UNIVERSITY OF SOUTHAMPTON

# Effects of the dinoflagellate parasite *Hematodinium* sp. on the immune response of its crustacean hosts

by

Jodi Leigh Hoppes

A thesis submitted in partial fulfillment for the
degree of Doctor of Philosophy

in the
Faculty of Natural and Environmental Sciences
School of Ocean and Earth Science

November 2011

*"Biology has at least 50 more interesting years."*

James D. Watson

UNIVERSITY OF SOUTHAMPTON

# Abstract

Faculty of Natural and Environmental Sciences

School of Ocean and Earth Science

Doctor of Philosophy

Effects of the dinoflagellate parasite *Hematodinium* sp. on the immune response of its crustacean hosts

by Jodi Leigh Hoppes

The aim of the present study was to investigate the immune response of two UK crustaceans, the edible crab *Cancer pagurus* and the Norway lobster *Nephrops norvegicus*, to the dinoflagellate parasite *Hematodinium* sp. *Hematodinium* sp. is an emerging global pathogen affecting a number of commercially important crustacean species. Parasites have been found to cause metabolic disruption, and mortality has generally been associated with a massive decrease in haemocyte numbers. Investigations to date have indicated the potential for the parasite to avoid or suppress the host immune system, as well as to increase the likelihood of secondary infection. *Hematodinium*-host interactions were examined using a combination of *in vitro* experiments, gene expression analysis and immune assays with the express purpose of determining if *a*) parasites were capable of avoiding the host immune response or *b*) parasites were suppressing the host immune response.

*In vitro* experiments were designed to study the response of individual haemocyte populations from *Cancer pagurus* to *Hematodinium* sp. in the absence of confounding factors such as secondary infection. Cell viability and gross morphology of separate hyalinocyte and granulocyte cultures were examined, though no significant differences were identified between challenged and naïve cultures. A differential display technique, used to identify differentially expressed genes between *Hematodinium*-exposed and naïve cultures, found two novel genes from naïve granulocyte cultures: a prophenoloxidase activating factor (*ppaf*) and an ATP-dependent RNA helicase DEAD-box protein (*ddx*). These genes, in addition to the immune-relevant gene for prophenoloxidase (*proPO*), were analysed for gene expression changes during *Hematodinium* sp. exposure. No change in gene expression was found for any of the genes, suggesting a lack of parasite recognition or the loss of a crucial humoral recognition component under *in vitro* conditions.

*In vivo* analysis of immune parameters of *N. norvegicus* found that, despite dramatically decreased total haemocyte counts, phenoloxidase activity increased in infected individuals. Haemagglutinin titres and intracellular superoxide (SO) anion activity were not significantly different between infected and control animals, however infected animals had much broader variability in SO activity. SO activity was negatively correlated with *Hematodinium* sp. presence in the haemolymph which suggested a potential mechanism of immune suppression via SO inhibition.

*In vivo* gene expression studies used differential display to identify differentially expressed genes in *Hematodinium*-infected and uninfected *N. norvegicus*. Novel genes were identified from *N. norvegicus* and coded for two crustin isoforms (*NnCrust1* and *NnCrust2*), a 40S ribosomal protein S12 (*NnrpS12*) and a chaperonin containing TCP1 protein (*NnCct7*). Gene expression analysis of these three genes and the immune-relevant *proPO* gene indicated that *NnrpS12* was significantly down-regulated in infected *N. norvegicus* and *proPO* was significantly up-regulated.

Results were discussed regarding the evidence of *Hematodinium* sp. to avoid recognition by the host immune system as well as the implications of actively suppressing host immune response by inhibiting the production of superoxide anions. Further consideration was given to the possibility of secondary infection during parasite infection.

# Contents

# List of Figures

# List of Tables

# Declaration of Authorship

I, JODI LEIGH HOPPES, declare that this thesis entitled, 'Effects of the dinoflagellate parasite *Hematodinium* sp. on the immune response of its crustacean hosts' and the work presented in it are my own. 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: _____

# *Acknowledgements*

I would like to thank my supervisors at the National Oceanography Centre, Dr. Chris Hauton and Dr. Lawrence E. Hawkins, for their steadfast support throughout my Ph.D. I would also like to thank my panel chair Dr. David S. M. Billett for his keen interest in my project. Particular thanks is due to Dr. Chris Hauton for his personal tuition in all things molecular and for his thorough reviews of my writing. His kindness and patience both in the lab and out were infallible to the end.

Thank you to the all of the people who helped me obtain samples for investigation. Thank you to Keith of Selsey Shellfish for providing *Cancer pagurus* and keeping watch for infections on the south coast. I thank the crews of the RV *Aora* and RV *Actinia* at the University Marine Biological Station, Millport for their assistance trawling for *Nephrops norvegicus*. Further thanks to the UMBS staff who made my visit a success, particularly Dr. Fiona Hannah who went to extreme lengths to post my samples.

Thank you to a great deal of support staff within NOCS for their time and knowledge, particularly Dr. John Gittins and Matt O'Shaughnessy. Further thanks to Jenny Mallinson for assisting in maintaining my research animals in the aquarium.

Lastly, I could not have completed my project or maintained my sanity without the support and encouragement of my family and friends. Thank you to my parents for their patience and understanding and to my brother for his strength and his ability to make me smile even when everything was falling apart. Thank you to Rob and his family for their continued support throughout my Ph.D. And extreme thanks to Kirsty, Anna and Frauke who held me up during the hard times and laughed with me when times were good.

# Abbreviations

| | |
|---|---|
| ACP | annealing control primer |
| AMP | antimicrobial peptide |
| BGBP | B1,3-glucan binding protein |
| cDNA | complementary DNA |
| CpG | oligodeoxynucleotide PAMP |
| Cq | quantification cycle |
| DEG | differentially expressed gene |
| DNA | deoxyribonucleic acid |
| ER | endogenous reference |
| GOI | gene of interest |
| ITS1 | internal transcribed spacer 1 |
| LGBP | LPS binding protein |
| LPS | lipopolysaccharide |
| mRNA | messenger RNA |
| NBT | nitroblue tetrazolium |
| NO | nitric oxide |
| ORF | open reading frame |
| PAMP | pathogen associated molecular pattern |
| PCR | polymerase chain reaction |
| PG | peptidoglycan |
| PGBP | peptidoglycan binding protein |
| PO | phenoloxidase |
| ppA | prophenoloxidase activating enzyme |
| ppaf | prophenoloxidase activating factor |
| proPO | prophenoloxidase |
| PRP | pattern recognition protein |
| qPCR | quantitative polymerase chain reaction |
| RACE | rapid amplification of cDNA ends |
| RBC | red blood cell |
| RNA | ribonucleic acid |
| ROS | reactive oxygen species |
| rRNA | ribosomal RNA |

| | |
|---|---|
| RT | reverse transcriptase |
| SO | superoxide |
| SOD | superoxide dismutase |
| sph | serine proteinase homologue |
| THC | total haemocyte count |
| Tm | melting temperature |
| UTR | untranslated region |
| WSSV | white spot syndrome virus |

*To my Parents and little Brother...*
*I've had the best days with you*

# Chapter 1

# Introduction

## 1.1 General Introduction

Crustaceans are an ancient subphylum of invertebrates that are one of the most abundant groups of animals on the planet and comprise around 52,000 currently identified species (Martin and Davis, 2001). They inhabit a wide range of ecosystems including marine, freshwater and terrestrial. Marine crustaceans are a popular food source and have become economically important as fishery species, both wild caught and farmed. UK fishermen in 2010 landed 28,000 tonnes of crab, primarily edible crab *Cancer pagurus*, worth £37 million in the UK and abroad, and 39,000 tonnes of Norway lobster *Nephrops norvegicus* worth £97 million (MMO, 2011). In recent years the fisheries for both species have increased as marine finfish stocks have decreased. Overall crab catch has increased by approximately 85% since 1994 and *N. norvegicus* catch has increased by 30% during the same time (MMO, 2011).

*Cancer pagurus* is a decapod crustacean of the infraorder Brachyura and the family Cancridae. *C. pagurus* inhabit coarse, rocky substrate typically at variable depths and have been fished along British coasts and throughout most European countries, with some of the most important fisheries occurring around Scotland and the south coasts of England (Edward, 1979). Catches have been constrained by seasonality, governed largely by weather and moulting, with most catches being landed between April and November (MMO, 2011) along England's south coast. Reduced mobility and metabolism due to cold water have led to poor winter catches (Edward, 1989).

*Nephrops norvegicus* is another decapod crustacean of the infraorder Astacidea and the family Nephropidae. *N. norvegicus* are burrowing animals that inhabit soft, muddy substrate and have been found at depths ranging from 10 to 900 m (Chapman *et al.*, 1975). Their geographical range extends from southern Portugal to Iceland and

northern Norway, though their strict habitat demands have led to a patchy distribution. *N. norvegicus* are predators and commonly scavenge during short excursions from their burrows (Atkinson and Naylor, 1976). Animals have typically been fished using bottom trawl and have had the highest landing rates during the summer months (June-Aug) (MMO, 2011). Until the 1950s the Norway lobster *Nephrops norvegicus* was considered a bycatch species and a nuisance by fishers because vast numbers of them clogged trawl and seine nets (Graham and Ferro, 2004). Increasing popularity of shellfish, however, has led to the fishery rapidly expanding throughout Europe and primarily in Scottish waters.

A large proportion of crustaceans caught by UK fishers are exported to Europe. *N. norvegicus* are primarily caught by trawl and tailed at sea before being sold as 'scampi,' though some animals are creel caught and transported live in vivier tanks (Graham and Ferro, 2004). Similarly, *C. pagurus* are transported by vivier tank to France and throughout Europe. The physiological stress caused by transport, in addition to that caused by capture and emersion, has been linked to poor meat quality and flavour (Stentiford and Neil, 2000). Furthermore, animals prestressed by disease or parasites are at a distinct disadvantage and are less likely to survive transportation causing economic losses for fishers (Stentiford and Neil, 2000).

## 1.2 Disease and crustaceans

Disease in fish populations has become an important topic for research as increased pressure on fish stocks has driven the need for effective management. Diseases have not only been found to reduce overall stock numbers, but have caused sublethal effects such as weight loss and carapace discolouration, rendering infected individuals unmarketable (Meyers *et al.*, 1987). Crustacean pathogens are numerous and affect both wild and farmed animals, and if an epizootic causes mass mortalities it has the potential to destroy entire populations leading to a fisheries crash (Sindermann, 1970). Non-infectious diseases can be caused by environmental factors such as temperature, hypoxia, nutrient deficiency and changes in salinity; cultured crustaceans are particularly susceptible to these diseases as a result of overcrowded systems and can suffer significant economic losses (Wickins and Lee, 2002). Other non-infectious diseases can be caused by pesticides, heavy metals and other pollutants (Wickins and Lee, 2002). For example, penaeid shrimp *Penaeus californiensis* fed a diet low in ascorbic acid suffered from a disease termed 'black death,' in which the animals developed blackened lesions in the subcuticular tissues caused by haemocyte aggregations in the absence of observable pathogens (Lightner *et al.*, 1977). Wild

crustacean populations, however, suffer primarily from pathogen-associated infectious diseases such as viruses, bacteria, fungi and protozoa.

Over 30 viruses have been described for crustaceans, mostly from penaeid shrimp, many of which have had significant impacts on crustacean farming (Chou *et al.*, 1995; Wickins and Lee, 2002; Lightner, 2011; Longshaw, 2011). White spot syndrome virus (WSSV), yellowhead virus (YHV) and Taura syndrome virus (TSV) have caused severe economic losses for many shrimp species and were able to wipe out an entire shrimp farm population in a matter of days (see Flegel (1997) for review). Despite the high prevalence of viruses in seawater ($>$ 10 million particles per millilitre seawater) (Breitbart *et al.*, 2007), Stentiford and Neil (2011) noted that few reports have been made on viruses in clawed lobsters, however a bacilliform virus has recently been reported to infect juvenile *C. pagurus* in the UK (Bateman and Stentiford, 2008).

Though not as numerous, bacteria play a substantial role in marine crustacean disease due to their ubiquitous presence in marine waters. The most commonly studied bacteria of marine crustaceans are the *Vibrio* species which cause shell disease and bacteraemia. Chitinolytic *Vibrio* spp. erode the chitinous exoskeleton causing 'black spot' (Vogan and Rowley, 2002) and excessive carapace lesions reduce the marketability of individuals (Woll, 2006). Furthermore, lesion sites can provide entry for other opportunistic pathogens causing septicaemia and other secondary infections (Vogan *et al.*, 2001). Outbreaks of the bacteria *Aerococcus viridans*, the causative agent of gaffkemia in lobsters, have caused mass mortality and significant economic losses in lobster pounds (Sindermann and Rosenfield, 1967; Wickins and Lee, 2002) and, similarly, farmed kuruma prawns *Penaeus japonicus* suffered mass mortalities from the bacteria *Vibrio nigripulchritudo* in Japan in 2005 (Sakai *et al.*, 2007).

Fungal diseases have also been reported for many crustacean species, notably the crayfish plague *Aphanomyces astaci* which affects the freshwater crayfish *Astacus astacus*, to the exclusion of most other hosts (Alderman, 1996). Rapid spread of *A. astacus* hyphae have typically been found to result in death within a couple of weeks and has significantly reduced native populations of European *Astacus astacus* (Alderman, 1996).

Parasites form a major group of pathogens affecting crustacean species. Stentiford (2008) suggested that protistan parasites have had the greatest detrimental effect on wild crustacean populations. Microsporidian parasites, *Enterospora* spp., have also recently been identified in *C. pagurus* and the hermit crab *Eupagurus bernhardus* from UK waters (Stentiford and Bateman, 2007; Stentiford *et al.*, 2007). The effect of *Enterospora* has yet to be determined although Stentiford *et al.* (2007) reported low prevalence. Alternately, the 'emerging disease' caused by the dinoflagellate parasite

*Hematodinium* sp. has had a severe impact on fisheries worldwide. *Hematodinium* epizootics have led to reduced fishing stocks, increased mortality in catches and, in extreme cases, fisheries closures (Meyers *et al.*, 1987). Furthermore, *Hematodinium*-infected animals have been reported to have a reduced meat yield and a bitter flavour (Meyers *et al.*, 1987; Taylor and Kahn, 1995).

## 1.3 *Hematodinium* spp.

*Hematodinium* sp. is a parasitic dinoflagellate of decapod crustaceans that was first reported by Chatton and Poisson (1931) and is a member of the Syndinial family (Hamilton *et al.*, 2007*b*). Syndiniales are obligate heterotrophs that are endoparasitic and can be cytoplasmic, intranuclear or reside in the haemolymph of their host organism (Coats, 1999). They include the *Amoebophyra* spp. which infect other dinoflagellate species, as well as several *Syndinium* spp. which infect copepods (Shields, 1994; Skovgaard *et al.*, 2005) and are part of the Group II marine alveolates (Skovgaard *et al.*, 2005; Saldarriaga *et al.*, 2004). Group I alveolates are comprised of a group of uncultured marine alveolates (alveoli-containing protists) which are phylogenetically linked to the Group II Syndinials, but whose morphology is still under investigation (Skovgaard *et al.*, 2005).

*Hematodinium* was first described from *Carcinus maenas* and *Liocarcinus depurator* in France by Chatton and Poisson (1931) and was named *Hematodinium perezi*. The prevalence was very low, however, (only 4 of 3570 crabs were infected) and no further reports were made on the parasite until the 1970s. The next report of *Hematodinium* sp. infection came from Newman and Johnson (1975) who identified a new disease from commercially important blue crabs *Callinectes sapidus* located in Florida, Georgia, North and South Carolina, USA and were able to match the description with that from Chatton and Poisson (1931). Three years later, further infections were reported in the cancer crabs *Cancer irroratus* and *Cancer borealis* as well as the portunid crab *Ovalipes ocellatus*; these reports extended the range northwards from North Carolina to Massachusetts (Maclean and Ruddell, 1978). *Hematodinium* spp. infection is now a globally recognised disease and has been located on both the east and west coast of the United States and Canada (Newman and Johnson, 1975; Maclean and Ruddell, 1978; Meyers *et al.*, 1987; Taylor and Kahn, 1995; Bower *et al.*, 2003), throughout the UK (Field *et al.*, 1992; Stentiford *et al.*, 2002; Stentiford and Feist, 2005) and Ireland (Chualain *et al.*, 2009), Russia (Ryazanova, 2008; Kharlamenko *et al.*, 2010), China (Li *et al.*, 2008*b*; Xu *et al.*, 2010), and Australia (Hudson and Shields, 1994; Hudson and Adlard, 1994) (Table 1.1).

TABLE 1.1: *Hematodinium* spp. and hosts

| **Crustacean Species** | **Location** | ***Hematodinium* species** | **Peak Infection Season** | **Reference** |
|---|---|---|---|---|
| *Cancer pagurus* | English Channel | *Hematodinium* sp. | | (Stentiford *et al.*, 2002) |
| | Bay of Biscay | *Hematodinium* sp. | Winter | (Latrouite *et al.*, 1988) |
| | Ireland | *Hematodinium* sp. | Late Autumn/Winter | (Chualain *et al.*, 2009) |
| | West Coast, Scotland | *Hematodinium* sp. | Autumn | (Hamilton *et al.*, 2009) |
| *Liocarcinus depurator* | French Coast | *Hematodinium perezi* | | (Chatton and Poisson, 1931) |
| | West Coast, Scotland | *Hematodinium* sp. | Spring | (Hamilton *et al.*, 2009) |
| | Denmark | *Hematodinium* sp. | | (Eigemann *et al.*, 2010) |
| *Nephrops norvegicus* | West Coast, Scotland | *Hematodinium* sp. | Spring Early Summer | (Field *et al.*, 1992, 1998) |
| | Irish Sea | *Hematodinium* sp. | Spring | (Briggs and McAliskey, 2002) |
| | Denmark | *Hematodinium* sp. | | (Eigemann *et al.*, 2010) |
| *Necora puber* | Mor-Braz, France | *Hematodinium* sp | Autumn | (Wilhelm and Mialhe, 1996) |
| | English Channel | *Hematodinium* sp. | | (Stentiford *et al.*, 2003) |
| | West Coast, Scotland | *Hematodinium* sp. | Autumn | (Hamilton *et al.*, 2009) |
| *Carcinus maenas* | French Coast | *Hematodinium perezi* | | (Chatton and Poisson, 1931) |
| | Southampton, UK | *Hematodinium perezi* | | (Stentiford and Feist, 2005) |
| | West Coast, Scotland | *Hematodinium* sp. | April | (Hamilton *et al.*, 2007*b*) |
| *Orchomene nanus* | West Coast, Scotland | *Hematodinium* sp. | | (Small *et al.*, 2006) |
| *Pagurus bernhardus* | West Coast, Scotland | *Hematodinium* sp. | April | (Hamilton *et al.*, 2009) |
| | Denmark | *Hematodinium* sp. | | (Eigemann *et al.*, 2010) |
| *Pagurus prideaux* | West Coast, Scotland | *Hematodinium* sp. | | (Hamilton *et al.*, 2009) |

TABLE 1.1: (continued)

| **Crustacean Species** | **Location** | ***Hematodinium* species** | **Peak Infection Season** | **Reference** |
|---|---|---|---|---|
| *Munida rugosa* | West Coast, Scotland | *Hematodinium* sp. | | (Hamilton *et al.*, 2009) |
| *Paralithodes camtschaticus* | Sea of Okhotsk | | August-mid October | (Ryazanova, 2008; Kharlamenko *et al.*, 2010) |
| *Paralithodes platypus* | Sea of Okhotsk | | August-mid October | (Ryazanova, 2008; Kharlamenko *et al.*, 2010) |
| *Callinectes sapidus* | Southeast Coast, USA | *Hematodinium perezi* | Late Autumn | (Newman and Johnson, 1975; Messick, 1994) |
| | Georgia, USA | *Hematodinium* sp. | Spring Autumn | (Sheppard *et al.*, 2003) |
| | MD & VA, USA | *Hematodinium* sp. (Portunoid Clade) | | (Jensen *et al.*, 2010) |
| *Libinia dubia* | Virginia, USA | *Hematodinium* sp. | | (Pagenkopp *et al.*, 2011) |
| *Eurypanopeus depressus* | Virginia, USA | *Hematodinium* sp. | | (Pagenkopp *et al.*, 2011) |
| *Pagurus pollicaris* | Virginia, USA | *Hematodinium* sp. | | (Pagenkopp *et al.*, 2011) |
| *Callinectes similus* | Georgia, USA | *Hematodinium* sp. | | (Sheppard *et al.*, 2003) |
| *Cancer irroratus* | East Coast, USA | *Hematodinium perezi* | | (Maclean and Ruddell, 1978) |
| *Cancer borealis* | East Coast, USA | *Hematodinium perezi* | All year | (Maclean and Ruddell, 1978) |
| *Ovalipes ocellatus* | East Coast, USA | *Hematodinium perezi* | | (Maclean and Ruddell, 1978) |
| *Hexapanopeus angustifrons* | East Coast, USA | *Hematodinium* sp. | | (Messick and Shields, 2000) |

TABLE 1.1: (continued)

| **Crustacean Species** | **Location** | ***Hematodinium* species** | **Peak Infection Season** | **Reference** |
|---|---|---|---|---|
| *Chionoecetes opilio* | Newfoundland, Canada | *Hematodinium* sp. | | (Taylor and Kahn, 1995; Dawe, 2002; Pestal *et al.*, 2003) |
| | Greenland | *Hematodinium* sp. | | (Eigemann *et al.*, 2010) |
| *Chionoecetes bairdi* | Alaska, USA | *Hematodinium* sp. | Summer | (Meyers *et al.*, 1987; Eaton *et al.*, 1991; Love *et al.*, 1993) |
| *Chionoecetes tanneri* | BC, Canada | *Hematodinium* sp. | | (Bower *et al.*, 2003) |
| *Chionoecetes angulatus* | BC, Canada | *Hematodinium* sp. (Chionoecetes Clade) | | (Jensen *et al.*, 2010) |
| *Lithodes couesi* | Vancouver Island, Canada | *Hematodinium* sp. (Chionoecetes Clade) | | (Jensen *et al.*, 2010) |
| *Hyas coarctatus* | Bering Sea | *Hematodinium* sp. (Chionoecetes Clade) | | (Jensen *et al.*, 2010) |
| *Hyas araneus* | Greenland | *Hematodinium* sp. | | (Eigemann *et al.*, 2010) |
| *Neopanope sayi* | Georgia, USA | *Hematodinium* sp. | Autumn | (Sheppard *et al.*, 2003) |
| *Libinia emarginata* | Georgia, USA | *Hematodinium* sp. | Autumn | (Sheppard *et al.*, 2003) |
| *Menippe mercenaria* | Georgia, USA | *Hematodinium* sp. | | (Sheppard *et al.*, 2003) |

TABLE 1.1: (continued)

| **Crustacean Species** | **Location** | ***Hematodinium* species** | **Peak Infection Season** | **Reference** |
|---|---|---|---|---|
| *Scylla serrata* | Australia<br>Southeastern China | *Hematodinium* sp | All year, low prevalence | (Hudson and Lester, 1994)<br>(Li *et al.*, 2008*b*) |
| *Portunus pelagicus* | Australia | *Hematodinium australis* | | (Hudson and Shields, 1994) |
| *Trapezia areolata* | Australia | *Hematodinium* sp. | | (Hudson *et al.*, 1993) |
| *Exopalaemon carinicauda* | Eastern China | *Hematodinium* sp. | | (Xu *et al.*, 2010) |

Epizootics of *Hematodinium* have led to reduced catch and fishery closures causing substantial economic losses. Alaskan Tanner crab *Chionoecetes bairdi* fisheries suffered an estimated 5% loss during the 1988/89 season (Meyers *et al.*, 1990) and prevalences reached 100% in some populations of blue crabs *Callinectes sapidus* in Maryland and Virginia during peak season (Messick, 1994). Infection prevalence has shown evidence of seasonality in the majority of host species although peak infection seasons vary by location (Table 1.1). Up to 90% infection prevalence was observed in late autumn in *C. sapidus* from the eastern United States (Messick, 1994; Messick and Shields, 2000), however Alaskan *Chionoecetes bairdi* suffered the most during the summer (Love *et al.*, 1993). Scottish crustacean populations had prevalence peaks in either the spring in *Nephrops norvegicus* (Field *et al.*, 1992) or autumn in *Cancer pagurus* (Hamilton *et al.*, 2009). Outbreaks of *Hematodinium* sp. have led to reduced catch and, in extreme cases such as the Alaskan Tanner crabs *Chionoecetes bairdi*, closure of the fishery (Meyers *et al.*, 1987). Infected animals have been unmarketable due to poor muscle quality and a bitter aspirin-like taste of tainted tissue, which consequently led to the name Bitter Crab Disease (BCD) (Meyers *et al.*, 1987; Taylor and Kahn, 1995; Ryazanova, 2008). Heavily infected animals often died quickly following handling and were incapable of surviving transportation in vivier tanks (Hudson and Shields, 1994; Stentiford *et al.*, 2002).

There are currently two described species of *Hematodinium*, though recent genetic data have shown that the species descriptions may warrant some redefinition. The type species *H. perezi*, which was originally described from *Car. maenas* and *Liocarcinus depurator* (Chatton and Poisson, 1931), was then ascribed to a dinoflagellate parasite found in *Cal. sapidus* and other crab species from the east coast of the US based on morphology (Newman and Johnson, 1975; Maclean and Ruddell, 1978). The second described species is that from the Australian sand crab, *Portunus pelagicus*, and due to some distinct morphological differences, was designated *Hematodinium australis* (Hudson and Shields, 1994). Following these initial morphological descriptions, *Hematodinium*-like infections were identified in numerous crustacean hosts, however some confusion has arisen over whether the parasite isolated from the US *Cal. sapidus* populations is, in fact, *H. perezi*. Recently, *Hematodinium*-like parasites from geographically distinct locations and between various host species have been tested using molecular techniques. Hudson and Adlard (1996) compared partial sequences of the small subunit (SSU) rDNA from *H. perezi* ex. *Callinectes sapidus*, and three *Hematodinium*-like dinoflagellates from *N. norvegicus*, *Chionoecetes bairdi* and *Ch. opilio* and confirmed that all parasites were from the genus *Hematodinium*. It was further suggested that *Hematodinium* from *N. norvegicus* should be a separate species from *Hematodinium* ex. *Ch. bairdi* and *Ch. opilio*, and that both were different from

'*H. perezi*' ex. *Cal. sapidus*. Further comparison of sequence variability of the first internal transcribed spacer region (ITS1) of *Hematodinium* ex. *Nephrops norvegicus*, *Cancer pagurus* and *Pagurus bernhardus* from four UK locations as well as *Chionoecetes opilio* from Newfoundland and Labrador, Canada, determined that *Hematodinium* within the UK was the same species, however there were large genetic differences from Canada (Small *et al.*, 2007*b*). Similarity of the parasite within UK host species was confirmed on a smaller scale by a study comparing the ITS1 and ITS2 regions of *Hematodinium* from *Carcinus maenas* and *N. norvegicus* from the Clyde Sea, Scotland (Hamilton *et al.*, 2007*a*). These reports were supported by Jensen *et al.* (2010) who found that *Cal. sapidus* harboured *Hematodinium* from a clade separate from that containing all other Northern Pacific and Atlantic species (*Chionoecetes tanneri*, *Ch. opilio*, *Ch. bairdi*, *Ch. angulatus*, *Hyas coactatus*, *Lithodes couesi* and *N. norvegicus*). However, Small *et al.* (2007*b*) found four microsatellites which caused some variation in sequence length and could suggest different strains of *Hematodinium*. Further studies by Small *et al.* (2007*c*) found that restriction enzyme digests were able to distinguish between *Hematodinium* sp. infection in *Cal. sapidus* vs. *Lio. depurator* and *P. trituberculatus*, identifying a possible method of differentiating *Hematodinium* species. A more recent study using both ITS1 and ITS2 regions to compare *Hematodinium* spp. has suggested that *Hematodinium* from *N. norvegicus* and other UK crabs reside in different clades (Hamilton *et al.*, 2009), however cross-infectivity between species remains untested. Overall, there appears to be a clear difference between the *Hematodinium* present in *C. sapidus* populations and that present in other crustaceans from both the North Pacific and the North Atlantic (Hamilton *et al.*, 2007*a*; Small *et al.*, 2007*b*; Jensen *et al.*, 2010), with the potential for differences in some of the North Atlantic species (Small *et al.*, 2007*b*; Hamilton *et al.*, 2009).

### 1.3.1 Pathology

Externally, *Hematodinium* infection has typically only been apparent in heavily infected animals where it has been diagnosed from hyperpigmentation of the carapace giving the host animal a 'cooked' appearance. The hyperpigmentation gave rise to the name Pink Crab Disease in *Cancer pagurus* (Stentiford *et al.*, 2002), however the change in carapace colour has been reported in most infected species. Further external evidence of infection has included identification of a yellowing of the arthrodial membranes due to excessive numbers of parasite cells in the haemolymph (Field *et al.*, 1992). Heavily infected *Chionoecetes bairdi* and *Nephrops norvegicus* were lethargic (Meyers *et al.*, 1987; Field *et al.*, 1992; Messick, 1994), and *Portunus pelagicus* and *C.*

*pagurus* were moribund and died quickly after handling (Hudson and Shields, 1994; Stentiford *et al.*, 2002).

The most common observation regarding internal pathology of infected animals has been the distention of haemal sinuses as a result of overwhelming numbers of parasite cells (Field *et al.*, 1992). The pressure from excessive parasite load was found to disrupt and degrade connective tissue (Meyers *et al.*, 1987) (pressure necrosis), and furthermore was found to restrict flow of haemocytes (Messick, 1994). Several studies have reported haemocytopoenia (a reduction in haemocytes) in infected animals including *N. norvegicus* (Field and Appleton, 1995), *Portunus pelagicus* (Hudson and Shields, 1994) and *Callinectes sapidus* (Shields *et al.*, 2003), however only Shields *et al.* (2003) have done a quantitative assessment of haemocyte numbers (50-70% decline in infected animals). Experimentally infected animals were also observed to have reduced haemocyte numbers and, in addition, haemocyte subpopulations fluctuated as well (Shields and Squyars, 2000).

Hepatopancreas and gill tissue have been severely altered following infection in numerous hosts of *Hematodinium* spp. The hepatopancreas, or digestive gland, in crustaceans is responsible for the production of digestive enzymes and glycogen storage. In *Hematodinium*-infected *Cancer pagurus*, hepatopancreatic tubules were degraded and lacked lipid reserves (Stentiford *et al.*, 2002). Tubule degeneration has also been observed in *N. norvegicus* (Field *et al.*, 1992), *C. bairdi* (Meyers *et al.*, 1987), and the ridgetail white prawn *Exopalaemon carinicauda* in China (Xu *et al.*, 2010). Wheeler *et al.* (2007) observed a reduction in reserve inclusion (RI) cells responsible for glycogen storage in *Chionoecetes opilio*, in addition to a loss of spongy connective tissue, similar to that observed in heavily infected *Cancer pagurus* (Stentiford *et al.*, 2002). *C. bairdi* from Alaska also had severe cell necrosis combined with increased vacuolation and occasional parasite cells contained within the tubule lumina (Meyers *et al.*, 1987). In some cases, parasite cells were found attached to hepatopancreatic tissue where it formed a multinucleate stage from which uninucleate cells detached into the haemolymph (Hamilton, 2007); Stentiford *et al.* (2002) hypothesised that the hepatopancreas could be a site of latent infection. Disruption of the hepatopancreas, an organ crucial to nutrient storage, in combination with reduced lipid reserves led Field *et al.* (1992) and Stentiford *et al.* (2002) to suggest that *Hematodinium* infection could cause physiological starvation in host animals, despite the fact that infected *N. norvegicus* were still observed to feed (Field *et al.*, 1992).

Disruption of gill tissue has been speculated to cause further physiological distress by impeding oxygen uptake (Field *et al.*, 1992; Field and Appleton, 1995; Wheeler *et al.*, 2007). During histological examination gill haemal sinuses have frequently been found

to be occluded by large numbers of parasite cells with very few haemocytes present (Field and Appleton, 1995; Sheppard *et al.*, 2003; Xu *et al.*, 2010; Chualain and Robinson, 2011). In animals where some haemocytes were present, they formed aggregations that contributed to and exacerbated the distention of gill filaments (Field *et al.*, 1992; Field and Appleton, 1995; Sheppard *et al.*, 2003). In *Chionoecetes opilio*, gill filaments had a noticeable loss of spongy connective tissue, compression of the epithelium and pressure necrosis (Wheeler *et al.*, 2007). Hamilton *et al.* (2007*b*) observed similar gill pathology in *C. maenas* and the distal ends of the gill lamellae were swollen and distended. Ultimately, parasite sporulation was found to rupture the gill cuticle to release parasite cells from *N. norvegicus* and *C. opilio*, an event which is reportedly fatal for *N. norvegicus* and *Chionoecetes bairdi* (Meyers *et al.*, 1987), and presumed fatal for *C. opilio* as well (Appleton and Vickerman, 1998; Wheeler *et al.*, 2007). Histological examination of gill filaments immediately prior to and during sporulation revealed that the epithelium was disrupted and the lamellae fused (Wheeler *et al.*, 2007).

Muscle tissue, the primary commercial product from crustaceans, has generally been found to be intact during *Hematodinium* infection, though parasite cells have been reported. Slight degradation of muscle cells was found in *Chionoecetes bairdi* (Meyers *et al.*, 1987), *Libinia emarginata* and *Menippe mercenaria*, and *Callinectes sapidus* was also found to have points of focal necrosis in the skeletal muscle (Sheppard *et al.*, 2003). *Nephrops norvegicus* was found to have some disruption of abdominal muscle tissue, particularly around the peripheral tissue, but was largely intact structurally (Field *et al.*, 1992; Field and Appleton, 1995; Stentiford *et al.*, 2000). In contrast, claw muscle of *Cancer pagurus* from the English Channel was found to be degraded and almost completely replaced with parasites (Stentiford *et al.*, 2002), however infected animals from Irish Sea populations reportedly had few parasites within their claws (Chualain and Robinson, 2011). Overall, tightly compacted muscle tissue, while suffering from a mild invasion of parasite cells, has reportedly maintained the bulk of its structural integrity.

Other tissues have been mentioned in conjunction with *Hematodinium* infection, though have not formed the primary basis of any studies to date. The antennal gland of heavily infected *N. norvegicus* was infiltrated with uninucleate parasite cells (Field and Appleton, 1995), causing potential disruption to the excretory system. Midgut tissue has also been found to contain considerable numbers of parasite cells and suffer severe disruption of the connective tissue (Field *et al.*, 1992; Field and Appleton, 1995); Field *et al.* (1992) suggested that the gut may pose a possible entry point for parasite infiltration, though this has not yet been confirmed. *Hematodinium* cells have also been identified in heart muscle tissue from *Carcinus maenas* (Hamilton *et al.*,

2007*b*; Hamilton, 2007), *Exopalaemon carinicauda* (Xu *et al.*, 2010) and *Chionoecetes opilio* (Wheeler *et al.*, 2007). *Chio. opilio* heart muscle had reduced connective tissue and heavily infected animals had severely disrupted muscle fibers (Wheeler *et al.*, 2007). Field and Appleton (1995) also examined brain and eyestalk tissue for the presence of parasite, but they were only found in haemal spaces and no overt parasite invasion was evident. Histologically, parasite cells appear to have been concentrated in areas of potential invasion (gill and gut tissue) or in tissues high in nutrients (hepatopancreas, muscle, haemolymph).

### 1.3.2 *Hematodinium* sp. life cycle and transmission

The entire life history of *Hematodinium* sp. has yet to be elucidated; however, several experiments have given indications of certain key stages. Eaton *et al.* (1991) were able to make several observations on life stages during the progression of *Hematodinium* infection in *Chionoecetes bairdi*. Appleton and Vickerman (1998) observed and documented several life cycles of *Hematodinium* sp. from *N. norvegicus* in culture (Fig 1.1) which identified many potential stages but may not represent an exact replication of the life history due to the lack of any essential nutritional elements or the potential presence of an intermediate host.

Based on observations, Appleton and Vickerman (1998) described the following life cycle: Parasites isolated from late-stage infections had the appearance of sporoblasts which gave rise to flagellated dinospores via sporogenesis or became a branched network of nucleated filaments (secondary sporonts). Uninucleate dinospores took the form of either macrospores (Fig 1.2A) or microspores (Fig 1.2B), both of which eventually became immotile and formed a monolayer in culture flasks producing multinucleate filaments. Filaments grew and branched forming trophont clouds or sheets extending into the medium. In less dense cultures filamentous colonies formed 'gorgonlocks' colonies (Fig 1.2D) (so named for the writhing movements of the filaments) with filaments radiating from a central point. If cultures were excessively diluted the trophonts adhered to the substratum and formed syncytial networks with anchoring points called arachnoid trophonts. Arachnoid trophonts transformed into arachnoid sporonts (Fig 1.2C) which adopted a volcano-like appearance from which sporoblasts (structurally identical to the circulating haemolymph stage) were generated.

Currently, disease transmission is still unclear for *Hematodinium* infection. Successful infections have been established using passage of infected haemolymph in *Callinectes sapidus* (Messick and Shields, 2000; Shields and Squyars, 2000) and *Chionoecetes bairdi*

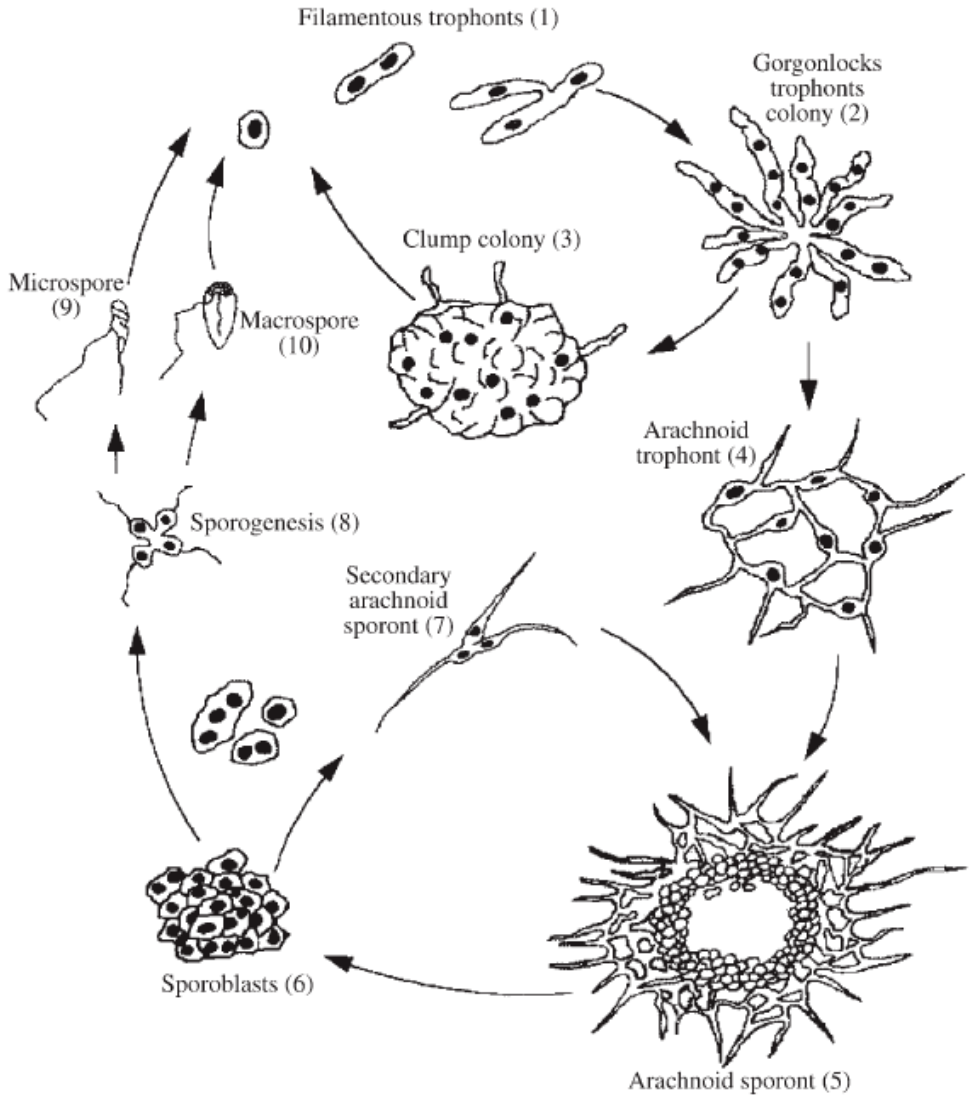



FIGURE 1.1: Schematic diagram of the *in vitro* development cycle of *Hematodinium* sp. ex-*N. norvegicus*. Figure from Appleton and Vickerman (1998).

(Eaton *et al.*, 1991), though, to date, no successful reports of infection by passage have been made for *N. norvegicus* or *Cancer pagurus*. Transmission by injection has been useful for experimental infection studies and the study of *Hematodinium* spp. life history stages, however it has not led to definite conclusions on the infective stage of the parasite. Appleton and Vickerman (1998) suggested that dinospores produced during sporulation events from *N. norvegicus* could be the primary infective state, and evidence of free-living dinospores has been found in water and suspended sediment samples from estuaries in Virginia, USA (Li *et al.*, 2010) as well as from plankton samples from the Clyde Sea, Scotland (Hamilton *et al.*, 2011). Additionally, Frischer *et al.* (2006) observed putative dinospores in the haemolymph of *Callinectes sapidus* from Georgia, USA. Further experimental exposure studies by Frischer *et al.* (2006) using

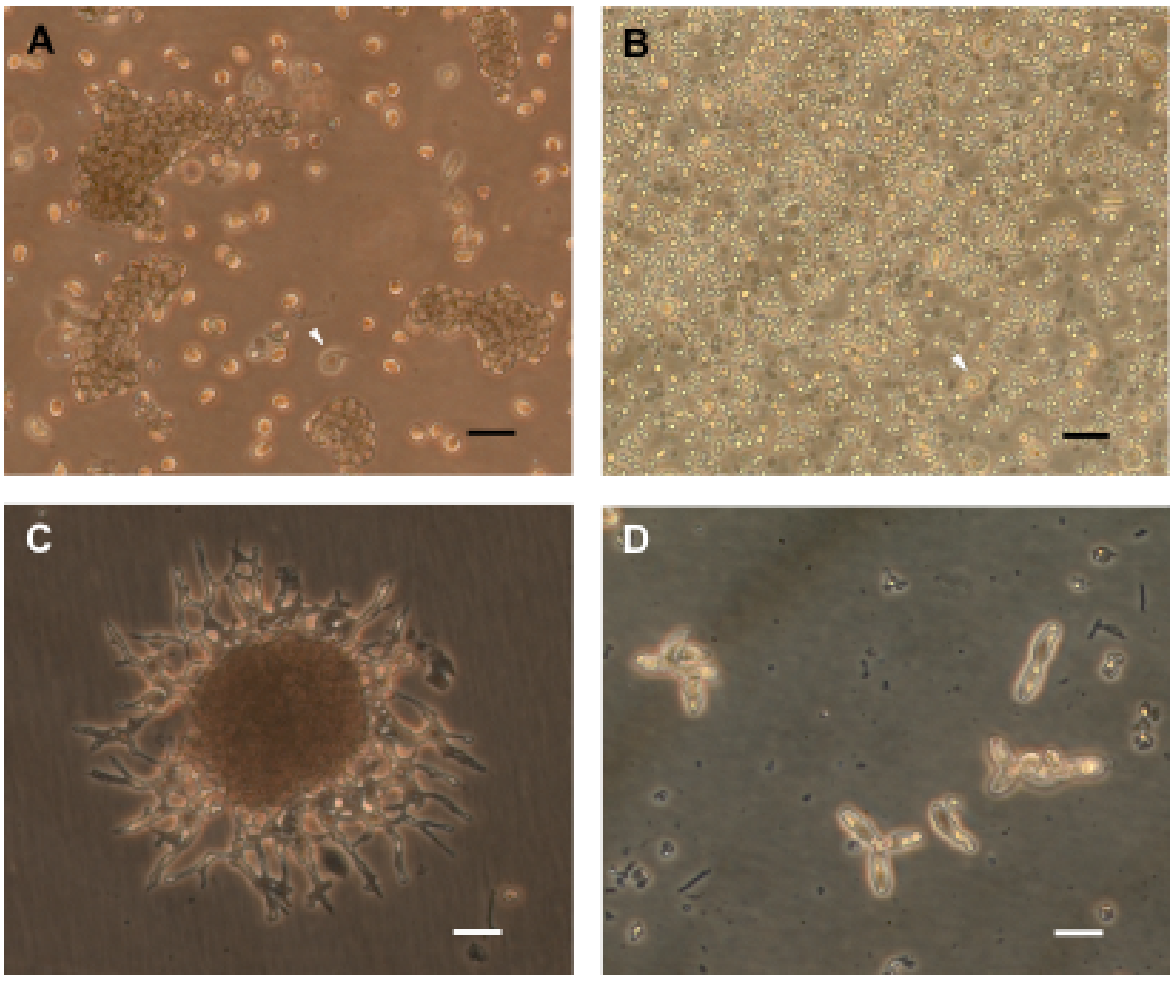



FIGURE 1.2: Micrograph images of *in vitro* life stages of *Hematodinium* sp. ex-*N. norvegicus*, including (A) macrospores, (B) microspores (host haemocytes indicated by white arrows), (C) arachnoid sporont and (D) gorgonlocks. Scale bar = 20 $\mu$m.

naïve *C. sapidus* found that animals could acquire infection by remaining in close proximity to infected crabs or by being placed in aquaria previously containing dinospore-infected crabs. The exact mode of transmission was not clearly determined however, and there was the possibility that infections could have developed from undetected latent infections. Furthermore, infected crabs reportedly died 4 days post-infection (Frischer *et al.*, 2006), which is inconsistent with disease successfully transmitted by injection of haemolymph containing parasite trophonts (Messick and Shields, 2000; Shields and Squyars, 2000). Following infection by passage, crabs did not begin to die until 14-17 post-inoculation (Messick and Shields, 2000; Shields and Squyars, 2000) and observations on naturally infected crabs found that they survived 14-35 days after capture (Messick and Shields, 2000).

Disease transmission through feeding has also been proposed and pathological observations of *Hematodinium* infiltration in the gut tissue of *N. norvegicus*, combined with the disruption of the connective tissue, support this idea (Field and Appleton,

1995). Several studies have suggested there may be an intermediate host (Appleton and Vickerman, 1998; Small *et al.*, 2006) which is consumed by crustacean hosts, however this has yet to be confirmed. Small *et al.* (2006) located *Hematodinium* ex *N. norvegicus* in the scavenging amphipod prey *Orchomene nanus* using PCR, however they were unable to confirm whether the cells were present in tissues, the gut or simply attached to the outer surface of the animal. Feeding studies of *O. nanus* found animals would feed indiscriminately on parasitised or unparasitised *N. norvegicus* carrion (Hamilton, 2007), so it is still a possible transmission route, however further investigation is required. Ongoing experiments in Alaskan tanner crabs *Chionoecetes bairdi* intend to use quantitative polymerase chain reaction (qPCR) as a method of identifying potential intermediate hosts and vectors which may store parasites (Crosson *et al.*, 2008).

Cannibalism has also been studied as a potential method of parasite infection as many crabs and other crustaceans are known to be opportunistic feeders. Recent experiments in which *Callinectes sapidus* were fed infected tissue found that *Hematodinium* could be detected in the haemolymph after only 16 hours (Walker *et al.*, 2009). These results were not entirely conclusive however, as the infection could have resulted from undetected latent infection or from release of spores from the tissue into the water column. Other studies have had difficulty using this method of transmission and Hudson and Shields (1994) were not able to establish infection in mud crabs *Scylla serrata* or sand crabs *Portunus pelagicus* fed infected tissue. An extensive study by Li *et al.* (2011) examined the development of *Hematodinium* infection in 120 adult and juvenile blue crabs *C. sapidus* fed infected tissue, however only two adult animals were found to contract an infection and were suspected to contain subpatent infections that were undetected during screening. Interestingly, *N. norvegicus* showed reluctance to feed on infected tissue when offered a choice (Hamilton, 2007), though further investigation would be required to establish the reason. Ultimately, while transmission via cannibalism can not be completely ruled out, it is considered unlikely to be the primary method of transmission.

### 1.3.3 Diagnostics

Initially, crustaceans infected with *Hematodinium* were identified by carapace and haemolymph colour. Infected animals showed hyperpigmentation of the carapace and the haemolymph appeared milky (Chatton and Poisson, 1931; Newman and Johnson, 1975; Maclean and Ruddell, 1978). This cursory external examination, however, only revealed heavily infected individuals and severely underestimated infection rates (Stentiford *et al.*, 2001*c*). Examination of stained haemolymph smears was more

definitive and showed the presence of often multinucleate cells similar in size to host haemocytes with densely stained chromosomes (Newman and Johnson, 1975; Maclean and Ruddell, 1978), although this technique was not ideal for field studies. Pleopod staging, in which pleopods from *N. norvegicus* were examined for parasite cells using light microscopy and graded from I-IV, was found to be a successful alternative field method (Field and Appleton, 1996; Stentiford *et al.*, 2001*c*) and gave some indication of infection level, though did not work on all crustaceans and still underestimated *Hematodinium* prevalence in the early season (Stentiford *et al.*, 2001*b*).

Laboratory approaches have been the most accurate for determining infection status and techniques for parasite detection have progressed rapidly during the last 15 years. In 1996 an indirect fluorescent antibody technique (IFAT) was developed for *Hematodinium* from *N. norvegicus* to calibrate field diagnostic results and was able to detect more subpatent infections than pleopod staging. Development of an immunoassay technique improved the sensitivity and was used to determine parasite load; the sensitivity limit was estimated at 204 parasites $mm^{-3}$ (Stentiford *et al.*, 2001*a*). An ELISA-based (enzyme-linked immunosorbent assay) method provided even higher sensitivity (5 parasites $mm^{-3}$) (Small *et al.*, 2002). All of these diagnostic techniques had the major advantage of being objective as researchers were not required to identify parasite cells from histological slides or blood smears.

Molecular detection has been particularly popular and with improved molecular techniques the diagnostics have gradually improved as well. The first use of polymerase chain reaction (PCR) for *Hematodinium* detection was by Hudson and Adlard (1994) who were able to isolate a 680 bp 'diagnostic' band from the internal transcribed spacer (ITS1) of small subunit (SSU) ribosomal DNA (rDNA) of *Hematodinium* sp. from *Chionoecetes opilio*, *Callinectes sapidus*, *N. norvegicus* and *Portunus pelagicus*. Hudson and Adlard (1994) based their diagnostics on the fact that DNA from host tissues amplified with the same primers resulted in a band of 1300 bp, however they later went on to sequence the band and confirmed that it was from *Hematodinium* sp. (Hudson and Adlard, 1996). The same gene has now been repeatedly used for diagnostics of infection status in haemolymph and tissue samples in all regions using various primers (Gruebl *et al.*, 2002; Small *et al.*, 2006, 2007*c*) and has a sensitivity limit of 0.6 parasites in a sample (which was equivalent to 1 ng DNA) (Small *et al.*, 2006). Furthermore, the technique has been used to detect *Hematodinium* in laboratory and environmental water samples (Li *et al.*, 2010).

Although many of these techniques can detect low levels of parasite in the haemolymph and tissue and can be high throughput, most of them are qualitative and, with the exception of direct cell counts, do not give sufficient information regarding the

quantity of parasites. Recent studies have developed real-time quantitative PCR (qPCR) assays to determine *Hematodinium* load in infected hosts (Nagle *et al.*, 2009). The assay was sensitive enough to detect 5 parasite cells $ml^{-1}$ and, furthermore, was able to quantify parasite numbers in tested animals. qPCR assays have also been developed to use on environmental samples (Frischer *et al.*, 2006; Donaldson *et al.*, 2009; Li *et al.*, 2010; Reece *et al.*, 2010) and to study life history progression of *Hematodinium* in Tanner crab populations from Alaska (Crosson *et al.*, 2008, 2009).

### 1.3.4 Effects of *Hematodinium* sp. on host physiology and biochemistry

In crustacean hosts, rapid exponential growth of *Hematodinium* populations has led to some severe physiological changes in haemolymph as well as crucial alterations to plasma and tissue biochemistry (Love *et al.*, 1996; Taylor *et al.*, 1996; Stentiford *et al.*, 1999, 2001*c*; Shields *et al.*, 2003). A more detailed discussion of physiological and biochemical effects of *Hematodinium* infection can be found in Chapter 5.1.2; however in general, high parasite numbers have been found to induce haemolymph acidosis, reduce oxygen carrying capacity (Love *et al.*, 1996; Shields, 2003) and deplete both glucose and glycogen levels (Stentiford *et al.*, 2001*c*; Shields *et al.*, 2003). To date, detailed studies of changes in host physiology and biochemistry have primarily focussed on *Callinectes sapidus* and *Nephrops norvegicus*. Major alterations to metabolic processes such as carbohydrate handling and glycogen storage have supported suggestions of physiological starvation during *Hematodinium* infection in *N. norvegicus* and *Callinectes sapidus* (Stentiford *et al.*, 2001*c*; Shields *et al.*, 2003). Furthermore, elevated concentrations of crustacean hyperglycaemic hormone (CHH), which controls the release of glucose from storage tissues during stress (Fanjul-Moles, 2006), were evident in *Hematodinium*-infected *N. norvegicus*, leading to the hypothesis that high glucose demands during heavy infection could interfere with the CHH negative feedback loop (Stentiford *et al.*, 2001*c*; Chang, 2005). Additional metabolic stress has been identified in the form of increased free amino acid concentrations in the haemolymph of *N. norvegicus*, likely the result of tissue degradation (Stentiford *et al.*, 1999, 2000).

Respiratory stress resulting from decreased plasma concentrations of the oxygen-carrying molecule haemocyanin and reduced carrying capacity in the remaining haemocyanin molecules in *N. norvegicus* has been found to increase hypoxia and acidosis of both tissue and haemolymph (Taylor *et al.*, 1996). Reduced haemocyanin concentrations have also been reported for *Chionoecetes bairdi* and *Callinectes sapidus* (Love *et al.*, 1996; Shields *et al.*, 2003). The combined effects of these physiological and

biochemical parameters have been found to impact total haemocyte counts (Cheng *et al.*, 2002; Verghese *et al.*, 2008) and thus severely immunocompromise crustacean hosts during infection. Furthemore, reduced immunocompetence could increase the risk of secondary infections and indeed, several reports have been made of secondary infections in *Hematodinium*-infected *Chionoecetes bairdi* (Meyers *et al.*, 1987) and *Cancer pagurus* (Stentiford *et al.*, 2003).

### 1.3.5 *Hematodinium* sp. effects on immunity

As previously mentioned, haemocytopaenia has been a primary pathological observation in crustaceans infected with *Hematodinium*-like parasites (Field *et al.*, 1992; Field and Appleton, 1995; Stentiford *et al.*, 2002; Shields, 2003). Circulating haemocytes (blood cells) in crustaceans, and indeed in all invertebrates, are the primary mediators of internal defense and play an active role in wound repair, phagocytosis, encapsulation and non-self recognition. Furthemore, they contain proteins and enzymes used to control the spread of pathogens and house the components necessary for the key internal defense system, the prophenoloxidase cascade.

Despite the clear decreases in haemocyte numbers and the implications this could have on host immunocompetence, very few studies have looked at the effects of *Hematodinium* infection on the immune response in crustaceans. To date, observations of immune response have only been made based on pathological investigations and the most common evidence of an internal defense reaction has been nodule formation and encapsulation reactions (Meyers *et al.*, 1987; Field and Appleton, 1995; Stentiford *et al.*, 2002; Sheppard *et al.*, 2003; Walker *et al.*, 2009). Melanised haemocytic encapsulations have been noted in the heart tissue and hepatopancreas of *Cancer pagurus* (Stentiford *et al.*, 2002) and *Callinectes sapidus* (Sheppard *et al.*, 2003; Walker *et al.*, 2009). Further encapsulations and granuloma-like foci have been found in haemal sinuses of *Cal. sapidus* (Messick, 1994) and *Chionoecetes bairdi* (Meyers *et al.*, 1987), and the claw muscle of *Can. pagurus* (Stentiford *et al.*, 2002). Importantly, these observations have not been confirmed to contain parasite cells (Field and Appleton, 1995) and numerous reports of secondary infection in *Hematodinium*-infected hosts (Meyers *et al.*, 1987; Field *et al.*, 1992; Stentiford *et al.*, 2003) make it possible that the observed responses are a result of colonisation by opportunistic pathogens.

A single report of swollen haemopoietic tissue (responsible for generation of new blood cells) was made by Field *et al.* (1992) who noted that the tissue in *Hematodinium*-infected *N. norvegicus* had a yellowish appearance and was clearly

visible to the naked eye, compared with uninfected individuals in which the haemopoietic organ could not be distiguished from surrounding tissue. In support of this observation, Ryazanova (2008) observed increased mitotic activity during histological investigation of haemopoietic tissue, suggesting a response to haemocytopaenia in both cases. Stentiford *et al.* (2002) questioned whether the alterations in haemopoietic tissue observed by Field *et al.* (1992) were in response to haemocytopoenia or whether the haemocytopaenia was due to parasite interference with the haemopoietic tissue. The evidence from Ryazanova (2008), combined with the rapid decline in haemocyte numbers following infection would suggest that it is the former.

Occasionally, fixed phagocytes in the hepatopancreas or heart tissue of *Chionoectes opilio* and *N. norvegicus* have been enlarged, however it has been unclear whether the response was to parasite cells, secondary infection or an increase in cell debris resulting from tissue degradation (Field and Appleton, 1995; Wheeler *et al.*, 2007). Field and Appleton (1995) also observed that fixed phagocytes of *N. norvegicus* containing parasite cells were necrotic.

As Wheeler *et al.* (2007) pointed out, secondary infection could account for all of the immune responses evident in infected animals, particularly due to the infrequency of reports of immune response. Indeed, many *Hematodinium*-infected crustacean hosts have been found to harbour secondary infections (Meyers *et al.*, 1987; Hudson and Shields, 1994; Sheppard *et al.*, 2003; Stentiford *et al.*, 2003). Experimental *Hematodinium* infection studies in sand crabs *Portunus pelagicus* resulted in death of all individuals within 16 days and all animals were found to contain bacteria in the haemolymph (Hudson and Shields, 1994). Of particular interest were reports from Stentiford *et al.* (2003) in which *Cancer pagurus* and *Necora puber* were co-infected with *Hematodinium* and a yeast-like organism. Crab haemocytes were observed to encapsulate budding yeast cells, however a similar response was not evident towards parasite cells. Based on the obvious capability of the host to mount an immune response, Stentiford *et al.* (2003) suggested that the parasite was suppressing or avoiding the immune system in crustaceans. Alternatively, Stentiford *et al.* (2003) suggested the possibility that the parasite may enhance the immune response towards opportunistic pathogens, thereby protecting both the parasite and its energy source, though no evidence was presented to support this hypothesis. Field *et al.* (1992) and Hamilton (2007) described haemocytes with a normal appearance in close proximity to parasite cells but with no apparent interaction. Several researchers have noted a distinct lack of response of host cells to parasite invasion (Field *et al.*, 1992; Shields, 2003; Wheeler *et al.*, 2007). Clearly further studies are necessary in order to determine the effect of *Hematodinium* sp. on the immune response of crustacean hosts.

## 1.4 Crustacean Immunity

Marine and freshwater crustaceans, as with many other invertebrates, are constantly surrounded by a myriad of pathogens and parasites awaiting the opportunity to invade a suitable host. The first line of defense for these invertebrates involves external physical and chemical barriers including exoskeletons, shells and mucus coverings. The hard cuticle of crustaceans is able to repel a great deal of infectious agents, however puncture or breakdown of this barrier exposes the body to internal infection by pathogens such as bacteria, fungi and parasites (eg. *Vibrio, Candida, Sacculina* or *Hematodinium*). An internal defense system is therefore necessary to deal with invading microorganisms.

Crustaceans, like other invertebrates, rely on an innate immune system for internal defense against foreign material. Innate immunity consists of both humoral and cellular factors mediated primarily by circulating haemocytes (blood cells). The innate immune system tends to recognise foreign material broadly based on molecular patterns, rather than using specific antibodies to target individual types of pathogens (Lee *et al.*, 2002; Medzhitov, 2007). Recognition molecules in crustacean haemocytes and haemolymph target non-self particles for removal, degradation and sequestration (Marques and Barracco, 2000). Once non-self particles are identified, a suite of responses are activated within the haemolymph including phagocytosis, encapsulation and melanisation (Bayne, 1990; Cerenius and Soderhall, 2004; Jiravanichpaisal *et al.*, 2006*a*). Numerous proteins and enzymes are involved in both the recognition and elimination of foreign material, the majority of which rely heavily on haemocytes for production (Battistella *et al.*, 1996; Johansson *et al.*, 2000; Vazquez *et al.*, 2009).

Traditionally, immune response was measured using haemocyte counts, microscopic observation of haemocyte responses to foreign pathogens and enzymatic assays, all of which are still currently relevant and provide a great deal of information regarding immunocompetence. During the last 15 years, rapid advances in molecular biology have opened new avenues of investigation by enabling the study of the expression of immune-relevant genes. Although by no means comprehensive, many of the enzymes and regulatory proteins involved in crustacean immune function have been sequenced and characterised, and their response to immunological challenge analysed.

### 1.4.1 Pathogen recognition and pathogen associated molecular patterns (PAMPs)

The initial recognition of foreign material during internal defense is crucial to mount an efficient and effective response. Crustaceans lack the pathogen-specific B- and T-cells found in the adaptive immune system of higher vertebrates and therefore must rely on microbial recognition factors within the haemolymph. Non-self recognition for crustaceans and other invertebrates is based on recognition of microbial patterns by a group of germ line encoded receptors called pattern recognition proteins (PRPs) (Vazquez *et al.*, 2009). PRPs are able to recognise microbe-specific molecules (or pathogen associated molecular patterns PAMPs) such as bacterial carbohydrates (lipopolysaccharide (LPS), mannose), bacterial or viral nucleic acids (CpG), bacterial peptides, peptidoglycans (particularly from Gram-positive bacteria), and fungal glucans (see Lee and Soderhall (2002); Dziarski (2004); Vazquez *et al.* (2009) for review).

Numerous PRPs have been identified for crustaceans, many of them referred to as binding proteins. PRPs act as mediators to alert and activate an immune response and are particularly responsible for activation of the proPO system (Soderhall, 1981; Lee *et al.*, 2000). PRP-PAMP complexes bind to receptor molecules on the surface of haemocytes and cause degranulation as well as instigating nodule formation (Lee *et al.*, 2000). $\beta$1,3-glucan binding proteins ($\beta$GBP) and LPS binding proteins (LGBPs) are two of the most commonly studied PRPs in crustaceans and have been identified in both shrimp and crabs (Vargas-Albores and Yepiz-Plascencia, 2000). $\beta$GBP from the crayfish *Pacifastacus leniusculus* induced spreading and degranulation in granular cells as well as acting as an opsonin increasing phagocytic activity (Barracco *et al.*, 1991; Cerenius *et al.*, 1994). Other $\beta$GBPs have been isolated from *Procambarus clarkii*, *Carcinus maenas* and *Litopenaeus vannamei* (Duvic and Soderhall, 1993; Thornqvist *et al.*, 1994; Vargas-Albores *et al.*, 1997). An LGBP from *Pac. leniusculus*, capable of binding both Gram-negative bacterial proteins and $\beta$1,3-glucans, was found to contribute to internal defense by activating the proPO system (Lee *et al.*, 2000).

Another major group of receptors involved in pathogen recognition are Toll and toll-like receptors (TLRs), several of which have been described in invertebrates, including crustaceans. The first Toll receptor from invertebrates was described from *Drosophila melanogaster* and was implicated in the control of dorsoventral polarity during embryogenesis (Stein *et al.*, 1991). Further research found that *Drosophila* Toll receptors had anti-fungal and anti-Gram positive bacteria immune function (Lemaitre *et al.*, 1996). Toll receptors from crustaceans have been isolated and characterised from the giant tiger shrimp *Penaeus monodon*, the white shrimp *Litopenaeus vannamei*, the

Chinese shrimp *Fenneropenaeus chinensis* and kuruma shrimp *Marsupenaeus japonicus* (Arts *et al.*, 2007; Yang *et al.*, 2007, 2008; Mekata *et al.*, 2008). In *F. chinensis*, Toll gene expression increased following bacterial challenge (*Vibrio anguillarum*), however decreased following viral challenge (white spot syndrome virus; WSSV) (Yang *et al.*, 2008).

Molecular analysis of many of the genes coding for pattern recognition proteins have found them to be similar to other invertebrate binding proteins (Padhi and Verghese, 2008) and $\beta$BGP from *P. leniusculus* had high similarity to other glucanases (glucan-degrading enzymes) (Lee *et al.*, 2000). Gene expression analysis of LGBP from the white shrimp *Litopenaeus vannamei* revealed an increase following injection challenge with the bacteria *Vibrio alginolyticus* (Cheng *et al.*, 2005). *L. vannamei* fed $\beta$1,3-glucan from *Schizophyllum commune* also had an up-regulation of $\beta$GBP-high density lipoprotein and LGBP, though the reaction was not observed until day 3 of the feeding trial (Wang *et al.*, 2008). Similarly, an LGBP cloned from the shrimp *Penaeus stylirostris* was up-regulated in animals infected with white spot syndrome virus (WSSV) (Roux *et al.*, 2002).

PRPs are also responsible for cell adhesion which is crucial for phagocytosis, encapsulation and nodule formation (see 1.4.4). Peroxinectin has been implicated as a primary cell-adhesive peroxidase in the freshwater crayfish *P. leniusculus* and the black tiger shrimp *Penaeus monodon*. Peroxinectin was found to bind to superoxide dismutase (SOD) in the cell-surface of haemocytes and respond to laminarin (Johansson *et al.*, 1999; Sritunyalucksana, 2001). Molecular studies have located the protein primarily in the granular and semi-granular cells of the white shrimp *Litopenaeus vannamei* (Liu *et al.*, 2005). Other cell adhesion molecules from crustaceans include calreticulin in *Fenneropenaeus chinensis* (Luana *et al.*, 2007) and a masquerade-like serine proteinase homologue from *P. leniusculus* (Huang *et al.*, 2000). An 80 kDa protein from *C. maenas* has also been identified in cell adhesion, but further research is required for full characterisation of the protein (Thornqvist *et al.*, 1994).

Recent work has focussed on Down Syndrome Cell Adhesion Molecule (Dscam) from invertebrates that is part of the immunoglobulin superfamily (IgSF) and has provided some evidence for specificity in the invertebrate immune response. Dscam is a cell adhesion molecule that was initially isolated from humans and was found to be associated with neural differentiation (Yamakawa *et al.*, 1998). When the first invertebrate Dscam was isolated from *Drosophila melanogaster* it was shown to be critical to the formation of axon pathways and embryonic central nervous system development (Schmucker *et al.*, 2000). Schmucker *et al.* (2000) also found that as a result of exon splicing, up to 38 000 Dscam isoforms were possible. Dscam was

associated with immune function when Watson *et al.* (2005) found that silencing Dscam in *Drosophila* using RNAi caused a significant reduction in phagocytosis. Reports that Dscam variants had variable binding interactions with bacteria (Watson *et al.*, 2005) and that isoform populations fluctuated between challenged and unchallenged mosquitos *Anopheles gambiae* (Dong *et al.*, 2006) led to the suggestion that Dscam may be involved in specific PAMP recognition and provided evidence for specificity in the invertebrate immune response. Crustacean Dscams have recently been identified from *Daphnia*, *Litopenaeus vannamei*, *Penaeus monodon* and *Pacifastacus leniusculus*(Brites *et al.*, 2008; Chou *et al.*, 2009, 2011; Watthanasurorot *et al.*, 2011).

### 1.4.2 Haemocytes and haemopoiesis

The crucial role of haemocytes to crustacean internal defense has led to numerous studies on haemocyte morphology and classification. The majority of crustacean species have been found to have three distinct classes of haemocytes: hyaline cells, granular cells and semi-granular cells (Bauchau, 1981). Development of a suitable anticoagulant and haemocyte cell separation techniques have enabled the study of each haemocyte class individually (Soderhall and Smith, 1983; Johansson and Soderhall, 1995; Vargas-Albores *et al.*, 2005) and therefore the primary immune functions for each class have been elucidated for several species.

**Hyaline cells (hyalinocytes):** *In vitro* studies of haemocytes from the shore crab *Carcinus maenas* identified hyaline cells as the primary phagocytic cells and that they were capable of engulfing both Gram-negative and Gram-positive bacteria (Smith and Ratcliffe, 1978). Further research has implicated hyaline cells in phagocytosis in the blue crab *Callinectes sapidus*, the crab *Carcinus aestuarii* and the harbour crab *Liocarcinus depurator* (Clare and Lumb, 1994; Walton and Smith, 1999; Matozzo and Marin, 2010), however in *Machrobrachium rosenbergii* phagocytosis was observed to be primarily carried out by granular cells (Vazquez *et al.*, 1997; Gargioni and Barracco, 1998). Hyaline cells have also been observed to initiate coagulation by cytolysis (Martin and Hose, 1992) and superoxide production in *C. maenas* elicited by bacterial cell wall components (Bell and Smith, 1993). Morphologically, hyaline cells are the smallest haemocytes, lack intracellular granules and have low contrast under light microscopy (Bauchau, 1981; Vargas-Albores *et al.*, 2005). When allowed to attach to slides in wet mounts, hyaline cells spread rapidly and extrude pseudopodia (Bauchau, 1981).

**Granular cells (granulocytes):** As their name implies, granulocytes have been found to contain substantial numbers of intracellular basophilic granules (Bauchau, 1981). Morphologically, crustacean granulocytes were larger than hyaline cells with a

centrally placed nucleus and the large numbers of refractile granules often obscured the nucleus under phase contrast microscopy (see Martin and Hose (1992) for review). Granulocytes have been reported to be capable of extruding pseudopodia when attached to slides, but not to the extent of hyaline cells (Vargas-Albores *et al.*, 2005). Most importantly, granulocytes have been found to be the principle producers of the phenoloxidase enzyme in *Car. maenas*, *Cancer pagurus*, (Soderhall and Smith, 1983) *Nephrops norvegicus* (Smith and Soderhall, 1991) and *Carcinus aestuarii* (Matozzo and Marin, 2010). Matozzo and Marin (2010) also reported that granulocytes from *Car. aestuarii* contain hydrolytic enzyme activity and granules from *Pacifastacus leniusculus* contained antimicrobial peptides (Sricharoen *et al.*, 2005) and superoxide dismutase (Wu *et al.*, 2008). Furthermore, in several species granulocytes have been found to play a role in phagocytosis (Gargioni and Barracco, 1998) and have been observed to aggregate and degranulate around fungal spores in crayfish (Persson *et al.*, 1987).

**Semi-granular cells (semigranulocytes; SGC):** Semi-granular cells are intermediate cells to hyaline and granular cells. Semigranulocytes have been found to contribute to phenoloxidase activity, but have been found equally important in encapsulation reactions (Persson *et al.*, 1987). Semi-granular cells from *Car. maenas* were found to contain variable numbers of cytoplasmic granules and easily degranulated during handling (Soderhall and Smith, 1983). Soderhall and Smith (1983) also detected phenoloxidase activity in the semi-granular cells of *Car. maenas*, *Can. pagurus*, *Liocarcinus depurator* and *Pagurus bernhardus*. Likewise, Vargas-Albores *et al.* (2005) identified phenoloxidase (PO) activity in semigranulocytes from the penaeid shrimp *Farfantepenaeus californiensis*, *Litopenaeus vannamei* and *L. stylirostris*, though the SGC contribution was lower than that for granulocyte cells. During exposure to foreign particles (fungal spores or charged Sepharose particles), semigranulocytes from the crayfish *Astacus leptodactylus* were observed to aggregate around the particles and form dense capsules of tightly packed flattened cells (Persson *et al.*, 1987).

