTG 3'	65
gapdh	5' GCTAAGGCTGTTGGCAAGGTCATCCCTGAACTGAATGGAAAGCTTACTGGCATGGCTTTCC 3'	61
peroxinectin	5' CACTGAGGGTTGATGCTCTTGAGTTGTCTGGCCACTGTGTTGTGTTGCCTCATCCAGATGGTGTGTATGGCTGTCAG 3'	77
prophenoloxidase	5' TGAAGCTAGTCGAGGCATTGACTTCAACTCCCCCAACCCTGTCATATTACGCCTCACCCATTTGG 3'	65
tubulin	5' GCCCTGAAGACAATCGCATTTTTCGGCTTCCTTGCGGACAACATCAAGGACGGAGTCTACAAGTTCA 3'	67

8.1.2 Protocols for non-molecular processing of samples

8.1.2.1 Preparation of marine agar plates

1. Add 55.1g of marine agar in 1 litre of deionised (R.O.) water and mix thoroughly.
2. Heat with agitation and boil for 1 minute to fully dissolve the marine agar powder.
3. Autoclave at 121°C for 15 minutes.
4. Pour onto sterile plates using flaming between plating.
5. Store at 4°C until use (use within 1 month).

8.1.2.2 Davidsons fixative reagents

SOP 2013, Revision No. 1, Community Reference Laboratory for Crustacean Diseases, CEFAS
Stock solution

1. Filtered seawater = 3340ml
2. 95% Ethanol = 3330ml
3. 36-40% Formaldehyde = 2220ml
4. Glycerin = 1110ml

Working solution

1. Stock solution = 720ml
2. Glacial acetic acid = 80ml

8.1.2.3 20% Seawater Formalin

1. Filtered seawater = 80ml
2. Formaldehyde (40%) = 20ml
3. 0.4g NaH_2PO_4
4. 0.65g Na_2HPO_4 (anhydrous)

8.1.2.4 Marine saline

Preparation

1. All glassware to be baked at 180°C for 5-6 hours to eliminate any pyrogens. Ensure any glass stoppers or open tops are covered with tin foil and wrap any small items completely in tin foil.
2. Autoclave any rubber items instead and autoclave deionised water.

Component ingredients

1. NaChl = 14.61g
2. $CaChl_2$ $6H_20$ = 2.19g or $CaChl_2$(anhydrous) = 1.1g
3. KCl = 0.41g
4. Tris = 2.6g
5. HCl = 18.23g (to adjust pH to 7.4)

Filter sterilise with 0.2μm filters.