Haemocyte counts have been found to vary significantly with pathogenic infection as well as with environmental factors such as temperature, salinity, hypoxia and pollution (Smith and Ratcliffe, 1980*b*; Le Moullac and Haffner, 2000). Furthermore, haemocyte counts can vary significantly between individuals of the same species due to moult status and diet (Johnson, 1980; Matozzo and Marin, 2010). Accordingly, haemocyte counts are considered an indicator of physiological state in crustaceans and are frequently used as an indicator of immune status in conjunction with other physiological and immune parameters (Le Moullac and Haffner, 2000).

Haemocytopaenia as a result of infection and general loss of haemocytes due to natural expiration require generation of new haemocytes (haemopoiesis). Haemopoiesis in crustaceans has largely been associated with a dedicated haemopoietic tissue (Ghiretti-Magaldi *et al.*, 1977; Johnson, 1980; Soderhall *et al.*, 2003) and only occasional reports have been made of cell division in circulating haemocytes (Sequeira *et al.*, 1996; Soderhall *et al.*, 2003; Roulston and Smith, 2011).

Haemopoietic tissue has been located in numerous crustacean species including blue crabs *C. sapidus*, American lobster *Homarus americanus*, penaeid shrimp *Penaeus monodon* and shore crabs *Carcinus maenas* (Ghiretti-Magaldi *et al.*, 1977; Johnson, 1980; Martin *et al.*, 1993; van de Braak *et al.*, 2000). Haemopoietic tissue has typically been found to be a sheet of tissue comprised of multiple lobules surrounding the dorsal side of the cardiac stomach (Johnson, 1980; Soderhall *et al.*, 2003). Additional locations for haemopoietic tissue have been identified around the antennal artery and at the base of the first three maxillipeds in penaeid shrimp (van de Braak *et al.*, 2000). Haemopoietic tissue is typically identified by dense aggregations of mitotic cells which increase in activity during infection or during other causes of haemocytopaenia (Martin and Hose, 1992).

There is some disagreement in the literature over how haemocytes proliferate from the haemopoietic tissue, though currently the general consensus is that haemocytes differentiate within the haemopoietic tissue before being released, possibly as precursor cells (Roulston and Smith, 2011), into circulation (see Johansson *et al.* (2000), Jiravanichpaisal *et al.* (2006*a*) and Soderhall and Lin (2011) for review). Hose *et al.* (1987) argued that haemocytes from the ridgeback prawn *Sicyonia ingentis* developed in circulation from hyaline cells to semi-granular cells and finally to granulocytes, however other studies have observed the release of differentiated cells from open areas of the haemopoietic tissue in *C. maenas*, *C. sapidus* and *P. leniusculus* (Ghiretti-Magaldi *et al.*, 1977; Johnson, 1980; Soderhall *et al.*, 2003).

The exact mechanisms involved in haemopoietic regulation have not been resolved, however recent studies have identified both a cytokine and a runt-like protein that appear to play key roles in the differentiation of haemocytes and mitotic index (Soderhall *et al.*, 2003; Soderhall and Lin, 2011). The role of astakine in haemopoiesis in the crayfish *Pacifastacus leniusculus* has been recently reviewed (Soderhall and Lin, 2011; Soderhall *et al.*, 2011) so will only be covered briefly here.

Astakines have been found to play a crucial role in the humoral regulation of crustacean haemopoiesis and cell differentiation. Two astakines from *P. leniusculus*, Ast1 and Ast2, have been isolated, cloned and characterised (Soderhall *et al.*, 2005; Lin *et al.*, 2010). Ast1 increased cell proliferation of haemopoietic tissue cells *in vitro*

and was found to be mainly secreted from semi-granular cells (Soderhall *et al.*, 2005). Further *in vivo* and *in vitro* experiments revealed that Ast1 was involved in differentiation of semi-granular cells and also inhibited apoptosis in haemopoietic cells (Lin *et al.*, 2010; Soderhall *et al.*, 2011). Furthermore, Ast1 plasma concentrations increased significantly following microbial challenge and prior to recruitment of new cells from the haemopoietic tissue (Soderhall *et al.*, 2005). Ast2 was not found to increase cell proliferation, however it increased the differentiation of granular cells (Lin *et al.*, 2010). Ast1 and Ast2 have both been cloned for use in molecular studies and, to date, have been found to be expressed in haemocytes and haemopoietic tissue (Lin *et al.*, 2010).

Although the transcriptional regulation of haemocyte release in crustaceans remains largely uncertain, recent studies using the crayfish *P. leniusculus* have made significant advances in the field (Soderhall *et al.*, 2003, 2005). Studies carried out in other invertebrates, particularly *Drosophila* (see Canon and Banerjee (2000) and Crozatier and Meister (2007) for review), have led the way for crustacean studies and have promoted interest for the Runx protein which is crucial for cell proliferation and differentiation (Crozatier and Meister, 2007). The *Runx* homologue in *Drosophila*, called *lozenge* (*lz*), has been found to be crucial to the differentiation of cells expressing *proPO* (Lebestky *et al.*, 2000). In the mosquito *Aedes aegypti* the Runx factor Runx4 was found to play a crucial role in the transcriptional regulation of four of the *proPO* genes (Zou *et al.*, 2008). Furthermore, in crayfish, a runt-like gene *Plrunt* isolated from haemocytes has been associated with differentiation of granular and semi-granular cells which are the primary sites of proPO generation (Soderhall *et al.*, 2003). The expression of *Plrunt* was upregulated prior to haemocyte release from the haemopoietic tissue. Similar results were found in the sea urchin *Strongylocentrotus purpuratus* where the highest levels of *runt* gene were found in the areas of highest mitotic activity (Stricker *et al.*, 2003).

### 1.4.3 Clotting and agglutination

For crustaceans living in what is essentially a microbial soup, coagulation is an important immune reaction in order to limit pathogen invasion during injury. The most well-characterised clotting system from invertebrates has been the haemocyte-driven clotting cascade from the horseshoe crab (chelicerate) *Tachypleus tridentatus* (see Theopold *et al.* (2004) for review). Almost as well characterised, and recently reviewed by Jiravanichpaisal *et al.* (2006*a*), is the transglutaminase-dependent clotting system from the freshwater crayfish *Pacifastacus leniusculus*. Transglutaminase (TGase) is an enzyme responsible for the cross-linking of clottable

proteins (coagulogens) found in the haemolymph plasma (Sritunyalucksana and Soderhall, 2000). TGase is released from haemocytes immediately following wounding and, in horseshoe crabs, when haemocytes detect bacterial LPS (see Iwanaga and Lee (2005) for review). Studies of the crustacean coagulation system have found that viruses and bacteria can interfere with TGase activity, confirming that, like horseshoe crabs, the clotting reaction plays an active role in crustacean non-self recognition and defense (Liu *et al.*, 2007*b*; Yeh *et al.*, 2009).

Another mechanical method of impeding the spread of pathogens internally is agglutination. Agglutination is an innate immune response whereby glycoproteins bind specific carbohydrates from non-self particles (Marques and Barracco, 2000; Raman *et al.*, 2008). The glycoproteins typically have at least two binding sites (bivalent) and are able to agglutinate foreign particles by binding to surface carbohydrates of multiple particles simultaneously. The most well-known form of agglutinins in crustaceans are lectins, found in the cell-free haemolymph, haemocyte membrane or cytosolic granules (Fragkiadakis and Stratakis, 1997; Raman *et al.*, 2008). Lectins have been identified in almost all crustaceans studied and have been active against *N*- and *O*-acetylated sugar residues commonly found in bacterial cell walls (see Vazquez *et al.* (2009) for review). Sera from the India river prawn *Macrobrachium malcolmsonii* was able to agglutinate Gram-negative *Aeromonas hydrophila* and had haemagglutinating activity against red blood cells from sheep, human, chicken and guinea pigs (Acharya *et al.*, 2004). Agglutination has been shown to play a crucial role in containing invading pathogens to a small area to facilitate a more efficient immune response in which haemocytes can concentrate a localised response. Additionally, the opsonising properties of many lectins have been found to bind foreign particles to individual haemocytes for phagocytosis (Ofek and Sharon, 1988; Thornqvist *et al.*, 1994).

### 1.4.4 Phagocytosis, nodule formation and encapsulation

Phagocytosis is a widely conserved process among both invertebrates and vertebrates (Vazquez *et al.*, 2009), and plays a primary role in internal defense. In crustaceans, despite claims that phagocytic rates have been relatively low (1-30%) (Smith and Ratcliffe, 1978; Smith and Soderhall, 1983, 1991), bacterial clearance has still been observed within a few hours (Smith and Ratcliffe, 1980*a*). In crustaceans, phagocytosis is typically carried out by circulating hyaline cells or fixed phagocytes in the hepatopancreas (Bayne, 1990; Iwanaga and Lee, 2005), however in some species granular and semi-granular cells have been observed to participate as well (Vazquez *et al.*, 1997).

Phagocytosis is initiated by non-self recognition and binding of the foreign particle to the phagocytic cell. The particle is then engulfed by the cell and sequestered intracellularly in a phagosome where it is enzymatically degraded (Bayne, 1990; Sagrista and Durfort, 1990). Target recognition can either occur through direct cell-target interaction or can be mediated by cell adhesion molecules such as peroxinectin found in the crayfish *P. leniusculus* (Johansson *et al.*, 1999).

In some instances, particularly with parasites and fungal hyphae, the invading particles are too large to be completely engulfed by a single haemocyte. In this case, an encapsulation response occurs whereby multiple haemocytes attach and spread across the surface of the particle to sequester the particle from the haemolymph. Following attachment and spreading the haemocytes begin to degrade leading to a localised melanisation reaction (Battistella *et al.*, 1996). Persson *et al.* (1987) observed encapsulation reactions in *P. leniusculus* to be primarily carried out by semi-granulocytes. A similar method of particle sequestration is nodule formation, which is different from encapsulation in that the haemocytes in nodule formation do not always form a tightly ordered mass around a central nucleus, but can be loosely formed aggregates. These aggregates form loosely associated haemocyte-particle networks to immobilise foreign material (Ratcliffe *et al.*, 1982).

### 1.4.5 Respiratory burst and reactive oxygen species (ROS)

Phagocytic cells from humans have been found to secrete lysozyme, peroxidase and reactive oxygen species (ROSs) such as superoxide, hydrogen peroxide and hydroxyl radicals in order to degrade invading microorganisms (see Rosen *et al.* (1995) for review). The reactive oxygen response has been evolutionarily conserved and in crustaceans respiratory burst has been (Fig 1.3) associated with the phagocytic cells in which reactive oxygen species including hydrogen peroxide, superoxide ions and hydroxyl radicals can be generated (Holmblad and Soderhall, 1999; Nappi and Ottaviani, 2000). Superoxide production in *C. maenas* has been reported from the hyaline cells which are responsible for phagocytosis and Jayasree (2009) has reported elevated superoxide levels during phagocytosis of *Vibrio* spp. in *Penaeus indicus*. Vibrio bacteria also induced superoxide production in *P. vannamei* (Muñoz *et al.*, 2000) and *L. vannamei* (Li *et al.*, 2008*a*), as well as elevated hydrogen peroxide in *C. maenas* (Hauton *et al.*, 1997*a*).

Despite the advantages of superoxide to microbial control, superoxide is a free radical capable of causing damage to the host as well as invading pathogens. To alleviate damage to the self-particles, organisms produce the superoxide-scavenging enzyme superoxide dismutase (SOD) which catalyses the dismutation of superoxide into

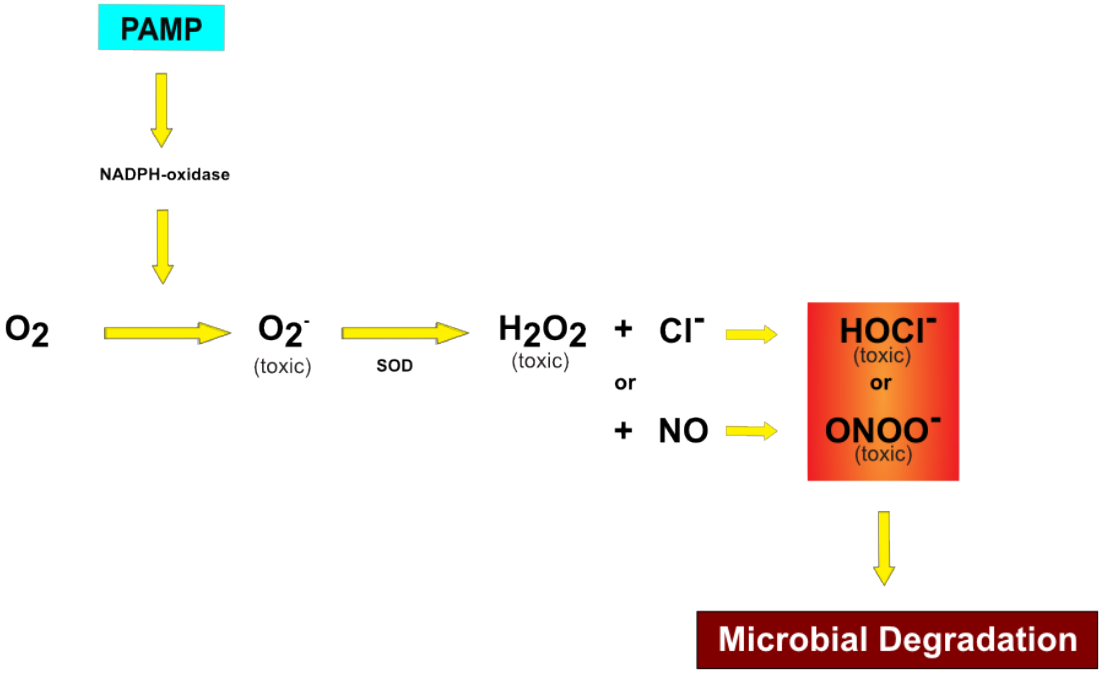



FIGURE 1.3: Respiratory burst is elicited by the activation of membrane-bound NADPH-oxidase complexes by PAMPs resulting in increased oxygen consumption. Excess oxygen molecules are then reduced by NADPH-oxidase leading to the production of superoxide ($O_2^-$). In the presence of SOD, superoxide is metabolised to hydrogen peroxide ($H_2O_2$), which reacts with chloride (using myeloperoxidase (MPO)) or nitric oxide (NO) to produce hypochlorite ($OCl^-$) or peroxynitrite ($ONOO^-$) radicals. This collection of reactive oxygen species is then able to oxidise key components of the cell membrane of foreign pathogens causing cell disruption and death (see Bachere *et al.* (1995), Rosen *et al.* (1995) and Nappi and Ottaviani (2000) for review).

oxygen and hydrogen peroxide. In crustaceans, molecular studies regarding respiratory burst have tended to focus on the gene for SOD rather than a direct measurement of reactive oxygen species. It is believed that increased SOD expression is an indication of increased oxidative stress (Wang *et al.*, 2007) and has in fact been found to increase in the crab *Scylla paramamosain* during challenge with LPS (Chen *et al.*, 2010*a*) and in mud crabs *S. serrata* following injection with $\beta$1,3-glucan and peptidoglycan (Lin *et al.*, 2008*b*).

### 1.4.6 Antimicrobial peptides

Antimicrobial peptides (AMPs) are generally small, cationic molecules that interact with the phospholipids commonly found in the cell membranes of foreign pathogens (Epand and Vogel, 1999) Though the exact method of microbial destruction for many AMPs remains unclear (Epand and Vogel, 1999; Zasloff, 2002), most have been found to increase plasma membrane permeability (Andreu and Rivas, 1998). Indeed, the crustin isoforms crustinPm1 and crustinPm7 from the black tiger shrimp *Penaeus*

*monodon* were found to cause inner membrane permeability in *Escherichia coli* (Krusong *et al.*, 2012). AMPs have been isolated from a variety of organisms and are used in host defense in bacteria, protozoa, invertebrates, vertebrates and plants (Epand and Vogel, 1999; Zasloff, 2002; Ghosh *et al.*, 2011). Many of the reported AMPs have been found in invertebrates and they have been proven to play a crucial role in host defense in many crustaceans, including penaeid shrimp, crabs and lobsters (Bartlett *et al.*, 2002; Hauton *et al.*, 2006; Brockton *et al.*, 2007). There are four major classes of AMPs: linear peptides forming $\alpha$-helices, cysteine-rich peptides containing $\beta$-sheets or $\alpha$-helical $\beta$ sheet mixtures, proline-rich peptides, or glycine-rich peptides (Dimarcq *et al.*, 1998; Epand and Vogel, 1999; Bartlett *et al.*, 2002).

Antimicrobial activity from crustacean haemocytes and haemolymph has been evident for many years though detailed studies into the proteins responsible have only occurred in the last 15 years. The first AMP characterised from crustaceans was a proline-rich antibacterial peptide from *Carcinus maenas* which was active against both Gram-positive and Gram-negative bacteria (Schnapp *et al.*, 1996). Shortly thereafter, a cysteine-rich AMP was also identified and found to be active against Gram-positive bacteria (Relf *et al.*, 1999). Since then, numerous AMPs have been identified in crustaceans, typically falling into the class of penaeidins or crustins, both of which have been recently reviewed (Destoumieux *et al.*, 2000; Smith *et al.*, 2008; Tassanakajon *et al.*, 2010). Crustin-like molecules from the American lobster *Homarus americanus* have been found to act against Gram-negative bacteria and protozoa (Battison *et al.*, 2008) and recombinantly expressed penaeidins from *Litopenaeus vannamei* were found to act against Gram-positive bacteria in addition to having antifungal activity (Destoumieux *et al.*, 1999). *P. monodon* crustins were found to bind to both Gram-positive and Gram-negative bacteria and to interact specifically with the cell wall components lipoteichoic acid and LPS (Krusong *et al.*, 2012). Furthermore, recent molecular studies have found expression levels of genes coding for antimicrobial peptides to fluctuate during microbial infection. *P. monodon* challenged with white spot syndrome virus (WSSV) and *L. vannamei* injected with LPS both exhibited a down-regulation in penaeidin mRNA levels (Okumura, 2007; Philip *et al.*, 2011). In contrast, a crustin-like gene sequenced from the lobster *Homarus gammarus* was found to increase in expression following challenge with Gram-positive *Aerococcus viridans* (Hauton *et al.*, 2006) and likewise, crustin from *Hyas araneus* increased following challenge with the Gram-negative bacteria *Listonella anguillarum*, however the increase was only evident in granular cells (Sperstad *et al.*, 2010).

### 1.4.7 Melanisation and the prophenoloxidase system

Melanisation has been observed as one of the key defense reactions in invertebrates to injury and pathogen invasion. Melanin synthesis is a major part of the sclerotisation process of the cuticle but has also been observed in wound healing and particularly in encapsulation and nodule formation (Soderhall, 1982). The primary enzyme responsible for catalysis of the melanisation pathway is phenoloxidase, which oxidizes monophenols to *o*-diphenols and further into *o*-quinones. The quinones are then converted into melanin. Due to the toxic nature of melanin and its byproducts during formation, the pathway is tightly regulated and the entire cascade has been termed the proPO-activating system (or proPO system) (Cerenius and Soderhall, 2004). There are extensive reviews on the importance of the proPO system in invertebrate immunity and the activation cascade (Fig 1.4) (Soderhall, 1982; Soderhall and Cerenius, 1998; Sritunyalucksana and Soderhall, 2000; Cerenius and Soderhall, 2004; Cerenius *et al.*, 2008).

The proPO system is instigated by the presence of minute amounts of microbial peptides, often termed pathogen associated molecular patterns (PAMPs). PAMPs include lipopolysaccharides (LPS) and peptidoglycans (PG) from bacterial cell walls and $\beta$1,3-glucans from fungal cells. PAMPs are recognised in the haemolymph by pattern-recognition proteins (PRPs) such as LPS-binding protein (LGBP) (Lee *et al.*, 2000; Du *et al.*, 2007), $\beta$1,3-glucan binding protein ($\beta$GBP) (Duvic and Soderhall, 1990) and PG binding protein (PGBP). PRP-PAMP complexes are then able to instigate an immune response by binding to a cell-surface protein such as superoxide dismutase (SOD) or $\beta$-integrin (Johansson *et al.*, 1999; Holmblad and Soderhall, 1999; Vargas-Albores and Yepiz-Plascencia, 2000) in the haemocyte membrane to trigger non-self recognition. This activation causes degranulation in granulocyte and semi-granular cells and the release of prophenoloxidase activating enzyme (ppA) (Aspan *et al.*, 1990; Barracco *et al.*, 1991).

Via limited proteolysis in the presence of calcium, ppA becomes active and cleaves proPO, generally at a conserved arginine-phenylalanine linkage (Cardenas and Dankert, 1997; Cerenius and Soderhall, 2004), into active phenoloxidase (PO). PO, a copper-containing enzyme that is part of the tyrosinase family, is then able to oxidize the amino acid tyrosine into dopa and further into dopaquinone. Dopaquinones undergo further conformational changes and oxidation (catecholoxidase reaction) into indolequinone which eventually polymerises into the purple-black heteropolymer eumelanin (Sritunyalucksana and Soderhall, 2000; Christensen *et al.*, 2005; Terwilliger, 2006). Melanin intermediates such as dihydroxyindole and quinones are cytotoxic and have been found to bind to nucleophiles on the surface of foreign pathogens and

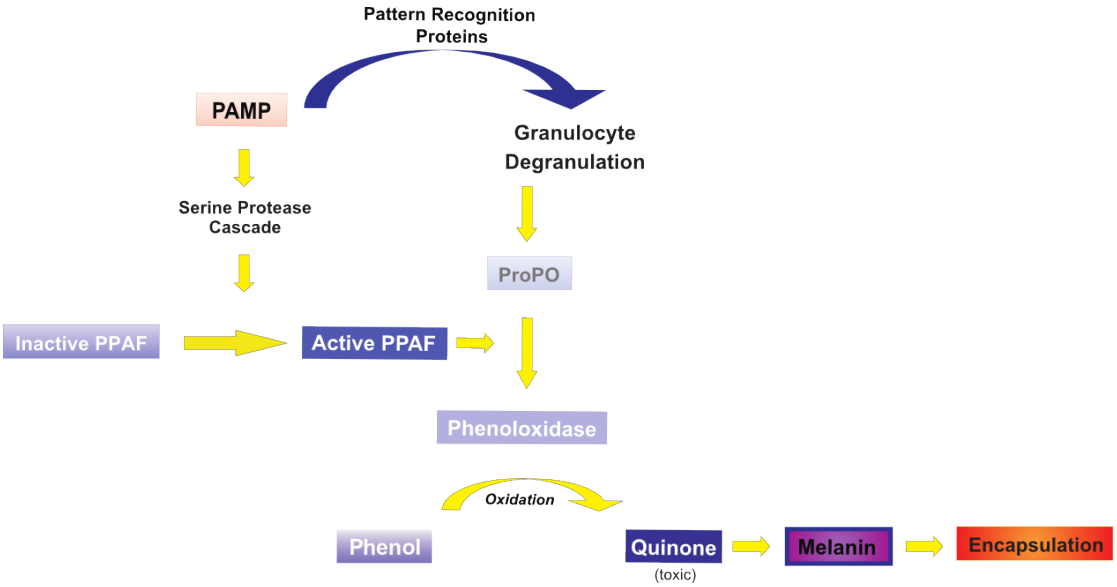



FIGURE 1.4: Crustacean prophenoloxidase internal defense cascade beginning with recognition of pathogen-associated molecular patterns (PAMPs) and terminating in the release of melanin and toxic byproducts (Hernandez-Lopez *et al.*, 1996; Sritunyalucksana and Soderhall, 2000; Cerenius and Soderhall, 2004).

immobilise endoparasites (Nappi and Ottaviani, 2000). Furthermore, melanin has been found to scavenge free radicals and the presence of melanin on the surface of a foreign particle could make it a target for cytotoxic molecules and assist in localising the immune response (Nappi and Ottaviani, 2000).

Generally, the proPO system has been found to be activated by Gram-negative and Gram-positive bacteria (Acharya *et al.*, 2004), fungi (Cerenius *et al.*, 2003) and viruses (Song *et al.*, 2003; Sarathi *et al.*, 2007). The antibacterial properties of phenoloxidase were confirmed when purified phenoloxidase was observed to inhibit *in vitro* bacterial growth of *Escherichia coli*, *Streptococcus pneumoniae* and *Aeromonas hydrophila* (Cerenius *et al.*, 2010). Injection with *Vibrio* species increased PO activity in *Litopenaeus vannamei*, *Fenneropenaeus indicus* and *Eriocheir sinensis* (Song *et al.*, 2003; Sarathi *et al.*, 2007; Gai *et al.*, 2008). Additionally, challenge with microbial cell wall components such as LPS and $\beta$1,3-glucans has also been found to increase PO activity in *Procambarus clarkii*, *Penaeus paulensis* and *Eriocheir sinensis* (Cardenas and Dankert, 1997; Perazzolo and Barracco, 1997; Zhang *et al.*, 2010), for example.

Molecular studies have analysed the transcriptional regulation of the *proPO* gene from crustacea in response to numerous pathogens. *proPO* expression was up-regulated following challenge with *Vibrio* spp. (Gai *et al.*, 2008; Chen *et al.*, 2010*b*), LPS (Ko *et al.*, 2007) and the endobacteria *Pasturia ramosa* (Labbe and Little, 2009). Furthermore, genetic knockout of *proPO* in *Pacifastacus leniusculus* was found to reduce PO enzyme activity, decrease nodule melanisation and increase bacterial growth

and mortality (Liu *et al.*, 2007*a*). The abundance of reports linking PO activity and *proPO* gene expression to pathogenic challenge have cemented the role of the proPO system in studies of crustacean internal defense. Assays for PO activity and real-time expression of *proPO* are key study parameters for indications of immune response and, when used in conjunction with other immune-related assays, have become widely accepted as an indicator of immunocompetence.

## 1.5 Apoptosis

Apoptosis is the process of programmed cell death used by metazoan organisms to eliminate unwanted or damaged cells. Unlike necrosis, where cells swell and rupture, triggering an inflammatory immune response, apoptosis maintains plasma membrane integrity and causes cells to shrink in order to be easily consumed by phagocytosis (Raff, 1998). Apoptosis plays a role in immunity by removing damaged cells and also by inhibiting the spread of intracellular pathogens. During viral infection, apoptosis is a key process to reducing viral replication by containing the virus within the cell and limiting the spread of viral particles (Liu *et al.*, 2009; Menze *et al.*, 2010). In crustaceans, recent research has focussed on apoptosis in relation to commercially important viruses such as White Spot Syndrome Virus (WSSV), Yellow Head Virus (YHV) and Taura syndrome virus (TSV) (Flegel, 2001, 2007; Liu *et al.*, 2009). Apoptosis occurred in black tiger shrimp *Penaeus monodon* infected with WSSV and reduced total haemocyte counts by 90% 60 hours post-infection (Wongprasert *et al.*, 2003). Similar results were found 36 hours post-infection in *P. monodon* exposed to YHV (Khanobdee *et al.*, 2002). Apoptosis is not limited to viral infection, however, and has also been implicated in bacterial and yeast infections in the giant freshwater prawn *Macrobrachium rosenbergii* (Hsu *et al.*, 2005). Yeast (*Debaryomyces hansenii*) and bacteria (*Aeromonas hydrophila* or *Enterococcus faecium* incubated *In vitro* with prawn haemocytes led to apoptosis in those haemocytes that engulfed the foreign pathogens and the haemocytes showed signs of cytoplasmic vacuoles and nucleic fragmenation. Importantly, fluctuations in apoptotic activity could lead to haemocytopaenia and compromised immune function.

## 1.6 *Cancer pagurus* and *Nephrops norvegicus*

Studies explicitly designed to explore immune parameters of *Cancer pagurus* and *Nephrops norvegicus* have been limited in past research, however generally accepted immune assays have been successfully used in a number of studies for both species

(Table 1.2). Haemocyte types have been categorised and phenoloxidase was associated with granulocyte populations in both cases (Smith and Soderhall, 1983, 1991). The assays used to explore specific immune responses in both species have included phenoloxidase, superoxide anion production and antimicrobial peptide activity (Chisholm and Smith, 1995; Vogan and Rowley, 2002). Haemocyte counts have frequently been used to assess physiological and immunological competence in response to stimulants such as LPS, manganese and trawl stress (Lorenzon *et al.*, 1999).

TABLE 1.2: Immune characteristics and parameters associated with *Cancer pagurus* and *Nephrops norvegicus*.

| Immune Mechanism/ Characteristic | Cancer pagurus | Nephrops norvegicus | References |
|---|---|---|---|
| Haemocytes | Hyaline | Hyaline | Smith and Soderhall (1983, 1991) |
| | Granular | Granular | |
| | Semi-granular | Semi-granular | |
| Phenoloxidase | Present in granular cells | Present in granular cells | Smith and Soderhall (1983, 1991) |
| Antimicrobial activity | Present against *Psychrobacter immobilis* | Present against *Psychrobacter immobilis* | Chisholm and Smith (1995); Vogan and Rowley (2002) |
| Reactive Oxygen Species (ROS) | MnSOD | | Marchand *et al.* (2009) |
| Total haemocyte counts (in response to) | Shell disease syndrome | Manganese | Lorenzon *et al.* (1999); Vogan and Rowley (2002); Costa-Ramos and Rowley (2004); Hernroth *et al.* (2004); Ridgway *et al.* (2006) |
| | *Pseudoalteromonas atlantica* | LPS | |
| | | Aerial exposure | |

## 1.7 The current study

Despite the growing body of literature discussing *Hematodinium* sp. and its effects on its crustacean hosts, there is a wide knowledge gap surrounding the host immune response. As discussed, pathological evidence has only indicated a mild immune response in crustacean hosts in the form of encapsulations, however it has been unclear whether the encapsulated particles have, in fact, been parasites (Field and Appleton, 1995) and the response could be due to secondary infection by opportunistic pathogens instead. Furthermore, studies of most crustaceans infected with *Hematodinium* have indicated a severe loss of haemocyte numbers which are crucial to internal defense. Biochemical and physiological parameters have suggested that infected animals are under considerable stress and these combined factors could contribute to a

compromised immune response. Generally, there has been speculation regarding whether host animals are able to recognise parasite cells and whether *Hematodinium* is able to avoid or suppress the immune system (Stentiford *et al.*, 2002). Stentiford *et al.* (2003) has also argued that parasite infection may enhance immune response in order to eliminate competition from opportunistic pathogens. The current study aims to investigate the immune response of two UK crustacean hosts to parasitic *Hematodinium* infection using a range of immune assays, *in vitro* challenge experiments and gene expression analysis. The overarching null-hypothesis being tested is that *Hematodinium* sp. has no effect on the immune response of its crustacean hosts.

The outline of the major objectives covered in this thesis are:

- To identify, clone and sequence immune-relevant genes from two crustaceans, *Cancer pagurus* and *Nephrops norvegicus* (Chapter 2), known to host *Hematodinium* sp. in the UK, and to develop and optimise assays for the gene expression of molecular markers for immune reaction (Chapter 3).
- To explore variation in immune gene expression of *Cancer pagurus* haemocytes to *Hematodinium* sp. exposure *in vitro* in the absence of confounding influences such as secondary infection (Chapter 4).
- To investigate the effect of *Hematodinium* sp. on multiple immune parameters of wild-caught *Nephrops norvegicus* from the Clyde Sea Area(Chapter 5).
- To investigate gene expression changes in immune-related genes from wild populations of *Nephrops norvegicus* (Chapter 6).

Based on analysis of the results obtained from the above studies, conclusions will be drawn regarding the immune response of crustacean hosts to *Hematodinium* sp. infection (Chapter 7).

# Chapter 2

# Molecular cloning of crustacean and *Hematodinium* sp. genes for gene expression studies

## 2.1 Introduction

### 2.1.1 Immune-related genes of interest

In this study, two genes were chosen *a priori* for study in response to *Hematodinium* sp. infection in crustacean hosts. The two genes of interest (GOIs), *prophenoloxidase* (*proPO*) and *runt*, were chosen for their role in innate immune function. *proPO* codes for the immune-relevant enzyme prophenoloxidase and *runt* codes for a runt-like protein involved in the regulation of haemopoiesis.

#### 2.1.1.1 Prophenoloxidase

Prophenoloxidase was chosen for expression analysis because the protein is an important precursor molecule in the melanisation pathway of invertebrates. Melanisation is an immune response commonly found in invertebrates in which melanin is secreted around foreign material found encapsulated internally or in wound repair (Cerenius and Soderhall, 2004; Cerenius *et al.*, 2008). Phenoloxidase (PO) controls the synthesis of melanin and is strictly regulated in crustacean haemocytes. Evidence for the role of phenoloxidase in the invertebrate internal defense system has come from two primary lines of research: enzymatic studies on phenoloxidase activity

and molecular studies of *prophenoloxidase*[1] genes. These parameters have been found to fluctuate under various conditions including pathogen invasion, environmental changes and physiological stress responses (Aspan *et al.*, 1995; Hauton *et al.*, 1997*b*; Sritunyalucksana *et al.*, 1999; Le Moullac and Haffner, 2000; Gao *et al.*, 2009; Zhu *et al.*, 2011). Due to the well-established methods for evaluating PO response, the study of PO activity and gene expression of *proPO* have become an important indicator of immunocompetence.

PO activity has been observed to increase under multiple conditions, including high population density (Reeson *et al.*, 1998), after wounding (Mucklow and Ebert, 2003), following injection with microbes and microbial components (Smith and Soderhall, 1991; Cardenas and Dankert, 1997), and after infection with parasites (Gomes *et al.*, 1999). PO is believed to increase at high population densities due to the increased need for pathogen resistance in crowded systems. Larvae of *Spodoptera exempta* were found to have increased resistance to bacteria when raised in high density situations compared to those raised at low densities (Reeson *et al.*, 1998). PO activity also significantly increased in the water flea *Daphnia magna* following wounding (Mucklow and Ebert, 2003), and has generally been found to increase locally around the wound site as well as in the circulating haemolymph (Mucklow and Ebert, 2003; Vafopoulou, 2009). Animals exposed to or injected with bacteria, fungi and other pathogens have been found to dramatically increase PO activity in the haemolymph. PO activity increased in *Macrobrachium rosenbergii* and *Eriocheir sinensis* following injection with CpG oligodeoxynucleotides (motifs associated with microbial genomes) (Lu *et al.*, 2006; Zhang *et al.*, 2010). Other microbial cell wall components such as lipopolysaccharides (LPS) and $\beta$1,3-glucans have also been found to elicit a PO response from haemocytes of the shore crab *Carcinus maenas*, swamp crayfish *Procambarus clarkii* and the Pacific white shrimp *Litopenaeus vannamei* (Smith and Soderhall, 1983; Cardenas and Dankert, 1997; Xian *et al.*, 2009). Furthermore, PO activity was elicited by both Gram-positive and Gram-negative bacteria in the Indian river prawn *Macrobrachium malcolmsonii* (Acharya *et al.*, 2004). These reports show a clear enzymatic response to foreign material.

The importance of *proPO* to crustacean immunity has been supported by numerous studies of pathogenic challenge. Genetic knockout of *proPO* in both *Pacifastacus leniusculus* and *Penaeus monodon* reduced PO enzyme activity and increased susceptibility to bacteria (Liu *et al.*, 2007*a*; Amparyup *et al.*, 2009). Nodule formation and encapsulation were also reduced in *P. leniusculus* (Liu *et al.*, 2007*a*). Additional studies have explored the direct interaction of purified *P. leniusculus* PO on bacterial

[1] A note on nomenclature: PO refers to the phenoloxidase enzyme, proPO refers to the prophenoloxidase protein, and *proPO* refers to the gene coding for proPO. All gene names will appear in *italics*.

growth *in vitro*. An antibacterial effect was evident for all six bacterial species studied (Cerenius *et al.*, 2010).

Several functional assays have been developed to examine prophenoloxidase fluctuations during immune responses, however more recently, the gene coding for proPO has been sequenced and molecular techniques have been developed for expression studies. Temporal expression of *proPO* mRNA following infection has been the focus of several immune studies including immune response variation and immunocompetence. Upregulation of *proPO* has been indicated in many infection studies, for example, during challenge with the bacteria *Vibrio anguillarum* (there was a concurrent increase in PO enzyme as well) (Gai *et al.*, 2008) or *Aeromonas hydrophila* (Liu *et al.*, 2007*a*) as well as challenge with the parasite *Aphanomyces astaci* (Cerenius *et al.*, 2003). An *in vitro* study by Hauton *et al.* (2005) also observed the upregulation of *proPO* in *Homarus gammarus* granulocytes 24 hours after challenge with heat-killed bacteria *Listonella anguillarum*. It is important to mention that not all infection studies have shown upregulation of *proPO*. No change was observed in blue shrimp *Litopenaeus stylirostris* infected with *Vibrio penaeicida* (de Lorgeril *et al.*, 2005) or Pacific white shrimp *Litopenaeus vannamei* exposed to LPS (Okumura, 2007). A further study examining the use of the immunostimulants MacroGard and Ergosan also failed to yield an upregulation in *proPO* (Hauton *et al.*, 2007).

Parasites, on the other hand, have been found to actively inhibit PO activity. For example, the native gammarid *Gammarus pulex* had decreased PO levels following invasion with the acanthocephalan parasites *Pomphorhynchus laevis* and *Polymorphus minutus* while the invasive host *Gammarus roeseli* had increased PO activity (Rigaud and Moret, 2003). Rigaud and Moret (2003) suggested that the parasites had adapted to suppress PO activity in their native host, but were still susceptible to the internal defense of the new host. In another study, fungal metabolites such as kojic acid from *Aspergillus* and *Penicillium* species also inhibited PO in the fall armyworm *Spodoptera frugiperda* (Dowd, 1999). PO inhibition has frequently been reported in invertebrate host-parasite interactions, however the actual mechanism of interference is unclear for most species.

Despite the fact that some studies found little change in *proPO*, it is widely accepted that the proPO system plays a key role in crustacean internal defense and has often shown an increase when animals are infected (de Lorgeril *et al.*, 2005; Liu *et al.*, 2007*a*; Okumura, 2007; Gai *et al.*, 2008) or faced with a risk of infection (Mucklow and Ebert, 2003). Based on the evidence of studies presented here, *proPO* was used as one of the genes of interest in the current study. Furthermore, if the *Hematodinium* parasite is

inhibiting or avoiding some part of the immune response, this study may yield important information regarding the mechanisms involved.

#### 2.1.1.2 Haemopoiesis

As previously mentioned (Chapter 1.4.2), haemopoiesis (or haematopoiesis) is the 'production of blood cells involving both the proliferation and differentiation from stem cells' (Brett *et al.*, 1989). Crustacean haemocytes are generally considered to arise from specialised haemopoeitic tissue containing undifferentiated haematopoietic cells (Jiravanichpaisal *et al.*, 2006*a*). It is via this tissue that old and damaged cells are replaced and the rate of synthesis can be influenced by microenvironmental factors regulated by cell-cell and cell-extracellular matrix interactions (Barreda and Belosevic, 2001) as well as season and moult stage (Johnson, 1980; Hose *et al.*, 1992).

Haemopoietic tissue has been identified in several crustacean species and is typically found on the dorsal and dorsolateral sides of the cardiac stomach. In blue crabs (*Callinectes sapidus*), American lobster (*Homarus americanus*) and freshwater crayfish (*Pacifastacus leniusculus*) the organ forms a sheet-like tissue composed of many small ovoid lobules (Martin *et al.*, 1993; Johnson, 1980; Soderhall *et al.*, 2003). The lobules, encased in a thin sheath of connective tissue, contain stem cells and developing haemocytes (Johnson, 1980) and vessels penetrating the tissue allow rapid release of new cells when required (Soderhall *et al.*, 2003). The ridgeback prawn *Sicyonia ingentis* was found to have a pair of nodules formed from branches of the haematopoietic artery situated on the dorsal surface of the hepatopancreas. The vessels had thick walls containing layers of haemocytes 6-10 cells deep in varying degrees of maturation (Martin *et al.*, 1987).

Due to the fact that crustacean haemocytes do not appear to divide in circulation, all haemocytes must proliferate from the haemopoietic tissue. Indeed, increased proliferation in the haemopoietic tissue has been observed following haemocyte depletion due to experimental bleeding and infection (Johnson, 1980; van de Braak *et al.*, 2002; Soderhall *et al.*, 2003). Increased stress due to moulting and environmental changes have also been observed to increase proliferation (Johnson, 1980). Little is known about humoral regulation of cell proliferation and differentiation in crustaceans, however regulation has recently been linked to a cytokine identified in the crayfish *P. leniusculus* and termed astakine (Soderhall *et al.*, 2005). Cytokines are small signal molecules typically associated with immune cell signaling, cell proliferation and cell differentiation (Brett *et al.*, 1989). Astakine was found to cause increased cell proliferation in *P. leniusculus* haematopoietic tissue *in vitro* as well as *in vivo* (Soderhall *et al.*, 2005).

As previously discussed (Chapter 1.4.2), the *runt* gene has been linked to haemopoiesis and has been shown to play an active role in the proliferation of cells expressing the *proPO* gene (Lebestky *et al.*, 2000). Furthermore, in crayfish, *Plrunt* increased in expression prior to the release of haemocytes from the haemopoietic tissue. The essential role of haemocytes to host defense and survival make them an ideal parameter to study for immunocompetence. Variations in total haemocyte counts have frequently been studied in response to pathogenic challenge in crustaceans, however the demand for new cells has only rarely been studied (Chen and Cheng, 2001; van de Braak *et al.*, 2002; Battison *et al.*, 2004; Brockton and Smith, 2008). Furthermore, reports of increased activity in the haemopoietic tissue of *Hematodinium*-infected animals (Field *et al.*, 1992; Ryazanova *et al.*, 2010) suggests there is increased demand for haemocytes during parasite invasion. The identification and characterisation of the *runx* gene from *P. leniusculus* implicate *runx* as an ideal target for gene expression studies during parasite invasion.

### 2.1.2 Quantifying *Hematodinium* sp. infection

*Hematodinium* sp. infection has traditionally been identified by pleopod staging, microscopy, ELISA, or PCR. Pleopod staging uses light microscopy to score infection status based on occlusion of the pleopods in *N. norvegicus* and is the most subjective and least sensitive method. Haemolymph smears, histology and PCR offer a more reliable method of assessing infection status, with haemolymph smears offering a way of quantifying infection and identifying parasite life stage. The small subunit (SSU) rDNA gene of *Hematodinium perezi* was initially sequenced in order to compare isolates from different hosts as well as to compare *Hematodinium* sp. to other protozoa (Hudson and Adlard, 1996). The same gene was later used to develop a PCR-based diagnostic for routine testing of *Hematodinium* hosts (Gruebl *et al.*, 2002). Recently, a successful quantitative PCR assay has been developed using 18S rRNA to accurately quantify parasite load in infected blue crabs *Callinectes sapidus* (Nagle *et al.*, 2009). This technique was able to quantify parasite load to a limit of 0.001 cell equivalents. In order to use this technique in the present study, the 18S rRNA gene was sequenced from the parasites used in these experiments.

### 2.1.3 Reference Genes

To use the relative quantification analysis method in quantitative PCR (qPCR), a suitable reference gene needed to be identified. Previously, several genes have been identified as potential reference genes for a number of species including $\beta$-actin,

GAPDH and tubulin (Thellin *et al.*, 1999; Garcia-Vallejo *et al.*, 2004). It has since been found that these genes are not always constitutively expressed under every experimental condition (Glare *et al.*, 2002; Dheda *et al.*, 2004), however with proper validation they are acceptable genes (Thellin *et al.*, 1999; Huggett *et al.*, 2005). The use of $\beta$-actin in previous crustacean studies (Hauton *et al.*, 2005) led to the selection of actin as the reference gene of choice in the following studies (see Chapter 3.5.1).

## 2.2 Isolation of species-specific mRNA transcripts

### 2.2.1 *Cancer pagurus*, *Nephrops norvegicus* and *Hematodinium* sp. collection

*Cancer pagurus* from the English Channel were obtained from Selsey Shellfish (Selsey, UK) during the winter of 2007 and placed in recirculating seawater aquaria at the National Oceanography Centre, Southampton. The aquaria were maintained at a salinity of 32.9 $\pm$ 1 and a temperature of 14.3°C $\pm$ 1.8°C. Healthy adult intermoult male and female crabs, identified by alertness and a clear carapace free of lesions, were sampled for these experiments.

Scottish *Nephrops norvegicus* were obtained from Portland Shellfish (Portland, UK) and kept in 150 l seawater aquaria with a Fluval 4 Plus filter pump (Rolf C. Hagen Ltd., Castelford, UK). Aquaria were maintained at 10°C in a controlled temperature room.

The parasitic dinoflagellate *Hematodinium* sp. was isolated from infected *Nephrops norvegicus* collected by trawl using the University Marine Biological Station, Millport vessel RV *Aplysia* in February 2009 near the Isle of Cumbrae, Scotland (55° 50.7 N, 04° 54.3 W). Before bleeding, each animal was surface-sterilized with 100% ethanol. Heavily infected animals were identified by the 'cooked colour' of their carapace. Between 0.5 ml and 2.5 ml of haemolymph was withdrawn from the base of the fifth pereiopod of each infected animal and placed immediately into 30 ml Appleton-Vickerman media (Appleton and Vickerman, 1998), (27.99 g $l^{-1}$ NaCl; 0.95 g $l^{-1}$ KCl; 2.01 g $l^{-1}$ CaCl2; 2.465 g $l^{-1}$ MgSO4; 0.554 g $l^{-1}$ Na2SO4; 1.92 g $l^{-1}$ HEPES; 10% foetal calf serum; 25 $\mu$l $ml^{-1}$ gentamycin; 200 $\mu$g $ml^{-1}$ penicillin; pH 7.8) in 25 $cm^2$ Corning®, non-vented culture flasks. In the laboratory, cultures were subcultured by at least half into fresh media with additional antibiotics (1% penicillin/streptomycin). Cultures were maintained at 6°C and subcultured every 3-4 weeks (Appleton and Vickerman, 1998).

### 2.2.2 Haemolymph collection

Haemolymph was extracted from the base of the fifth pereiopod (*N. norvegicus*) or the base of the third walking leg (*C. pagurus*) into an equal volume of ice cold marine anticoagulant (23.3 g $l^{-1}$ NaCl; 18.0 g $l^{-1}$ glucose; 8.82 g $l^{-1}$ trisodium citrate; 4.5 g $l^{-1}$ citric acid; 2.63 EDTA) at pH 4.6 (Soderhall and Smith, 1983) using a 25G needle and 2 ml sterile syringe. The haemolymph was placed in a 1.5 ml microcentrifuge tube and centrifuged at 2 500 *g* for 5 minutes and 4°C. The supernatant was discarded and the pellet used for RNA extraction.

### 2.2.3 *Haemopoietic tissue*

Haemopoietic tissue from the dorsal surface of the cardiac stomach was dissected from intermoult adult *C. pagurus*. Animals were anaesthetised by placing them on ice, followed with injection into the base of the third walking leg of 200 $\mu$l of 4% procaine in sterile seawater and incubation on ice for a further 20 min. The top of the cuticle was removed and the tissue from the top of the stomach extracted in order to ensure collection of the haemopoietic tissue. The tissue was divided in half, with one half being placed in fixative for histological examination and one half flash frozen in liquid nitrogen and stored in 1.5 ml microcentrifuge tubes at -80°C until required for RNA extraction.

In order to confirm the presence of haemopoietic tissue, histological sections were made of the fixed half of the dissected tissue. Dissected stomach was attached to a piece of dental wax using entomological pins to keep it flat and in the correct orientation and placed in Bouin's fixative overnight. Tissue was dehydrated through a graded ethanol series (40%, 70%, 96% x 2, 100%) then cleared in xylene and embedded in paraffin. Semi-thin tissue sections ($\sim$8 $\mu$m) were made using a Cambridge Rocking Microtome and were placed on clean glass slides and dried on a slide warmer overnight. Sections were deparaffinised in xylene and rehydrated through an ethanol series before haematoxalin and eosin (H & E) staining according to Luna (1969). Slides were mounted using DPX and visualised on an Olympus BH2 microscope.

Comparisons of putative haemopoietic tissue sections from *Cancer pagurus* coincided with images of haemopoietic tissue sections from *Callinectes sapidus* (Johnson, 1980) suggesting that the correct tissue was extracted (Fig 2.1). Johnson (1980) observed that the haemopoietic tissue of *Cal. sapidus* consisted of lobules located on a sheet of spongy connective tissue that was situated dorsally to the cardiac stomach. The lobules reportedly varied in thickness and could be as little as ten cells deep. The tissue sampled here from *Can. pagurus* appears to contain haemopoietic tissue

indicated by densely stained collections of cells situated adjacent to the epithelium of the cardiac stomach.

### 2.2.4 RNA Extraction

Total RNA was extracted from both cell pellets and haemopoietic tissue using TRI Reagent® Solution (Sigma-Aldrich, Dorset, UK). TRI Reagent® is a monophasic solution comprised of phenol and guanidine thiocyanate. Cells or tissue were disrupted in the solution and, following the addition of chloroform, the homogenate was separated into an aqueous and organic phase. RNA was located in the aqueous phase, DNA in the interphase and protein in the organic phase (Biosystems, 2010).

For total RNA extraction from haemocytes, TRI Reagent® was added to cell pellets immediately following removal of the supernatant in order to limit RNase activity in the sample. For haemopoietic tissue, TRI Reagent® was added to the tissue directly after removing the sample from -80°C. Both sample types were then disrupted using a sterile polypropylene tissue pestel.

Following homogenization 0.2 ml chloroform was added to each sample for every 1 ml TRI Reagent® used and the tubes vigorously shaken for 15 seconds. Samples were then centrifuged for 15 min, 12 000 *g*, 4°C. The clear aqueous layer containing total RNA was removed to a new eppendorf tube and 0.5 ml isopropanol added for every 1 ml TRI Reagent® used to precipitate the RNA. Samples were allowed to stand at room temperature for 10 minutes then centrifuged for 10 minutes at 12 000 *g*, 4°C. Isopropanol was carefully aspirated and the RNA pellet was washed in 75% molecular grade ethanol freshly prepared in diethyl polycarbonate (DEPC)-treated water (1 ml ethanol for every 1 ml TRI Reagent®). Samples were thoroughly mixed then centrifuged for 5 min at 7 500 *g*, 4°C. The ethanol was carefully removed and the pellet air dried before being redissolved in 20 $\mu$l DEPC-treated water. Total RNA quantity and quality were determined by measuring absorbance at 260 nm and 280 nm using a ND-1000 spectrophotometer (Nanodrop, Wilmington, USA). Extracted RNA samples were stored at -80° until needed.

### 2.2.5 cDNA Synthesis

In order to be used in PCR, total RNA was converted into complementary DNA (cDNA) using SuperScript™ II Reverse Transcriptase (SSII-RT; Invitrogen, Paisley, UK). The RT reaction was primed using oligo-dT primers which bind to the poly-A tail found at the end of messenger RNA (mRNA). This ensured that the cDNA library

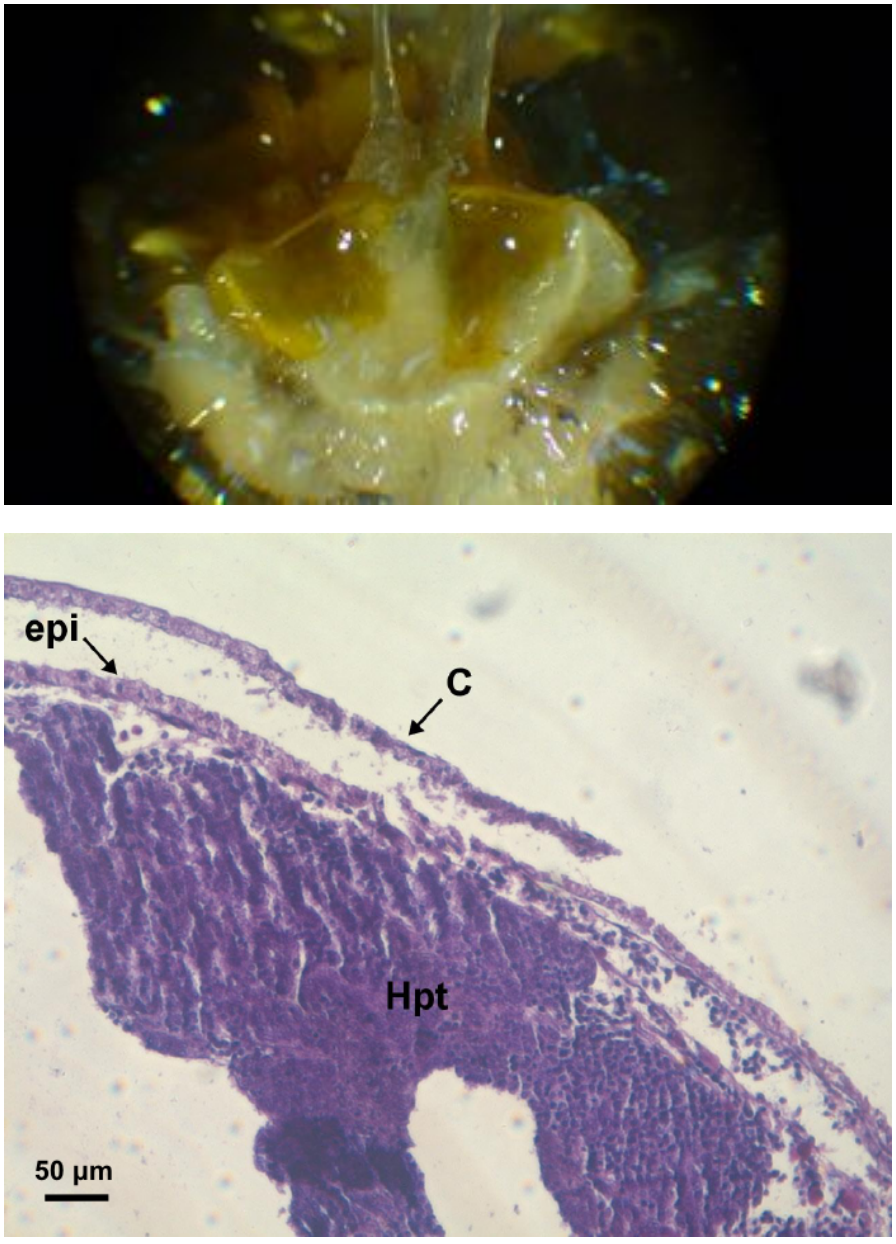



FIGURE 2.1: (a) Dorsal view of cardiac stomach, on top of which lies the haemopoietic tissue (b) Haemopoietic tissue, H&E stained showing haemopoietic tissue (hpt), epidermal cells (epi) and cuticle (C). The gap between the epidermal cells and cuticle is likely an artifact of the preservation technique.

was composed of only expressed genes. cDNA was generated by combining in a 0.2 ml PCR tube: 1 $\mu$l 10mM dNTP, 1 $\mu$l Oligo(dT)$_{23}$ (50 $\mu$M), 1-3 $\mu$g total RNA and sterile water to a total volume of 12 $\mu$l, and incubated at 65°C for 5 min. The samples were removed immediately onto ice and the following reagents were added: 4 $\mu$l 5X First Strand buffer, 2 $\mu$l 0.1M DTT, 1 $\mu$l RNA Inhibitor (40 units/$\mu$l) to a total volume of 19 $\mu$l. The samples were incubated at 42°C for 2 min, then 1 $\mu$l SSII-RT was added and the samples incubated for a further 60 min at 42°C. To stop the reaction, samples were heat-inactivated at 70°C for 15 min.

### 2.2.6 Degenerate Primer Development

Oligonucleotide primers are short nucleotide sequences used to prime the extension of longer strands of DNA in techniques such as polymerase chain reaction (PCR) and DNA sequencing. Primers are designed based on known sequences and are designed with the closest match to the parent gene as possible. If a new gene of unknown sequence is desired, such as in this project, the primers are designed based (a) on a peptide sequence derived from the purified peptide or (b) on a consensus sequence of multiple aligned amino acid sequences from closely related species (McPherson and Moller, 2000). For this project degenerate primers were already published for *proPO*, *runt* and crustacean *actin* and were used when possible. New degenerate primers were developed for *Hematodinium actin* genes and additional *runt* primers were also developed based on protein homologues from closely related species.

Translation of the amino acid sequence into the corresponding nucleotides results in a certain amount of degeneracy due to the fact that most amino acids are encoded by multiple codons and the degeneracy is typically located at the third position of the codon. This degeneracy can lead to non-specific binding of primers to undesirable genes, however careful primer design can reduce non-specific binding and improve specificity. By adhering to the following guidelines for primer design (McPherson and Moller, 2000; Biosoft, 2008) the majority of the genes of interest were able to be amplified and eventually sequenced, despite not knowing the species-specific sequences.

Degenerate primer design recommendations:

- Primer length: 18-22 bp
- Melting temperature ($T_m$): a $T_m$ of 52-58°C tends to be best and primer pairs should have similar $T_m$s
- GC content: 40-60%

- GC Clamp: the presence of G or C bases in the last five bases of the 3'-end of the primer can aid in specific binding due the bond strength between Gs and Cs, however more than 3 G's or C's is not recommended
- Avoid secondary structure and runs of a single base
- Avoid excessive degeneracy, particularly at the 3'-end of the primer

The degenerate primers used to look for crustacean *proPO* and crustacean *actin* (Table 2.1) were previously published in a study looking for the same genes in the lobster *Homarus gammarus* (Hauton *et al.*, 2005). Degenerate primers for dinoflagellate *actin* were designed based on an alignment of sequences from *Amphidinium carterae* (Accession: AAB62063.1), *Crypthecodinium cohnii* (Accession: AAM02969.1), *Prorocentrum minimum* (Accession: O15930) and *Heterocapsa triquetra* (Accession: Q84KN0).

TABLE 2.1: Degenerate primers designed to locate crustacean *proPO* and *actin* as well as dinoflagellate *actin*. Primers for *Hematodinium* sp. *18S* were species-specific.

| Primer Name | Tm (°C) | Primer Sequence (5' to 3') [1] | Fragment Length (bp) |
|---|---|---|---|
| **proPO**[2] | | | |
| proPO fwd | 54.0 | CAY CAY TGG CAY TGG CA | 1150 |
| proPO rev | 54.0 | CAN CCR CAN CCR CAR AA | |
| **Crustacean actin**[2] | | | |
| DG Act Fwd | 59.9 | GTC GGY GAY GAR GCN CAR A | 573 |
| Act Rev | 54.3 | CR TGN GGN ARY TCR TAN GA | |
| **Dinoflagellate actin** | | | |
| DinoActF | 59.8 | GAG AAG ATG ACN CAR ATH ATG TTY GA | 783 |
| DinoActR | 65.3 | G GCC TGG AAR CAY TTN CGR TGN AC | |
| ***Hematodinium* 18S** [3] | | | |
| HematF 1487 | 56.0 | CCT GGC TCG ATA GAG TTG | 1100 |
| WHemat | 54.5 | CC TCC GCT TAT TGA TAT GC | |

[1]IUPAC nucleotide code: Y = C/T; R = A/G; H = A/C/T; N = A/C/G/T
[2](Hauton *et al.*, 2005)
[3](White *et al.*, 1990; Gruebl *et al.*, 2002)

Soderhall *et al.* (2003) isolated the *runt* gene from haemocytes of the signal crayfish *Pacifasticus leniusculus* using degenerate primers containing the universal base deoxyinosine which can pair with any of the four bases A, C, T or G. These published primers were tested on haemocyte and haemopoietic tissue from *C. pagurus* and *N. norvegicus* and an additional set of primers were developed based on amino acid alignments from *P. leniusculus* (Q8MPK8, Q8MPK9), the lancelet *Branchostoma floridae* (Q6YI83) and Japanese pufferfish *Fugu rubripe* (A0A9P7) (Table 2.2). The reverse primers were weighted more towards the sequences from *P. leniusculus* due to the high variation between the three species and *P. leniusculus* being the the only

crustacean. All primers were ordered from Eurofins MWG Operon (Ebersberg, Germany).

Table 2.2: *Runt* primers

| Primer Name | Tm (°C) | Primer Sequence (5' to 3') | Fragment Length[1](bp) |
|---|---|---|---|
| F1a Runt | 56.7 | TN ACN RTN MGN GCN GGN AA | 1047 |
| F1b Runt | 57.3 | GTN ACN RTN ATG GCN GGN AA | |
| R1 Runt | 53 | TA NGG NCK CCA NAC NG | |
| F2 Runt | 56.6 | CN ATH AAR GTN ACN GTN GAY G | 768 |
| R2 Runt | 55.6 | T RTC YTC NGG NGT CAT RTG | |
| F3 Runt | 57.5 | TY AAY GAY YTN MGN TTY GTN GG | 963 |
| R3 Runt | 44 | TA NGG NCK CCA NAC | |
| F4 Runt [1] | 51.6 | GCN GGN AAY GAY GAR AA | 201 |
| R4a Runt[2] | 50.4 | GGI CCA TCI ACI GTI AC | |
| R4b Runt [2] | 52.8 | GGI CCG TCI ACI GTI AC | |

[1]*Runt* from *P. leniusculus* has two isoforms so fragment lengths are approximate
[2]Degenerate primers from Soderhall *et al.* (2003)

### 2.2.7 Polymerase Chain Reaction

Polymerase chain reaction (PCR) uses repeated cycles of heating and cooling in the presence of *Taq* DNA polymerase to amplify desired segments of DNA. The thermal cycles cause denaturation, annealing and extension of desired DNA segments specified by short priming sequences to yield near-exponential amplification. Qiagen reaction buffer and *Taq* DNA polymerase (Qiagen, Crawley, UK) were used in combination with the previously described degenerate primers to generate the desired amplicons. PCR reactions were performed in 25 $\mu$l volumes containing 2.5 $\mu$l 10X reaction buffer, 0.2 units *Taq* polymerase, 1 $\mu$l 10 mM dNTP (Invitrogen, Paisley, UK), 2 $\mu$l cDNA, 2 $\mu$l each primer (25 $\mu$M), and sterile water to final reaction volume. The reactions were mixed briefly then placed in a preheated MJ Research PTC-200 DNAEngine (Bio-Rad, Hemel Hempstead, UK) and run according to the following cycle:

Table 2.3: Degenerate PCR thermocycling conditions

| Step | Temp (°C) | Duration | No. Cycles |
|---|---|---|---|
| Initial Denaturation | 95 | 7 min | 1 |
| Denaturation | 95 | 30 sec | |
| Annealing | 45-61[1] | 30 sec | 30 |
| Extension | 72 | 90 sec | |
| Final Extension | 72 | 7 min | 1 |

[1]Optimal annealing temps: *proPO* 61°C; *actin* 54°C; *Hematodinium 18S* 55°C; *Hematodinium actin* 50°C

### 2.2.8 Degenerate PCR Results

Following PCR amplification, all samples were separated on a 1% agarose gel made in tris-acetate (TAE) buffer with 5.5 $\mu$l ethidium bromide (10 mg/ml) and run at 75 V for 45-50 min. Gels were visualised on a Gel Doc™ UV transilluminator (Bio-Rad Laboratories, Hemel Hempstead, UK) and images captured using the Quantity One® Software package (Bio-Rad, Hemel Hempstead, UK). Amplicon sizes for each sample were compared against a 100-bp ladder (Sigma-Aldrich, Dorset, UK) (Fig 2.2) and amplicons of the expected size were excised for extraction. Bands were successfully identified for *proPO* and *actin* for both *Cancer pagurus* and *Nephrops norvegicus* as well as the *Hematodinium actin* and *18S* genes.

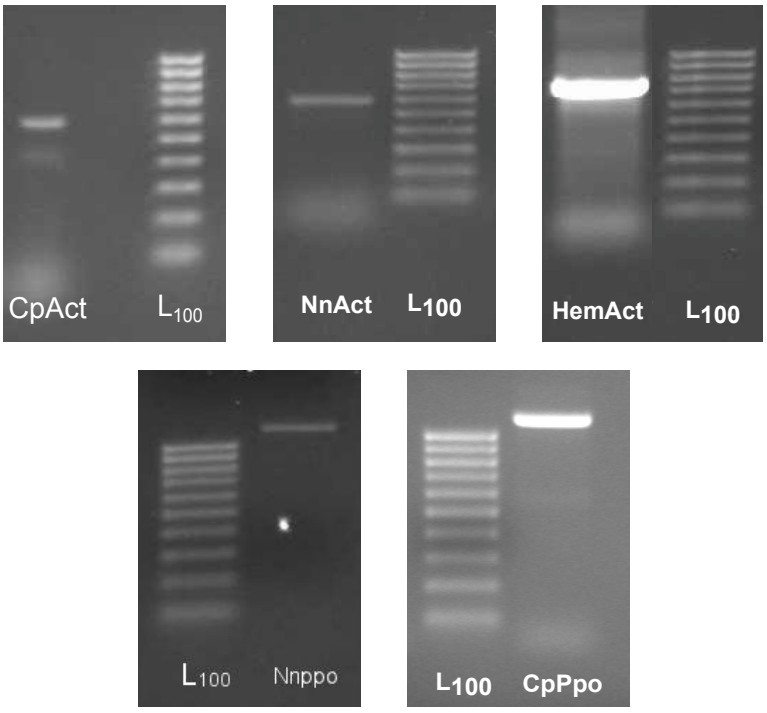



FIGURE 2.2: Ethidium bromide gel images of degenerate fragments generated for *actin* from *C. pagurus* (CpAct), *N. norvegicus* (NnAct), and *Hematodinium* (HemAct). *proPO* degenerate fragments *N. norvegicus* (Nnppo) and *C. pagurus* (CpPpo). $L_{100}$ = 100 bp ladder (Sigma-Aldrich).

All possible primer combinations for the *runt* gene were tried on haemocyte cDNA from *C. pagurus* using a gradient for annealing temperature between 45 and 61°C, however no bands were visualised based on electrophoresis gels. The published primers from Soderhall *et al.* (2003) which have been successful for *Pacifasticus leniusculus* were also used on haemocyte cDNA from *N. norvegicus* with no success. Further attempts were made using genomic DNA extracted from *C. pagurus* haemocytes as well as on cDNA synthesised from haemopoietic tissue RNA which also resulted in no evident bands.

Finally, *Not*I restriction digest (see Appendix A.1) of *C. pagurus* haemocyte genomic DNA was used to cut the DNA into smaller fragments. Restriction enzymes produced by bacteria cut double stranded DNA into fragments at specific nucleotide sequences. In this case, it was speculated that complex secondary structure of the genomic DNA could be inhibiting primer binding or extension; by digesting the DNA into smaller fragments, the complexity of the secondary structure could be reduced making the primer binding sites more accessible. PCR amplification of the restriction digest resulted in a potential band of the correct size for the *runt* gene using the primer combination F1b/R2 (1050 bp). Distinct bands were also identified using F4/R4a and F4/R4b which were larger than expected, but were sequenced based on the possibility of inserts in the sequence (Fig 2.3).

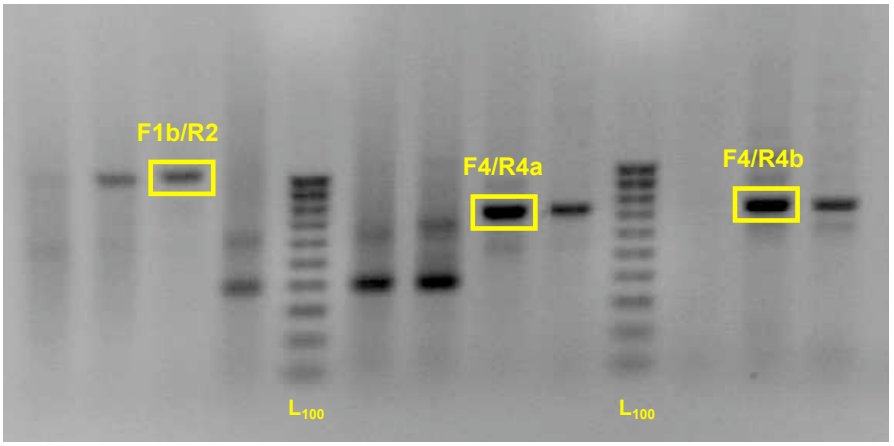



FIGURE 2.3: Ethidium bromide gel with PCR-amplified *runt* fragments following restriction digest of *C. pagurus* haemocyte genomic DNA with the *Not*I restriction enzyme. The fragment for F1b/R2 is the expected size for the fragments (approx. 1050 bp). The fragments for F4/R4a and F4/R4b are larger than expected, but could contain inserts. $L_{100}$ = 100 bp ladder (Sigma-Aldrich).

### 2.2.9 Cloning and Sequencing

Using a bacterial host to clone copies of a PCR fragment is a widely accepted method of obtaining pure single sequence transcripts of a gene for optimum sequencing. In this experiment, the positive PCR bands were excised from the agarose gel using a sterile scalpel blade and placed into a 1.5 ml microcentrifuge tube. The DNA bands were then extracted from the gel using a QIAquick® Gel Extraction Kit (Qiagen, Crawley, UK) according to the manufacturer's protocol (Appendix A.2). Following extraction, the fragments were cloned using a TOPO TA Cloning® Kit for Sequencing (Invitrogen, Paisley, UK) according to the manufacturer's instructions (see Appendix A.3 for full protocol). Briefly, the gene fragment was ligated into the pCR® 4-TOPO® vector which was used to transform the chemically competent One Shot®

TOP10 *Escherichia coli* by heat shock. Transformed cells were grown overnight on ampicillin-selective Luria Bertani (LB) agar plates containing 50 $\mu$g/ml ampicillin. A selection of colonies (up to ten) were replated on fresh LB-amp plates and the presence of the correct insert confirmed using PCR and M13 primers (Appendix 8), followed by visualisation on a 1% agarose gel. Positive clones, identified by the presence of a band of appropriate length, were cultured overnight in LB broth with 100 $\mu$g/ml ampicillin and the plasmids were extracted using a QIAprep® Spin Miniprep Kit (Qiagen, Crawley, UK) following the manufacturer's instructions (Appendix A.4). Plasmids were then sequenced by SourceBioscience (Department of Biochemistry, University of Oxford, UK) using vector-specific M13 primers. SourceBioscience uses Sanger sequencing technology on an Applied Biosystems 3739 DNA Analyzer.

### 2.2.10 Degenerate PCR sequence results and analysis

Digital sequence results from Geneservice were analysed using Geneious Basic software version 4.5.5-5.0.4 (Biomatters Ltd., Aukland, New Zealand). Sequences were edited to remove plasmid bases (see Appendix A.3.1 for the pCR® 4-TOPO® vector map) and reverse complemented where necessary. The chromatograms for each sequence were scrutinised for reading errors, and alignments for each clone were made to generate the full fragment length. Where available, multiple clones were aligned to generate a consensus sequence and the resulting sequences were searched against the NCBI database using BLASTX via Geneious.

### 2.2.11 Crustacean *actin*

The gene fragment sequenced from *Cancer pagurus* using degenerate primers for *actin* (*CpAct*; FR687021) contained 574 bp and a predicted 191 amino acid sequence (Fig 2.4). Sequence analysis using the BLAST algorithm identified the sequence most closely with *Triatoma matogrossensis* (98.4%; e-value = $5.43e^{-137}$; ADN29914), *Litopenaeus vannamei* (99%; e-value = $6.52e^{-137}$; AAR82845), *Marsupenaeus japonicus* (99.5%; e-value = $1.09e^{-136}$; ADG45307), *Hypochilus thorelli* (98.4%; e-value = $1.45e^{-136}$; ABZ91668), and *Limulus polyphemus* (97.9%; e-value = $3.06e^{-136}$; P41341). SMART™ analysis identified an actin domain (e-value = $4.74e^{-38}$), which corresponded with the Pfam analysis which identified the same domain (e-value = $2.80e^{-71}$). SMART™ also indicated the superfamily as an actin-like ATPase domain (d1j6za1 e-value = $2.00e^{-41}$; d1j6za2 e-value = $6.00e^{-36}$) via SCOP (Structural Classification of Proteins).