8.2 Chapter 3

8.2.1 qPCR optimising

8.2.1.1 Primer titrations for genes isolated from *Homarus gammarus* tissue

Endogenous Reference Genes

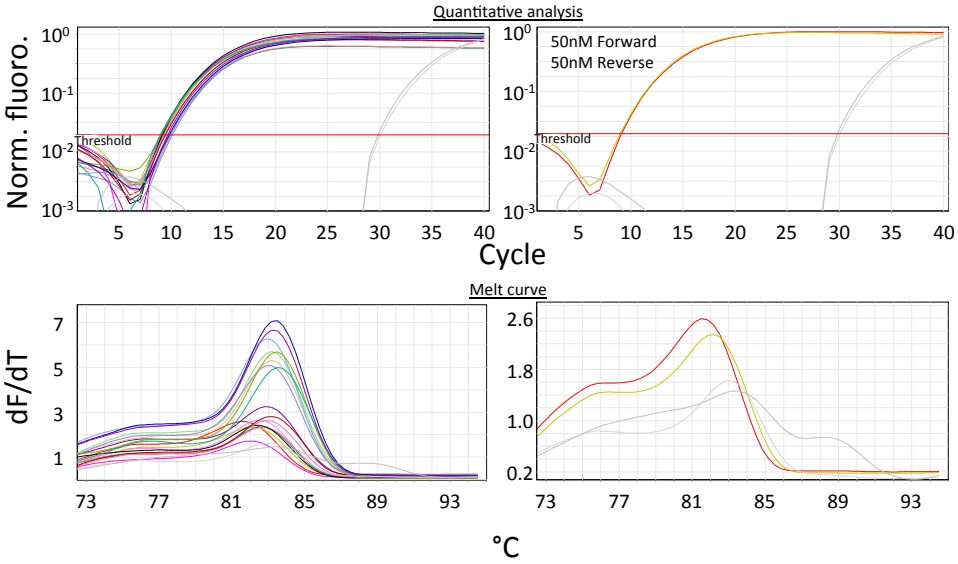


Figure 8.1: *Actin*

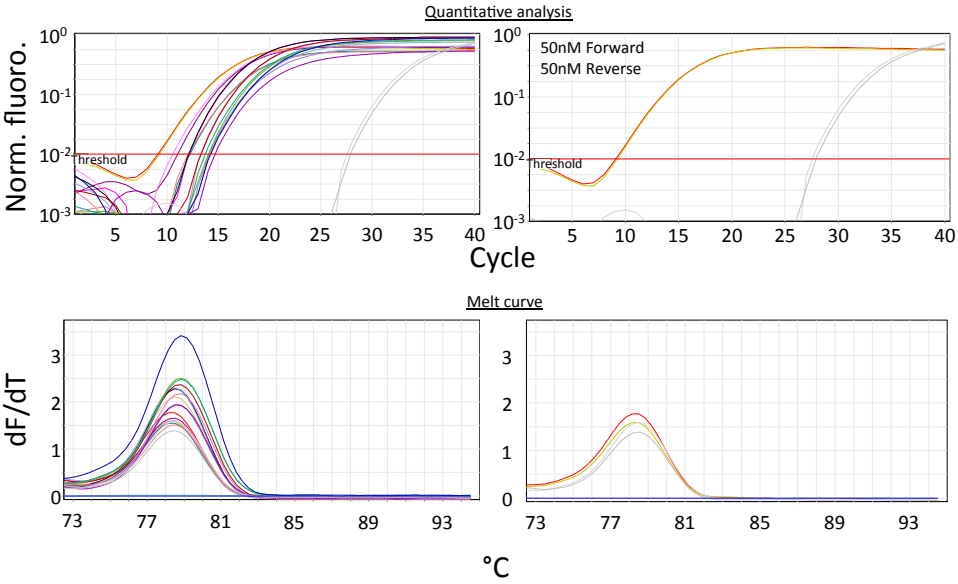


Figure 8.2: *eef1a*

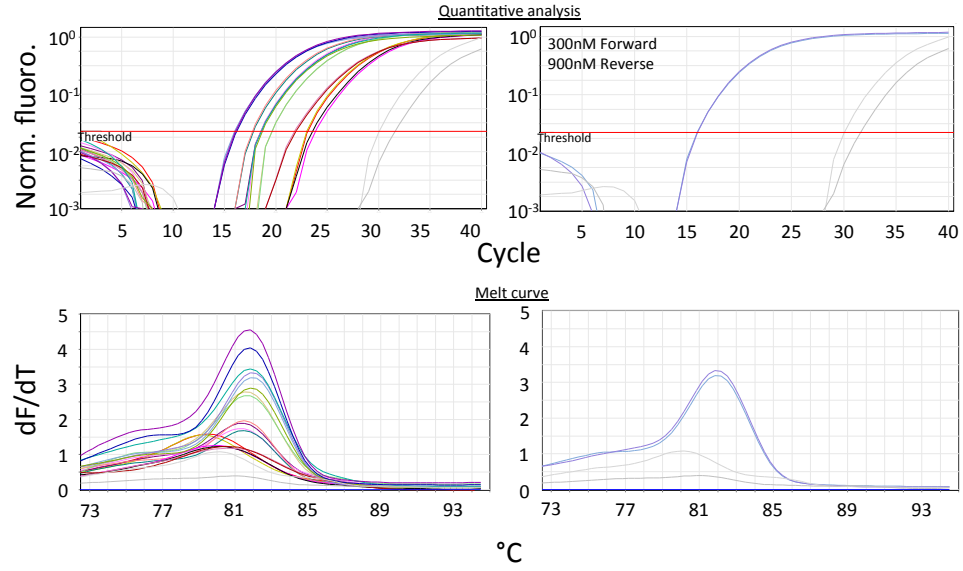


Figure 8.3: *gapdh*

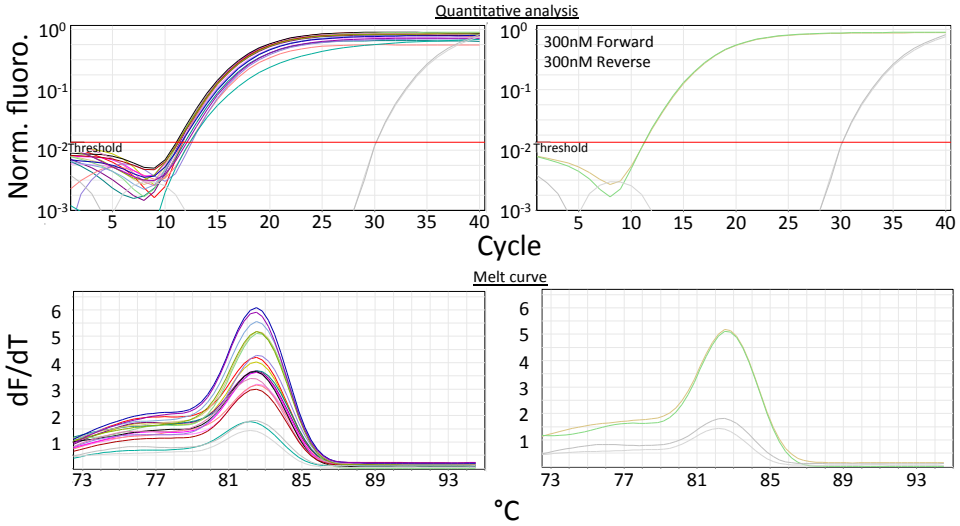


Figure 8.4: *Tubulin*

Immune genes

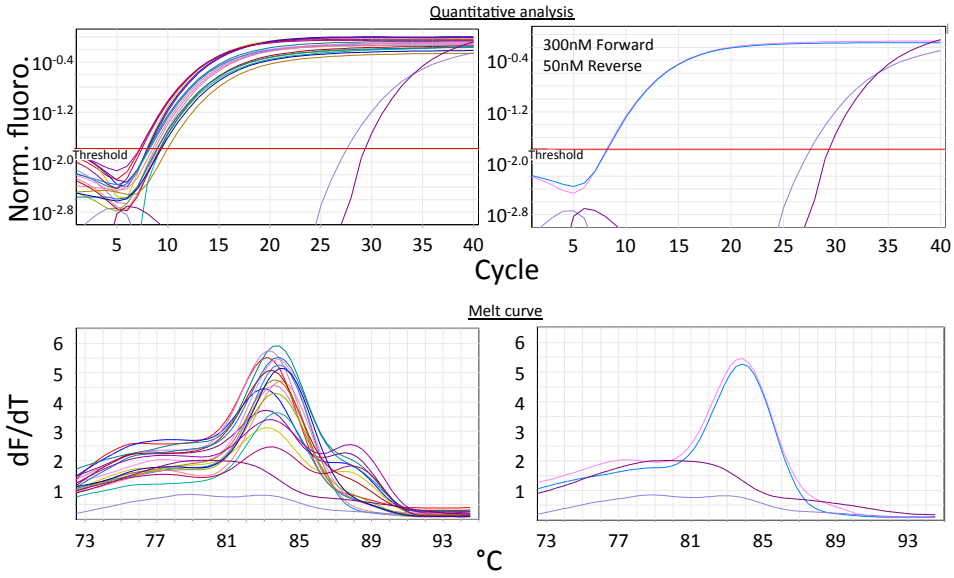


Figure 8.5: *Crustin*

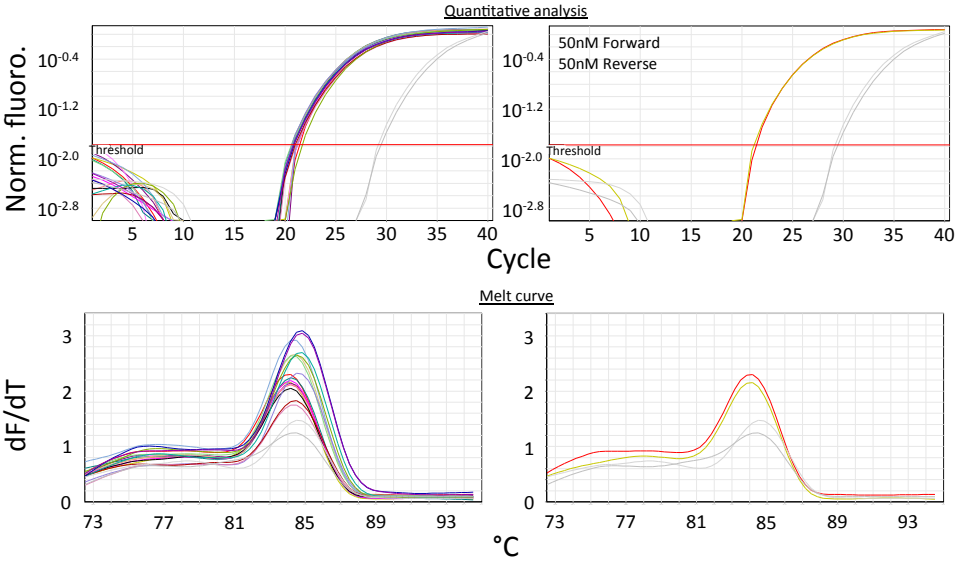


Figure 8.6: *Peroxinectin*

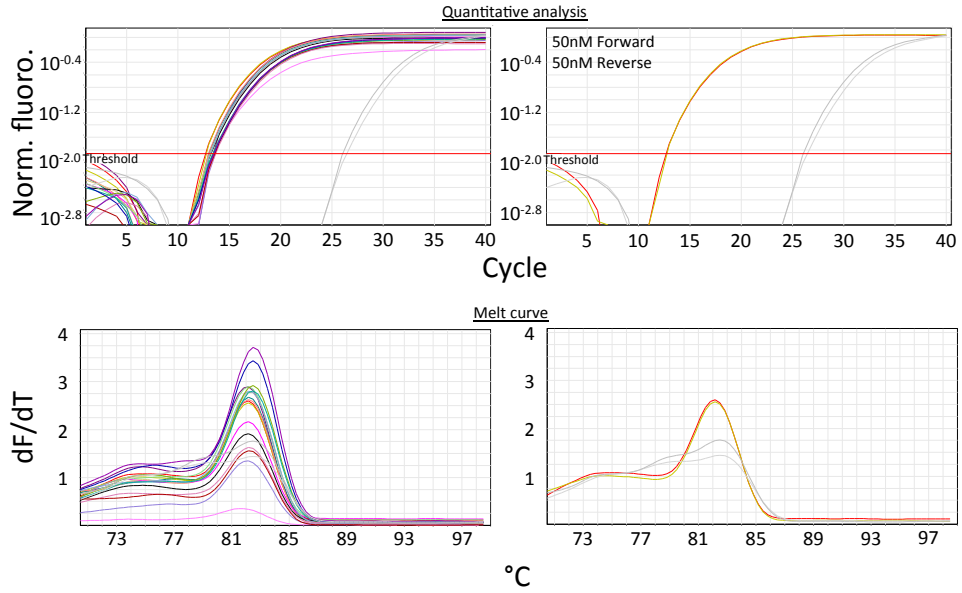


Figure 8.7: *Prophenoloxidase*

Viral genes

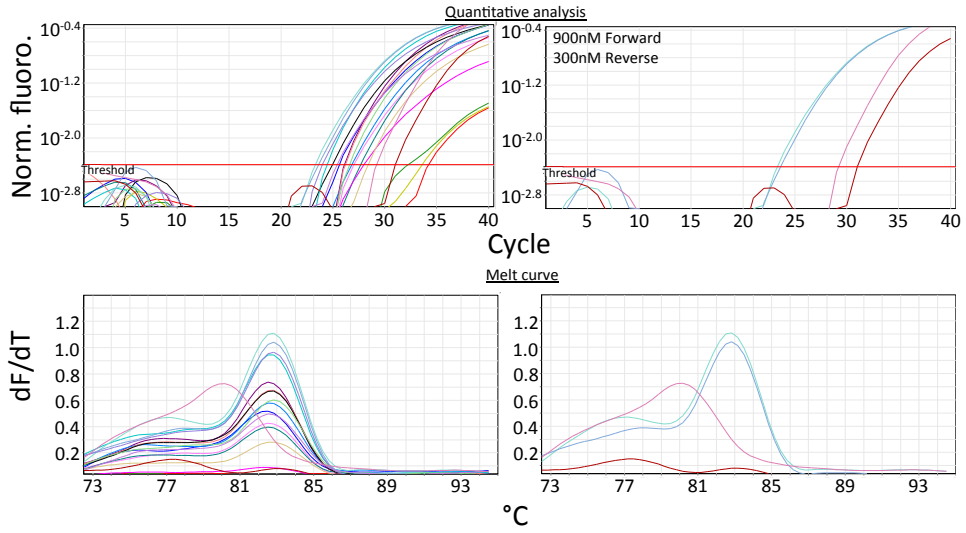


Figure 8.8: *ie1*

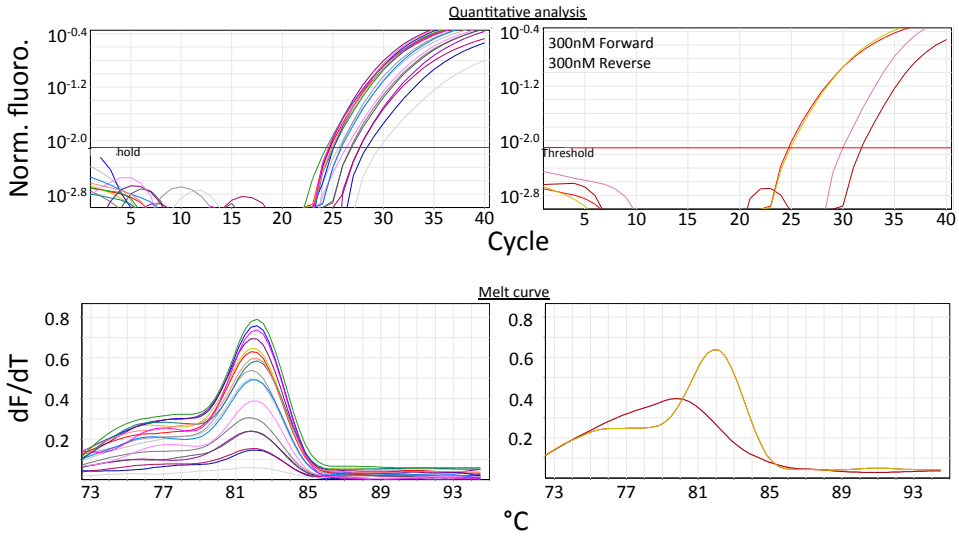


Figure 8.9: *dnapol*

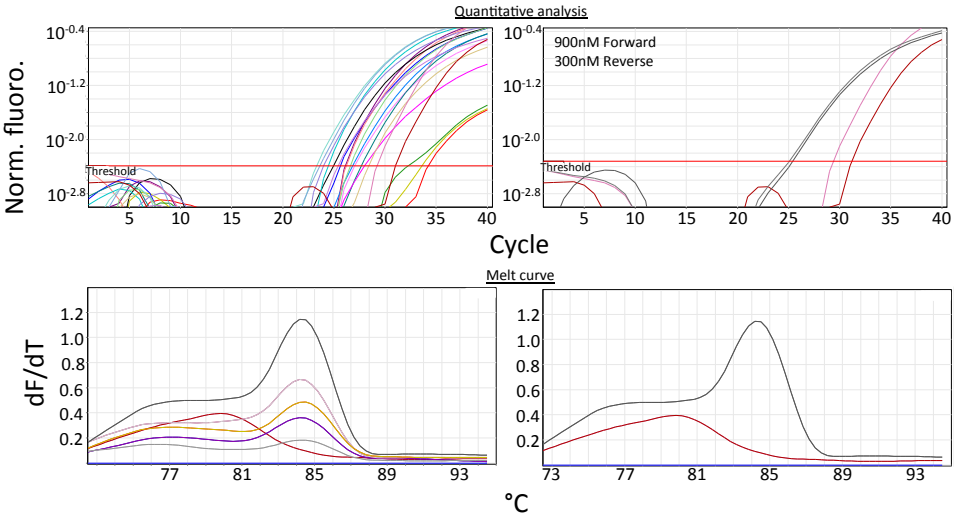


Figure 8.10: *vp28*

8.2.2 Standard curves for the genes isolated from *Homarus gammarus* tissue

Endogenous Reference Genes

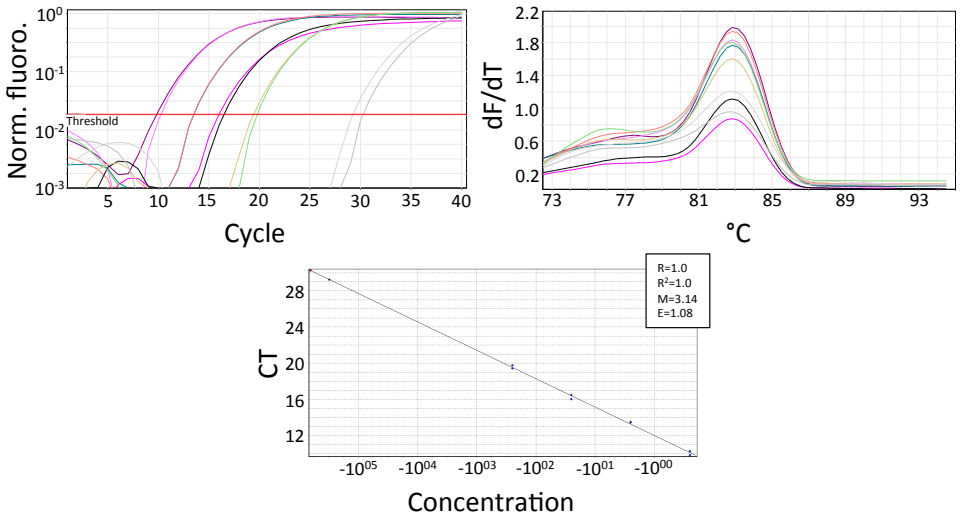


Figure 8.11: *Actin*

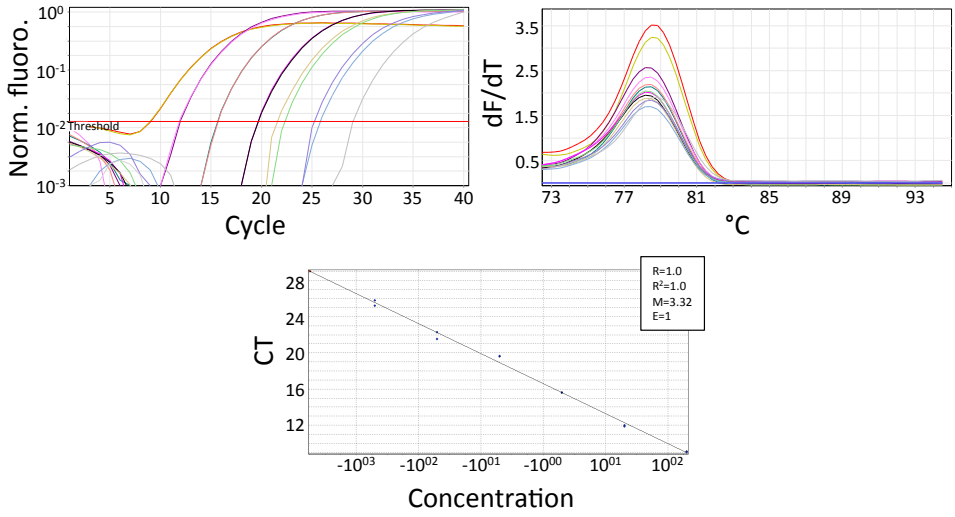


Figure 8.12: *eef1a*

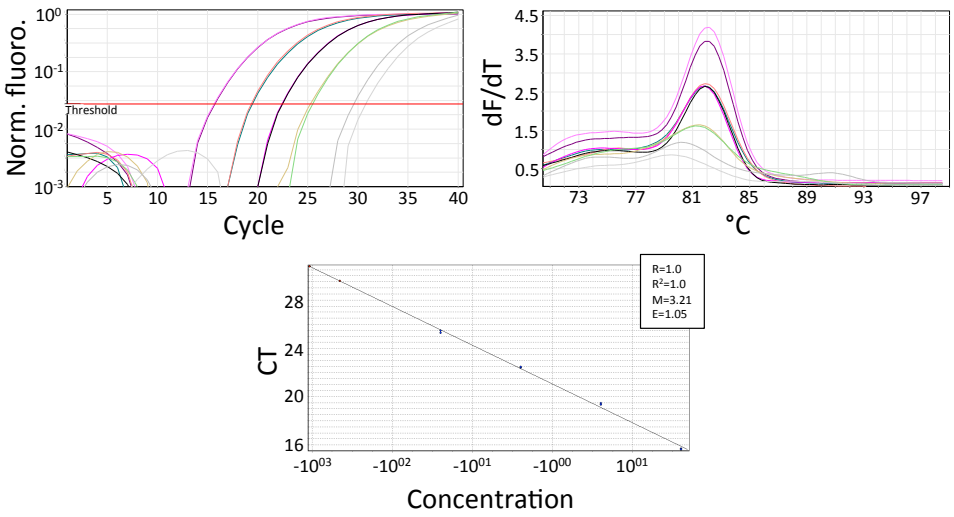


Figure 8.13: *gapdh*

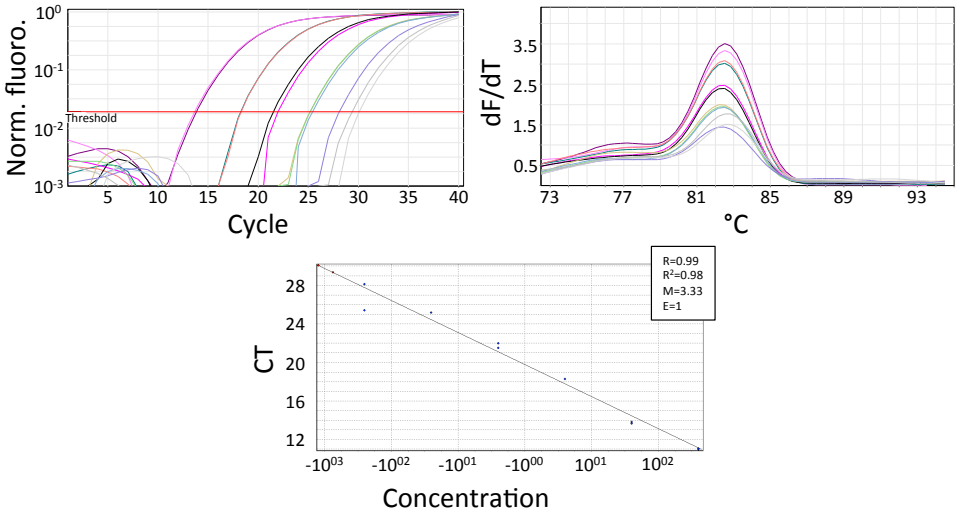


Figure 8.14: *Tubulin*

Immune genes

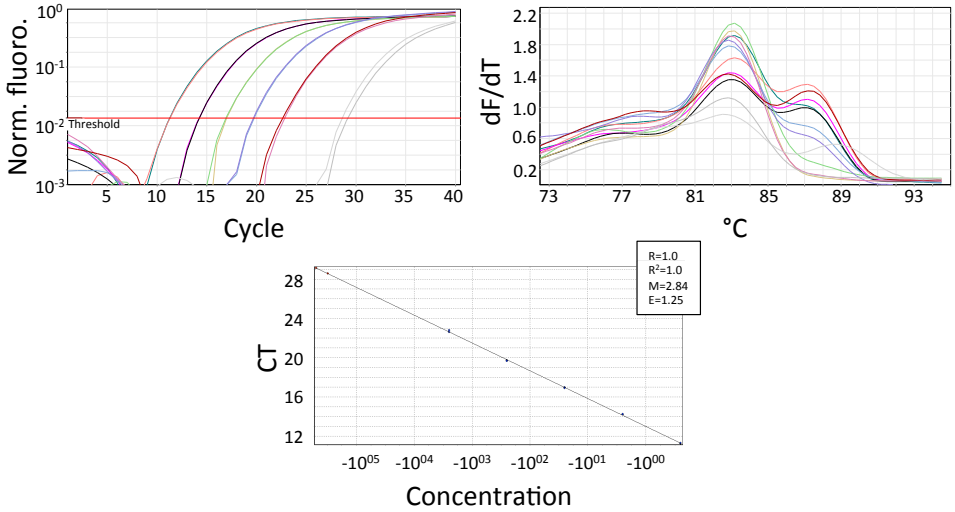


Figure 8.15: *Crustin*

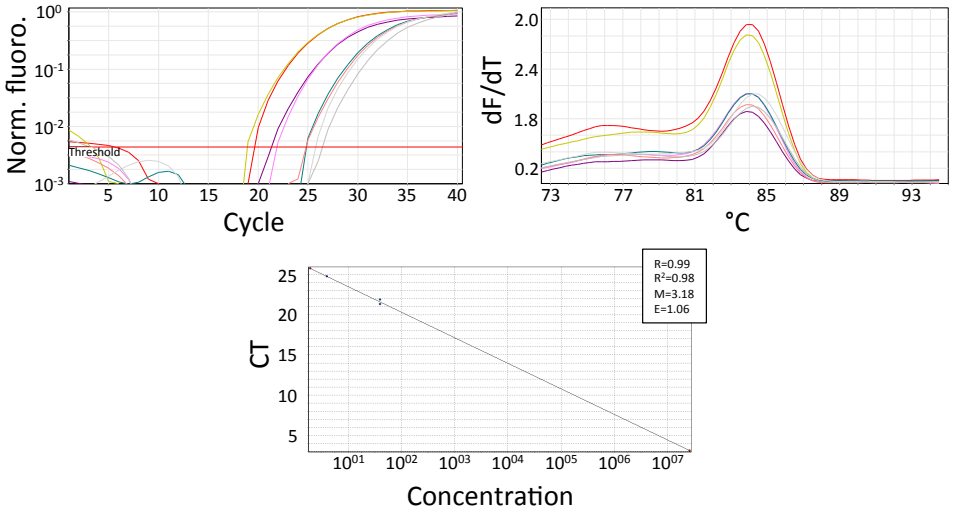


Figure 8.16: *Peroxinectin*

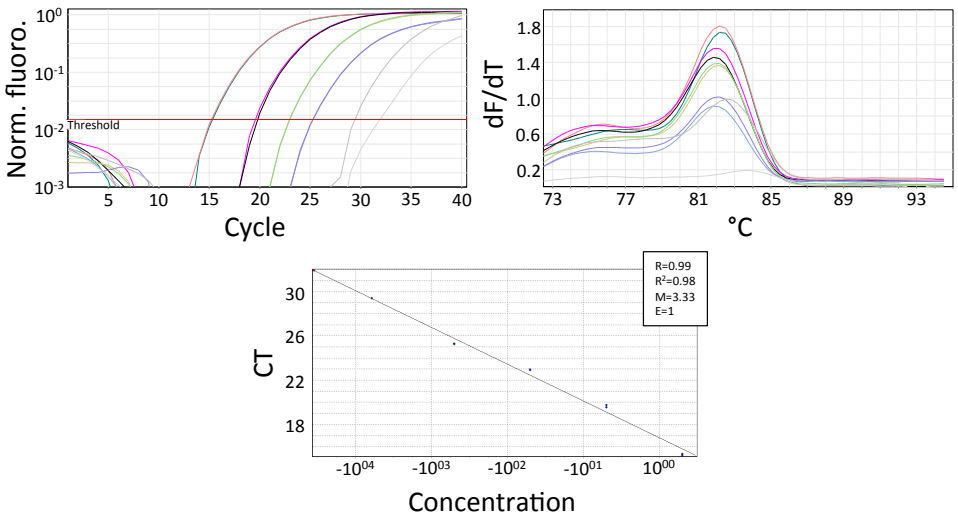


Figure 8.17: *Prophenoloxidase*

Viral genes

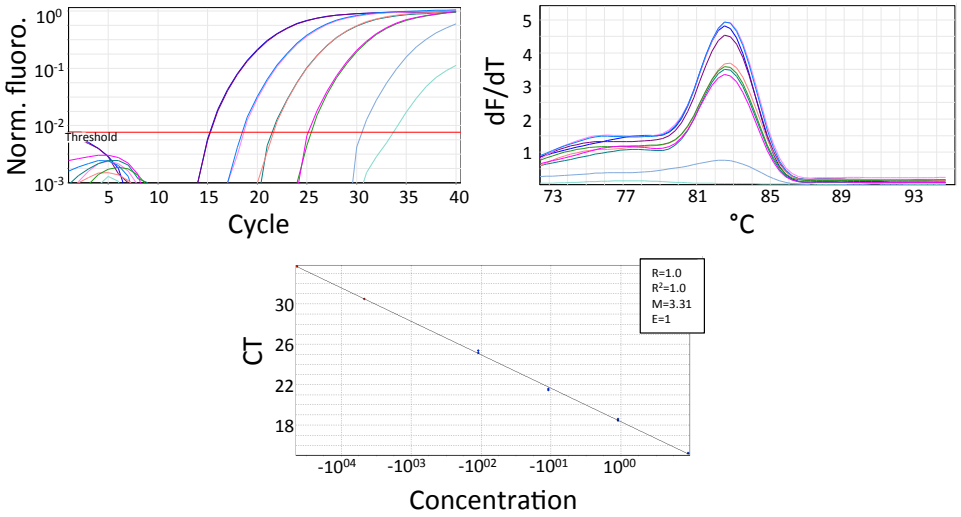


Figure 8.18: *ie1*

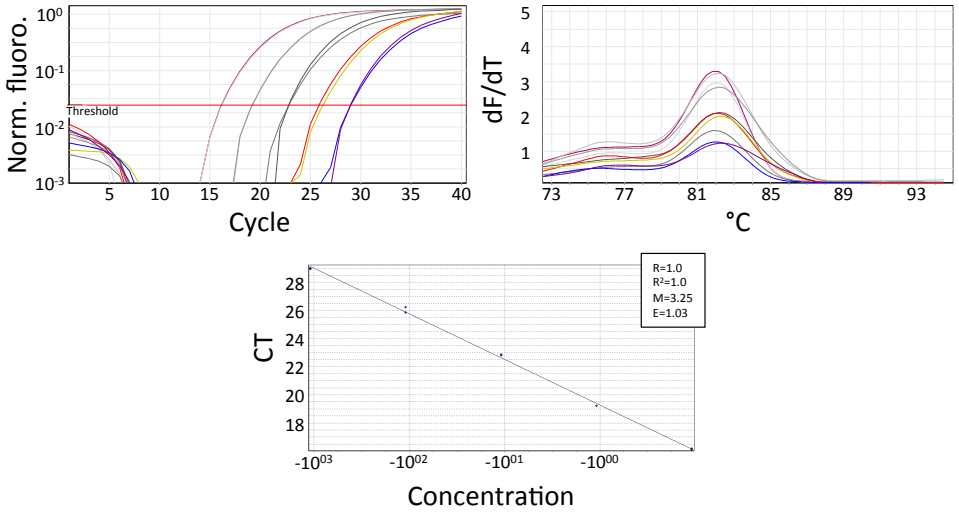


Figure 8.19: *dnapol*

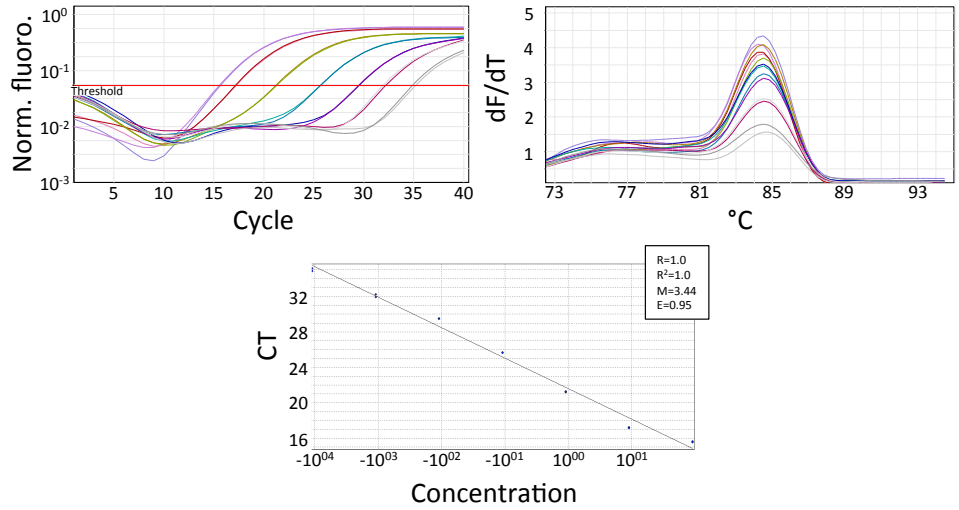


Figure 8.20: *vp28*

8.2.3 Assessment of normal distribution of data from the WSSV-infection challenge in *Homarus gammarus*

Table 8.9: Assessment of the normal distribution of data for *crustin*, *peroxinectin* and *prophenoloxidase* using the Shapiro test. p values highlighted in red denote the distribution of samples that were not normal.

Gene	***Crustin***						
Time	6 hours	12 hours	24 hours	36 hours	48 hours	7 days	10 days
SPF-fed (replicates)	W = 0.90 p >0.05	W = 0.89 p >0.05	W = 0.68 p <0.01	W = 0.91 p >0.05	W = 0.53 p <0.001	W = 0.98 p >0.05	W = 0.86 p >0.05
WSSV-fed (replcates)	W = 0.88 p >0.05	W = 0.49 p <0.001	W = 0.89 p >0.05	W = 0.82 p <0.05	W = 0.97 p >0.05	W = 0.84 p >0.05	W = 0.62 p >0.05
SPF-fed 6 hours – 10 days		W = 0.51 p <0.001		WSSV-fed 6 hours – 10 days		W = 0.62 p <0.001	
Gene	***Peroxinectin***						
Time	6 hours	12 hours	24 hours	36 hours	48 hours	7 days	10 days
SPF-fed (replicates)	W = 0.86 p >0.05	W = 0.58 p <0.001	W = 0.71 p <0.01	W = 0.91 p >0.05	W = 0.85 p >0.05	W = 0.86 p >0.05	W = 0.60 p <0.001
WSSV-fed (replcates)	W = 0.97 p >0.05	W = 0.96 p >0.05	W = 0.87 p >0.05	W = 0.84 p >0.05	W = 0.86 p >0.05	W = 0.95 p >0.05	W = 0.91 p >0.05
SPF-fed 6 hours – 10 days		W = 0.75 p <0.001		WSSV-fed 6 hours – 10 days		W = 0.96 p <0.05	
Gene	***Prophenoloxidase***						
Time	6 hours	12 hours	24 hours	36 hours	48 hours	7 days	10 days
SPF-fed (replicates)	W = 0.87 p >0.05	W = 0.81 p >0.05	W = 0.87 p >0.05	W = 0.90 p >0.05	W = 0.66 p <0.001	W = 0.95 p >0.05	W = 0.90 p >0.05
WSSV-fed (replcates)	W = 0.75 p <0.01	W = 0.95 p >0.05	W = 0.98 p >0.05	W = 0.86 p >0.05	W = 0.81 p <0.05	W = 0.77 p <0.05	W = 0.90 p >0.05
SPF-fed 6 hours – 10 days		W = 0.92 p <0.01		WSSV-fed 6 hours – 10 days		W = 0.67 p <0.001	

8.2.4 Homogeneity of variance of data from the WSSV-infection challenge in *Homarus gammarus*

Table 8.10: Assessment of equal variance across samples for *crustin*, *peroxinectin* and *prophenoloxidase* using the Bartlett test. p values highlighted in red denote the variance of samples that were not homogeneous.

Gene	***Crustin***						
Time	6 hours	12 hours	24 hours	36 hours	48 hours	7 days	10 days
SPF-fed v WSSV-fed	K^2 = 0.74 d.f. = 1 p >0.05	K^2 = 13.25 d.f. = 1 p <0.001	K^2 = 1.83 d.f. = 1 p >0.05	K^2 = 0.15 d.f. = 1 p >0.05	K^2 = 26.30 d.f. = 1 p <0.001	K^2 = 0.02 d.f. = 1 p >0.05	K^2 = 12.80 d.f. = 1 p <0.001
SPF-fed 6 hours – 10 days		K^2 = 84.84 d.f. = 6 p <0.001		WSSV-fed 6 hours – 10 days		K^2 = 69.77 d.f. = 6 p <0.001	
Gene	***Peroxinectin***						
Time	6 hours	12 hours	24 hours	36 hours	48 hours	7 days	10 days
SPF-fed v WSSV-fed	K^2 = 3.22 d.f. = 1 p >0.05	K^2 = 7.90 d.f. = 1 p <0.01	K^2 = 10.35 d.f. = 1 p <0.01	K^2 = 0.24 d.f. = 1 p >0.05	K^2 = 8.20 d.f. = 1 p <0.01	K^2 = 4.27 d.f. = 1 p <0.05	K^2 = 1.27 d.f. = 1 p >0.05
SPF-fed 6 hours – 10 days		K^2 = 47.73 d.f. = 6 p <0.001		WSSV-fed 6 hours – 10 days		K^2 = 15.21 d.f. = 6 p <0.05	
Gene	***Prophenoloxidase***						
Time	6 hours	12 hours	24 hours	36 hours	48 hours	7 days	10 days
SPF-fed v WSSV-fed	K^2 = 2.53 d.f. = 1 p >0.05	K^2 = 2.76 d.f. = 1 p >0.05	K^2 = 0.07 d.f. = 1 p >0.05	K^2 = 0.36 d.f. = 1 p >0.05	K^2 = 11.76 d.f. = 1 p <0.001	K^2 = 0.01 d.f. = 1 p >0.05	K^2 = 6.74 d.f. = 1 p <0.01
SPF-fed 6 hours – 10 days		K^2 = 11.92 d.f. = 6 p >0.05		WSSV-fed 6 hours – 10 days		K^2 = 52.67 d.f. = 6 p <0.001	

8.2.5 Raw CNRQ and RQI data for all immune and endogenous reference genes from the WSSV-infection challenge in *Homarus gammarus*

Table 8.11: CNRQ and RQI values at 6 hours

6 h			LOG10 CNRQ – ERG's				LOG10 CNRQ - GOI		
Treatment	**Sample**	**RQI**	***actin***	***eef1a***	***gapdh***	***tubulin***	***crustin***	***peroxinectin***	***prophenoloxidase***
SPF-fed	11	10	2.44E^{-02}	2.82E^{-01}	9.63E^{-02}	-1.21E^{-01}	-8.71E^{-01}	-3.63E^{-01}	-1.77E^{00}
	13	10	2.46E^{-01}	2.78E^{-01}	3.69E^{-03}	-2.49E^{-01}	-4.56E^{-01}	-4.28E^{-01}	-5.87E^{-01}
	14	10	2.83E^{-01}	2.41E^{-01}	-3.69E^{-02}	-2.46E^{-01}	-7.99E^{-01}	-5.86E^{-01}	-2.88E^{-02}
	15	10	2.51E^{-01}	2.90E^{-01}	-7.29E^{-02}	-1.78E^{-01}	-1.73E^{-01}	-4.84E^{-01}	-2.00E^{-02}
	17	10	2.05E^{-01}	5.60E^{-01}	-8.43E^{-02}	-1.20E^{-01}	-8.13E^{-01}	-3.88E^{-01}	-2.17E^{-01}
	18	10	2.11E^{-01}	3.83E^{-01}	1.91E^{-02}	-2.30E^{-01}	-7.02E^{-01}	-5.91E^{-01}	1.08E^{-02}
	19	10	1.69E^{-01}	-4.32E^{00}	2.90E^{-02}	-1.97E^{-01}	-5.27E^{-01}	-5.81E^{-01}	-3.85E^{-01}
	20	10	3.24E^{-01}	7.76E^{-01}	-6.15E^{-01}	2.91E^{-01}	7.74E^{-02}	/	1.77E^{00}
WSSV-fed	1	10	-1.32E^{-02}	1.72E^{-01}	1.39E^{-01}	-1.25E^{-01}	-5.49E^{-01}	-3.72E^{-01}	-1.32E^{-01}
	3	10	1.19E^{-01}	1.04E^{-02}	5.40E^{-02}	-1.73E^{-01}	-3.36E^{-01}	-4.95E^{-01}	-2.24E^{-01}
	4	10	4.56E^{-02}	1.85E^{-01}	6.88E^{-04}	-4.63E^{-02}	-5.59E^{-01}	-1.77E^{-01}	-3.72E^{00}
	5	10	8.69E^{-02}	4.43E^{-01}	2.57E^{-02}	-1.13E^{-01}	-6.11E^{-01}	-5.97E^{-01}	-8.06E^{-02}
	6	10	1.52E^{-01}	3.50E^{-01}	1.72E^{-01}	-3.24E^{-01}	-3.64E^{-01}	-7.56E^{-01}	-1.14E^{-01}
	7	10	-7.89E^{-02}	2.82E^{-01}	2.85E^{-01}	-2.06E^{-01}	-7.36E^{-01}	-4.80E^{-01}	-3.88E^{00}
	8	10	1.47E^{-01}	2.76E^{-01}	2.58E^{-01}	-4.05E^{-01}	-6.82E^{-01}	-8.67E^{-01}	1.95E^{-01}
	9	10	2.57E^{-02}	4.89E^{-01}	-2.32E^{-02}	-2.51E^{-03}	-4.64E^{-01}	-5.11E^{-01}	2.50E^{-01}
	10	10	3.80E^{-02}	9.20E^{-01}	-8.48E^{-01}	8.10E^{-01}	7.51E^{-02}	/	1.39E^{00}

Table 8.12: CNRQ and RQI values at 12 hours

12 h			LOG10 CNRQ – ERG's				LOG10 CNRQ - GOI		
Treatment	**Sample**	**RQI**	***actin***	***eef1a***	***gapdh***	***tubulin***	***crustin***	***peroxinectin***	***prophenoloxidase***
SPF-fed	21	10	1.02E^{-01}	3.76E^{-01}	-8.77E^{-02}	-1.39E^{-02}	-7.99E^{-01}	-2.57E^{-01}	5.32E^{-02}
	22	10	2.24E^{-02}	3.41E^{-01}	6.35E^{-02}	-8.59E^{-02}	-7.01E^{-01}	-2.71E^{-01}	6.99E^{-02}
	23	10	2.76E^{-01}	-2.96E^{-03}	-1.50E^{-01}	-1.26E^{-01}	-8.54E^{-01}	-4.28E^{-01}	/
	24	10	1.56E^{-01}	3.23E^{-01}	5.07E^{-02}	-2.06E^{-01}	-5.15E^{-01}	-3.83E^{-01}	-9.34E^{-03}
	27	9.3	3.30E^{-01}	1.53E^{-01}	-1.71E^{-01}	-1.59E^{-01}	-8.77E^{-01}	-2.86E^{-01}	-3.94E^{-01}
	29	10	1.07E^{-02}	2.07E^{-01}	-4.80E^{-01}	4.70E^{-01}	/	/	2.12E^{00}
	30	9.8	9.18E^{-02}	4.82E^{-01}	-5.48E^{-01}	4.56E^{-01}	-4.27E^{-01}	1.60E^{00}	6.86E^{-01}
WSSV-fed	11	10	4.47E^{-02}	3.30E^{-01}	-5.08E^{-02}	6.08E^{-03}	-2.15E^{-01}	-6.01E^{-01}	-1.78E^{-01}
	12	10	7.19E^{-02}	4.58E^{-01}	-8.71E^{-01}	7.99E^{-01}	5.33E^{-02}	/	1.28E^{00}
	13	10	-9.71E^{-02}	1.26E^{-01}	1.31E^{-01}	-3.35E^{-02}	-2.01E^{-01}	-3.25E^{-01}	5.66E^{-01}
	14	10	1.52E^{-01}	2.68E^{-01}	-5.30E^{-02}	-9.88E^{-02}	-3.70E^{-02}	-3.50E^{-01}	4.12E^{-01}
	15	10	3.55E^{-01}	6.34E^{-01}	-1.08E^{00}	7.25E^{-01}	-4.42E^{00}	1.32E^{-01}	1.43E^{00}
	16	10	-2.66E^{-02}	2.12E^{-01}	-2.61E^{-02}	5.28E^{-02}	4.67E^{-02}	1.01E^{-01}	7.44E^{-01}
	17	10	-2.33E^{-02}	2.70E^{-01}	1.07E^{-01}	-8.34E^{-02}	1.79E^{-01}	-1.15E^{-01}	4.28E^{-01}
	18	10	2.20E^{-01}	3.00E^{-01}	-2.24E^{-01}	4.24E^{-03}	3.72E^{-01}	-2.38E^{-01}	3.88E^{-01}
	19	10	-7.54E^{-02}	2.48E^{-01}	8.75E^{-02}	-1.21E^{-02}	7.04E^{-02}	-2.15E^{-01}	5.48E^{-01}
	20	10	6.04E^{-02}	2.10E^{-01}	-1.40E^{-02}	-4.64E^{-02}	2.34E^{-01}	1.57E^{-02}	8.84E^{-01}

Table 8.13: CNRQ and RQI values at 24 hours

24 h			LOG10 CNRQ – ERG's				LOG10 CNRQ - GOI		
Treatment	**Sample**	**RQI**	***actin***	***eef1a***	***gapdh***	***tubulin***	***crustin***	***peroxinectin***	***prophenoloxidase***
SPF-fed	31	9.7	$6.44E^{-02}$	$-5.14E^{-02}$	$-3.14E^{-01}$	$2.49E^{-01}$	$-5.19E^{-01}$	$-4.44E^{-01}$	/
	32	9.7	$4.90E^{-02}$	5.61E-01	$-3.53E^{-01}$	$3.04E^{-01}$	-4.84E-01	$7.07E^{-01}$	$1.64E^{00}$
	33	7.1	$-3.51E^{-01}$	/	$-4.46E^{-01}$	$7.97E^{-01}$	$-1.94E^{00}$	$9.69E^{-01}$	$1.40E^{00}$
	34	10	$3.50E^{-02}$	4.58E-01	$2.08E^{-01}$	$-2.43E^{-01}$	$-4.71E^{-02}$	$-1.06E^{-01}$	$2.92E^{-01}$
	35	10	$3.68E^{-01}$	6.55E-01	$-9.12E^{-01}$	$5.43E^{-01}$	$-1.87E^{-01}$	$6.33E^{-01}$	$1.66E^{00}$
	37	10	$1.40E^{-01}$	4.26E-01	$-1.87E^{-02}$	$-1.21E^{-01}$	$-4.42E^{-01}$	$-5.34E^{00}$	$2.70E^{-01}$
	38	10	$2.44E^{-01}$	3.68E-01	$-1.20E^{-02}$	$-2.31E^{-01}$	$-5.06E^{-01}$	$-1.91E^{-01}$	/
	39	9.2	$-2.76E^{-01}$	-9.67E-01	$-6.38E^{-01}$	$9.13E^{-01}$	/	$-1.13E^{00}$	$1.99E^{00}$
	40	9.5	$3.36E^{-01}$	5.90E-01	$-6.84E^{-01}$	$3.48E^{-01}$	$-4.83E^{-01}$	$8.70E^{-01}$	$1.20E^{00}$
WSSV-fed	22	10	$-1.05E^{-01}$	5.72E-01	$9.68E^{-02}$	$8.54E^{-03}$	$2.44E^{-01}$	$-2.03E^{-01}$	$6.71E^{-01}$
	23	10	$-5.84E^{-02}$	4.66E-01	$8.52E^{-02}$	$-2.67E^{-02}$	$2.26E^{-01}$	$-4.13E^{-01}$	$9.63E^{-01}$
	24	10	$2.33E^{-01}$	7.35E-01	$-6.56E^{-01}$	$4.22E^{-01}$	$8.70E^{-01}$	$4.48E^{-01}$	$1.53E^{00}$
	25	10	$8.20E^{-02}$	4.41E-01	$6.63E^{-02}$	$-1.48E^{-01}$	$-6.78E^{-02}$	$-5.19E^{-01}$	$-2.15E^{-01}$
	27	8.5	$-4.53E^{-02}$	6.67E-01	$3.07E^{-01}$	$-2.62E^{-01}$	$4.17E^{-01}$	$-3.70E^{-01}$	$3.61E^{-01}$
	29	10	$4.06E^{-03}$	4.66E-01	$1.75E^{-01}$	$-1.79E^{-01}$	$2.44E^{-01}$	$-8.04E^{-02}$	$2.78E^{-01}$

Table 8.14: CNRQ and RQI values at 36 hours

36 h			LOG10 CNRQ – ERG's				LOG10 CNRQ - GOI		
Treatment	**Sample**	**RQI**	***actin***	***eef1a***	***gapdh***	***tubulin***	***crustin***	***peroxinectin***	***prophenoloxidase***
SPF-fed	42	9.4	$1.49E^{-01}$	$6.58E^{-01}$	$-1.03E^{-01}$	$-4.59E^{-02}$	$-7.64E^{-01}$	$-5.50E^{-01}$	/
	45	10	$-2.98E^{-02}$	$6.39E^{-01}$	$1.96E^{-01}$	$-1.66E^{-01}$	$-4.10E^{-01}$	$-7.48E^{-02}$	$-2.19E^{-01}$
	46	10	$4.44E^{-01}$	$6.46E^{-01}$	$-4.39E^{-01}$	$-4.30E^{-03}$	$-7.20E^{-01}$	$-1.18E^{00}$	$-5.40E^{-01}$
	47	10	$-1.36E^{-01}$	$8.05E^{-01}$	$2.08E^{-01}$	$-7.24E^{-02}$	$-4.38E^{-01}$	/	$6.72E^{-01}$
	48	7.3	$-3.33E^{-02}$	$6.35E^{-01}$	$-7.76E^{-01}$	$8.10E^{-01}$	$-3.12E^{-02}$	$-4.69E^{-01}$	$7.90E^{-01}$
	49	8.7	$1.29E^{-01}$	$4.80E^{-01}$	$1.09E^{-01}$	$-2.37E^{-01}$	$-2.29E^{-01}$	$-3.79E^{-01}$	$-2.05E^{-01}$
	50	8.5	$9.17E^{-02}$	$5.56E^{-01}$	$2.60E^{-01}$	$-3.52E^{-01}$	$-4.85E^{-02}$	$-5.98E^{-01}$	$9.45E^{-02}$
WSSV-fed	31	10	$1.06E^{-03}$	$6.79E^{-01}$	$2.89E^{-01}$	$-2.90E^{-01}$	$3.71E^{-01}$	$2.08E^{-01}$	$6.48E^{-01}$
	32	10	$5.01E^{-02}$	$4.97E^{-01}$	$1.22E^{-01}$	$-1.72E^{-01}$	$8.06E^{-01}$	$3.59E^{-01}$	$1.06E^{00}$
	33	10	$-5.85E^{-02}$	$4.82E^{-01}$	$2.39E^{-01}$	$-1.81E^{-01}$	$2.40E^{-01}$	$-4.49E^{-01}$	$1.83E^{-01}$
	35	10	$9.41E^{-01}$	$9.92E^{-01}$	$7.54E^{-01}$	$-1.70E^{00}$	$1.17E^{00}$	$2.04E^{-01}$	$1.96E^{00}$
	36	10	$1.76E^{-02}$	$5.97E^{-01}$	$1.50E^{-01}$	$-1.68E^{-01}$	$4.02E^{-01}$	$-6.96E^{-02}$	$2.47E^{-01}$
	37	10	$4.16E^{-01}$	$9.54E^{-01}$	$-8.82E^{-01}$	$4.66E^{-01}$	$1.79E^{-01}$	$1.35E^{00}$	$2.26E^{00}$
	38	10	$-1.48E^{-01}$	$6.74E^{-01}$	$3.58E^{-01}$	$-2.10E^{-01}$	$2.74E^{-01}$	$1.72E^{-01}$	$6.71E^{-01}$
	39	10	$1.98E^{-02}$	$5.69E^{-01}$	$2.72E^{-01}$	$-2.92E^{-01}$	$1.55E^{-01}$	$-2.64E^{-02}$	$4.59E^{-01}$
	40	10	$-2.74E^{-03}$	$5.02E^{-01}$	$1.15E^{-01}$	$-1.12E^{-01}$	$4.58E^{-01}$	$-1.44E^{-01}$	$7.97E^{-01}$

Table 8.15: CNRQ and RQI values at 48 hours

48 h			LOG10 CNRQ – ERG's				LOG10 CNRQ - GOI		
Treatment	**Sample**	**RQI**	***actin***	***eef1a***	***gapdh***	***tubulin***	***crustin***	***peroxinectin***	***prophenoloxidase***
SPF-fed	52	10	$1.57E^{-01}$	$6.91E^{-01}$	$1.41E^{-01}$	$-2.98E^{-01}$	$-8.75E^{00}$	$-8.07E^{-02}$	$9.77E^{-02}$
	53	10	$-4.06E^{-01}$	$5.87E^{-01}$	$-1.18E^{-01}$	$5.24E^{-01}$	$-2.39E^{-01}$	$1.52E^{00}$	/
	54	10	$1.56E^{-01}$	$8.45E^{-01}$	$-1.82E^{-01}$	$2.57E^{-02}$	$3.86E^{-01}$	$-1.00E^{-01}$	$4.53E^{-01}$
	55	9.1	$5.12E^{-01}$	$5.10E^{-01}$	$-3.35E^{-01}$	$-1.77E^{-01}$	$-7.71E^{-01}$	$-7.38E^{-01}$	$-2.91E^{00}$
	56	7.6	$4.47E^{-01}$	$8.26E^{-01}$	$-6.09E^{-01}$	$1.62E^{-01}$	$5.98E^{-01}$	$1.04E^{00}$	$7.54E^{-01}$
	57	10	$7.52E^{-01}$	$7.39E^{-01}$	$-7.13E^{-01}$	$-3.95E^{-02}$	$1.37E^{-02}$	$-2.18E^{-01}$	$1.84E^{-01}$
	58	10	$2.73E^{-01}$	$7.19E^{-01}$	$-6.14E^{-02}$	$-2.11E^{-01}$	$7.03E^{-01}$	$-1.00E^{-01}$	$-3.41E^{-02}$
	59	9.2	$2.68E^{-01}$	$9.58E^{-01}$	$-1.15E^{-01}$	$-1.52E^{-01}$	$4.36E^{-01}$	$4.76E^{-02}$	$-1.17E^{-01}$
	60	9.2	$5.46E^{-01}$	$2.33E^{-01}$	$-8.82E^{-01}$	$3.36E^{-01}$	$-1.21E^{-01}$	$1.61E^{00}$	$2.85E^{-01}$
WSSV-fed	41	10	$8.60E^{-02}$	$6.81E^{-01}$	$1.17E^{-01}$	$-2.03E^{-01}$	$2.98E^{-01}$	$2.98E^{-01}$	$6.60E^{-01}$
	43	10	$3.46E^{-03}$	$1.45E^{-01}$	$3.23E^{-01}$	$-3.27E^{-01}$	$5.31E^{-01}$	$-2.46E^{-02}$	$7.96E^{-01}$
	45	10	$7.16E^{-02}$	$6.70E^{-01}$	$1.62E^{-01}$	$-2.34E^{-01}$	$3.86E^{-01}$	$3.63E^{-01}$	$8.71E^{-01}$
	46	10	$2.69E^{-01}$	$1.02E^{00}$	$2.06E^{-01}$	$-4.75E^{-01}$	$5.22E^{-01}$	$1.77E^{-01}$	$7.98E^{-01}$
	47	9.5	$2.29E^{-02}$	$7.72E^{-01}$	$-1.91E^{-02}$	$-3.73E^{-03}$	$2.19E^{-01}$	$3.39E^{-01}$	$9.95E^{-02}$
	48	9.7	$4.91E^{-02}$	$4.63E^{-01}$	$1.18E^{-01}$	$-1.67E^{-01}$	$4.05E^{-01}$	$1.92E^{-01}$	$6.35E^{-01}$
	49	10	$3.81E^{-01}$	$1.16E^{00}$	$-3.16E^{-01}$	$-6.48E^{-02}$	$7.83E^{-01}$	$8.36E^{-01}$	$7.41E^{-01}$
	50	10	$-2.98E^{-02}$	$7.81E^{-01}$	$1.64E^{-01}$	$-1.35E^{-01}$	$1.22E^{-01}$	$2.38E^{-01}$	$5.45E^{-01}$

Table 8.16: CNRQ and RQI values at 7 days

7 d			LOG10 CNRQ – ERG's				LOG10 CNRQ - GOI		
Treatment	**Sample**	**RQI**	***actin***	***eef1a***	***gapdh***	***tubulin***	***crustin***	***peroxinectin***	***prophenoloxidase***
SPF-fed	105	10	$-1.62E^{-01}$	$1.32E^{-01}$	$5.09E^{-03}$	$1.57E^{-01}$	$-1.88E^{-01}$	$-4.69E^{-01}$	$1.92E^{-01}$
	106	10	$2.53E^{-01}$	$2.27E^{-01}$	$-4.02E^{-01}$	$1.49E^{-01}$	$-3.96E^{-01}$	$1.19E^{-01}$	$-3.68E^{-01}$
	107	10	$-1.83E^{-01}$	$3.19E^{-01}$	$2.12E^{-01}$	$-2.83E^{-02}$	$1.32E^{-01}$	$-6.22E^{-02}$	$2.79E^{-01}$
	108	10	$4.13E^{-01}$	$4.94E^{-01}$	$-1.97E^{-01}$	$-2.16E^{-01}$	$2.65E^{-01}$	$-7.01E^{-02}$	$-1.58E^{-01}$
	109	9.9	$2.19E^{-01}$	$1.90E^{-01}$	$-3.87E^{-01}$	$1.67E^{-01}$	$-5.95E^{-02}$	$1.54E^{-01}$	$-8.41E^{-02}$
WSSV-fed	91	10	$-2.85E^{-01}$	$3.24E^{-01}$	$2.76E^{-01}$	$9.40E^{-03}$	$3.49E^{-01}$	$-7.09E^{-03}$	$4.49E^{-01}$
	92	10	$1.51E^{-01}$	$1.21E^{-01}$	$-2.81E^{-01}$	$1.31E^{-01}$	$-2.19E^{-01}$	$-7.18E^{-02}$	$-1.26E^{-01}$
	94	10	$-6.13E^{-02}$	$1.29E^{-01}$	$-8.61E^{-03}$	$7.00E^{-02}$	$1.82E^{-01}$	$-1.89E^{-01}$	$5.26E^{-01}$
	95	10	$-1.91E^{-01}$	$2.43E^{-01}$	$2.45E^{-01}$	$-5.37E^{-02}$	$4.44E^{-01}$	$-1.69E^{-01}$	$5.44E^{-01}$
	100	10	$-7.90E^{-02}$	$1.94E^{-01}$	$1.45E^{-01}$	$-6.62E^{-02}$	$4.57E^{-01}$	$-1.21E^{-01}$	$3.61E^{-01}$

Table 8.17: CNRQ and RQI values at 10 days

10 d			LOG10 CNRQ – ERG's				LOG10 CNRQ - GOI		
Treatment	**Sample**	**RQI**	***actin***	***eef1a***	***gapdh***	***tubulin***	***crustin***	***peroxinectin***	***prophenoloxidase***
SPF-fed	133	8.9	$-3.10E^{-01}$	$5.50E^{-01}$	$4.17E^{-01}$	$-1.08E^{-01}$	$5.66E^{-01}$	$1.85E^{-01}$	$7.49E^{-01}$
	135	10	$1.20E^{-01}$	$1.70E^{-01}$	$-2.27E^{-01}$	$1.07E^{-01}$	$-4.58E^{-01}$	$4.33E^{-01}$	$-1.08E^{-01}$
	136	10	$-1.77E^{-01}$	$6.00E^{-01}$	$3.35E^{-01}$	$-1.58E^{-01}$	$4.91E^{-01}$	$3.13E^{-01}$	$7.08E^{-01}$
	139	9.8	$-1.57E^{-01}$	$5.04E^{-01}$	$3.01E^{-01}$	$-1.44E^{-01}$	$4.74E^{-01}$	$3.72E^{-01}$	$7.33E^{-01}$
	141	10	$9.15E^{-02}$	$3.55E^{-01}$	$-2.14E^{-01}$	$1.22E^{-01}$	$5.16E^{-01}$	$3.75E^{-01}$	$8.95E^{-01}$
	142	8.7	$2.17E^{-01}$	$1.11E^{00}$	$-3.90E^{-01}$	$1.74E^{-01}$	$1.04E^{00}$	$4.97E^{-01}$	$1.29E^{00}$
	143	10	$1.92E^{-01}$	$2.93E^{-01}$	$-2.57E^{-01}$	$6.46E^{-02}$	$2.70E^{-01}$	$-1.16E^{00}$	$-3.55E^{-01}$
	146	9.4	$6.88E^{-02}$	$6.78E^{-01}$	$-4.40E^{-02}$	$-2.47E^{-02}$	$5.48E^{-01}$	$3.25E^{-01}$	$2.71E^{-01}$
	147	9.8	$6.40E^{-02}$	$8.79E^{-01}$	$-2.56E^{-02}$	$-3.84E^{-02}$	$8.92E^{-01}$	$1.95E^{-01}$	$9.40E^{-01}$
	148	9.3	$1.06E^{-01}$	$7.39E^{-01}$	$2.63E^{-02}$	$-1.32E^{-01}$	$7.35E^{-01}$	$1.12E^{-01}$	$7.04E^{-01}$
WSSV-fed	122	10	$-3.56E^{-01}$	$6.41E^{-01}$	$2.41E^{-01}$	$1.15E^{-01}$	$4.72E^{-01}$	$-1.13E^{-01}$	$6.78E^{-01}$
	123	10	$-2.65E^{-01}$	$5.90E^{-01}$	$2.54E^{-01}$	$1.06E^{-02}$	$5.52E^{-01}$	$3.67E^{-02}$	$5.35E^{-01}$
	125	10	$-1.09E^{-01}$	$7.26E^{-01}$	$1.31E^{-01}$	$-2.23E^{-02}$	$7.34E^{-01}$	$-6.27E^{-02}$	$4.42E^{-01}$
	127	10	$-1.46E^{-01}$	$5.05E^{-01}$	$1.84E^{-01}$	$-3.81E^{-02}$	$4.53E^{-01}$	$-6.39E^{-01}$	$1.12E^{-01}$
	128	10	$-2.47E^{-01}$	$6.31E^{-01}$	$2.66E^{-01}$	$-1.84E^{-02}$	$5.72E^{-01}$	$3.14E^{-01}$	$6.12E^{-01}$
	130	10	$-3.58E^{-01}$	$5.53E^{-01}$	$4.72E^{-01}$	$-1.14E^{-01}$	$5.30E^{-01}$	$4.91E^{-02}$	$7.51E^{-01}$
	131	10	$-6.27E^{-02}$	$7.44E^{-01}$	$1.21E^{-01}$	$-5.84E^{-02}$	$5.93E^{-01}$	$2.52E^{-01}$	$5.81E^{-01}$
	134	9.9	$-6.41E^{-02}$	$8.63E^{-01}$	$1.24E^{-01}$	$-5.96E^{-02}$	$4.18E^{-01}$	$1.54E^{-01}$	$5.11E^{-01}$
	136	10	$-2.59E^{-01}$	$4.25E^{-01}$	$2.16E^{-01}$	$4.27E^{-02}$	$5.41E^{-01}$	$-4.79E^{-01}$	$4.33E^{p}$

8.3 Chapter 4

8.3.1 Minimum, maximum and average temperatures for Weymouth

Table 8.18: Seawater temperature and salinity data for Weymouth from March 2012 until October 2012. T is temperature and S is salinity.

March	T °C	S	April	T °C	S	May	T °C	S	June	T °C	S
1	9.4		2	11.0		1	11.1		1	14.9	
2	9.4		3	10.9		2	11.3		6	15.1	
5	9.5		4	10.9		3	11.5		7	15.1	
6	9.3		5	10.8		4	11.7		8	15.1	
7	9.3		10	10.7	35	8	12.6	35	11	14.9	
8	9.4		11	10.5		9	12.7		12	14.9	34
9	9.3		12	10.6		10	12.7		13	14.9	
12	9.5		13	10.6		11	12.7		14	14.9	
13	9.7	34	16	10.9		14	12.7		15	14.9	
14	9.7		17	10.9		15	12.9		18	15.1	
15	9.8		18	10.9		16	12.7		19	15.1	
16	9.8		19	10.7		17	12.7		20	15.3	
19	9.6		20	10.6		18	12.7		21	15.4	
20	9.6		23	10.6		21	12.9		22	15.5	
21	9.6		24	10.7	35	22	13.1	35	25	15.6	
22	9.7		25	10.9		23	13.5		26	15.6	34
23	9.9		26	10.9		24	13.5		27	15.6	
26	10.7		27	11.1		25	13.5		28	15.6	
27	10.7	34	30	11.3		28	14.6		29	15.6	
28	11.1					29	14.9				
29	11.1					30	14.9				
30	11.1					31	14.9				

(a) Temperature and salinity data from March until June 2012

July	T °C	S	Aug	T °C	S	Sept	T °C	S	Oct	T °C	S
2	15.8		1	17.5		3	17.8		1	16.1	
3	15.8		2	17.5		4	17.8	35	2	16.1	35
4	15.8		3	17.5		5	17.8		3	16.4	
5	15.8		6	17.5		6	17.8		4	16.1	
6	16.0		7	17.6	35	7	17.9		5	16.1	
9	16.0		8	17.6		10	18.0		8	15.7	
10	16.0	34	9	17.6		11	18.0		9	15.5	
11	16.0		10	17.7		12	18.0		10	15.4	
12	16.1		13	18.4		13	17.8		11	15.4	
13	16.0		14	18.3		14	17.8		12	15.3	
16	16.0		15	18.3		17	17.6		15	15.1	
17	16.0		16	18.4		18	17.6	35	16	14.8	35
18	16.0		17	18.4		19	17.6		17	14.8	
19	16.1		20	18.4		20	17.4		18	14.8	
20	16.1		21	18.5	35	21	17.4		19	15.1	
23	16.5		22	18.4		24	17.1		22	14.8	
24	16.7	35	23	18.3		25	16.9		23	14.6	
25	16.9		24	18.2		26	16.7		24	14.7	
26	17.1		28	18.2		27	16.5		25	14.8	
27	17.3		29	18.0		28	16.5		26	14.8	
30	17.7		30	18.0					29	13.8	
31	17.7		31	18.0					30	13.6	35
									31	13.5	

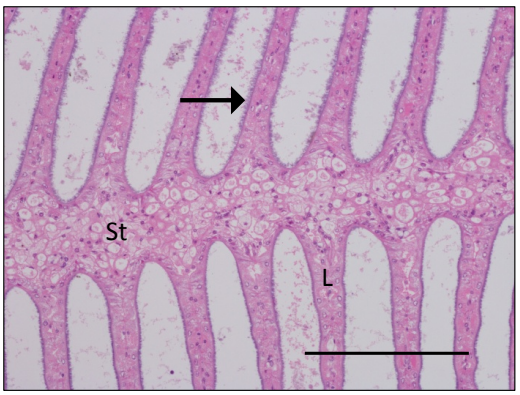
(b) Temperature and salinity data from July until October 2012

8.3.2 Sex and size of *Carcinus maenas* in Harbour and Newton's Cove

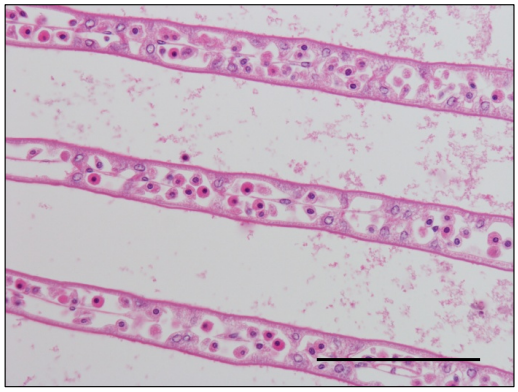
Table 8.19: Sex and size of *Carcinus maenas* in Harbour and Newton's Cove, Weymouth, UK.

Harbour								**Newton's Cove**							
Sex/Carapace width (mm)								Sex/Carapace width (mm)							
F	42	F	52	M	57	M	65	M	21	M	31	F	39	M	48
M	42	F	52	M	57	M	65	M	22	F	31	M	39	F	49
F	44	F	52	F	57	M	65	M	24	F	31	M	39	F	49
F	45	F	52	M	58	M	66	M	24	F	31	M	39	M	50
M	46	F	52	M	58	M	69	F	25	M	31	F	39	F	50
F	46	F	52	M	59	M	70	F	25	F	32	M	40	F	51
F	46	F	52	M	60	M	70	F	25	F	32	F	40	F	52
F	47	M	52	M	60	F	70	F	26	M	32	F	40	M	52
F	47	F	53	M	60	M	72	F	26	F	32	F	41	F	54
F	48	F	53	M	60	M	73	M	26	M	32	F	42	F	55
F	48	F	53	M	60	M	77	F	26	M	32	M	43	F	56
F	48	F	53	M	60	M		M	27	M	33	M	43	M	57
F	48	F	53	F	60	F		M	27	F	33	F	44	M	58
F	48	M	54	F	60			F	28	M	34	F	45	M	58
F	49	M	54	F	61			F	28	F	34	M	45	M	60
M	49	F	55	M	61			M	28	F	34	M	46	M	60
F	50	F	55	F	61			F	29	F	34	F	46		
F	50	M	56	F	62			M	29	M	34	F	46		
F	50	F	56	F	63			F	29	M	35	M	46		
M	50	F	56	F	63			M	29	F	35	M	47		
M	50	F	56	F	63			F	30	M	35	F	47		
M	51	F	56	F	63			M	30	M	35	M	47		
F	51	F	57	M	64			F	31	M	36	F	48		
F	52	F	57	M	64			F	31	M	36	F	48		
F	52	F	57	M	64			M	31	F	37	F	48		

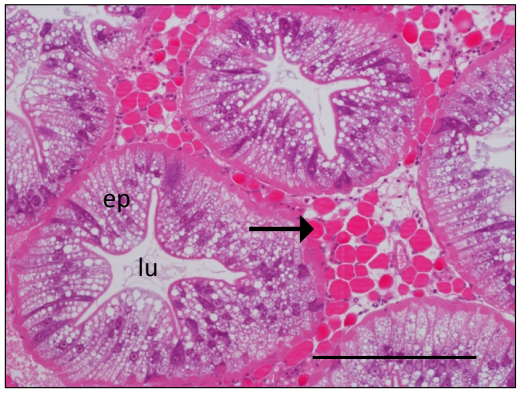
8.3.3 Normal histology



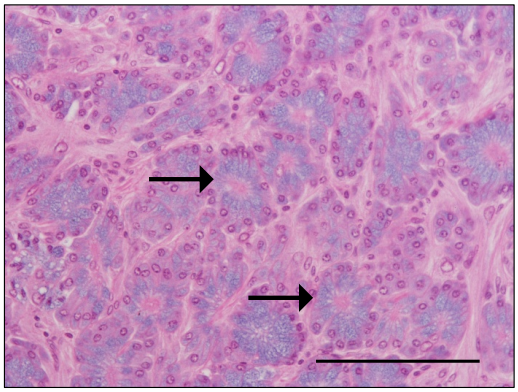
(a) Normal gill stem (St) and lamellae (L) in *Carcinus maenas*. Fouling of unidentified microbial epibionts observed on the gill lamellae (arrow). Scale bar 500μm. H&E stained.



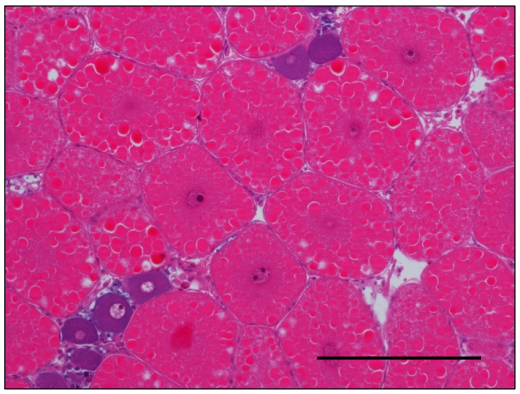
(b) Section of gill lamellae from *Carcinus maenas* containing haemocytes within the afferent and efferent vessels. Scale bar 200μm. H&E stained.



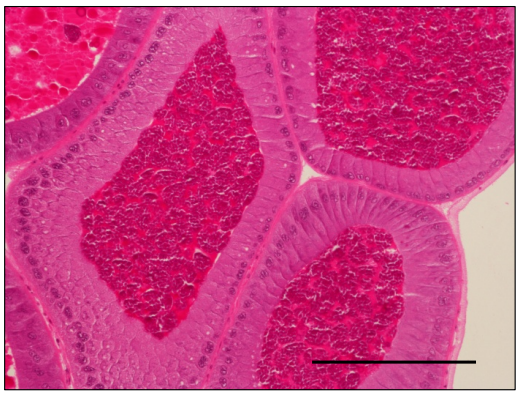
(c) Hepatopancreatic tubules with normal epithelial cells (ep) and lumens (lu). Reserve inclusions cells (arrow) observed within the haemal space surrounding the hepatopancreatic tubules. Scale bar 200μm. H&E stained.



(d) Tegmental glands from *Carcinus maenas* (arrows). Scale bar 200μm. H&E stained.



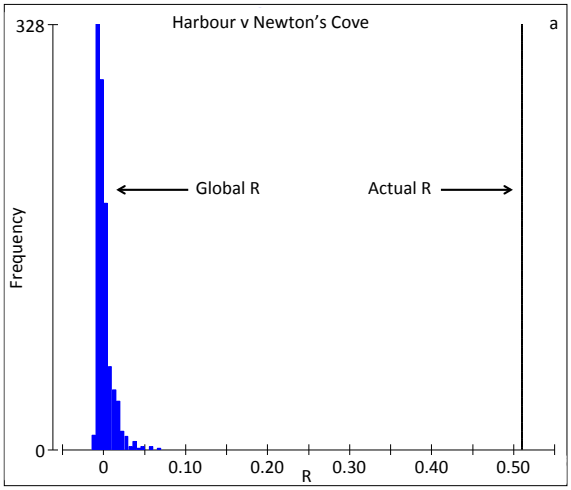
(e) Normal appearance of mature ovaries from *Carcinus maenas*. Scale bar 200μm. H&E stained.



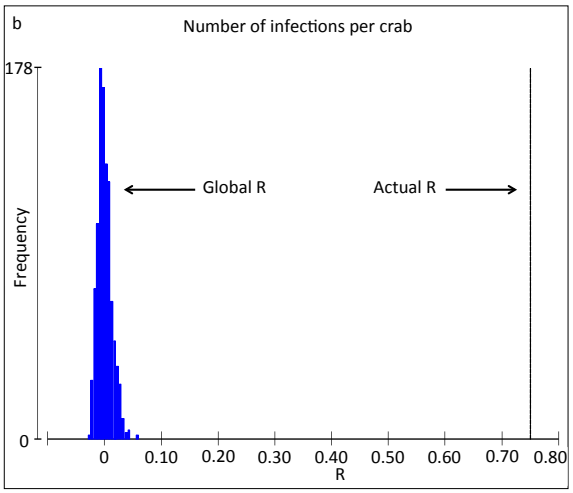
(f) Normal appearance of vas deferens from the testis in *Carcinus maenas*. Scale bar 500μm. H&E stained.

Figure 8.21: Normal histology of a variety of tissue types in *Carcinus maenas*.

8.3.4 Analysis of Similarity - Global R



(a) Global and actual R comparison of pathogen assemblages between locations.



(b) Global and actual R comparison of the number of infections per crab.

Figure 8.22: Global and actual R comparison. Comparison of the (dis)similarity of the pathogen assemblages in *Carcinus maenas* in Harbour and Newton's Cove and of the number of infections per crab. The actual R values (a. 0.503 or b. 0.751) were greater than the 999 permutations representing the global R value, therefore the ANOSIM was statistically significant in both cases.

8.3.5 Assessment of normal distribution of data

Absence and presence of pathogens in *Carcinus maenas*.

Table 8.20: Shapiro-Wilks test for normal distribution of data grouped as absence and presence of pathogens. The data not meeting the normal distribution requirements for parametric analysis are highlighted in red.

Carcinin		
	No pathogens	**Pathogens**
Harbour	W = 0.97, p >0.05	W = 0.96, p >0.05
Newton's Cove	W = 0.90, p >0.05	W = 0.96, p >0.05
Both locations	W = 0.98, p >0.05	W = 0.96, p >0.05
Peroxinectin		
Harbour	W = 0.85, p <0.001	W = 0.64, p <0.0001
Newton's Cove	W = 0.80, p >0.05	W = 0.97, p >0.05
Both locations	W = 0.87, p <0.001	W = 0.82, p <0.01^{-05}
Prophenoloxidase		
Harbour	W = 0.93, p <0.05	W = 0.74, p <0.01
Newton's Cove	W = 0.85, p >0.05	W = 0.90, p <0.001
Both locations	W = 0.95, p <0.05	W = 0.95, p <0.01

8.3.6 Frequency of infections and co-infections in the Harbour and Newton's Cove populations of *Carcinus maenas*.

Frequency of infections in relation to gene expression results - all RQI

Table 8.21: Frequency of pathogen infections and co-infections within the Harbour and Newton's Cove populations of *Carcinus maenas* - all RQI.

All RQI						
Pathogen	***Carcinin***		***Peroxinectin***		***Prophenoloxidase***	
	Harbour	**Newton's Cove**	**Harbour**	**Newton's Cove**	**Harbour**	**Newton's Cove**
Ameson, Microphallus	0	2	0	2	0	2
Ameson, Sacculina	0	1	0	1	0	1
CmBV, Microphallus	0	2	0	2	0	2
CmBV, Microphallus, Granuloma	0	0	0	1	0	1
Granuloma	9	0	9	0	9	0
Haplosporidia, Ameson, Microphallus	0	1	0	1	0	1
Haplosporidia, Granuloma	1	0	1	0	1	0
Hematodinium	1	0	1	0	1	0
Hematodinium, Microphallus	1	0	1	0	1	0
Hematodinium, Sacculina	1	0	1	0	1	0
Microphallus	3	35	3	34	3	34
Microphallus, Granuloma	1	11	1	10	1	10
Microphallus, HLV	0	6	0	6	0	6
Microphallus, MilkyDisease, Granuloma	0	4	0	4	0	4
MilkyDisease, HLV	1	0	1	0	1	0
Nematodes, Granuloma	1	0	1	0	1	0
No Pathogen	56	6	54	6	56	6
Sacculina	4	0	4	0	4	0
Sacculina, Microphallus	0	2	0	2	0	2
Sacculina, MilkyDisease	0	1	0	1	0	1

Frequency of infections in relation to gene expression results - high RQI

Table 8.22: Frequency of pathogen infections and co-infections within the Harbour and Newton's Cove populations of *Carcinus maenas* - high RQI only.

High RQI						
Pathogen	***Carcinin***		***Peroxinectin***		***Prophenoloxidase***	
	Harbour	**Newton's Cove**	**Harbour**	**Newton's Cove**	**Harbour**	**Newton's Cove**
Ameson, Microphallus	0	2	0	2	0	2
Ameson, Sacculina	0	1	0	1	0	1
CmBV, Microphallus	0	0	0	1	0	1
CmBV, Microphallus, Granuloma	0	0	0	1	0	1
Granuloma	6	0	6	0	6	0
Haplosporidia, Ameson, Microphallus	0	1	0	1	0	1
Haplosporidia, Granuloma	0	0	0	0	0	0
Hematodinium	0	0	0	0	0	0
Hematodinium, Microphallus	1	0	1	0	1	0
Hematodinium, Sacculina	0	0	0	0	0	0
Microphallus	0	32	0	31	0	31
Microphallus, Granuloma	0	9	0	8	0	8
Microphallus, HLV	0	2	0	2	0	2
Microphallus, MilkyDisease, Granuloma	0	1	0	1	0	1
MilkyDisease, HLV	0	0	0	0	0	0
Nematodes, Granuloma	1	0	1	0	1	0
No Pathogen	38	5	36	5	38	5
Sacculina	3	0	3	0	3	0
Sacculina, Microphallus	0	2	0	2	0	2
Sacculina, MilkyDisease	0	0	0	0	0	0

Frequency of infections in relation to gene expression results - low RQI

Table 8.23: Frequency of pathogen infections and co-infections within the Harbour and Newton's Cove populations of *Carcinus maenas* - low RQI only.

Low RQI (<7.6)						
Pathogen	***Carcinin***		***Peroxinectin***		***Prophenoloxidase***	
	Harbour	**Newton's Cove**	**Harbour**	**Newton's Cove**	**Harbour**	**Newton's Cove**
Ameson, Microphallus	0	0	0	0	0	0
Ameson, Sacculina	0	0	0	0	0	0
CmBV, Microphallus	0	1	0	1	0	1
CmBV, Microphallus, Granuloma	0	0	0	0	0	0
Granuloma	2	0	2	0	2	0
Haplosporidia, Ameson, Microphallus	0	0	0	0	0	0
Haplosporidia, Granuloma	1	0	1	0	1	0
Hematodinium	1	0	1	0	1	0
Hematodinium, Microphallus	0	0	0	0	0	0
Hematodinium, Sacculina	1	0	1	0	1	0
Microphallus	3	3	3	3	3	3
Microphallus, Granuloma	1	2	1	2	1	2
Microphallus, HLV	0	4	0	4	0	4
Microphallus, MilkyDisease, Granuloma	0	3	0	3	0	3
MilkyDisease, HLV	1	0	1	0	1	0
Nematodes, Granuloma	0	0	0	0	0	0
No Pathogen	18	1	18	1	18	1
Sacculina	1	0	1	0	1	0
Sacculina, Microphallus	0	0	0	0	0	0
Sacculina, MilkyDisease	0	1	0	1	0	1

8.4 Chapter 5

8.4.1 Log_{10} CNRQ and RNA quality values

Listonella anguillarum.

Table 8.24: Log_{10}CNRQ and RNA quality of *Listonella anguillarum* control samples at a) 0 hours and b) 72 hours. For the purpose of this investigation, both *actin* and *tubulin* were considered to be the least stable endogenous reference genes and were thereby omitted from the analysis.

Listonella anguillarum			**LOG_{10} CNRQ – ERG's**		**LOG_{10} CNRQ - GOI**		
Treatment	**Sample**	**RQI**	***eef1a***	***gapdh***	***carcinin***	***peroxinectin***	***prophenoloxidase***
0 hours	1	9.4	$1.16E^{-02}$	$-1.16E^{-02}$	$-7.07E^{-02}$	$-2.70E^{-01}$	$-2.71E^{-01}$
	2	/	/	/	/	/	/
	3	10	$-1.63E^{-02}$	$1.63E^{-02}$	$-6.47E^{-01}$	$2.45E^{-01}$	$-1.45E^{-02}$
	4	9.5	$-9.67E^{-03}$	$9.67E^{-03}$	$1.58E^{-01}$	$2.82E^{-01}$	$4.03E^{-01}$
	5	8.6	$-8.17E^{-02}$	$8.17E^{-02}$	$-5.06E^{-02}$	$-4.25E^{-03}$	$1.45E^{-01}$
	6	10	$1.43E^{-01}$	$-1.43E^{-01}$	$5.41E^{-01}$	$-1.38E^{-01}$	$5.44E^{-01}$
	7	10	$8.06E^{-02}$	$-8.06E^{-02}$	$-2.08E^{-01}$	$-1.31E^{-01}$	$-6.42E^{-01}$
	8	9.4	$-1.03E^{-01}$	$1.03E^{-01}$	$2.44E^{-01}$	$-5.48E^{-02}$	$-1.32E^{-01}$
	9	8.7	$-3.06E^{-02}$	$3.06E^{-02}$	$-2.55E^{-01}$	$-1.74E^{-01}$	$4.46E^{-01}$
	10	10	$-3.47E^{-02}$	$3.47E^{-02}$	$1.94E^{-01}$	$-1.97E^{-01}$	$3.70E^{-01}$
	11	10	$2.99E^{-03}$	$-2.99E^{-03}$	$-6.01E^{-01}$	$-8.20E^{-02}$	$-1.98E^{-01}$
	12	9.4	$-2.51E^{-03}$	$2.51E^{-03}$	$3.12E^{-01}$	$-5.99E^{-02}$	$-4.43E^{-01}$
	13	10	$-3.08E^{-02}$	$3.08E^{-02}$	$-1.40E^{-01}$	$-3.52E^{-02}$	$-4.74E^{-01}$
	14	10	$8.55E^{-02}$	$-8.55E^{-02}$	$1.04E^{-01}$	$-1.86E^{-01}$	$-2.52E^{-01}$
	15	9.6	$-1.53E^{-01}$	$1.53E^{-01}$	$1.91E^{00}$	$6.49E^{-02}$	$7.06E^{-01}$

(a) *Listonella anguillarum* control samples at 0 hours.

Listonella anguillarum			**LOG_{10} CNRQ – ERG's**		**LOG_{10} CNRQ - GOI**		
Treatment	**Sample**	**RQI**	***eef1a***	***gapdh***	***carcinin***	***peroxinectin***	***prophenoloxidase***
72 hours	1	9.8	$1.14E^{-01}$	$-1.14E^{-01}$	$8.27E^{-01}$	$-5.70E^{-01}$	$2.13E^{-01}$
	2	10	$1.34E^{-01}$	$-1.34E^{-01}$	$5.35E^{-01}$	$-1.11E^{-01}$	$1.56E^{-01}$
	3	10	$1.11E^{-01}$	$-1.11E^{-01}$	$3.89E^{-01}$	$-2.93E^{-01}$	$5.18E^{-01}$
	4	10	$7.85E^{-03}$	$-7.85E^{-03}$	$5.43E^{-01}$	$7.76E^{-01}$	$9.49E^{-01}$
	5	10	$5.20E^{-02}$	$-5.20E^{-02}$	$5.87E^{-01}$	$-4.19E^{-01}$	$6.05E^{-02}$
	6	8.0	$2.58E^{-01}$	$-2.58E^{-01}$	$4.30E^{-01}$	$-4.40E^{-01}$	$2.04E^{-01}$
	7	10	$1.69E^{-01}$	$-1.69E^{-01}$	$6.58E^{-01}$	$-6.33E^{-01}$	$2.12E^{-01}$
	8	/	/	/	/	/	/
	9	10	$3.16E^{-02}$	$-3.16E^{-02}$	$6.44E^{-01}$	$-3.60E^{-01}$	$4.46E^{-01}$
	10	10	$6.25E^{-02}$	$-6.25E^{-02}$	$7.35E^{-01}$	$-2.48E^{-01}$	$4.81E^{-01}$
	11	10	$1.50E^{-01}$	$-1.50E^{-01}$	$6.63E^{-01}$	$-4.25E^{-01}$	$6.23E^{-01}$
	12	10	$8.27E^{-02}$	$-8.27E^{-02}$	$9.66E^{-01}$	$-3.06E^{-01}$	$5.49E^{-01}$
	13	10	$1.89E^{-01}$	$-1.89E^{-01}$	$8.70E^{-01}$	$-1.78E^{-01}$	$4.26E^{-01}$
	14	10	$1.08E^{-01}$	$-1.08E^{-01}$	$7.54E^{-01}$	$-9.99E^{-02}$	$4.33E^{-01}$
	15	5.6	$6.54E^{-02}$	$-6.54E^{-02}$	$9.58E^{-01}$	$3.90E^{-02}$	$3.03E^{-01}$

(b) *Listonella anguillarum* control samples at 72 hours.

Table 8.25: Log_{10}CNRQ and RNA quality of *Listonella anguillarum* infected samples at a) 0 hours and b) 72 hours. As the least stable endogenous reference genes, *actin* and *tubulin* were omitted from this analysis.

Listonella anguillarum			**LOG_{10} CNRQ – ERG's**		**LOG_{10} CNRQ - GOI**		
Treatment	**Sample**	**RQI**	***eef1a***	***gapdh***	***carcinin***	***peroxinectin***	***prophenoloxidase***
0 hours	16	10	$9.53E^{-02}$	$-9.53E^{-02}$	$-4.72E^{-01}$	$2.42E^{-01}$	$4.00E^{-01}$
	17	10	$2.87E^{-02}$	$-2.87E^{-02}$	$2.03E^{-01}$	$2.60E^{-01}$	$3.87E^{-01}$
	18	10	$-1.48E^{-02}$	$1.48E^{-02}$	$-3.13E^{-01}$	$-2.73E^{-01}$	$1.37E^{-01}$
	19	10	$3.96E^{-02}$	$-3.96E^{-02}$	$4.37E^{-01}$	$2.64E^{-01}$	$7.19E^{-02}$
	20	/	/	/	/	/	/
	21	10	$1.12E^{-02}$	$-1.12E^{-02}$	$-6.19E^{-01}$	$6.55E^{-02}$	$-1.72E^{-01}$
	22	/	/	/	/	/	/
	23	10	$1.32E^{-01}$	$-1.32E^{-01}$	/	$1.15E^{00}$	$2.96E^{-01}$
	24	10	$3.72E^{-02}$	$-3.72E^{-02}$	$-1.19E^{-01}$	$-7.79E^{-02}$	$-3.12E^{-01}$
	25	/	$1.31E^{-01}$	$-1.31E^{-01}$	$-1.31E^{00}$	$-2.39E^{-01}$	$-3.36E^{-01}$
	26	10	$-6.16E^{-02}$	$6.16E^{-02}$	$-5.66E^{-01}$	$7.31E^{-02}$	$-9.40E^{-02}$
	27	10	$-1.78E^{-01}$	$1.78E^{-01}$	$2.75E^{-01}$	$-1.42E^{-02}$	$1.07E^{-01}$
	28	10	$-1.64E^{-02}$	$1.64E^{-02}$	$-4.37E^{-02}$	$-2.03E^{-01}$	$-1.70E^{-01}$
	29	10	$1.45E^{-02}$	$-1.45E^{-02}$	$6.33E^{-01}$	$-3.37E^{-01}$	$4.04E^{-02}$
	30	10	$-7.85E^{-02}$	$7.85E^{-02}$	$4.04E^{-01}$	$-1.72E^{-01}$	$-3.47E^{-01}$

(a) *Listonella anguillarum* infected samples at 0 hours.

Listonella anguillarum			**LOG_{10} CNRQ – ERG's**		**LOG_{10} CNRQ - GOI**		
Treatment	**Sample**	**RQI**	***eef1a***	***gapdh***	***carcinin***	***peroxinectin***	***prophenoloxidase***
72 hours	16	10	$2.19E^{-01}$	$-2.19E^{-01}$	$3.46E^{-01}$	$-1.12E^{-01}$	$6.64E^{-01}$
	17	10	$2.30E^{-01}$	$-2.30E^{-01}$	$1.24E^{00}$	$1.15E^{-02}$	$5.92E^{-01}$
	18	10	$1.07E^{-01}$	$-1.07E^{-01}$	$6.06E^{-01}$	$-4.05E^{-01}$	$3.18E^{-01}$
	19	10	$1.02E^{-01}$	$-1.02E^{-01}$	$7.85E^{-01}$	$-1.96E^{-01}$	$4.84E^{-01}$
	20	/	/	/	/	/	/
	21	10	$2.27E^{-01}$	$-2.27E^{-01}$	$6.62E^{-01}$	$-2.21E^{-01}$	$4.30E^{-01}$
	22	10	$2.76E^{-01}$	$-2.76E^{-01}$	$9.97E^{-01}$	$-1.15E^{-01}$	$3.66E^{-01}$
	23	8.9	$1.03E^{-01}$	$-1.03E^{-01}$	$1.16E^{00}$	$-2.96E^{-01}$	$1.41E^{-01}$
	24	10	$1.43E^{-01}$	$-1.43E^{-01}$	$9.40E^{-01}$	$-2.06E^{-02}$	$7.22E^{-01}$
	25	9.2	$1.26E^{-01}$	$-1.26E^{-01}$	$9.25E^{-01}$	$-8.72E^{-01}$	$-2.12E^{-01}$
	26	9.8	$6.83E^{-02}$	$-6.83E^{-02}$	$6.87E^{-01}$	$-9.30E^{-02}$	$2.36E^{-01}$
	27	10	/	/	/	/	/
	28	9.3	$1.51E^{-01}$	$-1.51E^{-01}$	$9.61E^{-01}$	$-2.96E^{-01}$	$7.63E^{-01}$
	29	10	$2.30E^{-01}$	$-2.30E^{-01}$	$1.15E^{00}$	$-3.40E^{-01}$	$5.40E^{-01}$
	30	9.8	$1.59E^{-01}$	$-1.59E^{-01}$	$6.06E^{-01}$	$-1.48E^{-01}$	$4.17E^{-01}$

(b) *Listonella anguillarum* infected samples at 72 hours.