Degenerate PCR for *actin* produced a 598 bp fragment from *Nephrops norvegicus* which was successfully cloned and sequenced (*NnAct*; HE608878). The nucleotide fragment generated a 199 aa sequence (Fig 2.4). Sequence analysis using the BLAST algorithm on the deduced amino acid sequence showed that *NnAct* had highest pairwise identity with *Litopenaeus vannamei* (99%; e-value = $3.61e^{-144}$; AAR82845), *Triatoma matogrossensis* (99%; e-value = $4.80e^{-144}$; ADN29914), *Ascaris suum* (99%; e-value = $5.98e^{-144}$; ADY48030), *Marsupenaeus japonicus* (99%; e-value = $6.66e^{-144}$; ADG45307), *Hypochilus thorelli* (99%; e-value = $9.30e^{-144}$; ABZ91668) and *Pacifasticus leniusculus* (99%; e-value = $2.79e^{-143}$; ABI34073). SMART™ analysis identified an actin domain (e-value = $2.96e^{-48}$), which corresponded with the Pfam analysis which identified the same domain (e-value = $5.80e^{-73}$). SMART™ also indicated the superfamily as an actin-like ATPase domain (d1j6za1; e-value = $1.00e^{-45}$; d1j6za2; e-value = $9.00e^{-37}$) via SCOP (Structural Classification of Proteins).

Multiple alignment of the predicted CpAct and NnAct protein sequences with other invertebrate actin sequences via Geneious identified conserved amino acids at putative binding sites for ATP, profilin and gelsolin (Fig 2.4). Gelsolin is an actin-binding protein found both intra- and extracellularly and is involved in the regulation of filament assembly and disassembly. The protein is able to generate actin filaments by joining monomeric actin molecules or sever filaments and control the polymerisation of barbed ends (Silacci *et al.*, 2004). Profilin is another actin-binding protein specifically involved in restructuring the actin cytoskeleton. It functions primarily in controlling the growth of actin filaments to aid in cellular movement and shape changes (Witke, 2004). As expected for actin, the sequences were highly conserved and had an overall pairwise identity of 99%.

### 2.2.12 Crustacean *prophenoloxidase*

The cloned sequences from *Cancer pagurus* and *Nephrops norvegicus* using degenerate primers for *proPO* contained 1151 bp and 1136 bp respectively and coded for an inferred protein sequence of 383 aa and 378 aa. Alignment of the two fragments determined that the sequences shared a pairwise identity of 61.1%. BLASTP analysis found that the amino acid sequence for *C. pagurus* proPO had highest identity with *Metacarcinus magister* (91.6%; e-value = $< 10^{-179}$; ABB59713), *Scylla serrata* (82.1%; e-value $< 10^{-179}$; ABD90511), *Portunus trituberculatus* (80.7%; e-value = $< 10^{-179}$; ACI46633), *Eriocheir sinensis* (73.4%; e-value = 0; ABS19633) and *Litopenaeus vannamei* (66.3%; e-value = $< 10^{-179}$; ABQ45957). *N. norvegicus* proPO had highest identity with *Homarus gammarus* (87.6%; e-value = $< 10^{-179}$; CAE46724), *Homarus americanus* (86.8%; e-value = $< 10^{-179}$; AAT73697), *Procambarus clarkii* (68.5%;

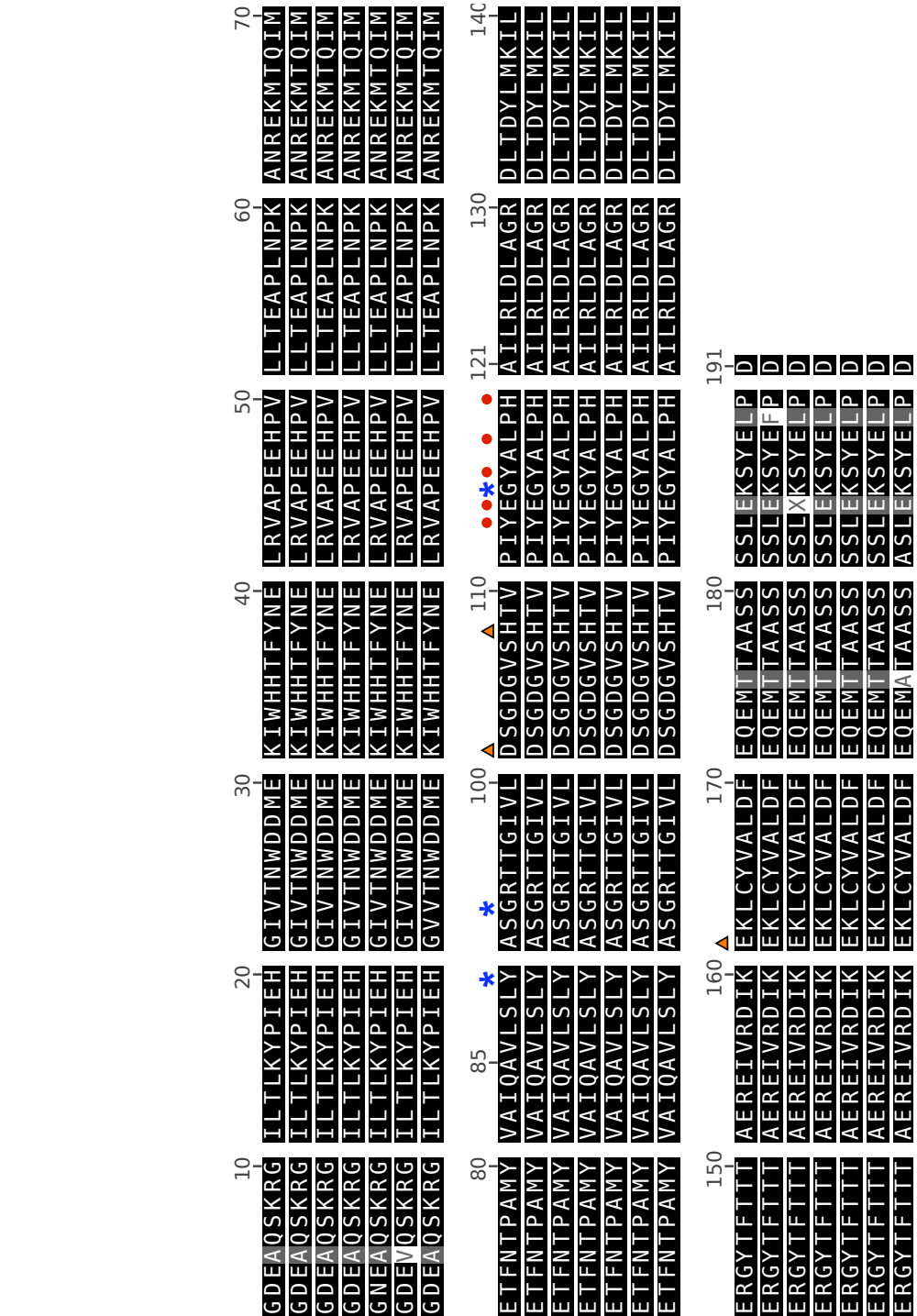



FIGURE 2.4: Multiple alignment of actin amino acid sequences from *N. norvegicus* and *C. pagurus* with actin from the crustaceans *L. vannamei* AAR82845, *M. japonicus* ADG45307, *P. leniusculus* ABI34073, *S. paramamosain* ADG03642, *A. franciscana* CAB55752. The gelsolin binding site is indicated by blue asterisks (*), the profilin binding site by red circles and the ATP-binding site by orange triangles. Overall pairwise identity for these sequences was 99%. Amino acid similarity is indicated by color: black - 100%, dark gray - 80-99%, light gray - 60-79%, white 0-59%.

e-value = 0; ABR12412), *Pacifasticus leniusculus* (68.5%; e-value = $< 10^{-179}$; CAA58471) and *Litopenaeus vannamei* (66.4%; e-value = $< 10^{-179}$; ABQ45957). Multiple sequence alignment of the top hits (Fig 2.5) resulted in an overall pairwise alignment of 67.7%. An incomplete copper binding site (copper-binding site A) and the complete copper-binding site B were identified from the inferred amino acid sequences. The sites also contained six conserved histidine residues commonly found in proPO amino acid sequences (Liu *et al.*, 2006; Terwilliger and Ryan, 2006; Labbe and Little, 2009). Furthermore, SMART™ analysis indicated a haemocyanin M (e-value = $1.60e^{-49}$ (CpPpo) and e-value = $6.10e^{-61}$ (NnPpo)) and haemocyanin C (e-value = $1.20e^{-42}$ (CpPpo) and e-value = $1.80e^{-79}$ (NnPpo)) pfam domain for both *C. pagurus* and *N. norvegicus* sequences. A pfam domain for tyrosinase was also indicated for both sequences that overlapped with the haemocyanin M domain (e-value = $4.80e^{-06}$ (CpPpo) and e-value = $3.10e^{-05}$ (NnPpo)). Sequence alignments and putative domains strongly indicated that fragments of the *proPO* gene had been isolated from both crustacean species.

### 2.2.13 *Hematodinium* actin (*HemAct*)

Degenerate PCR using the primers for dinoflagellate actin resulted in a fragment of 783 bp from *Hematodinium* which was successfully cloned and sequenced (*HemAct*; Accession number: HE609031). The nucleotide sequence coded for 261 amino acids and BLASTP analysis showed that *HemAct* had highest pairwise identity with *Symbiodinium* sp. CS-156 (84.6%; e-value = $3.34e^{-165}$; BAE79387), *Karlodinium veneficum* (84.2%; e-value = $5.66e^{-165}$; ACF32732), *Dinophysis caudata* (84.2%; e-value = $3.52e^{-164}$; ADU24822), *Karlodinium micrum* (84.2%; e-value = $4.19e^{-164}$; ABI14387), and *Amphidinium carterae* (83.4%; e-value = $1.02e^{-163}$; ACF28601) (Fig 2.6).

SMART™ analysis identified an actin domain (e-value = $3.60e^{-102}$), which corresponded with the Pfam analysis which identified the same domain (e-value = $5.40e^{-104}$). SMART™ also indicated the superfamily as having an actin-like ATPase domain (d1j6za2 e-value = $1.00e^{-86}$) via SCOP (Structural Classification of Proteins).

### 2.2.14 *Hematodinium* 18S

The *Hematodinium* 1,180 bp 18S ribosomal RNA (rRNA) fragment generated here comprised part of the 18S rRNA, internal transcribed spacer 1 (ITS1), 5.8S rRNA and part of internal transcribed spacer 2 (ITS2) (Accession number: HE609032). The ITS region refers to sections of non-functional RNA located between structural rRNAs.

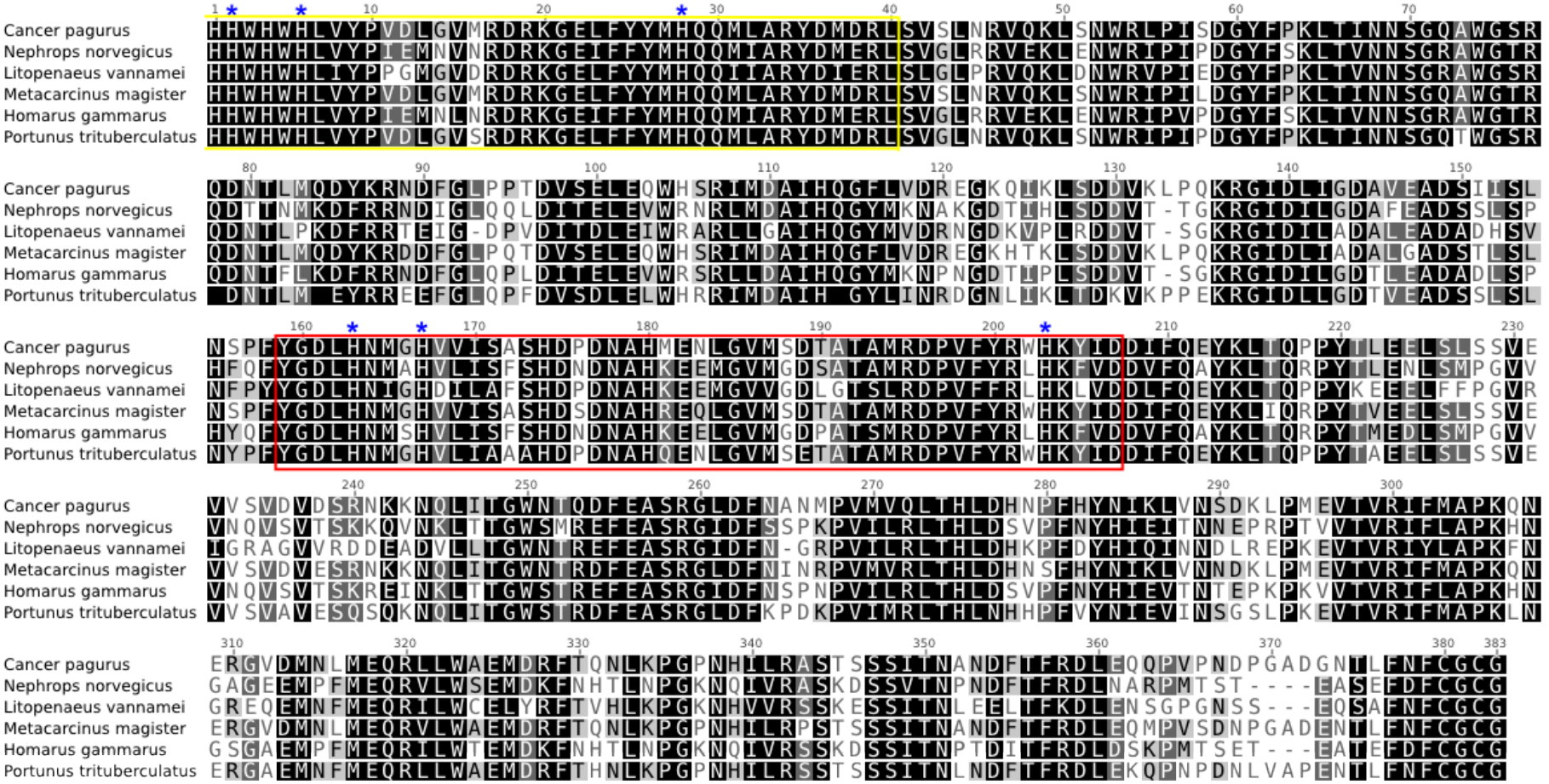



FIGURE 2.5: Multiple alignment of proPO amino acid fragments from *N. norvegicus* and *C. pagurus* with proPO from the crustaceans *L. vannamei* ABQ45957, *M. magister* ABB59713, *H. gammurus* , *P. trituberculatus* ACI46633. The six conserved histidine residues are indicated by blue asterisks (*), the partial copper binding site A is bounded by an open sided yellow box and copper binding site B by a red box. Overall pairwise identity for these sequences was 67.7%. Amino acid similarity is indicated by color: black - 100%, dark gray - 80-99%, light gray - 60-79%, white 0-59%.

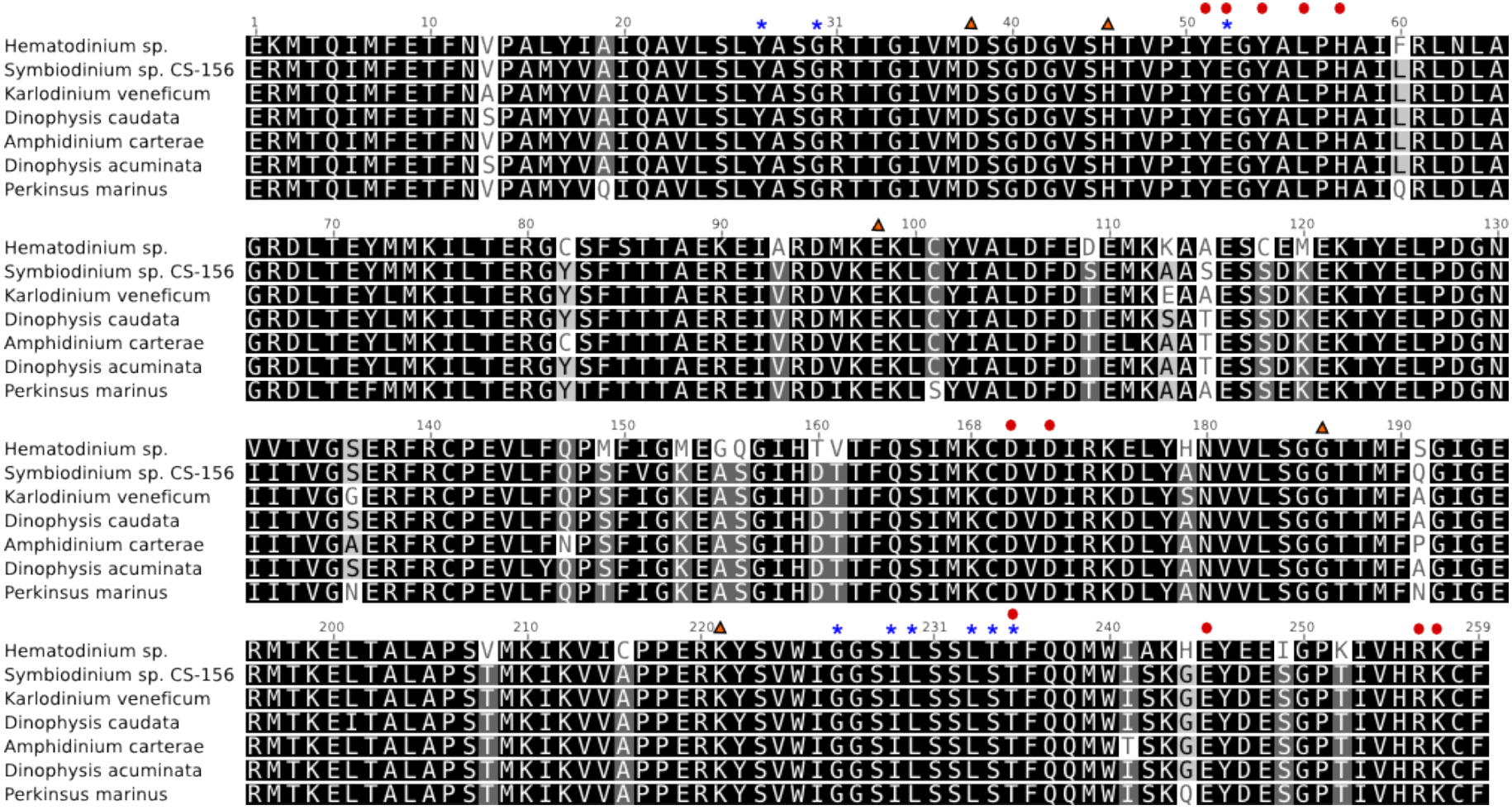



FIGURE 2.6: Multiple alignment of the actin amino acid sequence from *Hematodinium* sp. with actin from the dinoflagellates *Symbiodinium* sp. CS-156 BAE79387, *K. veneficum* ACF32732, *D. caudata* ADU24822, *A. carterae* ACF28601, *D. acuminata* ADU24811, and *Perkinsus marinus* AAR11391. The gelsolin binding site is indicated by blue asterisks (*), the profilin binding site by red circles and the ATP-binding site by orange triangles. Overall pairwise identity for these sequences was 92.4%. Amino acid similarity is indicated by color: black - 100%, dark gray - 80-99%, light gray - 60-79%, white 0-59%.

The complete transcript contains a 5' external transcribed sequence (5' ETS), 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA and a 3' ETS. The high variability of the ITS regions makes it a popular sequence for taxonomy and molecular phylogeny and it has been used to study differences in *Hematodinium* sp. populations for different hosts and geographic locations (Hudson and Adlard, 1996; Small *et al.*, 2007*b*; Hamilton *et al.*, 2007*a*). The nucleotide fragment isolated in this study had high pairwise identity (99%) with *Hematodinium* sp. extracted from other crustacean host species from the UK (Fig 2.7). BLAST analysis of the nucleotide sequence showed the first 129 hits as other *Hematodinium 18S* genes from numerous hosts, however the sequence fragment also had high identity with the dinoflagellate parasite *Syndinium turbo* (95.6%; e-value = $4.05e^{-141}$; DQ146405) and other dinoflagellates *Prorocentrum micans* (93.9%; e-value = $8.35e^{-131}$; EU780638), *Duboscquodinium collinii* (91.2%; e-value = $2.91e^{-130}$; HM483398), a *Pfiesteria*-like sp. (93.6%; e-value = $2.91e^{-130}$; AY590485) and *Gymnodinium aureolum* (92.9%; e-value = $5.27e^{-127}$; DQ779991). Hudson and Adlard (1996) used the hypervariable V9 domain to compare differences between *Hematodinium* and other dinoflagellate species and in this study the V9 domain appeared to be highly conserved in alignment of *Hematodinium* species but variable when compared with other dinoflagellate species.

As discussed in Chapter 1.3.3, the gene coding for *Hematodinium* sp. 18S rRNA is frequently used as a diagnostic for *Hematodinium* infection in crustacean hosts. In the present study, two sets of published primers were used to check for *Hematodinium* sp. infection in *Cancer pagurus* from the English Channel: those described in Table 2.1 (White *et al.*, 1990; Gruebl *et al.*, 2002) and a pair from Hudson and Adlard (1994) that were successfully used on *Hematodinium* sp. from the UK (Small *et al.*, 2007*b*) (SHemat Fwd: 5'-gtt ccc ctt gaa cga gga att c-3'; SHemat Rev: 5'-cgc att tcg ctg cgt tct tc-3'). Positive bands were found in all animals using the SHemat primers and no bands were found using those from White *et al.* (1990) and Gruebl *et al.* (2002). Sequencing revealed that the SHemat primers had, in fact, amplified 18S rRNA from *C. pagurus* rather than *Hematodinium* sp. It is imperative, therefore, that all positive bands be sequenced to confirm the amplification of parasite cDNA. The SHemat primers were not used for the remainder of the project.

### 2.2.15 Crustacean *runt*

Degenerate PCR using both published (Soderhall *et al.*, 2003) and manually designed primers for the *runt* gene only resulted in successful bands following restriction digest using *Not*I. The subsequent sequencing of several potential bands did not show that

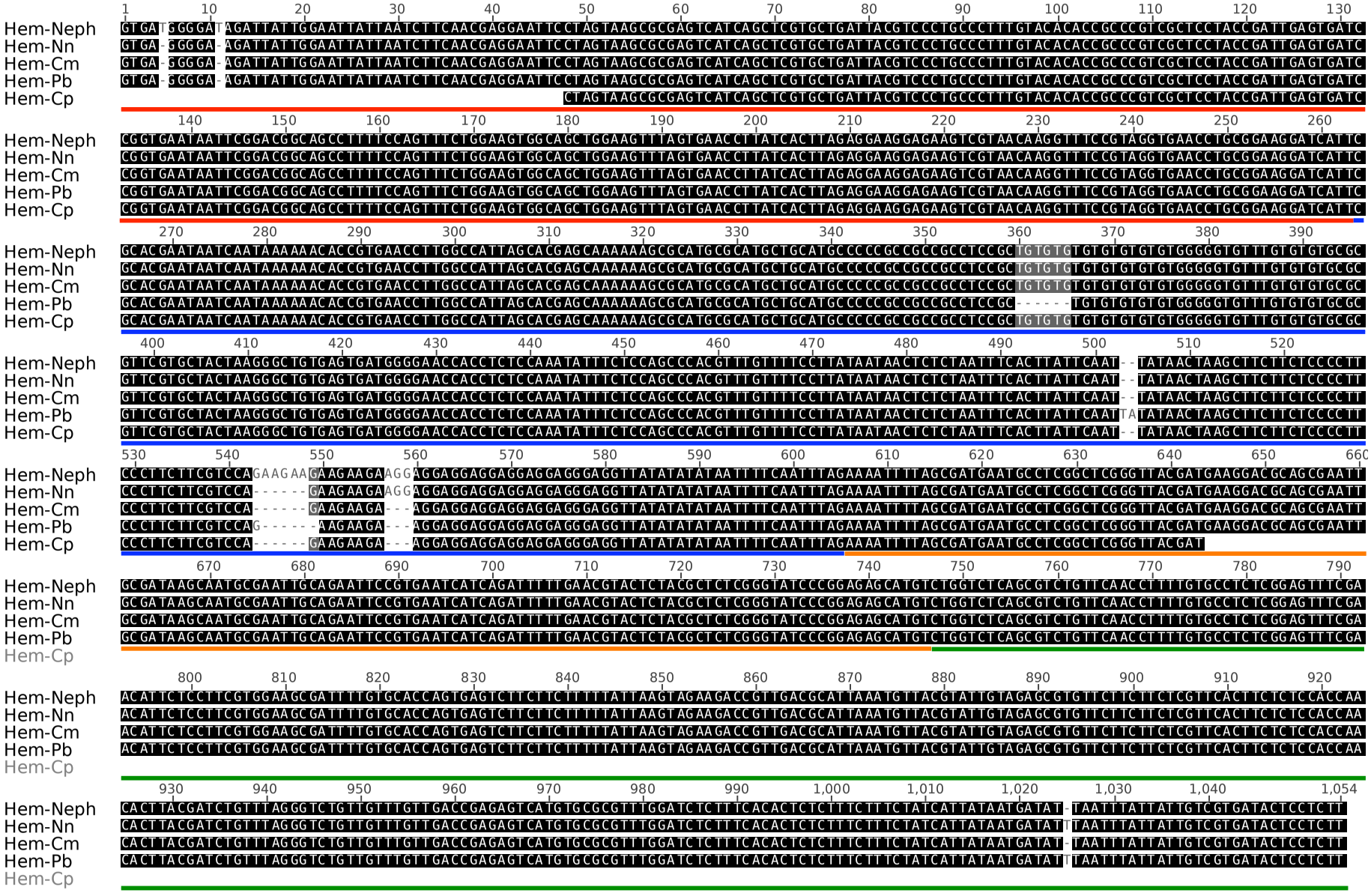



FIGURE 2.7: Multiple alignment of the *18S/ITS* nucleotide sequence from *Hematodinium* sp. ex. *N. norvegicus* (Hem-Neph) with *Hematodinium* sp. *18S/ITS* fragments from other UK crustacean hosts (Nn = *Nephrops norvegicus*; Cm = *Carcinus maenas*; Pb = *Pagurus bernhardus*; Cp = *Cancer pagurus*). The underlined areas are as follows: red = 18S rRNA, blue = ITS1 region, orange = 5.8S rRNA, green = ITS2 region. Overall pairwise identity for these sequences was 99%. Nucleotide similarity is indicated by color: black - 100%, dark gray - 80-99%, light gray - 60-79%, white 0-59%.

the extracted bands were the *runt* gene. BLASTP analysis resulted in no hits. As a result, it was decided that the *runt* gene would not be included in the present study.

### 2.2.16 Rapid amplification of 5' and 3' cDNA ends (RACE)

In order to complete the gene fragments previously attained, 5' and 3' rapid amplification of cDNA ends (RACE) was used to determine the sequences for the 5' and 3' regions of the *proPO* gene from *C. pagurus* and *N. norvegicus*. This technique allows the amplification of unknown regions of DNA from known adjacent regions. The 5'/3' RACE reactions for this project were carried out using a SMART™ RACE cDNA amplification kit from Clontech (Takara Bioscience, France). The SMART™ RACE kit uses a reverse transcriptase (RT) that is a variant of Maloney Murine Leukemia Virus RT (MMLV-RT) with a unique template transferase activity which, in combination with the SMART II™ A oligonucleotide, eliminates the need for adaptor ligation during 5' cDNA synthesis. The SMART™ RACE process can be divided into four major reactions: 5' cDNA synthesis, 3' cDNA synthesis, RACE PCR and nested RACE PCR (optional) (Clontech, 2006).

#### 2.2.16.1 SMART™ RACE protocol

**3' cDNA synthesis:** As before during cDNA synthesis for degenerate PCR, 3' first-strand cDNA synthesis exploits the poly-A tail of mRNA. In this protocol 1 $\mu$g RNA was combined with 1 $\mu$l 3'-CDS primer A and sterile water to a volume of 5 $\mu$l and incubated at 70°C for 2 min. The tubes were cooled and the following reagents added to each tube: 2 $\mu$l 5X First-Strand buffer, 1 $\mu$l 20mM DTT, 1 $\mu$l 10mM dNTP mix and 1 $\mu$l PowerScript™ Reverse Transcriptase. The samples were incubated at 42°C for 90 min in an air incubator. Each sample was diluted with 50 $\mu$l tricine-EDTA buffer and incubated at 72°C for 7 min. The samples were stored at -20°C until needed.

**5' cDNA synthesis:** 5' first-strand cDNA synthesis was carried out using a modified oligo-dT primer and PowerScript™ RT. The terminal transferase activity of the RT adds several (3-5) dC residues to the end of the cDNA strand when it reaches the end of the mRNA template. The SMART II™ A oligonucleotide contains a terminal stretch of G residues which were able to prime to the dC-rich cDNA tail and act as a template for reverse transcription. PowerScript™ RT was then able to switch templates and generate a copy of the RNA molecule using the cDNA as a template. The protocol for 5' cDNA synthesis is identical to that for 3' cDNA synthesis but uses 5'-CDS primer A and includes 1 $\mu$l SMART II™ A oligo during the initial reaction mixture.

Table 2.4: RACE PCR thermocycling conditions

| Step | Temp (°C) | Duration | No. Cycles |
|---|---|---|---|
| Initial Denaturation | 94 | 7 min | 1 |
| Denaturation | 94 | 30 sec | |
| Annealing | 67 | 30 sec | 35 |
| Extension | 72 | 3 min 30 sec[1] | |
| Final Extension | 72 | 7 min | 1 |

[1]Reduce to 2 min for nested RACE

**RACE PCR:** Primary PCR was carried out on first-strand cDNA ends using Advantage *Taq* polymerase, gene-specific primers (GSPs) and universal primer mix (UPM). The UPM binds to the SMART II™ A sequence contained in the SMART II™ A oligo from the 5' cDNA reaction and as part of the modified oligo-dT from the 3' cDNA reaction. The GSPs were manually designed based on the fragments generated during degenerate PCR (section 2.2.16.2). The PCR reaction was comprised of the following components: 2.5 $\mu$l RACE-ready cDNA, 5 $\mu$l UPM, 1 $\mu$l GSP, 5 $\mu$l 10X Advantage 2 PCR Buffer, 1 $\mu$l 10mM dNTP mix, 1 $\mu$l 50X Advantage 2 Polymerase mix and 34.5 $\mu$l sterile water to a total volume of 50 $\mu$l. Reactions were run according to the manufacturer-recommended cycle (Table **??**).

**Characterisation of RACE products (nested PCR):** To confirm that products of the desired size were being amplified RACE products were re-amplified using nested gene-specific primers (NGSP) and a nested universal primer (NUP) provided with the Clontech kit. The template for the nested PCR was generated by diluting 1.25 $\mu$l of primary PCR product in 61.25 $\mu$l tricine-EDTA. The final reaction contained: 1 $\mu$l diluted template, 0.5 $\mu$l NUP, 0.5 $\mu$l NGSP, 2.5 $\mu$l 10X Advantage 2 PCR buffer, 0.5 $\mu$l 50X dNTP, 0.5 $\mu$l 50X Advantage 2 Polymerase mix and 19.5 $\mu$l sterile water to a final volume of 25 $\mu$l. The PCR cycle was the same as that used for the primary PCR except that the extension time was reduced to two minutes (Table **??**).

#### 2.2.16.2 RACE Gene-specific primer design

Gene-specific primers for *proPO* from *C. pagurus* and *N. norvegicus* were designed based on the species-specific sequences generated from degenerate PCR (section 2.2.12). The primers were designed according to the guidelines set out in the Clontech SMART™ RACE user manual (Clontech, 2006). Primers were recommended to contain 23-28 nucleotides, contain 50-70% GC content and have a $T_m \geq 65$°C, and preferably $> 70$°C. All primers were designed to meet these recommendations and aimed to have a $T_m$ close to 70°C (Table 2.5). Furthermore, primers were screened using NetPrimer (http://www.premierbiosoft.com/netprimer/; PREMIER Biosoft, Palo Alto, US) to

avoid self-complementarity and secondary structure. Primers were ordered from Eurofins MWG Operon (previously MWG-Biotech; Ebersberg, Germany).

TABLE 2.5: *ProPO* gene-specific (GSP) and nested gene-specific primers (NGSP) designed for RACE PCR.

| Primer Name | $T_m$ (°C) | Primer Sequence (5' to 3') |
|---|---|---|
| Cp PPO GSP1 | 69.5 | GCT GAT GAT GCT GTC TGC CTC CAC TGC |
| Cp PPO GSP2 | 69.5 | GAG CCT CCA CTT CCT CTT CAA TCA CCA ATG |
| Cp PPO NGSP1 | 69.5 | GAG GTC AAT CCC ACG CTT CTG AGG CAG |
| Cp PPO NGSP2 | 69.5 | CCT CTT CAA CTT CTG TGG GTG CGG CTG |
| Nn PPO GSP1 | 66.4 | TCC TTC ATG TTG GTG GTG TCC TGA CG |
| Nn PPO GSP2 | 69.5 | GGG GAA CCC GTC AGG ACA CCA CCA A |
| Nn PPO NGSP1 | 68.0 | CGT CTA AGA CCA ACA CTC AGC CGT TCC |
| Nn PPO NGSP2 | 69.5 | GGA GAT TCA GCG ACC GCC ATG AGG GA |

## 2.3 Complete *proPO* sequence results

The total *proPO* sequence generated for *C. pagurus* was 2331 bp (Accession number: FR687020). When translated the sequence yielded an open reading frame (ORF) of 2013 bp (671 amino acids). The ORF began with the initiating methionine codon, ATG, at 58 bp and finished at 2071, leaving a 3' untranslated region (UTR) of 186 bp followed by the poly A tail indicative of the end of the mRNA. The calculated molecular weight for the proPO protein from *C. pagurus* was 77.138 kDa with an isoelectric point of 6.18 (as predicted by Geneious v. 4.6.5; Accession number CBW54878). The *N. norvegicus* mRNA sequence for *proPO* was 2825 bp in length (Accession number: HE608877), including a 68 bp 5' UTR and a 652 bp 3' UTR followed by the poly A tail. The 2046 bp coding sequence coded for a 683 aa protein sequence with a predicted molecular weight of 78.16 kDa and an isoelectric point of 7.27 (Geneious v. 5.0.4; Accession number: CCE46011.1).

Two hemocyanin domains, hemocyanin M (a copper-containing domain) and hemocyanin C (an Ig-like domain) were identified in a Pfam search (http://pfam.sanger.ac.uk) (Fig 2.8). Haemocyanin, the oxygen carrying molecule in crustaceans, and proPO proteins have conserved regions in their protein sequences and haemocyanin has been found to have a phenoloxidase-like function in the spiny lobster *Panulirus argus* and *Cancer magister* (Terwilliger and Ryan, 2006; Perdomo-Morales *et al.*, 2008), and is the primary molecule in melanin formation for horseshoe crabs (Iwanaga, 2002; Decker and Jaenicke, 2004). Additionally, two conserved copper binding sites (CuA and CuB) were found, including six highly conserved histidine residues. The two copper atoms located in the binding sites CuA and CuB are held in

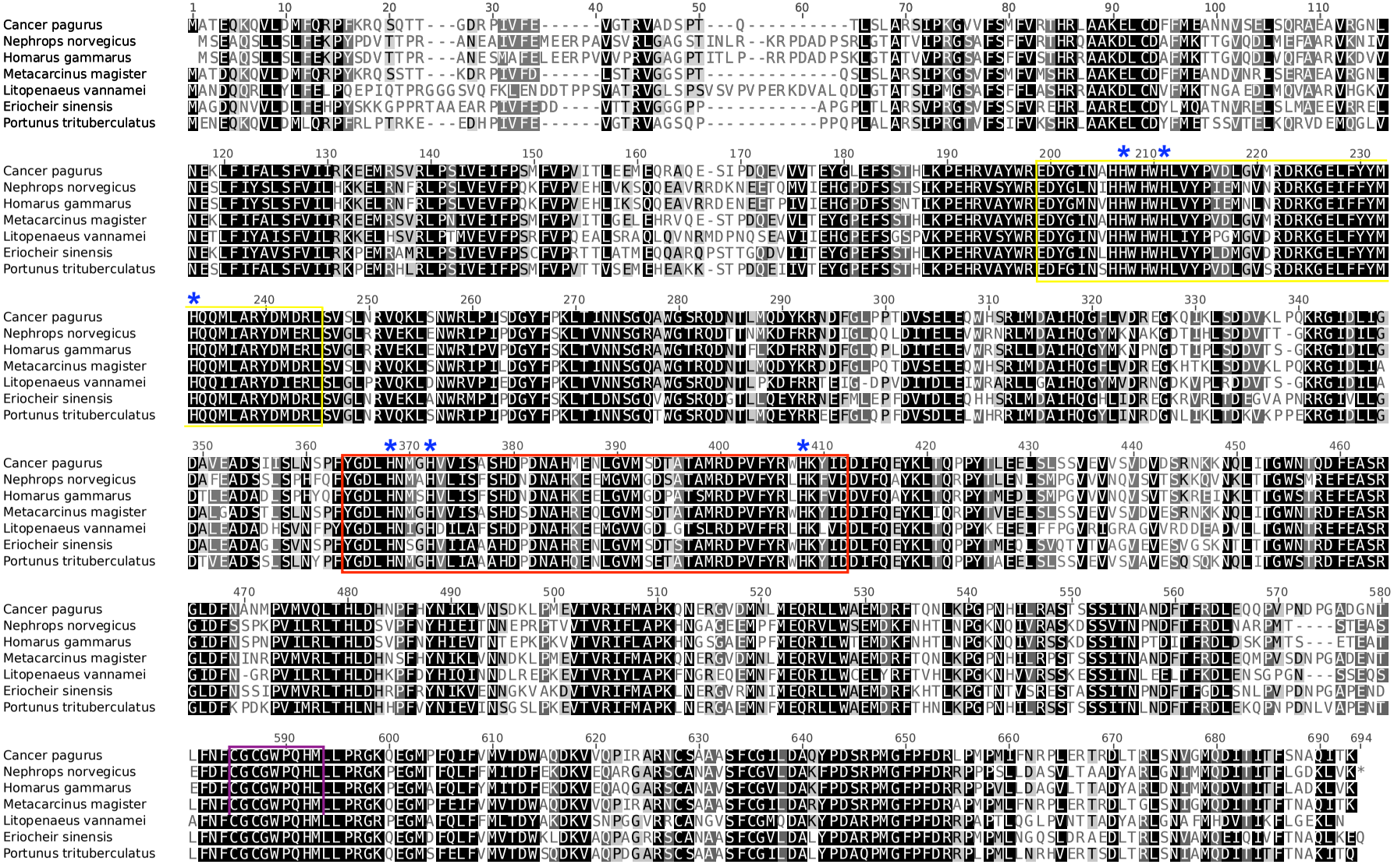



FIGURE 2.8: Multiple alignment of complete proPO amino acid sequences from *N. norvegicus* and *C. pagurus* with proPO from the crustaceans *H. gammarus* CAE46724.1, *M. magister* Q283K9, *L. vannamei* A0ASJ0, *E. sinensis* ABS19633.1, *P. trituberculatus* ACI46633.1. The six conserved histidine residues are indicated by blue asterisks (*), copper binding site A is boxed in yellow and copper binding site B is boxed in red. The thiol ester motif is boxed in purple. Overall pairwise identity for these sequences was 62.9%. Amino acid similarity is indicated by color: black - 100%, dark gray - 80-99%, light gray - 60-79%, white 0-59%.

place by three histidine residues each. In haemocyanin, the copper-binding sites then form an active site for binding dioxygen and transporting it, however proPO has been found to use one of the oxygen molecules for a chemical reaction (Decker and Terwilliger, 2000). Alignment with 7 other proPO protein sequences also showed a thiol ester-like motif (GCGWPQHM), commonly found in *proPO* genes (Ko *et al.*, 2007; Lai *et al.*, 2005; Terwilliger and Ryan, 2006). The thiol ester-like motif has been found in components C3 and C4 of the complement system from vertebrates suggesting a common evolutionary ancestry (Cerenius and Soderhall, 1995).

The fruit fly *Drosophila* (Ross *et al.*, 2003) and the mosquito *Anopheles gambiae* (Christophides *et al.*, 2002) are known to have multiple genes for proPO, however the sequences generated in this study did not show any signs of having isoforms so it was assumed that qPCR primers would only amplify a single gene. Based on the high pairwise identity of the *proPO* nucleotide and predicted amino acid sequences found for both *C. pagurus* and *N. norvegicus* with other crustacean *proPO*s, it is with high confidence that these sequences can be used for further investigation into gene expression.

## 2.4 Conclusions

The immunologically relevant gene for prophenoloxidase was successfully sequenced for both the edible crab *Cancer pagurus* and the Norway lobster *Nephrops norvegicus*. The combination of degenerate PCR and RACE PCR were used to generate the full sequence for both species and based on BLAST analysis it is with high confidence that both genes can be used in qPCR assays for gene expression studies.

Unfortunately, despite numerous attempts and extensive review and alteration of techniques and reaction conditions, it was not possible to isolate the *runt* gene from the haemocyte or haemopoietic tissue from either *C. pagurus* or *N. norvegicus*. Due to time constraints, it was unfeasible to continue searching for the gene and it was not included in this project.

The gene coding for actin, chosen as the reference gene for relative quantification in the qPCR assays for this project, was successfully cloned and sequenced for both *C. pagurus* and *N. norvegicus* as well as for *Hematodinium* sp. ex *N. norvegicus*. *Actin* fragments for all species were highly conserved and this was the first *actin* fragment to be generated for *Hematodinium* sp. With proper quantitative PCR optimisation and validation it was intended that this gene would serve as an appropriate endogenous reference for qPCR assays or parasite gene expression.

Finally, the gene coding for 18S rRNA in *Hematodinium* was cloned and sequenced with the aim of using qPCR assays to quantify parasite abundance within crustacean hosts, as carried out by Nagle *et al.* (2009) in the blue crab *Callinectes sapidus*. The gene was found to be highly conserved when compared to other *Hematodinium* sp. nucleotide sequences isolated from UK crustacean hosts, though it had some variation in the ITS regions when compared to other dinoflagellate species. These results indicate that carefully designed primers would be specific enough to amplify only genes from *Hematodinium* and limit interference from other dinoflagellate species that may be present.

# Chapter 3

# Quantitative PCR Methodology

## 3.1 Introduction

Conventional polymerase chain reaction (PCR) is the cornerstone of modern molecular biology (McPherson and Moller, 2000). PCR uses a thermostable DNA polymerase and repeated cycles of heating and cooling to amplify a desired segment of DNA or cDNA. In an ideal reaction, the amplification process is exponential, doubling the copies of the DNA fragment at each cycle; however, in reality the reaction goes through an exponential phase before plateauing as reaction components run low and the DNA polymerase wears out (McPherson and Moller, 2000). This conventional (end-point) method of PCR is a qualitative assessment, and the results are visualised using gel electrophoresis and image analysis making it a useful tool for identifying presence or absence of genes, but not sufficient to study changes in gene expression.

Quantitative PCR (qPCR) combines traditional end-point PCR with detection and quantification of amplified products in real-time. In qPCR, fluorescent dyes are used to bind to PCR products and the increase in fluorescence is measured after every cycle. The recorded fluorescence is proportional to the amount of DNA in each reaction. Fluorescence plots for each reaction are generated and the reactions are defined by the point at which they rise above a designated threshold value set above the background fluorescence (Fig 3.1). This point is termed the quantification cycle ($C_q$) and must always occur in the exponential phase of amplification (Bustin, 2000). The more template present at the beginning of the reaction, the sooner the fluorescence value will cross the threshold resulting in a lower $C_q$ value. $C_q$ values of a gene of interest (GOI) can then be compared to those of an endogenous reference (ER) gene to determine fold-change in experimental and control samples (relative quantification), or they can be compared against a standard curve of known copy numbers to determine

exact starting copies of the gene in each reaction (absolute quantification) (Arya *et al.*, 2005; Wong and Medrano, 2005).

In recent years, qPCR has become the 'gold standard' for studying gene expression (Bustin, 2000; Ginzinger, 2002; Pfaffl, 2010), however the numerous steps leading up to acquiring results leave room for a great deal of variability. Technical variations in sample storage, nucleic acid extraction and reverse transcription can lead to less efficient assays in downstream applications (Bustin, 2010). Furthermore, insufficient assay optimisation including poor primer design, insufficient primer concentrations, inefficient assays and poor reference gene choice can cause erroneous results (Glare *et al.*, 2002; Nolan, 2004; Bustin, 2010).

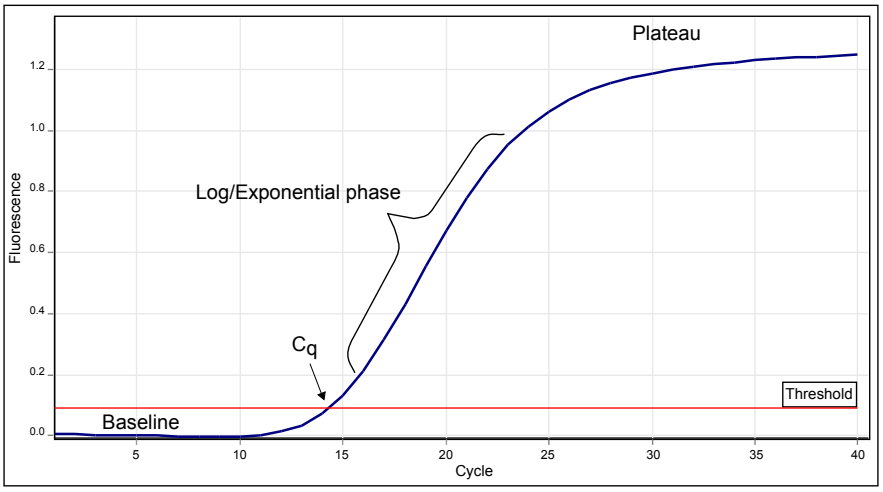



FIGURE 3.1: An illustration of a typical qPCR fluorescence plot. Fluorescence data is acquired during each reaction cycle of the PCR. Theoretically, the reaction curve should be exponential as the amount of DNA doubles during each cycle, however in reality the depletion in PCR components and decline in polymerase activity cause the reaction to lose efficiency. Setting a threshold above the baseline fluorescence and during the exponential phase of the reaction generates a $C_q$ value for every sample where it crosses the threshold. The $C_q$ values can then be compared between samples and starting copies of genetic material can be calculated.

Although the concept behind qPCR is quite simple, there are so many variations on methodology, reagents and instruments that it has become apparent that in order for experimental results to be considered robust and comparable there should be some minimum reporting standards. Furthermore, qPCR is used as a validation technique to corroborate data from other methods such as microarrays and differential display. This use as a reference technique makes it crucial that qPCR methodology be executed to exacting standards (Bustin, 2010). Bustin *et al.* (2009) have recently published their suggestions on what should be considered the Minimum Information for publication of Quantitative real-time PCR Experiments (MIQE) and generated an extensive checklist of essential and desirable information. The guidelines cover all aspects of qPCR (Fig 3.2) that may affect the downstream results and focus on four key areas: study design,

technical detail, analysis methods and statistics (Bustin, 2010). The aim of this chapter is to describe the methodologies used in the qPCR studies of this project. The methods covered will include the essential details of the key points of the MIQE guidelines, including sample handling, nucleic acid extraction, reverse transcription, assay optimisation, qPCR conditions and reagents, and data analysis.

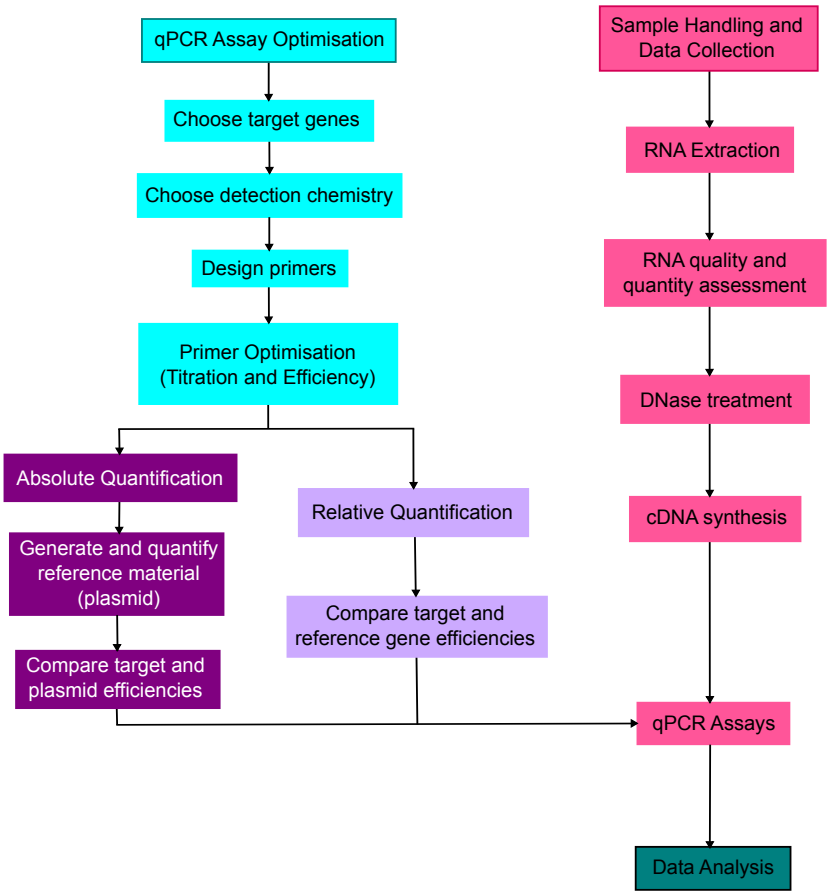



FIGURE 3.2: qPCR workflow requires numerous steps to generate a robust assay. All steps were followed in this order to optimise the qPCR assays.

## 3.2 Sample handling

Sampling technique is vital because it is the first source of variation in a qPCR experiment. Due to the fragile nature of nucleic acids, and specifically RNA, it is essential to record how samples were taken (micro- or macrodissection), how quickly they were processed and, if not processed immediately, how they were stored (Bustin *et al.*, 2009). The nucleic acid extraction method is also important and should be carried out as efficiently as possible to reduce processing time.

Haemolymph samples for this project were pelleted (see 5.2.2), the supernatant removed and then stored in RNAlater® (Applied Biosystems, Cheshire, UK) at 4°C overnight. Samples were then moved to -20°C until returning to the lab where the RNAlater® was removed and the samples flash frozen before storing at -80°C. Cell culture samples were lysed in the culture dish and the total RNA extracted immediately. RNA was initially extracted using TRI Reagent® (Sigma-Aldrich, Dorset, UK), however quality checks using the Experion™ indicated that this method was not suitable for use in qPCR. Samples were then extracted using a column-based Nucleospin® RNA II kit (Macherey-Nagel, Düren, Germany). To minimize handling time, samples were extracted in batches of 12 or less (Taylor *et al.*, 2010) and the extracted RNA stored at -80°C.

### 3.2.1 RNA quality and quantity

RNA quality is absolutely crucial to successful and relevant qPCR and has recently become a primary topic of discussion in the qPCR community. Degraded RNA is not acceptable for use in qPCR experiments as it can compromise expression results (Fleige and Pfaffl, 2006; Vermeulen *et al.*, 2011). Pure, intact RNA of known concentration is essential for expression studies.

There are numerous technologies available for assessing RNA quality and quantity. In this study, all samples were analysed for purity spectrometrically using a NanoDrop® ND-1000 (Thermo Scientific, Wilmington, USA) spectrophotometer (Fig 3.3B and E). RNA purity and quantity can be assessed spectrometrically by analysing $A_{260}/A_{280}$ ratios; however this method gives no indication of integrity and has been criticised for low accuracy (Bustin and Nolan, 2004*f*). Thermo Scientific recommend an $A_{260}/A_{280}$ ratio of 2.0 for 'pure' RNA' using the NanoDrop® system (Scientific, 2008), with lower values indicating contamination with protein or phenols which may inhibit downstream protocols.

Samples with suitable $A_{260}/A_{280}$ ratios (1.9-2.15) were then analysed for integrity and quantity using the Bio-Rad Experion™ system. Traditionally, RNA integrity had been measured using formaldehyde gel electrophoresis in which intact RNA can be identified by two distinct bands representing 18S and 28S ribosomal RNA (rRNA); however this method is somewhat subjective and requires a significant amount of RNA (Taylor *et al.*, 2010). More recent techniques use microfluidic electrophoresis such as the Agilent Bioanalyzer (Agilent Technologies, Palo Alto, CA) or the Bio-Rad Experion™ (Bio-Rad Laboratories, Hercules, CA) to analyse 28S/18S rRNA ratios and sample degradation. Microfluidics chip-based technologies, such as the Experion™ system used here, use an intercalating dye and laser-induced fluorescence to measure nucleic acid concentration

and quality on very small amounts of RNA (1 $\mu$l) (Woo and Strong, 2006). The software generated an electropherogram (Fig 3.3C and F) and a virtual gel image (Fig 3.3A and D) for each sample (Chang *et al.*, 2010). Electropherograms for intact samples showed two distinct peaks representing 28S and 18S rRNA and had an RNA quality indicator (RQI) values greater than 7.5. The Experion™ software calculates RQI values based on comparison of the sample electropherogram to a set degradation standard electropherograms (Denisov *et al.*, 2008). The RQI ranges between 10 (intact RNA) and 1 (degraded RNA) and, for this study, only samples with an RQI $\geq$ 7.5 were reverse transcribed for qPCR.

It should be mentioned that Experion™ (and Bioanalyzer) provides an indication of rRNA integrity which is not always representative of mRNA integrity (Bustin *et al.*, 2010) and this should be kept in mind when carrying out qPCR. Nevertheless, RNA integrity analysis is broadly considered to be essential to support robust qPCR quantification and remains a central requirment of the the published MIQE guidelines (Bustin *et al.*, 2009).

#### 3.2.1.1 DNase treatment

To eliminate genomic DNA from samples, all RNA samples were treated with an Ambion Turbo DNA-*free*™ kit (Applied Biosystems, Cheshire, UK). Reactions were carried out by combining 0.5 $\mu$l DNase, 2.5 $\mu$l 10X TURBO DNase Buffer, RNA (in equal ng amounts for each sample) and RNase-free water to 25 $\mu$l, then incubated at 37°C for 30 min. Resuspended DNase Stop Solution was added to each reaction (2.5 $\mu$l) and the reactions incubated for 5 min at room temperature and mixed intermittently. All samples were centrifuged at 10,000 *g* for 1.5 min and the supernatant removed to a new tube. Samples were stored at -80°C until cDNA synthesis.

### 3.2.2 cDNA preparation

Complimentary DNA (cDNA) was generated using SuperScript™ III (Invitrogen, Paisley, UK) reverse transcriptase (RT). SuperScript™ III RT enzyme is a Maloney murine leukemia virus (MMLV)-derived RT and is one of the most sensitive RT enzymes used to generate cDNA for qPCR (Bustin, 2002; Levesque-Sergerie *et al.*, 2007). MMLV-RT is known to have less RNase H activity than other RT enzymes, such as avian myeloblast virus (AMV) RT, which reduces RNA degradation during cDNA synthesis. In the past, MMLV-RT has only been capable of withstanding low temperatures (37°C) which reduces the ability for RT to read the RNA template (Bustin, 2002; Bustin and Nolan, 2004*a*); however, SuperScript™ III has been

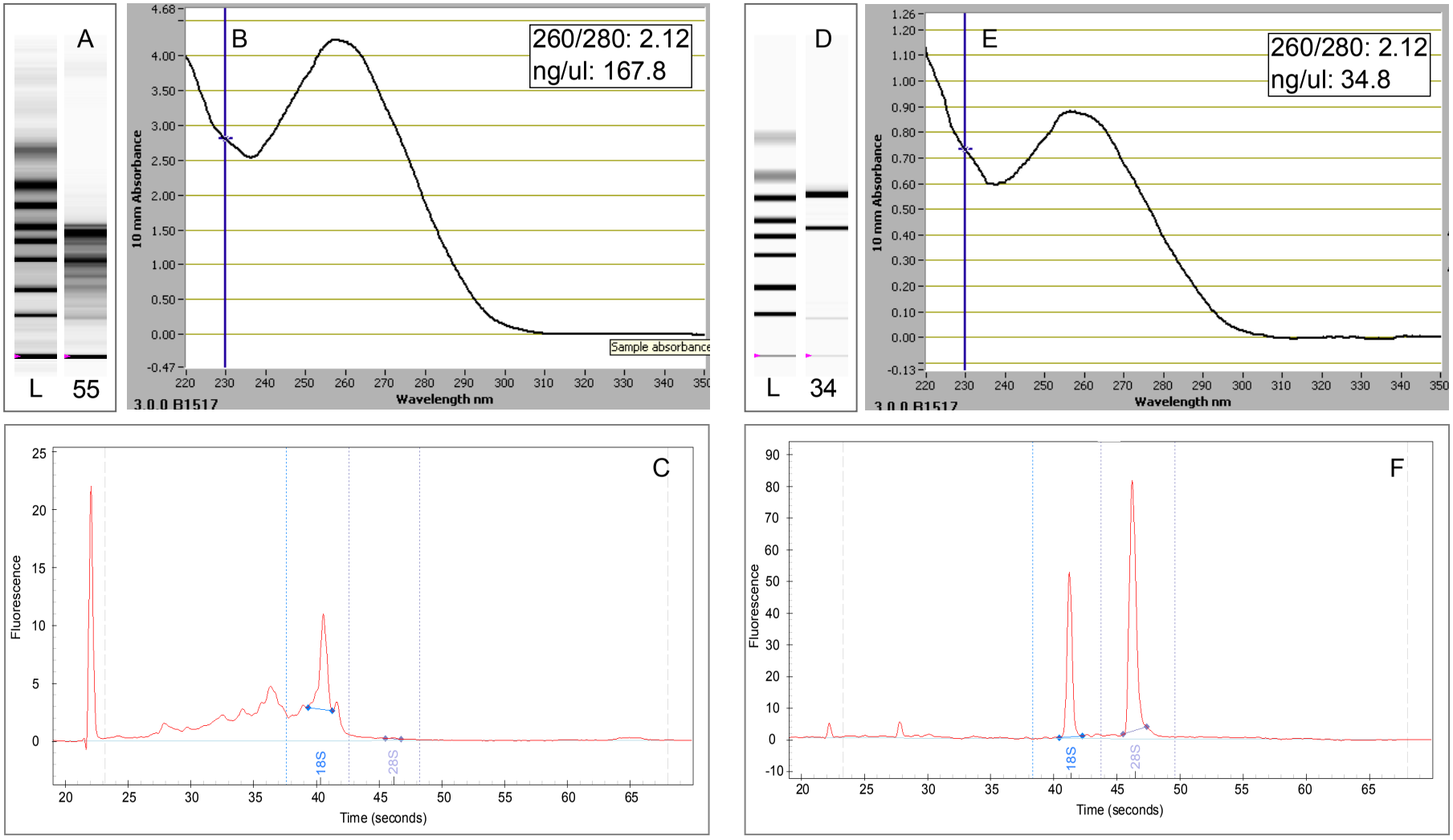



FIGURE 3.3: Comparison of degraded (Sample 55) and intact (Sample 34) RNA samples, but with identical Nanodrop® $A_{260}/A_{280}$ ratios (B and E). Experion™ electropherograms (C and F) showed that sample 34 had high quality RNA indicated by distinct 18S and 28S peaks visualised as two distinct bands on the virtual gel image (D). Sample 55 was partially degraded, indicated by a decrease in the 28S peak and a shift in RNA size toward smaller fragments (A and C). RQI values: Sample 55 = 3.4; Sample 34 = 10.

optimised to function at higher temperatures (up to 55°C) and has been proven to be one of the most efficient RT enzymes available (Stahlberg *et al.*, 2004*a*).

First strand cDNA was synthesised by combining in a 0.2 ml PCR tube: 1 $\mu$l dNTP mix (10 mM each), 1 $\mu$l Ambion RETROscript random decamers (Applied Biosystems, Cheshire, UK), total RNA (an equal amount of each sample) and RNase-free water to a final volume of 10 $\mu$l. The reaction was incubated at 65°C for 5 min, then chilled on ice for at least 1 min. To each tube was added 4 $\mu$l 5X First-Strand buffer, 1 $\mu$l DTT (0.1 M), 1 $\mu$l RNaseOUT™ (Invitrogen, Paisley, UK), and 1 $\mu$l SuperScript™ III. The reactions were incubated for 5 min at 25°C, then at 50°C for 55 min. The reactions were heat inactivated for 15 min at 70°C and stored at -20°C until needed. As stipulated by MIQE guidelines (Bustin *et al.*, 2009) and supported by experimental research (Stahlberg *et al.*, 2004*b*), all samples to be compared were reverse transcribed together to reduce variability.

After careful consideration of priming strategy options, random decamers were chosen for this study because they met both the technical and financial requirements of the project. Although oligo(dT) primers are often favoured for cDNA synthesis due to their theoretical specificity for mRNA, they are known to be biased toward the 3'-end of transcript copies. Furthermore, oligo(dT) primes from the 3' polyadenine tail of coding RNAs and complex secondary structure can interfere with complete transcription (Bustin and Nolan, 2004*d*; Bustin *et al.*, 2005). Use of the poly-A tail also prevents them from transcribing rRNA, such as 18S, which lacks a poly-A tail. Alternatively, random primers can prime anywhere along the RNA strand and are more tolerant to secondary structure (Bustin and Nolan, 2004*d*). Random primers generate cDNA from total RNA rather than just mRNA, enabling gene expression analysis of 18S. The most sensitive priming method uses target-specific primers, however to transcribe multiple targets simultaneously requires individual primer optimisation. Individual cDNA synthesis reactions can be carried out for each target gene, though this can prove problematic if there is limited sample available (Bustin *et al.*, 2005) and increases expenditure by requiring additional primers and reagents. Additionally, it is not possible to return to the same preparation at a later date if new target genes are identified (Bustin and Nolan, 2004*d*). Specific priming was unsuitable for this study due to limited amounts of RNA and would have resulted in unnecessary expenditure.

In this study, random decamers were chosen, not only to reduce 3' bias from oligo(dT), but because 18S transcripts were needed to quantify *Hematodinium* load. Random hexamers (6 bp) are the most commonly used random primers, however random decamers (10 bp) were chosen for this study because they generate longer cDNA strands and create a more faithful representation of the RNA present (Avison, 2007).

## 3.3 qPCR Assay Design and Validation

### 3.3.1 Dye and probe technology

Multiple technologies are available for detecting fluorescence in real time PCR, and are divided into non-specific and specific detection methods. Non-specific detection chemistries use intercalating dyes that bind to any double-stranded DNA. Specific chemistries use fluorophores attached to template-specific probes and must be custom-made for each target sequence (Bustin and Nolan, 2004*d*).

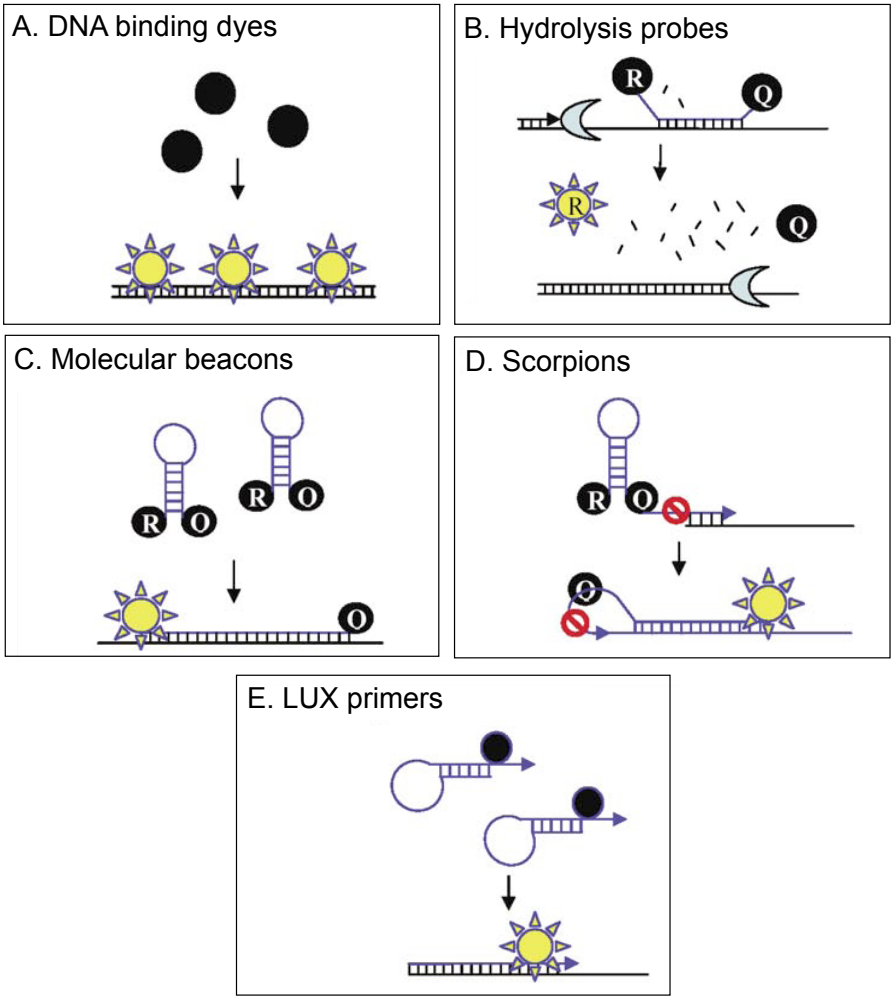



FIGURE 3.4: Detection chemistries for qPCR, see text for further explanation. Figure modified from Wong and Medrano (2005).

#### 3.3.1.1 Specific detection

Template-specific analysis uses fluorescent resonance energy transfer (FRET) involving a donor and a quencher molecule attached to an oligonucleotide probe. Oligonucleotide probes are designed to hybridise between the forward and reverse primers with the

major advantage that assay specificity no longer resides with the primers (Bustin and Nolan, 2004*b*). Primer-dimers and non-specific amplification will not generate any signal. Additionally, multiplex assays can be developed using different fluorophores for each target allowing many genes to be examined in a single reaction. Multiple probe types are available, mostly based around a quencher and reporter dye, though sometimes a quencher is unnecessary (Fig 3.4). Release of the reporter from the quencher results in increased fluorescence.

**Hydrolysis probes:** Hydrolysis probes are commonly known as TaqMan® probes. TaqMan® probes contain a fluorophore at the 5' end of the target-specific probe and a quencher at the 3' end. Whilst intact, the quencher is close enough to the reporter that fluorescence is limited. After annealing to the target sequence, the probe is then degraded by the 5' exonuclease activity of the *Taq* polymerase during the extension step of the PCR. This releases the fluorophore from the quencher, resulting in increased fluorescence (Fig 3.4B) (Bustin and Nolan, 2004*b*; Wong and Medrano, 2005).

**Molecular beacons:** Molecular beacons are a type of hairpin probe where the reporter and quencher are at opposite ends of the probe and are held in close proximity by the hairpin structure of the probe whilst free in solution (Fig 3.4C). Once bound to the target region, the reporter and quencher are separated enough to allow the reporter to emit a signal (Wong and Medrano, 2005).

**Scorpion:** Scorpions are another type of hairpin probe, but the primer and probe are combined. During annealing and extension, the primer binds to the target sequence and extends the new strand of DNA while the probe remains in its hairpin structure. An extra heating step denatures the new strand and the hairpin loop and during the following cooling step, the probe binds to the newly made DNA strand (Fig 3.4D).

**LUX™:** Another type of hairpin primer, LUX™ (light upon extension) primers use secondary structure of the 3' end of the probe to quench the fluorophore. The major advantage to this technology is the reduced cost due to lack of a quencher molecule (Fig 3.4E).

Increased specificity comes with increased price, however; separate probes must be designed for each target gene and multiple target detection can become costly. Furthermore, absence of detection of non-specific products does not mean that they are not there. Non-specific binding can interfere with reaction efficiency resulting in erroneous $C_q$ values. For this reason, specific detection chemistries should be optimised using intercalating dyes. Most importantly, assays should be optimised prior to probe

design. Primers are inexpensive and can easily be optimised using non-specific detection chemistries at reduced cost. Once a suitable primer pair is identified, the more expensive probes can be designed to bind to the amplicon.

#### 3.3.1.2 Non-specific detection

Due to the prohibitive cost associated with specific detection, it was decided to use the intercalating dye SYBR® Green I. Aside from reduced cost, SYBR® Green is also more versatile than specific probe technology because it does not require separate probes for each gene of interest. SYBR® Green binds to double stranded DNA (dsDNA) and only fluoresces when it is bound (Fig 3.4A). As the number of amplicons increase, the amount of bound dye causes an increase in fluorescence which is then detected by the qPCR instrument. A major advantage of using DNA-binding dyes is the ability to use them with any amplicon as they are simply added to a PCR reaction and can be used with previously established protocols (Bustin and Nolan, 2004*b*). However, indiscriminate binding to any dsDNA can be a disadvantage because it will bind to non-specific targets such as primer dimers if generated during the reaction. Furthermore, high concentrations of some dyes (SYBR® Green I, SYTOX Orange and TO-PRO-3) can inhibit the PCR reaction (Gudnason *et al.*, 2007). Specific amplification must be confirmed by melt curve analysis and sequencing. SYBR® Green assay optimisation for the present study was carried out using primer titrations (see 3.4.1.2), standard curve analysis for efficiency (see 3.4.1.3) and melt curve analysis for target specificity (see 3.4.1.1).

#### 3.3.1.3 qPCR Instrument

All qPCR reactions were run on a Corbett Rotor Gene 3000 (Qiagen (Corbett), Crawley, UK). Samples were run in 0.1 ml tubes designed for the 72-well rotor of the Rotor Gene 3000 and were analysed using the Rotor Gene Q series software v. 2.0.2 (Build 4).

## 3.4 Primer and amplicon design

qPCR primers are gene-specific and must meet certain criteria to be as efficient as possible. Primers can be designed manually following some general qPCR guidelines or can be designed using software specifically for primer design. PrimerExpress® (Applied Biosystems, Cheshire, UK) designs primers suitable for use with SYBR®

Green I or TaqMan® assays and was used to design primers in this study. Sequences from the desired genes of interest (GOI's) and reference genes were loaded into the software and a number of potential primer combinations were generated. Primer Express follows the guidelines below for melting temperature, amplicon length, primer length and G/C content, however it does not check for secondary structure or specificity (Theaker, 2004), so those were carried out manually.

**Amplicon Design:** Amplicon design for qPCR is important as assay efficiency can be impacted by length, G/C content and secondary structure. Bustin (2000) has recommended amplicons of approximately 80 bp in length and no more than 100, though others suggest amplicons up to 150 bp are suitable (Brisson *et al.*, 2004; Arya *et al.*, 2005). Short amplicons are more efficiently amplified because they are easily denatured during the denaturation step (92-95 °C). Due to the short time required for amplification, genomic DNA contaminants, if there are any left after DNase treating, are also less likely to be amplified, improving the assay quality (Bustin, 2000). G/C content should be less than 60% to enable easy denaturation and secondary structure should be examined to ensure that extension is not impeded during replication (Bustin and Nolan, 2004*e*; Derveaux *et al.*, 2010).

**Primer length:** Primers should be between 15 and 25 nucleotides in length.

**Primer G/C content:** The G/C content should be between 50-60%, optimally around 50% (Bustin and Nolan, 2004*e*; Taylor *et al.*, 2010). High G/C content may affect denaturing during thermal cycling or cause non-specific binding. Additionally, the 3' end of the primer should contain no more than two G or C bases in the last five nucleotides to limit non-specific priming (Bustin, 2000; Bustin and Nolan, 2004*e*). Long runs of G or C (more than 4) should also be avoided (Bustin and Nolan, 2004*e*).

**Melting temperature ($\mathbf{T}_m$):** The $T_m$ should be 58-60 °C for each primer and the primer pair should have as similar a melting temperature as possible. The $T_m$ of both primers should not be more than 1-2 °C different (Bustin, 2000).

**Secondary structure:** Primers were screened for secondary structure using NetPrimer (http://www.premierbiosoft.com/netprimer). NetPrimer analyzes primers for the likelihood to form self-dimers, cross-dimers and hairpins as well as indicating general primer characteristics such as melting temperature and molecular weight. A proprietary algorithm is used to generate a primer rating score (0-100) which is based on the thermodynamics of secondary structures that are identified. Primers scoring below 80 were not considered for use in qPCR.

**Specificity:** All primers were submitted to an NCBI BLAST (Basic Local Alignment Search Tool) search via Geneious v. 4.5.5-4.7.6 (Biomatters Ltd., Aukland, New Zealand) to ensure that they were specific to the genes of interest and would not misprime onto other genes (Bustin and Nolan, 2004*e*; Bustin *et al.*, 2010; Derveaux *et al.*, 2010).

### 3.4.1 qPCR assay optimisation

qPCR assay optimisation is an essential part of generating an efficient and robust qPCR assay. Using the correct primer concentrations can produce a more reliable assay as well as make use of the least amount of primer to be efficient. Primer optimisation is generally carried out using SYBR® Green I because it is sensitive, convenient and inexpensive (Nolan, 2004). Additionally, melt curve analysis can be used to ensure that a single product is being generated.

Lyophilised primers (from Eurofins MWG Operon, Ebersberg, Germany) were rehydrated in nuclease-free water to a stock concentration of 100 $\mu$M. Primers were then diluted to 5 $\mu$M and stored in aliquots for use in qPCR. All qPCR reactions were carried out by combining 25 $\mu$l Precision 2X real-time PCR MasterMix with SYBR Green (PrimerDesign Ltd, Southampton, UK), 4 $\mu$l cDNA, forward primer, reverse primer and sterile water to 50 $\mu$l total volume. The reactions were then divided into two 23-$\mu$l volumes in 0.1 ml qPCR strip tubes (Qiagen, West Sussex, UK). All samples were run according to the PrimerDesign recommended thermal cycling protocol (Table 3.2).

#### 3.4.1.1 Primer check and melt curve analysis

An initial primer check at a primer concentration of 300 nM for both forward and reverse primers was carried out to ensure that the primers amplified target fragments correctly before using valuable reagents on a full titration. During this check the amplification plots were examined for smooth, exponential amplification and melt curves were analysed for a single product.

Melt curves are an important tool for assay optimisation because they can indicate if there are multiple products being amplified. The melting temperature ($T_m$) of DNA is defined as the temperature at which half of the helical structure is lost; this temperature is defined by the nucleotide composition and size of the DNA fragment (Nolan *et al.*, 2006). Melt curves for this project were generated by gradually heating the PCR products from 72°C to 95°C and continuously recording fluorescence. By

TABLE 3.1: Details of qPCR primer pairs for predetermined genes of interest, including melting temperature ($T_m$), G/C content, NetPrimer rating and amplicon size.

| Gene | Primer Name | Sequence (5'-3') | $T_m$ (°C) | G/C content (%) | NetPrimer Rating | Amplicon size |
|---|---|---|---|---|---|---|
| *C. pagurus* prophenoloxidase | PPO qPCR Fwd | AGCAGCAGCCAGTCCCAAATGA | 62.1 | 55 | 100 | 99 |
| | PPO qPCR Rev | ACGTGGCAGCAGCATGTGTTGA | 62.1 | 55 | 88 | |
| *C. pagurus* actin | Cpag2 Act Fwd | CACGGCCAGCCAAGTCA | 57.6 | 65 | 83 | 56 |
| | Cpag2 Act Rev | CACACACTGTGTCCCCATCTACGA | 62.1 | 55 | 100 | |
| *N. norvegicus* prophenoloxidase | Neph PPOF qPCR | TGCTCCATGAAGGGCATCTC | 59.4 | 55 | 89 | 55 |
| | Neph PPOR qPCR | TCTTCCTGGCACCCAAACA | 56.7 | 53 | 100 | |
| *N. norvegicus* actin | Neph ActF qPCR | CCCCAAGGCTAACCGTGAA | 58.8 | 58 | 100 | 61 |
| | Neph ActR qPCR | GGCGGGAGTGTTGAAGGTTT | 59.4 | 55 | 100 | |
| *Hematodinium* sp. ITS2 | HemITS2 qPCRfwd | GGTCTCAGCGTCTGTTCAACCT | 62.1 | 55 | 90 | 85 |
| | HemITS2 qPCRev | GACTCACTGGTGCACAAAATCG | 60.3 | 50 | 82 | |
| *Hematodinium* sp. 18S | Hem18S qPCRfwd | GGTAATCTTCTGAAAACGCATCGT | 59.3 | 42 | 88 | 121 |
| | Hem18S qPCRrev | GTACAAAGGGCAGGGACGTAATC | 62.4 | 52 | 88 | |
| *Hematodinium* sp. actin | HemAct Fwd | CGCGATTTAACTGAATACATGATGA | 58.1 | 36 | 81 | 69 |
| | Hem ActRev | CTCCGCTGTCGTGCTGAAG | 61 | 63 | 100 | |

TABLE 3.2: Thermal cycling program for quantitative SYBR® Green I or TaqMan® assay. Acquiring data during last 20 sec of annealing/extension step.

| Temp (°C) | Hold | Cycles | Process |
|---|---|---|---|
| 95 °C | 10 min | 1 | Initial denaturation |
| 95 °C | 10 sec | 40 | Denature |
| 60 °C | 40 sec | | Anneal and extend |
| 60 °C | 20 sec | | Acquire |
| 72-95 °C | 3 min | 1 | Melt curve |

using the intercalating dye SYBR Green in which the dissociation of DNA double bonds corresponds to a decrease in fluorescence (Nolan, 2004), the $T_m$ of each PCR product was established. Examining the plot of the rate of change of fluorescence versus temperature revealed peaks where individual products dissociated (Nolan, 2004). In successful assays, there was a single distinct peak corresponding to a single product and occasionally a small hump at low temperature as a result of primer dimers (Fig 3.5A). Primer dimers were identified by comparing the sample melt curve to that of the non-template control (NTC) and the NTC melt curve was always examined for contamination. Multiple peaks or large wavy humps indicated multiple products (Fig 3.5C), likely due to non-specific binding, and new primers were designed and tested.

#### 3.4.1.2 Primer Titration

qPCR assay efficiency is dependent on the optimal hybridisation of primers which can be affected by hybridisation temperature or primer concentration (Nolan, 2004). Most qPCR instruments, including the RotorGene 3000, are not capable of running temperature gradients, therefore primers for this project were optimised by varying primer concentration (Nolan, 2004). All reactions were set up to contain exactly the same amount of target sample and the primer concentrations were varied from 50-900 nM according to a primer titration matrix (Table 3.3).

TABLE 3.3: Primer titration matrix for optimizing primer concentration in SYBR® Green I assays.

| | | Reverse Primer (nM) | | |
|---|---|---|---|---|
| | | 50 | 300 | 900 |
| Forward Primer (nM) | 50 | 50/50 | 50/300 | 50/900 |
| | 300 | 300/50 | 300/300 | 300/900 |
| | 900 | 900/50 | 900/300 | 900/900 |

The optimum primer concentration was chosen based on the primer combination with the lowest $C_q$ value. Melt curves were also examined to ensure specific target amplification. If multiple primer combinations shared the same $C_q$ value, the combination with the lowest concentration was chosen (Nolan, 2004).

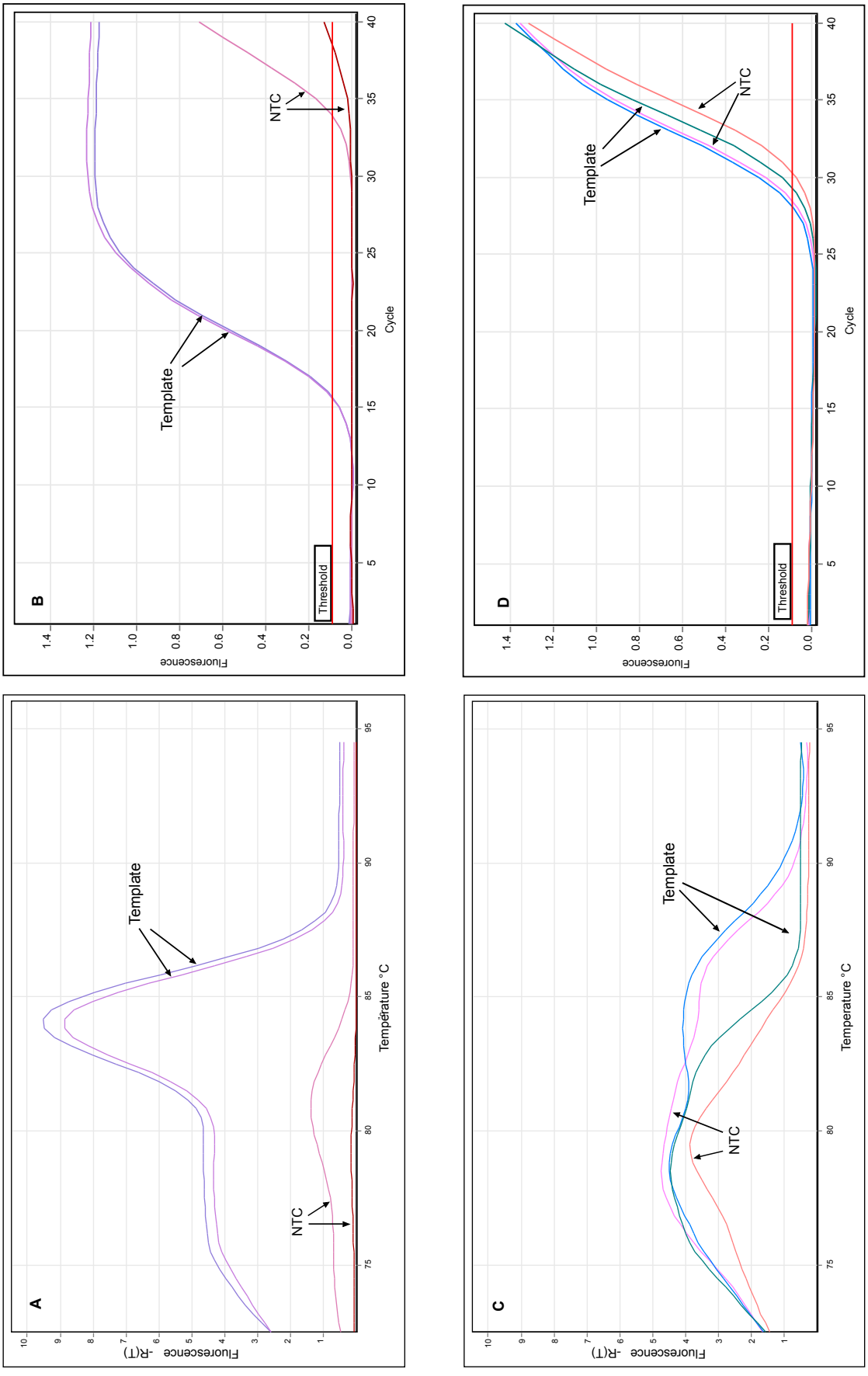



FIGURE 3.5: Melt curve (A) and amplification plot (B) for *N. norvegicus ppo* showing a single target product (purple lines) and a small amount of contamination in one of the NTCs (pink lines). The melt curve (C) and amplification plot (D) for *N. norvegicus cct7* shows multiple products in both the target samples (blue lines) and NTCs (pink lines), indicating a lack of specificity.

#### 3.4.1.3 Standard curve for assay efficiency

The final aspect of assay optimization was to determine assay efficiency and sensitivity using a standard curve. Standard curves were carried out using serial (10-fold) dilutions of cDNA template (Fig 3.6) for all GOIs and reference genes, as well as plasmid templates used in absolute quantification (see 3.5.3). Ideally, the linear regression of a plot of $C_q$ vs. log dilution should have a slope of -3.32 and an $R^2$ of 1. Standard curves were designed to cover up to 7 orders of magnitude and include the expected $C_q$ values of the experiment (Brisson *et al.*, 2004; Nolan, 2004; Nolan *et al.*, 2006). In these studies, the dynamic range for cDNA samples was linear for over 4-5 orders of magnitude and for over 6-7 orders of magnitude for the plasmid templates (Fig 3.6).

In order to compare GOIs and endogenous reference genes, the amplification efficiencies of each gene needed to be identical (Roche, 2001; Arya *et al.*, 2005). The same applied to GOIs compared to plasmid standard curves during absolute quantification. Amplification efficiency (E) was calculated based on the slope of the linear regression line of the standard curve (Brisson *et al.*, 2004), where:

$$E = 10^{-1/slope} - 1 \tag{3.1}$$

Standard curve linear regression plots provided a great deal of information on the performance of the reaction. The ideal slope of -3.32 would have indicated exact doubling of the reaction and therefore 100% reaction efficiency. The correlation coefficient ($R^2$) measures how well the data fit the standard curve, with $R^2 = 1$ indicating a perfect fit. Recommendations for acceptable slope and $R^2$ values vary throughout the literature. It is generally recommended that efficiency falls between 90-110% which corresponds to a slope between -3.58 and -3.10 (Arya *et al.*, 2005; Invitrogen, 2008). $R^2$ values are recommended to be greater than 0.98 (Nolan, 2004). $R^2$ values and amplification efficiencies were evaluated for all primer pairs designed for the present studies (Table 3.4).

## 3.5 Quantification method

### 3.5.1 Relative quantification

The *in vitro* study in this project used relative quantification to compare changes in gene expression of challenged and naive haemocyte cultures exposed to *Hematodinium*

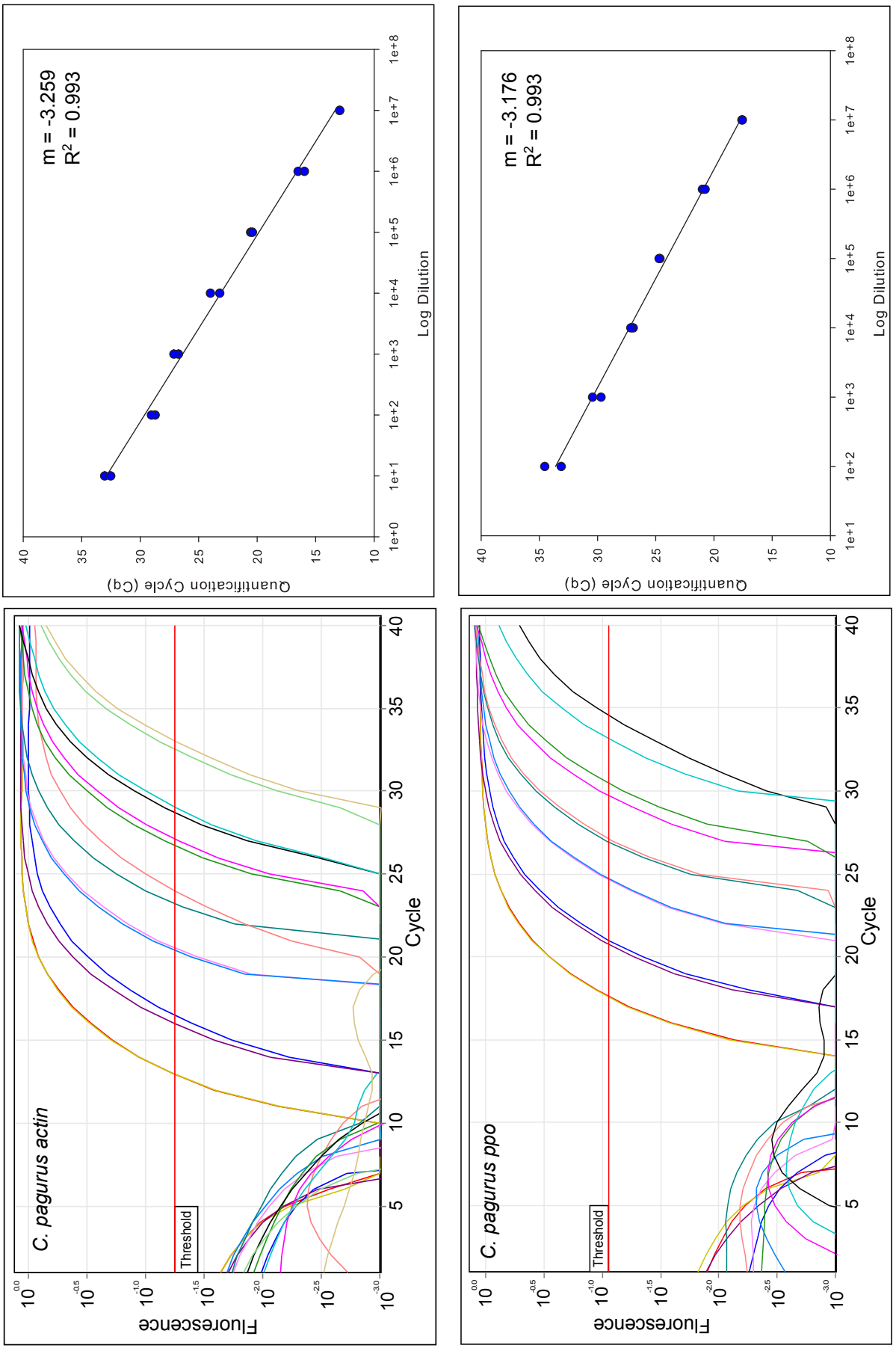



FIGURE 3.6: cDNA standard curves for *C. pagurus actin* (endogenous reference gene) and *prophenoloxidase* used in the *in vitro* study.

Table 3.4: Optimisation results of qPCR primer pairs for predetermined genes of interest and reference genes.

| Gene | Primer Name | Optimum primer concentration [nM] | $R^2$ | Amplification efficiency |
|---|---|---|---|---|
| *C. pagurus* prophenoloxidase | PPO qPCR Fwd<br>PPO qPCR Rev | 300<br>300 | 0.993 | 1.06 |
| *C. pagurus* actin | Cpag2 Act Fwd<br>Cpag2 Act Rev | 50<br>300 | 0.993 | 1.03 |
| *N. norvegicus* prophenoloxidase | Neph PPOF qPCR<br>Neph PPOR qPCR | 300<br>300 | 0.993 | 0.99 |
| *N. norvegicus* actin | Neph ActF qPCR<br>Neph ActR qPCR | 300<br>300 | 0.995 | 0.97 |
| *Hematodinium* sp. ITS2 | HemITS2 qPCRfwd<br>HemITS2 qPCRev | 900<br>300 | 0.991 | 1.58[1] |
| *Hematodinium* sp. 18S | Hem18S qPCRfwd<br>Hem18S qPCRrev | 900<br>300 | 0.995 | 1.11 |
| *Hematodinium* sp. actin | HemAct Fwd<br>Hem ActRev | 300<br>300 | 0.963 | 0.97 |

[1]This primer set was not used for this project due to its poor efficiency values

sp. cells (see Chapter 4.4.6). Relative quantification compares changes in expression levels of mRNA of a gene of interest (GOI) to that of an internal control RNA, an endogenous reference (ER) gene. The fold-change can then be compared between experimental and control samples. The GOI and ER can be co-amplified in the same tube (multiplexing) or can be amplified in separate tubes during the same run. The main advantages of using this method were that no external standard curve was required and normalisation by an internal reference reduced tissue-derived effects on the outcome.

In the *in vitro* study, *actin* was used as the endogenous reference gene. Previous *in vitro* studies using crustacean granulocytes have found *actin* to be normally expressed when exposed to immunostimulants (Hauton *et al.*, 2005) and this was supported by validation in this experiment for both granulocyte and hyaline cell cultures. Other crustacean studies have used *actin* as a reference gene to study expression changes of *crustin* in *Carcinus maenas* following bacterial challenge (Brockton and Smith, 2008) and to compare expression of nine immune-relevant genes from various tissues of white shrimp *Litopenaeus vannamei* (Wang *et al.*, 2007). The use of *actin* as an endogenous reference gene is not limited to crustaceans, it has been proven a useful reference gene in a variety of species over a wide range of experimental samples. The honeybee *Apis mellifera* had stable *actin* expression during challenge experiments with the bacteria *Escherichia coli* (Scharlaken *et al.*, 2008). In humans, *actin* and *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase) were found to be normally expressed in

human umbilical vein endothelial cells subjected to different stimuli (Garcia-Vallejo *et al.*, 2004) and FFPE (formalin-fixed paraffin embedded) prostate cancer samples (Mori *et al.*, 2008).

Recently, several of the genes traditionally used as reference genes ('housekeeping' genes), have been questioned and their expression found to alter under experimental conditions or between tissue types (Glare *et al.*, 2002; Garcia-Vallejo *et al.*, 2004). *Actin* was found to be affected by experimental conditions in asthma patients and both *GAPDH* and *actin* were found to vary in patients with pulmonary tuberculosis (Glare *et al.*, 2002; Dheda *et al.*, 2004), indicating that these genes were not suitable for the experimental conditions. These results have not rendered housekeeping genes useless as endogenous reference genes, but have underlined the need for validation of endogenous reference genes (Dheda *et al.*, 2004; Huggett *et al.*, 2005). In light of this fact, and the generally accepted requirements for qPCR, *actin* expression was validated using the REST© Software as described below (3.5.2.1).

### 3.5.2 Statistical analysis of relative expression qPCR data

Relative expression data was analysed using the Relative Expression Software Tool (REST©) 2009 (Pfaffl *et al.*, 2002). REST© uses randomisation tests and bootstrapping methods to test the statistical significance of gene expression ratios. Randomisation is used here because it avoids making assumptions about distribution. The quantities used in gene expression analysis are ratios, often with high variability, and would not be normally distributed (Pfaffl *et al.*, 2002).

Average $C_q$ values for each exposed and naive *in vitro* sample were input into the software for each GOI and the reference gene. Efficiency values (Eq (3.1)) calculated from the standard curves of each gene were also input into the software. 2000 randomisations were used to obtain a suitable p-value. Relative expression using the REST© software is calculated based on the following equation (3.2):

$$\text{Relative expression ratio} = \frac{(\text{E}_{\text{target}})^{\Delta C_{\text{q Target}}(\text{mean control-mean sample})}}{(\text{E}_{\text{ref}})^{\Delta C_{\text{q Ref}}(\text{mean control-mean sample})}} \quad (3.2)$$

#### 3.5.2.1 Reference gene validation

Normalisation to an endogenous reference gene requires that the reference gene be stably expressed for all tissue types being compared and over all experimental

conditions (Thellin *et al.*, 1999). Failure of the reference gene to remain constant can result in small variations of target gene being missed or, in the case of reference genes being affected by experimental conditions, can be the cause of apparent (and false) changes in gene expression (Glare *et al.*, 2002; Dheda *et al.*, 2004). REST© 2009 has built-in reference gene validation and the expression of *actin* in this case was examined for significant variation using the software's preprogrammed algorithms. In short, the mean $C_q$ values of the reference gene in control and experimental samples are compared and, if the means are the same, then the reference gene is considered stable and constant (Eq (3.3)) (Pfaffl *et al.*, 2002).

$$\Delta C_q ref_{(\text{mean control-mean sample})} = 0 \tag{3.3}$$

### 3.5.3 Absolute quantification

Absolute quantification was used on the *in vivo* studies of *Nephrops norvegicus* infected and uninfected with *Hematodinium* sp. Relative quantification was not suitable for this study because RNA was extracted simultaneously from haemocytes and parasites and therefore equal quantities of RNA would contain varying amounts of haemocyte and parasite RNA, causing the endogenous reference genes to vary in expression. Absolute quantification allowed gene copy numbers to be expressed per haemocyte number.

Absolute quantification is used to quantify copy numbers of the target gene which must then be related to a biological parameter (e.g., mg tissue, $\mu$g total RNA, cell number, etc.) (Bustin and Nolan, 2004*c*). In this sense, it is a form of relative quantification, though it yields a copy number value rather than a fold-change. Copy numbers are interpolated against a standard curve of known copy numbers of the target gene. In this study, standard curves were prepared using linearised plasmid DNA, though genomic DNA or amplicon-specific sense-strand oligonucleotides would also have been suitable (Bustin and Nolan, 2004*c*). The important aspect of using this method is that the standard curve template should have the same amplification efficiency as the target gene.

To prepare the plasmid DNA for this study specific gene fragments were identified using degenerate PCR and cloned using the TOPO® TA cloning kit (Invitrogen, Paisley, UK) and the pCR4-TOPO plasmid vector as described in Chapter 2. Positive clones were identified using colony PCR and the plasmids extracted were confirmed by sequencing (Source BioScience, Oxford, UK). Plasmid vectors were linearised using either *Not*I (Sigma-Aldrich, Dorset, UK) or *Nco*I (New England Biolabs, Hertfordshire, UK) restriction enzyme. For *Not*I, 1 $\mu$g plasmid was combined with 10

units *Not*I in the provided buffer solution to a final volume of 25 $\mu$l. The plasmid was incubated for 4 hours at 37°C to fully cleave all plasmids, then heat inactivated for 15 minutes at 65°C. To digest plasmids using *Nco*I, 1 $\mu$g of plasmid was combined with 1 $\mu$l *Nco*I (NEB R0193S), 2.5 $\mu$l 10X Buffer 3 (NEB B7003S) and sterile water to a final volume of 25 $\mu$l. Plasmids were incubated for 4 hours at 37°C and the restriction enzyme heat inactivated for 15 min at 65°C.

Linearised plasmids were quantified with a Quant-iT™ PicoGreen® dsDNA kit (P11496 Invitrogen, Paisley, UK) using the high-range standard curve method on a microplate. TE assay buffer was prepared in sufficient quantities for all samples by diluting the 20X concentrated buffer with sterile, DNase-free water. Quant-iT™ PicoGreen® reagent was made fresh by diluting the reagent 200-fold in TE buffer. A standard curve was prepared by preparing a 2$\mu$g ml$^{-1}$ stock solution of the provided $\lambda$ DNA standard in TE buffer and diluting it to a 5-point standard curve covering 1 ng ml$^{-1}$ to 1 $\mu$g ml$^{-1}$ (Table 3.5). Samples were diluted by placing 1 $\mu$l of sample in 99 $\mu$l TE buffer. PicoGreen® solution (100 $\mu$l) was added to each sample and each standard and mixed by vortexing. All samples and standards were loaded into a 96-well plate and read on a FLUOstar OPTIMA fluorescence plate reader (BMG Labtech, Aylesbury, UK) with excitation at 485 nm and emission at 520 nm. All samples and standards were run in triplicate.

Table 3.5: Reaction component volumes to generate a standard curve using Quant-iT™ PicoGreen® in a 96-well microplate.

| Volume ($\mu$l) TE Buffer | Volume ($\mu$l) $\lambda$ DNA stock (2 $\mu$g ml$^{-1}$) | Volume ($\mu$l) PicoGreen® | Final DNA concentration |
|---|---|---|---|
| 0 | 100 | 100 | 1 $\mu$g ml$^{-1}$ |
| 90 | 10 | 100 | 100 ng ml$^{-1}$ |
| 99 | 1 | 100 | 10 ng ml$^{-1}$ |
| 99.9 | 0.1 | 100 | 1 ng ml$^{-1}$ |
| 100 | 0 | 100 | blank |

To establish a standard curve using plasmid template a 10-fold dilution series over 6 orders of magnitude was created and run against a 10-fold dilution series of target cDNA for each gene of interest. The slopes and efficiency values were compared to determine whether absolute quantification using linearised plasmids would be appropriate for each gene of interest (Fig. 3.7).

Copy numbers of each target plasmid were calculated according to the following equation (Roche, 2000):

$$\frac{6 \times 10^{23}[copies/mol] \times \text{plasmid concentration}[g/\mu l]}{\text{plasmid molecular weight}} = copies/\mu l \qquad (3.4)$$

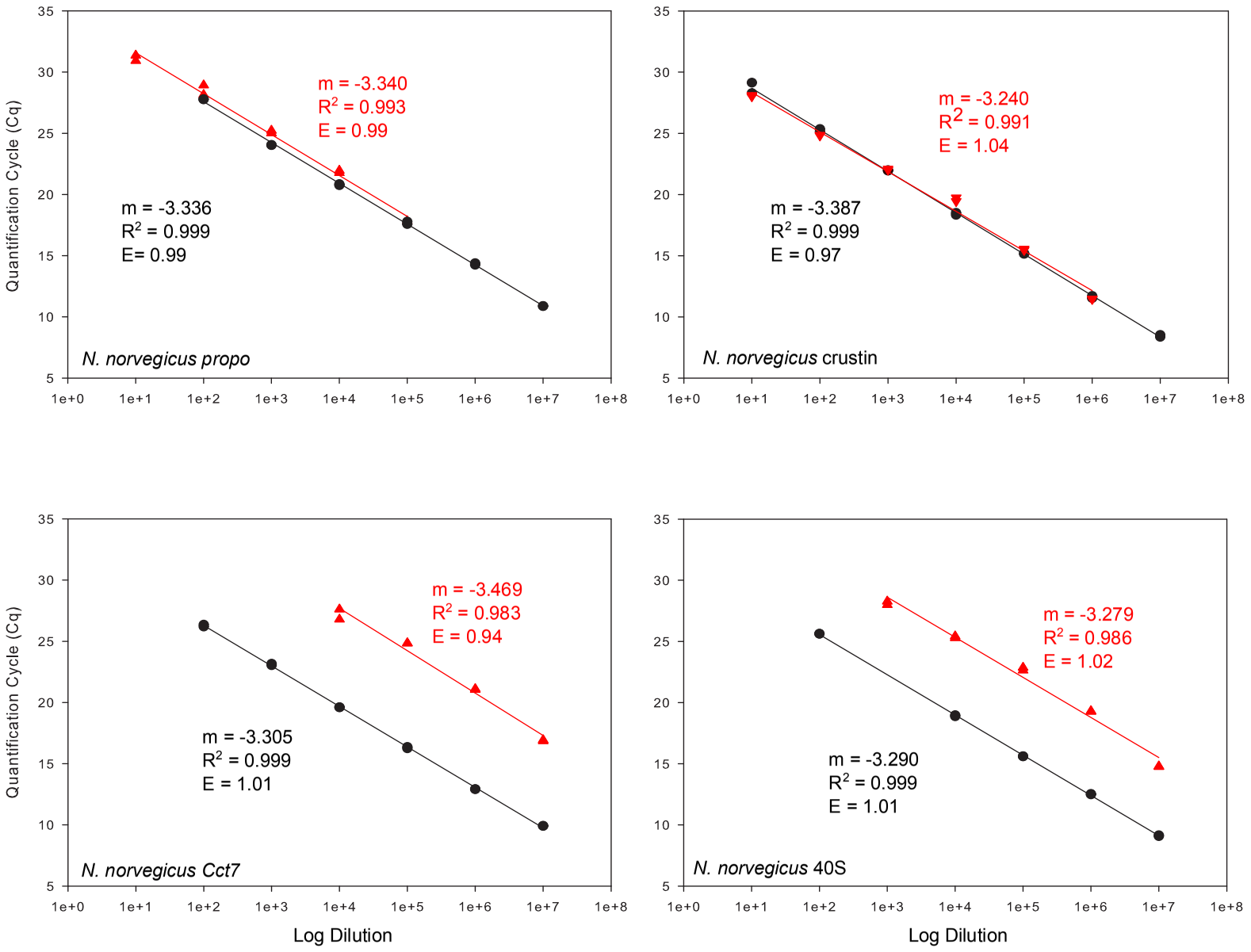



FIGURE 3.7: Comparison of the slopes of plasmid DNA (black) and target cDNA (red) standard curves. Slopes (m) and efficiencies (E) should be as similar as possible to ensure accurate quantification of gene copy number of the gene of interest.

Where:

Plasmid molecular weight = (3956 (plasmid bp) + x (fragment bp)) x 660 Da $bp^{-1}$

660 Da = average molecular weight of a nucleotide pair and 1 Da = 1 atomic mass unit (amu)

$6 \times 10^{23}[copies/mol]$ = Avagadro's number

Plasmid concentration = concentration defined by PicoGreen® analysis (g $\mu l^{-1}$)

Due to the combined presence of host and parasite cells in haemolymph samples, it was not possible to normalise gene copy numbers against total RNA because varying amounts of parasite RNA would confound the expression values. Therefore, gene copy numbers were normalised against total haemocyte number of each sample for statistical analysis (Bustin, 2000). Using this method, conventional statistical methods were able to be applied to the gene expression data. In this case, data were analysed using pairwise comparison tests ($t$-test or Mann-Whitney).

## 3.6 Summary

Extensive assay optimisation was carried out in preparation for gene expression studies using both absolute and relative quantification methods. Due care was given to all aspects of sample storage, handling and extraction to reduce variability between samples and RNA quality was confirmed using the Experion™ system. Furthermore, careful consideration was given to all reagents used, including RT enzymes, priming strategy and detection chemistry. Primer pairs were optimised for concentration and analysed for specificity and efficiency. Additionally, constitutive expression of the endogenous reference gene, *actin*, was first validated for the *in vitro* experiments, ensuring that it was suitable for normalisation in the studies with *Cancer pagurus*. Plasmid standard curves were also validated for use in absolute quantification by comparison to standard curves of cDNA for *in vivo* immune gene expression studies. Overall, robust assays were developed for both the *in vitro* and *in vivo* experiments which will be described in the following chapters.

# Chapter 4

# Expression of immune-related host genes in *Cancer pagurus* haemocytes during *in vitro* challenge with *Hematodinium* sp.

## 4.1 Introduction

In recent decades marine invertebrate cell culture has become more prominent in scientific research due to a need to study cellular interactions. Expansion of aquaculture has driven a need to study pathogenic diseases (Mourton *et al.*, 1992; Jiravanichpaisal *et al.*, 2006*b*; Shields *et al.*, 2007; Sperstad *et al.*, 2010) and to increase our knowledge of cellular immune responses (Smith and Soderhall, 1983; Muñoz *et al.*, 2002; Rodriguez-Ramos *et al.*, 2010). The use of a number of marine invertebrates as biomarkers (Hagger *et al.*, 2009; Oliver and Fisher, 1999) has also instigated *in vitro* cytotoxicity (Matozzo *et al.*, 2002; Sung *et al.*, 2003; Hauton and Smith, 2004) and stress-response studies (Webster, 1996). *In vitro* studies to elucidate direct interactions between invertebrate haemocytes and parasite cells have also been used (Gregorio and Ratcliffe, 1991; La Peyre *et al.*, 1995; Morga *et al.*, 2009).

### 4.1.1 Host-parasite immune interactions

In many arthropods, endoparasites have frequently been found to be encapsulated by haemocyte cells (Christensen *et al.*, 2005; Jiravanichpaisal *et al.*, 2006*a*) in a direct immune response; however, there are also several parasite species that have adapted to

their host immune system and are capable of mediating the immune response (Loker, 1994). Pathological investigation of the freshwater amphipod *Echinogammarus stammeri* revealed very few instances (<4%) of melanisation during parasitisation by the acanthocephalan parasite larvae *Pomphorhynchus laevis* which reside in the haemocoel. The parasite larvae were surrounded by a membranous layer composed of microvilli which maintains intimate contact with the host haemocytes and internal organs, however immune response was limited to occasional melanisation and there were frequent observations of disintigrated haemocytes in the vicinity of the larva (Dezfuli *et al.*, 2008). The authors have suggested that this indicates some method of immune avoidance or suppression, however the exact mechanisms are not understood. These observations were supported by further studies of *P. laevis* infecting *Gammarus pulex* where phenoloxidase activity was reduced, which was likely related to a significant decrease in total haemocyte count though again, the method of suppression was unclear (Cornet *et al.*, 2009; Cornet, 2010). Similarly, the nematode parasite *Steinernema feltiae* was found to reduce phenoloxidase activity, avoid encapsulation and inhibit phagocytosis in its lepidopteran host *Galleria mellonella* (Rigaud and Moret, 2003; Brivio *et al.*, 2006, 2010). In this case, the immune interference was linked to parasite surface lipids which bind opsonic host interacting proteins (HIPs). Pre-exposure, and therefore saturation, of the parasite to HIPs re-enabled host immune response, as did enzymatic interference with the parasite surface lipids (Brivio *et al.*, 2006, 2010). Sequestration of host HIPs allowed the parasite to survive unmolested.

As previously discussed (see Chapter 1.4.2), total and differential haemocyte numbers have been suggested to influence the capability of a crustacean to respond to invading pathogens (Soderhall and Cerenius, 1992) and have been found to vary in response to foreign material, environmental factors and moult stage (Persson *et al.*, 1987; Chen and Cheng, 2001; Hernroth *et al.*, 2004; Matozzo *et al.*, 2011*a*). Despite many pathological studies, there remains sparse evidence of an immune response in infected crustacean hosts to the presence of *Hematodinium* sp. Indeed, to date, few direct immune studies have been carried out on animals infected with *Hematodinium* and none with immunity as the primary focus. Studies focussing specifically on haematological reactions of crustacean hosts to *Hematodinium* have revealed a decrease in haemocyte concentrations with increased parasite load (Field *et al.*, 1992; Field and Appleton, 1995; Shields and Squyars, 2000; Meyers *et al.*, 1987; Love *et al.*, 1993) which suggests reduced immune function in invertebrates that rely on haemocytes to fight infection. Differential cell counts have revealed decreases in hyalinocytes, potentially due to host defense responses such as encapsulation (Shields and Squyars, 2000), however high variations in differential cell counts between individuals have led some researchers to consider this parameter less useful than total

haemocyte counts in determining crustacean health (van de Braak *et al.*, 2002). Limited reports have been made of the formation and activation and enlargement of fixed phagocytes within the hepatopancreas (Field and Appleton, 1995; Field *et al.*, 1992; Sheppard *et al.*, 2003; Stentiford *et al.*, 2002; Wheeler *et al.*, 2007); however only necrotic fixed phagocytes were proven to contain parasites (Field and Appleton, 1995). Meyers *et al.* (1987) observed occasional cell foci in the antennal gland and around the bladder, but noted a general lack of immune response in most tissues.

Co-infection of *Cancer pagurus* with a yeast-like organism revealed an abundance of haemocytic encapsulations associated with the yeast-like organism, but an apparent lack of response to the surrounding *Hematodinium* cells (Stentiford *et al.*, 2003). Further observations of co-infection with *Hematodinium* have led to speculation of the parasite being immunosuppressive (see Stentiford and Shields (2005)). Speculation regarding immunosuppression by the parasite and the minimal immune responses witnessed thus far reveal a knowledge gap in our understanding of *Hematodinium* disease progression and infection method. Due to the possibility of colonisation by opportunistic pathogens, such as that seen by Stentiford *et al.* (2003), it would be useful to examine the direct interaction of parasite cells and haemocytes to establish any immune response in isolation and without confounding factors such as secondary infection.

### 4.1.2 *In vitro* immune studies

*In vitro* immune studies in invertebrates have generally focussed on haemocytes and haemopoietic tissue as they are the functional unit of internal defense. Initial basic studies have primarily examined functional characteristics, such as phagocytosis, and gross morphology of haemocytes in response to pathogens, parasites, harmful algae or other immune stimulants such as pathogen-associated compounds (Smith and Ratcliffe, 1978; Smith and Soderhall, 1983; Ford *et al.*, 2008; Rodriguez-Ramos *et al.*, 2010; Hegaret *et al.*, 2011). Haemocyte separation has made it possible to establish individual cell population responses to stimulation (Soderhall and Smith, 1983; Vargas-Albores *et al.*, 2005) and haemocyte characterisation has been greatly enhanced by *in vitro* studies (Smith and Ratcliffe, 1978; Smith and Soderhall, 1983; Bell and Smith, 1993; Mortensen and Glette, 1996). In crustaceans, hyaline cells have been identified as primarily phagocytic in nature based on bacterial challenge (Smith and Ratcliffe, 1978; Smith and Soderhall, 1983). Hyaline cells from total haemocyte cultures from *Carcinus maenas* and *Liocarcinus depurator* were found to have increased vacuolation and increased phagocytosis when challenged with bacteria (Smith and Ratcliffe, 1978; Smith and Soderhall, 1983). Crustacean granulocyte cells

have been found to contain prophenoloxidase and could be stimulated by lipopolysaccharides (LPS; commonly found in bacterial cell walls) and $\beta$1,3-glucans (found in fungal cell walls) (Smith and Soderhall, 1983; Soderhall *et al.*, 1986). *In vitro* studies have also established that cell cooperation exists between populations; for example: amoebocytes, the phagocytic cells from the solitary tunicate *Ciona intestinalis*, were found to have reduced phagocytic ability when separated from total haemocyte cultures. When cell lysate from morula cells was introduced to the culture, phagocytic ability was restored, indicating that they strongly mediate a cooperative haemocyte response (Smith and Peddie, 1992).

Viability and gross morphology in response to pathogenic or stimulant challenge have also been studied *in vitro*. Whole blood monolayers of *Astacus astacus* and *C. maenas* exposed to $\beta$1,3-glucan resulted in degranulation and vacuolation combined with increased phagocytosis (Soderhall and Smith, 1983). Similarly, both crayfish (*Procambarus zonangulus*) and shrimp (*Litopenaeus vannamei*) haemocytes exposed to LPS decreased in size and suffered from increased mortality (Cardenas *et al.*, 2004; Xian *et al.*, 2009). Bivalve haemocytes showed a similar response when exposed to toxic algal species *in vitro*, with the added effects that phagocytic ability and cell adhesion were also inhibited (Hegaret *et al.*, 2011). Haemopoietic tissue cells from *Pacifastacus leniusculus* also had reduced cell adhesion when exposed to white spot syndrome virus (WSSV) *in vitro* and were observed to become rounded and detach from the culture well (Jiravanichpaisal *et al.*, 2006*b*). Cell rounding and size decrease has been proposed to be related to degranulation (Cardenas *et al.*, 2004; Xian *et al.*, 2009), followed shortly by cell death, all of which would lead to inhibition of an immune response.

Further studies have investigated the enzymatic and free radical response of haemocytes to pathogens. Enzymes such as phenoloxidase, and free radicals such as superoxide and nitric oxide (reactive oxygen species) are known to vary during response to foreign material and have been analysed during *in vitro* challenge (Johansson and Soderhall, 1985; Gregorio and Ratcliffe, 1991; Gomes *et al.*, 1999; Jayasree, 2009; Rodriguez-Ramos *et al.*, 2010; Hegaret *et al.*, 2011). Phenoloxidase (PO), as a key enzyme in invertebrate immune function, has commonly been studied in immune responses. *In vitro* studies have found PO activity levels to be increased in response to *Trypanosoma ragneli* epimsastigotes in the vector *Rhodnius prolixus* (Gomes *et al.*, 1999), however when combined with laminarin, PO was decreased (Gregorio and Ratcliffe, 1991). Other molecules often studied include reactive oxygen species such as nitric oxide and superoxide anion. Toxic algae were found to reduce reactive oxygen species in bivalves (Hegaret *et al.*, 2011). *Penaeus indicus*, however, was found to have increased superoxide anion production in response to bacterial (*Vibrio*) challenge (Jayasree, 2009).

Technological advances have led to the ability to detect internal defense responses at the molecular level and recent *in vitro* immunological studies have explored expression changes in immune-related genes (Hauton *et al.*, 2005; Rodriguez-Ramos *et al.*, 2010; Sperstad *et al.*, 2010). The *prophenoloxidase* gene (*proPO*) was identified for *Homarus gammarus* and gene expression analysed in response to immunostimulants. In granulocyte cultures a significant increase in expression was found after 24 hours of exposure to heat-killed *L. anguillarum* (Hauton *et al.*, 2005). *Hyas araneus* granulocytes in culture showed a similar response in *crustin* expression after 24 hours of exposure to *L. anguillarum*, with a decrease after 48 hours (Sperstad *et al.*, 2010). Gene expression for nitric oxide synthase (*NOS*) was correlated with increased enzyme activity in total haemocyte cultures from *Panulirus argus* and showed an increase in expression after 40 min of exposure to LPS from *E. coli* (Rodriguez-Ramos *et al.*, 2010).

It is important, however, that the limitations of these studies be recognised; cell culture studies occur in a plasma-free environment which may lack crucial humoral factors such as lectins and agglutinins involved in immune response (Smith and Chisholm, 1992; Gupta, 2001). Furthermore, communication and interaction between cell populations is ignored although it is known to be necessary for certain defense functions including inducing phagocytosis by hyalinocytes via granulocyte proPO release (Soderhall *et al.*, 1986; Smith and Chisholm, 1992; Johansson *et al.*, 2000). The *in vitro* studies described here were largely carried out in order to reduce confounding physiological and environmental effects (Smith and Ratcliffe, 1978; Hauton *et al.*, 2005; Jiravanichpaisal *et al.*, 2006*b*; Sperstad *et al.*, 2010; Hegaret *et al.*, 2011). Additionally, haemocyte populations were able to be isolated to examine the specific role or response of individual cell types (Sperstad *et al.*, 2010; Hegaret *et al.*, 2011). Cell culture also reduced inter-individual variability and cells from the same animal were able to be used for treatment and control (Hauton and Smith, 2004; Rodriguez-Ramos *et al.*, 2010). Most importantly, however, is that many of the causes of limitation in *in vitro* experiments (plasma factors and cell communication) were established from *in vitro* studies, without which the function of different cell types during infection or other immune challenge would be unknown. *In vitro* studies are extremely useful for studying cell-pathogen interactions and should be considered an important supplement to *in vivo* studies.

### 4.1.3 Crustacean cell culture

Throughout the last century cell culture techniques have progressed from the observation of frog embryo nerve fibres (Harrison, 1907) to the development of

continuous vertebrate cell lines (Gey *et al.*, 1952) and tissue engineering (Freshney, 2005). Though the culture of vertebrate cells has risen to the forefront of culture techniques, the advance of invertebrate *in vitro* studies during the last 50 years has led to the establishment of over 450 continuous cell lines for over 100 insect species (Lynn, 1999). Among the arthropod species there are immortal cell lines for the fall armyworm *Spodoptera frugiperda* (Vaughn *et al.*, 1977), *Drosophila melanogaster* (Schneider, 1972) and the European corn borer *Ostrinia nubilalis* (Belloncik *et al.*, 2007), however to date, there are no continuous cell lines for any marine invertebrates.

Crustacean cell culture has been developed using vertebrate and insect cell culture methods and it was initially believed that long-term culture techniques could be developed for crustaceans, however this has not been the case. Thus far, long-term culture has been limited to several months and cultures this long are generally from tissues rather than haemocytes (Walton and Smith, 1999). For this reason, crustacean cell culture studies are currently limited to primary cell culture. Haemocyte culture is still of great interest, however, due to viral propagation and the diversity of haemocyte functions, particularly in relation to immune response.

As discussed in Chapters 1 and 2, there is some disagreement over crustacean haemocyte proliferation, though many agree that proliferation occurs in the haemopoietic tissue and gives rise to differentiated cells (van de Braak *et al.*, 2002; Soderhall *et al.*, 2003). Mitosis has been clearly observed in the cells of haemopoietic tissue (Hose *et al.*, 1992; van de Braak *et al.*, 2002) and several studies have noted the presence of precursor or partially developed cells within the tissue (Ghiretti-Magaldi *et al.*, 1977; Martin *et al.*, 1993; van de Braak *et al.*, 2002; Soderhall *et al.*, 2003). Soderhall *et al.* (2003) observed distinct evidence of cell proliferation in the haemopoietic tissue (hpt) of the freshwater crayfish *Pacifasticus leniusculus*. Crayfish injected with laminarin (a $\beta$1,3-glucan) experienced a reduction in total haemocyte counts followed by a gradual recovery over 24-48 hours. To determine where the new blood cells came from, crayfish were injected with BrdU, which identifies cells replicating DNA during the active growth phase (S-phase) of the cell cycle, followed by laminarin injection. BrdU labeling revealed high proliferation rates (20-30% staining) within the hpt tissue and very little labeling in circulating haemocytes (1-2.5%) indicating that cells proliferate primarily in the tissue. van de Braak *et al.* (2002) found similar results in *Penaeus monodon* stimulated with lipopolysaccharide. Post injection, the mitotic index of the haemopoietic tissue was dramatically increased followed shortly by an increase in total haemocyte numbers. A small number of proliferating cells were observed in circulation using light microscopy, however their contribution to overall haemocyte numbers was deemed insignificant in this study. The researchers proposed that the dividing cells in circulation were young cells prematurely

released from the haemopoietic tissue in response to stimulation. Further confirmation of haemocyte generation within a tissue rather than in circulation was generated by Lang *et al.* (2002) when, after microscopic observation of haemocytes and multiple tissue cultures, only lymphoid and ovarian tissue were found to contain dividing cells. Haemocyte cultures were observed over one month, during which time they formed monolayers but showed no signs of cell division. Unfortunately this method of cell proliferation has negative implications for the development of a continuous cell line for crustacean haemocytes, however primary cell culture has still been valuable for observing short-term cellular interactions.

Evidence strongly supports haemopoietic tissue as the source of new haemocytes however continuing research into development of a continuous crustacean haemocyte cell line has been fueled by several reports of small percentages of proliferating haemocytes in circulation (Ellender *et al.*, 1992; Sequeira *et al.*, 1996; Hammond and Smith, 2002; Soderhall *et al.*, 2003; Roulston and Smith, 2011). Ellender *et al.* (1992) found a small number (1-2%) of proliferating cells in *P. vannamei* using the BrdU assay. Similarly, the penaeid shrimp *P. japonicus* had approximately 1% of circulating haemocytes in active growth phase which significantly increased to 4.6% during infection with *Fusarium* spp. fungus (Sequeira *et al.*, 1996). And as previously mentioned, Soderhall *et al.* (2003) also found a small percentage of BrdU-positive circulating cells in *P. leniusculus*. These studies were supported by similar studies in brachyuran crabs which have recently been found to show evidence of circulating haemocyte proliferation as well. The swimming crab *Liocarcinus depurator* exhibited cell proliferation in a small proportion of circulating haemocytes when examined using a BrdU assay (Hammond and Smith, 2002). Roulston and Smith (2011) have also identified a small population of proliferating cells (approximately 9% of total haemocyte count), termed prohaemocytes, in the spider crab *Hyas araneus*. The prohaemocytes were able to enter S-phase *in vitro* though the completion of mitosis was not observed so it is uncertain whether the cells would be capable of proliferation under culture conditions. To date, the most promising results for a continuous cell line were from George and Dhar (2010) who reported cell proliferation in haemocyte cultures from *Penaeus vannamei* based on increased cell counts over time. Additionally, lipopolysaccharide injection was found to increase cell proliferation during culture, however further investigation is required as cell proliferation was only calculated based on increased cell counts using the Trypan blue exclusion assay and provided no direct observations of dividing cells nor use of cell proliferation assays.

Despite this body of evidence for cell proliferation in circulating haemocytes, haemocyte proliferation *in vitro* continues to be elusive with only one report of cell growth in culture (George and Dhar, 2010). As pointed out by Soderhall *et al.* (2003),

although there is a small percentage of cells proliferating in circulation, this appears to be negligible when compared to the proliferation in haemopoietic tissue. It has been speculated that the limitations to proliferation arise from culture methods as, indeed, successful haemocyte culture has been established in several arthropods species including the European corn borer *Ostrinia nubilalis* (Belloncik *et al.*, 2007), the salt marsh moth *Estigmene acrea* (Wittwer *et al.*, 1997) and the beet armyworm *Spodoptera exigua* (Yasunaga-Aoki *et al.*, 2004). Even these cultures, however, have been reportedly more difficult to initiate than tissue cultures due to activation of the phenoloxidase cascade (Lynn, 2001). Additionally, culture media plays an important role in cell culture and it is possible that essential components are missing from the common media in use. With the exception of Grace's media which was developed for insect ovarian tissue *Antheraea eucalypti* (Grace, 1962), popular media for crustacean culture such as M199 and L-15 were originally developed for the culture of vertebrate cells and require the addition of essential amino acids and foetal bovine serum for cell survival (Walton and Smith, 1999). Although culture media is adjusted and tailored to each species as much as possible, the possibility of a missing essential factor cannot be ignored.

Despite the short-term limitations of *in vitro* haemocyte studies, they have become an established technique for studying immune function, immune stimulants, physiological cell response and cellular reactions to pollutants (Smith and Ratcliffe, 1978; Smith and Soderhall, 1983; Brody and Chang, 1989; Matozzo *et al.*, 2002; Hauton and Smith, 2004; Hauton *et al.*, 2005). They are also extremely useful in studying the role of different haemocyte types in isolation and due to the difficulty of differentiating haemocyte types in mixed cell culture, it has been recommended that *in vitro* analyses be made on isolated populations (Smith and Soderhall, 1986; Johansson *et al.*, 2000). Smith and Soderhall (1991) were able to separate the haemocytes of a number of crustacean species using Percoll™ gradients to enable the study of prophenoloxidase (proPO) localisation; other studies have successfully used separated cell populations on *in vitro* studies of superoxide production (Bell and Smith, 1993) and immunostimulants (Hauton *et al.*, 2005). The argument for cell separation is supported by Muñoz *et al.* (2002) who found that changes in mRNA of penaeidins in microbially challenged shrimp were due to changing haemocyte populations. Penaeidin-positive cells (granular cells) in challenged shrimp were found to decrease from 36% to 13% of total haemocyte population 12 hours post-injection and then increase to 50% after 48 hours. These changes in haemocyte population were concurrent with fluctuations in mRNA levels in which a strong decrease was evident after 12 hours and a 3-fold increase evident after 48 hours. These results support the

use of separated cell populations for *in vitro* challenge as differences in cell viability between hyaline cells and granulocytes *in vitro* could cause similar errors.

### 4.1.4 Aims of exposing *C. pagurus* haemocytes to *Hematodinium*

As previously discussed, crustaceans have an immune system mediated primarily by haemocytes making them the ideal target for initial immune response studies to pathogens such as *Hematodinium*. The aim of this study was to perform an *in vitro* experiment examining the early phase response of individual haemocyte populations of *Cancer pagurus* to the *Hematodinium* sp. parasite. Initial cell culture studies were used to examine gross morphology and viability of haemocyte populations exposed to *Hematodinium* sp., with the additional aim of determining suitable time for cell culture in the chosen media. Relative quantitative PCR was then used to determine the potential response of hyalinocytes and granulocytes to the parasite. The gene coding for the prophenoloxidase gene (*proPO*) was chosen *a priori* (see Chapter 2) and further genes were examined based on results of the differential display technique, GeneFishing™. The null-hypothesis being tested was that *Hematodinium* sp. would have no effect on the gross morphology or viability of separated haemocyte populations *in vitro* nor would it affect gene expression of immune-related genes. Studies to date have indicated a lack of immune response in *Hematodinium*-infected crustaceans, however this study presents the first direct investigation into immune response to *Hematodinium* sp. exposure.

## 4.2 Materials and Methods

### 4.2.1 *Cancer pagurus* and *Hematodinium* sp. collection

The parasitic dinoflagellate *Hematodinium* sp. was isolated from infected *Nephrops norvegicus* collected by trawl using the University Marine Biological Station, Millport vessel RV *Aplysia* near the Isle of Cumbrae, Scotland (55° 50.7 N, 04° 54.3 W). Before bleeding, each animal was surface-sterilized with 100% ethanol. Heavily infected animals were identified by the 'cooked' colour of their carapace. Between 0.5 ml and 2.5 ml of haemolymph was withdrawn from the base of the fifth pereiopod of each infected animal and placed immediately into 30 ml Appleton-Vickerman media (Appleton and Vickerman, 1998) (27.99 g $l^{-1}$ NaCl; 0.95 g $l^{-1}$ KCl; 2.01 g $l^{-1}$ $CaCl_2$; 2.465 g $l^{-1}$ $MgSO_4$; 0.554 g $l^{-1}$ $Na_2SO_4$; 1.92 g $l^{-1}$ HEPES; 10% foetal calf serum; 25 $\mu$l $ml^{-1}$ gentamycin; 200 $\mu$g $ml^{-1}$ penicillin; pH 7.8) in sterile 25 $cm^2$ Corning®, non-vented culture flasks. In the laboratory, cultures were subcultured by at least half into fresh

media with additional antibiotics (1% penicillin/streptomycin). Cultures were maintained at 6°C and subcultured every 3-4 weeks (Appleton and Vickerman, 1998).

*Cancer pagurus* from the English Channel were obtained from Selsey Shellfish in Selsey, UK during the summer of 2009 and placed in the recirculating seawater aquarium at the National Oceanography Centre, Southampton. The aquaria were maintained at a salinity of 32.8 ± 1 and a temperature of 14.3°C ± 1.8°C. Healthy adult intermoult male and female crabs, identified by alertness and an undamaged carapace free of lesions, were sampled for these experiments.

### 4.2.2 Haemocyte culture preparation

*C. pagurus* haemolymph was withdrawn from the base of the third pereiopod into ice cold marine anticoagulant (0.4M sodium chloride; 0.1M glucose; 30mM trisodium citrate; 25mM citric acid; 9mM EDTA; pH 4.6 (Soderhall and Smith, 1983)) in a ratio of 1:1.5 (haemolymph:anticoagulant) using a sterile 21G needle and 5 ml syringe. Hyaline and granulocyte cells (semigranular and granular) were separated using discontinuous gradients (45% - 65%) of Percoll™ (Amersham Pharmacia, Sweden) in 3.2% sodium chloride. Cell separation was carried out at 2500 *g* for 10 min at 5°C.

Haemocytes were separated into hyalinocytes (3 x $10^6$ cells culture$^{-1}$) and granulocytes (1 x $10^6$ cells culture $^{-1}$) based on location on the Percoll™ gradient and morphological identification using Nomarski contrast interference microscopy using an Olympus BH-2 microscope. Hyalinocytes were concentrated in the 50% Percoll™ layer and granulocytes were concentrated in the 55% Percoll™ layer. Each haemocyte type was removed from the gradient using pyrogen-free glass pasteur pipettes and the cell fractions washed with modified L15 culture media (13.8 g $l^{-1}$ L-15 powder; 14.6 g $l^{-1}$ sodium chloride; 51.5 mg $l^{-1}$ taurine; 37.6 mg l $^{-1}$; 1% penicillin/streptomycin; 10% foetal calf serum (Walton and Smith, 1999)). Separated haemocytes were divided into replicate control and experimental samples in 92 mm sterile Nunclon™ tissue culture dishes (Thermo Scientific Nunc, Langenselbold, Germany), containing 15 mL of culture media and incubated at 7°C.

### 4.2.3 Cell viability experiments

For the cell viability experiments, haemocytes were extracted from six individuals and separated as described (4.2.2) into two replicate hyaline and granulocyte cultures for each individual. Eight 22 x 22 mm coverslips were added to the bottom of each culture dish before the addition of either granulocytes or hyaline cells to allow the haemocytes

to attach. Cells were left for 30 min to attach to the coverslips. To the experimental culture dishes, 1.5 x $10^5$ *Hematodinium* macrospores ex. *N. norvegicucs* were added. Macrospores were identified as the single-celled motile stage of the *Hematodinium* life cycle (based on descriptions by Appleton and Vickerman (1998)) using phase contrast microscopy on an inverted microscope. The *Hematodinium* cells were washed three times in L-15 culture media by centrifuging at 500 *g* and resuspending in L-15 media before addition to the haemocyte cultures to remove residual calcium from the parasite media which caused clumping of the haemocytes. The cultures were placed in an incubator at 7°C and the culture media was replaced every 48 h. Cell viability was recorded using a trypan blue exclusion assay: a coverslip was removed and inverting onto 15 $\mu$l 0.4% trypan blue solution on a baked glass slide. Cells were visualised under a microscope and differential counts of 200 cells were carried out every 24 h for 7 days. Cells stained blue were recorded as non-viable.

Haemocytes were examined daily for overt changes in gross morphology. Specifically, cells were examined for signs of aggregation (especially around parasite cells), attempts at encapsulation or extreme lysing. Images for comparison were captured using an Axiovert 200 inverted microscope (Zeiss, Göttingen, Germany) with phase contrast.

### 4.2.4 Gene expression study

Following the cell viability experiments, it was determined that both granulocytes and hyaline cells should be kept in culture for a maximum of 48 hours to ensure enough viable cells for RNA extraction. Separated haemocyte cultures were set up as before, however no coverslips were added to the culture dishes. Individual granulocyte and hyaline cell cultures were set up in duplicate and maintained at 7°C. After 24 h the experimental dishes were inoculated with 5 x $10^5$ *Hematodinium* cells and incubated for a further 24 h before RNA extraction. A culture dish containing only *Hematodinium* sp. cells in modified L-15 media was also established at the time of inoculation as a control for the GeneFishing™ experiment.

### 4.2.5 RNA extraction and reverse transcription

Total RNA was initially extracted using TriReagent® (Sigma-Aldrich, Dorset, UK), however the RNA quality was not sufficient for use in qPCR. As an alternative, total RNA was extracted using a NucleoSpin® Extract II kit (Macherey-Nagel, Düren, Germany). Culture media was removed from each sample dish and 600 $\mu$l of the lysis buffer L1 from the Nucleospin RNA kit was placed in the dish and a cell scraper used to disrupt and remove the haemocytes. The culture media was centrifuged at 2000 *g*

for 5 min at 7°C to collect *Hematodinium* sp. cells which remained in suspension from infected samples and 350 $\mu$l L1 was added to the resulting *Hematodinium* cell pellet. RNA extractions from the haemocyte cultures and the pelleted *Hematodinium* sp. were carried out according to the manufacturer's protocol and eluted in 40 $\mu$l RNase free water. All samples were checked for purity using the A260/280 ratios using a NanoDrop® ND-1000 spectrophotometer (Wilmington, USA).

RNA integrity and quantity was determined using an Experion™ RNA StdSens Analysis Kit (Bio-Rad Laboratories, Hertfordshire, UK). All samples had an RQI value greater than 7.9 and were suitable for use in quantitative PCR (Denisov *et al.*, 2008). All samples were DNase treated using Ambion TURBO™ DNase (Ambion/Applied Biosystems, Cheshire, UK) as described in Chapter 3.2.1.1 and stored at -80°C until needed. First strand cDNA was synthesised using SuperScript™ III Reverse Transcriptase (Invitrogen Ltd., Paisley, UK) as described previously.

### 4.2.6 Genes of Interest, Endogenous Reference Genes and GeneFishing™

The gene coding for prophenoloxidase (*proPO*) was chosen *a priori* as a gene of interest in this study and was sequenced for *C. pagurus* as described in Chapter 2. Additionally, the gene for *Cancer pagurus* actin (*act*) was sequenced as previously described and used as a reference gene.

To explore other differentially expressed genes (DEGs) the primer-based Genefishing™ DEG101 and DEG102 Premix kit (K1021, Seegene, Seoul, South Korea) was used on a single replicate of challenged and naive hyaline and granulocyte cell cultures. GeneFishing™ is a differential display technique that uses a two-stage PCR (Table 4.1) to randomly amplify gene fragments and gel electrophoresis to visualise the results. The principle of the technique is based on the annealing control primers (ACPs™) used in both the reverse transcriptase and PCR steps. The ACPs are designed to contain two priming regions connected by a polydeoxyinosine regulator sequence (Fig 4.1) (Hwang *et al.*, 2003). The 5'-end of the primer contains a universal sequence and the 3'-end contains a short (10-mer) highly selective hybridising sequence (targeting core) (Kim *et al.*, 2004).

**Reverse transcription:** Reverse transcription uses dT-ACP1 to synthesize first-strand cDNA. dT-ACP1 contains a poly-T region at the 3'-end to exploit the poly-A tail of mRNA. This results in a cDNA template with a universal sequence at the 5'-end (Fig 4.2A).

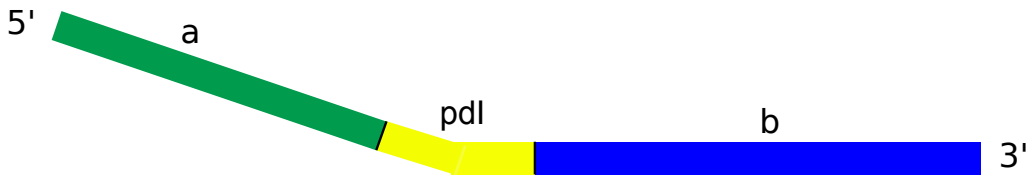



FIGURE 4.1: The tripartate structure of the annealing control primer (ACP™) contains a 5' universal sequence (a) and a 3' targeting core sequence (b) connected by a polydeoxyinosine regulator (pdI). The universal and targeting sequences are designed to anneal under different thermal conditions and the pdI regulator forms a bubble structure allowing the two sequences to operate independently. The core sequence is designed to anneal during the first PCR stage and the universal sequence to anneal during the second PCR stage.

**PCR Stage 1:** The resulting first-strand cDNA is combined with dT-ACP2 and an arbitrary ACP for the thermocycling reaction. The first PCR step is a single cycle during which the 3'-end targeting sequence of the arbitrary ACP binds to a suitable region of the cDNA template. A second-strand cDNA template is generated including the complementary sequence to dT-ACP1 at the 3'-end. PCR conditions during this step are favourable only to the 3'-end of the arbitrary ACP and not to dT-ACP2 (Fig 4.2B).

**PCR Stage 2:** During the second PCR stage conditions are favourable to annealing of the universal ends of both the arbitrary ACP and dT-ACP2 to the second-strand cDNA. This permits annealing of the arbitrary ACP to its complimentary sequence at the 3'-end and dT-ACP2 to its complimentary sequence on the 5'-end (Fig 4.2C).

TABLE 4.1: Reverse transcription (RT) and PCR thermocycling conditions for GeneFishing™.

| Step | No. of Cycles | Temperature (°C) | Duration | |
|---|---|---|---|---|
| RT | 1 | 80 | 3 min | |
| | 1 | 0 | 2 min | Add remaining reagents |
| | 1 | 42 | 90 min | dT-ACP1 3'-end polyT |
| | 1 | 94 | 2 min | Heat inactivation |
| PCR Stage 1 | 1 | 94 | 5 min | Denaturation |
| | 1 | 50 | 3 min | Arbitrary ACP annealing |
| | 1 | 72 | 1 min | Second-strand cDNA extension |
| PCR Stage 2 | 40 | 94 | 40 sec | Denaturation |
| | | 65 | 40 sec | Arbitrary ACP and dT-ACP2 universal sequence annealing |
| | | 72 | 40 sec | Extension |
| | 1 | 72 | 5 min | Final extension |

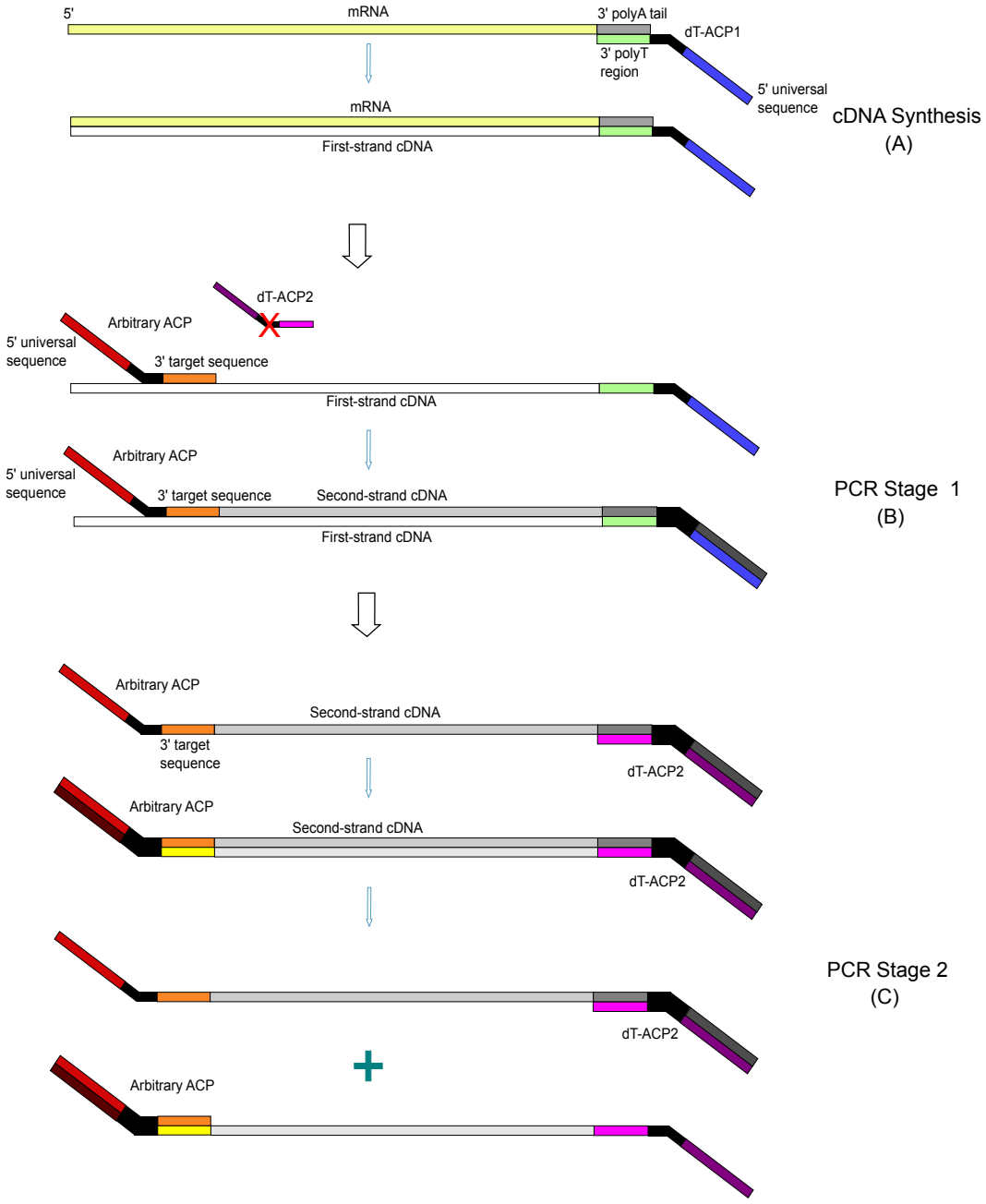



FIGURE 4.2: cDNA synthesis and GeneFIshing™ PCR flow chart. cDNA is synthesised by reverse transcription using dT-ACP1 which contains a polyT target priming region at the 3'-end of the primer (A). First-stage PCR generates second-strand cDNA using conditions optimal for the target sequence of the arbitrary ACP only (B). Second-stage PCR amplifies the desired product using annealing conditions optimal for the universal regions of the arbitrary ACP and dT-ACP (C). Figure based on GeneFishing™ Manual v.5.1 (Nov. 2007).

The use of ACPs™ ensures that non-specific priming does not occur during the PCR reaction. Although dT-ACP1 is still present in the reaction mixture, the PCR conditions are unfavourable to priming. Similarly, the targeting core region of the arbitrary ACP is only able to prime during second-strand cDNA synthesis as second-stage PCR conditions are unfavourable. The main advantage of using this type of primer is that they are designed to amplify only the targeted sequences and therefore reduce interference from primer dimers and eliminate false positives.

RNA was extracted from a single culture of challenged haemocytes for comparison to that from a single culture of naive (control) haemocytes. Reverse transcription was carried out on equal amounts of extracted RNA by combining 350 ng RNA in 7.5 $\mu$l volume with 2 $\mu$l dT-ACP1 and incubating at 80 °C for 3 min. Thereafter the following components were added to each RT reaction: 4 $\mu$l 5X RT buffer, 5 $\mu$l 2 mM dNTP (Invitrogen, Paisley, UK), 0.5 $\mu$l RNase Inhibitor (Sigma-Aldrich, Dorset, UK) and 1 $\mu$l SuperScript II RT, (Invitrogen, Paisley, UK). The tubes were incubated at 42°C for 90 min followed by 94 °C for 2 min. PCR amplifications were carried out according to the GeneFishing™ Kit protocol. Briefly, 8 ng cDNA were combined with 10 $\mu$mol arbitrary ACP, SeeAmp™ Master Mix and 10 $\mu$mol dT-ACP2 in a final volume of 20 $\mu$l. Samples were then run on an MJ Research PTC-200 thermal (Bio-Rad, Hemel Hempstead, UK) cycler using the previously described thermocycling parameters (Table 4.1). This process was repeated for both granulocyte and hyalinocyte cultures using each of the 20 arbitrary ACPs in separate reactions. The DNA fragments were separated using a 2% agarose gel and stained with ethidium bromide. Gels were examined for differentially expressed bands which were then extracted and cloned using a TOPO TA Cloning® kit and sequenced as described above. Sequences were aligned and submitted to BLAST analysis using Geneious v 4.7.6 (Biomatters Ltd., Auckland, NZ) to identify the closest homologs.