Planococcus citreus.

Table 8.26: Log_{10}CNRQ and RNA quality of *Planococcus citreus* control samples at a) 0 hours and b) 48 hours.

Planococcus citreus			**LOG_{10} CNRQ – ERG's**				**LOG_{10} CNRQ - GOI**		
Treatment	**Sample**	**RQI**	***actin***	***eef1a***	***gapdh***	***tubulin***	***carcinin***	***peroxinectin***	***prophenoloxidase***
0 hours	31	10	$2.58E^{-01}$	$-2.58E^{-01}$	$-1.16E^{-01}$	$-1.03E^{-01}$	$-1.47E^{-01}$	$-1.04E^{-01}$	$2.94E^{-01}$
	32	9.1	$2.00E^{-01}$	$-2.00E^{-01}$	$-3.39E^{-01}$	$-1.50E^{-01}$	$-4.98E^{-01}$	$-2.56E^{-01}$	$-9.80E^{-01}$
	33	10	$-3.61E^{-02}$	$3.61E^{-02}$	$-4.03E^{-01}$	$5.52E^{-02}$	$2.05E^{-01}$	$-2.54E^{-01}$	$7.83E^{-02}$
	34	10	$-1.09E^{-01}$	$1.09E^{-01}$	$-3.98E^{-01}$	$-2.85E^{-01}$	$2.45E^{-01}$	$2.04E^{-02}$	$-2.44E^{-01}$
	35	10	$-1.01E^{-01}$	$1.01E^{-01}$	$3.22E^{-03}$	$1.23E^{-01}$	$-5.73E^{-01}$	$-5.26E^{-02}$	$4.67E^{-01}$
	36	5.0	$-1.25E^{-01}$	$1.25E^{-01}$	$-4.98E^{-01}$	$-2.49E^{-01}$	$-8.60E^{-01}$	$-2.79E^{-01}$	$-6.23E^{-01}$
	37	10	$1.26E^{-01}$	$-1.26E^{-01}$	$-4.31E^{-01}$	$-1.99E^{-01}$	$-6.15E^{-01}$	$-3.84E^{-01}$	$-3.51E^{-01}$
	38	10	$1.09E^{-01}$	$-1.09E^{-01}$	$-2.72E^{-01}$	$9.31E^{-02}$	$-5.61E^{-03}$	$7.78E^{-02}$	$1.40E^{-01}$
	39	10	$2.25E^{-01}$	$-2.25E^{-01}$	$-1.80E^{-01}$	$-2.20E^{-02}$	$2.23E^{-01}$	$1.02E^{-01}$	$1.43E^{-01}$
	40	3.5	$-2.68E^{-02}$	$2.68E^{-02}$	$-1.61E^{-01}$	$-7.49E^{-02}$	$-3.23E^{-01}$	$-2.00E^{-01}$	$-1.75E^{-02}$
	41	10	$-2.89E^{-02}$	$2.89E^{-02}$	$-2.55E^{-01}$	$7.25E^{-03}$	$1.27E^{-01}$	$9.86E^{-02}$	$3.84E^{-01}$
	42	10	$-1.56E^{-01}$	$1.56E^{-01}$	$-2.74E^{-01}$	$6.65E^{-02}$	/	$1.01E^{-01}$	$1.27E^{-01}$
	43	10	$-2.33E^{-03}$	$2.33E^{-03}$	$-2.44E^{-01}$	$-4.43E^{-02}$	$1.91E^{-02}$	$5.44E^{-03}$	$8.53E^{-03}$
	44	10	$-5.56E^{-02}$	$5.56E^{-02}$	$-2.65E^{-01}$	$-8.41E^{-02}$	$2.55E^{-01}$	$-3.10E^{-02}$	$-1.59E^{-01}$
	45	4.2	$4.90E^{-02}$	$-4.90E^{-02}$	$-1.55E^{-01}$	$-8.41E^{-03}$	$5.09E^{-01}$	$-1.26E^{-01}$	$1.77E^{-01}$

(a) *Planococcus citreus* control samples at 0 hours.

Planococcus citreus			**LOG_{10} CNRQ – ERG's**				**LOG_{10} CNRQ - GOI**		
Treatment	**Sample**	**RQI**	***actin***	***eef1a***	***gapdh***	***tubulin***	***carcinin***	***peroxinectin***	***prophenoloxidase***
48 hours	31	10	$-4.68E^{-02}$	$4.68E^{-02}$	$-5.82E^{-01}$	$4.77E^{-01}$	$7.07E^{-02}$	$1.88E^{-01}$	$3.25E^{-02}$
	32	9.1	$1.02E^{-01}$	$-1.02E^{-01}$	$-3.23E^{-01}$	$5.87E^{-01}$	$9.75E^{-01}$	$3.43E^{-01}$	$1.54E^{-01}$
	33	10	$3.84E^{-02}$	$-3.84E^{-02}$	$-3.94E^{-01}$	$4.77E^{-01}$	$1.17E^{00}$	$4.44E^{-01}$	$2.37E^{-01}$
	34	10	$2.55E^{-02}$	$-2.55E^{-02}$	$-3.59E^{-01}$	$6.17E^{-01}$	$9.91E^{-01}$	$2.00E^{-01}$	$3.72E^{-01}$
	35	6.9	$-6.31E^{-02}$	$6.31E^{-02}$	$-4.76E^{-01}$	$4.22E^{-01}$	$5.64E^{-01}$	$2.02E^{-01}$	$1.59E^{-01}$
	36	9.9	$-4.71E^{-02}$	$4.71E^{-02}$	$-3.04E^{-01}$	$4.22E^{-01}$	$9.31E^{-01}$	$1.31E^{-01}$	$2.15E^{-01}$
	37	10	$7.20E^{-02}$	$-7.20E^{-02}$	$-6.51E^{-01}$	$4.30E^{-01}$	$8.14E^{-01}$	$8.75E^{-02}$	$-2.81E^{-01}$
	38	8.7	$2.37E^{-02}$	$-2.37E^{-02}$	$-5.51E^{-01}$	$7.35E^{-01}$	$8.23E^{-01}$	$6.17E^{-02}$	$9.43E^{-02}$
	39	10	$1.19E^{-01}$	$-1.19E^{-01}$	$-3.51E^{-01}$	$5.14E^{-02}$	$1.40E^{00}$	$6.82E^{-02}$	$-8.80E^{-02}$
	42	10	$-2.74E^{-02}$	$2.74E^{-02}$	$-3.91E^{-01}$	$3.89E^{-01}$	$7.90E^{-01}$	$3.19E^{-01}$	$4.66E^{-01}$
	43	10	$-6.69E^{-02}$	$6.69E^{-02}$	$-3.92E^{-01}$	$4.01E^{-01}$	$9.44E^{-01}$	$4.61E^{-01}$	$2.64E^{-02}$
	44	10	$8.44E^{-02}$	$-8.44E^{-02}$	$-6.13E^{-01}$	$5.35E^{-01}$	$7.11E^{-01}$	$5.61E^{-01}$	$3.26E^{-01}$
	45	10	$-1.02E^{-01}$	$1.02E^{-01}$	$-4.25E^{-01}$	$4.82E^{-01}$	$-2.53E^{-01}$	$-7.41E^{-03}$	$-3.35E^{-01}$

(b) *Planococcus citreus* control samples at 48 hours.

Table 8.27: Log_{10}CNRQ and RNA quality of *Planococcus citreus* infected samples at a) 0 hours and b) 48 hours.

Planococcus citreus			**LOG_{10} CNRQ – ERG's**				**LOG_{10} CNRQ - GOI**		
Treatment	**Sample**	**RQI**	***actin***	***eef1a***	***gapdh***	***tubulin***	***carcinin***	***peroxinectin***	***prophenoloxidase***
0 hours	46	10	$3.46E^{-02}$	$-3.46E^{-02}$	$1.37E^{-01}$	$1.33E^{-01}$	$1.54E^{-01}$	$2.55E^{-01}$	$-1.69E^{-01}$
	47	10	$-1.34E^{-01}$	$1.34E^{-01}$	$1.41E^{-01}$	$-5.64E^{-02}$	$3.57E^{-01}$	$1.30E^{-01}$	$-2.12E^{-01}$
	48	3.5	$-2.08E^{-02}$	$2.08E^{-02}$	$2.41E^{-01}$	$-5.30E^{-02}$	$4.34E^{-01}$	$1.99E^{-01}$	$2.12E^{-01}$
	49	3.1	$2.20E^{-01}$	$-2.20E^{-01}$	$1.11E^{-01}$	$-4.16E^{-02}$	$-1.29E^{00}$	$-2.12E^{-01}$	$-2.89E^{-01}$
	50	10	$2.77E^{-02}$	$-2.77E^{-02}$	$2.51E^{-01}$	$2.42E^{-02}$	$5.88E^{-01}$	$2.96E^{-01}$	$5.04E^{-03}$
	51	3.2	$-3.44E^{-01}$	$3.44E^{-01}$	$8.52E^{-01}$	$5.62E^{-01}$	$8.78E^{-02}$	/	$1.22E^{00}$
	52	10	$-1.19E^{-01}$	$1.19E^{-01}$	$1.42E^{-01}$	$1.92E^{-01}$	$3.26E^{-01}$	$-5.94E^{-01}$	$2.31E^{-01}$
	53	10	$-5.36E^{-02}$	$5.36E^{-02}$	$1.23E^{-01}$	$-7.70E^{-02}$	$-8.51E^{-01}$	$1.09E^{-01}$	$-2.92E^{-01}$
	54	9.7	$6.29E^{-02}$	$-6.29E^{-02}$	$2.64E^{-01}$	$8.92E^{-02}$	$2.41E^{-01}$	$1.75E^{-02}$	$1.60E^{-01}$
	55	10	$-1.13E^{-01}$	$1.13E^{-01}$	$2.00E^{-01}$	$6.60E^{-02}$	$1.57E^{-01}$	$2.32E^{-01}$	$-1.74E^{-01}$
	56	10	$4.11E^{-02}$	$-4.11E^{-02}$	$2.87E^{-01}$	$2.29E^{-02}$	$8.70E^{-01}$	$1.67E^{-01}$	$1.02E^{-01}$
	57	3.7	$1.64E^{-02}$	$-1.64E^{-02}$	$1.95E^{-01}$	$2.74E^{-02}$	$-7.00E^{-01}$	$2.44E^{-01}$	$-1.18E^{-01}$
	58	3.0	$8.88E^{-03}$	$-8.88E^{-03}$	$4.72E^{-01}$	$-9.86E^{-02}$	$9.75E^{-02}$	$2.69E^{-01}$	$1.06E^{-01}$
	59	/	$6.33E^{-02}$	$-6.33E^{-02}$	$3.37E^{-01}$	$1.24E^{-01}$	$8.52E^{-01}$	$2.31E^{-01}$	$3.87E^{-02}$
	60	9.5	$-1.86E^{-02}$	$1.86E^{-02}$	$2.34E^{-01}$	$-4.03E^{-02}$	$1.16E^{-01}$	$-6.11E^{-02}$	$-2.69E^{-01}$

(a) *Planococcus citreus* infected samples at 0 hours.

Planococcus citreus			**LOG_{10} CNRQ – ERG's**				**LOG_{10} CNRQ - GOI**		
Treatment	**Sample**	**RQI**	***actin***	***eef1a***	***gapdh***	***tubulin***	***carcinin***	***peroxinectin***	***prophenoloxidase***
48 hours	46	2.2	$-5.35E^{-02}$	$5.35E^{-02}$	$-2.67E^{-01}$	$9.58E^{-02}$	$1.57E^{-01}$	$2.65E^{00}$	/
	47	5.8	$3.28E^{-02}$	$-3.28E^{-02}$	$-2.40E^{-01}$	$3.96E^{-01}$	$1.67E^{00}$	$-9.84E^{-02}$	$-2.50E^{-02}$
	48	8.9	$2.72E^{-02}$	$-2.72E^{-02}$	$-1.43E^{-01}$	$5.84E^{-01}$	$2.08E^{00}$	$4.97E^{-01}$	$1.85E^{-01}$
	49	9.5	$-3.51E^{-02}$	$3.51E^{-02}$	$9.61E^{-02}$	$2.34E^{-01}$	$1.07E^{00}$	$-3.30E^{-01}$	$7.74E^{-02}$
	50	9.3	$-9.45E^{-02}$	$9.45E^{-02}$	$1.50E^{-01}$	$3.10E^{-01}$	$1.90E^{00}$	$3.54E^{-01}$	$2.28E^{-01}$
	51	9.9	$1.55E^{-02}$	$-1.55E^{-02}$	$-8.69E^{-02}$	$3.90E^{-01}$	$1.12E^{00}$	$1.16E^{-02}$	$8.55E^{-02}$
	52	4.7	$1.81E^{-01}$	$-1.81E^{-01}$	$-2.20E^{-01}$	$7.27E^{-01}$	$3.08E^{00}$	$3.34E^{-01}$	$1.02E^{00}$
	53	10	$-1.00E^{-02}$	$1.00E^{-02}$	$-2.63E^{-01}$	$7.07E^{-01}$	$7.44E^{-01}$	$2.60E^{-02}$	$-2.08E^{-01}$
	54	10	$1.46E^{-01}$	$-1.46E^{-01}$	$3.34E^{-01}$	$8.12E^{-01}$	$1.28E^{00}$	$-8.61E^{-03}$	$1.07E^{-01}$
	56	10	$-5.44E^{-02}$	$5.44E^{-02}$	$1.63E^{-01}$	$2.94E^{-01}$	$1.08E^{00}$	$1.07E^{-01}$	$-3.25E^{-01}$
	57	9.4	$-8.23E^{-02}$	$8.23E^{-02}$	$8.49E^{-04}$	$5.71E^{-02}$	$2.18E^{00}$	$-2.58E^{-01}$	$2.16E^{-01}$
	58	10	$-6.24E^{-02}$	$6.24E^{-02}$	$2.41E^{-01}$	$6.00E^{-01}$	$2.42E^{00}$	$2.23E^{-01}$	$6.08E^{-01}$
	59	9.7	$-4.60E^{-03}$	$4.60E^{-03}$	$-2.62E^{-02}$	$3.79E^{-01}$	$1.91E^{00}$	$1.59E^{-01}$	$-1.19E^{-01}$
	60	10	$1.13E^{-01}$	$-1.13E^{-01}$	$-2.40E^{-01}$	$5.24E^{-01}$	$1.47E^{00}$	$-8.83E^{-01}$	$-8.47E^{-02}$

(b) *Planococcus citreus* infected samples at 48 hours.

8.4.2 Assessment of normal distribution and equal variance of gene transcription data

The normality test for the data was carried out using the Shapiro-Wilk test for normality and homogeneity of variance was assessed using Bartlett's test. p values highlighted in red indicate data that is not normally distributed or equal in variance.

Table 8.28: Assessment of normal distribution of immune gene expression data for the *Listonella anguillarum* infection trial.

Immune gene	Treatment	0 hours	72 hours
Carcinin	Control	W = 0.83, p <0.05	W = 0.98, p >0.05
	Listonella anguillarum	W = 0.95, p >0.05	W = 0.96, p >0.05
Peroxinectin	Control	W = 0.89, p >0.05	W = 0.76, p <0.01
	Listonella anguillarum	W = 0.80, p <0.01	W = 0.83, p <0.05
Prophenoloxidase	Control	W = 0.95, p >0.05	W = 0.93, p >0.05
	Listonella anguillarum	W = 0.93, p >0.05	W = 0.94, p 0.05

Table 8.29: Assessment of normal distribution of immune gene expression data for the *Planococcus citreus* infection trial.

Immune gene	Treatment	0 hours	72 hours
Carcinin	Control	W = 0.92, p >0.05	W = 0.92, p >0.05
	Planococcus citreus	W = 0.88, p <0.05	W = 0.99, p >0.05
Peroxinectin	Control	W = 0.92, p >0.05	W = 0.91, p >0.05
	Planococcus citreus	W = 0.77, p <0.01	W = 0.72, p <0.001
Prophenoloxidase	Control	W = 0.86, p >0.05	W = 0.98, p >0.05
	Planococcus citreus	W = 0.74, p <0.001	W = 0.89, p >0.05

Table 8.30: Assessment for homogeneity of variance of the gene expression data from the *Listonella anguillarum* infection trial.

Immune gene	Treatment or Time	O hours	48 hours
Carcinin	*Listonella anguillarum* v Control	K^2 = 0.2, d.f. = 1, p >0.05	K^2 = 2.2, d.f. = 1, p >0.05
Peroxinectin		K^2 = 7.5, d.f. = 1, p <0.01	K^2 = 2.1, d.f. = 1, p >0.05
Prophenoloxidase		K^2 = 2.6, d.f. = 1, p >0.05	K^2 = 0.1, d.f. = 1, p >0.05
		Control	***Listonella anguillarum***
Carcinin	O hours v 48 hours - Control	K^2 = 16.3, d.f. = 1, p >0.0001	K^2 = 5.9, d.f. = 1, p <0.05
Peroxinectin		K^2 = 5.9, d.f. = 1, p <0.05	K^2 = 3.2, d.f. = 1, p >0.05
Prophenoloxidase		K^2 = 3.7, d.f. = 1, p >0.05	K^2 = 3^{-04}, d.f. = 1, p <0.001

Table 8.31: Assessment for homogeneity of variance of the gene expression data from the *Planococcus citreus* infection trial.

Immune gene	**Treatment or Time**	**0 hours**	**48 hours**
Carcinin	*Planococcus citreus* v Control	$K^2 = 1.4$, d.f. = 1, $p > 0.05$	$K^2 = 8.8$, d.f. = 1, $p < 0.01$
Peroxinectin		$K^2 = 1.3$, d.f. = 1, $p > 0.05$	$K^2 = 11$, d.f. = 1, $p < 0.001$
Prophenoloxidase		$K^2 = 0.3$, d.f. = 1, $p > 0.05$	$K^2 = 2.9$, d.f. = 1, $p > 0.05$
		Control	***Planococcus citreus***
Carcinin	0 hours v 48 hours	$K^2 = 2.3$, d.f. = 1, $p > 0.05$	$K^2 = 0.6$, d.f. = 1, $p > 0.05$
Peroxinectin		$K^2 = 0.2$, d.f. = 1, $p > 0.05$	$K^2 = 14.2$, d.f. = 1, $p < 0.001$
Prophenoloxidase		$K^2 = 4.7$, d.f. = 1, $p < 0.05$	$K^2 = 0.04$, d.f. = 1, $p > 0.05$

8.4.3 Assessment of normal distribution and equal variance of THC data

Table 8.32: Assessment of normal distribution of THC data using the Shapiro-Wilk test. Results highlighted in red with a $p < 0.05$ were not normally distributed.

	0 hours	**3 hours**	**72 hours**
Control	W = 0.88, $p > 0.05$	W = 0.90, $p > 0.05$	W = 0.96, $p > 0.05$
Listonella anguillarum	W = 0.74, $p < 0.001$	W = 0.84, $p < 0.05$	W = 0.93, $p > 0.05$
	0 hours	**3 hours**	**24 hours**	**48 hours**
Control	W = 0.98, $p > 0.05$	W = 0.94, $p > 0.05$	W = 0.95, $p > 0.05$	W = 0.86, $p < 0.05$
Planococcus citreus	W = 0.78, $p < 0.01$	W = 0.92, $p > 0.05$	W = 0.90, $p > 0.05$	W = 0.58, $p < 0.0001$

Table 8.33: Assessment for homogeneity of variance of THC data using Bartlett's test. The variance between the control and bacterial infected *Carcinus maenas* were assessed in each infection trial. Data highlighted in red did not show equal variance.

	0 hours	**3 hours**	**72 hours**
Control v *Listonella anguillarum*	$K^2 = 0.28$, d.f. = 1, $p > 0.05$	$K^2 = 0.09$, d.f. = 1, $p > 0.05$	$K^2 = 0.22$, d.f. = 1, $p > 0.05$
	0 hours	**3 hours**	**24 hours**	**48 hours**
Control v *Planococcus citreus*	$K^2 = 3.80$, d.f. = 1, $p > 0.05$	$K^2 = 0.37$, d.f. = 1, $p > 0.05$	$K^2 = 8.18$, d.f. = 1, $p < 0.01$	$K^2 = 1.46$, d.f. = 1, $p > 0.05$

8.4.4 Assessment of normal distribution and equal variance of bacterial load data

Table 8.34: Assessment of normal distribution of bacterial load using the Shapiro-Wilk test. Red highlights non-normal distribution of data.

	0 hours	**3 hours**	**72 hours**
Control	W = 0.60, $p < 0.0001$	W = 0.53, $p < 0.0001$	W = 0.70, $p < 0.001$
Listonella anguillarum	W = 0.72, $p < 0.001$	W = 0.86, $p < 0.05$	W = 0.79, $p < 0.01$
	0 hours	**3 hours**	**48 hours**
Control	W = 0.61, $p < 0.0001$	W = 0.65, $p < 0.001$	W = 0.87, $p > 0.05$
Planococcus citreus	W = 0.42, $p < 0.00001$	W = 0.90, $p > 0.05$	W = 0.70, $p < 0.001$

Table 8.35: Assessment for homogeneity of variance of bacterial load data using Bartlett's test. The variance between the control and bacterial infected *Carcinus maenas* were assessed in each infection trial. Data highlighted in red did not show equal variance.

	0 hours	**3 hours**	**72 hours**
Control v *Listonella anguillarum*	K^2 = 1.94, d.f. = 1, p >0.05	K^2 = 6.88, d.f. = 1, p <0.05	K^2 = 3.82, d.f. = 1, p >0.05
	0 hours	**3 hours**	**72 hours**
Control v *Planococcus citreus*	K^2 = 0.20, d.f. = 1, p >0.05	K^2 = 7.61, d.f. = 1, p <0.01	K^2 = 2.48, d.f. = 1, p >0.05

Table 8.36: Acclimation and experimental conditions for the *Listonella anguillarum* and *Planococcus citreus* infection trials showing mean salinity, temperature, oxygen concentration and pH values in the tank seawater.

	Day	**Salinity**	**Temperature (°C)**	**O2 (mg/l)**	**% O2**	**pH**
Aquarium	1	30.4	14.3	10.02	/	/
	2	30.8	14.0	10.2	97.7	/
	3	31.0	14.1	9.9	97.3	/
	4	31.0	13.9	10.0	97.6	/
Acclimation	5	31.1	13.8	10.3	98.6	/
	6	31.5	13.8	10.2	99.6	/
	7	30.8	12.2	10.3	97.6	/
	8	30.9	12.2	10.3	99.5	7.9
	9	30.1	12.7	10.0	97.7	/
	10	30.0	12.3	10.2	98.1	/
	11	30.1	12.2	10.3	98.3	/
	12	30.4	12.3	10.3	98.9	/
	13	30.4	12.4	10.5	99.6	/
	14	30.8	11.5	10.9	99.9	/
	15	31.0	12.2	10.8	99.9	8.1
	16	30.5	12.5	10.8	100.1	/
	17	30.3	12.8	10.2	98.7	8.1
	18	30.8	12.1	10.6	100.1	/
Experiment	19	31.3	12.6	10.5	99.3	/
	21	30.4	12.6	10.4	97.2	/

8.4.5 Bacterial load in the control and *Listonella anguillarum* infected *Carcinus maenas*

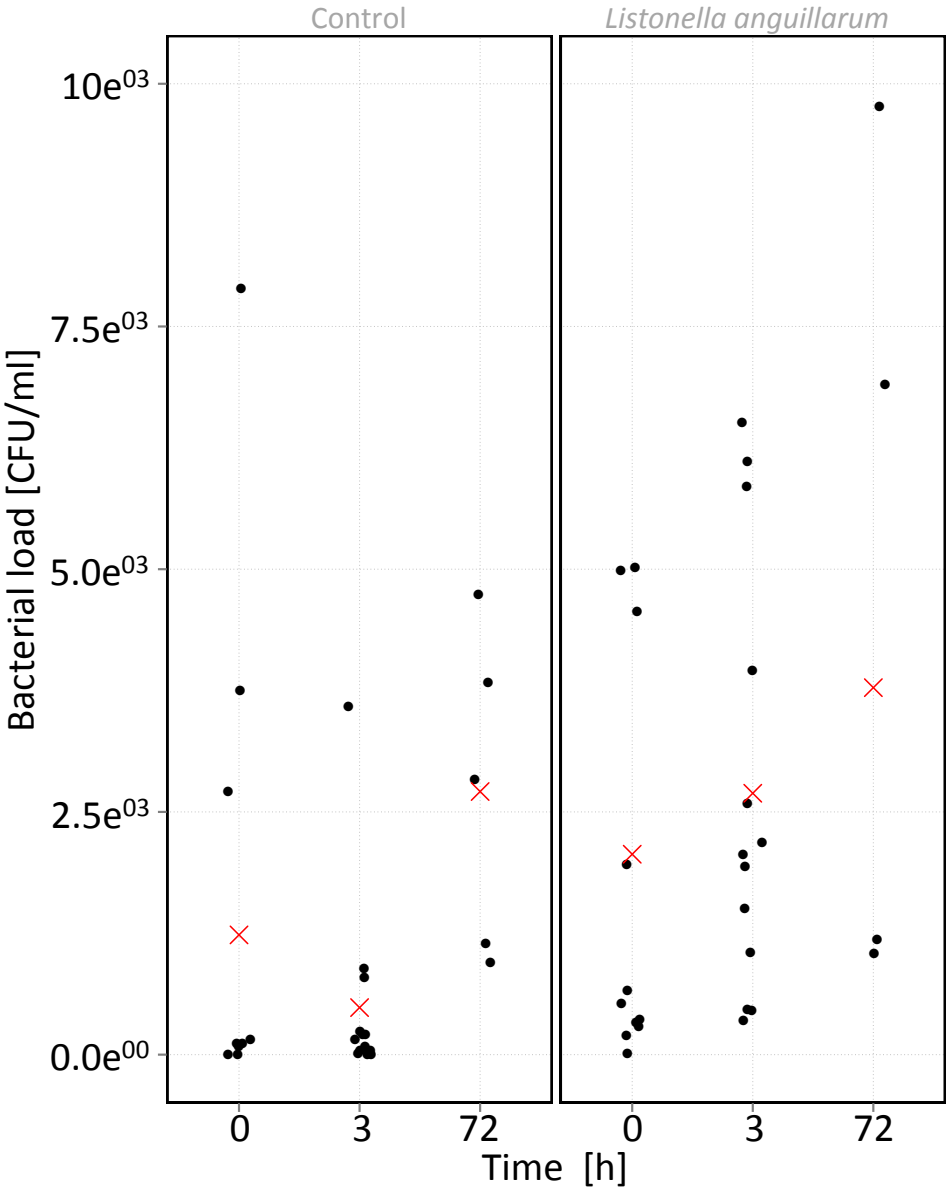
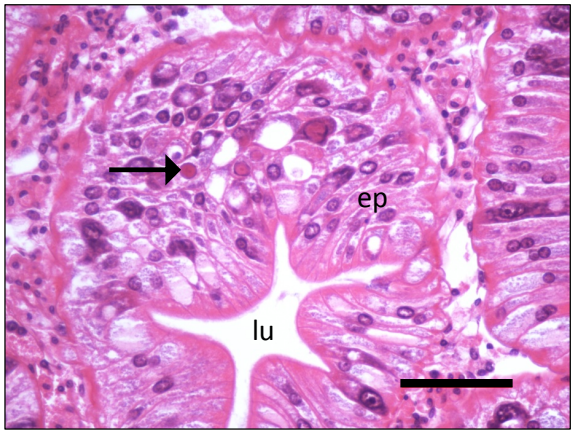


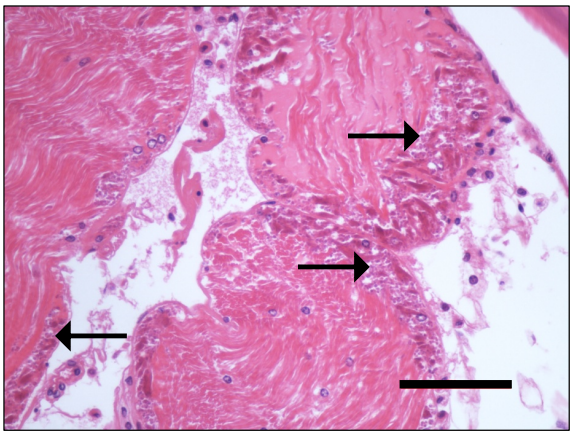
Figure 8.23: Bacterial load between control and *Listonella anguillarum* infected *Carcinus maenas*. Y axis scale changed to illustrate the significant differences (p=<0.001) in bacterial load at 3 hours.

8.5 Chapter 6

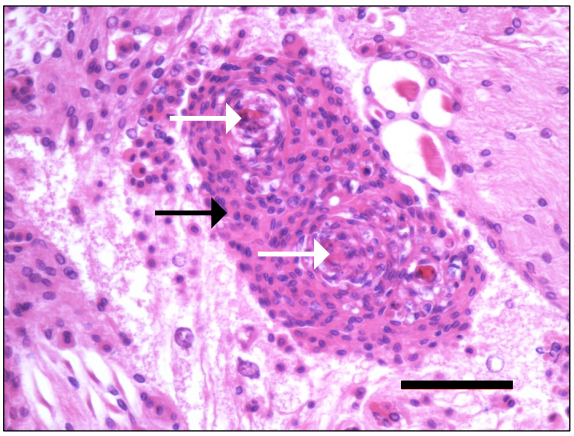
8.5.1 Histology



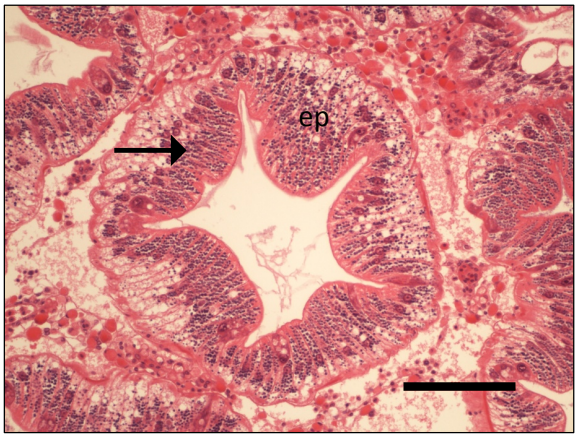
(a) Hepatopancreatic tubule with normal lumen (lu) and presumptive CmBV infection of some epithelial cells (ep) (arrow). Scale bar 100μm. H&E stained.



(b) *Ameson pulvis* infection of the muscle of *Carcinus maenas*. (Reviewed in (Stentiford et al., 2013a)). Microsporidian spores evident infecting the periphery of the musculature (arrows). Scale bar 100μm. H&E stained.



(c) Nodule formation (white arrows) and encapsulation (black arrow) of unidentified pathogen. Scale bar 100μm. H&E stained.



(d) Pre-moult *Carcinus maenas* with calcium inclusion bodies (arrow) within the epithelial cells (ep) of the hepatopancreas. Scale bar 200μm. H&E stained.

Figure 8.24: Histology of hepatopancreatic and connective tissue in *Carcinus maenas* showing (a) viral (CmBV) and (b) microsporidium infection (*Ameson pulvis*), (c) nodule formation and encapsulation by haemocytes and (d) a non-infected pre-moult crab.

8.5.2 Log_{10} CNRQ and RNA quality values

Table 8.37: Log_{10}CNRQ and RNA quality of control samples at 11 hours.

			LOG$_{10}$ CNRQ – ERG's				LOG$_{10}$ CNRQ - GOI		
Treatment	**Sample**	**RQI**	***actin***	***eef1a***	***gapdh***	***tubulin***	***carcinin***	***peroxinectin***	***prophenoloxidase***
Control 11 hours	31	9.3	8.66E^{-02}	6.82E^{-04}	-1.66E^{-01}	7.85E^{-02}	1.63E^{-01}	-1.67E^{-01}	3.75E^{-02}
	32	10	1.22E^{-02}	-5.50E^{-02}	1.06E^{-01}	-6.31E^{-02}	1.36E^{-02}	-1.63E^{-02}	3.19E^{-02}
	33	9.7	3.54E^{-02}	7.46E^{-02}	-1.88E^{-01}	7.76E^{-02}	-1.61E^{-01}	-1.31E^{-01}	-1.08E^{-01}
	34	9.9	1.58E^{-01}	6.75E^{-02}	-1.08E^{-01}	-1.18E^{-01}	-1.54E^{-02}	7.75E^{-02}	7.44E^{-02}
	35	9.2	1.49E^{-02}	1.56E^{-01}	-2.53E^{-02}	-1.46E^{-01}	1.29E^{-01}	2.27E^{-01}	2.21E^{-01}
	36	7.5	5.52E^{-02}	-6.28E^{-03}	1.13E^{-01}	-1.61E^{-01}	-2.78E^{-02}	7.44E^{-02}	-4.09E^{-02}
	37	8.1	-6.62E^{-03}	-5.45E^{-02}	8.22E^{-02}	-2.10E^{-02}	1.03E^{-01}	4.77E^{-02}	1.14E^{-01}
	38	5.4	-1.44E^{-01}	-1.48E^{-02}	7.04E^{-02}	8.88E^{-02}	-1.19E^{-01}	-5.62E^{-03}	8.09E^{-02}
	39	9.3	-1.22E^{-01}	-1.66E^{-01}	-1.57E^{-02}	3.04E^{-01}	-3.08E^{-01}	-2.70E^{-01}	-2.13E^{-01}
	40	9.4	-8.95E^{-02}	-2.31E^{-03}	1.31E^{-01}	-3.95E^{-02}	2.23E^{-01}	1.63E^{-01}	-1.98E^{-01}

Table 8.38: Log_{10}CNRQ and RNA quality of crowding samples at 11 hours.

			LOG$_{10}$ CNRQ – ERG's				LOG$_{10}$ CNRQ - GOI		
Treatment	**Sample**	**RQI**	***actin***	***eef1a***	***gapdh***	***tubulin***	***carcinin***	***peroxinectin***	***prophenoloxidase***
Crowding 11 hours	1	10	-4.39E^{-02}	4.93E^{-02}	5.05E^{-02}	-5.59E^{-02}	-3.34E^{-01}	-1.21E^{-01}	4.13E^{-01}
	2	10	-4.39E^{-02}	1.67E^{-01}	1.11E^{-01}	-1.71E^{-01}	-2.04E^{-01}	7.61E^{-02}	2.20E^{-01}
	3	10	-1.06E^{-01}	8.14E^{-02}	1.57E^{-01}	-1.33E^{-01}	-1.90E^{-01}	1.16E^{-01}	1.26E^{-01}
	4	10	-3.97E^{-02}	1.73E^{-02}	2.66E^{-01}	-2.44E^{-01}	-6.14E^{-01}	-5.96E^{-02}	1.29E^{-02}
	5	9.5	-8.43E^{-02}	9.43E^{-02}	1.57E^{-01}	-1.67E^{-01}	-4.74E^{-01}	1.21E^{-01}	8.09E^{-02}
	6	10	-6.60E^{-02}	-3.54E^{-02}	2.09E^{-01}	-1.07E^{-01}	-1.02E^{-01}	3.57E^{-01}	2.56E^{-01}
	7	10	-4.03E^{-02}	-5.70E^{-02}	2.51E^{-01}	-1.54E^{-01}	5.76E^{-02}	-3.73E^{-02}	9.88E^{-02}
	8	/	-2.36E^{-01}	7.87E^{-02}	2.31E^{-01}	-7.34E^{-02}	-3.42E^{-01}	-1.30E^{-01}	-1.35E^{-01}
	9	/	-1.72E^{-01}	2.99E^{-03}	3.75E^{-01}	-2.07E^{-01}	-1.02E^{00}	-2.44E^{-01}	-3.38E^{-01}

Table 8.39: Log_{10}CNRQ and RNA quality of control samples at 7 days.

			LOG$_{10}$ CNRQ – ERG's				LOG$_{10}$ CNRQ - GOI		
Treatment	**Sample**	**RQI**	***actin***	***eef1a***	***gapdh***	***tubulin***	***carcinin***	***peroxinectin***	***prophenoloxidase***
Control 7 days	21	10	4.33E^{-02}	-3.26E^{-02}	3.63E^{-02}	-4.69E^{-02}	-4.70E^{-02}	2.77E^{-02}	6.41E^{-01}
	22	9.6	-8.77E^{-02}	-2.06E^{-02}	3.45E^{-01}	-2.36E^{-01}	-1.19E^{-01}	-2.01E^{-01}	2.09E^{-01}
	23	8.5	-3.20E^{-02}	1.67E^{-01}	2.74E^{-01}	-4.10E^{-01}	5.30E^{-01}	-1.54E^{-01}	2.70E^{-01}
	24	9.4	-7.26E^{-02}	3.10E^{-03}	3.05E^{-01}	-2.36E^{-01}	-1.20E^{-01}	-1.76E^{-01}	1.53E^{-01}
	25	10	-1.34E^{-01}	5.74E^{-02}	3.06E^{-01}	-2.29E^{-01}	1.52E^{-01}	7.50E^{-02}	2.72E^{-01}
	26	9.7	-4.07E^{-02}	-3.36E^{-02}	1.61E^{-01}	-8.70E^{-02}	-9.17E^{-02}	-1.44E^{-01}	4.16E^{-01}
	27	9.2	-7.00E^{-02}	4.55E^{-02}	1.88E^{-01}	-1.64E^{-01}	-4.21E^{-01}	-1.84E^{-01}	5.39E^{-02}
	28	9.5	-4.19E^{-02}	9.93E^{-02}	1.06E^{-01}	-1.63E^{-01}	-8.49E^{-01}	-3.41E^{-01}	-9.93E^{-02}
	29	10	-1.47E^{-02}	-5.72E^{-03}	1.85E^{-01}	-1.64E^{-01}	-5.50E^{-02}	-1.64E^{-01}	4.29E^{-01}

Table 8.40: Log_{10}CNRQ and RNA quality of crowding samples at 7 days.

			LOG$_{10}$ CNRQ – ERG's				LOG$_{10}$ CNRQ - GOI		
Treatment	**Sample**	**RQI**	***actin***	***eef1a***	***gapdh***	***tubulin***	***carcinin***	***peroxinectin***	***prophenoloxidase***
Crowding 7 days	11	10	-1.35E^{-01}	9.60E^{-02}	1.30E^{-01}	-9.11E^{-02}	5.20E^{-01}	-1.90E^{-01}	2.18E^{-01}
	12	10	-7.32E^{-02}	-6.50E^{-02}	2.29E^{-01}	-9.10E^{-02}	-4.61E^{-01}	-4.39E^{-01}	2.57E^{-01}
	13	10	-1.24E^{-01}	-1.81E^{-02}	1.70E^{-01}	-2.74E^{-02}	-1.27E^{-01}	1.47E^{-01}	6.01E^{-02}
	14	9.3	-1.13E^{-01}	3.63E^{-02}	2.02E^{-01}	-1.26E^{-01}	-2.46E^{-02}	1.12E^{-01}	2.80E^{-01}
	15	10	-1.81E^{-01}	6.93E^{-02}	1.24E^{-01}	-1.23E^{-02}	2.93E^{-01}	-1.57E^{-02}	1.48E^{-01}
	16	10	-9.52E^{-02}	-9.44E^{-02}	2.65E^{-01}	-7.56E^{-02}	1.08E^{-01}	-1.43E^{-01}	2.80E^{-01}
	17	9.0	-3.04E^{-02}	-9.66E^{-02}	1.56E^{-01}	-2.85E^{-02}	-2.69E^{-01}	-3.05E^{-01}	2.76E^{-01}
	18	8.7	-1.31E^{-01}	-1.35E^{-01}	2.79E^{-01}	-1.24E^{-02}	2.92E^{-01}	-4.17E^{-02}	4.87E^{-01}
	19	8.8	-4.12E^{-02}	3.79E^{-02}	5.88E^{-02}	-5.54E^{-02}	2.13E^{-02}	-3.15E^{-01}	1.31E^{-01}
	20	9.4	-5.84E^{-02}	8.13E^{-02}	1.75E^{-01}	-1.98E^{-01}	-6.42E^{-01}	-5.09E^{-02}	4.21E^{-01}

Immune gene expression

Table 8.41: Assessment of normal distribution of gene expression data using the Shapiro-Wilks test. The data is normally distributed.

Immune gene	**Treatment**	**11 hours**	**7 days**
Carcinin	Control	W = 0.98, p >0.05	W = 0.92, p >0.05
	Crowding	W = 0.94, p >0.05	W = 0.98, p >0.05
Peroxinectin	Control	W = 0.97, p >0.05	W = 0.88, p >0.05
	Crowding	W = 0.95, p >0.05	W = 0.96, p >0.05
Prophenoloxidase	Control	W = 0.95, p >0.05	W = 0.99, p >0.05
	Crowding	W = 0.97, p >0.05	W = 0.95, p >0.05

Table 8.42: Assessment of equal variance of gene transcription data using Bartlett's test. Data not displaying equal variance is highlighted in red.

Immune gene	**Treatment**	**11 hours**	**7 hours**
Carcinin	Control v Crowding	K^2 = 3.40, d.f. = 1, p >0.05	K^2 = 0.02, d.f. = 1, p >0.05
Peroxinectin		K^2 = 0.20, d.f. = 1, p >0.05	K^2 = 1.41, d.f. = 1, p >0.05
Prophenoloxidase		K^2 = 1.67, d.f. = 1, p >0.05	K^2 = 2.20, d.f. = 1, p >0.05
		Control	**Crowding**
Carcinin	11 hours v 7 days	K^2 = 5.16, d.f. = 1, p <0.05	K^2 = 0.12, d.f. = 1, p >0.05
Peroxinectin		K^2 = 0.35, d.f. = 1, p >0.05	K^2 = 0.03, d.f. = 1, p >0.05
Prophenoloxidase		K^2 = 1.62, d.f. = 1, p >0.05	K^2 = 2.26, d.f. = 1, p >0.05

THC

Table 8.43: Assessment of normal distribution of gene expression data using the Shapiro-Wilks test. The data is normally distributed.

	Control	**Crowding**
11 hours	W = 0.95, p >0.05	W = 0.93, p >0.05
7 days	W = 0.86, p >0.05	W = 0.89, p >0.05

Table 8.44: Assessment of equal variance of total haemocyte count using Bartlett's test. All data had homogeneous variance.

	11 hours	7 days
Control v Crowding	K2 = 2.94, d.f. = 1, p >0.05	K2 = 0.23, d.f. = 1, p >0.05

Bibliography

Abt, M. C., Osborne, L. C., Monticelli, L. A., Doering, T. A., Alenghat, T., Sonnenberg, G. F., Paley, M. A., Antenus, M., Williams, K. L., Erikson, J., Wherry, E. J., and Artis, D. (2012). Commensal bacteria calibrate the activation threshold of innate antiviral immunity. *Immunity*, 37:158–170.

Adachi, K., Hirata, T., Nishioka, T., and Sakaguchi, M. (2003). Hemocyte components in crustaceans convert hemocyanin into a phenoloxidase-like enzyme. *Comparative Biochemistry and Physiology Part B*, 134:135–141.

Adamo, S. A. (2008). Norepinephrine and octopamine: Linking stress and immune function across phyla. *Invertebrate Survival Journal*, 5:12–19.

Adamo, S. A. (2012). The effects of the stress response on immune function in inverterbrates: An evolutionary perspective on an ancient connection. *Hormones and Behavior*, 62:324–330.

Adamo, S. A., Roberts, J. L., Easy, R. H., and Ross, N. W. (2008). Competition between immune function and lipid transport for the protein apolipophorin III leads to stress-induced immunosuppression in crickets. *Journal of Experimental Biology*, 211:531–538.

Agnalt, A.-L., Kistiansen, T. S., and Jorstad, K. E. (2006). Growth, reproductive cycle, and movement of berried European lobsters (*Homarus gammarus*) in a local stock off southwestern Norway. *ICES Journal of Marine Science*, 64:288–297.

Aiken, D. E. (1980). Molting and Growth. In Cobb, J. S. and Phillips, B. F., editors, *The Biology and Management of Lobsters*, pages 91–163. Academic Press, New York, USA.

Aiken, D. E. and Waddy, S. L. (1986). Environmental influence on recruitment of the American lobster *Homarus americanus*: A perspective. *Canadian Journal of Fisheries and Aquatic Sciences*, 43:2258–2270.

Alderman, D. J. (1996). Geographical spread of bacterial and fungal diseases of crustaceans. *Revue Scientifique et Technique de l'Office International des Opizooties*, 15:603–632.

Algers, B., Blokhuis, H. J., Broom, D. M., Costa, P., Domingo, M., Greiner, M., Guemene, D., Hartung, J., Koenen, F., Muller-Graf, C., Morton, D. B., Osterhaus, A., Pfeiffer, D. U., Roberts, R., Sanaa, M., Salman, M., Sharp, J. M., Vannier, P., and Wierup, M. (2008). Aquatic species susceptible to diseases listed in Directive 2006:88:EC. *The EFSA Journal*, 808:1–144.

Alvarez, R. J. and Koburger, J. A. (1979). *Planococcus citreus* - Its potential for shrimp spoilage. *Proceedings of the Annual Tropical and Subtropical Fisheries Technological Conference of the Americas*, pages 79–90.

Ambion (2012). TURBO DNase treatment and removal reagents. *Life Technologies*, `http://tools.lifetechnologies.com/content/sfs/manuals/cms_055740.pdf`, [Accessed;10/10/2013].

Ameyaw-Akumfi, C. and Hughes, R. N. (1987). Behaviour of *Carcinus maenas* feeding on large *Mytilus edulis*. How do they assess the optimal diet? *Marine Ecology Progress Series*, 38:213–216.

Amparyup, P., Donpudsa, S., and Tassanakajon, A. (2008). Shrimp single WAP domain (SWD)-containing protein exhibits proteinase inhibitory and antimicrobial activities. *Developmental and Comparative Immunology*, 32:1497–1509.

Anderson, K. (2010). Globalization's effects on world agricultural trade, 1960-2050. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, 365:3007–3021.

Anonymous (2004). Projections to 2050. *United Nations Department of Economic and Social Affairs/Population Division*, `http://www.un.org/esa/population/publications/longrange2/WorldPop2300final.pdf`, [Accessed;28/07/2014].

Anonymous (2011). Green grab. `http://beauty-animal.blogspot.co.uk/2011/06/green-crab.html`, [Accessed;10/01/2015].

Antony, S. P., Singh, I. S., Sudheer, N. S., Vrinda, S., Priyaja, P., and Philip, R. (2011a). Molecular characterization of a crustin-like antimicrobial peptide in the giant tiger shrimp, *Penaeus monodon*, and its expression profile in response to various immunostimulants and challenge with WSSV. *Immunobiology*, 216:184–194.

Antony, S. P. P., Philip, R., Joseph, V., and Singh, I. S. B. (2011b). Anti-lipopolysaccharide factor and *crustin-III*, the anti-White Spot Virus peptides in *Penaeus monodon*: Control of viral infection by up-regulation. *Aquaculture*, 319:11–17.

Aparicio-Simòn, B., Piñó, M., Racotta, R., and Racotta, I. S. (2010). Neuroendocrine and metabolic responses of Pacific whiteleg shrimp *Litopenaeus vannamei* exposed to acute handling stress. *Aquaculture*, 298:308–314.

Appleton, P. L. and Vickerman, K. (1996). Presence of apicomplexan-type micropores in a parasitic dinoflagellate, *Hematodinium* sp. *Parasitology Research*, 82:279–282.

Ariki, S., Koori, K., Osaki, T., Motoyama, K., Inamori, K., and Kawabata, S. (2004). A serine protease zymogen functions as a pattern-recognition receptor for lipopolysaccharides. *Proceedings of the National Academy of Sciences of the United States of America*, 101:953–958.

Arnold, K. E., Findlay, H. S., Spicer, J. I., Daniels, C. L., and Boothoyd, D. (2009). Effect of CO_2-related acidification on aspects of the larval development of the European lobster, *Homarus gammarus* (L.). *Biogeosciences Discussions*, 6:3087–3107.

Arslan, D., Legendre, M., Seltzer, V., Abergel, C., and Claverie, J. M. (2011). Distant Mimivirus relative with a larger genome highlights the fundamental features of Megaviridae. *Proceedings of the National Academy of Sciences*, 108:17486–17491.

Ashida, M. and Söderhäll, K. (1984). The prophenoloxidase activating system in crayfish. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry*, 77:21–26.

Aspan, A., Hall, M., and Söderhäll, K. (1990). The effect of endogeneous proteinase inhibitors on the prophenoloxidase activating enzyme, a serine proteinase from crayfish haemocytes. *Insect Biochemistry*, 20:485–492.

Aspan, A. and Söderhäll, K. (1991). Purification of prophenoloxidase from crayfish blood cells, and its activation by an endogenous serine proteinase. *Insect Biochemistry*, 21:363–373.

Audemard, C., Sajus, M.-C., Barnaud, A., Sautour, B., Sauriau, P.-G., and Berthe, F. J. C. (2004). Infection dynamics of *Marteilia refringens* in flat oyster *Ostrea edulis* and copepod *Paracartia grani* in a claire pond of *Marennes-oléron* bay. *Diseases of Aquatic Organisms*, 61:103–111.

Bachère, E., Gueguen, Y., Gonzalez, M., de Lorgeril, J., Garnier, J., and Romestand, B. (2004). Insights into the antimicrobial defense of marine invertebrates-the penaeid shrimps and the oyster *Crassostrea gigas*. *Immunological Reviews*, 198:149–168.

Bachère, E., Mialhe, E., and Rodriguez, J. (1995). Identification of defence effectors in the haemolymph of crustaceans with particular reference to the shrimp *Penaeus japonicus* (Bate): Prospects and applications. *Fish and Shellfish Immunology*, 5:597–612.

Barracco, M. A., Duvic, B., and Söderhäll, K. (1991). The β-l,3-glucan-binding protein from the crayfish *Pacifastacus leniusculus*, when reacted with a β-l,3-glucan, induces spreading and degranulation of crayfish granular cells. *Cell and Tissue Research*, 266:491–497.

Barreda, D. R. and Belosevic, M. (2001). Transcriptional regulation of hemopoeisis. *Developmental and Comparative Immunology*, 25:763–789.

Barrento, S., Marques, A., Teixeira, B., Vaz-Pires, P., and Nunes, M. L. (2009). Nutritional quality of the edible tissues of European lobster *Homarus gammarus* and American lobster *Homarus americanus*. *Journal of Agriculture and Food Chemistry*, 57:3645–3652.

Barrento, S., Marques, A., Vaz-Pires, P., and Nunes, M. L. (2010). Live shipment of immersed crabs *Cancer pagurus* from England to Portugal and recovery in stocking tanks: Stress parameter characterization. *ICES Journal of Marine Science*, 67:435–443.

Bartlett, J. M. S. and Stirling, D. (2003). A short history of the polymerase chain reaction. *Methods in Molecular Biology*, 226:3–6.

Bartlett, T. C., Cuthbertson, B. J., Shepard, E. F., Chapman, R. W., Gross, P. S., and Warr, G. W. (2002). Crustins, homologues of an 11.5-kDa antibacterial peptide, from two species of penaeid shrimp, *Litopenaeus vannamei* and *Litopenaeus setiferus*. *Marine Biotechnology*, 4:278–293.

Barton, B. A. (2002). Stress in fishes: A diversity of responses with particular reference to changes in circulating corticosteroids. *Intergrative and Comparative Biology*, 42:517–525.

Bateman, K. S., Munro, J., Uglow, B., Small, H. J., and Stentiford, G. D. (2012a). Susceptibility of juvenile European lobster *Homarus gammarus* to shrimp products infected with high and low doses of White Spot Syndrome Virus. *Diseases of Aquatic Organisms*, 100:169–184.

Bateman, K. S. and Stentiford, G. D. (2008). *Cancer pagurus* bacilliform virus (*Cp*BV) infecting juvenile European edible crabs *C. pagurus* from UK waters. *Diseases of Aquatic Organisms*, 79:147–51.

Bateman, K. S., Tew, I., French, C., Hicks, R. J., Martin, P., Munro, J., and Stentiford, G. D. (2012b). Susceptibility to infection and pathogenicity of White Spot Disease (WSD) in non-model crustacean host taxa from temperate regions. *Journal of Invertebrate Pathology*, 110:340–351.

Beale, K. M., Towle, D. W., Jayasundara, N., Smith, C. M., Shields, J. D., Small, H. J., and Greenwood, S. J. (2008). Anti-lipopolysaccharide factors in the American lobster *Homarus americanus*: Molecular characterization and transcriptional response to *Vibrio fluvialis* challenge. *Comparative Biochemistry and Physiology. Part D, Genomics and Proteomics*, 3:263–9.

Bean, A. G., Baker, M. L., Stewart, C. R., Cowled, C., Deffrasnes, C., Wang, L. F., and Lowenthal, J. W. (2013). Studying immunity to zoonotic diseases in the natural host - Keeping it real. *Nature Reviews. Immunology*, 13:851–61.

Belak, S. and Ballagi-Pordany, A. (1993). Application of the polymerase chain reaction (PCR) in veterinary diagnostic virology. *Veterinary Research Communications*, 17:55–72.

Bensch, K. W., Raida, M., Hans-Jürgen, M., Schulz-Knappe, P., and Forssmann, W.-G. (1995). hBD-1: A novel β-defensin from human plasma. *FEBS Letters*, 368:331–335.

Bergmann, M., Beare, D. J., and Moore, P. G. (2000). Damage sustained by epibenthic invertebrates discarded in the Nephrops fishery of the Clyde Sea area, Scotland. *Journal of Sea Research*, 45:105–118.

Bernasconi, C. J. and Uglow, R. F. (2008). Effects of emersion and re-immersion on physiological and immunological variables in creel-caught and trawled Norway lobster *Nephrops norvegicus*. *Diseases of Aquatic Organisms*, 81:241–247.

Binggeli, O., Neyen, C., Poidevin, M., and Lemaitre, B. (2014). Prophenoloxidase activation is required for survival to microbial infections in *Drosophila*. *PLOS Pathogens*, 10:e1004067.

Bolinches, J., Toranzo, A. E., Silva, A., and Barja, J. L. (1986). Vibriosis as the main causative factor of heavy mortalities in the oyster culture industry in northwestern Spain. *Bulletin of the European Association of Fish Pathologists*, 6:1–4.

Bostock, J., McAndrew, B., Richards, R., Jauncey, K., Telfer, T., Lorenzen, K., Little, D., Ross, L., Handisyde, N., Gatward, I., and Corner, R. (2010). Aquaculture: Global status and trends. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, 365(1554):2897–912.

Braker, G., Fesefeldt, A., and Witzel, K.-P. (1998). Development of PCR primer systems for amplification of nitrite reductase genes (*nitK* and *nitS*) to detect denitrifying bacteria in environmental samples. *Applied and Environmental Microbiology*, 64:3769–3775.

Breteler, W. C. M. K. (1975). Laboratory experiments on the influence of environmental factors on the frequency of moulting and the increase in size at moulting of juvenile shore crabs, *Carcinus maenas*. *Netherlands Journal of Sea Research*, 9:100–120.

Brockton, V., Hammond, J. A., and Smith, V. J. (2007). Gene characterisation, isoforms and recombinant expression of *carcinin*, an antibacterial protein from the shore crab, *Carcinus maenas*. *Molecular Immunology*, 44:943–949.

Brockton, V. and Smith, V. J. (2008). Crustin expression following bacterial injection and temperature change in the shore crab, *Carcinus maenas*. *Developmental and Comparative Immunology*, 32:1027–33.

Broderick, N. A., Raffa, K. F., and Handelsman, J. (2010). Chemical modulators of the innate immune response after gypsy moth larval susceptibility to *Bacillus thuringiensis*. *BMC Microbiology*, 10:129.

Brogden, K. A. (2005). Antimicrobial peptides: Pore formers or metabolic inhibitors in bacteria? *Nature Reviews Microbiology*, 3:238–250.

Brown, R. J., Galloway, T. S., Lowe, D., Browne, M. A., Dissanayake, A., Jones, M. B., and Depledge, M. H. (2004). Differential sensitivity of three marine invertebrates to copper assessed using multiple biomarkers. *Aquatic Toxicology*, 66:267–278.

Bulet, P., Hetru, C., Dimarcq, J.-L., and Hoffman, D. (1999). Antimicrobial peptides in insects; Structure and function. *Developmental and Comaprative Immunology*, 23:329–344.

Burge, C. A., Mark Eakin, C., Friedman, C. S., Froelich, B., Hershberger, P. K., Hofmann, E. E., Petes, L. E., Prager, K. C., Weil, E., Willis, B. L., Ford, S. E., and Harvell, C. D. (2014). Climate change influences on marine infectious diseases: Implications for management and society. *Annual Review of Marine Science*, 6:249–277.

Burge, E. J., Burnett, L. E., and Burnett, K. G. (2009). Time-course analysis of peroxinectin mRNA in the shrimp *Litopenaeus vannamei* after challenge with *Vibrio campbellii*. *Fish and Shellfish Immunology*, 27:603–609.

Burkholder, J. M., Jr., H. B. G., and Hobbs, C. W. (1995). Fish kills linked to a toxic ambush-predator dinoflagellate: Distribution and environmental conditions. *Marine Ecology Progress Series*, 124:43–61.

Burreson, E. M., Stokes, N. A., and Friedman, C. S. (2000). Increased virulence in an introduced pathogen: *Haplosporidium nelson* (MSX) in the Eastern oyster *Crassostrea virginica*. *Journal of Aquatic Animal Health*, 12:1–8.

Bustin, S. A., Beaulieu, J. F., Huggett, J., Jaggi, R., Kibenge, F. S., Olsvik, P. A., Penning, L. C., and Toegel, S. (2010). MIQE precis: Practical implementation of minimum standard guidelines for fluorescence-based quantitative real-time PCR experiments. *BMC Molecular Biology*, 11:74.

Bustin, S. A., Benes, V., Garson, J., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M. W., Shipley, G., Wittwer, C. T., Schjerling, P., Day, P. J., Abreu, M., Aguado, B., Beaulieu, J. F., Beckers, A., Bogaert, S., Browne, J. A., Carrasco-Ramiro, F., Ceelen, L., Ciborowski, K., Cornillie, P., Coulon, S., Cuypers, A., De Brouwer, S., De Ceuninck, L., De Craene, J., De Naeyer, H., De Spiegelaere, W., Deckers, K., Dheedene, A., Durinck, K., Ferreira-Teixeira, M., Fieuw, A., Gallup, J. M., Gonzalo-Flores, S., Goossens, K., Heindryckx, F., Herring, E., Hoenicka, H., Icardi, L., Jaggi, R., Javad, F., Karampelias, M., Kibenge, F., Kibenge, M., Kumps, C., Lambertz, I., Lammens, T., Markey, A., Messiaen, P., Mets, E., Morais, S., Mudarra-Rubio, A., Nakiwala, J., Nelis, H., Olsvik, P. A., Perez-Novo, C., Plusquin, M., Remans, T., Rihani, A., Rodrigues-Santos, P., Rondou, P., Sanders, R., Schmidt-Bleek, K., Skovgaard, K., Smeets, K., Tabera, L., Toegel, S., Van Acker, T., Van den Broeck, W., Van der Meulen, J., Van Gele, M., Van Peer, G., Van Poucke, M., Van Roy, N., Vergult, S., Wauman, J., Tshuikina-Wiklander, M., Willems, E., Zaccara, S., Zeka, F., and Vandesompele, J. (2013). The need for transparency and good practices in the qPCR literature. *Nature Methods*, 10:1063–1067.

Bustin, S. A., Benes, V., Garson, J. A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M. W., Shipley, G. L., Vandesompele, J., and Wittwer, C. T. (2009). The MIQE guidelines: Minimum information for publication of quantitative real-time PCR experiments. *Clinical Chemistry*, 55:611–622.

Bustin, S. A. and Nolan, T. (2004). Chemistries. In Bustin, S. A., editor, *A-Z of quantitative PCR*, IUL Biotechnology Series, pages 217–278. International University Line, La Jolla, California, USA, 1st edition.

Calfee, C. S., Ware, L. B., Glidden, D. V., Eisner, M. D., Parsons, P. E., Thompson, B. T., and Matthay, M. A. (2011). Use of risk reclassification with multiple biomarkers improves mortality prediction in acute lung injury. *Critical Care Medicine*, 39:711–777.

Calvez, J. C. l. (1987). Location of the shore crab, *Carcinus maenas* L., in the food web of a managed estuary ecosystem. The Rance basin (Brittany, France). *Inv. Pesq. Barcelona*, 51:431–442.

Carlton, J. T. (1989). Man's role in changing the face of the ocean: Biological invasions and implications for conservation of near-shore environments. *Conservation Biology*, 3:265–273.

Carlton, J. T. and Cohen, A. N. (2003). Episodic global dispersal in shallow water marine organisms: The case history of the European shore crabs *Carcinus maenas* and *C. aesstuarii*. *Journal of Biogeography*, 30:1809–1820.

Cassels, F. J., Marchalonis, J., and Vasta, G. R. (1986). Heterogeneous humoral and hemocyte-associated lectins with N-acylaminosugar specificities from the blue crab, *Callinectes sapidus* Rathbun. *Comparative Biochemistry and Physiology Part B*, 85:23–30.

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

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

Cefas (2011). Cefas Stock Status 2011- European lobster (*Homarus gammarus*) in the southwest. *Southwest Lobster Stock Assessment for 2012*, `http://www.cefas.defra.gov.uk/media/580130/lobster%20south%20west%202011.pdf`, [Accessed;24/04/2014].

Cefas (2013). Method for testing crustaceans for White Spot Disease, Yellowhead Disease and Taura Syndrome by histological analysis. *Standard Operating Proceedure, Revision Number 1*, `http://www.crustaceancrl.eu/sops/2013.pdf`, [Accessed;02/01/2014].

Celi, M., Filiciotto, F., Parrinello, D., Buscaino, G., Damiano, M. A., Cuttitta, A., D'Angelo, S., Mazzola, S., and Vazzana, M. (2013). Physiological and agonistic behavioural response of *Procambarus clarkii* to an acoustic stimulus. *Journal of Experimental Biology*, 216:709–718.

Cerenius, L., Bangyeekhun, E., Keyser, P., Söderhäll, I., and Söderhäll, K. (2003). Host prophenoloxidase expression in freshwater crayfish is linked to increased resistance to the crayfish plague fungus, *Aphanomyces astaci*. *Cellular Microbiology*, 5:353–357.

Cerenius, L., Jiravanichpaisal, P., Liu, H.-P., and Söderhäll, K. (2010). Crustacean immunity. *Advances in Experimental Medicine and Biology*, 708:239–259.

Cerenius, L., Lee, B. L., and Söderhall, I. (2008). The proPO system: Pros and cons for its role in invertebrate immunity. *Trends in Immunology*, 29:263–271.

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

Cesar, E., B. Nadala, J., Tapay, L. M., and Loh, P. C. (1998). Characterization of a non-occluded baculovirus-like agent pathogenic to penaeid shrimp. *Diseases in Aquatic Organisms*, 33:221–229.