### 4.2.7 Rapid amplification of 5' and 3' cDNA ends (RACE)

The 5' ends of the gene fragments identified using GeneFishing™ were generated using the SMART™ RACE cDNA amplification kit from Clontech (Takara Bioscience, Saint-Germain-en-Laye, France) as previously described (Chapter 2.2.16). The dT-ACP2 primer uses a universal sequence attached to the 3' poly-A tail to generate fragments for GeneFishing™, therefore it is not necessary to perform RACE on the 3' end of the fragment. Primers for 5' RACE were designed according to the conditions described in Chapter 2.2.16.2 (Table 4.2) (Eurofins MWG Operon, Ebersberg, Germany).

The RACE fragments were cloned using the pGEM®-T Easy vector system (Promega, Southampton) according to the manufacturer's recommendations. Briefly, the ligation reaction was composed of 5 $\mu$l 2X Rapid Ligation Buffer, 1 $\mu$l pGEM®-T Easy vector, 1 $\mu$l T4 DNA ligase, PCR product in a 1:1 molar ratio with the vector and nuclease-free water to a final volume of 10 $\mu$l. The reaction was incubated at 4°C overnight. JM109 High Efficiency Competent Cells were transformed by combining 2 $\mu$l of the ligation reaction with 50 $\mu$l defrosted cells and incubating for 20 min on ice. The reaction was then heat-shocked for 45 sec at 42°C and immediately placed on ice for 2 min. SOC medium (950 $\mu$l) was added to each tube and the tubes placed in a shaking incubator for 1.5 hrs at 37°C, 150 rpm. Transformed cultures (100 $\mu$l) were plated on duplicate LB/ampicillin/IPTG/X-gal plates and incubated overnight at 37°C. White colonies were screened for inserts using colony PCR and positive colonies sent for sequencing (Source BioScience, Oxford, UK).

TABLE 4.2: Gene-specific (GSP) and nested gene-specific (NGSP) 5' RACE primers for genes identified by GeneFishing™ (see 4.3.2.1), including melting temperature ($T_m$), G/C content, length (bp) and NetPrimer rating.

| Primer Name | $T_m$ °C | Sequence | bp | G/C | NetPrimer Rating |
|---|---|---|---|---|---|
| Cpag DEAD GSP1 | 66.1 | 5'-TTG CCT CCA CCA CCA CGA GCC ATA-3' | 24 | 58.3 | 100 |
| Cpag DEAD NGSP1 | 68.1 | 5'-GTA GGC TGT TCC AGT CTT GTC TGA TCG G-3' | 28 | 53.6 | 89 |
| paf GSP1 | 68 | 5'-CGC TCA GAC ACA TCA GTG GGG AAC CT-3' | 26 | 57.7 | 100 |
| paf NGSP1 | 67.8 | 5'-AGA GTC CTT GCC CGC CTC ACC GT-3' | 23 | 65 | 100 |

### 4.2.8 Quantifying changes in gene expression

Samples for quantitative real-time PCR (qPCR) analysis were run as duplicates using a Corbett Rotor Gene 3000 (Corbett Life Science, New South Wales, Australia) according to the methods described in Chapter 3 using SYBR Green chemistry (Primer Design, Southampton, UK). Primers for the genes found during GeneFishing™ were designed and optimised using the same methodology as for *proPO* and *β-act* (Table 4.3) (Eurofins MWG Operon, Ebersberg, Germany).

TABLE 4.3: qPCR primer pairs for genes identified by GeneFishing™, including optimised primer concentrations, amplification efficiency and $R^2$ values

| Primer Name | Tm (°C) | Sequence | Optimised primer concentration (nM) | Amplification Efficiency | $R^2$ |
|---|---|---|---|---|---|
| PAF qPCR Fwd | 57.1 | 5'-TTG GAA GAG GCA AAC AAG TTG TT-3' | 300 | 1.05 | 0.99488 |
| PAF qPCR Rev | 61.0 | 5'-CTC TGA GGA CAA CTG TGC ATG TTA-3' | 300 | | |
| DEAD qPCR Fwd | 61.0 | 5'-CCA TCA TGC TCT GAG GAC TAT GTT-3' | 300 | 0.97 | 0.99784 |
| DEAD qPCR Rev | 62.7 | 5'-GTA GGC TGT TCC AGT CTT GTC TGA-3' | 50 | | |

### 4.2.9 Data Analysis

Cell viability data were expressed as percent (%) viable cells ± standard deviation of the mean. Data for comparison between challenged and naive cultures were arcsin transformed and statistically analysed using a General Linear Model ANOVA on SPSS Statistics 17.0 software (Chicago, USA). The *post hoc* Bonferroni test was used to analyse any significant changes from the effects of time or infection independent of each other.

After the completion of the PCR runs, raw fluorescent data were processed using the RotorGene Q-Series software (v6.0.33). $C_q$ values for each gene in all samples were then compared using the REST (Relative Expression Software Tool) 2009 software (Pfaffl *et al.*, 2002). Before analysis of the genes of interest, the endogenous reference gene ($\beta$-*act*) was examined for consistent expression as described in Chapter 3.

## 4.3 Results

### 4.3.1 Preliminary cell viability and gross morphology experiments

Cell viability assays revealed a declining trend in viability over 7 days, with no evidence of cell division when cells were examined microscopically. Naive hyaline cell cultures declined from 87% viability on day 1 to 65% viability on day 7, while granulocyte cultures declined from 78% to 38%. Granulocyte cultures declined significantly in viability by day 6 ($p \leq 0.05$). From this experiment it was evident that subsequent cell culture experiments needed to be short and it was decided to carry out gene expression studies with a 48 hour total culture time and incubate with *Hematodinium* parasites for the final 24 hours.

The comparison of cell viability in challenged and naive cultures of both haemocyte types showed no significant difference ($p \geq 0.5$) (Fig 4.3).

Observations of gross morphology between challenged and naive granulocyte cultures showed no obvious, distinguishing differences such as cell clumping or aggregation (Fig **??**). Hyaline cultures appeared to have a slight aggregation or cell clumping response on day 7 in challenged cultures, though there were no signs of phagocytosis of parasite cells. Cell size and adhesion did not appear to be affected for any of the cultures.

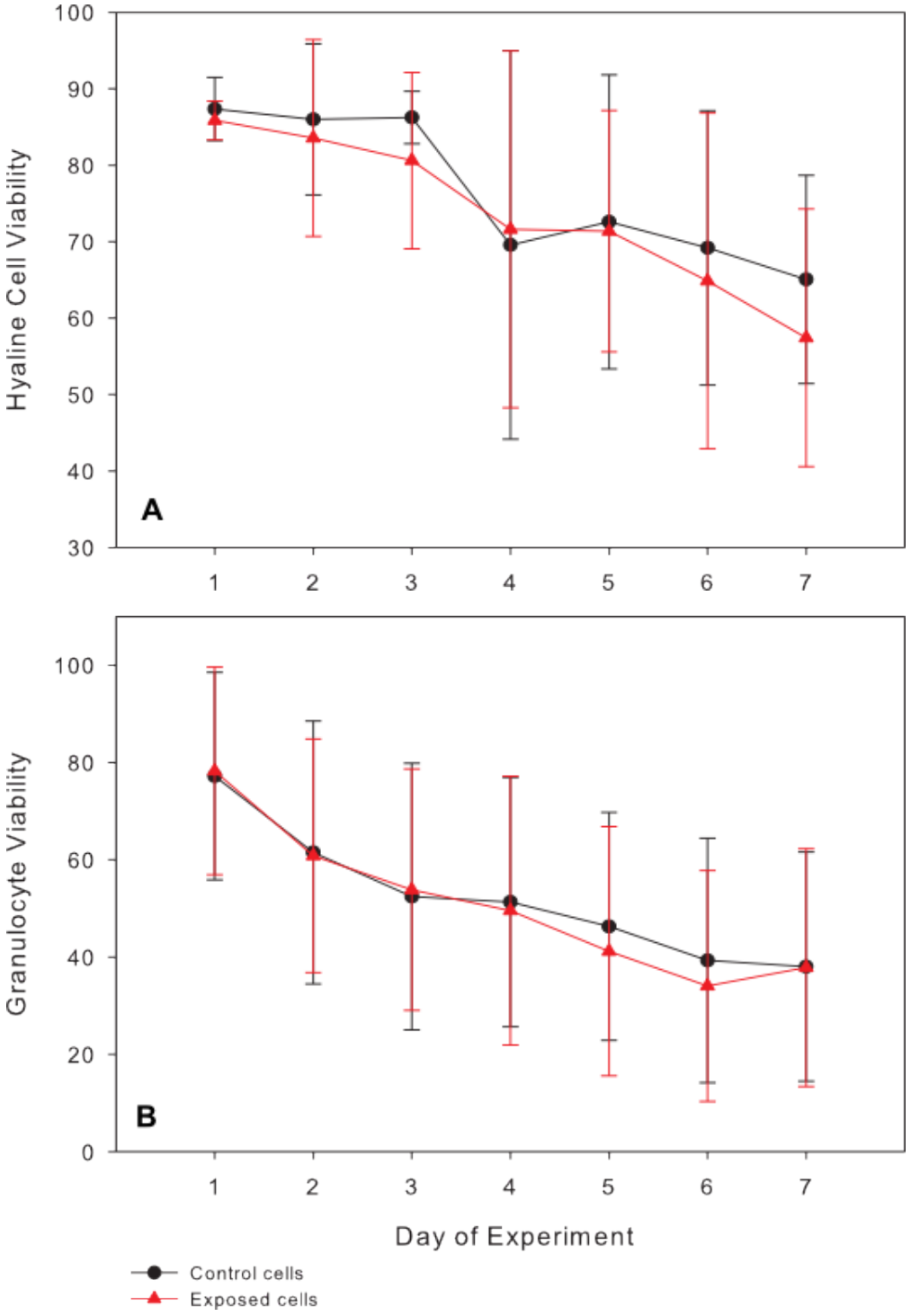



FIGURE 4.3: Mean cell viability of cultured haemocytes exposed to *Hematodinium* sp. and naive cultures; scored using the trypan blue exclusion assay (± SD; n = 6 animals). Haemocytes were separated into hyaline cell (A) and granulocyte (B) cultures.

### 4.3.2 Gene Identification

#### 4.3.2.1 GeneFishing™ Results

Based on fragments observed on agarose gels (representative image shown in Fig 4.5), GeneFishing™ analysis using 20 arbitrary primers revealed seven differentially expressed genes during *in vitro* infection with *Hematodinium* sp. Three genes were identified as being differentially expressed in granulocyte cultures and three genes were identified in hyalinocyte cultures. A further gene was identified from *Hematodinium*. All DEGs were cloned and the sequences compared with GenBank sequence data to determine putative gene identity (one gene from hyaline cells and the gene from *Hematodinium* had unsuccessful sequence results). Two of the cloned sequences from granulocytes were found to have high identity to known genes, but none of the hyalinocyte sequences shared any significant identity with known genes (Appendix C.1).

Sequence DEG16 from granulocyte cultures produced a fragment of 399 bp (Accession number: FR687023) and 5' RACE completed the gene generating a total fragment 1393 bp in length (Accession number: HE608875). The sequence contained a 5' untranslated region (UTR) of 130 bp, a 3' UTR of 132 bp and an open reading frame (ORF) of 1,131 bp which encoded a protein sequence of 377 amino acids. Comparison of the deduced amino acid sequence using NCBI BLASTP analysis showed that DEG16 had high identity to prophenoloxidase activating factor (PPAF) from *Portunus trituberculatus* (74.5% ACN87221; e-value = $3.06e^{-140}$), *Callinectes sapidus* (75.5%, AAS60227; e-value = $4.87e^{-140}$), *Eriocheir sinensis* (70.3%, ACU65942; e-value = $2.57e^{-130}$) and *Penaeus monodon* (52.4%, ABE03741; e-value = $2.03e^{95}$), as well as serine proteinase-like proteins from *Scylla paramamosain* (75.5%, ADG83846; e-value = $5.22e^{-144}$), *P. monodon* (52.1%, ABD62888; e-value = $9.45e^{96}$) and *Pacifastacus leniusculus* (52.7%, ACB41379; e-value = $8.44e^{89}$). SMART (Simple Modular Architectural Research Tool; http://smart.embl-heidelberg.de) analysis identified a putative tryspsin-like serine proteinase (Schnipsel database) which was supported by the Structural Classification of Proteins (SCOP; http://scop.mrc-lmb.cam.ac.uk/scop), indicating the same domain (e-value < $5.95e^{-18}$). SMART warned that this domain was probably not catalytically active due to the absence of some of the required catalytic sites, and therefore a serine proteinase homologue (SPH). A further query against the PDBsum database (http://www.ebi.ac.uk/pdbsum) revealed strongest identity with the prophenoloxidase activating factor from the beetle *Holotrichia diomphalia* (PDB id: 2b9l) (Letunic *et al.*, 2009). Sequence alignment with other crustacean PPAFs revealed common structural features of PPAFs and serine proteinase homologs, including an N-terminal clip-domain containing six highly

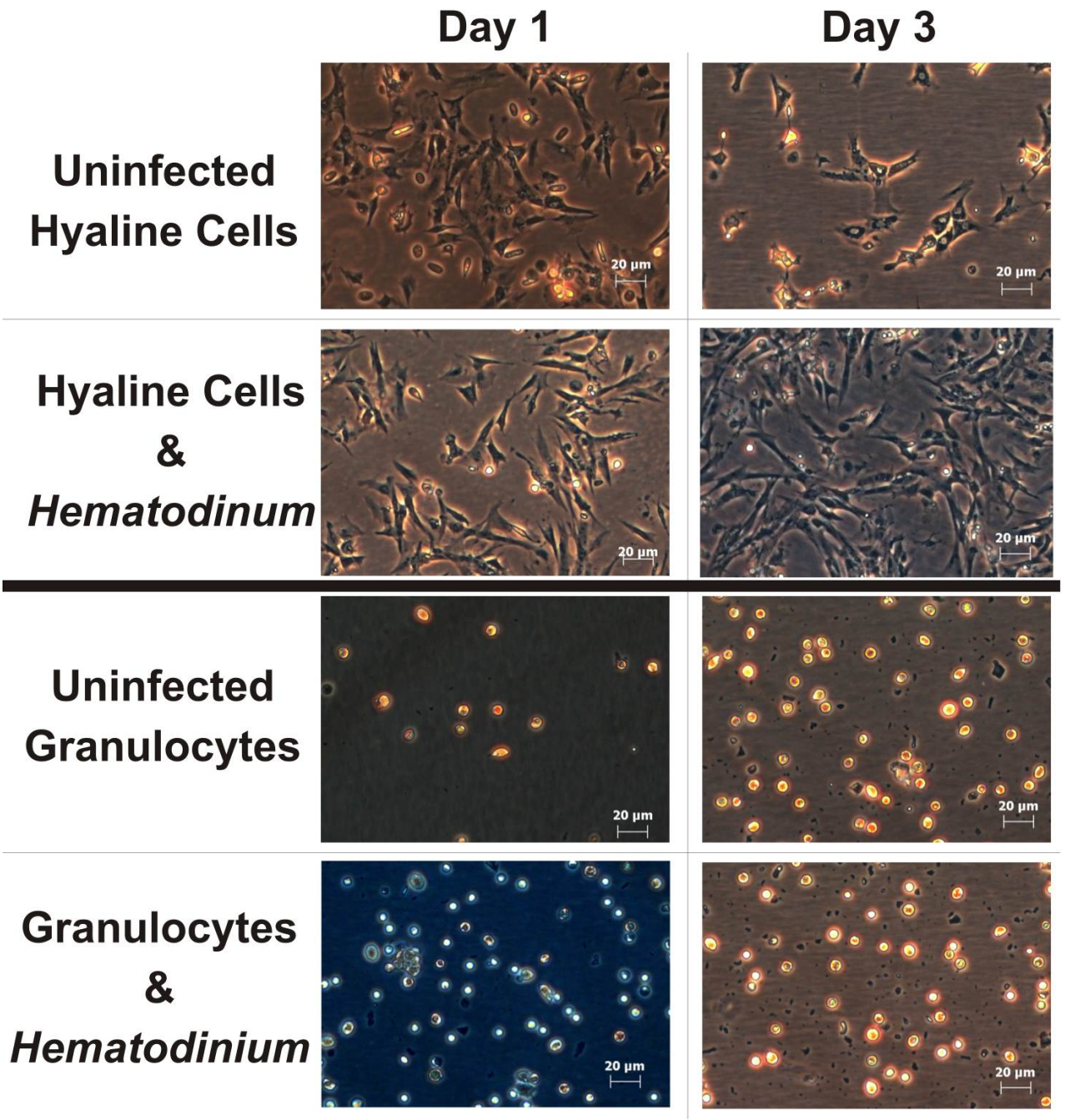
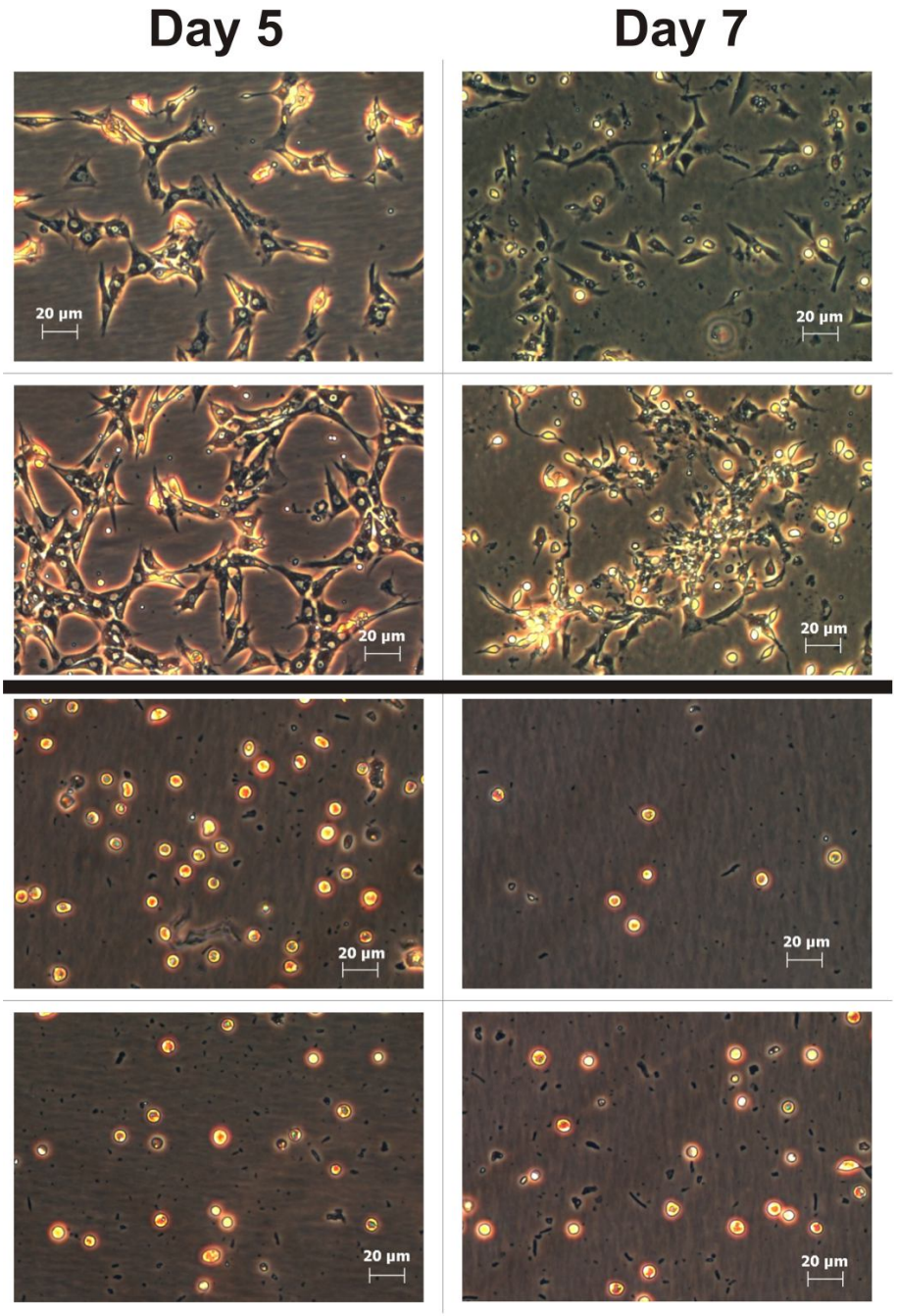
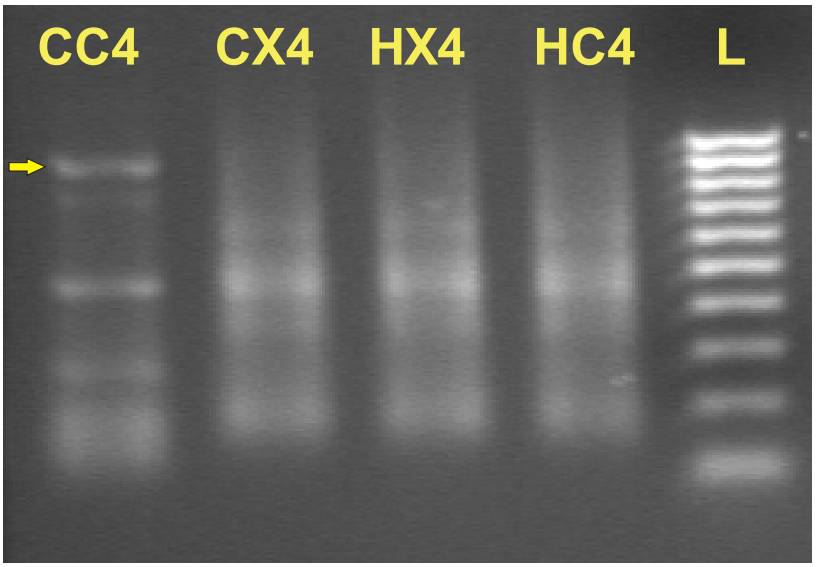
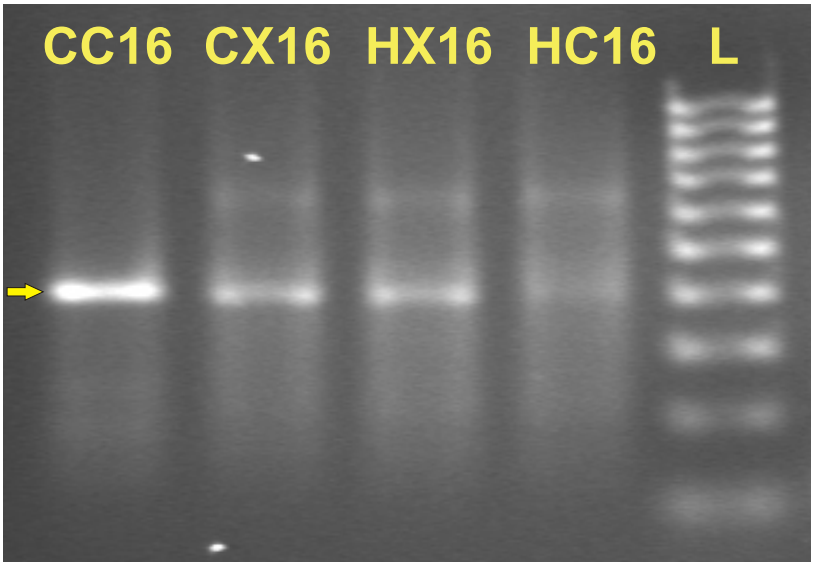



FIGURE 4.4: Comparison of haemocyte gross morphology in uninfected and *Hematodinium*-infected cell cultures. Scale bars = 20 $\mu$m.


Day 5
Day 7
20 µm
20 µm
20 µm
20 µm
20 µm
20 µm
20 µm
20 µm




(a) DEG4




(b) DEG16

FIGURE 4.5: Electrophoresis gel of GeneFishing™ results for DEG 4 and DEG 16 comparing unexposed granulocytes (control cells: CC), exposed granulocytes (exposed cells: CX), exposed *Hematodinium* (HX), and unexposed *Hematodinium* (*Hematodinium* control: HC), compared against a 100-basepair ladder (L) (Sigma-Aldrich). Arrows in CC4 and CC16 indicate potential hits for differentially expressed genes.

conserved cysteine residues and the catalytic triad His-Asp-Ser located within the C-terminal trypsin-like serine proteinase domain (Fig 4.6). In non-catalytic serine proteinase homologs the serine from the catalytic triad is replaced with glycine, as found in this sequence and identified by SMART.

Sequence DEG4 from granulocyte culture produced a fragment of 833 bp (Accession numer: FR687022) and 5' RACE extended the gene sequence to a total of 2,257 bp (Accession number: HE608876). The ORF was comprised of 1,644 bp, coding for an amino acid sequence of 548 aa, with a 5' UTR of 175 bp and a 3' UTR of 438 bp. Comparison of the predicted amino acid sequence to the NCBI BLASTP database indicated high identity with a number of ATP-dependent RNA helicases, many from the DEAD-box family. Primary hits were from a number of invertebrates including the European honey bee *Apis mellifera* (75%, XP001122489; e-value $< 10^{-180}$), the California sea hare *Aplysia californica* (71.4%, NP001191665; e-value $< 10^{-180}$), the leaf-cutter ant *Acromyrmex echinatior* (74.8%, EGI60733; e-value $< 10^{-180}$), and the jumping ant*Harpegnathos saltator* (73.9%, EFN89962; e-value $< 10^{-180}$). SMART analysis of the predicted amino acid sequence identified a DEAD-like helicase (DEXDc) (e-value = 2.45 $e^{-62}$) and helicase C domain in the Pfam database (e -value = 2.82 $e^{-30}$) and in PDBsum the highest identities were to a human ATP-dependent RNA helicase ddx5 (3fe2) (76% target sequence; z-score = 1448.5), *Drosophila* vasa (40.7% target identity; z-score = 1146.1) and human ddx3 (47.7% target identity; z-score = 1110.1). Alignment with other invertebrate DEAD-box ATP-dependent helicases (Fig 4.7) revealed that all nine conserved motifs (Table 4.4), including the DEAD-box (motif II), were present in the sequence generated from *C. pagurus*.

The *Hematodinium* sp. cells separated from the haemocytes were examined in the GeneFishing™ experiment and run against *Hematodinium* sp. cultures to control for any parasite genes that were present in challenged haemocyte cultures due to incomplete separation. This process also provided the opportunity to look for any differential expression of parasite-specific genes, however the single band identified as differentially expressed did not result in any successful hits during a BLAST search.

FIGURE 4.6 *(facing page)*: Multiple alignment of amino acid sequences of PPAFs and SPHs: *C. pagurus* PPAF, *Scylla paramamosain* SPH (ADG83846), *Portunus trituberculatus* PPAF (ACN87221), *Callinectes sapidus* PPAF (AAS60227), *Eriocheir sinensis* PPAF (ACU65942), *Penaeus monodon* SPH (ABD6288) and PPAF (ABE03741), and *Pacifasticus leniusculus* SPH (ACB41379). The pairwise % identity is 61.4% for these sequences. The six conserved cysteine residues from the clip-domain are numbered 1-6. The catalytic triad is marked with blue asterisks (*); the serine has been replaced with glycine, rendering the protein catalytically inactive. The trypsin-like serine proteinase domain is double underlined in blue. Amino acid similarity is indicated by color: black - 100%, dark gray - 80-99%, light gray - 60-79%, white 0-59%.

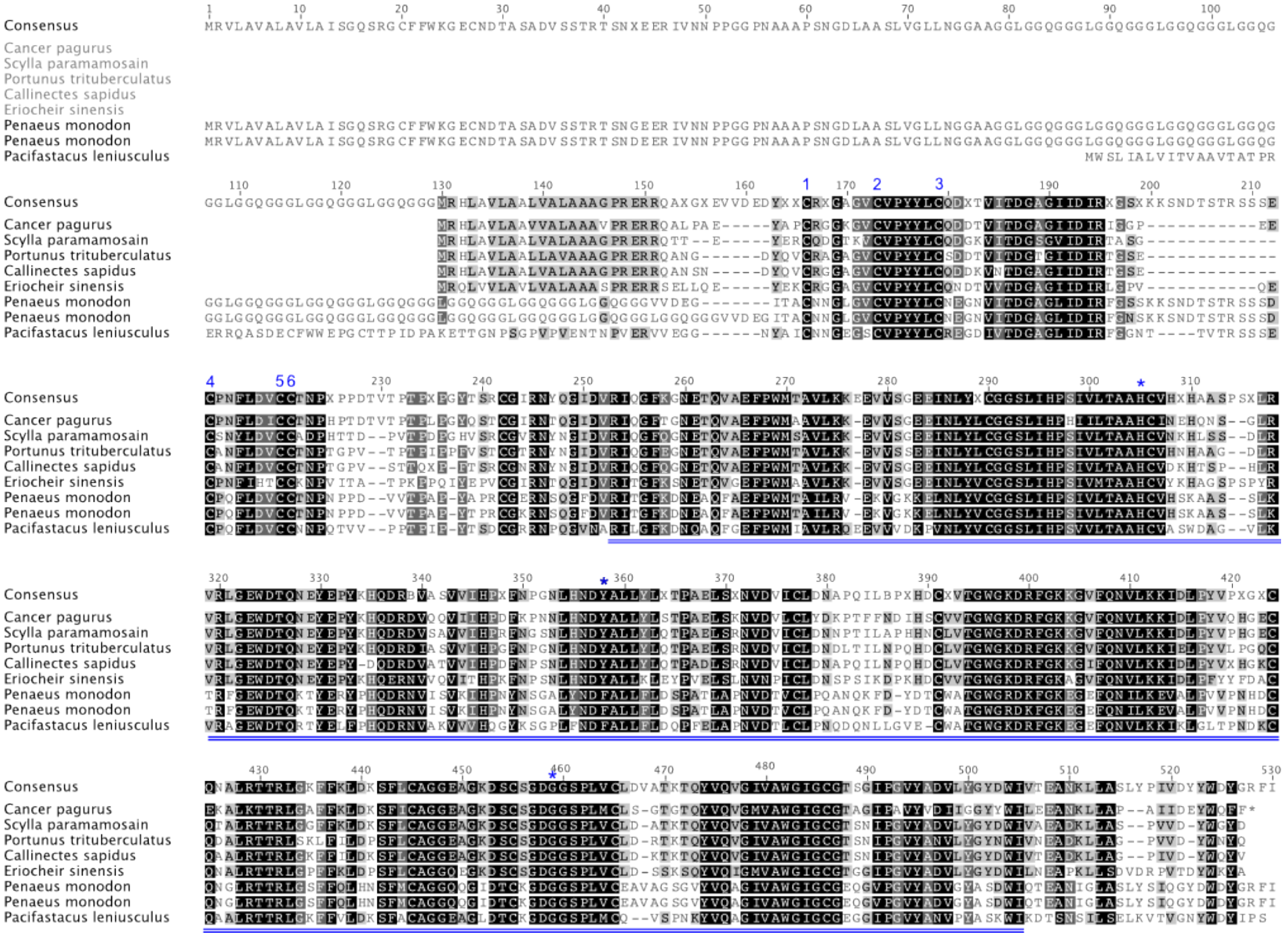

Consensus
Cancer pagurus
Scylla paramamosain
Portunus trituberculatus
Callinectes sapidus
Eriocheir sinensis
Penaeus monodon
Penaeus monodon
Pacifastacus leniusculus

TABLE 4.4: Putative functions of the conserved motifs of DEAD-box RNA helicases as described by Cordin *et al.* (2006)

| Motif | Conserved Region | Putative function |
|---|---|---|
| Q motif | GaaccPohIQ | Highly conserved adenine recognition and ATP-binding site that interacts with motif I; necessary for conformational changes within protein; unique to DEAD-box proteins |
| Motif I (Walker A motif) | AxTGoGKT | Crucial to ATPase activities, common to all helicases |
| Motif Ia<br>Motif Ib | PTRELA<br>TPGRI | Necessary for RNA binding in combination with motifs IV and V |
| Motif II (DEAD-box or Walker B motif) | DEAD | Coordinates magnesium ions and hydrolises $\beta$-$\gamma$ phosphoanhudride bonds of bound ATP; necessary for ATPase activity |
| Motif III | SAT | Necessary for linking ATPase and helicase activities; can interact with motif I and II |
| Motif IV | IIFhxT+cx | Possibly binds ssRNA with RR residues when present; precise function unclear at present |
| Motif V | TDVuARGID | RNA-binding motif, works with motif Ia, Ib and IV; implicated in signal transmission of RNA binding to ATPase domain; possibly regulates hydrolysis |
| Motif VI | HRIGRTGR | Implicated in binding and stablising ATP for hydrolysis. |

FIGURE 4.7 *(facing page)*: Multiple alignment of amino acid sequences of ATP-dependent RNA helicases (DEAD-box): *C. pagurus* ddx, *Apis mellifera* (XP001122489), *Aplysia californica* (NP001191665), *Harpegnathos saltator* (EFN89962), *Acromyrmex echinator* (EGI60733). Motif II (DEAD-box) is boxed in red, eight other conserved motifs are boxed in black. The DEAD/DEAH box helicase domain is double underlined in blue. Amino acid similarity is indicated by color: black - 100%, dark gray - 80-99%, light gray - 60-79%, white 0-59%.

Consensus
Cancer pagurus
Apis mellifera
Aplysia californica
Harpegnathos saltator
Acromyrmex echinatior

### 4.3.3 Quantification of gene expression during *Hematodinium* sp. infection

Analysis of the host reference gene, *β-act*, using REST 2009 software found no significant difference in expression between control and experimental samples, which indicated that it was suitable for use as an endogenous reference gene in this experiment.

REST 2009 was used to compare the relative change in gene expression of *ppaf* and *ddx* (and *proPO* for granulocytes) between control haemocyte cultures and those exposed to *Hematodinium* sp. cells. For challenged hyaline cultures, a slight increase was found in *ppaf* expression and a slight decrease in *ddx* expression, however neither were significant. Challenged granulocytes had decrease in all genes of interest compared to the reference gene, however none were significant (Table 4.5).

TABLE 4.5: Relative gene expression of exposed cell cultures compared to control cell cultures

| | Gene Expression Ratio[a] | | |
|---|---|---|---|
| Cell Type | *ppaf* | *ddx* | *proPO* |
| Hyaline Cells | ↑1.19 | ↓1.17 | - |
| Granulocytes | ↓5.08 | ↓4.74 | ↓4.58 |

[a] Genes up- or down-regulated relative to the reference gene *β-act* (n = 6)

## 4.4 Discussion

Similar to other invertebrates, crustaceans lack an adaptive immune system and rely on innate processes mediated by haemocytes for internal defense. The two primary haemocyte classes, hyalinocytes and granulocytes, carry out numerous immune functions such as pathogen recognition, phagocytosis and nodule formation. Hyalinocytes are largely involved in phagocytosis and nodule formation, while granulocytes are predominantly in charge of agglutinin release and melanin formation (Ratcliffe and Rowley, 1979; Smith and Soderhall, 1983, 1991; Battistella *et al.*, 1996). A key component of the crustacean immune response is the prophenoloxidase (proPO) activating system, which converts the proenzyme proPO to its active form, phenoloxidase (PO). PO is then able to catalyze the oxidation of phenols into quinones, forming fungistatic melanin (used in encapsulation and nodule formation)

and releasing toxic quinone byproducts (Cerenius and Soderhall, 2004). ProPO activation is mediated by a proteinase cascade and proPO is ultimately cleaved by a serine proteinase which is part of a set of prophenoloxidase activating factors (PPAFs). The cascade is triggered by microbial products such as lipopolysaccharides in bacteria (Xian *et al.*, 2009) and $\beta$-1, 3-glucans present in fungal cell walls (Smith and Soderhall, 1983).

### 4.4.1 *In vitro* crustacean haemocyte viability

Although continuous cell lines for insect haemocytes have been established (Belloncik *et al.*, 2007; Mitsuhashi and Shozawa, 1985; Carter *et al.*, 1994; Yasunaga-Aoki *et al.*, 2004) crustacean haemocyte culture is currently limited to primary culture of short duration. The current study found hyaline cells to be more robust than granulocyte cells and retained 65% viability after seven days *in vitro*, whereas granulocyte cells decreased to 38% viability after seven days. Smith and Ratcliffe (1978) found that granulocytes were extremely fragile and easily lysed, particularly in cell clumps, but that hyalinocytes rarely lysed and could be examined *in vitro* for up to 7 hours and retain 98% viability. Walton and Smith (1999) reported keeping viable hyaline cells in culture for 14 days with viability remaining above 70% at the end of the culture period and, furthermore, they retained functionality throughout the culture period, measured by phagocytic activity towards marine bacteria. Extended hyaline cell viability was supported by hyaline cell cultures from the spiny lobster *Panulirus argus* that retained 80% viability after 18 days in modified L-15 culture media (Li and Shields, 2007), whereas granular cells were found to decrease to less than 10% viability after 5-7 days. Li and Shields (2007) also found that separating granulocytes from hyaline cells improved the viability of hyaline cultures; degranulation appeared to reduce the viability of hyaline cells. The seven-day experiment in this study found hyaline cell viability to decrease to 65% at the end of the experiment. Granulocytes, though reduced in viability compared to hyaline cells (38%), retained higher viability than that observed in other crustacean cultures (Li and Shields, 2007), however the decrease in viability was significant after six days in culture and therefore imposed limitations on culture studies. For use in expression studies, such low viability is undesirable and therefore it was decided that culture challenge experiments would be kept to a maximum time of 48 hours.

### 4.4.2 *In vitro* exposure experiment

Throughout the majority of the exposure experiment, little obvious change in gross morphology was observed between challenged and naive cultures of granulocytes or hyaline cells. The single exception was a mild aggregation response forming on day seven in challenged hyaline cultures. Localised haemocyte recruitment is an important immune response in which haemocytes aggregate around foreign material to either phagocytose it or encapsulate it to prevent spread of the infection (Takle, 1986). No signs of phagocytosis or encapsulation were apparent in the cultures, although encapsulation was one of the few observations of immune response observed in the Tanner crab *Chionecetes bairdi* in response to *Hematodinium* sp. infection. Cell rounding has been associated with aggregation responses in cockroach haemocytes in culture as the cells round up to increase motility (Takle, 1986), however there was no evidence of this trait in *C. pagurus* hyaline cells *in vitro*.

Changes in cell morphology have been observed for a number of invertebrates in response to pathogens, parasites, viruses or harmful algal toxins ((Strand, 1994; Humbert and Coustau, 2001; Hegaret *et al.*, 2011) and see Loker (1994) for review). Haemocytes from Manila clams *Ruditapes phillipinarum* exposed *in vitro* to the toxic dinoflagellate *Karenia selliformis* showed a decrease in haemocyte complexity, reactive oxygen species production and cell adhesion. Additionally, there was a significant decrease in haemocyte viability (Hegaret *et al.*, 2011). *In vitro* exposure of haemocytes from the snail *Biomphalaria glabrata* to excretory-secretory products of the trematode parasite *Echinostoma caproni* resulted in inhibited haemocyte adhesion and phagocytosis, indicating a potential mechanism of immune resistance by the parasite (Humbert and Coustau, 2001). Primary culture of soybean looper moth *Chrysodeixis includens* (syn: *Pseudoplusia includens*) haemocytes also showed loss of cell adhesion as well as apoptosis and melanisation when exposed to parasites or MdBV (*Microplitis demolitor* bracovirus)-infected larvae (Strand and Noda, 1991; Strand, 1994; Strand and Pech, 1995). In the present study, apart from the slight hyaline response on day seven, no other evidence of cell rounding, degranulation or loss of adhesion was observed in any of the cultures, indicating that short-term haemocyte response is limited in *Cancer pagurus* during *in vitro* challenge with *Hematodinium* sp.

A potential alternative to crustacean haemocyte culture is to use tissue culture instead. Indeed, it has been noted by researchers using continuous cell lines from insect haemocytes that the cells do not retain their original function for very long (Belloncik *et al.*, 2007) and tissue cultures may be more reliable. Furthermore, in continuous cell lines generated from other insect tissues such as ovaries and embryos, there have been many observations of the cells having immunoreactive traits and responding in a

similar manner to haemocytes. The mosquito *Anopheles gambiae* continuous cell line 4a-3B, originated from whole *A. gambiae* larvae, was found to secrete prophenoloxidase and has therefore been used as a tool for studying the immune response to the malarial parasite *Plasmodium falciparum* (Muller *et al.*, 1999). The same cell line also showed expression of defensin and Gram-negative bacteria-binding protein, leading the researchers to believe the cell line is haemocyte-derived. Crustacean tissues such as haemopoietic tissue and lymphoid tissue may serve as suitable alternatives to haemocytes for immune studies. Haemopoietic tissue contains precursor cells to circulating haemocytes and has been maintained in culture for up to three months (Brody and Chang, 1989). Furthermore, recent research has identified a cytokine in *Pacifastacus leniusculus*, called astakine, which has been found to increase cell proliferation and differentiation in haemopoietic tissue cell in culture (Soderhall *et al.*, 2005; Lin *et al.*, 2008*a*; Soderhall and Lin, 2011). The addition of astakine to haemopoietic tissue cultures challenged with WSSV was found to increase the infection, leading the authors to suggest that WSSV favours dividing cells (Jiravanichpaisal *et al.*, 2006*b*).

Recent work has also looked into the use of alternate species to study immune response to viruses. Sriton *et al.* (2009) used common and readily available continuous insect cell lines and infected them with white spot syndrome virus (WSSV) and yellow head virus (YHV). Both viruses are frequently found in crustaceans and outbreaks are a threat to shrimp culture, however lack of a continuous cell line to propagate the virus has led to difficulty studying their interactions with immune cells. Insect cells were readily infected by the viruses, though they showed no morphological signs of infection, and were able to be passaged extensively. Although it was thought that specific receptors were required for virus invasion, it appeared that was not the case. It may be possible to use established insect cell cultures to study crustacean pathogens, though this has not been tried and receptors may prove more crucial for bacterial or fungal pathogens.

### 4.4.3 Differentially expressed haemocyte genes during *Hematodinium* sp. exposure *in vitro*

In this study a differential display technique (GeneFishing™) was used to identify potentially relevant changes in gene expression of haemocytes from *Cancer pagurus* exposed to the dinoflagellate parasite *Hematodinium* sp. This technique, involving conventional PCR and gel electrophoresis, identified two relevant genes of interest coding for a prophenoloxidase activating factor (*ppaf*) and a DEAD-box ATP-dependent RNA helicase (*ddx*). Five further genes were cloned and sequenced however they were not successfully identified using BLAST.

#### 4.4.3.1 Prophenoloxidase Activating Factor (*ppaf*)

DEG16, selected by GeneFishing™, was identified in a BLAST search as a prophenoloxidase activating factor (PPAF). PPAFs encompass the set of molecules involved in activating the prophenoloxidase cascade, including the prophenoloxidase activating enzyme (PPAE) and additional cofactors. All of these molecules are referred to as PPAFs and contain an N-terminus clip domain and a highly conserved C-terminus proteinase domain. PPAF cofactors include a group of serine proteinases and serine proteinase homologues (SPHs) which are separated by their proteolytic activity. SPHs are non-proteolytic because the serine residue at the catalytic site has been replaced with glycine (Fig 4.6).

PPAFs have been found in numerous insects including *Manduca sexta* (Yu *et al.*, 2003), *Holotrichia diomphalia*(Lee *et al.*, 1998*b*,*a*) and *Bombyx mori* (Satoh *et al.*, 1999), as well as in a number of crustaceans such as *Portunus trituberculatus* (Cui *et al.*, 2010), *Penaeus monodon* (Charoensapsri *et al.*, 2009) and *Callinectes sapidus* (Buda and Shafer, 2005). The role of SPHs in invertebrates is typically associated with immune response, though it is not limited to this function. Many SPHs are found in haemocytes (Qin *et al.*, 2010; Cui *et al.*, 2010; Okumura, 2007; Charoensapsri *et al.*, 2009), however other tissues such as hepatopancreas and eyestalk (Cui *et al.*, 2010; Qin *et al.*, 2010) have shown high expression levels and the *ppaf* isolated from *C. sapidus* was upregulated in the hypodermal tissue of pre-moult animals and is predicted to contribute to tanning of the exoskeleton (Buda and Shafer, 2005).

Although PO activity is not limited to immune function, it is well-established that the PO cascade plays a primary role in immune response (Soderhall and Cerenius, 1998; Cerenius and Soderhall, 2004). It is likely that SPHs isolated from haemocytes are most likely acting in a defensive capacity, particularly during intermolt periods. A large body of research is focussed on the immune involvement of SPHs, and particularly in relation to the proPO pathway. *M. sexta* required an SPH to be present for the prophenoloxidase activating protein (PAP) to cleave proPO into its active form (Yu *et al.*, 2003) and similarly *H. diomphalia* required a 45-kDa masquerade-like SPH for PPAF-I to activate proPO (Kwon *et al.*, 2000). In addition to assisting in PPO activation, crustacean SPHs have been implicated in opsonin-like activity and pattern recognition (Huang *et al.*, 2000; Jitvaropas *et al.*, 2009). Shrimp injected with bacteria pretreated with purified masquerade-like serine proteinase were able to clear the treated bacteria significantly faster than untreated control bacteria, indicating an opsonic characteristic. Furthermore, the ability for the protein to bind to both *Vibrio harveyi* and LPS implicates the protein as a pattern recognition molecule (Jitvaropas *et al.*, 2009). Both Jitvaropas *et al.* (2009) and Huang *et al.* (2000) have shown cell

adhesion properties for masquerade-like serine proteinases. Although GeneFishing™ can not give an indication as to the specific role of the *ppaf* in this experiment, it is likely to be immune-related. It is evident that crustacean serine proteinase homologues function in a variety of immune capacities, though further investigation is required to determine the exact role of the *ppaf* found in this study.

#### 4.4.3.2 DEAD-box ATP-dependent RNA helicase (*ddx*)

A DEAD-box ATP-dependent RNA helicase was also identified by differential display as being involved in an early granulocyte response to the parasite. DEAD-box proteins are named from the conserved motif Asp-Glu-Ala-Asp (DEAD in IUPAC one-letter code) (Fig 4.7) which is required for ATP hydrolysis (Linder *et al.*, 1989; Rocak and Linder, 2004). Cellular functions undertaken by DEAD-box proteins include, but are not limited to, transcription, RNA transport, pre-mRNA splicing and RNA decay (Py *et al.*, 1996; Tseng *et al.*, 1998; Cordin *et al.*, 2006). The gene fragment generated from this experiment had high identity with *ddx17*, which codes for both p72 and p82 RNA helicase, as well as to *ddx5*, which codes for p68. *Ddx5* and *ddx17* are the most closely related members in the family and have been proven to carry out a number of functions such as unwinding RNA and initiating gene transcription (Janknecht, 2010). Furthermore, *ddx17* and *ddx5* have been found to be crucial to cell proliferation and survival (Jalal *et al.*, 2007) which could have a knock-on effect for efficient immune response in a haemocyte-driven immune system. Individual knock-down of p68, p72 or p82 caused only a slight decrease in the proliferation of HeLa cells, however when all three were simultaneously knocked down, cell proliferation ceased and DNA replication was blocked (Jalal *et al.*, 2007). The DEAD-box protein 3 (ddx3), to which *C. pagurus ddx* had high identity with using PDBsum, has also been implicated as an activator of interferon regulatory proteins in the innate immune response to the vaccinia virus (Schroder *et al.*, 2008) and other DEAD box proteins have been identified as crucial elements in the proliferation of HIV as mRNA transporters (Yedavalli *et al.*, 2004).

The expression of *ddx* in this culture is quite remarkable for the fact that, in invertebrates, it is typically studied in embryonic and germ cells (Schupbach and Wischaus, 1986; Aflalo *et al.*, 2007; Nakkrasae and Damrongphol, 2007; Shukalyuk *et al.*, 2007) and is particularly related to cellular growth and division. To date, studies of crustacean DEAD-box proteins and gene expression have been limited to the *vasa*-like gene involved in germ cell development (Aflalo *et al.*, 2007; Nakkrasae and Damrongphol, 2007; Shukalyuk *et al.*, 2007). The *vasa* gene is a DEAD-box family member originally studied in posterior development of *Drosophila melanogaster* embryos (Schupbach and Wischaus, 1986). *Vasa*-like genes have now been identified in

the Pacific white shrimp *Litopenaeus vannamei* (Aflalo *et al.*, 2007), the giant freshwater prawn *Macrobrachium rosenbergii* (Nakkrasae and Damrongphol, 2007), the amphipod *Parhyale hawaiensis* (Ozhan-Kizil *et al.*, 2009) and the green mud crab *Scylla paramamosain* (Wang *et al.*, 2011). All of these studies have located *vasa* in ovary and testis, but not in other tissues such as heart, stomach or hepatopancreas. Importantly, however, *vasa* appears to be essential to cell differentiation (Aflalo *et al.*, 2007; Nakkrasae and Damrongphol, 2007) and proliferation and maintenance (Ozhan-Kizil *et al.*, 2009). Similar to *ddx5* and *ddx17* knock-down in HeLa cells (Jalal *et al.*, 2007), knock-down of *vasa* in *P. hawaiensis* stopped cell division, indicating that *vasa* is necessary for cell proliferation (Ozhan-Kizil *et al.*, 2009). Based on current knowledge regarding the limited observations of crustacean cell proliferation *in vitro*, gene expression studies using DEAD-box genes could be useful in optimising cell culture parameters. Upregulation of DEAD-box genes could indicate stimulation of cell division and therefore act as an indicator of cell culture conditions. It is clear that further investigation is needed into the role of DEAD-box proteins in crustacean haemocytes kept in culture to determine their exact function and determine their efficacy as culture condition monitors.

### 4.4.4 Gene Expression

Having sequenced the aforementioned genes, the ultimate goal of this study was to explore the changes in expression of each gene in response to parasite infection. Due to the fact that crustacean immunity is based primarily on the response of haemocytes to invading pathogens, it is likely that this would be the first place to see evidence of changes in gene expression of relevant immune genes. *Ppafs* and *proPO* have been found to vary during infection both *in vitro* and *in vivo* (Cerenius *et al.*, 2003; Hauton *et al.*, 2005; Ai *et al.*, 2008; Charoensapsri *et al.*, 2009; Liu *et al.*, 2010).

To validate the differential display results from GeneFishing™, gene expression analysis was carried out on a broader sample set ($n = 6$ for each haemocyte type) and the analysis of the *proPO* gene was included for granulocyte cultures as well. The main finding in the gene expression portion of this study was that no significant change was observed in the immune-related genes *proPO* and *ppaf* as well as the ATP-dependent RNA helicase *ddx*. These findings indicate that *Hematodinium* sp. does not elicit a transcriptional response in individual haemocyte populations for the genes examined in *C. pagurus*.

Similarly, *proPO* and serine proteinase expression in Pacific white shrimp *Litopenaeus vannamei* was found to remain stable when individuals were injected with lipopolysaccharide (LPS) from *Escherichia coli* although genes coding for the

antimicrobial peptides penaeidin (PEN2, PEN3, PEN4) and crustin were significantly decreased (Okumura, 2007). Okumura (2007) proposed that this could be due to proPO enzyme already being present and merely needing serine proteinases to initialise the cascade rather than altering gene expression, however many studies have observed changes in *proPO*, including exposure to LPS.

Crustacean immune gene responses have been found to fluctuate dramatically over time, with expression changes varying from 1-hour up to 96 hours post-challenge (Cerenius *et al.*, 2003; Gai *et al.*, 2008; Liu *et al.*, 2010). Crustacean proPO enzyme activity is frequently found to change when exposed to various pathogens and pathogen associated stimulants (Sung *et al.*, 1996; Hauton *et al.*, 1997*b*; Cerenius *et al.*, 2003; Perazzolo and Barracco, 1997; Ji *et al.*, 2011). More recently, this has been observed using *proPO* gene expression analysis. After observing an increase in *proPO* expression in *Macrobrachium rosenbergii* one hour post-injection with the pathogen associated molecular pattern (PAMP) CpG oligodeoxynucleotide Lu *et al.* (2006) proposed that *in vitro* studies longer than a few hours may miss the *proPO* expression response, which if true, could be a possible reason for the lack of change during this study. However, many immune gene expression changes have been observed after 12 to 48 hours, meaning 24 hours would be an optimal time to observe a response to *Hematodinium*. Hauton *et al.* (2005) found *proPO* to be elevated only after 24 hours and no significant expression changes after three hours. A similar result was found in crustin expression of *Hyas araneus* granulocytes exposed to *L. anguillarum* where expression was increased after 24 hours, but no significant change was observed after 5 hours and expression levels had decreased again after 48 hours (Sperstad *et al.*, 2010). *In vivo* studies support the observed *in vitro* timelines. Mud crabs *Scylla serrata* injected with LPS had increased *proPO* expression after 12 hours and were still elevated after 48 hours compared to saline-injected control animals (Ko *et al.*, 2007). Similarly, *Eriochier sinensis* showed expression peaks after 2, 12 and 48 hours post-injection with the bacteria *Vibrio anguillarum*, the most marked increase being after 12 hours (Gai *et al.*, 2008). The freshwater crayfish *Astacus astacus* also showed increased *proPO* expression 12 hours post-laminarin injection (Cerenius *et al.*, 2003).

*ppaf* gene expression has shown comparable results to *proPO* by showing upregulation in haemocytes and subcuticular epidermis of the crab *Scylla paramamosain* during bacterial challenge with *Vibrio parahaemolyticus* after 24 hours and remaining elevated up to 96 hours (Liu *et al.*, 2010). *Eriocheir sinensis* and *Portunus trituberculatus* SPHs were similarly affected by bacterial challenge (Qin *et al.*, 2010; Cui *et al.*, 2010) and the masquerade-like SPH from *P. monodon* showed upregulation during *Vibrio harveyii* challenge (Jitvaropas *et al.*, 2009).

It is clear that proPO and PPAFs respond to numerous invading pathogens during internal defense reactions in crustaceans, however it is not a consistent reaction to all pathogens across all crustacean species. The lack of response to LPS in *L. vannamei* (Okumura, 2007) and the stability of expression in granulocytes of *H. gammarus* to certain immunostimulants (Hauton *et al.*, 2005) suggests a certain specificity must be met. The nature of the *Hematodinium* sp. surface coat is unknown and it is also unclear whether the parasites secrete any enzymes or proteins that may interfere with internal defense. Further investigation into these parameters is needed to define the interactions between the parasite and *C. pagurus* haemocytes.

This is the first report of *ddx* being identified in crustacean haemocytes and the specific functional role of of this gene is unknown for *C. pagarus* making speculation regarding gene expression changes, even if not significant, difficult. Sequence similarity to *ddx5* and *ddx17* would indicate that it is likely that *C. pagurus ddx* is involved in cell proliferation (Jalal *et al.*, 2007). In mice, the protozoan parasite *Trypanosoma cruzi* has been found to inhibit T cell proliferation via a complex pathway of molecular activation and inhibition of interleukin 2 (IL-2) synthesis (Alcaide and Fresno, 2004). In short, parasite molecules activate T-cells which in turn activate myeloid cells to produce large amounts of nitric oxide (NO). NO enhances T-cell proliferation at low concentrations, however high levels of NO are cytotoxic and cause a decrease in T-cell proliferation (Niedbala *et al.*, 2006). Although DEAD-box proteins have not been implicated in this method of suppression, it is clear that parasites are capable of intricate methods of immune suppression and are capable of acting at a transcriptional level. It was not within the scope of the current study, however further investigation into the role of *ddx* in the haemopoietic tissue of crustacean hosts during parasite infection may help to establish whether *Hematodinium* sp. is capable of affecting cell proliferation.

The data reported here are from separated haemocyte cultures and *in vitro* limitations may have played a role in the resultant gene expression. Although cell culture has its advantages in controlling experimental parameters, the full complement of plasma factors and interactions with other tissue cell types are absent. Hauton *et al.* (2005) recognised this limitation when exposing *H. gammurus* granulocytes to immunostimulants and Lu *et al.* (2006) suggested that this may be the case for LPS and laminarin-like plasma factors. Furthermore, individual cell types have been found to communicate in response to pathogens (Johansson and Soderhall, 1989; Soderhall *et al.*, 1990). For example, semigranular cells from the crayfish *Pacifastacus leniusculus* had an enhanced encapsulation response towards glass beads when they had been previously coated with haemocyte lysate supernatant in which haemocytes had previously degranulated (Kobayashi *et al.*, 1990).

#### 4.4.4.1 Limitations of differential display

Despite GeneFishing™ evidence that *ppaf* and *ddx* were more highly expressed in uninfected cultures, this was not supported by qPCR. False positives, such as the ones found here, are quite frequent for a variety of differential display techniques and therefore require validation by another method, such as qPCR. Differential display-PCR is particularly susceptible to non-specific primer binding and a bias towards high copy number mRNAs (Ledakis *et al.*, 1998) and Rajeevan *et al.* (2001) found that genes identified using DNA array with a 2- to 4-fold change were not reliable and required validation. GeneFishing™ is designed to reduce false positives by using annealing control primers, however this study was also limited by the use of cell cultures from an individual animal as opposed to pooled cell culture. Physiological differences in a single animal (ie. varying stress levels, secondary infection) may not transfer to an entire population and therefore the differences seen when cultures from several animals are analysed together result in no significant change.

### 4.4.5 Haemocyte response to *Hematodinium* sp. challenge *in vitro*

Based on previous observations of animals infected with *Hematodinium* sp., the current *in vitro* results support previous evidence of a limited host immune response. Very few studies have noted an immunological response to *Hematodinium* and no research to date has specifically targeted immune function. The few studies that have mentioned an immune response have been discovered primarily during histological examination of infected animals or from haemocyte counts. Several researchers have noted decreases in haemocyte numbers, possibly due to host cell lysis while fighting infection (Field and Appleton, 1995; Shields and Squyars, 2000; Meyers *et al.*, 1987). Shields and Squyars (2000) also observed an increase in the proportion of granulocytes to hyalinocytes, implying that hyalinocytes were being consumed to encapsulate parasite cells. This could lead to an increased need for cell proliferation to maintain a sufficient immune response.

A cell proliferation response is supported by evidence of increased size of the haemopoietic tissue of infected *N. norvegicus*, possibly triggered by parasite invasion. Haemopoietic tissue was affected enough to be visible to the naked eye and was swollen and yellow (Field and Appleton, 1995; Field *et al.*, 1992). Despite the increased activity, however, very few newly differentiated cells were observed and most of the nearby haemal space was occupied by parasite cells (Field and Appleton, 1995). A more detailed study involving gene expression changes of *ddx* in the haemopoietic

tissue may provide more insight into how cell proliferation varies during parasite infection.

As mentioned earlier, immune suppression and avoidance is a common trait of internal parasites that wish to keep their host alive yet also avoid detection by host internal defense mechanisms. Immune suppression has the associated risk of allowing co-infection causing competition for host resources. It is possible this is the case for *Hematodinium* sp. As yet there have been no studies directly examining the method of avoidance or suppression, however GeneFishing™ results in the present study have indicated a differentially expressed fragment from *Hematodinium* sp. The gene fragment was not able to be identified in the present study, however further examination of differentially expressed parasite genes could prove worthwhile insight into *Hematodinium*-host interactions.

Furthermore, it has been suggested that *Hematodinium* sp. may be able to suppress or avoid the immune system of its crustacean hosts. Stentiford et al. (Stentiford *et al.*, 2003) found that *C. pagurus* co-infected with *Hematodinium* and a yeast-like organism produced an immune response to the yeast-like organism while the *Hematodinium* cells remained largely unaffected. Yeast-like cells were contained in large haemocyte encapsulations in the haemal sinuses of the hepatopancreas and there were melanised encapsulations found within the gill lamellae and connective tissue of the midgut wall. There were no signs of encapsulations or melanisation events containing *Hematodinium*. Immune suppression is reported from other parasites as well and the Ichneumonid parasitoid *Campoletis sonorensis* has been found to inhibit PO activity in its moth larvae host *Heliothis virescens* (Shelby *et al.*, 2000). Parasitoid wasps infect their hosts with polydnaviruses, causing loss of haemocytic adhesive capabilities, inhibition of melanisation pathways or cell lysis (see review Beckage (1998)). While it is unlikely that *Hematodinium* is using a method as extreme as polydnaviruses it is possible there may be some form of immune suppression involved or that the cell wall components of *Hematodinium* may not be recognised as non-self as is often found in bacteria or fungi. The results of the present study (stable expression of all genes, consistent morphology and viability between exposed and control cultures) provide stronger evidence for avoidance of the immune response by *Hematodinium* sp. cells, however this requires further investigation and the observations of *in vitro* studies must be supported by *in vivo* studies.

Alternatively, there is the potential for *Hematodinium* sp. to interfere in host internal defense at a post-transcriptional level. The polydnavirus *Cs*PDV injected into *H. viriscens* with *C. sonorensis* larvae has been found to inhibit translation of the host's growth proteins (Shelby and Webb, 1997). Infected host larvae were found to have

reduced plasma concentrations of the growth-related storage proteins p82 (Riboflavin binding hexamer) and p74/p76 (arylphorin) although mRNA analysis found no change in expression levels of the genes coding for them. This indicated that the inhibition was occurring post-transcriptionally and the authors hypothesised that parasites were inhibiting translation of these proteins in order to commandeer the resources for parasite development (Shelby and Webb, 1997). In addition to being classed as a parasite, *Hematodinium* is also a dinoflagellate, of which there are several toxic species known to cause alterations to immune parameters in bivalves (Hegaret and Wikfors, 2005; Hegaret *et al.*, 2011). The dinoflagellate *Prorocentrum minimum* was found to decrease cell complexity in quahogs *Mercenaria mercenaria* and phagocytosis in soft shell clams *Mya arenaria*; *Karenia selliformis* also inhibited cell adhesion and decreased reactive oxygen species formation and phagocytosis in Manila clams *Ruditapes philippinarum* (Hegaret *et al.*, 2011). It is possible that *Hematodinium* sp. secrete toxins or inhibitory molecules which interact directly with host proteins or other immune factors and would not be evident during gene expression studies. There is a need, therefore, to examine host immune parameters using immune assays in order to support gene expression analysis.

### 4.4.6 Conclusions

The lack of response of either granulocytes or hyaline cells to the presence of *Hematodinium* sp. *in vitro* suggests limited recognition of the parasite whilst under culture conditions. It is possible that *in vitro* conditions removed an essential humoral recognition factor, however haemocyte cells were in direct contact with parasite cells and still no positive signs of recognition were present. *Hematodinium* sp. also did not appear to directly interfere with haemocyte morphology as no difference in cell appearance (cell adhesion, cell rounding or degranulation) was apparent between exposed and control cultures. Additionally, no differences were found in haemocyte viability indicating that parasite cells were not directly reducing haemocyte numbers.

The differential display technique, GeneFishing™, identified two novel genes in uninfected granulocyte cultures: a prophenoloxidase activating factor (*ppaf*) and a DEAD-box ATP-dependent RNA helicase (*ddx*). Gene expression analysis of a larger sample set of cultures was unable to confirm a change in expression of either gene between exposed and control cultures from either granulocyte or hyaline cells. Expression of the prophenoloxidase gene (*proPO*) in granulocytes also did not vary between exposed and control cultures. The stable expression of these genes shows that *Hematodinium* sp. does not interfere at the trasncriptional level with the

prophenoloxidase system of separated haemocyte populations *in vitro*, nor does the parasite act on the mRNA expression of *ddx*.

*In vitro* experiments were an incredibly useful tool for studying direct cell-cell interactions between *Hematodinium* sp. and separated haemocyte populations and had the added advantage of removing confounding factors such as secondary infections from opportunistic pathogens. However, *in vitro* experiments are not a replacement for *in vivo* studies and it is important to examine the interactions of *Hematodinium* sp. with its host under natural conditions. In order to further explore the effects of *Hematodinium* sp. on immune function, *in vivo* studies of naturally infected crustacean hosts were undertaken using both immune assays (Chapter 5) and gene expression analysis (Chapter 6). Due to difficulty in obtaining infected *Cancer pagurus* and the lack of success with experimentally instigated infections, *Nephrops norvegicus* from the Clyde Sea Area, Scotland were used for the *in vivo* studies.

# Chapter 5

# Effect of *Hematodinium* sp. infection on multiple immune parameters of *Nephrops norvegicus* from the Clyde Sea Area, Scotland

## 5.1 Introduction

### 5.1.1 *Hematodinium* in the Clyde Sea

*Nephrops norvegicus* with an unusual pathology were originally observed in the Firth of Clyde in the 1980s during a routine biological investigation. Animals were observed to have opaque joints, creamy haemolymph and 'watery' muscle structure. The animals were also very lethargic and the carapace colour was a dull orange. Initial investigations determined that the symptoms were related to moult status as infected individuals were usually encountered seasonally. However, by 1987 fishers and processors had raised more widespread concern and further investigations were instigated (Field, 1992). The results of the investigation revealed a non-motile protistan parasite in the haemal spaces that was formally identified as *Hematodinium* sp. in 1992 (Field *et al.*, 1992).

Prevalence of *Hematodinium* sp. infection in Clyde Sea populations of *N. norvegicus* has been well-studied and there is an obvious seasonality to the disease. Patent

infections have been observed from December until June with peaks usually occurring between March and May (Field *et al.*, 1992, 1998; Stentiford *et al.*, 2001*c*,*b*). More sensitive detection methods, including Western blot and ELISA, were able to detect subpatent infections earlier in the season (Stentiford *et al.*, 2001*c*; Small, 2004). PCR detection of *Hematodinium* infection in several other crustacean species in the Clyde Sea found similar seasonality of infection prevalence with peaks during February and April. *Cancer pagurus* and *Carcinus maenas* were also found to have a prevalence peak in November (Hamilton *et al.*, 2009). Prevalence in *N. norvegicus* has been found to be higher in both females and smaller males (Field *et al.*, 1992, 1998; Stentiford *et al.*, 2001*b*). Stentiford *et al.* (2001*b*) suggested that female lobsters may provide a proliferative advantage for parasite cells due to the higher proportion of hepatopancreatic tissue found in females. Hepatopancreas has been identified as a nutrient storage organ and found to contain high concentrations of glycogen and lipid which are likely to be necessary for rapid parasite growth. Similarly, smaller animals also have a higher proportion of hepatopancreatic tissue which could account for higher infection prevalence in smaller individuals Stentiford *et al.* (2001*b*). Moult status has also been implicated in prevalence studies and Stentiford *et al.* (2001*b*) suggested that mobilisation of stored reserves from lobster hepatopancreas into the haemolymph to sustain the animal through late pre-moult and post-moult fasting may provide enhanced conditions for parasite proliferation. Field *et al.* (1992) found the highest infection prevalence to be in intermoult animals; however, recently moulted animals were not examined. Furthermore, infection status was determined using pleopod staging which may not have been sensitive enough to detect infections in recently moulted individuals.

### 5.1.2 Physiological and biochemical impacts of *Hematodinium* infection on its hosts

As suggested by Stentiford *et al.* (2001*b*), host physiology may play an important role in infection status; extreme increases in crustacean hyperglycemic hormone (which stimulates glycogen release) are evident immediately before moulting which is coincident with the seasonal infection peak of *Hematodinium* sp. in *N. norvegicus*. It is therefore possible that moult physiology creates a favourable environment for the rapid growth of parasite cells. More relevant to this study, however, is that host physiology has also been found to be affected as a result of parasite infection (Love *et al.*, 1996; Taylor *et al.*, 1996; Stentiford *et al.*, 1999, 2000, 2001*c*; Shields *et al.*, 2005; Hamilton *et al.*, 2010) and physiological factors can strongly influence immune response. Physiological studies have included measurements of oxygen consumption (Taylor *et*

*al.*, 1996), tissue protein and glycogen (Stentiford *et al.*, 2000, 2001*c*; Shields, 2003), free amino acids (Stentiford *et al.*, 1999, 2000), stress hormones (Stentiford *et al.*, 2001*c*) and haemolymph components (Love *et al.*, 1996; Stentiford *et al.*, 1999; Shields, 2003; Hamilton *et al.*, 2010). These studies have indicated that crustaceans infected with *Hematodinium* sp. suffer significant metabolic and respiratory stress. All of these stressors have the potential to impact immune response, however to date, no studies have explored the direct impacts on immune responses such as phenoloxidase activity and superoxide production.

Of the physiological studies to date, two parameters were strongly immune related: haemagglutinin capabilities and haemocyanin availability. As previously mentioned in Chapter 1.4.3, agglutination is an immune response in which foreign material, recognised by surface carbohydrates, is clumped together to prevent it spreading further throughout the organism and to facilitate a localised immune response. Previous studies have found no significant change in agglutinin activity between uninfected blue crabs *Callinectes sapidus* and those with varying levels of *Hematodinium* infection, suggesting that agglutination does not play a role in *Hematodinium* sp. recognition. Measures of agglutinating activity in the presence of pathogens have been recorded for several crustacean species with varying results (Murali *et al.*, 1994; Sritunyalucksana *et al.*, 1999; Song *et al.*, 2003) and several pathogens, particularly viruses, have been found to inhibit agglutinin activity (Pais *et al.*, 2007). A haemagglutinin inhibition assay on the hermit crab *Diogenes affinis* found that haemagglutination was inhibited in the presence of lipopolysaccharide (LPS) from *Salmonella abortus* and Murali *et al.* (1999) suggested that *D. affinis* agglutinins were likely to have a specificity for the acetyl groups found in the LPS coats of bacteria. In contrasting observations, haemagglutinin activity was increased in the freshwater prawn *Macrobrachium rosenbergii* following injection with white spot syndrome virus (WSSV), however decreased in the tiger shrimp *Penaeus monodon* (Pais *et al.*, 2007). These results suggested that agglutinins played a role in immune defense of *M. rosenbergii* towards WSSV, but that agglutinin activity was potentially virus-inhibited in *P. monodon*. Conversely, several immunostimulants and bacterial components were found to have no effect on agglutinin activity in haemolymph from *P. monodon* (Sritunyalucksana *et al.*, 1999). So far it is unclear whether *Hematodinium* sp. possess surface carbohydrates that hosts recognition molecules identify as non-self, however agglutination studies could shed some light on whether agglutination plays a role on *Hematodinium* sp. recognition.

In further plasma physiology studies, haemocyanin concentrations were found to significantly decrease in *Hematodinium*-infected male blue crabs, *Callinectes sapidus*, however concentrations remained stable in infected females (Shields, 2003). Heavily

infected Tanner crabs *Chionoecetes bairdi* also had significantly reduced haemocyanin levels (Love *et al.*, 1996). Haemocyanin is a copper-containing protein primarily involved in carrying oxygen, however it has also been implicated in the immune response as sharing phenoloxidase-like capabilities (Adachi *et al.*, 2003; Perdomo-Morales *et al.*, 2008) and as a source of antimicrobial peptides (Destoumieux-Garzon *et al.*, 2001; Lee *et al.*, 2003; Decker and Jaenicke, 2004). Antimicrobial peptides such as astacidin from crayfish and anti-fungal peptides from shrimp have been found to have identical sequences to haemocyanin and are believed to be derived from the haemocyanin peptide by proteolytic cleavage in the presence of PAMPs (pathogen-associated molecular patterns) such as LPS and $\beta$1,3-glucans (Destoumieux-Garzon *et al.*, 2001; Lee *et al.*, 2003).

Several other physiological studies have examined parameters that affect whole animal health and thereby have indirect effects on host immunity. Haemocyanin constitutes up to 90% of haemolymph proteins and, as expected due to the decrease in haemocyanin in infected animals, male blue crabs had significantly reduced total serum proteins during heavy *Hematodinium* infection (Shields, 2003). Shields (2003) suggested that this may have been the result of the log-phase growth period of *Hematodinium* rapidly consuming *Callinectes sapidus* haemolymph components. Interestingly, these responses were only evident in male crabs, females showed no significant change in haemocyanin or total serum protein concentration (Shields, 2003) and *N. norvegicus* abdominal muscle protein concentrations also remained stable in both sexes (Stentiford *et al.*, 2000). Female Tanner crabs *Chionoecetes bairdi*, however, were reported to respond similarly to male blue crabs and had reduced haemocyanin levels during heavy infection (Love *et al.*, 1996). Disruption of the hepatopancreatic tissue, where haemocyanin is synthesised, has frequently been observed during pathological studies of infected animals and could be a cause for the reduction in haemocyanin levels.

Carbohydrate levels have frequently been studied as an indicator of physiological stress in crustaceans in response to infection and other external stressors such as hypoxia and emersion (Oliveira *et al.*, 2001; Ridgway *et al.*, 2006). In *Hematodinium*-infected crustaceans, studies have been made on glucose and glycogen concentrations (Love *et al.*, 1996; Stentiford *et al.*, 2000, 2001*c*; Shields, 2003) as well as the crustacean hyperglycemic hormone (CHH) (Stentiford *et al.*, 2001*c*). Glucose and glycogen are important energy reserves for crustaceans. Glucose is a simple sugar used as the primary energy source for cells and is generated from dietary carbohydrates. The conversion of glucose to pyruvate via the glycolytic pathway in the cell's cytosol releases free energy which is retained in the form of ATP (adenosine triphosphate). When glucose is plentiful it is stored as glycogen (a homopolymer of glucose), primarily in the hepatopancreas, and released when haemolymph glucose levels are low

(Berg *et al.*, 2007). In *Nephrops norvegicus* infected with *Hematodinium* sp. haemolymph glucose was significantly reduced when compared to uninfected animals (Stentiford *et al.*, 2001*c*). Conversely, Hudson (1995) reported an increase in glucose in *Hematodinium*-infected *Portunus pelagicus*, however the report was based on a single infected animal compared to the 'normal' range of several uninfected animals and can not be considered a representative sample. Tissue glycogen levels from both muscle tissue and the hepatopancreas were reduced in both *C. sapidus* and *N. norvegicus* during *Hematodinium* infection (Stentiford *et al.*, 2001*c*; Shields, 2003). Furthermore, CHH levels in infected *N. norvegicus* steadily increased with increased infection (Stentiford *et al.*, 2001*c*). CHH is a neuropeptide responsible for activating the release of glycogen from storage and is triggered by plasma glucose levels (Fanjul-Moles, 2006). Despite the increasing CHH levels of infected animals, glucose and glycogen levels continued to drop and Stentiford *et al.* (2001*c*) suggested that it is likely the parasites consume glucose and so interfere with the negative feedback system for CHH. Alternatively, Stentiford *et al.* (2001*c*) suggested that increased levels of CHH before moulting cause an increase in plasma glucose levels providing an ideal growth medium for parasites during which they proliferate extensively and overcome their host. Thus, the parasite could either be the cause of CHH increase or as a result of CHH increase.

Further reported signs of metabolic stress have included changes in enzyme constituents (Shields, 2003), and free amino acid availability (Stentiford *et al.*, 1999, 2000). Acid phosphatase levels in haemolymph of infected *C. sapidus* were significantly higher than uninfected animals, as was napthol AS-BI phosphohydrolase and $\beta$-galactosidase (Shields, 2003). $\alpha$-fucosidase was also reported to be higher in uninfected animals. Stentiford and Shields (2005) suggested that these biochemical changes are likely due to a collapse of metabolic homeostasis and may be markers of virulence. Total free amino acid profiles of haemolymph and deep abdominal flexor muscle of *N. norvegicus* were significantly increased in late stage *Hematodinium* infection (Stentiford *et al.*, 1999, 2000). Specifically, taurine and serine were increased as infection increased, associated with, and likely due to, the release of amino acids as tissues and haemocytes degraded during the progress of infection. Taurine is particularly linked with hepatopancreatic tissue which is known to suffer severe degradation during infection (Meyers *et al.*, 1987; Field *et al.*, 1992; Stentiford *et al.*, 2002; Xu *et al.*, 2010) and, as serine is required to synthesise taurine, it is probable the levels of both are linked (Stentiford *et al.*, 1999).

In addition to metabolic stress, respiratory stress is equally affected by *Hematodinium* infection. Oxygen consumption of infected *N. norvegicus* increased with infection stage, though the carrying capacity was decreased. A simultaneous increase in lactate concentration and haemolymph acidosis was also observed in stage III and IV infected

animals, likely in response to hypoxia caused either by high oxygen demand by the increasing parasite load, occluded haemal spaces or reduced haemocyanin levels (Taylor *et al.*, 1996). In contrast, lactate levels of the hermit crab *Pagurus bernhardus* were found to decrease during *Hematodinium* infection and were correlated with an increase in haemolymph pH, while no change in lactate levels was apparent in *C. pagurus* and *C. maenas* (Hamilton *et al.*, 2010). It is possible the discrepancy between species lies with infection stage as Hamilton *et al.* (2010) did not report infection stage for tested animals; stage I and II infections for *N. norvegicus* had no significant change in lactate or pH levels (Taylor *et al.*, 1996).

### 5.1.3 Immune Response to Pathogens and Parasites

As discussed in Chapter 1.7, invertebrates have a suite of innate immune functions to respond to internal invasion by foreign material. Haemocytes are generally one of the first indicators of changes to immune function as they are the primary acting body of internal defense in invertebrates. Changes in haemocyte counts, either up or down, are known to occur during bacterial challenge, parasite infection, stress and other physiological changes such as moulting (Le Moullac *et al.*, 1998; Chen and Cheng, 2001; Humbert and Coustau, 2001; Cheng *et al.*, 2002; Song *et al.*, 2003; Battison *et al.*, 2004; Sarathi *et al.*, 2007; Li *et al.*, 2008*a*). Prawns *Macrobrachium rosenbergii* had reduced total haemocyte numbers following injection with *Enterococcus* and exhibited a slow recovery compared with those injected with saline (Chen and Cheng, 2001). Similar results were found in white shrimp *Litopenaeus vannamei* following injection with *Vibrio alginolyticus* (Li *et al.*, 2008*a*) and lobsters *Homarus americanus* inoculated with the Gram-positive bacteria *Aerococcus viridans*, however in lobsters haemocyte counts did not recover and the animals ultimately died (Battison *et al.*, 2004). The gammarid intermediate host *Gammarus pulex* infected with the acanthocephalan parasites *Pomphorhynchus laevis*, *Polymorphus minutus* and *Pomphorhynchus tereticollis* were all found to have significantly reduced haemocyte numbers (Cornet *et al.*, 2009). Tussock moths parasitised with braconid wasp larvae *Cotesia melanoscela* also had reduced haemocyte numbers combined with suppression of nodulation and encapsulation (Guzo and Stoltz, 1987). In contrast, however, total haemocyte counts of the soybean looper moth *Pseudoplusia includens* were increased during infection with another braconid wasp *Microplitis demolitor*, although spreading behaviour of the haemocytes was impaired causing a reduction in the encapsulation response (Strand and Noda, 1991). Haemocyte count has been been associated with improved defense against parasites in several *Drosophila* species. *Drosophila* spp. with naturally lower total haemocyte counts were found to have a reduced ability to

encapsulate parasitoid eggs (Eslin and Prevost, 1998). The key roles of haemocytes in internal defense, both in terms of direct action against foreign material (phagocytosis and encapsulation) as well as the production of anti-pathogenic molecules (prophenoloxidase, superoxide production, etc.), make haemocyte concentration a primary study parameter for immunocompetence during infection.

Haemocyte numbers have been observed to be reduced in several *Hematodinium*-infected host species based on haemolymph smears and pathology (Hudson and Shields, 1994; Messick, 1994; Wheeler *et al.*, 2007; Xu *et al.*, 2010), however only a few studies have measured total haemocyte numbers in response to infection (Field and Appleton, 1995; Love *et al.*, 1996; Shields and Squyars, 2000; Walker *et al.*, 2009). Specific investigations of total haemocyte counts have generally found that increased parasite number resulted in reduced haemocyte numbers (Field and Appleton, 1995; Love *et al.*, 1996; Shields and Squyars, 2000). In one exception of experimental infection by cannibalism, haemocyte numbers were found to initially increase during the first 16 h post-infection, however numbers decreased substantially throughout disease progression (Walker *et al.*, 2009).

The haemocyte-mediated phenoloxidase system plays a key role in crustacean internal defense and has come to be viewed as an important measure of immunocompetence (Sritunyalucksana and Soderhall, 2000). Phenoloxidase (PO) activity is well-characterised in crustaceans and other invertebrates in response to numerous bacteria, parasites and fungi, as well as to pathogen cell wall components (Smith and Soderhall, 1983; Cardenas and Dankert, 1997; Perazzolo and Barracco, 1997; Franssens *et al.*, 2008; Xian *et al.*, 2009). Black tiger prawns, *Penaeus monodon* and white shrimp *Litopenaeus vannamei* had significantly increased PO activity in response to lipopolysaccharide (LPS) (Sritunyalucksana *et al.*, 1999; Xian *et al.*, 2009) and peptidoglycan (PG) (Sritunyalucksana *et al.*, 1999), while shore crabs *Carcinus maenas* responded with a four-fold increase in PO activity following $\beta$1,3-glucan challenge (Smith and Soderhall, 1983). Similarly, the desert locust *Schistocerca gregaria* and the shrimp *Penaeus paulensis* had increased PO activity following injection with LPS and laminarin (Perazzolo and Barracco, 1997; Franssens *et al.*, 2008). However, PO activity is not always enhanced in the presence of invading pathogens and some parasites have adapted to be able to inhibit the PO activating pathway (Strand and Noda, 1991). Phenoloxidase activity was suppressed in the soybean looper moth *Pseudoplusia includens* larvae when infected with the braconid parasite *Microplitis demolitor* (Strand and Noda, 1991). Similar results were found in the blackfly *Simulium ornatum* in response to the filarial parasite *Onchocerca lienalis*; sham-injected flies showed much higher PO activity than infected flies in comparison to non-injected control flies, suggesting a suppression of PO activity by the parasite (Hagen *et al.*, 1994).

Parasites have also been implicated in suppression of superoxide production in invertebrate hosts (Remaley *et al.*, 1984). Intracellular superoxide anion assays using the reduction of nitroblue tetrazolium (NBT) have become a common immune assay for numerous invertebrates to measure the production of superoxide in response to pathogens (Muñoz *et al.*, 2000; Li *et al.*, 2008*a*; Jayasree, 2009). Superoxide anions are produced as part of the respiratory burst and are generally associated with the breakdown of foreign material during phagocytosis and in crustaceans superoxide has been specifically associated with hyaline cells (Bell and Smith, 1993). Numerous hosts have been observed to increase superoxide production in response to pathogens, particularly in response to *Vibrio* spp. (Li *et al.*, 2008*a*; Jayasree, 2009). Two shrimp species *Litopenaeus vannamei* and *Penaeus indicus* increased superoxide production in response to *Vibrio* both *in vivo* and *in vitro* (Muñoz *et al.*, 2000; Li *et al.*, 2008*a*; Jayasree, 2009). In the shore crab *Carcinus maenas* Hauton *et al.* (1997*b*) used hydrogen peroxide ($H_2O_2$), an intermediate in the superoxide reduction pathway, as an indicator of respiratory burst in response to sublethal bacterial infection with *Listonella anguillarum*. Crabs were found to have increased $H_2O_2$ seven days post-infection which suggested increased phagocytosis of the invading bacteria.

Reports on various pathogens have suggested that superoxide production can be modulated by acid phosphatase (AP), an enzyme secreted by many parasites (Remaley *et al.*, 1984) and identified in *Hematodinium* from *C. sapidus* and *N. norvegicus* (Shields *et al.*, 2003; Small *et al.*, 2007*a*). Shields *et al.* (2003) initially reported the presence of acid phosphatase in *Hematodinium* sp. ex-*C. sapidus* and determined that the enzyme was primarily located intracellularly as it was found in whole haemolymph samples from infected animals but was absent from cell-free sera. Further investigation into AP localisation in *Hematodinium* sp. revealed that, in *N. norvegicus* AP was present in parasite granules and in the nuclear membrane, however high levels were also found in parasite culture media indicating that the parasite was secreting AP (Small *et al.*, 2007*a*).

### 5.1.4 Aim of immune assay studies on *N. norvegicus*

Previous *in vitro* results found that immune-related gene expression in separated granulocyte and hyaline cells from *Cancer pagurus* was not affected by *Hematodinium* sp. challenge, however there is still a need to explore the *in vivo* response of host animals to parasite infection. In this study, the primary aim was to determine the *in vivo* effects of *Hematodinium* sp. infection on various immune parameters of wild-caught *Nephrops norvegicus* from the Clyde Sea Area. Total and differential haemocyte counts were examined to give an indication of immune competence, and

phenoloxidase activity and superoxide anion production were used to gain knowledge regarding the immune response towards invading parasites. Haemagglutination assays were also used to study changes in agglutinating properties between infected and control animals. The null-hypothesis being tested was that *Hematodinium* sp. infection would have no significant effect on immune parameters compared to control animals. The previous lack of modulation in gene expression suggested that *Hematodinium* sp. does not illicit an immune response, however *in vitro* limitations demand further investigation into the natural state of host-parasite interaction.

## 5.2 Materials and Methods

### 5.2.1 Animals

*Nephrops norvegicus* were collected by trawl from the Clyde Sea (55° 46.8 N, 4° 58.5 W to 55° 44.8 N, 4° 59.2 W) from a depth between 80 and 100 m in late March 2011. Heavily infected animals were identified by carapace colour (hyperpigmentation) and opaque joints, and were separated from healthy looking individuals on board the RV *Actinia*. A random selection of healthy animals with an undamaged carapace were also collected for comparison. All animals were maintained in flowing seawater aboard the vessel until arrival at the University Marine Biological Station, Millport where they were kept in flowing seawater aquaria until needed. Sex and carapace length were recorded for each individual sampled.

### 5.2.2 Haemolymph collection, haemocyte and *Hematodinium* sp. cell counts

Haemolymph (0.75 ml) was extracted from the base of the fifth pereiopod into 0.75 ml ice cold marine anticoagulant (see 4.2.2) using a 25G needle and 2 ml sterile syringe. From this, 0.5 ml was placed in a 1.5 ml eppendorf tube and centrifuged at 300 $g$ to generate a cell pellet for storage in Ambion RNALater® (Applied Biosystems, Cheshire, UK) for use in qPCR analysis (see 6.2). A further 10$\mu$l was removed and examined using an improved Neubauer Haemocytometer under phase contrast. Total haemocyte counts and *Hematodinium* sp. counts were recorded for each animal.

Infection status was determined using cell counts of *Hematodinium* sp. cells present in the haemolymph. Animals were separated into 'infected' (*Hematodinium* sp. $> 35\%$ of total cell count) and 'control' animals (no *Hematodinium* sp. cells or $< 10\%$ total cell count). As discussed in Chapter 1.3.3, attempts have been made to quantify

*Hematodinium* sp. load using qPCR (Nagle *et al.*, 2009), however the efficiency (E) of the qPCR assay was not sufficient to support reliable quantification values (Chapter 3.4.1.3).

### 5.2.3 Phenoloxidase Assay

Phenoloxidase activity was determined according to Hauton *et al.* (1995) (modified from Smith and Soderhall (1983)) in which the intracellular inactive zymogen prophenoloxidase was activated using trypsin, with L-dopa as substrate. Haemocyte lysate supernatant (HLS) was generated by centrifuging 1 ml of haemolymph/anticoagulant at 300 *g* for 15 min at 4°C. The supernatant was removed and the cell pellet washed twice with 1 ml sodium cacodylate citrate buffer (10 mM sodium cacodylate, 0.45 M sodium chloride, 100 mM trisodium citrate). Cell pellets were then disrupted using a tissue pestle and 1.5 ml sodium cacodylate buffer (10 mM sodium cacodylate, 150 mM sodium chloride, 10 mM hydrated calcium chloride) was added to each sample. Samples were centrifuged again at 1000 *g* for 25 min at 4°C and the resulting supernatant was designated HLS. HLS (400 $\mu$l) was placed in two separate 1.5 ml eppendorf tubes. To one tube 400 $\mu$l 0.1% trypsin (from beef pancreas; Fisher Scientific, Loughborough, UK) was added and to the other 400 $\mu$l of sodium cacodylate buffer. A control containing 400 $\mu$l 0.1% trypsin and 400 $\mu$l sodium cacodylate buffer was also made. All samples and controls were incubated at 17°C for 1 hr. Following incubation, 400 $\mu$l freshly prepared L-DOPA (3,4-dihydroxy-L-phenylalanine; Sigma-Aldrich, Dorset, UK) was added to each sample and the samples read at 490 nm on a Thermo Spectronic He$\lambda$ios $\gamma$ spectrophotometer (Thermo Fisher, Loughborough, UK). Readings were taken at time 0 and at 5 min. Phenoloxidase activity was expressed in units 100 $000^{-1}$ haemocytes, where one unit represented an increase in absorbance of 0.001 $min^{-1}$.

### 5.2.4 Intracellular Superoxide Anion Assay

Intracellular superoxide anion activity was measured according to the assay developed by Pipe *et al.* (1995). In each of two 1.5 ml eppendorf tubes, 200 $\mu$l of haemolymph was centrifuged at 300 *g* for 10 min at 4°C. While the samples were centrifuging, nitroblue tetrazolium (NBT; Sigma-Aldrich, Dorset, UK) solution was made by dissolving 1 mg NBT in 5 ml TBS (0.05M Tris/HCl, 0.034M NaCl) and then filtered using a Nalgene 0.45 $\mu$m syringe filter (Thermo Fisher, Loughborough, UK). The supernatant was removed from all samples and 100 $\mu$l TBS added to each cell pellet. Each of the two replicate pellets received separate treatments, either 100 $\mu$l NBT (baseline) or 100 $\mu$l

NBT + 300U $ml^{-1}$ superoxide dismutase (inhibited) (SOD from horseradish; Sigma-Adlrich, Dorset, UK). Cell-free controls consisted of 100 $\mu$l NBT + 100 $\mu$l TBS. All samples and controls were incubated for 1 hr at room temperature in the dark. Following incubation, all samples were centrifuged at 300 *g* for 10 min at 4°C. The supernatant was removed and all samples were fixed in 200 $\mu$l 100% methanol for 10 min in the dark; samples were then centrifuged at 600 *g* for 5 min. Cell pellets were washed two times with 70% methanol, and centrifuged at 1000 *g* for 5 min in between each. All methanol was removed from each cell pellet and the pellets resuspended in 140 $\mu$l dimethyl sulfoxide (DMSO; Sigma-Aldrich, Dorset, UK) and 120 $\mu$l 2M potassium hydroxide. Samples were loaded onto a 96-well plate and read at 620 nm using a Labsystems Multiskan RC plate reader (Fisher Scientific, Loughborough, UK). Superoxide data were expressed as units 1 000 000 $cells^{-1}$, where one unit represented a difference in optical density of 0.001 between baseline and inhibited values.

### 5.2.5 Haemagglutination Assay

Haemagglutination is the agglutination of erythrocytes, or red blood cells (RBCs), and is used to study the agglutinating properties of serum or haemocyte lysate (HLS) components (Smith and Soderhall, 1983; Fragkiadakis and Stratakis, 1997). The haemagglutinin assay for this study was carried out on haemocyte lysate supernatant (HLS) using a protocol modified from Smith and Soderhall (1983). Cell pellets from infected and control animals were generated by centrifuging 1 ml of haemolymph:marine anticoagulant (1:1) at 300 *g* for 10 min at 4°C. The supernatant was removed and the pellets stored at -80°C until required. HLS was made by adding TBS to each sample (TBS volume was adjusted to have equivalent haemocyte concentrations across all samples) and the pellet disrupted using a tissue pestle. The samples were then centrifuged at 16000 *g* for 10 min at 4°C. Fifty microlitres of each sample was placed in the first well of a round-bottomed Sterilin® 96-well plate (Fisher Scientific, Loughborough, UK) and then serially diluted 1:1 using TBS. Chicken red blood cells (RBCs) in Alsever's (SeraLab, West Sussex, UK) or glutaraldehyde-fixed sheep RBCs (Fisher Scientific, Loughborough, UK) were washed three times in TBS (centrifuged 1000 *g* for 5 min at 4°C) then diluted to 3% v/v. Each HLS sample had 50 $\mu$l RBC added to each well and the plates were incubated at room temperature. The plates were examined at 2 h and 24 h for signs of agglutination and the haemagglutinin titre recorded as the reciprocal of the highest dilution to cause agglutination of RBCs.

### 5.2.6 Data Analysis

Where data were normally distributed and had equal variance, analysis was carried out using t-tests to compare differences between infected and control *Nephrops norvegicus*. Phenoloxidase activity data were transformed ($log\ x + 1$) to produce a normal distribution and equal variance while total haemocyte data were square root transformed. Differential haemocyte counts (DHC) were expressed as percent (%) hyaline cells and were not normally distributed so were analysed using a Mann-Whitney Rank Sum test. All tests were carried out using SigmaStat v. 3.5 (Systat Software, Inc.; San Jose, CA).

## 5.3 Results

### 5.3.1 Haemocyte count

Total haemocyte counts (THCs) were significantly decreased in *N. norvegicus* infected with *Hematodinium* sp. ($p < 0.001$). THCs in infected individuals decreased to 48% of THCs in control animals. Differential haemocyte counts (DHCs; expressed as % hyalinocytes), however, were not found to be significantly different between infected and control individuals ($p = 0.508$) (Fig 5.1).

### 5.3.2 Phenoloxidase Assay

Phenoloxidase activity was measured as the change in optical density over a five minute incubation period. Phenoloxidase activity per 100,000 haemocytes was significantly increased in *N. norvegicus* infected with *Hematodinium* sp. ($p = 0.041$) (Fig 5.2). Examination of a correlation effect between phenoloxidase activity and *Hematodinium* count revealed no significant correlation ($p > 0.05$; Pearson correlation, data not shown).

### 5.3.3 Superoxide Anion Assay

There was no significant difference in superoxide activity between control *N. norvegicus* and those infected with *Hematodinium*, though the variability was much higher in infected animals (SD = 23.0) compared with control animals (SD = 6.0) (Fig 5.3). Further investigation revealed that superoxide activity was negatively correlated with *Hematodinium* load (correlation coefficient = -0.775, $p = 0.005$; Pearson correlation) (Fig 5.4).

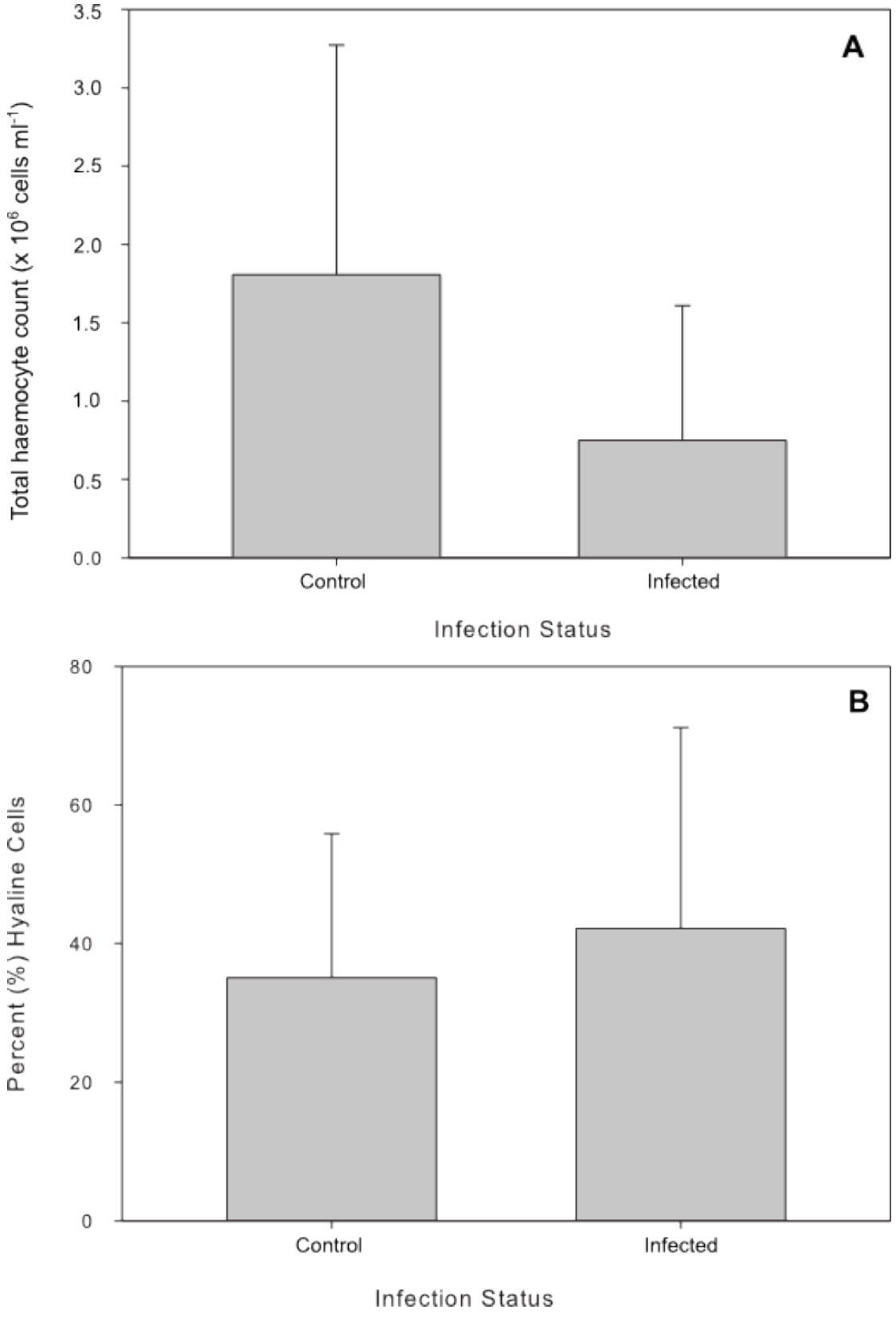



FIGURE 5.1: (A) Difference in total haemocyte counts between control *N. norvegicus* and those infected by *Hematodinium* expressed as mean ± SD of $n$ observations ($n$ = 50 uninfected; 33 infected); total haemocytes were significantly decreased in infected animals ($p < 0.001$). (B) Difference in percent (%) hyaline cells (differential cell count) between control and infected *N. norvegicus* expressed as mean % ± SD ($n = 46$ uninfected; 25 infected); difference not significant between infected and control individuals ($p > 0.05$).

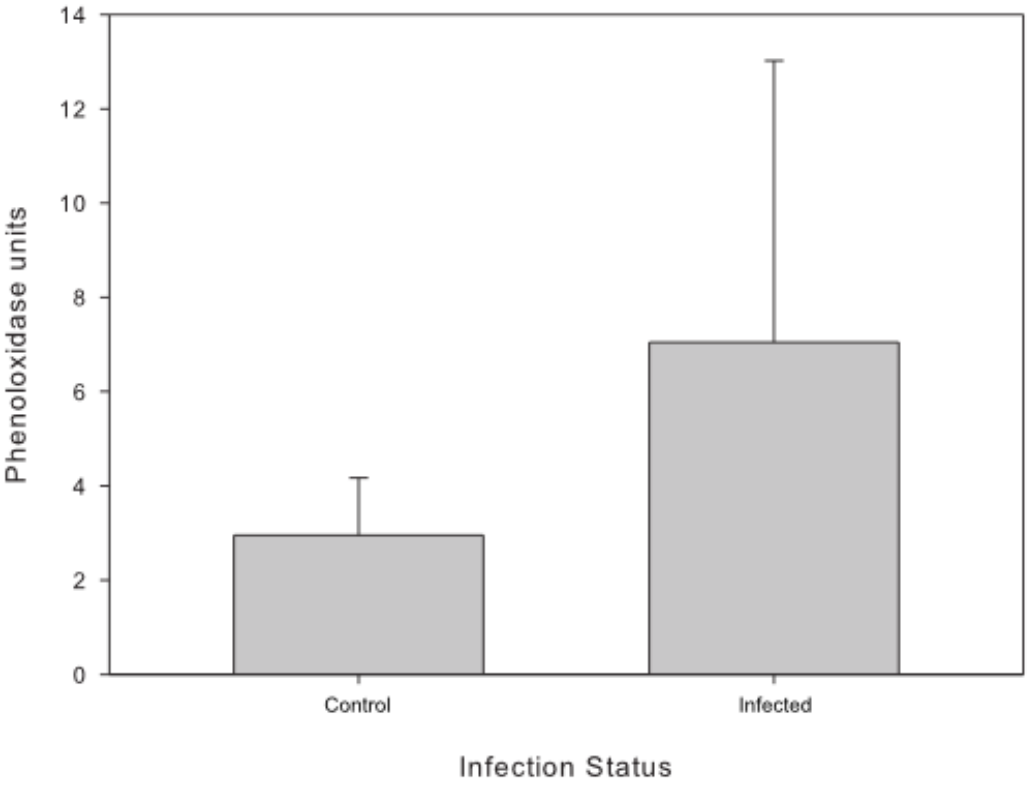



FIGURE 5.2: Difference in phenoloxidase activity between control *N. norvegicus* and those infected with *Hematodinium* expressed as mean change in units per 100 000 haemocytes ± SD of *n* observations (n = 11 uninfected; 11 infected). The difference between control and infected animals was significant (p = 0.041; *t*-test of log-transformed data)

### 5.3.4 Haemagglutination Assay

*N. norvegicus* haemocyte lysate supernatant was able to agglutinate chicken red blood cells (RBCs), however had no response towards glutaraldehyde-treated sheep RBCs. High titres (1:128) were observed in some infected individuals, however there was no significant difference between mean titres of control versus infected animals. The variation in titres was much more broad for infected animals than control animals with SD = 53 (infected) compared to SD = 20 (control) (Fig 5.5).

## 5.4 Discussion

This study represents the first examination of direct immune response of a crustacean host to the dinoflagellate parasite *Hematodinium* sp. using phenoloxidase activity and superoxide anion assay in addition to haemocyte counts and agglutination response.

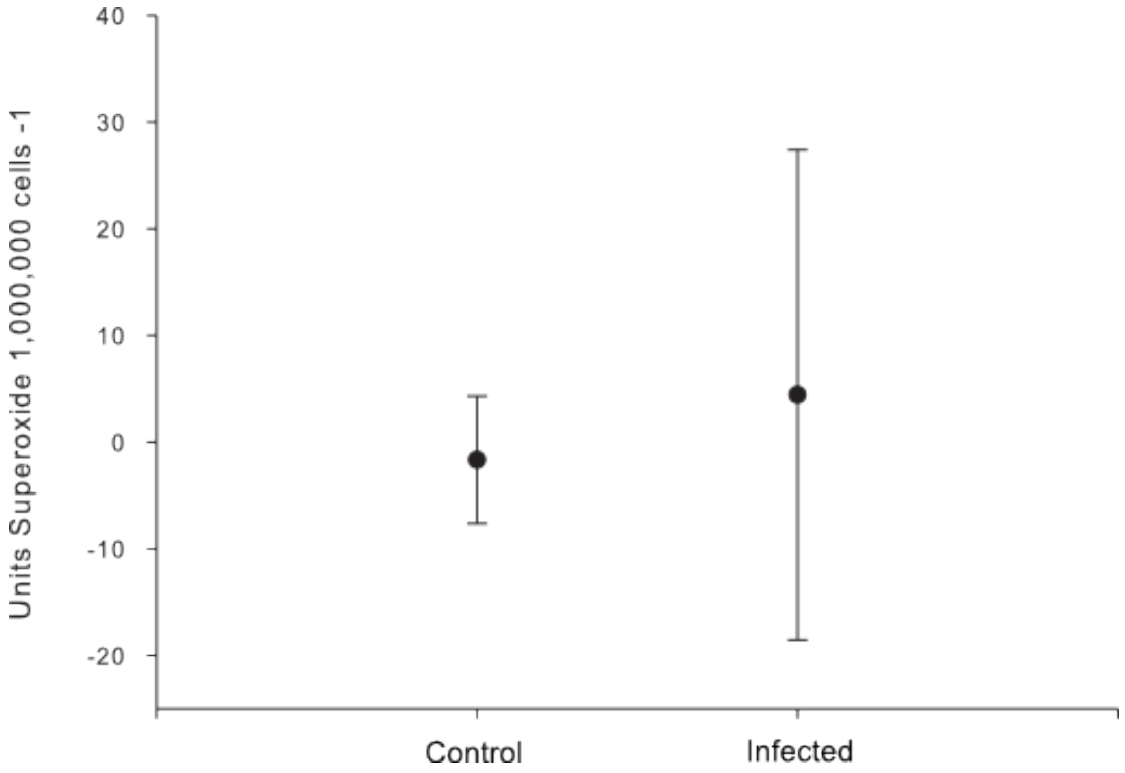



FIGURE 5.3: Difference in superoxide activity between control *N. norvegicus* and those infected with *Hematodinium* expressed as mean change in units per 1 000 000 haemocytes ± SD of $n$ observations ($n = 12$ uninfected; 12 infected). There was no significant difference ($p = 0.346$ t-test), however the standard deviation in the infected animals (SD = 23.0) is much greater than that from the control animals (SD = 6.0).

Phenoloxidase activity was observed to increase in *Nephrops norvegicus* infected with *Hematodinium* despite a considerable decrease in total haemocyte counts. No significant differences were observed in superoxide anion production or haemagglutination titres between infected and control animals though the variability of response was clearly increased in infected individuals. Superoxide anion production was, however, correlated with *Hematodinium* sp. count in infected animals. The combination of these results indicate that immune response, while potentially compromised by reduced haemocytes, is not entirely defunct during parasite infection.

### 5.4.1 Haemocyte Counts

Total haemocyte counts (THCs) in this study were found to significantly decline in infected *N. norvegicus* compared to control animals. Haemocyte numbers have frequently been associated with the ability of invertebrates to fight infection. *Nephrops norvegicus* exposed to sublethal doses of manganese (Mn) were found to have reduced total haemocyte counts and consequently were found to have significantly lower PO activity in response to lipopolysaccharide injection (Hernroth *et al.*, 2004). When

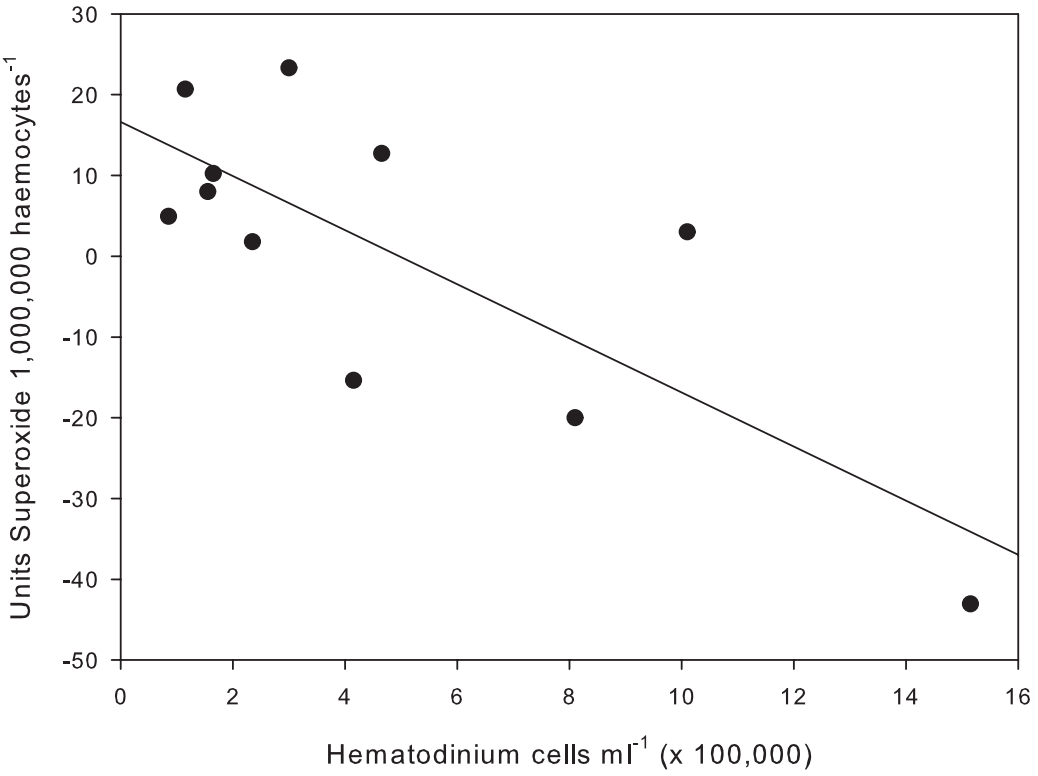



FIGURE 5.4: Correlation between superoxide activity and *Hematodinium* load in infected *N. norvegicus* of $n$ observations ($n$ = 11). Superoxide activity was negatively correlated with parasite load (correlation coefficient = -0.775, p = 0.005; Pearson correlation)

larvae from six *Drosophila* species infected with the parasitoid *Asobara tabida* were compared, four of them responded with increased THCs and it was found that those with the highest haemocyte load were the most successful at encapsulating parasitic eggs (Eslin and Prevost, 1998). Species with the most successful encapsulation rates also had increased lamellocytes (cells specifically functioning in encapsulation), while individuals from species with low haemocyte concentrations were inefficient at encapsulation. Interestingly, although *D. melanogaster* larvae had increased lamellocytes, they were unable to mount a successful encapsulation response. Larvae from *D. yakuba*, *D. teissieri* and *D. simulans*, however, were able to both react and successfully encapsulate the parasites. Eslin and Prevost (1998) suggested that successful defense was based on both the ability to respond to parasite infection by increasing haemocyte numbers as well as to cross a minimum threshold of haemocyte count in order to have sufficient haemocytes for encapsulation. Of the limited observations of immune response of crustacean hosts to *Hematodinium* infection, encapsulation and nodule formation make up the majority of the reports. It is possible that, similar to the *Drosophila* species which mount insufficient haemocyte responses

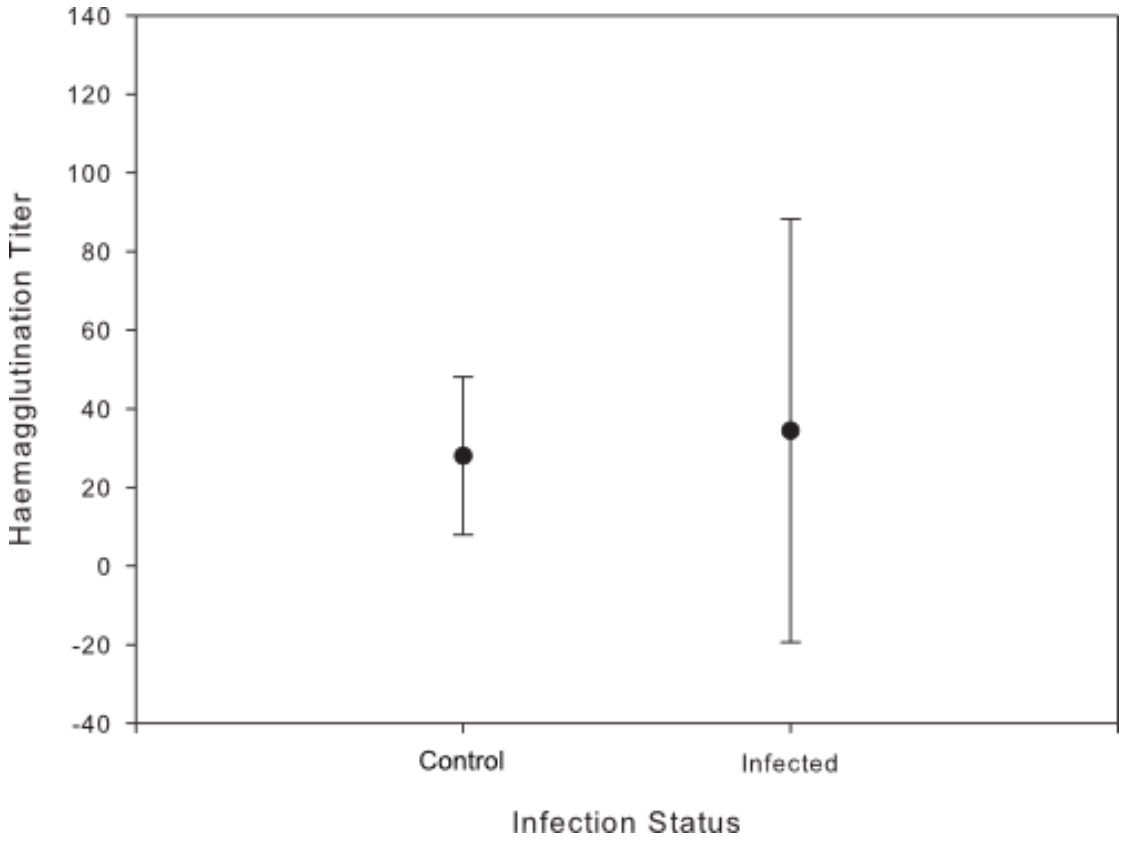



FIGURE 5.5: Difference in haemagglutination between control *N. norvegicus* and those infected with *Hematodinium* expressed as mean of the inverse titer ± SD of $n$ observations ($n = 7$ uninfected; 4 infected). Titre means were similar between infected and control animals, however standard deviation of infected animals was greater than in control animals.

to parasitoids, many crustaceans are incapable of reaching the necessary threshold to successfully encapsulate *Hematodinium* cells.

To date, no studies have reported a definitive increase in total haemocyte counts in *Hematodinium* hosts and, indeed, most studies have reported haemocytopaenia even when not specifically examining haemocyte counts (Hudson and Shields, 1994; Messick, 1994; Wheeler *et al.*, 2007; Walker *et al.*, 2009; Xu *et al.*, 2010). Shields and Squyars (2000) recorded the first haemocyte count following infection in *Callinectes sapidus* three days post-injection with *Hematodinium* and haemocyte numbers were already significantly decreased; THCs remained depressed for the remainder of the infection until death. Similarly, THCs were reduced in *Hematodinium*-infected *N. norvegicus* and *Chionoecetes bairdi* (Field and Appleton, 1995; Love *et al.*, 1996). Walker *et al.* (2009) gave the only report of increased haemocytes in haemal spaces of *Cal. sapidus* three and six hours after being fed *Hematodinium*-infected tissue, however the observation was based on histology rather than quantitative data. Further investigation into early stage infection status of host haemocyte counts may yield more

information on initial haemocyte response. Unfortunately, attempts to experimentally infect *Nephrops norvegicus* and *Cancer pagurus* by passage of infected haemolymph or *Hematodinium* in culture were unsuccessful during this study, therefore early-stage infection and disease progression were not examined here.

In this study, differential cell counts did not change significantly between control and infected *N. norvegicus*, which is in contrast to observations by Shields (1999) in which granulocyte populations increased in infected *Callinectes sapidus*. Shields (1999) suggested that the change in proportions could either be due to a proliferative response in granulocytes specifically, or a result of sequestration of semigranulocytes and hyaline cells for encapsulation or other defensive response. As previously mentioned, encapsulation has been observed in *Hematodinium*-infected animals (Meyers *et al.*, 1987; Field and Appleton, 1995; Stentiford *et al.*, 2002; Sheppard *et al.*, 2003; Walker *et al.*, 2009) and it is possible hyaline cells were removed from circulation as a result. The study by Shields (1999) had the advantage of observing animals over the progression of the disease, while the present study examined naturally infected individuals with heavy parasite loads. It is possible that fluctuations of haemocyte populations as a direct immune response were more evident during subpatent infection and therefore were missed in this study.

### 5.4.2 Phenoloxidase activity

This study found significantly increased intracellular stores of prophenoloxidase (hereafter termed phenoloxidase (PO) activity) in the haemocyte lysate supernatant (HLS) from *Hematodinium*-infected *N. norvegicus*. The increase in activity despite the significant decrease in total haemocyte counts suggests an enhanced immune response in infected individuals compared to lightly and uninfected control animals. To further emphasise the importance of the increased PO activity, physiological stress such as starvation and hypoxia, which have been reported in *Hematodinium*-infected animals (Taylor *et al.*, 1996; Stentiford *et al.*, 2001*c*), has generally been associated with reduced immunocompetence in the form of reduced PO activity. For example, juvenile *Litopenaeus vannamei* that were starved for 21 days were found to have reduced PO activity (Pascual *et al.*, 2006), and spiny lobsters *Panulirus homarus* had reduced PO enzyme concentrations in the serum following seven days of starvation (Verghese *et al.*, 2008). Experimentally-induced hypoxia resulted in significantly decreased PO activity in *L. vannamei* during the first 12-24 hours in hypoxic conditions, which then stabilised at reduced levels for the remainder of the experiment (up to 72 hrs) (Hu *et al.*, 2009). In spite of these observations and previous reports of physiological starvation and

functional hypoxia in *Hematodinium* hosts, the observed increase in PO activity in the present study make a clear case for a competent response from the proPO system.

Generally, bacterial infection and injection with bacterial components have resulted in increased PO activity, measured both as enzyme activity and as change in gene expression (Gai *et al.*, 2008; Rodriguez-Ramos *et al.*, 2008; Chen *et al.*, 2010*b*). Increased PO activity has been reported for southern white shrimp *Litopenaeus schmitii* following injection with *E. coli* LPS with a concurrent drop in total haemocyte counts (Rodriguez-Ramos *et al.*, 2008). Furthermore, increased bacterial abundance in the water column has been found to correlate with increased PO activity of HLS from *Carcinus maenas* (Hauton *et al.*, 1997*b*). Conversely, some infections have led to decreased PO activity as well. White shrimp *Litopenaeus vannamei* infected with the bacterium *Vibrio alginolyticus* had reduced PO activity in combination with reduced total haemocyte counts 24-48 hours post-injection but levels recovered to control levels after 72 hours (Li *et al.*, 2008*a*).

As previously mentioned, fluctuations in PO activity in response to parasite invasion have been variable amongst invertebrates. The PO activity of two gammarid species, *Gammarus pulex* (native) and *Gammarus roeseli* (invasive), were studied for their response towards local acanthocephalan parasites and PO activity was found to be significantly reduced in native hosts, but significantly increased in invasive hosts (Rigaud and Moret, 2003). Rigaud and Moret (2003) suggested that the parasites had adapted to their native host in order to suppress the immune response and improve the success of transmission to the final host, whereas new invasive hosts were still capable of mounting an immune response towards the parasites. Based on the observed increase in PO activity in the present study, it is evident that *Hematodinium* sp. is not inhibiting PO activity in *Nephrops norvegicus*.

As Loker (1994) pointed out in his review of invertebrate parasites, several parasite species are able to coexist with their host without causing undue harm to host haemocytes. Loker (1994) advocated that the advantage to this approach was that the parasite was still protected by the host internal defense system from opportunistic pathogens. A possible example of this in *Hematodinium*-infected animals was previously described by Stentiford *et al.* (2003) who observed *Cancer pagurus* and *Necora puber* to be co-infected with *Hematodinium* sp. and a yeast-like organism. A clear immune response was directed towards the yeast-like cells though not towards *Hematodinium* sp. cells. In addition to *Cancer pagurus* (Stentiford *et al.*, 2003), secondary infections have been observed in *Hematodinium*-infected hosts including *Nephrops norvegicus* (Field *et al.*, 1992) and *Chionoecetes bairdi* (Meyers *et al.*, 1987). Meyers *et al.* (1987) reported bacteraemia in several *Hematodinium*-infected *Ch. bairdi*

prior to death and suggested that bacterial and ciliate protozoal infection may also be a contributing factor to host mortality. Similarly, bacteria and ciliate protozoa were observed as secondary infections in *N. norvegicus* (Field *et al.*, 1992), though neither Field *et al.* (1992) nor Meyers *et al.* (1987) reported further details of the infections. A study by Ridgway *et al.* (2006) found that aerial exposure increased bacteraemia in *N. norvegicus* and functional hypoxia caused by parasite infection may contribute to the likelihood of increased secondary infection. The presence of opportunistic pathogens in *Hematodinium*-infected crustacean hosts could account for the variable response in PO activity reported for infected animals. Varying intensity of bacterial infection could create a broad distribution of PO response in infected animals. Indeed, in the present study, microbes were observed in the haemolymph of infected animals during haemocyte counts; however, due to time constraints, the nature of the concurrent infections were not examined further.

### 5.4.3 Superoxide anion activity

In this study superoxide anion production was not found to vary between *N. norvegicus* infected with *Hematodinium* compared with lightly infected and uninfected control animals, however the variation in response was much more broad in infected animals. Further investigation revealed a significant negative correlation between parasite load and superoxide production (ie. increased *Hematodinium* sp. load was correlated with a decrease in intracellular superoxide anion production.) Previous studies have implicated the secretion of acid phosphatase (AP) by *Hematodinium* sp. ex. *N. norvegicus* as a possible mechanism of immune suppression (Small *et al.*, 2007*a*). AP secreted by the bacteria *Coxiella burnetti* and *Francisella*, the causative agents of Q fever and tularemia in humans, has been found to inactivate NADPH oxidase (nicotinamide adenine dinucleotide phosphate-oxidase) which prevents the generation of reactive oxygen species (Baca *et al.*, 1993; Siemsen *et al.*, 2009; Mohapatra *et al.*, 2010; Hill and Samuel, 2011). The present results suggest a dose-dependent response between parasite load and superoxide (SO) inhibition, and Small *et al.* (2007*a*) found that increased concentrations of *Hematodinium* sp. in culture resulted in an increase in AP concentrations in the culture media.

Previous studies have shown evidence of the importance of superoxide anion production to immune response in pathogen-challenged crustaceans and have generally indicated an increase in superoxide anions in response to bacterial challenge (Muñoz *et al.*, 2002; Sarathi *et al.*, 2007; Jayasree, 2009). Haemocytes from the Indian white shrimp *Penaeus indicus* were found to have increased intracellular superoxide production in response to challenge with *Vibrio alginolyticus*, *V. parahaemolyticus* and *V.*

*anguillarum* in a dose-dependent manner (ie. increased bacterial concentration resulted in increased SO production) (Jayasree, 2009). Similar results were found for *Penaeus vannamei* haemocytes exposed to *V. alginolyticus* and *V. anguillarum* (Muñoz *et al.*, 2002). Sarathi *et al.* (2007) reported increased intracellular SO anion activity in *Fenneropenaeus indicus* in response to *Vibrio* and white spot syndrome virus (WSSV) and, although activity was not reported per cell or per mg protein, the concurrent decrease in total haemocyte counts suggest that the SO increase was genuine.

In contrast to bacteria, several parasite species, have been found to interfere directly with superoxide anion production in their hosts (Remaley *et al.*, 1984). The intracellular protozoan parasite *Perkinsus marinus* has been implicated in the reduction of reactive oxygen species (ROS) in haemocytes from the Eastern oyster *Crassostrea virginica* (Volety and Chu, 1995; Anderson, 1999*a*,*b*) and, although the exact mechanism has yet to be elucidated, *P. marinus* were found to secrete high concentrations of acid phosphatase which was suggested to play a primary role in ROS inhibition (Volety and Chu, 1997). Similar results were found in the clam *Ruditapes decussatus* and the mussel *Mytilus galloprovincialis* in response to *Perkinsus atlanticus* though, again, the exact mechanism of inhibition has not been conclusively defined (Ordas *et al.*, 1999). *Bonamia ostreae* has also been found to modulate the production of reactive oxygen species of haemocytes from the flat oyster *Ostrea edulis in vitro* (Morga *et al.*, 2009). The mammalian protozoan parasite *Leishmania* is particularly well-known for it's use of acid phosphatase to inhibit reactive oxygen species in neutrophils of mammals (Remaley *et al.*, 1984). Although the use of acid phosphatase has not been proven for all examples of the suppression of reactive oxygen species during parasite infection, it has been strongly implicated in several studies. It has been previously shown that *Hematodinium* sp ex. *N. norvegicus* contain high intracellular concentrations of acid phosphatase and also excrete it into culture media (Small *et al.*, 2007*a*). Furthermore, infected *N. norvegicus* were found to contain high concentrations of acid phosphatase in cell-free sera (Small *et al.*, 2007*a*), so it is possible that acid phosphatase is playing an active role in superoxide anion suppression in *Hematodinium*-infected *N. norvegicus*.

Clearly, the inhibition of reactive oxygen species including superoxide anions is a common mechanism of immune suppression utilised by parasites. In the present study, the clear correlation between superoxide production and parasite load suggest that *Hematodinium* sp. has the potential to suppress superoxide production. Evidence from other parasites indicate that the method of suppression is likely due to acid phosphatase secretion, however this must be confirmed by further examination.

### 5.4.4 Haemagglutination

*N. norvegicus* haemocyte lysate was able to agglutinate chicken RBCs, however had no response to gluteraldehyde-fixed sheep RBCs. This agrees with Shields (2003) who found that *C. sapidus* haemolymph had very low titers for sheep RBCs and only moderate agglutination for rabbit RBCs, however a much stronger response was found for chicken RBCs with titers up to 1:64 in uninfected animals and 1:128 in moderately infected animals. This study used haemocyte lysate supernatant (HLS) to study haemagglutination as agglutinating lectins have been identified intracellularly in the harbour crab *Liocarcinus depurator* (Fragkiadakis and Stratakis, 1997). Smith and Soderhall (1983) also studied HLS haemagglutination response in *Carcinus maenas*, however found no response to sheep, horse or human RBCs and only weak agglutination response in *C. maenas* sera. *Macrobrachium malcolmsonii* was found to agglutinate a wide range of RBCs, including those from human, sheep, chicken and guinea pig (Acharya *et al.*, 2004), and it has become clear that there are wide species-specific differences in agglutinating properties. Researchers have found haemagglutinin titres to vary widely among individuals as well (Sritunyalucksana *et al.*, 1999).

No significant difference was found in haemagglutinin titers for any RBCs between control and*Hematodinium*-infected animals in this study which supports results from Shields (2003) who examined haemagglutination response over varying levels of *Hematodinium* infection in *C. sapidus*. Based on the present results, it would appear that intracellular agglutinins do not have a strong role in the crustacean immune response to *Hematodinium* sp. infection. Agglutination is based on the ability of agglutinins (lectins) to recognise specific carbohydrate molecules on the surface of invading pathogens and bind them together to facilitate haemocyte attachment and phagocytosis (see Marques and Barracco (2000) for review). To date, no studies have explicitly examined the surface coat of *Hematodinium* sp., however it is possible that either agglutinins from *Nephrops norvegicus* are unable to recognise the carbohydrates present in the *Hematodinium* sp. surface coat or that the parasite is able to mask its carbohydrate surface. Parasites have been found to mimic host molecules in an effort to avoid detection by host immune factors, and have also been found to disguise themselves with host molecules (see Loker (1994) for review). Additionally, the fungal pathogen *Metarhizium anisopliae* secretes a collagenous coat shortly after entering the haemocoel of its insect host, blocking the recognition of non-self molecules and successfully colonizing the host (Wang and Leger, 2006). It is possible that *Hematodinium* sp. uses a cloaking mechanism to avoid detection by crustacean hosts, however further studies into surface coat composition would be required.

### 5.4.5 Conclusions

For the first time, results were presented on the analysis of multiple immune parameters from the crustacean host *Nephrops norvegicus* to the dinoflagellate parasite *Hematodinium* sp. measured using a variety of immune assays. Total haemocyte counts indicated that *Hematodinium*-infected *N. norvegicus* were immunocompromised by the overall decrease in haemocyte counts, though there was no difference in differential haemocyte counts. Despite the significant decrease in THC, however, intracellular proPO stores (PO activity) per cell increased in infected animals, which showed that they were still capable of mounting an immune response. Interestingly, the PO activity was highly variable in infected *N. norvegicus* compared to control animals, but further analysis showed that PO activity was not correlated with *Hematodinium* load. It is possible that the high variability in infected animals was a result of secondary infection by opportunistic pathogens. Varying intensities of secondary infection could therefore be contributing the variable PO activity.

Haemagglutinin titres of haemocyte lysate and intracellular superoxide (SO) anion production were not significantly different between infected and control *N. norvegicus*. Similar to PO activity, SO production showed high variability in infected *N. norvegicus*. In this case, SO production showed significant negative correlation with *Hematodinium* sp. abundance. Previous research by Small *et al.* (2007*a*) suggested that acid phosphatase secreted by *Hematodinium* sp. ex *N. norvegicus* could inhibit superoxide production and the evidence presented here supports that hypothesis. This is the first evidence for a mechanism of immune suppression used by *Hematodinium* sp.

Results from the previous chapter (Chapter 4) found no significant change in gene expression for *proPO*, however the present study found that intracellular stores of proPO were increased. These combined results imply that there could be a difference between the transcriptional and translational expression of prophenoloxidase during *Hematodinium* sp. infection, and underlines the need to corroborate the results of *in vitro* results. However, due to the limitations discussed regarding *in vitro* experiments, it is necessary to further verify the results of the *in vivo* immune assays using gene expression analysis (Chapter 6). This will provide a more direct comparison of gene expression results between *in vitro* and *in vivo* methods as well as between gene expression and protein expression *in vivo*.

# Chapter 6

# Effects of *Hematodinium* sp. infection on immune-related gene transcription in *Nephrops norvegicus* from the Clyde Sea Area, Scotland

## 6.1 Introduction

As discussed previously, pathological investigations to date have noted little immune response to parasite infection (Stentiford *et al.*, 2003) and gene expression analysis of *in vitro* exposure of *Cancer pagurus* haemocytes to *Hematodinium* sp. cells (see Chapter 4) upheld those observations. However, *in vivo* immune assays on *Nephrops norvegicus* indicated parameters with significant changes in *Hematodinium*-infected individuals. Total haemocyte counts were found to significantly decrease and phenoloxidase activity increased during infection. In order to determine whether *Hematodinium* was affecting phenoloxidase at the transcriptional level, gene expression was studied on the same population of animals. To further explore the physiological responses of infection on *N. norvegicus*, the differential display technique GeneFishing™ was again used to identify any other potential genes specifically affected by *Hematodinium* sp.

The use of molecular techniques to study immune responses in invertebrates has gained momentum in the last decade. Gene expression studies have allowed researchers to look at which genes are being transcriptionally regulated in response to infection,

pollution and stress (de Lorgeril *et al.*, 2011; Leu *et al.*, 2011). Crustacean immune studies in response to pathogens have generally focussed on genes for antimicrobial peptides, phagocytosis activation and, crucially, genes involved in the prophenoloxidase activation cascade (Cerenius *et al.*, 2003; Deachamag *et al.*, 2006; Okumura, 2007; Smith *et al.*, 2008; Gai *et al.*, 2008). The most common pathogens investigated in these studies are bacteria, viruses and pathogen-associated molecules such as lipopolysaccharides and $\beta$1,3-glucans. As previously discussed, the responses to these pathogens seldom follow a specific pattern and vary highly with host and pathogen species. Similarly, immune response to parasite infection is variable in invertebrates (see 4.1.3), though many parasites have been observed to inhibit or suppress phenoloxidase (PO) activity (Rigaud and Moret, 2003; Brivio *et al.*, 2006; Dezfuli *et al.*, 2008; Cornet *et al.*, 2009). A recent study has found calyx fluid from the parasitic wasp *Cotesia glomerata* to reduce both PO activity and *proPO* expression in the Small White butterfly *Pieris rapae* larvae, suggesting that inhibition occurs at the transcriptional level (Zhu *et al.*, 2011). *In vivo* PO activity was observed to increase during *Hematodinium* infection in *N. norvegicus* and the *proPO* expression pattern of individuals from the same population can now be determined.

Phenoloxidase, while a key component of crustacean immunity, is merely a member of an entire host of cellular and humoral functions in internal defense (Ratcliffe *et al.*, 1982; Smith and Soderhall, 1986; Smith and Chisholm, 1992). Differential display techniques have expanded the variety of genes studied during experimental conditions as they explore a random subset of genes throughout the transcriptome. A recent study has used suppressive subtractive hybridisation (SSH) to examine differentially expressed genes (DEGs) in haemocytes of the penaeid shrimp *Litopenaeus stylirostris* in response to *Vibrio* infection (de Lorgeril *et al.*, 2005). The study specifically looked at shrimp able to survive *Vibrio* infection in order establish which genes allowed individuals to survive vibriosis. DEGs were sorted into biological function and were found to cover cell cycle/proliferation, cell motility/communication, cell growth/death, signal transduction and response to stimulus. From the subset of DEGs immune genes of particular interest were able to be targeted for further analysis using quantitative polymerase chain reaction (qPCR).

### 6.1.1 Aim of gene expression studies on *N. norvegicus*

The primary aim of this study was to examine the effects of *Hematodinium* infection on gene expression of wild-caught *Nephrops norvegicus* from the Clyde Sea Area. To achieve this, the differential display technique GeneFishing™ was used to identify potentially differentially expressed genes during heavy parasite infection. The genes

identified were then analysed, in addition to the immune-relevant gene *proPO*, using quantitative real-time PCR to determine up- or down-regulation during infection. The null-hypothesis being tested was that *Hematodinium* sp. infection in *N. norvegicus* would have no significant effect on gene expression when compared to uninfected and lightly infected control animals.

## 6.2 Materials and Methods

### 6.2.1 Animals

*Nephrops norvegicus* were collected by trawl from the Clyde Sea (55° 46.8 N, 4° 58.5 W to 55° 44.8 N, 4° 59.2 W) from a depth between 80 and 100 m in late March 2011. Heavily infected animals were identified by carapace colour (hyperpigmentation) and opaque joints, and were separated from healthy looking individuals on board the RV *Actinia*. A random selection of healthy animals with an undamaged carapace were also collected for comparison. All animals were maintained in flowing seawater aboard the vessel until arrival at the University Marine Biological Station, Millport where they were kept in flowing seawater aquaria until needed. As described in Chapter 5.2.2, total and differential haemocyte counts, in addition to *Hematodinium* sp. counts, were carried out for each individual using an improved Neubauer Haemocytometer under phase contrast microscopy. The samples used in this study came from the same animals used in the phenoloxidase assay and intracellular superoxide anion assay and the same description of infection status applies here: 'infected' animals contained $>$ 35% *Hemaotodinium* sp. concentration in the total haemolymph and 'control' animals contained no or $<$ 10% *Hematodinium* sp. cells.

### 6.2.2 GeneFishing™

GeneFishing™ was carried out on cDNA from haemolymph extracted from an infected and an uninfected *Nephrops norvegicus* to determine any differentially expressed genes (see Chapter 4.2.6). The present study used GeneFishing™ DEG103 and DEG104 Premix kit (K1022, Seegene, Seoul, South Korea) containing a set of 20 random primers different from those used in Chapter 4. Briefly, haemolymph was extracted from the base of the fifth pereiopod and into an equal volume of ice cold marine anticoagulant and pelleted at 300 *g* 10 min, 4°C. The supernatant was removed and the RNA extracted using a Nucleospin RNA II extraction kit (Macherey-Nagel, Düren, Germany). Additionally, a sample of cultured *Hematodinium* sp. ex. *N. norvegicus*, collected during the same trip and maintained in Appleton-Vickerman media

(Appleton and Vickerman, 1998) at 7°C, was pelleted and extracted using the same method to determine any differentially expressed parasite genes between *in vitro* and *in vivo* parasite cells.

RNA was reverse transcribed according the manufacturer's recommendations for GeneFishing™ (see Chapter 4.2.6) using SuperScript™ III (Invitrogen, Paisley, UK). PCR amplifications were carried out as described in the GeneFishing™ manual and run on a Bio-Rad MyCycler (Bio-Rad, Hemel Hempstead, UK) (see Chapter 4.2.6). Products were separated on a 2% agarose gel stained with ethidium bromide and visualised using a Gel Doc™ UV transilluminator (Bio-Rad, Hemel Hempstead, UK). Bands that were identified as being differentially expressed were excised, extracted using a QIAquick® Gel Extraction kit (Qiagen, Crawley, UK; Appendix A.2) and cloned using a TOPO TA Cloning® kit according to the manufacturer's protocol (Appendix A.3). Successful clones were cultured overnight in LB broth with 100 $\mu$g/ml ampicillin and the plasmids were extracted using a QIAprep® Spin Miniprep Kit (Qiagen, Crawley, UK) following the manufacturer's instructions (Appendix A.4). Plasmids were sequenced by SourceBioscience (Department of Chemistry, University of Oxford, UK) using vector-specific M13 primers.

### 6.2.3 Gene Expression Study

Gene expression analysis was carried out on control and *Hematodinium*-infected *Nephrops norvegicus* using quantitative polymerase chain reaction (qPCR) as described in Chapter 3. Briefly, haemolymph was collected from the base of the fifth pereiopod 1:1 in marine anticoagulant and pelleted 300 *g* 10 min, 4°C at the Marine Station. The supernatant was removed and 0.5 ml RNAlater® (Ambion, Cheshire, UK) added to each pellet. Pellets were stored at 4°C while at the marine station and transported on ice in cool boxes to Southampton where the RNAlater® was removed and the samples flash-frozen in liquid nitrogen before storing at -80°C until needed. RNA was extracted as previously described following the manufacturer's protocol using a Nucleospin RNA II extraction kit (Macherey-Nagel, Düren, Germany) and DNase treated using Ambion TURBO™ DNase (Applied Biosystems, Cheshire, UK). All RNA samples were checked for quality using the Bio-Rad Experion™ system (Bio-Rad, Hemel Hempstead, UK) as previously described (Chapter 3.2.1) and only samples with an RQI $\geq$7.5 were reverse transcribed for qPCR. cDNA was synthesised using SuperScript™ III (Invitrogen, Paisley, UK) according to the manufacturer's recommendations (see Chapter 3) and stored at -20°C until needed. qPCR reactions contained: 25 $\mu$l PrimerDesign MasterMix with SYBR Green (PrimerDesign, Southampton), 3.5 ng cDNA, 4 $\mu$l forward primer, 4 $\mu$l reverse primer (Table 6.1) and

sterile RNase free water to 50 $\mu$l total reaction volume. Samples were divided into 23 $\mu$l volumes and run in 0.1 ml qPCR tubes designed for use in a 72-well rotor of the Qiagen (Corbett) Rotor Gene 3000 (Qiagen, Crawley, UK). Assays for all qPCR primers were optimised as described in Chapter 3.

TABLE 6.1: qPCR primer pairs for genes identified by GeneFishing,™ including optimised primer concentrations, amplification efficiency and $R^2$ values

| Primer Name | Tm (°C) | Sequence | Optimised primer concentration (nM) | Amplification Efficiency | $R^2$ |
|---|---|---|---|---|---|
| Neph 40S Fwd | 59.7 | 5'-GCT GGC CTG TGC AAG ATT G-3' | 300 | 1.02 | 0.986 |
| Neph 40S Rev | 60.9 | 5'-CAT GAG CAG CCA ACG ACC TT-3' | 300 | | |
| Neph Cct7-2 Fwd | 60.5 | 5'-GAC GTG GAA GGC CCA TGT AA-3' | 300 | 1.01 | 0.983 |
| Neph Cct7-2 Rev | 60.0 | 5'-TGT TGA GTC ACT GTG TTC AGA GAG TT-3' | 300 | | |
| Neph Crust2 Fwd[1] | 60.7 | 5'-CAC TCG GCA GCC AGA CCT T-3' | 300 | 1.04 | 0.991 |
| Neph Crust2 Rev | 59.7 | 5'-TCG ACC TTC TGA CCC AAA AGA-3' | 300 | | |

[1]The primers for *NnCrust* amplify both *crustin* isoforms

Genes were quantified using absolute quantification against a standard curve generated using linearised plasmid DNA (see Chapter 3). As previously mentioned (Chapter 3.5.3), RNA extracted from *Hematodinium*-infected animals contained varying amounts of parasite RNA, making it impractical to normalise gene expression against $\mu$g total RNA or to use relative quantification. Instead, gene copy numbers were quantified against total haemocyte counts (Bustin, 2000).

### 6.2.4 Data Analysis

In the present study, gene expression data were not normally expressed or lacked equal variance, so the non-parametric Mann-Whitney rank sum test was used to compare differences between infected and control *Nephrops norvegicus*. All statistics were carried out using SigmaStat v. 3.5 (Systat Software, Inc.; San Jose, CA).

## 6.3 Results

### 6.3.1 GeneFishing™

GeneFishing™ analysis identified 14 differentially expressed genes between infected and control *Nephrops norvegicus*, one from infected animals and 13 from control animals. A further three genes were identified from *Hematodinium* sp. Of the 17 total bands, 12 were successfully cloned and sequenced, nine were from *N. norvegicus* (Appendix C.2.1) and three were from *Hematodinium* sp. (Appendix C.2.2). All sequences were searched against the BLAST database (Altschul *et al.*, 1990) and three differentially

expressed genes were putatively identified, all from control *N. norvegicus*: DEG 201-21, DEG 201-26 and DEG 201-28 (Fig 6.1).

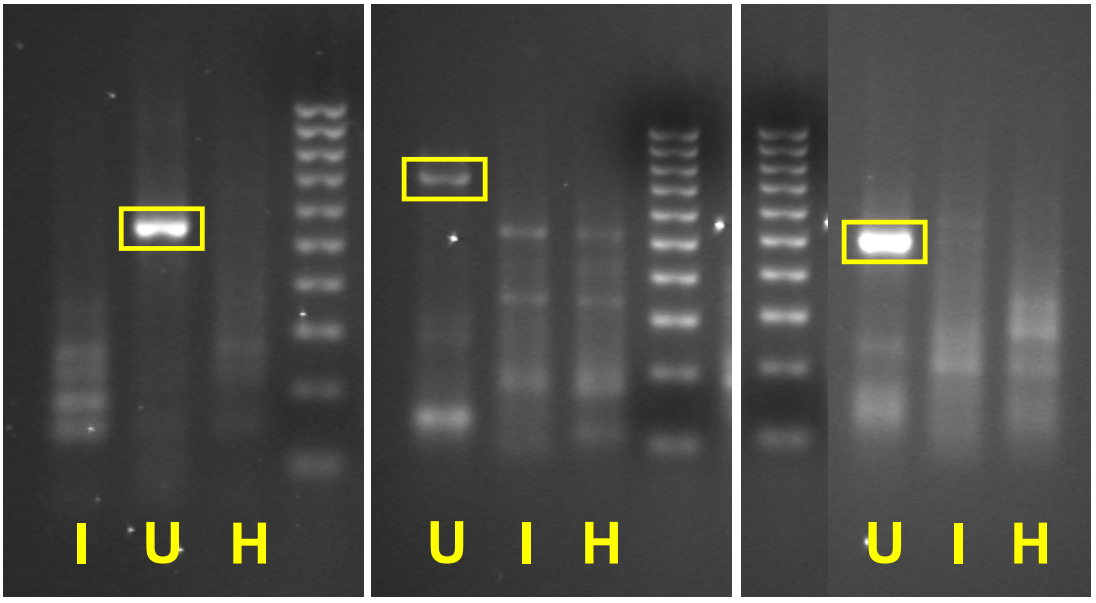



FIGURE 6.1: Electrophoresis gels of GeneFishing™ results comparing haemolymph of uninfected (U) and infected (I) *N. norvegicus* and cultured *Hematodinium* cells (H). Boxed bands indicated differentially expressed genes DEG201-21 (a), DEG201-26 (b) and DEG201-28 (c). All bands are compared to a 100-bp ladder (Sigma-Aldrich).