Chaga, O., Lignell, M., and Söderhall, K. (1995). The haematopoeitic cells of the freshwater crayfish, *Pacifastacus leniusculus*. *Animal Biology*, 4:59–70.

Chang, E. S., Keller, R., and Chang, S. A. (1998b). Quantification of Crustacean Hyperglycemic Hormone by ELISA in hemolymph of the Lobster, *Homarus americanus*, following various stresses. *General and Comparative Endocrinology*, 111:359–366.

Chang, P.-S., Lo, C.-F., Wang, Y.-C., and Kou, G.-H. (1996). Identification of White Spot Syndrome associated baculovirus (WSBV) target organs in the shrimp *Penaeus monodon* by *in situ* hybridization. *Diseases of Aquatic Organisms*, 27:131–139.

Chang, Y. S., Chen, T. C., Liu, W. J., Hwang, J. S., Kou, G. H., and Lo, C. F. (2011). Assessment of the roles of copepod *Apocyclops royi* and bivalve mollusk *Meretrix lusoria* in White Spot Syndrome Virus transmission. *Marine Biotechnology*, 13:909–17.

Chaplin, D. D. (2010). Overview of the immune response. *Journal of Allergy and Clinical Immunology*, 125:S3–23.

Charmandari, E., Tsigos, C., and Chrousos, G. (2005). Endocrinology of the stress response. *Annual Reviews of Physiology*, 67:259–284.

Chen, J. J. (2007). Key aspects of analyzing microarray gene-expression data. *Pharmacogenomics*, 8:473–482.

Chen, J. Y., Chuang, H., Pan, C. Y., and Kuo, C. M. (2005). cDNA sequence encoding an antimicrobial peptide of chelonianin from the tiger shrimp *Penaeus monodon*. *Fish and Shellfish Immunology*, 18:179–183.

Chen, L. (2002). Transcriptional analysis of the DNA polymerase gene of shrimp White Spot Syndrome Virus. *Virology*, 301:136–147.

Chen, L. L., Leu, J. H., Huang, C. J., Chou, C. M., Chen, S. M., Wang, C. H., Lo, C. F., and Kou, G. H. (2002). Identification of a nucleocapsid protein (*VP35*) gene of shrimp White Spot Syndrome Virus and characterization of the motif important for targeting *VP35* to the nuclei of transfected insect cells. *Virology*, 293:44–53.

Chen, S.-C., Lin, Y.-D., Liaw, L.-L., and Wang, P.-C. (2001). *Lactococcus garvieae* infection in the giant freshwater prawn *Macrobranchium rosenbergii* confirmed by polymerase chain reaction and 16S rDNA sequencing. *Diseases of Aquatic Organisms*, 45:45–52.

Cheng, W., Chen, S.-M., Wang, F.-I., Hsu, P.-I., Liu, C.-H., and Chen, J.-C. (2003). Effects of temperature, pH, salinity and ammonia on the phagocytic activity and clearance efficiency of giant freshwater prawn *Macrobrachium rosenbergii* to *Lactococcus garvieae*. *Aquaculture*, 219:111–121.

Cheng, W., Wang, L.-U., and Chen, J.-C. (2005b). Effect of water temperature on the immune response of white shrimp *Litopenaeus vannamei* to *Vibrio alginolyticus*. *Aquaculture*, 250:592–601.

Cherbas, L. and Cherbas, P. (1993). The arthropod initiator: The capsite consensus plays an important role in transcription. *Insect Biochemistry and Molecular Biology*, 23:81–90.

Chien, A., Edgar, D. B., and Trela, J. M. (1976). Deoxyribonucleic acid polymerase from the extreme thermophile *Thermus aquaticus*. *Journal of Bacteriology*, 127:1550–1557.

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

Chou, H.-Y., Huang, C.-Y., Wang, C.-H., Chiang, H.-C., and Lo, C. F. (1995). Pathogenicity of a baculovirus infection causing White Spot Syndrome in cultured penaeid shrimp in Taiwan. *Diseases of Aquatic Organisms*, 23:165–173.

Christie, A. E., Rus, S., Goiney, C. C., Smith, C. M., Towle, D. W., and Dickinson, P. S. (2007). Identification and characterization of a cDNA encoding a crustin-like, putative antibacterial protein from the American lobster *Homarus americanus*. *Molecular Immunology*, 44:3333–3337.

Chrousos, G. P. (1998). Stressors, stress and neuroendocrine integration of the adaptive response. The 1997 Hans Seyle Memorial lecture. *Annals of the New York Academy of Sciences*, 851:311–335.

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

Clark, K. F., Acorn, A. R., and Greenwood, S. J. (2013a). A transcriptomic analysis of American lobster (*Homarus americanus*) immune response during infection with the bumper car parasite *Anophryoides haemophila*. *Developmental and Comparative Immunology*, 40:112–122.

Clark, K. F., Greenwood, S. J., Acorn, A. R., and Byrne, P. J. (2013b). Molecular immune response of the American lobster (*Homarus americanus*) to the White Spot Syndrome Virus. *Journal of Invertebrate Pathology*, 114:298–308.

Clarke, K. R. and Gorley, R. N. (2006). Primer v6: User manual/tutorial. PRIMER-E.

Cohen, A. N., Carlton, J. T., and Fountain, M. C. (1995). Introduction, dispersal and potential impacts of the green crab *Carcinus maenas* in San Francisco Bay, California. *Marine Biology*, 122:225–237.

Cook, T., Folli, M., Klinch, J., Ford, S., and Miller, J. (1998). The relationship between increasing sea-surface temperature and the northward spread of *Perkinsus marinus* (Dermo) disease epizootics in oysters. *Estuarine, Coastal and Shelf Science*, 46:587–597.

Cooper, M. and Herrin, B. (2010). How did our complex immune system evolve? *Nature Reviews Immunology*, 10:2–3.

Copois, V., Bibeau, F., Bascoul-Mollevi, C., Salvetat, N., Chalbos, P., Bareil, C., Candeil, L., Fraslon, C., Conseiller, E., Granci, V., Maziere, P., Kramar, A., Ychou, M., Pau, B., Martineau, P., Molina, F., and Del Rio, M. (2007). Impact of RNA degradation on gene expression profiles: Assessment of different methods to reliably determine RNA quality. *Journal of Biotechnology*, 127:549–59.

Corbel, V., Zuprizal, Shi, Z., Sumartono, Archer, J.-M., and Bonami, J.-R. (2001). Experimental infection of European crustaceans with White Spot Syndrome Virus (WSSV). *Journal of Fish Diseases*, 24:377–382.

Cornish-Bowden, A. (1985). Nomenclature for incompletely specified bases in nucleic acid sequences - recommendations 1984. *Nucleic Acids Research*, 13:3021–3030.

Coyle, S. D., Tidwell, J. H., Danaher, J., Yasharian, D. K., and Bright, L. A. (2006). The effect of biomass density, salinity, and substrate on transport survival of juvenile freshwater prawns *Macrobrachium rosenbergii* in continuously oxygenated, vented containers. *North American Journal of Aquaculture*, 68:271–275.

Cuthbertson, B. J., Shepard, E. F., Chapman, R. W., and Gross, P. S. (2002). Diversity of the penaeidin antimicrobial peptides in two shrimp species. *Immunogenetics*, 54:442–445.

Dall, W. (1974). Indices of nutritional state in the western rock lobster, *Panulirus longipes* (Milne Edwards). I. blood and tissue constituents and water content. *Journal of Experimental Biology and Ecology*, 16:167–180.

Damman, P., Beijk, M. A., Kuijt, W. J., Verouden, N. J., van Geloven, N., Henriques, J. P., Baan, J., Vis, M. M., Meuwissen, M., van Straalen, J. P., Fischer, J., Koch, K. T., Piek, J. J., Tijssen, J. G., and de Winter, R. J. (2011). Multiple biomarkers at admission significantly improve the prediction of mortality in patients undergoing primary percutaneous coronary intervention for acute ST-segment elevation myocardial infarction. *Journal of the American College of Cardiology*, 57:29–36.

Darling, J. A., Bagley, M. J., Roman, J., Tepolt, C. K., and Geller, J. B. (2008). Genetic patterns across multiple introductions of the globally invasive crab genus *Carcinus*. *Molecular Ecology*, 17:4992–5007.

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

de la Vega, E., Hall, M. R., Wilson, K. J., Reverter, A., Woods, R. G., and Degnan, B. M. (2007). Stress-induced gene expression profiling in the black tiger shrimp *Penaeus monodon*. *Physiological Genomics*, 31:126–138.

Decorte, R. and Cassiman, J.-J. (1993). Forensic medicine and the polymerase chain reaction technique. *Journal of Medical Genetics*, 30:625–633.

Deerenberg, C., Arpanius, V., Daan, S., and Bos, N. (1997). Reproductive effort decreases antibody responsiveness. *Proceedings of the Royal Society B*, 264:1021–1029.

Destoumieux, D., Bulet, P., Loew, D., A. van, D., Rodriguez, J., and Bachere, E. (1997). Penaeidins, a new family of antimicrobial peptides isolated from the shrimp *Penaeus vannamei* (Decapoda). *The Journal of Biological Chemistry*, 272:28398–28406.

Destoumieux, D., Bulet, P., Strub, J.-M., van Dorsselaer, A., and Bachere, E. (1999). Recombinant expression and range of activity of penaeidins, antimicrobial peptides from penaeid shrimp. *European Journal of Biochemistry*, 266:335–346.

Destoumieux, D., Muñoz, M., Cosseau, C., Rodriguez, J., Bulet, P., Comps, M., and Bachere, E. (2000). Penaeidins, antimicrobial peptides with chitin-binding activity, are produced and stored in shrimp granulocytes and released after microbial challenge. *Journal of Cell Science*, 113:461–468.

Devine, D. A. and Hancock, R. E. W. (2002). Cationic peptides: Distribution and mechanisms of resistance. *Current Pharmaceutical Design*, 8:703–714.

Dhabhar, F. S. (2002). Stress-induced augmentation of immune function: The role of stress hormones, leukocyte trafficking, and cytokines. *Brain, Behavior, and Immunity*, 16:785–798.

D'Haene, B. and Hellemans, J. (2010). The importance of quality control during qPCR data analysis. *International Drug Discovery*, August/September:18–24.

D'Haene, B., Vandesompele, J., and Hellemans, J. (2010). Accurate and objective copy number profiling using real-time quantitative PCR. *Methods*, 50:262–270.

Diaz, F., Re, A. D., Sanchez, A., Cruz, H., Gonzalez, R. A., Sanchez, L. N., Licea, A., Ponce-Rivas, E., Muniz-Marquez, M. E., Giffard, I., and Rosas, C. (2013). The effects of exposure to critical thermal maxima on the physiological, metabolic and immunological responses in adult white shrimp *Litopennaeus vannamei* (Boone). *Marine and Freshwater Behaviour and Physiology*, 45:365–374.

Dobson, A. (2004). Population dynamics of pathogens with multiple host species. *The American Naturalist*, 164:S64–S78.

Doma, M. K. and Parker, R. (2007). RNA quality control in eukaryotes. *Cell*, 131:660–8.

Doney, S. C., Ruckelshaus, M., Duffy, J. E., Barry, J. P., Chan, F., Englsih, C. A., Galindo, H. M., Grenmeier, J. M., Hollowed, A. B., Knowlton, N., Polovina, J., Rabalais, N. N., Sydeman, W. J., and Talley, L. D. (2012). Climate change impacts on marine ecosystems. *Marine Science*, 4:11–37.

Dong, B., Liu, F., Gao, H., Wang, B., and Xiang, J. (2009). cDNA cloning and gene expression pattern following bacterial challenge of *peroxinectin* in Chinese shrimp *Fenneropenaeus chinensis*. *Molecular Biology Reports*, 36:2333–2339.

Donpudsa, S., Rimphanitchayakit, V., Tassanakajon, A., Söderhäll, I., and Söderhäll, K. (2010). Characterization of two crustin antimicrobial peptides from the freshwater crayfish *Pacifastacus leniusculus*. *Journal of Invertebrate Pathology*, 104:234–238.

d'Or Bocuse (2013). It was a big scoop at the end of 2012: Rougié is getting into lobster! `http://www.bocusedor-winners.com/uk/actualite/was-big-scoop-the-end-2012-rougie-getting-into-lobster,224.html`, [Accessed;05/01/2015].

Dove, A. D. M., Allam, B., Powers, J. J., and Sokolowski, M. S. (2005). A prolonged thermal stress experiment on the American lobster, *Homarus americanus*. *Journal of Shellfish Research*, 24:761–765.

Downs, J. A., Lowndes, N. F., and Jackson, S. P. (2000). A role for *Saccharomyces cerevisiae* histone H2A in DNA repair. *Nature*, 408:1001–1004.

Du, Z.-Q., Qian, R., Huang, A.-M., Fang, W.-H., Zhou, J.-F., Gao, L.-J., and Li, X.-C. (2013). A novel *peroxinectin* involved in antiviral and antibacterial immunity of mud crab, *Scylla paramomosain*. *Molecular Biology Reports*, 40:6873–6881.

Dunoyer, P. and Voinnet, O. (2005). The complex interplay between plant viruses and host RNA-silencing pathways. *Current Opinion in Plant Biology*, 8:415–23.

Durand, S., Lightner, D. V., Nunan, L. M., Redman, R. M., Mari, J., and Bonami, J.-R. (1996). Application of gene probes as diagnostic tools for White Spot Baculovirus (WSBV) of penaeid shrimp. *Diseases of Aquatic Organisms*, 27:59–66.

Durand, S., Lightner, D. V., Redman, R. M., and Bonami, J.-R. (1997). Ultrastructure and morphogenesis of White Spot Syndrome Baculovirus (WSSV). *Diseases of Aquatic Organisms*, 29:205–211.

Durand, S. V., Redman, R. M., Mohney, L., Tang-Nelson, K., Bonami, J. R., and Lightner, D. V. (2003). Qualitative and quantitative studies on the relative virus load of tails and heads of shrimp acutely infected with WSSV. *Aquaculture*, 216:9–18.

Durand, S. V., Tang, K. F. J., and Lightner, D. V. (2000). Frozen commodity shrimp: Potential avenue for introduction of White Spot Syndrome Virus and Yellow Head Virus. *Journal of Aquatic Animal Health*, 12:128–135.

Eddy, F. Powell, A., Gregory, S., Nunan, L. M., Lightner, D. V., Dyson, P. J., Rowley, A. F., and Shields, R. J. (2007). A novel bacterial disease of the European shore crab, *Carcinus maenas* molecular pathology and epidemiology. *Microbiology*, 153:2839–2849.

Edgerton, B. (1996). A new bacilliform virus in Australian *Cherax destructor* (Decapoda-Parastacidae) with notes on *Cherax quadricarinatus* bacilliform virus (Cherax baculovirus). *Diseases of Aquatic Organisms*, 27:43–52.

Eleftherianos, I., Baldwin, H., ffrench Constant, R. H., and Reynolds, S. E. (2008). Developmental modulation of immunity: Changes within the feeding period of the fifth larval stage in the defence reactions of *Manduca sexta* to infection by *Photorhabdus*. *Journal of Insect Physiology*, 54:309–318.

Elner, R. W. and Hughes, R. N. (1978). Energy maximisation in the diet of the shore crab, *Carcinus maenas*. *Journal of Animal Ecology*, 47:103–116.

Elston, R. A., Hasegawa, H., Humphrey, K. L., Polyak, I. K., and Hase, C. C. (2008). Re-emergence of *Vibrio tubiashii* in bivalve shellfish aquaculture: Severity, environmental drivers, geographic extent and management. *Diseases of Aquatic Organisms*, 82:119–134.

Engelhardt, M. A., Daly, K., Swannell, R. P. J., and Head, I. M. (2001). Isolation and characterization of a novel hydrocarbon-degrading, Gram-positive bacterium, isolated from intertidal beach sediment, and description of *Planococcus alkanoclasticus* sp. nov. *Journal of Applied Microbiology*, 90:237–247.

Escobedo-Bonilla, C. M., Alday-Sanz, V., WIlle, M., Sorgeloos, P., and Pensaert, M. B. (2008). A review on the morphology, molecular characterization, morphogenesis and pathogenesis of White Spot Syndrome Virus. *Journal of Fish Diseases*, 31:1–18.

EURL (2008). White Spot Disease. *European Community Reference Laboratory for Crustacean Diseases, Weymouth, UK*, `http://www.crustaceancrl.eu/diseases/WhiteSpot.pdf`, [Accessed;02/02/2014].

European Union (2006). Council Directive 2006:88:EC. *Official Journal of the European Union*, 49:14–56.

Fall, J., Kono, T., Tanekhy, M., Itami, T., and Sakai, M. (2010). Expression of innate immune-related genes of Kuruma shrimp, *Marsupenaeus japonicus*, after challenge with *Vibrio nigripulchritudo*. *African Journal of Microbiology Research*, 4:2426–2433.

FAO (2014). Global capture production. *Food and Agriculture Organization of the United Nations*, `http://www.fao.org/fishery/statistics/global-capture-production/en`, [Accessed;07/08/2014].

Farrow, J. A. E., Wallbanks, S., and Collins, M. D. (1994). Phylogenetic interrelationships of round-spore-forming bacilli containing cell walls based on lysine and the non-spore forming genera, *Caryophanon*, *Exiguobacterium*, *Kurthia* and *Planococcus*. *International Journal of Systematic Bacteriology*, 44:74–82.

Fernandez-Capetillo, O., Lee, A., Nussenzweig, M., and Nussenzweig, A. (2004). H2AX: The histone guardian of the genome. *DNA Repair*, 3:959–67.

Field, R. H. and Appleton, P. L. (1995). A *Hematodinium*-like dinoflagellate infection of the Norway lobster *Nephrops norvegicus* - observations on pathology and progression of infection. *Diseases of Aquatic Organisms*, 22:115–128.

Filee, J. and Chandler, M. (2008). Convergent mechanisms of genome evolution of large and giant DNA viruses. *Research in Microbiology*, 159:325–31.

Fillingham, J., Keogh, M. C., and Krogan, N. J. (2006). γH2AX and its role in DNA double-stranded break repair. *Biochemistry and Cell Biology*, 84:568–577.

Findley, A. M., Jr., E. W. B., and Weidner, E. H. (1981). *Ameson michaelis* (Microsporidia) in the blue crab, *Callinectes sapidus*: Parasite-induced alterations in the biochemical composition of host tissues. *Biological Bulletin*, 161:115–125.

Fleige, S. and Pfaffl, M. W. (2006). RNA integrity and the effect on the real-time qRT-PCR performance. *Molecular Aspects of Medicine*, 27:126–39.

Foster, E. R. and Downs, J. A. (2005). Histone H2A phosphorylation in DNA double-strand break repair. *Federation of the Societies of Biochemistry and Molecular Biology*, 272:3231–3240.

Fotedar, S. and Evans, L. (2011). Health management during handling and live transport of crustaceans: A review. *Journal of Invertebrate Pathology*, 106:143–52.

Fotedar, S., Evans, L., and Jones, B. (2006). Effect of holding duration on the immune system of western rock lobster, *Panulirus cygnus*. *Comparative Biochemistry and Physiology. Part A, Molecular and Integrative Physiology*, 143:479–87.

Francki, R. I., Fauquet, C. M., Knudson, D. L., and Brown, F. (1991). Classification and nomenclature of viruses. In *Archives of Virology, Supplementa*. Springer-Verlag, Vienna, 5th edition.

Frans, I., Michiels, C. W., Bossier, P., Willems, K. A., Lievens, B., and Rediers, H. (2011). *Vibrio anguillarum* as a fish pathogen: Virulence factors, diagnosis and prevention. *Journal of Fish Diseases*, 34:643–661.

Frohlich, E., Schlagenhauff, B., Mohrle, M., Weber, E., Klessen, C., and Rassner, G. (2001). Activity, expression, and transcription rate of the cathepsins B, D, H, and L in cutaneous malignant melanoma. *Cancer*, 91:972–982.

Ganal, S. C., Sanos, S. L., Kallfass, C., Oberle, K., Johner, C., Kirschning, C., Lienenklaus, S., Weiss, S., Staeheli, P., Aichele, P., and Diefenbach, A. (2012). Priming of natural killer cells by nonmucosal mononuclear phagocytes requires instructive signals from commensal microbiota. *Immunity*, 37:171–86.

Gao, H., Li, F., Dong, B., Zhang, Q., and Xiang, J. (2009). Molecular cloning and characterisation of *prophenoloxidase* (ProPO) cDNA from *Fenneropenaeus chinensis* and its transcription injected by *Vibrio anguillarum*. *Molecular Biology Reports*, 36:1159–1166.

Garcia-Garcia, E., Galindo-Villegas, J., and Mulero, V. (2013). Mucosal immunity in the gut: The non-vertebrate perspective. *Developmental and Comparative Immunology*, 40:278–288.

Gaylord, B. and Gaines, S. D. (2000). Temperature or transport? Range limits in marine species mediated solely by flow. *The American Naturalist*, 155:769–789.

Ghosh, J., Lun, C. M., Majeske, A. J., Sacchi, S., Schrankel, C. S., and Smith, L. C. (2011). Invertebrate immune diversity. *Developmental and Comparative Immunology*, 35:959–974.

Gillespie, J. P., Kanost, M. R., and Trenczek, T. (1997). Biological mediators of insect immunity. *Annual Review of Entomology*, 42:611–643.

Ginzinger, D. G. (2002). Gene quantification using real-time quantitative PCR - An emerging technology hits the mainstream. *Experimental Haemotology*, 30:503–512.

Gismondi, E., Rigaud, T., Beisel, J.-N., and Cossu-Leguille, C. (2012). Effect of multiple parasitic infections on the tolerance to pollutant contamination. *PLoS One*, 7:e41950.

Gjerde, J. (1984). Occurrence and characterization of *Aerococcus viridans* from lobsters, *Homarus gammarus* L., dying in captivity. *Journal of Fish Diseases*, 7:355–362.

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

Glenner, H. and Werner, M. (1998). Increased susceptibility of recently moulted *Carcinus maenas* (L.) to attack by the parasitic barnacle *Sacculina carcini* Thompson 1836. *Journal of Experimental Biology and Ecology*, 228:29–33.

Glittenberg, M. T., Kounatidis, I., Christensen, D., Kostov, M., Kimber, S., Roberts, I., and Ligoxygakis, P. (2011). Pathogen and host factors are needed to provoke a systemic host response to gastrointestinal infection of *Drosophila* larvae by *Candida albicans*. *Disease Models and Mechanisms*, 4:515–525.

Glude, J. B. (1955). The effects of temperature and predators on the abundance of the soft-shell clam, *Mya arenaria*, in New England. *Transactions of the American Fisheries Society*, 84:13–26.

Gomez-Jimenez, S., Uglow, R. F., and Gollas-Galvan, T. (2000). The effects of cooling and emersion on total haemocyte count and *phenoloxidase* activity of the spiny lobster *Panulirus interruptus*. *Fish and Shellfish Immunology*, 10:631–635.

GoogleEarth (2014). Weymouth, UK. `https://www.google.com/maps/place/Weymouth,+Dorset,+UK/@50.6079515,-2.4497135`, [Accessed;04/04/2014].

Gordon, S., Todd, J., and Cohn, Z. A. (1974). *In vitro* synthesis and secretion of lysozyme by mononuclear phagocytes. *Journal of Experimental Medicine*, 139:1228–1248.

Gotto, R. V. (1954). A copepod new to the British isles and others hitherto unrecorded from Irish coastal waters. *The Irish Naturalists' Journal*, 11:133–135.

Green, B. S., Gardner, C., Hochmuth, J. D., and Linnane, A. (2014). Environmental effects on fished lobsters and crabs. *Reviews in Fish Biology and Fisheries*, 24:613–638.

Greenwood, S. J., Deprés, B. M., Cawthorn, R. J., Lavallée, J., Groman, D. B., and Desbarats, A. (2005). Case report - Outbreak of bumper car disease caused by *Anophryoides haemophila* in a lobster holding facility in Nova Scotia, Canada. *Journal of Aquatic Animal Health*, 17:345–352.

Grosholz, E. D. and Ruiz, G. M. (1995). Spread and potential impact of the recently introduced European green crab, *Carcinus maenas*, in central California. *Marine Biology*, 122:239–247.

Grosholz, E. D., Ruiz, G. M., Dean, C. A., Shirley, K. A., Maron, J. L., and Connors, P. G. (2000). The impacts of a nonindigenous marine predator in a California Bay. *Ecology*, 81:1206–1224.

Grunstein, M. (1990). Histone function in transcription. *Annual Review of Cell Biology*, 6:643–678.

Guhaniyogi, J. and Brewer, G. (2001). Regulation of mRNA stability in mammalian cells. *Gene*, 265:11–23.

Hall, M., Wang, R., van Antwerpen, R., Sottrup-Jensen, L., and Söderhäll, K. (1999). The crayfish plasma clotting protein-A vitellogenin-related protein responsible for clot formation in crustacean blood. *Proceedings of the National Academy of Sciences, USA*, 96:1965–1970.

Hall, M. R. (1998). The effects of different types of stress on blood glucose in the giant tiger prawn *Penaeus monodon*. *Journal of the World Aquaculture Society*, 29:290–299.

Hamilton, K. M., Shaw, P. W., and Morritt, D. (2009). Prevalence and seasonality of *Hematodinium* (Alveolata- Syndinea) in a Scottish crustacean community. *ICES Journal of Marine Science*, 66:1837–1845.

Han-Ching Wang, K., Tseng, C. W., Lin, H. Y., Chen, I. T., Chen, Y. H., Chen, Y. M., Chen, T. Y., and Yang, H. L. (2010). RNAi knock-down of the *Litopenaeus vannamei* Toll gene (LvToll) significantly increases mortality and reduces bacterial clearance after challenge with *Vibrio harveyi*. *Developmental and Comparative Immunology*, 34:49–58.

Hancock, R. E. W. (2001). Cationic peptides: Effectors in innate immunity and novel antimicrobials. *The Lancet Infectious Diseases*, 1:156–164.

Hardy, K. M., Burnett, K. G., and Burnett, L. E. (2013). Effect of hypercapnic hypoxia and bacterial infection (*Vibrio campbellii*) on protein synthesis rates in the Pacific whiteleg shrimp, *Litopenaeus vannamei*. *American Journal of Physiology: Regulatory, Integrative and Comparative Physiology*, 305:R1356–66.

Harley, C. D. G., Hughes, A. R., Hultgren, K. M., Miner, B. G., Sorte, C. J. B., Thornber, C. S., Rodriguez, L. F., Tomanek, L., and Williams, S. L. (2006). The impacts of climate change in coastal marine systems. *Ecological Letters*, 9:228–241.

Harvell, C. D. (1999). Emerging marine diseases: Climate links and anthropogenic factors. *Science*, 285:1505–1510.

Harvell, D., Aronson, R., Baron, N., Connell, J., Dobson, A., Ellner, S., Gerber, L., Kim, K., Kuris, A., McCallum, H., Lafferty, K., McKay, B., Porter, J., Pascual, M., Smith, G., Sutherland, K., and Ward, J. (2004). The rising tide of ocean diseases: Unsolved problems and research priorities. *Frontiers in Ecology and the Environment*, 2:375–382.

Hasson, K. W., Fan, Y., Reisinger, T., Venuti, J., and Varner, P. (2006). White Spot Syndrome Virus (WSSV) introduction into the Gulf of Mexec and Texas freshwater systems through imported, frozen bait-shrimp. *Diseases of Aquatic Organisms*, 71:91–100.

Hauton, C. (2012). The scope of the crustacean immune system for disease control. *Journal of Invertebrate Pathology*, 110:251–260.

Hauton, C., Brockton, V., and Smith, V. J. (2006). Cloning of a crustin-like, single whey-acidic-domain, antibacterial peptide from the haemocytes of the European lobster, *Homarus gammarus*, and its response to infection with bacteria. *Molecular Immunology*, 43:1490–1496.

Hauton, C., Brockton, V., and Smith, V. J. (2007). Changes in immune gene expression and resistance to bacterial infection in lobster (*Homarus gammarus*) post-larval stage VI following acute or chronic exposure to immune stimulating compounds. *Molecular Immunology*, 44:443–450.

Hauton, C., Hudspith, M., and Gunton, L. (2015). Future prospects for prophylactic immune stimulation in crustacean aquaculture - the need for improved metadata to address immune system complexity. *Developmental and Comparative Immunology*, 48:360–368.

Hauton, C. and Smith, V. J. (2004). *In vitro* cytotoxicity of crustacean immunostimulants for lobster (*Homarus gammarus*) granulocytes demonstrated using the neutral red uptake assay. *Fish and Shellfish Immunology*, 17:65–73.

Hauton, C., Williams, J. A., and Hawkins, L. E. (1997). The effects of a live *in vivo* pathogenic infection on aspects of the immunocompetence of the common shore crab, *Carcinus maenas* (L.). *Journal of Experimental Marine Biology and Ecology*, 211:115–128.

He, F. and Kwang, J. (2008). Identification and characterization of a new E3 ubiquitin ligase in White Spot Syndrome Virus involved in virus latency. *Virology Journal*, 5:151.

Hebel, D. K., Jones, M. B., and Depledge, M. H. (1997). Responses of crustaceans to contaminant exposure: A holistic approach. *Estuarine, Coastal and Shelf Science*, 44:177–184.

Hellemans, J., Mortier, G., De Paepe, A., Speleman, F., and Vandesompele, J. (2007). qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Genome biology*, 8:R19.

Hennig, O. L. and Andreatta, E. R. (1998). Effect of temperature in an intensive nursery system for *Penaeus paulensis* (Perez Farfante, 1967). *Aquaculture*, 164:167–172.

Hergenhahn, H.-G., Aspan, A., and Soderhall, K. (1987). Purification and characterization of a high-M_r proteinase inhibitor of pro-phenol oxidase activation from crayfish plasma. *Biochemical Journal*, 248:223–228.

Hergenhahn, H.-G., Hall, M., and Soderhall, K. (1988). Purification and characterization of an α_2-macroglobulin-like proteinase inhibitor from plasma of the crayfish *Pacifastacus leniusculus*. *Biochemical Journal*, 255:801–806.

Hernroth, B., Baden, S. P., Holm, K., Andre, T., and Soderhall, I. (2004). Manganese induced immune suppression of the lobster, *Nephrops norvegicus*. *Aquatic Toxicology*, 70:223–231.

Hidalgo, F. J., Baron, P. J., and Orensanz, J. M. (2005). A prediction come true: The green crab invades the Patagonian coast. *Biological Invasions*, 7:547–552.

Hirano, M., Das, S., Guo, P., and Cooper, M. D. (2011). Chapter 4 - The evolution of adaptive immunity in vertebrates. *Advances in Immunology*, 109:125–157.

Høeg, J. T. (1995). The biology and life cycle of the Rhizocephalan (Cirripedia). *Journal of the Marine Biological Association of the UK*, 75:517–550.

Holman, J. D., Burnett, K. G., and Burnett, L. E. (2004). Effects of hypercapnic hypoxia on the clearance of *Vibrio campbellii* in the Atlantic Blue Crab, *Callinectes sapidus*. *Biological Bulletin*, 206:188–196.

Holt, J., Wakelin, S., Lowe, J., and Tinker, J. (2010). The potential impacts of climate change on the hydrography of the northwest European continental shelf. *Progress in Oceanography*, 86:361–379.

Hose, J. E., Martin, G. C., Tiu, S., and McKrell, N. (1992). Pattern of hemocyte production and release throughout the moult cycle in the penaeid shrimp *Sicyonia ingentis*. *Biological Bulletin*, 183:185–199.

Howard, A. E. and Bennett, D. B. (1979). The substrate preference and burrowing behaviour of juvenile lobsters (*Homarus gammarus* L.). *Journal of Natural History*, 13:433–438.

Howard, J., Babij, E., Griffis, R. B., Helmuth, A., Himes-Cornell, P., and Petes, L. (2013). Ocean and marine resources in a changing climate. *Oceanography and Marine Biology: An Annual Review*, 51:71–192.

Huang, C.-H., Zhang, L.-R., Zhang, J.-H., Xiao, L.-C., Wu, Q.-J., Chen, D.-H., and Li, J. K. K. (2001). Purification and characterization of White Spot Syndrome Virus (WSSV) produced in an alternate host: Crayfish, *Cambarus clarkii*. *Virus Research*, 76:115–125.

Huang, H. W. (2000). Action of antimicrobial peptides: Two-state model. *Biochemistry*, 39:8347–8352.

Hulka, B. S. (1990). Overview of biological markers. In Hulka, B. S., Griffith, J. D., and Wilcosky, T. C., editors, *Biological Markers in Epidemiology*, pages 3–15. Oxford University Press, New York.

Ichinohe, T., Pang, I. K., Kumamoto, Y., R.Peaper, D., Ho, J. H., Murray, T. S., and Iwasaki, A. (2011). Microbiota regulates immune defense against respiratory tract influenza A virus infection. *Proceedings of the National Academy of Sciences*, 108:5354–5359.

Imjongjirak, C., Amparyup, P., Tassanakajon, A., and Sittipraneed, S. (2007). Antilipopolysaccharide factor (ALF) of mud crab *Scylla paramamosain*: Molecular cloning, genomic organization and the antimicrobial activity of its synthetic LPS binding domain. *Molecular Immunology*, 44:3195–3203.

Itami, T., Asano, M., Tokushige, K., Kubono, K., Nakagawa, A., Takeno, N., Nishimura, H., Maeda, M., Kondo, M., and Takahashi, Y. (1998). Enhancement of disease resistance of kuruma shrimp, *Penaeus japonicus*, after oral administration of peptidoglycan derived from *Bifidobacterium thermophilum*. *Aquaculture*, 164:277–288.

IUCN (2013). The IUCN Red List of Threatened Species™. *Red List*, `http://www.iucnredlist.org/details/169955/0`, [Accessed;08/08/2014].

Iwanaga, S., Kawabata, S., and Muta, T. (1998). New types of factors and defense molecules found in horseshoe crab hemolymph, their structures and functions. *Journal of Biochemistry*, 123:1–15.

Iwanaga, S. and Lee, B. L. (2005). Recent advances in the innate immunity of invertebrate animals. *Journal of Biochemistry and Molecular Biology*, 38:128–150.

Iwanaga, S., Miyata, T., Tokunaga, F., and Muta, T. (1992). Molecular mechanisms of hemolymph clotting in *Limulus*. *Thrombosis Research*, 68:1–32.

Jacklin, M. and Combes, J. (2007). The good practice guide to handling storing live Crustacea. *Seafish*, `http://www.seafish.org/media/Publications/CrustaceaGPG_0505.pdf`, [Accessed;10/09/2014].

Janeway Jr., C. A., Travers, P., and Walport, M. (2012). The adaptive immune response. In Murphy, K., editor, *Immunobiology*, pages 335–428. Garland Science, New York, 8th edition.

Jiménez-Vega, F., Yepiz-Plascencia, G., Söderhäll, K., and Vargas-Albores, F. (2004). A single WAP domain-containing protein from *Litopenaeus vannamei* hemocytes. *Biochemical and Biophysical Research Communications*, 314:681–687.

Jiravanichpaisal, P., Lee, B. L., and Söderhäll, K. (2006). Cell-mediated immunity in arthropods: Hematopoiesis, coagulation, melanization and opsonization. *Immunobiology*, 211:213–36.

Jiravanichpaisal, P., Lee, S. Y., Kim, Y.-A., Andren, T., and Söderhäll, I. (2007). Antibacterial peptides in hemocytes and hematopoietic tissue from freshwater crayfish *Pacifastacus leniusculus*: Characterization and expression pattern. *Developmental and Comparative Immunology*, 31:441–455.

Johansson, M. W., Hind, M. I., Holmblad, T., Thönqvist, P.-O., and Söderhäll, K. (1995). Peroxinectin, a novel cell adhesion protein from crayfish blood. *Biochemical and Biophysical Research Communications*, 216:1079–1087.

Johansson, M. W., Holmblad, T., Thörnqvist, P. O., Cammarata, M., Parrinello, N., and Söderhäll, K. (1999). A cell-surface superoxide dismutase is a binding protein for peroxinectin, a cell-adhesive peroxidase in crayfish. *Journal of Cell Science*, 112:917–925.

Johansson, M. W., Keyser, P., Sritunyalucksana, K., and Söderhäll, K. (2000). Crustacean haemocytes and haematopoiesis. *Aquaculture*, 191:45–52.

Johansson, M. W. and Söderhäll, K. (1985). Exocytosis of the prophenoloxidase activating system from crayfish haemocytes. *Journal of Comparative Physiology B: Biochemical, Systemic and Environmental Physiology*, 156:175–181.

Johansson, M. W. and Söderhäll, K. (1988). Isolation and purification of a cell adhesion factor from crayfish blood cells. *The Journal of Cell Biology*, 106:1795–1803.

Johansson, M. W. and Söderhäll, K. (1989). Cellular immunity in crustaceans and the proPO system. *Parasitology Today*, 5:171–176.

Johnson, N. G., Burnett, L., and Burnett, K. G. (2011). Properties of bacteria that trigger hemocytopenia in the Atlantic Blue Crab, *Callinectes sapidus*. *Biological Bulletin*, 221:164–175.

Johnson, P. T. (1976). Bacterial infection in the blue crab, *Callinectes sapidus*: Course of infection and histopathology. *Journal of Invertebrate Pathology*, 28:25–36.

Johnson, P. T. (1978). Viral diseases of the blue crab, *Calllinecfes sapidus*. *Marine Fisheries Review*, 40:13–15.

Jørstad, K. E., Prodöhl, P. A., Agnalt, A.-L., Hughes, M., Apostolidis, A. P., Triantafyllidis, A., Farestveit, E., Kristiansen, T. S., Mercer, J., and Svasland, T. (2004). Sub-arctic populations of European lobster, *Homarus gammarus*, in northern Norway. *Environmental Biology of Fishes*, 69:223–231.

Jussila, J., Jago, J., Tsvetnenko, E., Dunstan, B., and Evans, L. H. (1997). Total and differential haemocyte counts in western rock lobsters (*Panulirus cygnus* George) under post-harvest stress. *Marine and Freshwater Research*, 48:863–867.

Jussila, J., McBride, S., Jago, J., and Evans, L. H. (2001). Hemolymph clotting time as an indicator of stress in western rock lobster (*Panulirus cygnus* George). *Aquaculture*, 199:185–193.

Jussila, J., Paganini, M., Mansfield, S., and Evans, L. H. (1999). On physiological responses, plasma glucose, total hemocyte counts and dehydration of Marron *Cherax tenuimanus* (Smith) to handling and transportation under simulated conditions. *Freshwater Crayfish*, 12:154–167.

Kadowaki, T., Inagawa, H., Kohchi, C., Nishizawa, T., Takahashi, Y., and Soma, G.-I. (2011). Anti-lipopolysaccharide factor evokes indirect killing of virulent bacteria in kuruma prawn. *In Vivo*, 25:741–744.

Karvonen, A., Rintamaki, P., Jokela, J., and Valtonen, E. T. (2010). Increasing water temperature and disease risks in aquatic systems: Climate change increases the risk of some, but not all, diseases. *International Journal for Parasitology*, 40:1483–1488.

Kautsky, N., Roönnbäck, P., Tedengren, M., and Troell, M. (2000). Ecosystem perspectives on management of disease in shrimp pond farming. *Aquaculture*, 191:145–161.

Kawasaki, H., Nose, T., Muta, T., Iwanaga, S., Shimohigashi, Y., and Kawabata, S. (2000). Head-to-tail polimerization of coagulin, a clottable protein of the horseshoe crab. *Journal of Biological Chemistry*, 275:35297–35301.

Kearney, J. (2010). Food consumption trends and drivers. *Philosophical transactions of the Royal Society of London. Series B, Biological Sciences*, 365:2793–807.

Kinnebrew, M. A. and Pamer, E. G. (2012). Innate immune signaling in defense against intestinal microbes. *Immunological Reviews*, 245:113–131.

Kleppe, K., Ohtsuka, E., Kleppe, R., Molineux, I., and Khorana, H. G. (1971). Studies on polynucleotides: XCVI. Repair replication of short synthetic DNA's as catalyzed by DNA polymerase. *Journal of Molecular Biology*, 56:341–361.

Ko, C. F., Chiou, T. T., Vaseeharan, B., Lu, J. K., and Chen, J. C. (2007). Cloning and characterisation of a *prophenoloxidase* from the haemocytes of mud crab *Scylla serrate*. *Developmental and Comparative Immunology*, 31:12–22.

Kobayashi, M., Johansson, M. W., and Söderhäll, K. (1990). The 76 kD cell-adhesion factor from crayfish haemocytes promotes encapsulation *in vitro*. *Cell Tissue Research*, 260:13–18.

Kromer, E., Nakakura, N., and Lagueux, M. (1994). Cloning of a Locusta cDNA encoding a precursor peptide for two structurally related proteinase inhibitors. *Insect Biochemistry and Molecular Biology*, 24:329–331.

Kubaryk, J. and Harper, C. (2001). Optimizing waterless shipping conditions for *Macrobrachium rosenbergii*. *Marketing and Shipping Live Aquatic Products*, University of Alaska Sea Grant:Report No AK–SG–01–03.

Kuris, A. M. (2005). Trophic transmission of parasites and host behavior modification. *Behavioural Processes*, 68:215–7.

Lackie, A. M. (1988). Hemocyte behaviour. *Advances in Insect Physiology*, 21:85–178.

Lan, Y., Xu, X., Yang, F., and Zhang, X. (2006). Transcriptional profile of shrimp White Spot Syndrome Virus (WSSV) genes with DNA microarray. *Archives of Virology*, 151:1723–33.

Lang, R. P., Bayne, C. J., Camara, M. D., Cunningham, C., Jenny, M. J., and Langdon, C. J. (2009). Transcriptome profiling of selectively bred Pacidic oyster *Crassostrea gigas* families that differ in tolerance of heat shock. *Marine Biotechnology*, 11:650–68.

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

Le Moullac, G., Soyez, C., Saulnier, D., Ansquer, D., Avarre, J. C., and Levy, P. (1998). Effect of hypoxic stress on the immune response and the resistance to vibriosis of the shrimp *Penaeus stylirostris*. *Fish and Shellfish Immunology*, 8:621–629.

Lee, S. Y. and Söderhäll, K. (2001). Characterization of a pattern recognition protein, a masquerade-like protein, in the freshwater crayfish *Pacifastacus leniusculus*. *Journal of Immunology*, 166:7319–7326.

Lee, W. J. and Brey, P. T. (2013). How microbiomes influence metazoan development: Insights from history and *Drosophila* modeling of gut-microbe interactions. *Annual Review of Cell and Developmental Biology*, 29:571–592.

Leight, E. R. and Sugden, B. (2000). EBNA-1- a protein pivotal to latent infection by Epstein-Barr virus. *Reviews in Medical Virology*, 10:83–100.

Leonardo, V. A. D., Bonnichon, V., Roch, P., Parrinello, N., and Bonami, J.-R. (2005). Comparative WSSV infection routes in the shrimp genera *Marsupenaeus* and *Palaemon*. *Journal of Fish Diseases*, 28:565–569.

Levin, D. M., Breuer, L. N., Zhuang, S., Anderson, S. A., Nardi, J. B., and Kanost, M. R. (2005). A hemocyte-specific integrin required for hemocytic encapsulation in the tobacco hornworm, *Manduca sexta*. *Insect Biochemistry and Molecular Biology*, 35:369–380.

Lewis, F. and Maughan, N. J. (2004). Extraction of total RNA from formalin-fixed paraffin-embedded tissue. In Bustin, S. A., editor, *A-Z of Quantitative PCR*, pages 591–604. International Unversity Line, La Jolla, California.

Li, F., Li, M., Ke, W., Ji, Y., Bian, X., and Yan, X. (2009). Identification of the immediate-early genes of White Spot Syndrome Virus. *Virology*, 385:267–74.

Li, K., Zheng, T., Tian, Y., Xi, F., Yuan, J., Zhang, G., and Hong, H. (2007). Beneficial effects of *Bacillus licheniformis* on the intestinal microflora and immunity of the white shrimp, *Litopenaeus vannamei*. *Biotechnology Letters*, 29:525–530.

Li, S., Guo, S., and Xiang, J. (2014). Characterization and function analysis of an anti-lipopolysaccharide factor (ALF) from the Chinese shrimp *Fenneropenaeus chinensis*. *Developmental and Comparative Immunology*, 46:349–355.

Li, Y., Deng, W., Yang, K., and Wang, W. (2012). The expression of prophenoloxidase mRNA in red swamp crayfish, *Procambarus clarkii*, when it was challenged. *Genomics*, 99:355–360.

Lightner, D. V. (1996). *A handbook of shrimp pathology and diagnostic procedures for diseases of cultured penaeid shrimp*. World Aquaculture Society, Baton Rouge, Louisiana, USA.

Lightner, D. V. (2011). Virus diseases of farmed shrimp in the Western Hemisphere (the Americas): A review. *Journal of Invertebrate Pathology*, 106:110–30.

Lightner, D. V. and Redman, R. M. (1994). An epizootic of necrotizing hepatopancreatitis in cultured penaeid shrimp (Crustacea: Decapoda) in northwest Peru. *Aquaculture*, 122:9–18.

Lightner, D. V., Redman, R. M., and Bell, T. A. (1983). Infectious hypodermal and hematopoietic necrosis, a newly recognized virus disease of penaeid shrimp. *Journal of Invertebrate Pathology*, 42:62–70.

Lima, S. L. and Dill, L. M. (1990). Behavioural decisions made under the risk of predation: A review and prospectus. *Canadian Journal of Zoology*, 68:619–640.

Lin, F., Huang, H., Xu, L., Li, F., and Yang, F. (2011). Identification of three immediate-early genes of White Spot Syndrome Virus. *Archives of Virology*, 156:1611–1614.

Liu, C., Cheng, W., Kuo, C.-M., and Chen, J.-C. (2004). Molecular cloning and characterisation of a cell adhesion molecule, peroxinectin from the white shrimp *Litopenaeus vannamei*. *Fish and Shellfish Immunology*, 17:13–26.

Liu, C. H., Cheng, W., and Chen, J. C. (2005). The *peroxinectin* of white shrimp *Litopenaeus vannamei* is synthesised in the semi-granular and granular cells, and its transcription is up-regulated with *Vibrio alginolyticus* infection. *Fish and Shellfish Immunology*, 18:431–444.

Liu, C. H., Yeh, S. P., Hsu, P. Y., and Cheng, W. (2007a). Peroxinectin gene transcription of the giant freshwater prawn *Macrobrachium rosenbergii* under intrinsic, immunostimulant, and chemotherapeutant influences. *Fish and Shellfish Immunology*, 22:408–417.

Liu, H., Jiravanichpaisal, P., Cerenius, L., Lee, B. L., Söderhäll, I., and Söderhäll, K. (2007b). Phenoloxidase is an important component of the defense against *Aeromonas hydrophila* infection in a crustacean, *Pacifastacus leniusculus*. *The Journal of Biological Chemistry*, 282:33593–33598.

Liu, H., Jiravanichpaisal, P., Söderhäll, I., Cerenius, L., and Söderhäll, K. (2006). Antilipopolysaccharide factor interferes with White Spot Syndrome Virus replication *in vitro* and *in vivo* in the crayfish *Pacifastacus leniusculus*. *Journal of Virology*, 80:10365–10371.

Liu, H., Wu, C., Matsuda, Y., Kawabata, S., Lee, B. L., Söderhäll, K., and Söderhäll, I. (2011). Peptidoglycan activation of the proPO-system without a peptidoglycan receptor protein (PGRP)? *Developmental and Comparative Immunology*, 35:51–61.

Liu, W. J., Chang, Y. S., Wang, H. C., Leu, J. H., Kou, G. H., and Lo, C. F. (2008). Transactivation, dimerization, and DNA-binding activity of White Spot Syndrome Virus immediate-early protein *IE1*. *Journal of Virology*, 82:11362–11373.

Livak, K. J. and Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative RT-PCR and the $2^{-\delta\delta c_t}$ method. *Methods*, 25:402–408.

Lizárraga-Cubedo, H. A., Tuck, I., Bailey, N., Pierce, G. J., and Kinnear, J. A. M. (2003). Comparisons of size at maturity and fecundity of two Scottish populations of the European lobster, *Homarus gammarus*. *Fisheries Research*, 65:137–152.

Loc, N. H., Macrae, T. H., Musa, N., Bin Abdullah, M. D., Abdul Wahid, M. E., and Sung, Y. Y. (2013). Non-lethal heat shock increased Hsp70 and immune protein transcripts but not *Vibrio* tolerance in the white-leg shrimp. *PLoS One*, 8:e73199.

Lodeiros, C., Bolinches, J., Dopazo, C. P., and Toranzo, A. E. (1987). Bacillary necrosis in hatcheries of *Ostrea edulis* in Spain. *Aquaculture*, 65:15–29.

Löfgren, S. E., Miletti, L. C., Steindel, M., Bachere, E., and Barracco, M. A. (2008). Trypanocidal and leishmanicidal activities of different antimicrobial peptides (AMPs) isolated from aquatic animals. *Experimental Parasitology*, 118:197–202.

Löfgren, S. E., Smania, A., Smania, E. F. A., Bachere, E., and Barracco, M. A. (2009). Comparative activity and stability under salinity conditions of different antimicrobial peptides isolated from aquatic animals. *Aquaculture Research*, 40:1805–1812.

Lohner, K. and Prenner, E. J. (1999). Differential scanning calorimetry and X-ray diffraction studies of the specificity of the interaction of antimicrobial peptides with membrane-mimetic systems. *Biochimica et Biophysica Acta*, 1462:141–156.

Lorenzon, S., Edomi, P., Giulianini, P. G., Mettulio, R., and Ferrero, E. A. (2005). Role of biogenic amines and cHH in the crustacean hyperglycemic stress response. *Journal of Experimental Biology*, 208:3341–3347.

Lorenzon, S., Giulianini, P. G., Libralato, S., Martinis, M., and Ferrero, E. A. (2008). Stress effect of two different transport systems on the physiological profiles of the crab *Cancer pagurus*. *Aquaculture*, 278:156–163.

Lorenzon, S., Giulianini, P. G., Martinis, M., and Ferrero, E. A. (2007). Stress effect of different temperatures and air exposure during transport on physiological profiles in the American lobster *Homarus americanus*. *Comparative Biochemistry and Physiology. Part A, Molecular and Integrative Physiology*, 147:94–102.

Lorenzon, S., Guarrini, S. d., Smith, V. J., and Ferrero, E. A. (1999). Effects of LPS injection on circulating haemocytes in crustaceans *in vivo*. *Fish and Shellfish Immunology*, 9:31–50.

Lotz, J. M. (2012). Shrimp Disease Research. *The University of Southern Mississippi*, `http://www.usm.edu/gcrl/research/shrimp_disease.php`, [Accessed;05/01/2012].

Maloy, W. L. and Kari, U. P. (1995). Structure-activity studies on magainins and other host defense peptides. *Biopolymers*, 37:105–122.

Maltby, L. (1999). Studying stress: The importance of organismal-level responses. *Ecological Applications*, 9:431–440.

Marks, H. (2005). Genomics and transcriptomics of White Spot Syndrome Virus. Ph.D thesis. Wageningen University.

Martin, G. G., Kay, J., Poole, D., and Poole, C. (1998). *In vitro* nodule formation in the ridgeback prawn, *Sicyonia ingentis*, and the American lobster, *Homarus americanus*. *Invertebrate Biology*, 117:155–168.

Martins, A., Ribeiro, G., Marques, M. I., and Costa, J. V. (1994). Genetic identification and nucleotide sequence of the DNA polymerase gene of African swine fever virus. *Nucleic Acids Research*, 22:208–213.

Mayeux, R. (2004). Biomarkers: Potential uses and limitations. *NeuroRx®: A New Journal Focusing on Critical Reviews Relating to Neurotherapeutics*, 1:182–188.

Mayfield, S., Branch, G. M., and Cockcroft, A. C. (2000). Relationships among diet, growth rate and food availability for the South African rock lobster, *Jasus lalandii* (Decapoda, Palinuridea). *Crustaceana*, 73:815–834.

Mayo, M. A. (2002). A summary of taxonomic changes recently approved by ICTV. *Virology Division News*, 147:1655–1656.

McKay, D. and Jenkin, C. R. (1969). Immunity in the invertebrates. *Immunology*, 17:127–137.

Medzhitov, R. and Janeway Jr., C. A. (1997). Innate immunity: The virtues of a nonclonal system of recognition. *Cell*, 91:295–298.

Mejia-Ruiz, C. H., Vega-Pena, S., Alvarez-Ruiz, P., and Escobedo-Bonilla, C. M. (2011). Double-stranded RNA against White Spot Syndrome Virus (WSSV) *vp28* or *vp26* reduced susceptibility of *Litopenaeus vannamei* to WSSV, and survivors exhibited decreased susceptibility in subsequent re-infections. *Journal of Invertebrate Pathology*, 107:65–8.

Mente, E., Legeay, A., Houlihan, D. F., and Massabuae, J.-C. (2003). Influence of oxygen partial pressure on protein synthesis in feeding crabs. *American Journal of Physiology: Regulatory, Integrated and Comparative Physiology*, 284:R500–R510.

Mercier, L., Palacios, E., Campa-Cørdova, A. I., Tovar-Ramırez, D., Hernández-Herrera, R., and Racotta, I. S. (2006). Metabolic and immune responses in Pacific whiteleg shrimp *Litopenaeus vannamei* exposed to a repeated handling stress. *Aquaculture*, 258:633–640.

Meyer, R. and Candrian, U. (1996). PCR-based DNA analysis for the identification and characterization of food components. *Lebensmittel-Wissenschaft Technologie*, 29:1–9.

Meyers, T. R., Koeneman, T. M., Botelho, C., and Short, S. (1987). Bitter crab disease: A fatal dinoflagellate infection and marketing problem for Alaskan Tanner crabs *Chionoecetes bairdi*. *Diseases of Aquatic Organisms*, 3:195–216.

Mor, A. and Nicolas, P. (1994). The NH_2-terminal α-helical domain 1-18 of dermaseptin is responsible for antimicrobial activity. *The Journal of Biological Chemistry*, 269:1934–1939.

Morado, F. J., Siddeek, M. S., Mullowney, D. R., and Dawe, E. G. (2012). Protistan parasites as mortality drivers in cold water crab fisheries. *Journal of Invertebrate Pathology*, 110:201–210.

Morgan, D. E., Goodsell, J., Mattiessen, G. C., Garey, J., and Jacobson, P. (1980). Release of hatchery-reared bay scallops (*Argopecten irradians*) onto a shallow coastal bottom in Waterford, Connecticut. *Journal of the World Aquaculture Society*, 11:247–261.

Mori, K. and Stewart, J. E. (2006). Immunogen-dependent quantitative and qualitative differences in phagocytic responses of the circulating hemocytes of the lobster *Homarus americanus*. *Diseases of Aquatic Organisms*, 69:197–203.

Moriarty, D. J. W. (1998). Control of luminous *Vibrio* species in penaeid aquaculture ponds. *Aquaculture*, 164:351–358.

Mu, C., Zheng, P., Zhao, J., Wang, L., Qiu, L., Zhang, H., Gai, Y., and Song, L. (2011). A novel type III crustin (CrusEs2) identified from Chinese mitten crab *Eriocheir sinensis*. *Fish and Shellfish Immunology*, 31:142–147.

Mullis, K. B. (1990). The unusual origin of the polymerase chain reaction. *Scientific American*, 262:56–65.

Muñoz, M., Cedeño, R., Rodriguez, J., Knapp, W. P. W. v. d., Mialhe, E., and Bachère, E. (2000). Measurement of reactive oxygen intermediate production in haemocytes of the penaeid shrimp, *Penaeus vannamei*. *Aquaculture*, 191:89–107.

Nagoshi, H., Inagawa, H., Morii, K., Harada, H., Kohchi, C., Nishizawa, T., Taniguchi, Y., Uenobe, M., Honda, T., Kondoh, M., Takahashi, Y., and Soma, G. (2006). Cloning and characterization of a LPS-regulatory gene having an LPS binding domain in kuruma prawn *Marsupenaeus japonicus*. *Molecular Immunology*, 43:2061–2069.

NanoDrop (2007). Technical Support Bulletin T009. *Thermo Scientific*, `http://www.nanodrop.com/Library/T009-NanoDrop%201000-&-NanoDrop%208000-Nucleic-Acid-Purity-Ratios.pdf`, [Accessed;03/11/2013].

Naylor, S. (2003). Biomarkers: Current perspectives and future prospects. *Expert Review of Molecular Diagnostics*, 3:525–529.

Neil, D. M. (2012). Ensuring crustacean product quality in the post-harvest phase. *Journal of Invertebrate Pathology*, 110:267–275.

Nga, B. T., Lürling, M., Peeters, E. T. H. M., Roijackers, R., Scheffer, M., and Nghia, T. T. (2005). Chemical and physical effects of crowding on growth and survival of *Penaeus monodon* Fabricius post-larvae. *Aquaculture*, 246:455–465.

Nikolov, D. B. and Burley, S. K. (1997). RNA polymerase II transcription initiation: A structural view. *Proceedings of the National Academy of Science, USA*, 94:15–22.

Nolan, T. (2004). Getting started - The basics of setting up a qPCR assay. In Bustin, S. A., editor, *A-Z of Quantitative PCR*, pages 529–543. International University Line, La Jolla, California.

Nowak, B. F. (2007). Parasitic diseases in marine cage culture - An example of experimental evolution of parasites. *International Journal for Parasitology*, 37(6):581–588.

Oh, C.-W. and Hartnoll, R. G. (2000). Effects of food consumption on the growth and survival of the common shrimp, *Crangon crangon* (Linnaeus, 1758) (Decapoda, Caridea). *Crustaceana*, 73:83–99.

Oidtmann, B. and Stentiford, G. D. (2011). White Spot Syndrome Virus (WSSV) concentrations in crustacean tissues: A review of data relevant to assess the risk associated with commodity trade. *Transboundary and Emerging Diseases*, 58:469–82.

OIE (2009). Manual of diagnostic tests for aquatic animals. *World Organisation for Animal Health*, `http://www.oie.int/international-standard-setting/aquatic-manual/`, [Accessed;15/10/2010].

OIE (2014). Aquatic code. *World Organisation for Animal Health*, `http://www.oie.int/international-standard-setting/aquatic-code/`, [Accessed;09/09/2014].

Padhi, A. and Verghese, B. (2008). Detecting molecular adaptation at individual codons in the pattern recognition protein, lipopolysaccharide- and β-1,3-glucan-binding protein of decapods. *Fish and Shellfish Immunology*, 24:638–648.

Pálffy, R., Gardlik, R., Behuliak, M., Kadasi, L., Turna, J., and Celec, P. (2009). On the physiology and pathophysiology of antimicrobial peptides. *Molecular Medicine*, 15:51–59.

Pascual, C., Sánchez, A., Zenteno, E., Cuzon, G., Gabriela, G., Brito, R., Gelabert, R., Hidalgo, E., and Rosas, C. (2006). Biochemical, physiological, and immunological changes during starvation in juveniles of *Litopenaeus vannamei*. *Aquaculture*, 251:416–429.

Paterson, B. D. and Spanoghe, P. T. (1997). Stress indicators in marine decapod crustaceans, with particular reference to the grading of western rock lobsters (*Panulirus cygnus*) during commercial handling. *Marine and Freshwater Research*, 48:829–834.

Pearce, J. and Balcom, N. (2005). The 1999 Long Island Sound lobster mortality event: Findings of the comprehensive research initiative. *Journal of Shellfish Research*, 24:691–697.

Peeler, E. J. (2012). Costs and benefits of freedom from shrimp diseases in the European Union. *Journal of Invertebrate Pathology*, 110:188–195.

Perez-Novo, C. A., Claeys, C., Speleman, F., Cauwenberge, P. v., Bachert, C., and Vandesompele, J. (2005). Impact of RNA quality on reference gene expression stability. *BioTechniques*, 39:52–56.

Persson, M., Cerenius, L., and Söderhäll, K. (1987). The influence of haemocyte number on the resistance of the freshwater crayfish, *Pacifasticus leniusculus* Dana, to the parasitic fungus *Aphanomyces astaci*. *Journal of Fish Diseases*, 10:471–477.

Pestal, G. P., Taylor, D. M., Hoenig, J. M., Shields, J. D., and Pickavance, R. (2003). Monitoring the prevalence of the parasitic dinoflagellate *Hematodinium* sp. in snow crabs *Chionoecetes opilio* from Cinception Bay, Newfoundland. *Diseases of Aquatic Organisms*, 53:67–75.

Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research*, 29:e45.

Pfaffl, M. W. (2004). Quantification strategies in real-time PCR. In Bustin, S. A., editor, *A-Z of quantitative PCR*, IUL Biotechnology Series, pages 89–120. International University Line, La Jolla, California, USA, 1st edition.

Philippe, N., Legendre, M., Doutre, G., Coute, Y., Poirot, O., Lescot, M., Arslan, D., Seltzer, V., Bertaux, L., Bruley, C., Garin, J., Claverie, J. M., and Abergel, C. (2013). Pandoraviruses: Amoeba viruses with genomes up to 2.5 Mb reaching that of parasitic eukaryotes. *Science*, 341:281–286.

Popova, T., Mennerich, D., Weith, A., and Quast, K. (2008). Effect of RNA quality on transcript intensity levels in microarray analysis of human post-mortem brain tissues. *BMC Genomics*, 9:91.

Pörtner, H. (2008). Ecosystem effects of ocean acidification in times of ocean warming: A physiologist's view. *Marine Ecology Progress Series*, 373:203–217.

Prapavorarat, A., Pongsomboon, S., and Tassanakajon, A. (2010). Identification of genes expressed in response to yellow head virus infection in the black tiger shrimp, *Penaeus monodon*, by suppression subtractive hybridization. *Developmental and Comparative Immunology*, 34:611–617.

Primavera, J. H. (1997). Socio-economic impacts of shrimp culture. *Aquaculture Research*, 28:815–827.

Prodöhl, P. A., Jørstad, K. E., Triantafyllidis, C., Katsares, V., and Triantafyllidis, C. (2007). European lobster - *Homarus gammarus*. *IGENIMPACT, 6th Framework plan of the European Commission*.

Pushpanathan, M., Gunasekaran, P., and Rajendhran, J. (2013). Antimicrobial peptides: Versatile biological properties. *International Journal of Peptides*, 2013:675391.

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

Råberg, L., Grahn, M., Hasselquist, D., and Svensson, E. (1998). On the adaptive significance of stress-induced immunosuppression. *Proceedings of the Royal Society B*, 265:1637–1641.

Rad, M. and Shahsavani, D. (2008). Isolation and characterization of *Vibrio* (*Listonella*) *anguillarum* from catfish. *Turkish Journal of Veterinary and Animal Sciences*, 34:413–415.

Rajan, B., Lokesh, J., Kiron, V., and Brinchmann, M. F. (2013). Differentially expressed proteins in the skin mucus of Atlantic cod (*Gadus morhua*) upon natural infection with *Vibrio anguillarum*. *BMC Veterinary Research*, 9:103.

Ramirez, J. L., Souza-Neto, J., Torres Cosme, R., Rovira, J., Ortiz, A., Pascale, J. M., and Dimopoulos, G. (2012). Reciprocal tripartite interactions between the *Aedes aegypti* midgut microbiota innate immune system and dengue virus influences vector competence. *PLoS Neglected Tropical Diseases*, 6:e1561.

Ranganathan, S., Simpson, K. J., Shaw, D. C., and Nicholas, K. R. (1999). The whey acidic protein family: A new signature motif and three-dimensional structure by comparative modeling. *Journal of Molecular Graphics and Modelling*, 17:106–113.

Reiss, J. (1991). The polymerase chain reaction and its potential role in clinical diagnostics and research. *Journal of Internal Medicine*, 230:391–395.

Relf, J. M., Chisholm, J. R. S., Kemp, G. D., and Smith, V. J. (1999). Purification and characterization of a cysteine-rich 11.5-kDa antibacterial protein from the granular haemocytes of the shore crab, *Carcinus maenas*. *European Journal of Biochemistry*, 264:350–357.