Sequence DEG201-21 from control *N. norvegicus* produced a fragment of 484 nucleotides in length including the poly-A tail. The full gene was generated using RACE and two isoforms were identified. The first isoform was 854 nucleotides in length and the second was 844 nucleotides. Isoform1 was comprised of an open reading frame (ORF) of 342 nucleotides coding for a sequence of 114 amino acids, with a 5' UTR of 113 bp and a 3' UTR of 399 bp (Accession number: HE608880). Isoform2 was comprised of an ORF of 330 nucleotides coding for 110 amino acids, with a 5' UTR of 115 bp and a 3' UTR of 399 bp (Accession number: HE608881). A BLAST search of both amino acid sequences showed that both sequences had highest pairwise identity with a carcinin-like antibacterial peptide from *Homarus gammarus* (CAH10349; 75.7%, e-value = $1.16e^{-43}$ Isoform1 and 73.6%, e-value = $1.39e^{-39}$ Isoform2) and a crustin-like protein precursor from *Homarus americanus* (ABM92333; 72.6%, e-value = $3.87e^{-42}$ Isoform1 and 71.9%, e-value = $3.35e^{-40}$ Isoform2). Pairwise identity then decreased to 35-40% identity for several other crustacean species, but was still identified as a crustin in *Pacifasticus leniusculus* (ABP88043; 38.4%, e-value = $1.59e^{-11}$ Isoform1; 40.6%, e-value = $3.25e^{-12}$ Isoform2), *Procambarus clarkii* (ACY64752; 37.7%, e-value = $2.09e^{-09}$ Isoform1; 39.4%, e-value = $7.18e^{-10}$ Isoform2), *Scylla paramamosain* (ABY20727; 38.6%, e-value = $1.20e^{-05}$ Isoform1; 37.2%, e-value = $5.31e^{-08}$ Isoform2) and *Carcinus maenas* (CAH25399; 39.5%, e-value = $5.21e^{-06}$ Isoform1; 38.1%, e-value

= 4.79e$^{-07}$ Isoform2). The isoforms were named *NnCrust1* and *NnCrust2*. SMART (Simple Modular Architectural Research Tool; http://smart.embl-heidelberg.de/) analysis identified a whey-acidic protein (WAP) domain (e-value = 1.76 e$^{-1}$) with a 4-disulfide core (Fig 6.2). A signal sequence MLRLLVVMTVVTVALG with a cleavage site was identified using Geneious v. 4.8.5 (based on the EMBOSS sigcleave program). The cleavage site was between glycine and serine (G$^{21}$/S$^{22}$) for *NnCrust1* and between two glycine residues (G$^{21/22}$) for *NnCrust2*. Geneious also predicted that *NnCrust1* had a molecular weight of 12.5 kDa and an isoelectric point of 7.85 and *NnCrust2* had a molecular weight of 12 kDa and an isoelectric point of 7.62.

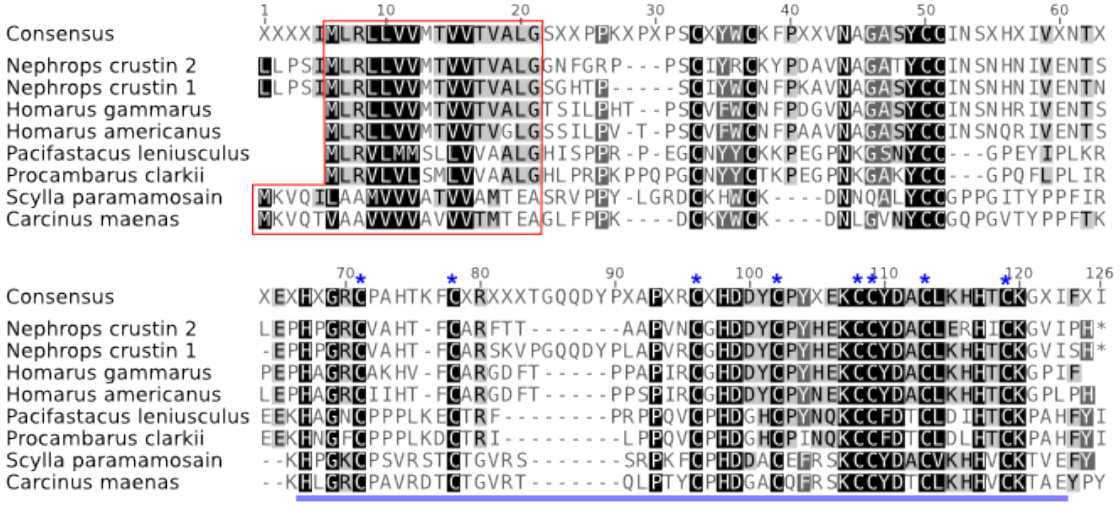



FIGURE 6.2: Multiple alignment of the two isoforms of *Nephrops norvegicus* crustin-like protein with crustin and crustin-like proteins from the crustaceans: *Homarus gammarus* CAH10349, *Homarus americanus* ABM92333, *Pacificasticus leniusculus* ABP88043, *Procambrus clarkii* ACY64752, *Scylla paramamosain* ABY20727, *Carcinus maenas* CAH25399. The pairwise identity for all of these sequences is 47.9%. The WAP domain is double underlined in blue and the eight highly conserved cysteine-residues are marked with blue asterisks (*). C96, C102, C108 and C109 comprise the basis of the 4-disulfide core of the WAP domain. The signal sequence is boxed in red. Amino acid similarity is indicated by color: black - 100%, dark gray - 80-99%, light gray - 60-79%, white 0-59%.

DEG201-26 was identified from control *N. norvegicus* and produced a fragment of 718 nucleotides including the poly-A tail. An open reading frame of 423 nucleotides coding for 141 amino acids was identified (Accession number: HE608882). A BLASTP search showed that DEG201-26 had highest pairwise identity with 40S ribosomal protein S12 from *Eurythoe complanata* (78.8% ABW23206, e-value = 6.12e$^{-67}$), *Branchiostoma belcheri* (83.2% AF4706871, e-value = 1.36e$^{-66}$), *Perinereis aibuhitensis* (77.5% AAO43049, e-value = 4.72e$^{-66}$), *Saccoglossus kowalevskii* (75.5% XP002739754, e-value = 2.30e$^{-65}$), *Dermacentor variabilis* (81.6% AAP04352, e-value = 7.55e$^{-65}$), *Ixodes scapularis* (80.8% AAY66899, e-value = 2.30e$^{-64}$), *Rhipicephalus sanguineus*

(80.8% ACX53878, e-value = $4.01e^{-64}$), *Pinctada maxima* (75.7% AS72284, e-value = $1.42e^{-62}$), *Novocrania anomala* (76.4% ACD65102, e-value = $2.59e^{-62}$), and *Strongylocentrotus purpuratus* (71.8% XP795427, e-value = $2.88e^{-62}$). The sequence was termed *NnrpS12*. SMART analysis indicated a ribsomal L7Ae domain (e-value = $6.6e^{-29}$) (Fig 6.3).

The final differentially expressed gene was DEG201-28 identified from control *N. norvegicus*. The nucleotide sequence fragment was 453 nucleotides in length containing 45 amino acids at the end of the open reading frame (Accession number: HE608879). The sequence was identified using a BLASTP search as a chaperonin containing T-complex protein subunit 7 (eta) (*NnCct7*). *NnCct7* had highest pairwise identity with *Acromyrmex echinatior* (86.0% EGI1148, e-value = $2.35e^{-21}$), *Camponotus floridanus* (85.7% EFN60224, e-value = $4.11e^{-21}$), *Xenopus laevis* (83.0% AAH42312, e-value = $1.71e^{-20}$), *Callithrix jacchus* (73.6% XP002757673, e-value = $1.07e^{-20}$) and *Anolis carolinensis* (77.3% XP003229175, e-value = $4.75e^{-19}$). SMART analysis of the amino acid sequence identified the end of a chaperonin containing sequence (Cpn60_TCP1; e-value = $9.20e^{-02}$) via Pfam which comprised the first 29 amino acids of the fragment isolated in this study (Fig 6.4). Furthermore, comparison with multiple cct7 amino acid sequences indicated a conserved site (SVDETIK(R)) as part of the ATP/$Mg^{+}$ binding site.

### 6.3.2 RNA integrity

As previously described in Chapter 3, all RNA samples were tested for quality using the BioRad Experion™ system which analyses sample quality based on 18S and 28S ribosomal RNA ratios. Interestingly, the banding patterns observed on the virtual gel image for infected and control animals were different (Fig 6.5). Control animals had second strong peaks very close to the initial 18S peak and therefore two dark bands are very close on the virtual gel (approximately 1800 and 2000). Heavily infected animals with very few haemocytes had two bands (18S and 28S) further apart and at the expected locations (approximately 1800 and 3300). Infected animals with sufficient haemocyte counts contained three bands at 1800, 2000 and 3300. The banding pattern for *Cancer pagurus* haemocytes used in Chapter 4 was similar to that for control *N. norvegicus*. It is therefore evident that the banding patterns using the Experion™ system can act as a marker for *Hematodinium* infection.

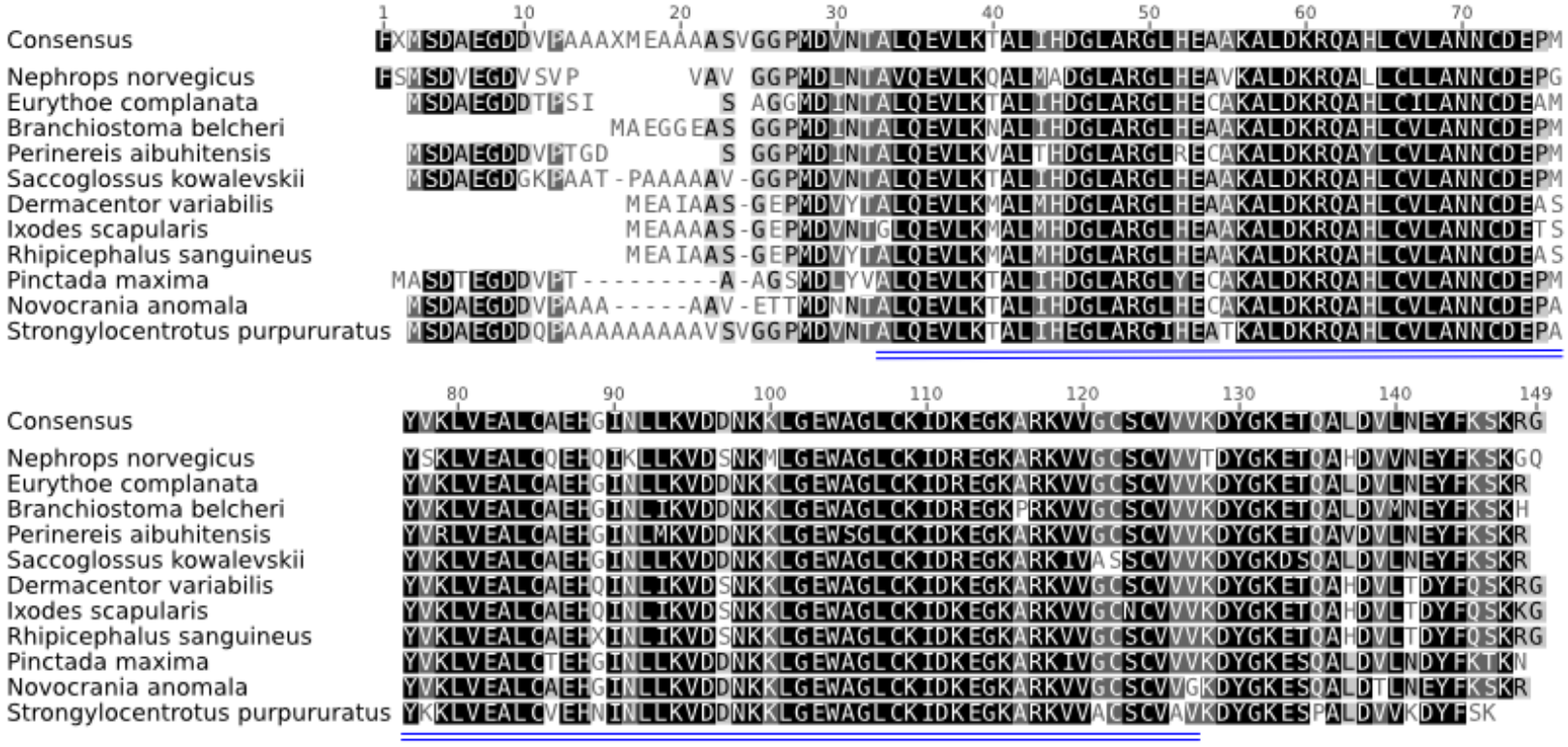



FIGURE 6.3: Multiple alignment of *Nephrops norvegicus* 40S ribosomal protein S12 with other invertebrate 40S ribosomal proteins *Eurythoe complanata* ABW23206, *Branchiostoma belcheri* AF4706871, *Perinereis aibuhitensis* AAO43049, *Saccoglossus kowalevskii* XP002739754, *Dermacentor variabilis* AAP04352, *Ixodes scapularis* AAY66899, *Rhipicephalus sanguineus* ACX53878, *Pinctada maxima* AS72284, *Novocrania anomala* ACD65102, and *Strongylocentrotus purpuratus* XP795427. The pairwise identity for all of these sequences is 81.0%. The L7Ae domain is marked with double blue underline. Amino acid similarity is indicated by color: black - 100%, dark gray - 80-99%, light gray - 60-79%, white 0-59%.

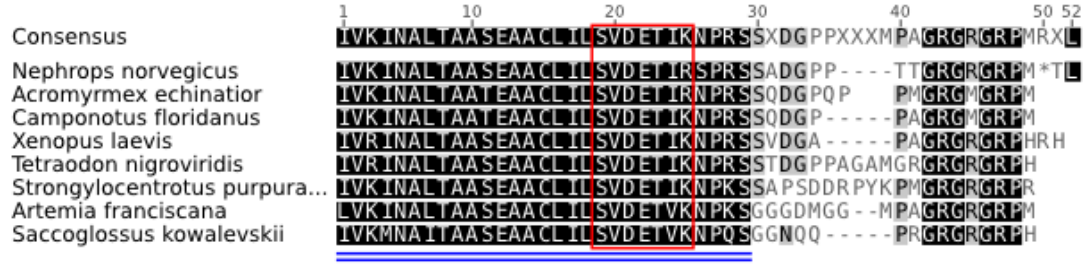



FIGURE 6.4: Multiple alignment of *Nephrops norvegicus* chaperonin containing T-complex protein subunit 7 with other Cct7 proteins: *Acromyrmex echinatior* (EGI61148), *Camponotus floridanus* (EFN60224), *Xenopus laevis* (AAH45074), *Tetradon nigroviridis* (CAG05730), *Strongylocentrotus purpuratus* (XP782448), *Artemia franciscana* (AF4275971), and *Saccoglossus kowalevskii* (XP002734944). The double underlined region represents the end of the chaperonin-containing sequence (Cpn60_TP1) identified by Pfam. The red boxed area (SVDETIK(R)) indicates a portion of the conserved ATP/$Mg^{+}$ binding site. The pairwise % identity for this alignment was 75.9%. Amino acid similarity is indicated by color: black - 100%, dark gray - 80-99%, light gray - 60-79%, white 0-59%.

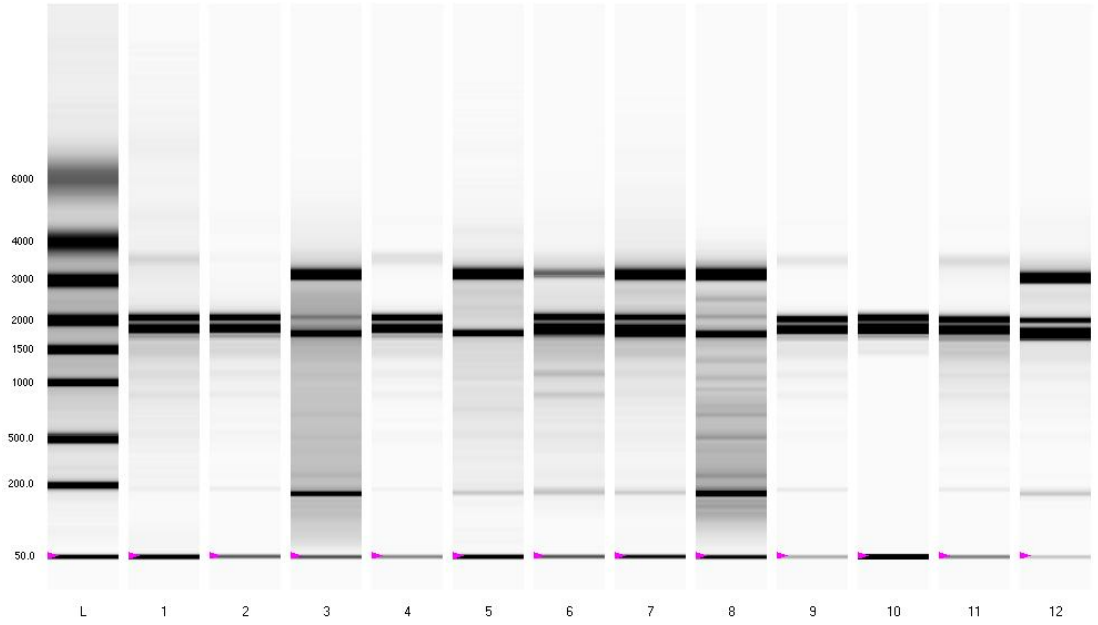



FIGURE 6.5: Bio-Rad Experion™ virtual gel image for 12 *N. norvegicus* RNA samples. Lanes 3, 5, 6, 7, 8 and 12 contain samples from animals infected with *Hematodinium*. All infected samples have a strong band at 3300 and heavily infected animals with low haemocyte counts have little evidence of a band at 2000 (lanes 5 and 8).

### 6.3.3 Gene Expression Results

The prophenoloxidase gene (*proPO*) was significantly upregulated in *N. norvegicus* infected with *Hematodinium* sp. (Fig 6.6). Copies of *proPO* increased from a mean of 4,327 copies 1000 haemocytes$^{-1}$ ($\pm$ 5,835 SD) to 10,969 copies 1000 haemocytes$^{-1}$ ($\pm$

12,461 SD). No significant correlation between *proPO* and *Hematodinium* load was evident ($p > 0.05$; Pearson's correlation).

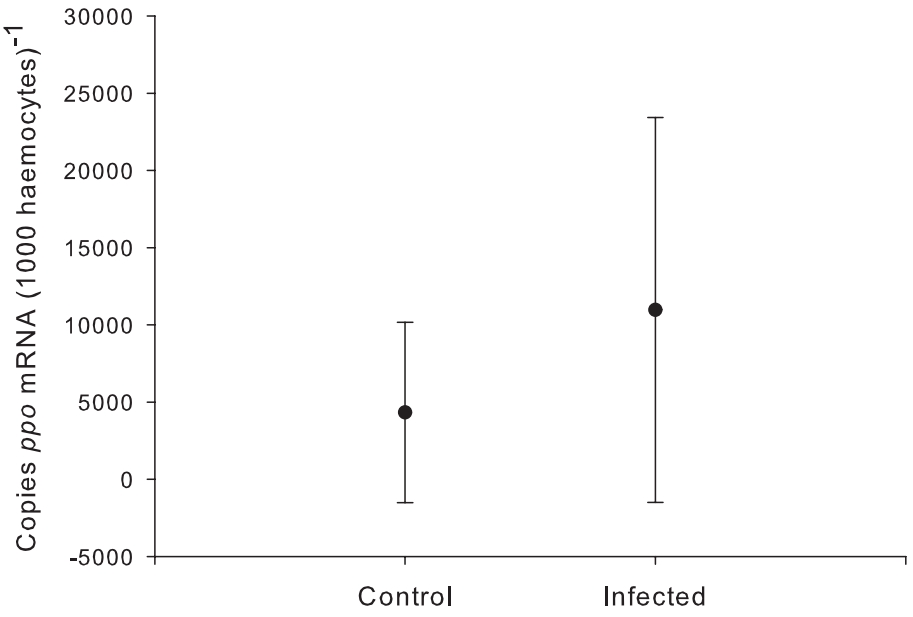



FIGURE 6.6: Comparison of *proPO* mRNA copy numbers (1000 haemocytes)$^{-1}$ ±SD in control *N. norvegicus* ($n = 22$) and those infected with *Hematodinium* ($n = 21$). Gene expression was significantly upregulated in infected animals ($p = 0.038$; Mann-Whitney rank sum test).

The primers designed for *NnCrust* amplified both isoforms and resulted in the highest expression levels of all genes analysed during this study, however expression levels did not vary between infected and control *N. norvegicus* ($p > 0.05$; Mann-Whitney Rank Sum test) (Fig 6.7).

The gene encoding 40S ribosomal protein S12 *NnrpS12* was found to be significantly down-regulated in *N. norvegicus* infected with *Hematodinium* compared with control individuals (Fig 6.8). Copy numbers decreased from 10,646 ± 5,356 SD copies 1000 haemocytes$^{-1}$ in control animals to 4,679 ± 2,669 SD copies 1000 haemocytes$^{-1}$ in infected animals.

The gene coding for the chaperonin containing TCP1 subunit 7 *NnCct7* did not vary significantly in expression between *N. norvegicus* infected with *Hematodinium* and control animals ($p = 0.078$). Copy numbers for *NnCct7* were much lower than the other genes examined during this study (Fig 6.9).

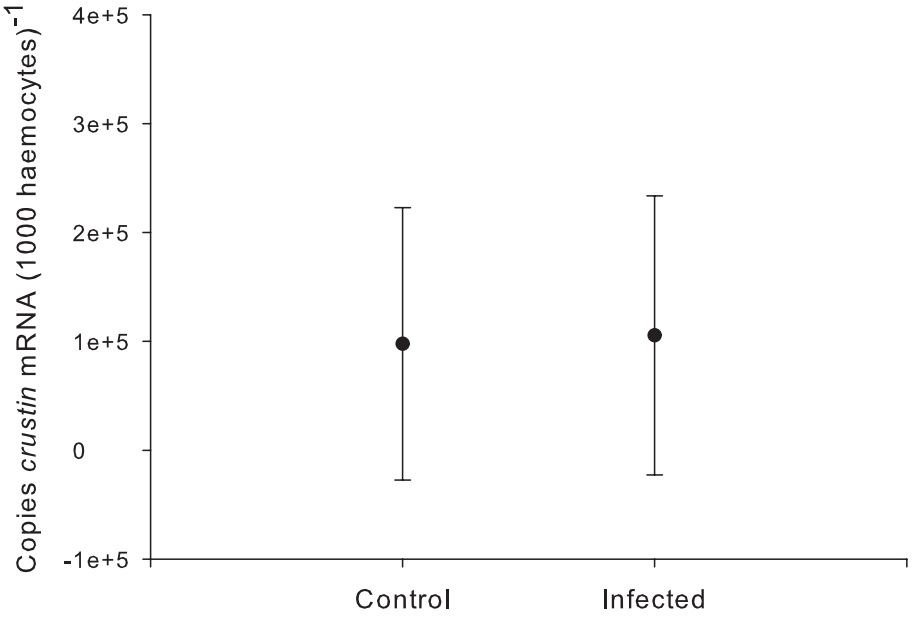



FIGURE 6.7: Comparison of combined *NnCrust1* and *NnCrust2* isoform mRNA copies (1000 haemocytes)$^{-1}$ ±SD between control *N. norvegicus* ($n = 22$) and those infected with *Hematodinium* ($n = 21$). Expression levels were not significantly different ($p = 0.990$; Mann-Whitney Rank Sum test).

## 6.4 Discussion

There were two main objectives to this study. The first objective was to use a differential display technique, GeneFishing,™ to identify differentially expressed genes in wild-caught *Nephrops norvegicus* during *Hematodinium* infection. The second goal was to use quantitative real-time PCR to explore the changes in any genes identified using differential display, as well as any changes to the immune-relevant gene *proPO* coding for prophenoloxidase. To that end, three genes were successfully cloned and identified using GeneFishing™, and qPCR indicated that one of the genes was significantly down-regulated in *Hematodinium*-infected animals, in addition to the significant up-regulation of *proPO*.

### 6.4.1 GeneFishing™

GeneFishing™ resulted in the identification of 14 differentially expressed genes between *Hematodinium*-infected and control *N. norvegicus*, three of which were successfully sequenced and used in gene expression studies. Of the three genes identified, one of them had high similarity to the immune-relevant *crustin* and *crustin*-like genes (*NnCrust*) identified in other crustaceans. Two other genes were identified as those

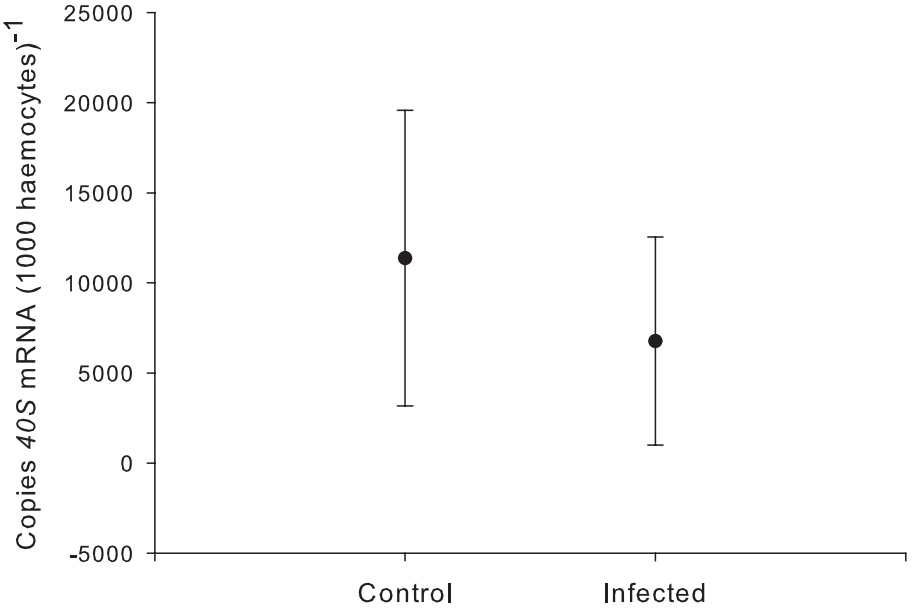



FIGURE 6.8: Comparison of the 40S ribosomal protein S12 *NnrpS12* mRNA copy numbers (1000 haemocytes)$^{-1}$ ±SD between control *N. norvegicus* ($n$ = 22) and *N. norvegicus* infected with *Hematodinium* ($n$ = 21). Copy numbers were significantly down-regulated in infected animals (p = 0.042; Mann-Whitney Rank Sum Test).

necessary to protein translation and folding and had highest identity with a 40S ribosomal protein S12 (*NnrpS12*) and a chaperonin-containing T-complex protein 1 subunit 7 (*NnCct7*).

#### 6.4.1.1 *Crustin*

DEG 201-21 was identified using BLASTP analysis as a crustin-like protein, of which two putative isoforms were sequenced: *NnCrust1* (HE608880) and *NnCrust2* (HE608881). This is the first report of a crustin-like protein from *Nephrops norvegicus* and was most similar to the crustin-like proteins sequenced from *Homarus gammarus* (CAH10349) and *Homarus americanus* (ABM92333) (Hauton *et al.*, 2006; Christie *et al.*, 2007). Crustins are generally defined as cysteine-rich antimicrobial polypeptides (AMPs) with a molecular weight of 7-14kDa, typically characterised by a whey-acid protein (WAP) domain at the carboxyl terminus and have an isoelectric point (pI) between 7 and 8.7 (Hauton *et al.*, 2006; Smith *et al.*, 2008). All of these criteria were met in the crustin sequences identified in this study.

Based on descriptions by Dimarcq *et al.* (1998) and Bartlett *et al.* (2002), the crustin found here putatively belongs to the cysteine-rich class of AMPs which are characterised by the formation of $\beta$-sheets or $\alpha$-helical $\beta$-sheet mixed structures and

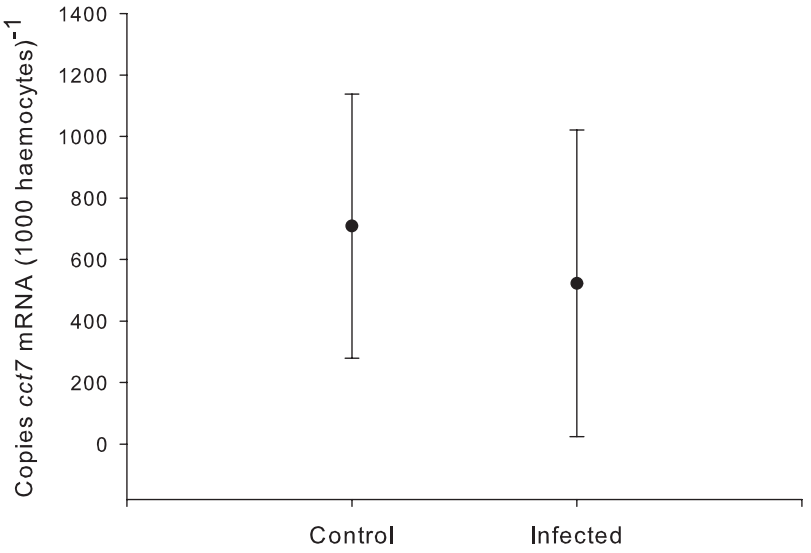



FIGURE 6.9: Comparison of *NnCct7* mRNA copy numbers (1000 haemocytes)$^{-1}$ ±SD between control *N. norvegicus* ($n = 22$) and *N. norvegicus* infected with *Hematodinium* ($n = 21$). Copy numbers did not vary significantly between infected and control animals (p = 0.078; Mann-Whitney Rank Sum Test).

contain disulphide bonds. Defensins comprise a large part of this group and have been isolated from a wide variety of organisms including invertebrates, vertebrates and plants (Bartlett *et al.*, 2002). Defensins are generally active towards Gram-positive bacteria, but have been found to act against Gram-negative bacteria and viruses as well (Dimarcq *et al.*, 1998). Defensins typically act by forming pores in invading bacteria and disrupting the bacterial cell membrane (Dimarcq *et al.*, 1998). Crustins have previously been found to act against Gram-positive (Relf *et al.*, 1999; Mu *et al.*, 2010) and Gram-negative bacteria (Hauton *et al.*, 2006; Battison *et al.*, 2008), though to date, only limited evidence for activity towards a parasite has been found (Battison *et al.*, 2008).

Following RACE, two clones were sequenced which revealed two crustin isoforms for *Nephrops norvegicus*. Isoform 1 (*NnCrust1*) contains an insert of seven amino acids (PGQQDYP)$^{109-115}$ in the WAP domain along with several amino acid variations throughout the length of the sequence. Both isoforms had high identity to multiple crustin amino acid sequences and both *NnCrust* isoforms met the general molecular weight and pI characteristics of previous crustins. Following the guidelines proposed by Smith *et al.* (2008) the present crustins fall into the Type I class of crustins typically found in lobsters, crabs and crayfish and loosely conform to the suggested framework:

-C-X(3)-C-X(8-12)-C-C-X(16-17)-C-X(6)-C-(9-10)-C-X(5)-C-X(5)-C-C-X(3-5)-C-X(3-4)-C-

where the underlined region represents the cysteines of the WAP domain that form the 4-disulfide core (Ranganathan *et al.*, 1999).

WAP domains have been associated with proteinase inhibition and antibacterial activity (Smith *et al.*, 2008). The residues following the second cysteine in the WAP domain are particularly important in determining overall protein function in WAP-containing proteins as they are the site for proteinase inhibition or antibacterial functionality. Proteinase activity is associated with the presence of a methionine residue at the scissile bond following the second cysteine residue (Hagiwara *et al.*, 2003) while a replacement with a hydrophobic or cationic amino acid enables protein insertion into microbial cell walls, thereby functioning as an antibacterial peptide (Zasloff, 2002; Hagiwara *et al.*, 2003). The presence of alanine (A) and arginine (R) following the $C^{103/107}$ in both *N. norvegicus* isoforms suggests that this protein functions primarily as an antibacterial peptide.

As previously mentioned, two putative isoforms were identified in this study. Isoforms have been found in several crustacean crustins (Brockton *et al.*, 2007), however the isoforms typically only vary by 1-4 amino acids. In this case, an insert of seven amino acids was identified in *NnCrust1* in addition to 17 other aa variations. Crucially, the 7 aa insert is contained within the WAP domain. The WAP domain is speculated to play a key role in the antibacterial functionality of crustin proteins (Smith *et al.*, 2008), and indeed, the single amino acid change from a methionine to a cationic or hydrophobic amino acid is the difference between proteinase inhibition and antibacterial function (Zasloff, 2002). An insert of this magnitude could cause severe structural changes in protein tertiary structure and could render this protein non-functional. Alternatively, this protein could simply have another role in crustacean physiology. For example, the WAP-domain containing protein Lustrin A from the red abalone*Haliotis rufescens* was found to have proteinase inhibitory properties that protect the shell from degradation (Shen *et al.*, 1997) and caltrin II from guinea pigs is a calcium transporter inhibitor (Furutani *et al.*, 2004). The two isoforms in this study were from two separate clones from a single animal, however, so further sequences are required to definitively ascertain the presence of multiple isoforms. If these are established, then further studies will be necessary to determine functional differences.

#### 6.4.1.2 Chaperonin containing T-complex protein 1 subunit 7 (*NnCct7*)

DEG201-28 was identified using BLASTP analysis as a chaperonin containing T-complex protein 1 (cct7) (HE608879) which had highest identity with amino acid sequences from *Acromyrmex echinatior* (EGI1148) and *Camponotus floridanus* (EFN60224). Initially, peptide folding was thought to be due solely to the peptide

primary sequence, but it was later found that extra proteins are necessary for correct folding (Ellis, 1996). Chaperonins are cytosolic and mitochondrial proteins used to stabilise folding of newly synthesised polypeptides in crowded cellular environments where there is the potential for unformed proteins to adversely aggregate. Additionally, these (helper) proteins are required for stabilisation and protein refolding during stress response, particularly during heat-shock conditions (van der Vies and Georgopoulos, 1996). *NnCct7* codes for the T-complex protein 1 subunit $\eta$ (eta/7), a member of the Group II chaperonins, and is one of eight related subunits forming a double ringed particle. The stacked rings are responsible for folding several major cytoskeletal proteins, including actin and tubulin, in an ATP-dependent manner (Grantham *et al.*, 2002) (reviewed in Liang and MacRae (1997)). All of the subunits share highly conserved motifs for ATP binding, however their amino acid sequence identities are quite low ($\sim$30%) and it is believed that each subunit has an additional function which is subunit-specific, though these roles are still uncertain (Kubota *et al.*, 1995).

Several molecular chaperones are stress-inducible and respond to heat-shock. CCT chaperonins are believed to be derived from the same ancestor as heat shock protein 60 (Hsp60) and another heat-inducible chaperone from *E. coli*, GroEL, based on amino acid sequence, oligomeric structure and chaperone activity (Gupta, 1990). GroEL and heat-shock proteins are members of the Group I chaperonins found in prokaryotes and the mitochondria of eukaryotes (Ranford and Henderson, 2002). The present study supports the relationship of CCT7 to these chaperonins by the SMART identification of a Cpn60 TCP1 Pfam domain. Cpn60 is also part of the Group I chaperonins which includes Hsp60 and GroEL (Ranford and Henderson, 2002).

In addition to heat-shock, many chaperonins have been found to play a role in the stress-response to numerous physiological and environmental stressors (see Morimoto and Santoro (1998) for review) and have recently been implicated to play a role in the innate immune response in humans (reviewed in Ranford and Henderson (2002) and Tsan and Gao (2004)). A Cpn60 chaperonin from *Mycobacterium leprae* was found to induce cytokine secretion from human monocytes (Coates, 1996). Coates (1996) suggested that although chaperonins are intracellular proteins, it is possible that the release of these proteins during cell death and lysis could induce immune activation. A more recent study by Basu *et al.* (2001) found that heat shock proteins Hsp70 and Hsp90 were able to form complexes with antigenic peptides and present them for recognition to T cells. Basu *et al.* (2001) suggested that the Hsps involved in this process were released from cells as a result of necrotic cell death in response to pathogenic invasion or disease.

In crustaceans, heat shock proteins (Hsps) have been found to vary in expression during viral and bacterial infection (Rungrassamee *et al.*, 2010; Liu *et al.*, 2011). Proteomic analysis of differentially expressed proteins in the mud crab *Scylla serrata* infected with white spot syndrome virus (WSSV) found Hsp70 to be up-regulated and Hsp90 to be down-regulated compared to uninfected animals, and the authors suggested that both Hsps play a role during viral immune response in *S. serrata* (Liu *et al.*, 2011). Additionally, the gene expression response of three Hsps (*hsp21*, *hsp70* and *hsp90*) were analysed in the gill tissue of the penaeid shrimp *Penaeus monodon* following injection with the bacteria *Vibrio harveyi* (Rungrassamee *et al.*, 2010). All three hsps were up-regulated 3-24 hours post-injection and Rungrassamee *et al.* (2010) speculated that Hsps played a role in protein stabilisation during cell damage as a result of infection as well as having a possible role as signaling molecules during the immune response. Furthermore, Rungrassamee *et al.* (2010) suggested that accumulation of damaging reactive oxygen species (ROS) such as superoxide, hydrogen peroxide and nitric oxide (Nappi and Ottaviani, 2000) in host cells resulting from pathogen phagocytosis could also increase the demand for protein repair by chaperonins. A specific role for the Cct7 subunit as a mediator of nitric oxide (NO) production was also previously reported (Hanafy *et al.*, 2004). Cct7 was implicated as a co-factor and inhibitor for soluble guanylyl cyclase (sGC), an intracellular mediator of nitric oxide (NO) and down-regulation of Cct7 resulted in an increase in sGC and an ultimate increase in NO.

Based on the role of chaperonins in stress-response and the observed responses to pathogen challenge, *NnCct7* is a promising gene for expression studies between control *Nephrops norvegicus* and ones infected with *Hematodinium* sp. As previously discussed *Hematodinium* sp. infection results in a great deal of physiological stress including hypoxia and physiological starvation, as well as tissue degradation, all of which could lead to protein unfolding and increased need for protein stabilisation.

#### 6.4.1.3 40S ribosomal protein S12 *NnrpS12*

DEG201-26 was identified during BLASTP analysis as a 40S ribosomal protein S12 (rpS12) (HE608882) and was most similar to sequences isolated from the polychaete orange fire worm *Eurythoe complanata* (78.8% ABW23206, e-value = $6.12e^{-67}$) and the lancelet *Branchiostoma belcheri* (83.2% AF4706871, e-value = $1.36e^{-66}$). Ribosomes are ribonucleoproteins (complexes of RNAs and proteins) that are the key cellular component for translation of RNA into proteins. Each ribosome is comprised of a large and a small subunit; during translation the small subunit binds to messenger RNA (mRNA) and the large subunit binds to transfer RNA (tRNA) and the amino

acids. In eukaryotes, the 40S subunit is the small subunit (the large is 60S), and is comprised of a 1900-nucleotide (18S) RNA and approximately 33 proteins (Alberts *et al.*, 2008). The reaction site for protein synthesis is comprised primarily of ribosomal RNA (rRNA) and the small individual proteins stabilise the ribosomal structure (Alberts *et al.*, 2008).

The Pfam domain identified for rpS12 was L7Ae. L7A is a domain from metazoa, but is part of a family that includes 40S ribosomal protein S12 and ribosomal protein L30 from eukaryotes, as well as ribosomal L8-A and L8-B from fungi and Gadd45 and MyD118. The family falls under the RNA ribose binding superfamily, a clan whose members are involved in binding the ribose sugar of RNA (Caban *et al.*, 2007).

Ribosomal proteins are typically found to be associated with energy production and are frequently up-regulated during cell proliferation (Chen and Ioannou, 1999). Cancer cells, in particular, have been found to have high expression levels of ribosomal proteins associated with rapid cell proliferation (Kim *et al.*, 2004). A microarray study to examine differential gene expression during the moult cycle of the crab *Portunus pelagicus* found ribosomal and mitochondrial proteins to be up-regulated during pre-moult and post-moult and were concurrent with periods of high energy requirement (Kuballa *et al.*, 2011). Similarly, during the energy-demanding phase of vitellogenesis the ribosomal protein RPL10a was significantly up-regulated in the banana prawn *Fenneropenaeus merguiensis* de Man (Wonglapsuwan *et al.*, 2009). In contrast, hypoxic stress was found to down-regulate the ribosomal proteins S15 and L23 in blue crabs *Callinectes sapidus* held in conditions of chronic hypoxia for five days (Brown-Peterson *et al.*, 2005). Brown-Peterson *et al.* (2005) argued that the inhibition of protein synthesis, which has been observed in vertebrates (Hochachka and Lutz, 2001) as a mechanism to reduce energy demand during hypoxia, was responsible for the down-regulation. Ribosomal protein expression has been clearly implicated in relation to energy demand and GeneFishing™ results indicated that gene expression levels of *NnrpS12* were reduced in *Hematodinium*-infected *Nephrops norvegicus*. Based on the observations of respiratory stress and fluctuations in haemolymph pH in crustaceans infected with *Hematodinium* (Taylor *et al.*, 1996; Hamilton *et al.*, 2010) (see 5.1.2 for further description) there is potential for protein inhibition in infected animals as an energy conservation mechanism.

### 6.4.2 RNA Integrity

As mentioned previously (6.3.2), the measurement of RNA quality using the Experion™ System revealed a difference in band pattern on the virtual gel between crustaceans heavily infected and those lightly infected or uninfected with *Hematodinium* sp. The

reason behind this pattern difference is unclear at present, however it was not found to affect the RQI value for any samples. All samples used had RQI values ≥7.5 regardless of the prevalence of *Hematodinium* sp. Furthermore, the bands for all samples were clear and distinct with little evidence of degradation in either the infected or uninfected animals. In a study comparing the quality of RNA extracted from frozen and live samples of the gorgonian coral *Pseudopterogorgia elisabethae* and its dinoflagellate symbiont *Symbiodinium* sp., an extra set of bands were evident on an electrophoresis gel in the live samples (Santiago-Vazquez *et al.*, 2006). Santiago-Vazquez *et al.* (2006) suggested that the extra bands are 16S and 23S ribosomal RNA belonging to cohabiting prokaryotes in *P. elisabethae* and prokaryotic genes were amplified from the sample, however the prokaryotes were not identified and it is unlikely that this is the case for *Hematodinium* sp. In the present study, it is likely that the size of the ribosomal subunits is distinct between *Hematodinium* sp. and their crustaceans hosts, however complete gene sequences for 18S and 28S ribosomal subunits are not currently available on NCBI (http://www.ncbi.nlm.nih.gov/guide/) for *Hematodinium* sp., *Nephrops norvegicus* or *Cancer pagurus*.

### 6.4.3 Gene Expression during *Hematodinium* infection

#### 6.4.3.1 *Prophenoloxidase*

The *proPO* gene was found to be significantly different between infected and control *N. norvegicus* and on average had a 2.5 fold increase in expression level in infected animals. The increase in *proPO* gene expression supports the observed increase in PO enzyme activity in infected animals from the same population. Invertebrate *proPO* has been observed to increase in response to numerous pathogens and their associated compounds, particularly bacteria and viruses (Lu *et al.*, 2006; Ko *et al.*, 2007; Ai *et al.*, 2008; Gai *et al.*, 2008; Labbe and Little, 2009; Zhang *et al.*, 2010).

Numerous *proPO* studies have been previously discussed (Chapter 4) and *proPO* expression has been clearly linked to pathogen susceptibility (Cerenius *et al.*, 2003; Liu *et al.*, 2007*a*; Amparyup *et al.*, 2009). The link has been formed to the extent that silencing of *proPO* using RNA interference (RNAi) in *Penaeus monodon* was found to reduce PO enzyme activity by up to 88% and increase susceptibility of hosts to *Vibrio harveyi* infection (Amparyup *et al.*, 2009). A similar study using RNA interference (RNAi) found that crayfish *Pacifastacus leniiusculus* with inhibited *proPO* had reduced PO activity and higher bacterial growth (Liu *et al.*, 2007*a*). Additionally, they suffered from reduced phagocytosis and nodule formation and increased mortality. Further studies have indicated that generally higher levels of *proPO* were beneficial in

crayfish *P. leniusculus* that are resistant to crayfish plague *Aphanomyces astaci* in comparison to susceptible crayfish *Astacus astacus* (Cerenius *et al.*, 2003) which contained lower transcript numbers.

*ProPO* responses have been variable and no consistent pattern in expression is evident. Crustaceans exposed to *Vibrio* sp., *Pasteuria ramosa* and lipopolysaccharide (LPS) have been shown to have short-term (2-48 hours post-infection) up-regulation of *proPO*, typically returning to pre-infection levels around 72 hours post-infection (Ko *et al.*, 2007; Gai *et al.*, 2008; Labbe and Little, 2009; Chen *et al.*, 2010*a*). Other studies have explored the effects of LPS injection and $\beta$1,3-glucan feeding on crustaceans and found no change in *proPO* expression (Okumura, 2007; Wang *et al.*, 2008, 2010). Similarly, a selection of immunostimulants including $\beta$1,3-glucan, a *Vibrio* vaccine, liquid extract of marine algae, CpG 1668 oligonucleotide (a pathogen-associated molecular pattern associated with microbes) and heat-killed *Listonella anguillarum* (Gram-negative bacteria) found that only heat-killed *L. anguillarum* was able to induce *proPO* expression in lobster granolocytes in culture, all other immunostimulants did not affect *proPO* expression (Hauton *et al.*, 2005). And finally, white spot syndrome virus (WSSV) and CpG-C injection were both found to down-regulate *proPO* expression (Ai *et al.*, 2008; Zhang *et al.*, 2010) although, interestingly, during the CpG-C study, PO activity was found to increase despite gene down-regulation (Zhang *et al.*, 2010).

Importantly, some of these studies have only used semi-quantitative PCR to analyse expression change and have not validated their reference genes; and, as Sperstad *et al.* (2010) and de Lorgeril *et al.* (2008) point out, many studies neglect to take into account changing haemocyte populations. ProPO is known to be primarily contained within granulocyte cells (Smith and Soderhall, 1983; Matozzo and Marin, 2010) and haemocyte populations change during infection. It is possible that the changes in gene expression following microbial challenge are due to fluctuations in granulocyte numbers. The present study found no significant change in haemocyte populations during *Hematodinium* sp. infection of *N. norvegicus* (see Chapter 5.3.1), therefore it is not believed that fluctuating haemocyte populations contributed to gene expression variations.

Additionally, the majority of the studies reported here used experimental infections rather than wild-caught infected animals. With this in mind, the researchers had a greater degree of control over sampled individuals and experimental conditions. All individuals were able to be treated identically to reduce confounding environmental effects. Furthermore, infection responses could be explored at specific times post-infection and the progression of disease monitored. The present study is a

snapshot of individuals from their natural environment that were potentially subject to numerous unknown stressors, in addition to the stresses trawling imposes on crustaceans. The duration of infection is also unknown when using wild-caught animals, though based on experimental infection studies in crabs from cold-water climates such as those from Alaska, it is possible chronically infected animals have suffered for many months (Meyers *et al.*, 1987).

Parasites have been found to have a unique interaction with their invertebrate hosts and several parasite species have been found to deliberately inhibit phenoloxidase activity to avoid melanisation (Beckage, 1998; Shelby *et al.*, 2000; Brivio *et al.*, 2002; Zhu *et al.*, 2011). Wasps and nematodes have been found to actively inhibit PO activity using a variety of methods. Parasitic wasps are particularly notorious for phenoloxidase interference and use polydnaviruses to reduce PO activity, inhibit melanisation pathways and suppress haemocyte adhesion (Beckage, 1998; Shelby *et al.*, 2000). Nematodes infecting larvae of the greater wax moth *Galleria mellonella* were also found to inhibit proPO activity and it was speculated that cuticle components were able to interfere with LPS-binding proteins reducing the activation of phenoloxidase cascade (Brivio *et al.*, 2002). Despite the large body of evidence for phenoloxidase suppression during parasite infection in invertebrates, the results from this study (increased *proPO* expression and PO activity) do not support PO inhibition as a method of immune interaction between *Hematodinium* and *N. norvegicus*. The lack of observed encapsulation and melanisation response (Stentiford *et al.*, 2003) combined with the high variability in PO activity (Chapter 5) suggest that parasites are likely avoiding recognition by the internal defense system and increases in *proPO* are a result of opportunistic secondary infections. This method of avoidance is supported by the *in vitro* study previously described (Chapter 4).

As previously discussed, *proPO* expression has also been observed to vary during *in vitro* exposure to pathogens, indicating direct interaction between haemocytes and foreign particles (Sritunyalucksana *et al.*, 1999; Hauton *et al.*, 2005). In this project, previous exposure of *Cancer pagurus* haemocytes resulted in no change in *proPO* expression, however *in vivo* studies indicate that *proPO* has increased during infection. This underlines the need to use *in vitro* and *in vivo* studies in conjunction with one another. In this case, the *in vitro* study provides support for the idea that *Hematodinium* may avoid the immune system altogether. Although *proPO* was increased in infected *N. norvegicus in vivo*, there is no conclusive evidence that this is a direct immune response to *Hematodinium* infection. It has been proven that certain physiological stressors such as starvation and hypoxia can increase PO enzyme activity (Le Moullac *et al.*, 1998; Matozzo *et al.*, 2011*b*). Previous studies suggesting physiological starvation in *Hematodinium*-infected animals (Stentiford *et al.*, 2000,

2001*c*) in combination with the limited pathological evidence of an immune response at the haemocyte level (Stentiford *et al.*, 2003) and the complete lack of a haemocyte response seen here *in vitro* suggests that phenoloxidase changes at both the transcriptional and translational level could be due to an indirect response to *Hematodinium* infection.

Alternatively, *Hematodinium* hosts have been known to suffer from secondary infections including bacteria, ciliates and yeast (Meyers *et al.*, 1987; Love *et al.*, 1993; Stentiford *et al.*, 2003; Hamilton, 2007). As previously mentioned, numerous bacterial species, particularly *Vibrio* spp. are known to increase PO activity (Gai *et al.*, 2008; Wang *et al.*, 2010) and *proPO* mRNA levels (Chen *et al.*, 2010*b*; Gai *et al.*, 2008). The ciliates and bacteria observed in *Chionocetes bairdii* were suggested to be those frequently found in previously compromised hosts (Meyers *et al.*, 1987; Stentiford *et al.*, 2003; Stentiford and Shields, 2005). In *Cancer pagurus* co-infected with *Hematodinium* sp. and a yeast-like organism, no immune response (recorded as degree of encapsulation) was evident towards *Hematodinium*, however nodule formation was observed around the yeast (Stentiford *et al.*, 2003). Nodule formation indicated that an immune response was still possible in *Hematodinium*-infected animals, however the degree of variation in immune response to the yeast was extremely high between individuals. This makes it clear that further investigation into the cause of increased *proPO* expression is required as the increase may be due to secondary infection by opportunistic pathogens.

#### 6.4.3.2 *Crustin*

Antimicrobial responses in crustaceans have been observed many times in the past, but only recently have researchers identified the individual peptides and corresponding nucleotide sequences involved in antibacterial response (Schnapp *et al.*, 1996; Relf *et al.*, 1999; Bartlett *et al.*, 2002; Hauton *et al.*, 2006; Brockton *et al.*, 2007; Battison *et al.*, 2008; Mu *et al.*, 2010). As previously described, in this study a crustin-like gene was identified via GeneFishing™ as being differentially expressed between a Norway lobster *N. norvegicus* infected with the parasite *Hematodinium* sp. and a control individual. To date, only limited evidence has been found for crustin activity towards a parasite. A crustin-like peptide (CAP-2) was isolated from the lobster *Homarus americanus*; recombinant expression of CAP-2 was found to inhibit growth of the ciliate parasite *Anophyroides haemophila*, though was not able to kill the parasite outright as was found for a temporin-like peptide isolated during the same study (Battison *et al.*, 2008). Other invertebrates have been found to contain AMPs against parasites: four cecropin and four defensin genes (both coding for their respective

antimicrobial peptides) have been found to increase expression in *Anopheles gambiae* in response to malaria infection (Christophides *et al.*, 2002). Furthermore, *A. gambiae* inoculated with *E. coli* expressing a cecropin-like immunotoxin were able to decrease the transmission of malaria by 95% (Yoshida *et al.*, 2001).

In this study, further investigation into *NnCrust* expression in a wider sample (n = 45) of *N. norvegicus*, however, revealed little variation in gene copy numbers normalised against haemocyte counts. Numerous studies have explored changes in crustin gene expression and have found varying results in response to a variety of pathogens. *Vibrio* spp., Gram-negative bacteria commonly found in fish and shellfish, have been used in multiple challenge studies with crustins. Exposure of the penaeid shrimp *Litopenaeus vannamei* and *Penaeus monodon* to *Vibrio* spp. caused a down-regulation in crustin expression six hours post-infection followed by a gradual recovery (Vargas-Albores *et al.*, 2004; Supungul *et al.*, 2004). In contrast, Amparyup *et al.* (2008) reported an increase in crustin expression from *P. monodon* 24 hours post-infection with *V. harveyi*. A further study reported a decrease in crustin 12 hours post-infection followed by an increase above normal expression by 24 hours (de Lorgeril *et al.*, 2008). Crustin expression changes have also been observed towards other Gram-negative bacteria including *Escherichia coli* (Jiravanichpaisal *et al.*, 2007), *Listonella anguillarum* (Hauton *et al.*, 2006; Sperstad *et al.*, 2010), *Aeromonas hydrophila*, *Acinetobacter* sp. (Jiravanichpaisal *et al.*, 2007), and the Gram-negative cell wall component lipopolysaccharide (Okumura, 2007). Results varied from increased expression towards *E. coli*, *A. hydrophila* and *Acinetobacter* sp. (Jiravanichpaisal *et al.*, 2007), decreased expression towards *L. anguillarum* (Hauton *et al.*, 2006) and LPS (Okumura, 2007) and no response to *L. anguillarum* in *Eriocheir sinensis* (Mu *et al.*, 2010). Isolated granulocytes showed a slight increase in expression when exposed to *L. anguillarum in vitro* (Sperstad *et al.*, 2010).

Responses to Gram-positive bacteria have been similarly variable. *Homarus gammarus* crustin was up-regulated following exposure to *Aerococcus viridans* var. *homari* and *Marsupenaeus japonicus* crustin was also up-regulated following injection with peptidoglycan (a cell wall component of Gram-positive bacteria) (Fagutao *et al.*, 2008). Other crustacean crustins from *Eriocheir sinensis* and *Carcinus maenas* were down-regulated following Gram-positive bacteria exposure (Mu *et al.*, 2010; Brockton and Smith, 2008); and studies on *Hyas araneus* and *Homarus americanus* showed no response towards Gram-positive bacteria (Sperstad *et al.*, 2010; Battison *et al.*, 2008).

It is clear that the gene expression response of crustins vary widely between both host and pathogen species. The different results observed between these studies could merely be a result of functional diversity due to varying crustin isoforms. It has been

reported several times that isoforms respond differently to identical experimental conditions, and in fact, a single amino acid change can be the difference between down-regulated gene expression and no response at all (Vatanavicharn *et al.*, 2009). Although the functional domains are often conserved in crustins across many crustacean species the amino acid sequences are actually highly variable. In this study two putative isoforms were identified, one of them including an insert of seven amino acids in the WAP domain, possibly having an effect on functionality of the entire protein. Due to the location of the qPCR primers in the conserved region of the nucleotide sequence, the present study quantified combined expression of both *crustin* isoforms, therefore it is unclear if there is, indeed, functional diversity between *NnCrust1* and *NnCrust2*. In future, expression studies could be improved by designing qPCR primers to target the differing amino acid region to measure expression separately.

As to the implication of the differential expression of *NnCrust* during GeneFishing,™ it could be a result of bacterial infection in the control *N. norvegicus* used in the experiment. Only a single animal was examined with differential display and individual immune responses to any foreign pathogens would be evident during this portion of the experiment that would not be observed in the entire population. As with all differential display techniques, it is essential to validate the results using a quantitative technique such as quantitative PCR, over a representative sample size. This ensures that the expression results are genuine and represent a universal response rather than that of an individual.

#### 6.4.3.3 Chaperonin containing T-complex protein 1 subunit 7 (*NnCct7*)

This is the first reported sequence for a chaperonin containing T-complex protein subunit 7 (*NnCct7*) from crustacean haemocytes. CCT proteins are known to be primarily involved in the folding of the cytoskeletal proteins actin and tubulin, however no functional studies have been carried out on these proteins in crustaceans, so the specific role is still unclear. In the present study, despite the difference in expression indicated by GeneFishing™, further investigation using qPCR found no significant difference in expression between control *Nephrops norvegicus* and those infected with *Hematodinium* sp.

A study of CCT subunits in HeLa cells and human erythroleukemia cells found that CCT subunits decreased slightly in concentration during chemical stress before increasing transiently during the recovery period (Yokota *et al.*, 2000). During a chronic infection such as found in *Hematodinium*-infected animals a recovery period may not be strongly evident and therefore a change in expression would not be

recorded for *cct* genes. An additional study by Yokota *et al.* (1999) linked CCT subunit mRNA expression to cell growth in which CCT was upregulated during early S phase in cultures of human and mouse cells. However, due to the nature of cell proliferation in crustaceans (arising from designated haemopoietic tissue), it is unlikely that gene expression change resulting from cell growth would be evident in circulating haemocytes. Although gene expression did not change significantly in the present study, *NnCct7* could still prove a useful gene for studying stress response, particularly during recovery, in crustaceans and further investigation would be useful to fully understand the functional role of this particular chaperonin subunit. Furthermore, due to its speculated link to cell growth, *cct* could be a useful gene for expression studies in haemopoietic tissue.

#### 6.4.3.4 40S ribosomal protein S12 (*NnrpS12*)

The gene coding for the 40S ribosomal protein S12 in *Nephrops norvegicus* (*NnrpS12*) was significantly down regulated in infected animals compared to control animals. Ribosomal proteins are generally considered the stabilising units of the ribosomal subunits and are frequently identified during differential gene expression studies (Tassanakajon *et al.*, 2006; Lee *et al.*, 2009; Li *et al.*, 2011). Their function in protein synthesis means they make up a high proportion of cellular molecules and can account for up to 50% of total cellular transcription in rapidly dividing yeast cells (Warner, 1999), but their commonality seems to make them often ignored or merely used as reference genes in gene expression studies. Genes coding for ribosomal RNA and proteins were found to comprise up to 30% of a suppressive subtractive hybridisation (SSH) library generated from a study investigating gene expression changes in Chinese mitten crabs *Eriocheir japonica sinensis* with white spot syndrome virus (WSSV) (Li *et al.*, 2011). The percentage of ribosomal proteins identified using ESTs from *Penaeus monodon* haemocytes increased from 8.2% in control animals to 17.6% in those challenged with *Vibrio harveyi* and 23.9% WSSV-challented animals (Tassanakajon *et al.*, 2006).

In general, ribosomal proteins are up-regulated during periods of high cell division when there are optimal growth conditions and are down-regulated during stress or during limited nutrient availability. This change in ribosomal protein gene expression has frequently been observed in yeast, bacteria and plants (Cardenas *et al.*, 1999; Betts *et al.*, 2002; Volkov *et al.*, 2003; Marion *et al.*, 2004). As previously mentioned, *N. norvegicus* suffer many forms of stress in response to *Hematodinium* infection, including physiological starvation and hypoxia (Taylor *et al.*, 1996; Stentiford *et al.*, 2001*c*; Shields, 2003). The decrease in *NnrpS12* expression supports previous evidence

of reduced serum protein concentration in *Hematodinium*-infected *Callinectes sapidus* (Shields, 2003). Haemocyanin, the major contributor to serum protein, was also reduced in infected *N. norvegicus* (Taylor *et al.*, 1996). Further reports have observed decreased glycogen and glucose concentrations and it has been speculated that parasites are consuming glycogen stores and proteins, causing the physiological starvation state. Infected *Callinectes sapidus* suffered chronic anorexia and progressive lethargy (Meyers *et al.*, 1987), suggesting depleted energy levels. Based on this evidence, in combination with evidence that starvation in yeast cells causes down-regulation in ribosomal protein expression (Marion *et al.*, 2004), there is reason to believe that physiological starvation caused by *Hematodinium* energy demands could decrease the expression of *NnrpS12*. Despite this evidence, it is also important to mention that although the primary role of ribosomal proteins is to provide structural support during protein synthesis, many individual proteins have been found to carry out separate functions (Lee *et al.*, 2009).

Without further examination, the role of *NnrpS12* during *Hematodinium* infection can not be determined. Decreases in translational components could be a result of decreased physiological competence due to severe parasite infection. Heavily parasitised animals were frequently lethargic and it is likely the drain on internal resources had rendered the animals incapable of directing energy towards protein synthesis.

## 6.5 Conclusions

In conclusion, the use of the differential display technique GeneFishing™ in this study has identified three new genes from *Nephrops norvegicus*: *NnrpS12*, *NnCct7* and two isoforms of *NnCrust*. Of these three genes, only *NnrpS12* was found to change expression, in addition to the *proPO* gene, during *Hematodinium* sp. infection and the null hypothesis (6.1.1) was rejected for these genes. The ribosomal protein *NnrpS12* was significantly down-regulated in *Hematodinium*-infected animals and is likely a result of physiological starvation. Simultaneously, the *proPO* gene was found to be up-regulated in infected animals, confirming that prophenoloxidase is up-regulated at both the transcriptional and translational level, and placing a further demand on energy reserves. Increased expression of *proPO* mRNA in combination with decreased haemocyte counts (Chapter 5) indicate that an internal defense response is present in *Hematodinium*-infected animals, though it can not at present be confirmed that the response is directed towards *Hematodinium* sp. cells. The lack of response by haemocyte *proPO in vitro* (Chapter 4) suggest that the change in gene expression observed in the present study is not in response to parasite cells and could possibly be

in response to secondary infection by opportunistic pathogens. In light of these results it is evident that, despite the apparent reduction in immunocompetence, evidenced by a significant decrease in haemocyte numbers (Chapter 5) and down-regulation of *NnrpS12* (known to decrease during stress and starvation), *Hematodinium*-infected *Nephrops norvegicus* are still capable of mounting an immune response.

# Chapter 7

# Conclusions

The increasing importance of crustaceans in both fisheries and aquaculture has driven the need to expand our understanding of their general biology, with particular interest directed towards disease control for management purposes. Crustaceans can suffer from both non-infectious diseases precipitated by physiological stressors such as hypoxia and nutrient deficiency, as well as from pathogen-associated infectious diseases including viruses, bacteria, fungi and protozoan parasites. Disease control is only possible when there is a clear understanding of host-pathogen interaction, and a great deal of the current research focusses on the host internal defense system. The dinoflagellate parasite *Hematodinium* spp. is an emerging pathogen of decapod crustaceans worldwide and has been reported in blue crabs *Callinectes sapidus* along the East coast of the US (Newman and Johnson, 1975; Messick, 1994; Sheppard *et al.*, 2003), in Tanner crabs *Chionoecetes bairdi* in Alaska (Meyers *et al.*, 1987) and the mud crab *Scylla serrata* from Australia and Southeastern China (Hudson and Lester, 1994; Li *et al.*, 2008*b*), among others. In the UK, *Hematodinium* sp. has been reported from several species, including hermit crabs *Pagurus bernhardus* (Hamilton *et al.*, 2009) and velvet swimming crabs *Necora puber* (Stentiford *et al.*, 2003; Hamilton *et al.*, 2009) as well as the commercially important Norway lobsters *Nephrops norvegicus* and edible crabs *Cancer pagurus* (Field *et al.*, 1992; Stentiford *et al.*, 2002).

Infection with *Hematodinium* spp. has been found to cause metabolic disruption and extreme lethargy in host animals (Field *et al.*, 1992; Taylor *et al.*, 1996; Stentiford *et al.*, 2001*c*). Pathological investigation has found reduced haemocyte numbers in infected animals in combination with tissue disruption of hepatopancreas, gill and muscle (Meyers *et al.*, 1987; Shields, 2003; Wheeler *et al.*, 2007). Interestingly, several studies have noted a distinct lack of immune response to *Hematodinium* presence. There are a few reports of haemocytic nodules and encapsulation responses in infected

animals, though none were able to confirm that the response was directed toward parasite cells (Meyers *et al.*, 1987; Field and Appleton, 1995; Stentiford *et al.*, 2002). The same reports noted the presence of secondary bacterial and ciliate infections in a number of *Hematodinium*-infected individuals and suggested that *Hematodinium* sp. may increase the likelihood of colonisation by opportunistic pathogens. In a specific observation by Stentiford *et al.* (2003) *Cancer pagurus* were co-infected with *Hematodinium* sp. and a yeast-like organism. Histological investigation revealed that an immune response towards the yeast-like organisms was present in the form of encapsulation and nodule formation, however no response was directed towards parasite cells. These observations led to speculation that *Hematodinium* sp. was able to either avoid or suppress the immune system of its host. A later study by Small *et al.* (2007*a*) found that *Hematodinium* sp. ex *N. norvegicus* secreted acid phosphatase into the culture media and suggested that this may be a mechanism of immune suppression used by *Hematodinium* sp. Despite the hypotheses that *Hematodinium* sp. either avoids or suppresses the internal defense of its host, to date no studies have specifically investigated host response towards the parasite and there is a current knowledge gap in *Hematodinium*-host immune interaction. The present study aimed to investigate if *a*) there is no immune response from crustacean hosts towards *Hematodinium* sp. or *b*) the parasite actively suppresses or inhibits the immune response. To achieve this aim, the immune response of two UK crustacean hosts, *Cancer pagurus* and *Nephrops norvegicus*, to *Hematodinium* sp. infection was investigated using gene expression analysis and immune assays to achieve three primary objectives:

1. *In vitro* experiments in which individual haemocyte populations from *Cancer pagurus* were exposed to *Hematodinium* sp. cells aimed to determine gene expression responses of specific haemocyte types during host-parasite cell interaction (Chapter 4).

2. Immune assays aimed to investigate *in vivo* changes in immune parameters of wild-caught *Nephrops norvegicus* (Chapter 5).

3. Gene expression analysis of naturally infected wild-caught *Nephrops norvegicus* aimed to examine *in vivo* immune response of a host to *Hematodinium* sp. infection (Chapter 6).

Before gene expression studies could commence, gene sequences had to be generated for all genes of interest and reference genes, and quantitative PCR assays had to be optimised. In Chapter 2, the methods for gene identification and molecular cloning utilised throughout this thesis were described. The gene coding for the immune-relevant proenzyme prophenoloxidase (*proPO*) was chosen for its key role in crustacean internal defense and the first published *proPO* sequences were generated

here for *Cancer pagurus* (*CpproPO*; FR687020) and *Nephrops norvegicus* (*NnproPO*; HE608877). Both *CppropPO* and *NnproPO* had high identity with other crustacean *proPO* genes and the translated amino acid sequences contained two copper-binding sites and a thiol ester-like motif, all of which are conserved in proPO proteins. The sequences generated in the present study did not show signs of having isoforms and, based on their high identity with other crustacean proPOs, were deemed suitable for gene expression analysis.

Partial sequences for the *actin* gene were also generated for the first time for *C. pagurus* (*CpAct*; FR687021), *N. norvegicus* (*NnAct*; HE608878) and *Hematodinium* sp. ex *N. norvegicus* (*HemAct*; HE609031) for use as endogenous reference genes, though due to the lack of *Hematodinium* sp. genes for expression analysis and the use of absolute quantification during the *Nephrops norvegicus* studies, only the gene from *C. pagurus* was used during this project for relative quantification of *C. pagurus* host gene expression. An additional gene from *Hematodinium* sp. (*Hem18S*; HE609032), comprising a partial sequence of the 18S ribosomal RNA (rRNA), internal transcribed spacer 1 (ITS1; complete), 5.8S rRNA (complete) and a partial sequence of ITS2, was sequenced with the intention of quantifying parasite abundance in the haemolymph of wild-caught *N. norvegicus*. Unfortunately, after considerable effort, the *runt* gene involved in haemopoiesis was not able to be isolated and sequenced from either *Cancer pagurus* or *Nephrops norvegicus* and due to time constraints was left out of the present study.

Quantitative PCR (qPCR) has become the 'gold standard' for studying gene expression and is extremely sensitive to fluctuations in gene copy numbers (Bustin, 2000; Ginzinger, 2002). High sensitivity, however, is accompanied by a high susceptibility to error due to technical variations in sample handling and poor assay optimisation (Bustin, 2010). Chapter 3 described the rigorous optimisation methods that were carried out on all genes of interest and endogenous reference genes in this project to reduce the possibility of error and to ensure that results were robust and comparable. Suitable assays were developed for all genes except for *Hem18S* which had a poor efficiency value and was therefore discarded.

The PCR-based differential display technique GeneFishing™ (Chapter 4) was used to identify differentially expressed genes between control cultures and those exposed to *Hematodinium* sp. GeneFishing™ identified two novel *C. pagurus* genes, one coding for a prophenoloxidase activating factor (*ppaf*) and another coding for a DEAD-box ATP-dependent RNA helicase (*ddx*) in unexposed granulocyte cultures. The full sequence obtained for *ppaf* (HE608875) had high identity with other crustacean *ppaf* genes and several serine proteinase homologues which are closely related. Six conserved

cysteine residues were present and a catalytic triad was identified, though (as in other closely related sequences) the serine was replaced with a glycine rendering the protein catalytically inactive. The full sequence of *ddx* (HE608876) had high identity with other invertebrate *ddx* genes and contained nine conserved motifs found in DEAD-box proteins.

The data presented in Chapter 4 indicated no sign of immune response in the measured parameters from individual haemocyte types (granulocytes or hyaline cells) from *Cancer pagurus* to *Hematodinium* sp. exposure *in vitro*. Microscopic observations of individual granulocyte and hyalinocyte cultures exposed to *Hematodinium* sp. cells gave no indication of an overt immune response such as aggregation, cell rounding or loss of adhesion, and no difference could be observed when compared to control haemocyte cultures. Furthermore, viability assays indicated no difference in viability between exposed and control cultures.

Gene expression analysis using relative quantification found no significant difference in expression for any of the genes of interest in either granulocyte or hyaline cell cultures. *Hematodinium* sp. did not elicit a transcriptional response for *proPO*, *ppaf* or *ddx* from separated haemocytes *in vitro*. These results indicated that under *in vitro* conditions, separated haemocyte populations had a limited defense response to parasite cells. Importantly, the advantages and limitations of *in vitro* conditions must be considered as they could be contributing to the observed response. The primary advantage of *in vitro* experimentation is the use of a controlled environment in which sterile and nutrient-stable conditions can be maintained, and therefore direct cell-cell interactions can be observed without other confounding factors. In crustaceans, cell separation and *in vitro* study have proven invaluable to understanding the individual role of haemocyte types and to reduce confusion over which haemocytes are contributing to an observed transcriptional response (Smith and Ratcliffe, 1978; Muñoz *et al.*, 2002). However, crustacean internal defense is based on a combination of humoral and cellular components and in the present study the humoral factors (eg. lectins and agglutinins) were removed during cell separation. Furthermore, crustacean cells have been shown to communicate during defense reactions and the separation of granulocytes and hyaline cells may have had a limiting effect on the cellular response. With this in mind, it was imperative that *in vitro Hematodinium* sp. exposure studies be supported by *in vivo* analysis as well. Due to difficulty obtaining infected specimens of *Cancer pagurus*, *in vivo* studies were carried out on the Norway lobster, *Nephrops norvegicus*.

*In vivo* studies in this project commenced with the comparison of multiple immune parameters between *Hematodinium*-infected *Nephrops norvegicus* and those that were uninfected or lightly infected based on parasite counts in the haemolymph (Chapter 5).

Total haemocyte counts were significantly reduced in infected animals which is a strong indication of reduced immune capacity. However, infected animals had significantly enhanced prophenoloxidase (proPO) stores (termed 'phenoloxidase (PO) activity'), showing that an immune response was still possible during heavy parasite infection and under conditions of haemocytopoenia. PO activity was much more variable in infected animals than control animals, however correlation tests between PO activity and parasite load were not significant. Changes in PO activity as a result of fluctuating granulocyte counts were ruled out because differential cell counts were not signficiantly different between infected and control animals. Occasional observations of concurrent bacterial infection during haemocyte counts in infected individuals suggest secondary infections could have contributed to the increased variability of PO activity, however data were not specifically collected for bacterial analysis during this study so further investigations must be carried out to confirm this. Secondary infections have frequently been recorded in *Hematodinium*-infected crustaceans (Meyers *et al.*, 1987; Stentiford *et al.*, 2003; Small, 2004) and the interactions of *Hematodinium* sp. and opportunistic pathogens could shed further light on crustacean immune response during parasite infection.

Haemocyte lysate from *Nephrops norvegicus* did not show any significant difference in haemagglutinating properties between infected and control animals. Likewise, intracellular superoxide (SO) anion production was not significantly different between infected and control animals. Interestingly, SO production varied considerably in infected animals (evidenced by a large standard deviation) compared to control animals. Further investigation revealed a significant correlation between SO concentration and *Hematodinium* sp. abundance. This correlation supported a hypothesis by Small *et al.* (2007*c*) who suggested that acid phosphatase (AP) secreted by *Hematodinium* sp. ex *N. norvegicus* could be used to inhibit SO production. Studies linking the inhibition of reactive oxygen species (such as SO) to acid phosphatase production by bacteria (Baca *et al.*, 1993; Siemsen *et al.*, 2009) indicate that a similar mechanism of immune suppression could be in use by *Hematodinium* sp.