Rengpipat, S., Rukpratanporn, S., Piyatiratitivorakul, S., and Menasaveta, P. (2000). Immunity enhancement in black tiger shrimp (*Penaeus monodon*) by a probiont bacterium (*Bacillus* s11). *Aquaculture*, 191:271–288.

Reynolds, J. (2013). Serial dilution protocols. *ASM MicrobeLibrary*, `http://www.microbelibrary.org/library/laboratory-test/2884-serial-dilution-protocols`, [Accessed;27/06/2014].

Riddell, C. E., Sumner, S., Adams, S., and Mallon, E. B. (2011). Pathways to immunity: Temporal dynamics of the bumblebee (*Bombus terrestris*) immune response against a trypanosomal gut parasite. *Insect Molecular Biology*, 20:529–540.

Robalino, J., Payne, C., Parnell, P., Shepard, E., Grimes, A. C., Metz, A., Prior, S., Witteveldt, J., Vlak, J. M., Gross, P. S., Warr, G., and Browdy, C. L. (2006). Inactivation of White Spot Syndrome Virus (WSSV) by normal rabbit serum: Implications for the role of the envelope protein VP28 in WSSV infection of shrimp. *Virus Research*, 118:55–61.

Rosa, R. D. and Barracco, M. A. (2010). Antimicrobial peptides in crustaceans. *Invertebrate Survival Journal*, 7:262–284.

Rout, N., Kumar, S., Jaganmohan, S., and Murugan, V. (2007). DNA vaccines encoding viral envelope proteins confer protective immunity against WSSV in black tiger shrimp. *Vaccine*, 25:2778–86.

Roux, M. M., Pain, A., Klimpel, K. R., and Dhar, A. K. (2002). The lipopolysaccharide and β-1,3-glucan binding protein gene is upregulated in White Spot Virus-infected shrimp (*Penaeus stylirostris*). *Journal of Virology*, 76:7140–7149.

Ryu, J. H., Ha, E. M., and Lee, W. J. (2010). Innate immunity and gut-microbe mutualism in *Drosophila*. *Developmental and Comparative Immunology*, 34:369–376.

Safeena, M. P., Rai, P., and Karunasagar, I. (2012). Molecular biology and epidemiology of hepatopancreatic parvovirus of penaeid shrimp. *Indian Journal of Virology*, 23:191–202.

Saha, S. (2011). Innate immune source and functional machinery in decapods of Crustacea. *Indian Journal of Fundamental and Applied Life Sciences*, 1:310–324.

Sanchez, A., Pascual, C., Sanchez, A., Vargas-Albores, F., Moullac, G. d., and Rosas, C. (2001). Hemolymph metabolic variables and immune response in *Litopenaeus setiferus* adult males: The effect of acclimation. *Aquaculture*, 198:13–28.

Sánchez-Martínez, J. G., Aguirre-Guzmán, G., and Mejía-Ruíz, H. (2007). White Spot Syndrome Virus in cultured shrimp: A review. *Aquaculture Research*, 38:1339–1354.

Sanchez-Paz, A. (2010). White Spot Syndrome Virus: An overview on an emergent concern. *Veterinary Research*, 41:43.

Sangamaheswaran, A. P. and Jeyaseelan, M. J. P. (2001). White Spot Viral Disease in penaeid shrimp: A review. *The ICLARM Quarterly*, 24:16–22.

Saville, D. H. and Irwin, S. W. B. (1991). *In ova* cultivation of *Microphallus primas* (Trematoda: Microphallidae) metacercariae to ovigerous adults and the establishment of the life-cycle in the laboratory. *Parasitology*, 103:479–484.

Schnapp, D., Kemp, G. D., and Smith, V. J. (1996). Purification and characterization of a proline-rich antibacterial peptide, with sequence similarity to bactenecin-7, from the haemocytes of the shore crab, *Carcinus maenas*. *European Journal of Biochemistry*, 240:532–539.

Schulte, P. A. (1993). A conceptual and historical framework for molecular epidemiology. In Schulte, P. A. and Perera, F. P., editors, *Molecular Epidemiology: Principles and Practices*, pages 3–44. Academic Press, San Diego.

Sengupta, D., Leontiadou, H., Mark, A. E., and Marrink, S. J. (2008). Toroidal pores formed by antimicrobial peptides show significant disorder. *Biochimica et Biophysica Acta*, 1778:2308–2317.

Sheehan, E. V., Thompson, R. C., Coleman, R. A., and Attrill, M. J. (2008). Positive feedback fishery: Population consequences of 'crab-tiling' on the green crab *Carcinus maenas*. *Journal of Sea Research*, 60:303–309.

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

Shields, J. D. and Squyars, C. M. (2000). Mortality and hematology of blue crabs, *Callinectes sapidus*, experimentally infected with the parasitic dinoflagellate *Hematodinium perezi*. *Fishery Bulletin*, 98:139–152.

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

Shockey, J. E., O'Leary, N. A., Vega, E. d. l., Browdy, C. L., Baatz, J. E., and Gross, P. S. (2009). The role of crustins in *Litopenaeus vannamei* in response to infection with shrimp pathogens: An *in vivo* approach. *Developmental and Comparative Immunology*, 33:668–673.

Shokralla, S., Spall, J. L., Gibson, J. F., and Hajibabaei, M. (2012). Next-generation sequencing technologies for environmental DNA research. *Molecular Ecology*, 21:1794–1805.

Sinclair, J. and Sissons, P. (2006). Latency and reactivation of human cytomegalovirus. *The Journal of General Virology*, 87:1763–79.

Sindermann, C. J. and Rosenfield, A. (1967). Principle diseases of commercially important marine bivalve Mollusca and Crustacea. *Fishery Bulletin*, 66:335–385.

Slifka, M. K., Homann, D., Tishon, A., Pagarigan, R., and Oldstone, M. B. A. (2003). Measles virus infection results in suppression of both innate and adaptive immune responses to secondary bacterial infection. *Journal of Clinical Investigation*, 111:805–810.

Small, H. J. (2012). Advances in our understanding of the global diversity and distribution of *Hematodinium* spp. - Significant pathogens of commercially exploited crustaceans. *Journal of Invertebrate Pathology*, 110:234–246.

Smith, I. P., Collins, K. J., and Jensen, A. C. (1998). Movement and activity patterns of the European lobster, *Homarus gammarus*, revealed by electromagnetic telemetry. *Marine Biology*, 132:611–623.

Smith, V. J. (1996). The prophenoloxidase activating system: A common defence pathway for deuterostomes and protosomes? In Cooper, E. L., editor, *Comparative and Environmental Physiology*, volume 23, pages 75–114. Springer, Berlin Heidelberg New York, 1st edition.

Smith, V. J. (2011). Phylogeny of whey acidic protein (WAP) four-disulfide core proteins and their role in lower vertebrates and invertebrates. *Biochemical Society Transactions*, 39:1403–8.

Smith, V. J., Fernandes, J. M., Kemp, G. D., and Hauton, C. (2008). Crustins: Enigmatic WAP domain-containing antibacterial proteins from crustaceans. *Developmental and Comparative Immunology*, 32:758–772.

Smith, V. J. and Söderhäll, K. (1983). β-l,3 Glucan activation of crustacean hemocytes *in vitro* and *in vivo*. *Biological Bulletin*, 164:299–314.

Smith, V. J. and Söderhäll, K. (1983). Induction of degranulation and lysis of haemocytes in the freshwater crayfish, *Astacus astacus* by components of the prophenoloxidase activating system *in vitro*. *Cell and Tissue Research*, 233:295–303.

Smith, V. J., Swindlehurst, R. J., Johnston, P. A., and Vethaak, A. D. (1995). Disturbance of host defence capability in the common shrimp, *Crangon crangon*, by exposure to harbour dredge spoils. *Aquatic Toxicology*, 32:43–58.

Smits, K., Goossens, K., Van Soom, A., Govaere, J., Hoogewijs, M., Vanhaesebrouck, E., Galli, C., Colleoni, S., Vandesompele, J., and Peelman, L. (2009). Selection of reference genes for quantitative real-time PCR in equine *in vivo* and fresh and frozen-thawed *in vitro* blastocysts. *BMC Research Notes*, 2:246.

Söderhäll, K. and Cerenius, L. (1998). Role of the prophenoloxidase-activating system in invertebrate immunity. *Current Opinion in Immunology*, 10:23–38.

Söderhäll, K., Cerenius, L., and Johansson, M. W. (1994). The prophenoloxidase activating system and its role in invertebrate defense. *Annual NY Academy of Science*, 712:155–161.

Söderhäll, K. and Smith, V. J. (1983). Separation of the haemocyte populations of *Carcinus maenas* and other marine decapods, and prophenoloxidase distribution. *Developmental and Comparative Immunology*, 7:229–239.

Somboonwiwat, K., Marcos, M., Tassanakajon, A., Klinbunga, S., Aumelas, A., Romestand, B., Gueguen, Y., Boze, H., Moulin, G., and Bachere, E. (2005). Recombinant expression and anti-microbial activity of anti-lipopolysaccharide factor (ALF) from the black tiger shrimp *Penaeus monodon*. *Developmental and Comparative Immunology*, 29:841–851.

Somero, G. N. (2011). Comparative physiology: A "crystal ball" for predicting consequences of global change. *American Journal of Physiology Regulatory Integrative and Comparative Physiology*, 301:R1–14.

Soonthornchai, W., Rungrassamee, W., Karoonuthaisiri, N., Jarayabhand, P., Klinbunga, S., Söderhäll, K., and Jiravanichpaisal, P. (2010). Expression of immune-related genes in the digestive organ of shrimp, *Penaeus monodon*, after an oral infection by *Vibrio harveyi*. *Developmental and Comparative Immunology*, 34:19–28.

Sparks, A. K. and Morado, J. F. (1986). A herpes-like virus disease in the blue king crab *Paralithodes platypus*. *Diseases of Aquatic Organisms*, 1:115–122.

Sperstad, S. V., Haug, T., Paulsen, V., Rode, T. M., Strandskog, G., Solem, S. T., Styrvold, O. B., and Stensvag, K. (2009). Characterization of crustins from the hemocytes of the spider crab, *Hyas araneus*, and the red king crab, *Paralithodes camtschaticus*. *Developmental and Comparative Immunology*, 33:583–591.

Sperstad, S. V., Smith, V. J., and Stensvåg, K. (2010). Expression of antimicrobial peptides from *Hyas araneus* haemocytes following bacterial challenge *in vitro*. *Developmental and Comparative Immunology*, 34:618–624.

Sritunyalucksana, K., Sithisarn, P., Withayachumnarnkul, B., and Flegel, T. W. (1999). Activation of *prophenoloxidase*, agglutinin and antibacterial activity in haemolymph of the black tiger prawn, *Penaeus monodon*, by immunostimulants. *Fish and Shellfish Immunology*, 9:21–30.

Sritunyalucksana, K. and Söderhäll, K. (2000). The proPO and clotting system in crustaceans. *Aquaculture*, 191:53–69.

Sritunyalucksana, K., Wongsuebsantati, K., Johansson, M. W., and Soderhall, K. (2001). Peroxinectin, a cell adhesive protein associated with the proPO system from the black tiger shrimp, *Penaeus monodon*. *Developmental and Comparative Immunology*, 25:353–363.

Stentiford, G. D., Bateman, K. S., Feist, S. W., Chambers, E., and Stone, D. M. (2013a). Plastic parasites: Extreme dimorphism creates a taxanomic conundrum in the Phylum Microsporidia. *International Journal for Parasitology*, 43:339–352.

Stentiford, G. D., Bateman, K. S., Stokes, N. A., and Carnegie, R. B. (2013b). *Haplosporidium littorals* sp. nov.: A crustacean pathogen within the Haplosporida (Cercozoa, Ascetosporea). *Diseases of Aquatic Organisms*, 105:243–52.

Stentiford, G. D., Evans, M., Bateman, K., and Feist, S. W. (2003). Co-infection by a yeast-like organism in *Hematodinium*-infected European edible crabs *Cancer pagurus* and velvet swimming crabs *Necora puber* from the English Channel. *Diseases of Aquatic Organisms*, 54:195–202.

Stentiford, G. D. and Feist, S. W. (2005). A histopathological survey of shore crab (*Carcinus maenas*) and brown shrimp (*Crangon crangon*) from six estuaries in the United Kingdom. *Journal of Invertebrate Pathology*, 88:136–46.

Stentiford, G. D., Feist, S. W., Bateman, K. S., and Hilne, P. M. (2004). Haemolymph parasite of the shore crab *Carcinus maenas* - Pathology, ultrastructure and observations on crustacean haplosporidians. *Diseases of Aquatic Organisms*, 59:57–68.

Stentiford, G. D., Feist, S. W., Stone, D. M., Peeler, E. J., and Bass, D. (2014). Policy, phylogeny, and the parasite. *Trends in Parasitology*, 30:274–281.

Stentiford, G. D. and Lightner, D. V. (2011). Cases of White Spot Disease (WSD) in European shrimp farms. *Aquaculture*, 319:302–306.

Stentiford, G. D., Neil, D. M., Peeler, E. J., Shields, J. D., Small, H. J., Flegel, T. W., Vlak, J. M., Jones, B., Morado, F., Moss, S., Lotz, J., Bartholomay, L., Behringer, D. C., Hauton, C., and Lightner, D. V. (2012). Disease will limit future food supply from the global crustacean fishery and aquaculture sectors. *Journal of Invertebrate Pathology*, 110:141–157.

Stentiford, G. D. and Shields, J. D. (2005). A review of the parasitic dinoflagellates *Hematodinium* species and *Hematodinium*-like infections in marine crustaceans. *Diseases of Aquatic Organisms*, 66:47–70.

Storni, T., Kundig, T. M., Senti, G., and Johansen, P. (2005). Immunity in response to particulate antigen-delivery systems. *Advanced Drug Delivery Reviews*, 57:333–355.

Strand, C., Enell, J., Hedenfalk, I., and Ferno, M. (2007). RNA quality in frozen breast cancer samples and the influence on gene expression analysis - A comparison of three evaluation methods using microcapillary electrophoresis traces. *BMC Molecular Biology*, 8:38.

Sugumaran, M. (2002). Comparative biochemistry of eumelanogenesis and the protective roles of phenoloxidase and melanin in insects. *Pigment Cell and Melanoma Research*, 15:2–9.

Sugumaran, M. and Nellaiappan, K. (2000). Characterization of a new phenoloxidase inhibitor from the cuticle of *Manduca sexta*. *Biochemical and Biophysical Research Communications*, 268:379–383.

Supungul, P., Tang, S., Maneeruttanarungroj, C., Rimphanitchayakit, V., Hirono, I., Aoki, T., and Tassanakajon, A. (2008). Cloning, expression and antimicrobial activity of *crustinPm1*, a major isoform of crustin, from the black tiger shrimp *Penaeus monodon*. *Developmental and Comparative Immunology*, 32:61–70.

Suttle, C. A. (2007). Marine viruses - Major players in the global ecosystem. *Nature reviews. Microbiology*, 5:801–12.

Takaki, Y., Seki, N., Kawabata, S., Iwanaga, S., and Muta, T. (2002). Duplication binding sites for (1-3)-β-D-glucan in the horseshoe crab coagulation factor G: Implications for a molecular basis of the pattern recognition in innate immunity. *Journal of Biological Chemistry*, 277:14281–14287.

Tanaka, S., Nakamura, T., Morita, T., and Iwanaga, S. (1982). *Limulus* anti-LPS factor: An anticoagulant which inhibits the endotoxin-mediated activation of *Limulus* coagulation system. *Biochemical and Biophysical Research Communications*, 105:717–723.

Tang, X., Zhou, L., and Zhan, W. (2008). Isolation and characterization of pathogenic *Listonella anguillarum* of diseased half-smooth tongue sole (*Cynoglossus semilaevis* Günther). *Journal of Ocean University of China*, 7:343–351.

Taylor, D. M. and Khan, R. A. (1995). Observations of the occurrance of *Hematodinium* sp. (Dinoflagellata: Syndinidae), the causative agent of Bitter Crab Disease in Newfoundland snow crab (*Chionoecetes opilio*). *Journal of Invertebrate Pathology*, 65:283–288.

Taylor, E. W. and Whiteley, N. M. (1989). Oxygen transport and acid-base balance in the haemolymph of the lobster, *Homarus gammarus*, during aerial exposure and resubmersion. *Journal of Experimental Biology*, 144:417–436.

Taylor, S., Mississauga, D., Buchanan, M., and Basik, M. (2011). A MIQE Case Study - Effect of RNA sample quality and reference gene stability on gene expression data. *Bio-Rad Laboratories Canada, Bulletin 6245*.

Taylor, S., Wakem, M., Dijkman, G., Alsarraj, M., and Nguyen, M. (2010). A practical approach to RT-qPCR-publishing data that conform to the MIQE guidelines. *Methods*, 50:S1–5.

Teixeira, L., Ferreira, A., and Ashburner, M. (2008). The bacterial symbiont *Wolbachia* induces resistance to RNA viral infections in *Drosophila melanogaster*. *PLoS Biology*, 6(12):e2.

Tharntada, S., Ponprateep, S., Somboonwiwat, K., Liu, H., Söderhäll, I., Söderhäll, K., and Tassanakajon, A. (2009). Role of anti-lipopolysaccharide factor from the black tiger shrimp, *Penaeus monodon*, in protection from White Spot Syndrome Virus infection. *Journal of General Virology*, 90:1491–1498.

Thermo Scientific (2013). Basic principles of qPCR. *Life Technologies*, `http://www.thermoscientificbio.com/applications/pcr-and-qpcr/introduction-to-qpcr/`, [Accessed;12/02/2015].

Thompson, K. L., Pine, P. S., Rosenzweig, B. A., Turpaz, Y., and Retief, J. (2007). Characterization of the effect of sample quality on high density oligonucleotide microarray data using progressively degraded rat liver RNA. *BMC Biotechnology*, 7:57.

Thörnqvist, P.-O., Johansson, M. W., and Söderhäll, K. (1994). Opsonic activity of cell adhesion proteins and β-1, 3-glucan binding proteins from two crustaceans. *Developmental and Comparative Immunology*, 18:3–12.

Thresher, R. E., Werner, M., Høeg, T. J., Svane, I., Glenner, H., Murphy, N. E., and Wittwer, C. (2000). Developing the options for managing marine pests- specificity trials on the parasitic castrator, *Sacculina carcini*, against the European crab, *Carcinus maenas*, and related species. *Journal of Experimental Biology and Ecology*, 254:37–51.

Toranzo, A. E. and Barja, J. L. (1990). A review of the taxonomy and seroepizootiology of *Vibrio anguillarum*, with special reference to aquaculture in the northwest of Spain. *Diseases of Aquatic Organisms*, 9:73–82.

Towle, D. W. and Smith, C. M. (2006). Gene discovery in *Carcinus maenas* and *Homarus americanus* via expressed sequence tags. *Integrative and Comparative Biology*, 46:912–918.

Triantafyllidis, A., Apostolidis, A. P., Katsares, V., Kelly, E., Mercer, J., Hughes, M., Jørstad, K. E., Tsolou, A., Hynes, R., and Triantaphyllidis, C. (2005). Mitochondrial DNA variation in the European lobster (*Homarus gammarus*) throughout the range. *Marine Biology*, 146:223–235.

Truscott, R. and White, B. L. (1990). The influence of metal and temperature stress on the immune system of crabs. *Functional Ecology*, 4:455–461.

Trussell, G. C., Ewanchuk, P. J., and Matassa, C. M. (2006). The fear of being eaten reduces the energy transfer in a simple food chain. *Ecology*, 87:2979–2984.

Tsai, J. M., Wang, H. C., Leu, J. H., Hsiao, H. H., Wang, A. H., Kou, G. H., and Lo, C. F. (2004). Genomic and proteomic analysis of thirty-nine structural proteins of shrimp White Spot Syndrome Virus. *Journal of Virology*, 78:11360–70.

Tsai, M. F., Yu, H. T., Tzeng, H. F., Leu, J. H., Chou, C. M., Huang, C. J., Wang, C. H., Lin, J. Y., Kou, G. H., and Lo, C. F. (2000). Identification and characterization of a shrimp White Spot Syndrome Virus (WSSV) gene that encodes a novel chimeric polypeptide of cellular-type thymidine kinase and thymidylate kinase. *Virology*, 277:100–10.

Tsvetnenko, E., Fotedar, S., and Evans, L. (2001). Antibacterial activity in the haemolymph of western rock lobster, *Panulirus cygnus*. *Marine and Freshwater Research*, 52:1407–1412.

Tully, O., O'Donovon, V., and Fletcher, D. (2000). Metabolic rate and lipofuscin accumulation in juvenile European lobster (*Homarus gammarus*) in relation to simulated seasonal changes in temperature. *Marine Biology*, 137:1031–1040.

Universiteit Gent (1999). Principle of the PCR. `http://users.ugent.be/~avierstr/principles/pcr.html`, [Accessed;09/01/2014].

Vadas, R. L., Burrows, M. T., and Hughes, R. N. (1994). Foraging strategies of dogwhelks, *Nucella lapillus* (L.): Interacting effects of age, diet and chemical cues to the threat of predation. *Oecologia*, 100:439–450.

Valasek, M. A. and Repa, J. J. (2005). The power of real-time PCR. *Advances in Physiology Education*, 29:151–9.

van Hulten, M. C., Witteveldt, J., Peters, S., Kloosterboer, N., Tarchini, R., Fiers, M., Sandbrink, H., Lankhorst, R. K., and Vlak, J. M. (2001a). The White Spot Syndrome Virus DNA genome sequence. *Virology*, 286:7–22.

van Hulten, M. C., Witteveldt, J., Snippe, M., and Vlak, J. M. (2001b). White Spot Syndrome Virus envelope protein VP28 is involved in the systemic infection of shrimp. *Virology*, 285:228–33.

van Hulten, M. C. W., Reijns, M., Vermeesch, A. M. G., Zandbergen, F., and Vlak, J. M. (2002). Identification of *VP19* and *VP15* of White Spot Syndrome Virus (WSSV) and glycosylation status of the WSSV major structural proteins. *Journal of General Virology*, 83:257–267.

van Hulten, M. C. W. and Vlak, J. M. (2001). Identification and phylogeny of a protein kinase gene of White Spot Syndrome Virus. *Virus Genes*, 22:201–207.

Vandesompele, J., Kubista, M., and Pfaffl, M. (2009). Reference gene validation software for improved normalization. In Logan, J., Edwards, K., and Saunders, N., editors, *Real-time PCR: Current Technology and Applications*, pages 47–64. Caister Academic Press, Norfolk, England.

Vandesompele, J., Preter, K. D., Pattyn, F., Poppe, B., Roy, N. V., Paepe, A. D., and Speleman, F. (2002). Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biology*, 3:research0034.1–0034.11.

Vargas-Albores, F., Hinojosa-Baltazar, P., Portillo-Clark, G., and Magallón-Barajas, F. (1998). Influence on temperature and salinity on the yellowleg shrimp, *Penaeus californiensis* Holmes, prophenoloxidase system. *Aquaculture Research*, 29:549–553.

Vargas-Albores, F., Hinojosa-Baltazar, P., Portillo-Clark, G., and Magallon-Barajas, F. (2008). Influence of temperature and salinity on the yellowleg shrimp, *Penaeus californiensis* Holmes, prophenoloxidase system. *Aquaculture Research*, 29:549–553.

Vargas-Albores, F., Yepiz-Plascencia, G., Jimenez-Vega, F., and Avila-Villa, A. (2004). Structural and functional differences of *Litopenaeus vannamei* crustins. *Comparative Biochemistry and Physiology. Part B, Biochemistry and Molecular Biology*, 138:415–22.

Vatanavicharn, T., Supungul, P., Puanglarp, N., Yingvilasprasert, W., and Tassanakajon, A. (2009). Genomic structure, expression pattern and functional characterization of crustin*Pm5*, a unique isoform of crustin from *Penaeus monodon*. *Comparative Biochemistry and Physiology. Part B, Biochemistry & Molecular Biology*, 153:244–52.

Vazquez, L., Alpulche, J., Maldonado, G., Agundis, C., Pereya-Morales, A., and Zenteno, E. (2009). Immunity mechanisms in crustaceans. *Innate Immunity*, 15:179–188.

Vermeulen, J., De Preter, K., Lefever, S., Nuytens, J., De Vloed, F., Derveaux, S., Hellemans, J., Speleman, F., and Vandesompele, J. (2011). Measurable impact of RNA quality on gene expression results from quantitative PCR. *Nucleic Acids Research*, 39:e63.

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

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

Walker, P. J., Gudkovs, N., Mohan, C. V., Raj, V. S., Pradeep, B., Sergeant, E., Mohan, A. B. C., Ravibabu, G., Karunasagar, I., and Santiago, T. C. (2011). Longitudinal disease studies in small-holder black tiger shrimp (*Penaeus monodon*) ponds in Andhra Pradesh, India. II. Multiple WSSV genotypes associated with disease outbreaks in ponds seeded with uninfected postlarvae. *Aquaculture*, 319:18–24.

Walker, P. J. and Mohan, C. V. (2009). Viral disease emergence in shrimp aquaculture: Origins, impact and the effectiveness of health management strategies. *Reviews in Aquaculture*, 1:125–154.

Wang, C.-H., Lo, C.-F., Leu, J.-H., Chou, C.-M., Yeh, P.-Y., Chou, H.-Y., Tung, M.-C., Chang, C.-F., Su, M.-S., and Kou, G.-H. (1995). Purification and genomic analysis of baculovirus associated with White Spot Syndrome (WSBV) of *Penaeus monodon*. *Diseases of Aquatic Organisms*, 23:239–242.

Wang, H. C., Ko, T. P., Lee, Y. M., Leu, J. H., Ho, C. H., Huang, W. P., Lo, C. F., and Wang, A. H. (2008). White Spot Syndrome Virus protein ICP11: A histone-binding DNA mimic that disrupts nucleosome assembly. *Proceedings of the National Academy of Sciences of the United States of America*, 105:20758–63.

Wang, L. U. and Chen, J. C. (2005). The immune response of white shrimp *Litopenaeus vannamei* and its susceptibility to *Vibrio alginolyticus* at different salinity levels. *Fish and Shellfish Immunology*, 18:269–278.

Wang, R., Lee, S. Y., Cerenius, L., and Söderhäll, K. (2001). Properties of the prophenoloxidase activating enzyme of the freshwater crayfish, *Pacifastacus leniusculus*. *European Journal of Biochemistry*, 268:895–902.

Webster, S. G. (1996). Measurement of crustacean hyperglyceamic hormone levels in the edible crab *Cancer pagurus* during emersion stress. *The Journal of Experimental Biology*, 199:1579–1585.

Werner, E. E. and Anholt, B. R. (1993). Ecological consequences of the trade-offs between growth and mortality rates mediated by foraging activity. *American Naturalist*, 142:242–272.

Werner, M. (2001). Prevalence of the parasite *Sacculina carcini* Thompson 1836 (Crustacea, Rhizocephala) on its host crab *Carcinus maenas* (L.) on the west coast of Sweden. *Ophelia*, 55:101–110.

Whiteley, N. M. and Taylor, E. W. (1992). Oxygen and acid-base disturbances in the hemolymph of the lobster *Homarus gammarus* during commercial transport and storage. *Journal of Crustacean Biology*, 12:19–30.

Wilcox, R. M. and Fuhrman, J. A. (1994). Bacterial viruses in coastal seawater - lytic rather than lysogenic production. *Marine Ecology Progress Series*, 114:35–45.

Wilhelm, G. and Mialhe, E. (1996). Dinoflagellate infection associated with the decline of *Necora pubes* crab populations in France. *Diseases of Aquatic Organisms*, 26:231–219.

Williams, B. G. and Naylor, E. (1967). Spontaneously induced rhythm of tidal periodicity in laboratory-reared *Carcinus*. *Journal of Experimental Biology*, 47:229–234.

Winkler, A., Siebers, D., and Becker, W. (1988). Osmotic and ionic regulation in shore crabs *Carcinus maenas* inhabiting a tidal estuary. *Helgolander Meeresunters*, 42:99–111.

Wittwer, C. T., Herrmann, M. G., Moss, A. A., and Rasmussen, R. P. (1997). Continuous fluorescence monitoring of rapid cycle DNA amplification. *BioTechniques*, 22:130–138.

Wongteerasupaya, C., Vickers, J. E., Sriurairatana, S., Nash, G. L., Akarajamorn, A., Boonsaeng, V., Panyim, S., Tassanakajon, A., Withyachumnarnkul, B., and Flegel, T. W. (1995). A non-occluded, systemic baculovirus that occurs in cells of ectodermal and mesodermal origin and causes high mortality in the black tiger prawn *Penaeus monodon*. *Diseases of Aquatic Organisms*, 21:69–77.

Wootton, E. C., Pope, E. C., Vogan, C. L., Roberts, E. C., Davies, C. E., and Rowley, A. F. (2011). Morphology and pathology of the ectoparasitic copepod, *Nicothoe astaci* ('lobster louse') in the European lobster, *Homarus gammarus*. *Parasitology*, 138:1285–1295.

Wootton, E. C., Woolmer, A. P., Vogan, C. L., Pope, E. C., Hamilton, K. M., and Rowley, A. F. (2012). Increased disease calls for a cost-benefits review of marine reserves. *PloS One*, 7:e51615.

Woramongkolchai, N., Supungul, P., and Tassanakajon, A. (2011). The possible role of penaeidin5 from the black tiger shrimp, *Penaeus monodon*, in protection against viral infection. *Developmental and Comparative Immunology*, 35:530–536.

Wu, W., Wang, L., and Zhang, X. (2005). Identification of White Sspot Syndrome Virus (WSSV) envelope proteins involved in shrimp infection. *Virology*, 332:578–83.

Xu, H., Yan, F., Deng, X., Wang, J., Zou, T., Ma, X., Zhang, X., and Qi, Y. (2009). The interaction of White Spot Syndrome Virus envelope protein *VP28* with shrimp *Hsc70* is specific and ATP-dependent. *Fish and Shellfish Immunology*, 26:414–21.

Yamada, S. B. and Hauck, L. (2001). Field identification of the European green crab species: *Carcinus maenas* and *Carcinus aestuarii*. *Journal of Shellfish Research*, 20:905–912.

Yang, F., He, J., Lin, X., Li, Q., Pan, D., Zhang, X., and Xu, X. (2001). Complete genome sequence of the shrimp White Spot Bacilliform Virus. *Journal of Virology*, 75:11811–20.

Yedery, R. D. and Reddy, K. V. (2009). Identification, cloning, characterization and recombinant expression of an anti-lipopolysaccharide factor from the hemocytes of Indian mud crab, *Scylla serrata*. *Fish and Shellfish Immunology*, 27:275–284.

Yi, G., Wang, Z., Qi, Y., Yao, L., Qian, J., and Hu, L. (2004). *VP28* of shrimp White Spot Syndrome Virus is involved in the attachment and penetration into shrimp cells. *Journal of Biochemistry and Molecular Biology*, 37:726–734.

Young, L. C., Campling, B. G., Cole, S. P. C., Deeley, R. G., and Gerlach, J. H. (2001). Multidrug resistance proteins MRP3, MRP1, and MRP2 in lung cancer - Correlation of protein levels with drug response and messenger RNA levels. *Clinical Cancer Research*, 7:1798–1804.

Young, R. A. (1991). RNA polymerase II. *Annual Review of Biochemistry*, 60:689–715.

Zasloff, M. (2002). Antimicrobial peptides of multicellular organisms. *Nature*, 415:389–395.

Zhang, J., Li, F., Wang, Z., and Xiang, J. (2007). Expression, purification and characterization of recombinant Chinese shrimp crustin-like protein (CruFc) in *Pichia pastoris*. *Biotechnology Letters*, 29:813–817.

Zheng, X. and Xia, Y. (2012). β-1,3-Glucan recognition protein (βGRP) is essential for resistance against fungal pathogen and opportunistic pathogenic gut bacteria in *Locusta migratoria manilensis*. *Developmental and Comparative Immunology*, 36:602–609.