The data presented in Chapter 6 indicated that the presence of *Hematodinium* sp. *in vivo* could influence the transcription of the immune-relevant *proPO* gene in addition to genes involved in protein synthesis. To begin with, GeneFishing™ identified 14 differentially expressed genes between naturally infected and control wild-caught *Nephrops norvegicus* and three genes from *Hematodinium* sp. Following cloning and sequencing, three genes were successfully identified from uninfected *N. norvegicus*. The novel *N. norvegicus* genes were submitted to GenBank and coded for an immune-relevant crustin-like antimicrobial peptide (AMP) (*NnCrust*), a 40S ribosomal protein S12 (*NnrpS12*) and a chaperonin containing T-complex protein subunit 7

($\eta$/eta) (*NnCct7*). The full sequence generated for *NnCrust* revealed two isoforms of the gene (*NnCrust1* HE608880 and *NnCrust2* HE608881), both of which had high identity with other crustacean crustin-like proteins and contained a whey-acid protein (WAP) domain linked with protein inhibition or antibacterial activity (Smith *et al.*, 2008). *NnCrust1* contained an insert in the functional WAP domain however, and it is unclear if this isoform is an active antimicrobial peptide and if it has a separate function. *NnrpS12* (HE608882) had high identity with several invertebrate 40S ribosomal proteins and most ribosomal proteins are typically linked with energy production (Chen and Ioannou, 1999). The final gene, *NnCct7* (HE608879), had high identity with several invertebrate cct7 proteins and contained a conserved site that plays a role in ATP/$Mg^{+}$ binding for protein folding and stabilisation.

Gene expression analysis showed *proPO* to be up-regulated in *Hematodinium*-infected *N. norvegicus* and *NnrpS12* was down-regulated. No significant response was observed for *NnCct7* or *NnCrust*. The increase in *proPO* expression corroborated the increase in PO activity and further confirmed that the internal defense system was not completely defunct in infected animals. It was also the first indication in the present study of an active immune response. Based on reports from the gene expression of other ribosomal proteins, the decrease in *NnrpS12* was indicative of depleted energy reserves and/or increased stress (Cardenas *et al.*, 1999; Betts *et al.*, 2002; Marion *et al.*, 2004), both of which have been reported for crustaceans infected with *Hematodinium* sp. (Taylor *et al.*, 1996; Stentiford *et al.*, 2001*c*; Shields, 2003). No change in expression was found for *NnCrust*, which indicated that *Hematodinium* sp. was not altering the transcriptional regulation of the crustin-like protein, though further protein studies would be required to determine whether the parasite was actually avoiding detection by the peptide or interacting only at the translational level. Additionally, the *NnCrust* primers designed for the present study encompassed both isoforms and examination of the individual isoforms may result in observable changes in expression. *NnCct7* also did not show any change in expression between infected and control *N. norvegicus*, which underlined the need for validation of differential display techniques. Despite several genes being identified as differentially expressed in both *in vivo* and *in vitro* experiments, the majority of them were not supported when examined across multiple animals. Differential display is frequently carried out on only a small sample set and individual variations in physiology can indicate changes in expression that are not upheld upon further investigation. As evidenced here, it is essential to validate all differentially expressed genes using qPCR to gain a firm understanding of the overall expression response.

The gene expression results from the *in vivo* portion of this study, in combination with the *in vitro* experiment expression results, emphasise the need for validation between

the two methods. As previously mentioned, *in vitro* experiments are extremely valuable as tools to understand cell function in the absence of external influences and where strict environmental control is desired. They are also useful to establish cell function of individual cell populations, though they lack essential humoral components and separated cell populations eliminate cellular communication between cell types. *proPO* expression from haemocytes *in vitro* did not vary in expression when exposed to *Hematodinium* sp., but a significant increase in *proPO* expression was found *in vivo*. Clearly there were additional factors or secondary infections present in the *in vivo* system which were altering expression and further studies are required to determine the exact details.

## 7.1 *Hematodinium* sp. and crustacean immune response

The success of an internal parasite is based largely on its ability to circumvent the host internal defense system long enough to complete its life cycle. Stentiford and Shields (2005) previously suggested that *Hematodinium* sp. may be avoiding or suppressing the host immune system, which is common in a number of internal parasites (Loker, 1994). From the data presented in this thesis it is evident that *Hematodinium* sp. has a significant impact on immune parameters within its crustacean hosts, with evidence for both avoidance and possible mechanisms for suppression.

The significant decrease in total haemocyte counts observed in *Nephrops norvegicus* invariably has a severe impact on immune competence as it is the primary unit of internal defense. Several previous studies have suggested that the decrease is due to encapsulation, agglutination and other immune responses (Field and Appleton, 1995; Stentiford *et al.*, 2002; Shields, 2003); however, *in vitro* observations of *C. pagurus* haemocyte-parasite interactions during the present study did not show any signs of encapsulation or any overt response to parasite cells, nor was there any difference in haemocyte viability as a result of parasite exposure. An alternative hypothesis is that total haemocytes decrease as a result of physiological starvation caused by excessive parasite load (Stentiford and Shields, 2005), with the knock-on effect of suppressing the immune response. Haemocytes are likely being outcompeted for nutrients and this is supported by the down-regulation of the ribosomal protein *NnrpS12*. Members of this gene family of ribosomal proteins have been shown to be down-regulated during periods of stress or limited nutrient availability (Cardenas *et al.*, 1999; Betts *et al.*, 2002).

The strongest evidence for active immune suppression found in the present study was the negative correlation between intracellular superoxide anion production and *Hematodinium* sp. presence in the haemolymph. It is currently unknown whether

*Hematodinium* sp. secrete acid phosphatase (AP) solely as a mechanism of protection against crustacean defense molecules or as a mediator of nutrient acquisition (Small, 2004). Even if the primary function is to aid in nutrient acquisition, however, the reduction in SO production likely aids in parasite survival.

The stable expression of *proPO* during *in vitro* challenge experiments using *C. pagurus* haemocytes provided evidence for immune avoidance, however the results were contradicted by *in vivo* studies of naturally infected *N. norvegicus*. Despite the decrease in total haemocytes *in vivo*, both PO activity and *proPO* expression were significantly increased in *Hematodinium*-infected *N. norvegicus*, which provided evidence that infected animals were still capable of mounting an immune response as well as confirming that proPO was enhanced at both the transcriptional and translational level. The contradiction in response between *in vitro* and *in vivo* responses could be explained by limitations imposed by *in vitro* conditions and the absence of crucial plasma factors, however the role of opportunistic pathogens should also not be ignored. Several reports of secondary infection have been made from *Hematodinium*-infected crustacean hosts (Meyers *et al.*, 1987; Stentiford *et al.*, 2003) and it is likely the increase in PO activity and *proPO* are, in fact, a result of secondary infection. Secondary infections would also explain the increased variability in PO activity in infected animals due to the possibility of a varied range of pathogenic species as well as a variable intensity of infection.

Further evidence of immune avoidance were the non-significant results from the haemagglutinin assays and the stable expression of the crustin-like AMP *NnCrust*. Both of these studies suggest a lack of recognition of *Hematodinium* sp. Haemagglutinin assays rely on recognition of surface carbohydrates (Marques and Barracco, 2000) and, although *N. norvegicus* haemocytes were capable of agglutinating chicken red blood cells, there was no difference between infected and control animals, indicating that intracellular agglutinins do not play a strong role in parasite recognition. Crustins and other AMPs interact with phospholipids in pathogen cell membranes (Epand and Vogel, 1999), and the stable expression of *NnCrust* suggests that phospholipid recognition is not part of the immune response to *Hematodinium* sp. either. Pathogen recognition is a key component of the internal defense response, however very little evidence was found in the present study to indicate any form of parasite cell recognition, suggesting that the parasite cells may lack recognisable cell surface molecules or be able to cloak them under a surface coat.

## 7.2 Future Research Considerations

The present study has initiated the complex task of analyzing the relationship between an internal dinoflagellate parasite *Hematodinium* sp. and its crustacean host's internal defense system. Essential progress has been made and, in the process, has highlighted future avenues of research.

Pathogen recognition is imperative in instigating an immune response in all organisms and the limited evidence for *Hematodinium* sp. recognition infers that immune avoidance may be a key mechanism to successful host colonisation for this parasite. The present study has focussed primarily on cellular immune components (intracellular proPO, intracellular SO, intracellular agglutinins, haemocyte RNA), though many pathogen recognition molecules are humoral and circulate freely in the haemolymph. Further studies regarding the specific interactions of pathogen recognition receptors and *Hematodinium* sp. cells would be invaluable in elucidating mechanisms of avoidance. For example, genes coding for several pattern recognition proteins have been sequenced from crustaceans and used for gene expression analysis during infection studies (Lee and Soderhall, 2001; Cheng *et al.*, 2005; Du *et al.*, 2007) and binding assays using recombinant proteins have been used to which pathogens are recognised by individual PRPs (Lee and Soderhall, 2001; Du *et al.*, 2007). Furthermore, pathogens are recognised based on pathogen associated molecular patterns (lipopolysaccharides, $\beta$1,3-glucans, peptidoglycans, etc.), however, to date, few studies have specifically analysed *Hematodinium* sp. for the presence of these molecules in the cell membrane. It is possible that *Hematodinium* sp. lacks these molecules or is able to mask them and thus evade detection.

Further investigation into the role of phenoloxidase during *Hematodinium* sp. infection is necessary to determine if the increased PO activity and *proPO* gene expression is, indeed, due to *Hematodinium* sp. presence or if it is a result of secondary infection. Controlled infection studies would be extremely useful to progress these investigations and would require successful passage of *Hematodinium* sp. infection. To date, *Hematodinium* sp. has been successfully passaged in a few crustaceans, particularly the blue crab *Callinectes sapidus* (Messick and Shields, 2000; Shields and Squyars, 2000) and development of a similar method for *Nephrops norvegicus* would significantly aid *in vivo* immune studies. Co-infection studies could then be used to elucidate whether prophenoloxidase is varying due to parasite infection or as a result of opportunistic pathogens. Instigation of *in vivo Hematodinium* sp. infections would also aid further exploration of the interaction between parasite-produced acid phosphatase and host superoxide anion production.

In conclusion, it can be stated that *Hematodinium* sp. has shown signs of both avoiding the host immune response as well as possessing a potential mechanism for immune suppression. At present, *Hematodinium* sp. has been found to elicit little immune response although explicit mechanisms of avoidance were not determined. It is possible that molecules typically recognised by host pattern recognition proteins are absent from the plasma membrane of the parasite or, like trypanosomes, a surface coat is secreted to prevent host molecules from coming in contact with parasite PAMPs (Zarnbrano-Villa *et al.*, 2002). However, a mechanism for immune suppression, suggested by Small *et al.* (2007*a*) was supported here by the clear correlation between superoxide anion production and *Hematodinium* sp. abundance in the haemolymph. The suppression of reactive oxygen species by phosphatase has been found to be a common trait of protozoan parasites and is likely a key strategy for parasite success. The use of acid phosphatase was particularly noted from *Leishmania* which inhibits the respiratory burst of the phagocytes it infiltrates during infection (Remaley *et al.*, 1984). The mechanism is also common from a number of protozoan parasites of marine shellfish such as the intracellular parasites *Bonamia ostreae* infecting *Ostrea edulis* (Hervio *et al.*, 1991; Morga *et al.*, 2009) and *Perkinsus marinus* infecting *Crassostrea virginica* (Volety and Chu, 1995; Anderson, 1999*a*,*b*). Overall, the results of this thesis provide valuable information and guidance towards the continued endeavor to understand host-parasite internal defense interactions.

As a matter of personal opinion, and based on the knowledge gained and observations made during the course of my research, it is apparent that *Hematodinium*-infected *N. norvegicus* suffer a slowly debilitating disease that consumes energy and leaves the individual susceptible to a multitude of opportunistic pathogens. The lack of observed direct immune response to date indicates that *Hematodinium* likely avoiding detection by host molecules, possibly by masking any PAMPs present on the cell surface or by not having proteins recognisable by the host. At present, *Hematodinium* has only caused one major fishery closure, however some shellfish stocks suffer significant losses during peak infection season. I believe that the importance of this disease will only increase as fishing pressure increases on rapidly diminishing stocks. Based on the information presented here it is hoped that future research will further expand our knowledge regarding the mechanisms utilised by *Hematodinium* sp. to avoid or suppress its host's immune system. A complete understanding of disease progression and methods of infection can only lead to improved management methods to minimize the impact of *Hematodinium* infection on commercially important species.

# Appendix A

# Molecular Protocols

## A.1 *Not*I Restricion Digest

Table A.1: Components for *Not*I restriction digest

| **Component** | **Volume** |
|---|---|
| Plasmid | 1 $\mu$g |
| 10X Buffer SH | 2.5 $\mu$l |
| *Not*I | 1 $\mu$l |
| Sterile water | $*$ |
| **Final volume** | 25 $\mu$l |

**Method**

1. All reaction components listed above (Table A.1) were combined in a 200 $\mu$l thin-walled PCR tube and incubated for 1 hr at 37°C.
2. Following incubation, the reaction was heat inactivated at 65°C for 15 min.
3. Samples were then stored at -20°C until needed.

## A.2 Extraction of nucleic acids from agarose gel using Qiagen QIAquick® Gel Extraction kit

This protocol was used to extract and purify DNA from an agarose gel after electrophoresis using the Qiagen QIAquick® Gel Extraction kit. All centrifugation steps were carried out at 17 900 $g$ at room temperature in a conventional tabletop microcentrifuge.

**Method**

1. Using appropriate UV protection, the desired bands were identified using UV light and excised from the gel using a sterile scalpel blade and removing a minimum of excess gel. The gel fragments were then placed in individual sterile 1.5 ml microcentrifuge tubes.

2. To each sample, 500 $\mu$l of Buffer QG was added and the samples incubated at 50°C for 10 min. To ensure the gel completely dissolved samples were vortexed every 2-3 min. (Buffer QG contains a pH indicator which is yellow when the pH is $\leq$ 7.5. If the colour turns orange or violet following incubation, add 10 $\mu$l of 3M sodium acetate pH 5.0 and mix.)

3. 200 $\mu$l of 2-propanol (Sigma-Aldrich, Dorset, UK) were added to each reaction and mixed.

4. Samples were then pipetted into individual spin columns in collection tubes and centrifuged for 1 min to bind the DNA to the column. The flow through was discarded and the column replaced in the collection tube.

5. The column was washed with 500 $\mu$l Buffer QG and centrifuged for 1 min. The flow through was again discarded and the column replaced in the collection tube.

6. To wash, 750 $\mu$l of Buffer PE were added to each collection tube and allowed to stand for 5 min at room temperature. Columns were then centrifuged for 1 min and the flow through discarded.

7. The columns were replaced in the collection tubes and centrifuged for a further 1 min to remove residual ethanol from Buffer PE.

8. The QIAquick columns were then placed into clean 1.5 $\mu$l microcentrifuge tubes and the DNA eluted with 30 $\mu$l Buffer EB. Columns were allowed to stand for 1 min before being centrifuged for 1 min.

9. Samples were stored at -20°C.

## A.3 Cloning PCR products using TOPO TA Cloning® Kit for Sequencing

This protocol was used to clone *Taq* polymerase-amplified PCR products using the TOPO TA Cloning® kit for Sequencing (Invitrogen, Paisley, UK), following the manufacturers recommendations. The cloning reaction requires the presence of deoxyadenosine (As) overhangs at the 3' end of the PCR product in to ligate. Proofreading *Taq* polymerase, such as that used in the SMART RACE protocol, does not add A-overhangs but they can be added prior to beginning cloning.

**Addition of 3' A-overhangs**

1. Following gel extraction of the desired product, 28 $\mu$l of product were combined in a 200 $\mu$l PCR tube with 0.5 $\mu$l *Taq* polymerase (Qiagen, Crawley, UK), 0.5 $\mu$l dATP and 3.2 $\mu$l *Taq* buffer.

2. Samples were incubated at 72°C for 20 min and were then ready for cloning.

**TOPO Cloning reaction**

1. In a 200 $\mu$l PCR the reagents listed in Table A.2 were combined in the order listed and mixed gently without vortexing.

Table A.2: Components for TOPO cloning reaction

| Component | Volume |
|---|---|
| PCR product | 1 $\mu$l |
| Salt Solution | 1 $\mu$l |
| (1.2 M NaCl, 0.06 M $MgCl_2$) | |
| Sterile water | |
| TOPO vector | 1 $\mu$l |
| **Final volume** | 6 $\mu$l |

2. Samples were incubated at room temperature for up to 30 min and then placed on ice.

3. Fifteen minutes prior to use, One Shot TOP10 chemically competent *E. coli* (one vial per reaction) was removed from -80°C storage and placed on ice to thaw.

4. To transform the *E. coli*, 2 $\mu$l of the TOPO cloning reaction was added to the 50 $\mu$l *E. coli* vial and the reaction gently mixed. Samples were incubated on ice for 30 min.

5. *E. coli* were then heat-shocked in a water bath at 42°C for 30 seconds and then placed immediately on ice.

6. After cooling on ice, 250 $\mu$l room temperature SOC medium (Super Optimal broth with Catabolite repression) was added to each reaction and the tube caps tightly secured.

7. All samples were placed in a shaking incubator at 37°C for 1 hour (200 rpm).

8. Following incubation, 30-50 $\mu$l *E. coli* were spread onto pre-warmed selective plates (Luria agar treated with 50-100 $\mu$g ml$^{-1}$ ampicillin sodium salts) and incubated at 37°C overnight. Only *E. coli* containing the vector and insert can have ampicillin resistance.

**Selection of positive *E. coli* transformants**

9. After incubation, 8-10 colonies were selected for further analysis by colony PCR.

10. Colony PCR master mix was prepared by combining in a PCR tube the reagents listed in Table A.3.

Table A.3: Components for colony PCR

| **Component** | **Volume** |
|---|---|
| 10X *Taq* Buffer | 2 $\mu$l |
| M13 Forward primer | 0.5 $\mu$l |
| M13 Reverse primer | 0.5 $\mu$l |
| dNTP | 0.4 $\mu$l |
| Sterile water | 16.1 $\mu$l |
| *Taq* | 0.5 $\mu$l |
| **Final volume** | 20 $\mu$l |

11. Selected colonies were picked from the Luria agar plate using a sterile cocktail stick and swirled gently in the master mix. The cells were then streaked onto a fresh Luria agar plate and the plate placed in the incubator overnight at 37°C overnight.

12. The PCR reactions were then placed in a thermal cycler using the following cycling conditions:

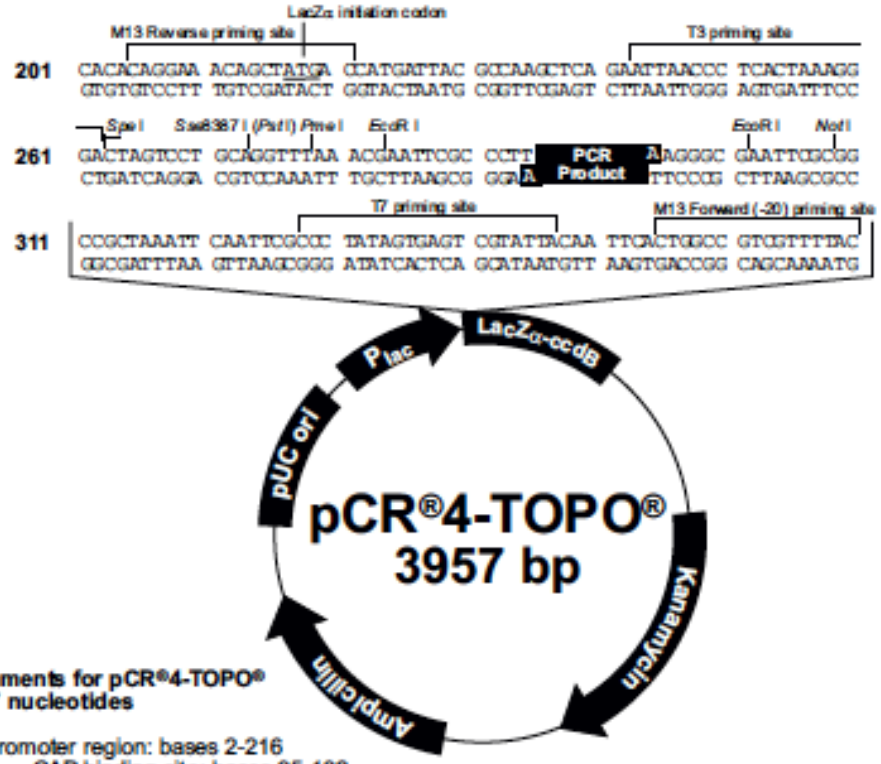
Table A.4: Colony PCR thermocycling conditions

| Step | Temp (°C) | Duration | No. Cycles |
|---|---|---|---|
| Initial Denaturation | 95 | 10 min | 1 |
| Denaturation | 95 | 30 sec | |
| Annealing | 55 | 30 sec | 30 |
| Extension | 72 | 90 sec | |

13. The resulting PCR products were visualised on a 1% agarose gel containing 5.5 $\mu$l ethidium bromide (10 mg ml$^{-1}$). Colonies containing fragments of the expected size were cultured overnight in preparation for plasmid extraction.

### A.3.1 TOPO® Cloning Vector Map

The map below shows the features of pCR®4-TOPO® and the sequence surrounding the TOPO® Cloning site. Restriction sites are labeled to indicate the actual cleavage site. **The complete sequence of pCR®4-TOPO® is available for downloading from our Web site (www.invitrogen.com) or by contacting Technical Service (page 25).**




**Comments for pCR®4-TOPO®**
**3957 nucleotides**

*lac* promoter region: bases 2-216
- CAP binding site: bases 95-132
- RNA polymerase binding site: bases 133-178
- Lac repressor binding site: bases 179-199

Start of transcription: base 179
M13 Reverse priming site: bases 205-221
LacZα-*ccdB* gene fusion: bases 217-810
- LacZα portion of fusion: bases 217-497
- *ccdB* portion of fusion: bases 508-810

T3 priming site: bases 243-262
Polylinker: bases 262-312
TOPO® Cloning site: bases 294-295
T7 priming site: bases 328-347
M13 Forward (-20) priming site: bases 355-370
*neo* (kanamycin) promoter region: bases 1021-1070
*neo* (kanamycin) resistance gene (ORF): bases 1159-1953
*bla* promoter region: bases 2062-2156
- RNA polymerase binding site: bases 2062-2143
- -35 region: bases 2087-2093
- *bla* promoter (P3): bases 2116-2122

Start of transcription: base 2122
Ribosome binding site: bases 2145-2149
*bla* (ampicillin) resistance gene (ORF): bases 2157-3017
pUC origin: bases 3162-3835

FIGURE A.1: pCR®4-TOPO® cloning vector map

## A.4 Plasmid extraction

Plasmid vectors were extracted from *E. coli* using a Qiagen QIAprep® Miniprep Kit following the manufacturer's recommendations. The protocol used here was designed to extract plasmids from *E. coli* grown overnight in 5 ml of Luria broth containing 100 $\mu$g ml$^{-1}$ ampicillin.

**Method**

1. Falcon tubes containing *E. coli* cultures were removed from the incubator and centrifuged at 3000 *g* for 10 min at 25°C.
2. The culture broth was removed from the cell pellet and disposed of in the appropriate biohazard waste bins designated for autoclaving.
3. Buffer P1 (250 $\mu$l) containing LyseBlue indicator was added to each cell pellet and the pellet gently resuspended in the buffer. The resulting suspension was then removed to a sterile 1.5 ml microcentrifuge tube.
4. Buffer P2 (250 $\mu$l) was added to each sample and the samples gently inverted until thoroughly mixed and the colour was homogenous (blue due the LyseBlue indicator). The reactions were allowed to stand for up to but not more than 5 min.
5. Buffer N3 (350 $\mu$l) was then added to each sample and the tubes mixed immediately by inverting until all trace of blue was gone and a white suspension formed.
6. Samples were centrifuged for 10 min at 17 900 *g* at room temperature.
7. The supernatant from each sample was moved to individual QIAprep spin columns in 2 ml collection tubes and the columns centrifuged for 1 min at 17 900 *g*.
8. The flow through was discarded and the columns washed with 500 $\mu$l Buffer PB. Samples were centrifuged for 1 min at 17 900 *g*.
9. The flow through was again discarded and the columns washed with 750 $\mu$l Buffer PE.
10. Samples were centrifuged for 1 min at 17 900 *g*, the flow through discarded and the samples centrifuged for a further 1 min at 17 900 *g* to dry the column and remove residual wash buffer.

11. The columns were removed from the collection tubes and placed in clean 1.5 ml microcentrifuge tubes. Samples were eluted by adding 40 $\mu$l Buffer EB to each sample and letting them stand for 1 min at room temperature. Samples were then centrifuged for 1 min at 17 900 *g*.

12. Plasmid DNA was analysed for purity and quantity using a NanoDrop ND-1000 spectrophotometer and stored at -20°C until needed.

# Appendix B

# Sequence Translations

## B.1 DEAD-box ATP-dependent RNA helicase

```
GGCCGCGAATTCGCCCTTAAGCAGTGGTATCAACGCAGAGTACGCGGGGCAAGTCAAAATGGCGACCGAGCAGAAGCAAGTGCTGGACATGTTCCAGCGGCCCTTTAAGCGCCAGTCTCAAAC
                                                          M  A  T  E  Q  K  Q  V  L  D  M  F  Q  R  P  F  K  R  Q  S  Q  T

AACAGGGGATCGTCCCATCGTGTTCGAAGTGGGTACCCGGGTTGCAGACTCCCCAACTCAGACGTTGTCCCTCGCCCGCTCGATTCCTAAGGGCGTAGTCTTCTCCATGTTCGTTAGGACTCA
  T  G  D  R  P  I  V  F  E  V  G  T  R  V  A  D  S  P  T  Q  T  L  S  L  A  R  S  I  P  K  G  V  V  F  S  M  F  V  R  T  H

TCGCCTAGCTGCCAAGGAACTCTGCGACTTCTTTATGGAGGCCAACAACGTGAGTGAGCTGAGTCAGAGAGCAGAGGCGGTGCGGGGCAACCTCAACGAGAAGCTCTTCATCTTTGCCCTGTC
  R  L  A  A  K  E  L  C  D  F  F  M  E  A  N  N  V  S  E  L  S  Q  R  A  E  A  V  R  G  N  L  N  E  K  L  F  I  F  A  L  S

CTTCGTAATCATCAGGAAGGAGGAGATGAGAAGTGTGCGTCTACCCAGCATCGTGGAGATCTTCCCGAGTATGTTTGTGCCGGTTATCACCCTAGAAGAAATGGAACAGAGGGCACAGGAATC
  F  V  I  I  R  K  E  E  M  R  S  V  R  L  P  S  I  V  E  I  F  P  S  M  F  V  P  V  I  T  L  E  E  M  E  Q  R  A  Q  E  S

CATCCCTGACCAAGAAGTGGTAGTAACTGAATATGGGCTGGAGTTCTCCAGCACTCACTTGAAGCCTGAGCACCGTGTAGCCTATTGGCGTGAAGACTATGGCATCAACGCCCACCACTGGCA
  I  P  D  Q  E  V  V  V  T  E  Y  G  L  E  F  S  S  T  H  L  K  P  E  H  R  V  A  Y  W  R  E  D  Y  G  I  N  A  H  H  W  H

CTGGCATCTTGTCTACCCCGTGGATCTTGGTGTGATGAGGGACCGGAAGGGGGAGCTGTTCTACTACATGCACCAGCAGATGCTTGCTCGTTACGACATGGATCGCCTGAGTGTCAGCCTCAA
  W  H  L  V  Y  P  V  D  L  G  V  M  R  D  R  K  G  E  L  F  Y  Y  M  H  Q  Q  M  L  A  R  Y  D  M  D  R  L  S  V  S  L  N

TCGGGTGCAGAAACTGAGTAACTGGCGCCTCCCTATTTCTGATGGCTACTTCCCCAAGCTGACCATTAACAATTCAGGCCAGGCATGGGGCTCCCGGCAGGACAACACACTAATGCAGGACTA
  R  V  Q  K  L  S  N  W  R  L  P  I  S  D  G  Y  F  P  K  L  T  I  N  N  S  G  Q  A  W  G  S  R  Q  D  N  T  L  M  Q  D  Y

CAAACGGAACGACTTTGGGCTGCCACCAACTGACGTCTCTGAGCTGGAGCAGTGGCACAGTAGGATCATGGACGCCATCCATCAGGGCTTCCTTGTTGATCGTGAAGGTAAGCAGATAAAACT
  K  R  N  D  F  G  L  P  P  T  D  V  S  E  L  E  Q  W  H  S  R  I  M  D  A  I  H  Q  G  F  L  V  D  R  E  G  K  Q  I  K  L

TTCCGATGACGTCAAGCTGCCTCAGAAGCGTGGGATTGACCTCATTGGGGATGCAGTGGAGGCAGACAGCATCATCAGCCTCAACAGTCCCTTCTATGGTGACCTGCACAACATGGGTCACGT
  S  D  D  V  K  L  P  Q  K  R  G  I  D  L  I  G  D  A  V  E  A  D  S  I  I  S  L  N  S  P  F  Y  G  D  L  H  N  M  G  H  V

GGTCATCTCAGCTTCTCATGATCCGGACAATGCTCACATGGAGAATCTTGGAGTGATGTCGGACACAGCCACAGCCATGAGGGACCCAGTGTTCTATCGCTGGCACAAGTACATTGATGACAT
  V  I  S  A  S  H  D  P  D  N  A  H  M  E  N  L  G  V  M  S  D  T  A  T  A  M  R  D  P  V  F  Y  R  W  H  K  Y  I  D  D  I

CTTCCAGGAGTACAAGCTCACTCAGCCGCCCTACACTCTTGAGGAACTGTCCCTGTCGTCAGTTGAGGTGGTGTCGGTGGATGTGGATAGCCGAAATAAGAAGAACCAGYTGATCACTGGCTG
  F  Q  E  Y  K  L  T  Q  P  P  Y  T  L  E  E  L  S  L  S  S  V  E  V  V  S  V  D  V  D  S  R  N  K  K  N  Q  L  I  T  G  W

GAACACCCAGGACTTTGAGGCGAGTCGTGGACTGGACTTCAATGCCAACATGCCAGTGATGGTGCAACTCACCCACCTTGACCACAACCCCTTCCACTACAACATCAAGTTGGTCAACAGCGA
  N  T  Q  D  F  E  A  S  R  G  L  D  F  N  A  N  M  P  V  M  V  Q  L  T  H  L  D  H  N  P  F  H  Y  N  I  K  L  V  N  S  D

CAAGTTACCAATGGAAGTGACTGTGAGGATCTTCATGGCTCCAAAGCAAAATGAGAGAGGAGTGGATATGAACCTCATGGAACAGCGTCTCTTGTGGGCAGAGATGGACCGCTTTACCCAGAA
  K  L  P  M  E  V  T  V  R  I  F  M  A  P  K  Q  N  E  R  G  V  D  M  N  L  M  E  Q  R  L  L  W  A  E  M  D  R  F  T  Q  N

CTTGAAGCCTGGCCCAAACCACATACTGCGAGCCTCCACTTCCTCTTCAATCACCAATGCAAATGACTTTACCTTCCGTGACTTGGAGCAGCAGCCAGTCCCAAATGATCCAGGTGCCGATGG
  L  K  P  G  P  N  H  I  L  R  A  S  T  S  S  S  I  T  N  A  N  D  F  T  F  R  D  L  E  Q  Q  P  V  P  N  D  P  G  A  D  G

AAACACCCTCTTCAACTTCTGTGGGTGCGGCTGGCCTCAACACATGCTGCTGCCACGTGGGAAACAGGAGGGAATGCCATTTCAGATCTTTGTCATGGTCACGGATTGGGCACAGGATAAGGT
  N  T  L  F  N  F  C  G  C  G  W  P  Q  H  M  L  L  P  R  G  K  Q  E  G  M  P  F  Q  I  F  V  M  V  T  D  W  A  Q  D  K  V

AGTACAGCCAATCCGTGCACGTAACTGTTCTGCTGCTGCCTCATTCTGCGGTATATTGGATGCCCAGTATCCTGATTCCCGGCCCATGGGTTTCCCCTTCGACCGCCTTCCCATGCCCATGAT
  V  Q  P  I  R  A  R  N  C  S  A  A  A  S  F  C  G  I  L  D  A  Q  Y  P  D  S  R  P  M  G  F  P  F  D  R  L  P  M  P  M  I

ATTCAACAGACCTCTGGAAAGAACCCGAGACCTAACACGCCTCAGCAACGTAGGCATGCAGGACATCACCATCACTTTCAGCAATGCTCAGATCACCAAGTAAGAGTGGAATGACCACTTGTC
  F  N  R  P  L  E  R  T  R  D  L  T  R  L  S  N  V  G  M  Q  D  I  T  I  T  F  S  N  A  Q  I  T  K  *

CTCTCACAGCCCTGGATGGTGTGGCTTGGGGAAGAAGGAAAGTTGAAAAATGAGATGGCATTGCTGCAGTTTGGTTAAGACTAGTTGGTATATGTGGGTAGGTCTCTATATATAAAACCAAGA

AGACTATTTGCAAAATTATATAGTTCAATAAAGCATTTTTCCTAAAAAAAAAAAAAAAAAAAAAAAAAANNAGTACTCTGCGTTGATACCACTGCTTAAGGGCGAATTCGCGGCCGCT
```

FIGURE B.1: Complete nucleotide sequence and amino acid translation for *Cancer pagurus* prophenoloxidase

```
GTCGGTGATGAGGCACAAAGCAAGCGAGGTATCCTCACCCTCAAATACCCCATCGAGCACGGTATCGTCACCAACTGG
  V   G   D   E   A   Q   S   K   R   G   I   L   T   L   K   Y   P   I   E   H   G   I   V   T   N   W

GACGACATGGAGAAGATCTGGCATCACACTTTCTACAATGAGCTGCGTGTGGCCCCCAGAGGAGCACCCCAGTCCTGCTG
  D   D   M   E   K   I   W   H   H   T   F   Y   N   E   L   R   V   A   P   E   E   H   P   V   L   L

ACGGAGGCTCCTCTCAACCCCCAAGGCCAACCGCGAAAAAGATGACCCCAGATCATGTTTGAAACCTTCAACACACTCCCGCC
  T   E   A   P   L   N   P   K   A   N   R   E   K   M   T   Q   I   M   F   E   T   F   N   T   P   A

ATGTACGTGGCCATCCAGGCCGTGTCTGTACGCCTCCGGCCGTACCACCGGTATTGTGCTCGACTCCGGTGAC
  M   Y   V   A   I   Q   A   V   L   S   L   Y   A   S   G   R   T   T   G   I   V   L   D   S   G   D

GGCGTTTCACACACTGTCCCCATCTACGAGGGGATATGCTCTTCCTCACGCTATCCTTCGTCTTGACTTGGCCGT
  G   V   S   H   T   V   P   I   Y   E   G   Y   A   L   P   H   A   I   L   R   L   D   L   A   G   R

GACCTCACCGACTACTTGATGAAAATCCTGAGCGAGGCTACACCTTCACAACCACCGCCGAGCGAGAAATCGTA
  D   L   T   D   Y   L   M   K   I   L   T   E   R   G   Y   T   F   T   T   A   E   R   E   I   V

CGTGACATCAAGGAAAAGCTGTGTTACGTCGCCCTTGACTTCGAGCAGGAGATGACCACACTGCCGCTTCCTCCTCTTCA
  R   D   I   K   E   K   L   C   Y   V   A   L   D   F   E   Q   E   M   T   T   A   S   S   S   S

CTGGAGAAGTCTTACGAGTTCCCCCGACG
  L   E   K   S   Y   E   F   P   D
```

FIGURE B.2: Complete nucleotide sequence and amino acid translation for *Cancer pagurus* actin

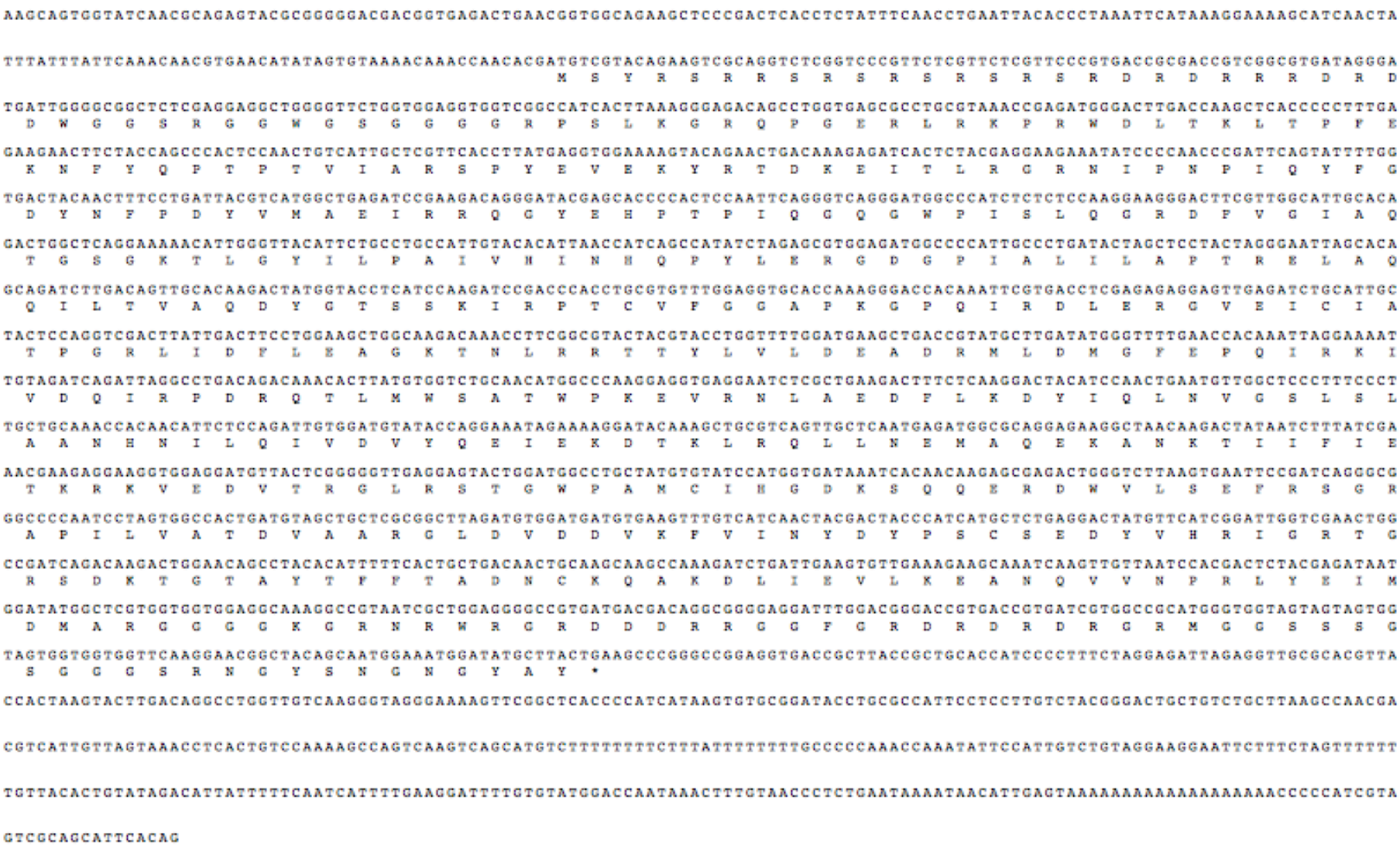



FIGURE B.3: Complete nucleotide sequence and amino acid translation for *Cancer pagurus* DEAD-box ATP-dependent RNA helicase

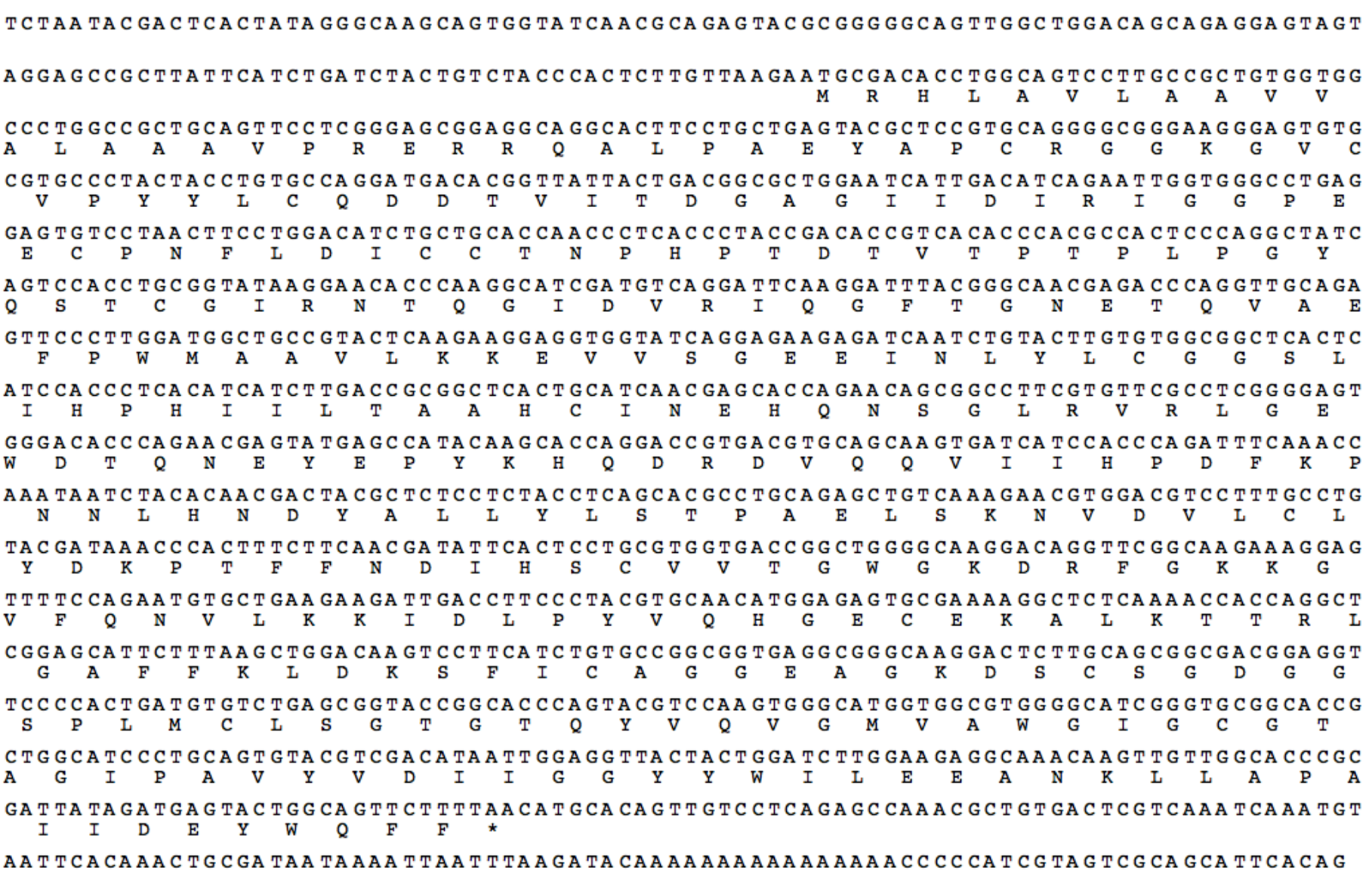



FIGURE B.4: Complete nucleotide sequence and amino acid translation for *Cancer pagurus* prophenoloxidase activating factor

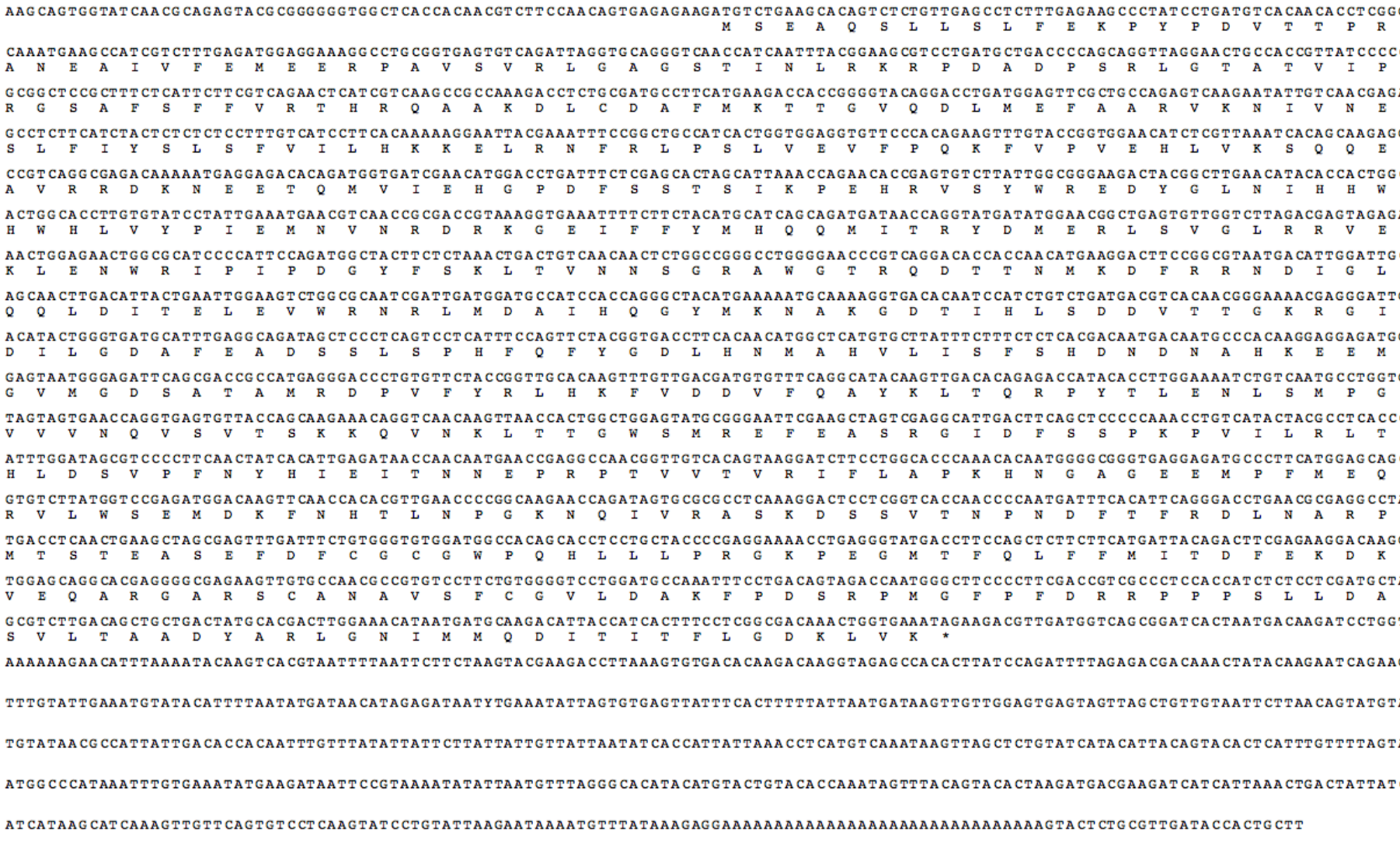


FIGURE B.5: Complete nucleotide sequence and amino acid translation for *Nephrops norvegicus* prophenoloxidase

```
TTAAGCGTGTTATTGTGAACGTTGACGATCGAGGACGTCCAATAGTTCAGCATGTCTGATGTGGAAGGAGACGTTAGC
                                                    M   S   D   V   E   G   D   V   S

GTTCCCGTGGCCGTCGGTGGCCCCATGGACTTGAACACTGCCGTCCAGGAAGTGCTCAAACAGGCCCTTATGGCTGAT
 V   P   V   A   V   G   G   P   M   D   L   N   T   A   V   Q   E   V   L   K   Q   A   L   M   A   D

GGTCTTGCTCGTGGACTTCATGAAGCAGTCAAGGCTCTTGACAAGCGTCAGGCTCTCTTGTGCCTGTTGGCAAACAAC
 G   L   A   R   G   L   H   E   A   V   K   A   L   D   K   R   Q   A   L   L   C   L   L   A   N   N

TGCGATGAGCCTGGCTACTCCAAGCTGGTCGAGGCCCTTTGCCAGGAGCACCAGATCAAACTCCTGAAAGTTGACTCA
 C   D   E   P   G   Y   S   K   L   V   E   A   L   C   Q   E   H   Q   I   K   L   L   K   V   D   S

AACAAGATGTTGGGAGAATGGGCTGGCCTGTGCAAGATTGATCGCGAAGGAAAGGCTCGCAAGGTCGTTGGCTGCTCA
 N   K   M   L   G   E   W   A   G   L   C   K   I   D   R   E   G   K   A   R   K   V   V   G   C   S

TGTGTTGTTGTCACTGATTATGGCAAGGAGACCCAAGCCCATGATGTTGTCAATGAGTACTTCAAGAGCAAAGGACAG
 C   V   V   V   T   D   Y   G   K   E   T   Q   A   H   D   V   V   N   E   Y   F   K   S   K   G   Q

TAAGGAATCACATGTCCCATTTGTATGCAGAGAATCAGCCAGGAAGTCCAGATCAAAGATGACCGAGTAATGATGAAC
 *

ATTCAACACTGGGAGTCCATGAAAAGGATGACAAACCACAGCTGTAAGATCCTGCTTAGCCTTTTCAAGGGGCCACTG

ATTCCAAATAATTAAAATAAAGTAAAAAAAAATTGAAAAAAAAAAAAAAAAAAA
```

FIGURE B.6: Complete nucleotide sequence and amino acid translation for *Nephrops norvegicus* 40S ribosomal protein S12

```
TCTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGTACGCGGGATACTTGGCCTGACAACCTCGTCGT

CTACACTGCTCCACCAACGTCTCCTGCCATCCATAATGCTGCGTCTGTTGGTAGTGATGACCGTCGTGACGGTGGCTC
                                      M   L   R   L   L   V   V   M   T   V   V   T   V   A

TTGGCTCCGGTCACACTCCCAGCTGTATCTACTGGTGTAATTTCCCAAAAGCTGTCAACGCTGGTGCCAGCTACTGCT
L   G   S   G   H   T   P   S   C   I   Y   W   C   N   F   P   K   A   V   N   A   G   A   S   Y   C

GCATTAATTCAAACCATAATATTGTCGAAAATACAAATGAACCCCATCCAGGTAGATGTGTAGCCCATACCTTCTGCG
C   I   N   S   N   H   N   I   V   E   N   T   N   E   P   H   P   G   R   C   V   A   H   T   F   C

CAAGAAGCAAAGTTCCTGGACAACAAGACTATCCTCTCGCTCCTGTCAGATGCGGTCATGATGACTATTGTCCCTACC
A   R   S   K   V   P   G   Q   Q   D   Y   P   L   A   P   V   R   C   G   H   D   D   Y   C   P   Y

ATGAAAAATGCTGTTATGACGCCTGTTTGAAGCACCATACCTGCAAGGGTGTAATATCCCATTAACACCTTCCACAAG
H   E   K   C   C   Y   D   A   C   L   K   H   H   T   C   K   G   V   I   S   H   *

ATCAGTGTTCCACCCGCTGCCCATCTCGGAGTCACACCTAAAACAATTATCATATAAACAACGTCAATACGAGCACTC

GGCAGCCAGACCTTCCTCCCCACCCAGTAATGCAGTTCTCGAGAAAGTTCTTTTGGGTCAGAAGGTCGAGACAAGATG

GAAGGAACACTTTAGTTGGCTCAAAATAGAACTTGGAACGTGATCCACTCGAGTACCACCAAATGTTACACATGATGA

ACTGTGTATTATTATTGCTGTTATTATGTTCTTCAGGTTTGTTGTATCTGCTTTATGTTCATTAGAGAGTATTGTTTC

TTACAATGAAGTTGTTGTCATCAGTTACAAGGTTTTACAATAAACTTGTCATCAGTTAAAAAAAAAAAAAAAAA
```

FIGURE B.7: Complete nucleotide sequence and amino acid translation for *Nephrops norvegicus* crustin isoform 1

```
TCTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGTACGCGGGGGATACTTGGCCTGACAACCTCGTC

GTCTACACTGCTCCACCAACGTCTCCTGCCATCCATAATGCTGCGTCTGTTGGTAGTGATGACCGTCGTGACGGTGGC
                                        M   L   R   L   L   V   V   M   T   V   V   T   V   A

TCTTGGCGGTAACTTCGGTCGCCCTCCCAGCTGTATCTACCGGTGCAAATACCCAGACGCTGTCAACGCTGGTGCCAC
  L   G   G   N   F   G   R   P   P   S   C   I   Y   R   C   K   Y   P   D   A   V   N   A   G   A   T

CTACTGCTGCATTAATTCAAACCATAATATTGTCGAAAATACAAGTCTAGAACCCCATCCAGGTAGATGTGTAGCCCA
  Y   C   C   I   N   S   N   H   N   I   V   E   N   T   S   L   E   P   H   P   G   R   C   V   A   H

TACCTTCTGCGCAAGGTTCACTACTGCAGCTCCTGTCAATTGCGGTCATGATGACTATTGTCCCTACCATGAAAAATG
  T   F   C   A   R   F   T   T   A   A   P   V   N   C   G   H   D   D   Y   C   P   Y   H   E   K   C

CTGTTATGACGCCTGTTTGGAGCGCCACATCTGCAAGGGTGTAATTCCCCATTAACACCTTCCACAAGATCAGTGTTC
  C   Y   D   A   C   L   E   R   H   I   C   K   G   V   I   P   H   *

CACCCGCTGCCCATCTCGGAGTCACACCTAAAACAATTATCATATAAACAACGTCAATACGAGCACTCGGCAGCCAGA

CCTTCCTCCCCACCCAGTAATGCAGTTCTCGAGAAAGTTCTTTTGGGTCAGAAGGTCGAGACAAGATGGAAGGAACAC

TTTAGTTGGCTCAAAATAGAACTTGGAACGTGATCCACTCGAGTACCACCAAATGTTACACATGATGAACTGTGTATT

ATTATTGCTGTTATTATGTTCTTCAGGTTTGTTGTATCTGCTTTATGTTCATTAGAGAGTATTGTTTCTTACAATGAA

GTTGTTGTCATCAGTTACAAGGTTTTACAATAAACTTGTCATCAGTTAAAAAAAAAAAAAAAAA
```

FIGURE B.8: Complete nucleotide sequence and amino acid translation for *Nephrops norvegicus* crustin isoform 2

```
CAATTGTGAAGATCAATGCCCTAACTGCTTCGGAGGCGGCTTGTCTAATCCTGTCCGTCGATGAAACGATCAGAT
  I   T   F   I   L   A   R   V   A   A   E   S   A   A   Q   R   I   R   D   T   S   S   V   I   L   D
CACCACGATCGAGTGCCGACGGTCCACCCACGACCGGCAGGGGACGGTGGAAGGCCCATGTAAACATTATGGACCGATG
  G   R   D   L   A   S   P   G   G   V   V   P   L   P   R   P   L   G   M
GTTTAACTCTCTGAACACAGTGACTCAACAAGTTAAGACTAAAATACTGAAAGTCAAACTAACTCATTTGCTTATTCAGA

TACATTCTAAAAATTATAAGGACTCTAATGTGGCTGTATTAGCACATTAAAGCTGTAAACTGTTGAAGTTGAGGACAG

CTCGGTTAAGTCACAAAAGCTTACTAAACACTCTTAAATATTTTCTCTCACCTCTCGAGTCCAGTGATGTCTCGTCA

TCTATGATGAGAACCGTGCTTATTATTAAAGGATTTAACGATAAAAAAAAAAAAAAAAAAAA
```

FIGURE B.9: Complete nucleotide sequence and amino acid translation for *Nephrops norvegicus* chaperonin containing T-complex protein subunit 7

```
GCGGCCGCGAATTCGCCCTTGGTATGGGTCAAAAGGACTCGTACGTAGGTGACGAGGCACAGAGCAAGAGAGGTATCC
   G   R   E   F   A   L   G   M   G   Q   K   D   S   Y   V   G   D   E   A   Q   S   K   R   G   I

TCACCCTCAAATATCCCATCGAGCACGGTATCGTTACCAACTGGGACGACATGGAAAAGATCTGGCATCACACTTTCT
L   T   L   K   Y   P   I   E   H   G   I   V   T   N   W   D   D   M   E   K   I   W   H   H   T   F

ACAATGAGCTGCGCGTTGCCCCAGAGGAGCACCCCGTCCTGTTGACAGAGGCTCCCCTCAACCCCAAGGCTAACCGTG
Y   N   E   L   R   V   A   P   E   E   H   P   V   L   L   T   E   A   P   L   N   P   K   A   N   R

AAAAGATGACCCAAATTATGTTTGAAACCTTCAACACTCCCGCCATGTACGTCGCTATCCAGGCTGTGCTCTCCCTGT
E   K   M   T   Q   I   M   F   E   T   F   N   T   P   A   M   Y   V   A   I   Q   A   V   L   S   L

ACGCTTCCGGCCGTACCACCGGTATTGTCTTGGACTCTGGTGATGGCGTGTCACACACTGTTCCTATCTACGAGGGAT
Y   A   S   G   R   T   T   G   I   V   L   D   S   G   D   G   V   S   H   T   V   P   I   Y   E   G

ACGCCCTTCCCCATGCTATCCTCCGTCTGGACTTGGCTGGACGTGACCTTACTGACTACCTGATGAAGATCCTGACTG
Y   A   L   P   H   A   I   L   R   L   D   L   A   G   R   D   L   T   D   Y   L   M   K   I   L   T

AGCGTGGCTACACCTTCACTACCACCGCTGAGCGAGAAATCGTTCGTGACATTAAGGAAAAGTTGTGCTATGTTGCCC
E   R   G   Y   T   F   T   T   T   A   E   R   E   I   V   R   D   I   K   E   K   L   C   Y   V   A

TAGACTTCGAGCAGGAAATGACCACTGCTGCGTCGTCCTCCTCCCTAGAGAAGTCCTATGAACTACCCGACGAAGGGC
L   D   F   E   Q   E   M   T   T   A   A   S   S   S   S   L   E   K   S   Y   E   L   P   D   E   G

GAAT
R
```

FIGURE B.10: Complete nucleotide sequence and amino acid translation for *Nephrops norvegicus* actin

```
GAGAAGATGACGCAGATTATGTTTGAAACTTTCAATGTTCCCGCTTTATACATCGCCATCCAGGCGGTCCTCTCCTTA
 E   K   M   T   Q   I   M   F   E   T   F   N   V   P   A   L   Y   I   A   I   Q   A   V   L   S   L

TACGCCTCAGGGCGCACCACCGGCATCGTGATGGACAGCGGCGACGGCGTGTCTCATACAGTGCCTATTTACGAGGGC
 Y   A   S   G   R   T   T   G   I   V   M   D   S   G   D   G   V   S   H   T   V   P   I   Y   E   G

TATGCGCTCCCTCACGCCATTTTCCGCTTGAACTTAGCTGGTCGCGATTTAACTGAATACATGATGAAAATTCTGACC
 Y   A   L   P   H   A   I   F   R   L   N   L   A   G   R   D   L   T   E   Y   M   M   K   I   L   T

GAACGCGGGTGCTCCTTCAGCACGACAGCGGAGAAGGAAATCGCGCGTGACATGAAGGAGAAGTTGTGCTACGTGGCG
 E   R   G   C   S   F   S   T   T   A   E   K   E   I   A   R   D   M   K   E   K   L   C   Y   V   A

CTTGATTTTGAGGATGAAATGAAGAAAGCGGCGGAGTCCTGCGAAATGGAAAAAACGTACGAGTTACCAGACGGCAAT
 L   D   F   E   D   E   M   K   K   A   A   E   S   C   E   M   E   K   T   Y   E   L   P   D   G   N

GTTGTGACCGTGGGAAGCGAGCGCTTTCGCTGCCCAGAAGTGCTCTTTCAGCCTATGTTCATCGGCATGGAAGGACAA
 V   V   T   V   G   S   E   R   F   R   C   P   E   V   L   F   Q   P   M   F   I   G   M   E   G   Q

GGCATCCATACCGTCACCTTCCAGTCCATCATGAAATGCGATATTGACATCCGTAAGGAGTTATACCACAACGTCGTG
 G   I   H   T   V   T   F   Q   S   I   M   K   C   D   I   D   I   R   K   E   L   Y   H   N   V   V

CTCTCTGGTGGCACCACGATGTTCTCCGGTATTGGCGAACGCATGACAAAGGAACTCACTGCGCTGGCGCCTTCAGTG
 L   S   G   G   T   T   M   F   S   G   I   G   E   R   M   T   K   E   L   T   A   L   A   P   S   V

ATGAAGATTAAAGTGATATGCCCGCCTGAACGCAAGTACAGCGTCTGGATCGGCGGCTCCATCCTGTCCTCCTTGACC
 M   K   I   K   V   I   C   P   P   E   R   K   Y   S   V   W   I   G   G   S   I   L   S   S   L   T

ACATTTCAGCAGATGTGGATCGCGAAACACGAGTACGAGGAAATCGGGCCCAAGATAGTCCACCGCAAGTGCTTCCAG
 T   F   Q   Q   M   W   I   A   K   H   E   Y   E   E   I   G   P   K   I   V   H   R   K   C   F   Q

GCC
 A
```

FIGURE B.11: Complete nucleotide sequence and amino acid translation for *Hematodinium* actin

# Appendix C

# GeneFishing™ sequence results

## C.1 *In vitro* GeneFishing™ sequence results: *Cancer pagurus*

CC4

TGCATGGGCAGCTCACTGGGACCCATGGACCTTCTCTCTGACTCTCACCT
CGACAGCGACGCCTCATCCTTGGACAAGCACTCCACGGGCAGCGATGCCT
CTCGGGAATCAGAATCAGAGTTTTGACGGCCGGGAGGAGGGTAGTTTGCT
GATATCGTCGTCAGTCCCCCAACCTCTTCTAGTTGTATTTCCCGCCCAAA
GGCGCTGGTCGCCATACCTCGCAGCTCCGCCCCTGGCCCCCGTCTCCCAG
GTGTCGGGGCGGCGCCCTCAGGCTTGTCCTGAACGGTGCTACCCCTCTCC
CTCAAACCCCCCCCCAAGGTGCCTCGTGCACGCTAATCTCACCAAAGCCTC
AAAACATTGTCTGCCTCTGGGTTTGGGCAATGTTATTATATTTTCTYTGA
GAGAAATTGTATGTAATGTAATTTAAAATTATATTTATATTAGGTTATAT
TGTACATATTGTATGCAATTAAGATTCGCCTGACTTGTCAGGTATTGATG
CCATCAATGCAACCCAATTTTTTGTATTAAATGTTTATACAAGAATATTT
TTTGTAAAAAATCAAAAAATAGCGCAAATAAAATTCATGAACTTTAAAAA
AAAAAAAAAAAAAAAAAAAA

CC16

GGCGGGCAAGGACTCTTGCAGCGGCGACGGAGGTTCCCCACTGATGTGTC
TGAGCGGTACCGGCACCCAGTACGTCCAAGTGGGCATGGTGGCGTGGGGC
ATCGGGTGCGGCACCGCTGGCATCCCTGCAGTGTACGTCGACATAATTGG
AGGTTACTACTGGATCTTGGAAGAGGCAAACAAGTTGTTGGCACCCGCGA
TTATAGATGAGTACTGGCAGTTCTTTTAACATGCACAGTTGTCCTCAGAG

CCAAACGCTGTGACTCGTCAAATCAAATGTAATTCACAAACTGCGATAAT
AAAATTAATTTAAGATACAAAAAAAAAAAAAAA

CC7

TTAAAAGCGACAAAAAGGGATGGAGCGAGGGGTCTGGAAAGCCTCTCCTC
TATATTCTCTCCTACAAAAATATATGGCACAGGGGATGGGAGAATAAAGT
CTCCCTGAACCCTAAATGCCTTGTCTTGGTTAACCTGTTTTAACAGGGAT
ATATATATGTATGCATATGTATATATATTAACCAAAGGTTACAAAGAGTA
CAGGTGAGATATGTGCTTTGTGTTGCACAATATCCTTCGTACAATCATGT
TTACTTACTTAAGCTCTGAGCATTTCTTATAAAACACCAAAGATTCCGTC
CGTAACAAGCTTATAAAGCACGTGTGGTTTTATTTTCCTTATCTTGCCTT
ACCAAGGGAAGGAATGGATAATAGAAACAGTACATTGACTGTTTCCTTGT
TGGAGACTTTTTATTAAAGAAAATTCACCCACTAGTAAAAAAAAAAAAAA
AAAAAA

CX6

TGCATGGGCAGCTCACTGGGACCCATGGACCTTCTCTCTGACTCTCACCT
CGACAGCGACGCCTCATCCTTGGACAAGCACTCCACGGGCAGCGATGCCT
CTCGGGAATCAGAATCAGAGTTTTGACGGCCGGGAGGAGGGTAGTTTGCT
GATATCGTCGTCAGTCCCCCAACCTCTTCTAGTTGTATTTCCCGCCCAAA
GGCGCTGGTCGCCATACCTCGCAGCTCCGCCCCTGGCCCCCGTCTCCCAG
GTGTCGGGGCGGCGCCCTCAGGCTTGTCCTGAACGGTGCTACCCCTCTCC
CTCAAACCCCCCCCCAAGGTGCCTCGTGCACGCTAATCTCACCAAAGCCTC
AAAACATTGTCTGCCTCTGGGTTTGGGCAATGTTATTATATTTTCTYTGA
GAGAAATTGTATGTAATGTAATTTAAAATTATATTTATATTAGGTTATAT
TGTACATATTGTATGCAATTAAGATTCGCCTGACTTGTCAGGTATTGATG
CCATCAATGCAACCCAATTTTTTGTATTAAATGTTTATACAAGAATATTT
TTTGTAAAAAATCAAAAAATAGCGCAAATAAAATTCATGAACTTTAAAAA
AAAAAAAAAAAAAAAAAAAA

CC19

GGCGGGCAAGGACTCTTGCAGCGGCGACGGAGGTTCCCCACTGATGTGTC
TGAGCGGTACCGGCACCCAGTACGTCCAAGTGGGCATGGTGGCGTGGGGC
ATCGGGTGCGGCACCGCTGGCATCCCTGCAGTGTACGTCGACATAATTGG
AGGTTACTACTGGATCTTGGAAGAGGCAAACAAGTTGTTGGCACCCGCGA
TTATAGATGAGTACTGGCAGTTCTTTTAACATGCACAGTTGTCCTCAGAG
CCAAACGCTGTGACTCGTCAAATCAAATGTAATTCACAAACTGCGATAAT
AAAATTAATTTAAGATAAAAAAAAAAAAAAAAAA

## C.2 *In vivo* GeneFishing™ sequence results: *Nephrops norvegicus*

### C.2.1 Uninfected *Nephrops norvegicus*

201-21 (Crustin)

TGATGACTATTGTCCCTACMATGAAAAATGCTGTTATGACGCCTGTTTGG
AGCGCCACATCTGCAAGGGTGTAATTCCCCATTAACACCTTSCACAAGAT
CAGTGTTCCACCCSCTGCCCATCTCGGAGTCACACCTAAAACAATTATCA
TATAAACAACGTCAATACGAGCACTCGGCAGCCAGACCTTCCTCCCCACC
CAGTAATGCAGTTCTCGAGAAAGTTCTTTTGGGTCAGAAGGTCGAGACAA
GATGGAAGGAACACTTTAGTTGGCTCAAAATAGAACTTGGAACGTGATCC
ACTCGAGTACCACCAAATGTTACACATGATGAACTGTGTATTATTATTGC
TGTTATTATGTTCTTCAGGTTTGTTGTATCTGCTTTATGTTCATTAGAGA
GTATTGTTTCTTACAATAAAGTTGTTGTCATCAGTTACAAGGTTTTACAA
TAAACTTAGTCATCAGAAAAAAAAAAAAAAAAAAAAAA

201-23

GCATCAATTTGTCAATTAGATGTGAAAATGCGCTCACAGAAGTCTTTGAT
TTAAATAAAAATTTGCTTAGATGGATGTACAAGGGTCGAAATGTAGGTGT
ACTCTTTAGTCAGTGCNNAAAAAAAAAAAAAAAAA

201-26 (40S ribosomal protein S12)

TTAAGCGTGTTATTGTGAACGTTGACGATCGAGGACGTCCAATAGTTCAG
CATGTCTGATGTGGAAGGAGACGTTAGCGTTCCCGTGGCCGTCGGTGGCC
CCATGGACTTGAACACTGCCGTCCAGGAAGTGCTCAAACAGGCCCTTATG
GCTGATGGTCTTGCTCGTGGACTTCATGAAGCAGTCAAGGCTCTTGACAA
GCGTCAGGCTCTCTTGTGCCTGTTGGCAAACAACTGCGATGAGCCTGGCT
ACTCCAAGCTGGTCGAGGCCCTTTGCCAGGAGCACCAGATCAAACTCCTG
AAAGTTGACTCAAACAAGATGTTGGGAGAATGGGCTGGCCTGTGCAAGAT
TGATCGCGAAGGAAAGGCTCGCAAGGTCGTTGGCTGCTCATGTGTTGTTG
TCACTGATTATGGCAAGGAGACCCAAGCCCATGATGTTGTCAATGAGTAC
TTCAAGAGCAAAGGACAGTAAGGAATCACATGTCCCATTTGTATGCAGAG
AATCAGCCAGGAAGTCCAGATCAAAGATGACCGAGTAATGATGAACATTC
AACACTGGGAGTCCATGAAAAGGATGACAAACCACAGCTGTAAGATCCTG
CTTAGCCTTTTCAAGGGGCCACTGATTCCAAATAATTAAAATAAAGTAAA
AAAAAATTGAAAAAAAAAAAAAAAAAAA

201-28 (Chaperonin-containing T-complex 1 protein)

CAATTGTGAAGATCAATGCCCTAACTGCTGCTTCGGAGGCGGCTTGTCTA
ATCCTGTCCGTCGATGAAACGATCAGATCACCACGATCGAGTGCCGACGG
TCCACCCACGACCGGCAGGGGACGTGGAAGGCCCATGTAAACATTATGGA
CCGATGGTTTAACTCTCTGAACACAGTGACTCAACAAGTTAAGACTAAAT
ACTGAAAGTCAACTAACTCATTTGCTTATTCAGATACATTCTAAAAATTA
TAAGGACTCTAATGTGGCTGTATTAGCACATTAAAGCTGTAAACTGTTGA
AGTTGAGGACAGCTCGGTTAAGTCACAAAAGCTTACTAAACACTCTTAAA
TATTTTTCTCTCACCTCTCGAGTCCAGTGATGTCTCGTCATCTATGATGA
GAACCGTGCTTATTATTAAAGGATTTAACGATAAAAAAAAAAAAAAAAAA
AAA

201-32

CTATGAGGCTACAGTTGCTGAATGCCGCATAGACATGCGGCTCAACTAAT
TCAACACAGTTAGGATAGCGAATAATGTATTAGGATATTCAGTATTAATA
AAAAAAAAAAAAAAA

201-33

GGACTTGAGAATGTATCGTGACCGGGCGAAGGATGTCAATGCCCAGACAA
TAAAGAAGGTGGTTGAGGCAAAGGCTCGCAAGCAGCGGCGTGTGAAGAAG
CGAATGGAAAGGGCACGGAAGACGGCAGAAAATGTGACAAACAATGCTGA
TATGTCAGAGAGAGAAAAGGCACAGGAAGTGAAGAAGTTGTACAAGAAAG
CCTTGACACCATTAAAGAAGAAAGAAACAACTTACGTTGTGATGAAGAAA
AGGCATACTGGAAAGAAGCCAAAAGGAACCAAGGGTCCTTACAAACTGGT
TGACAAGAGAATGAAAAAGGATGCAAGAGCAATGAAGAAGTCTGATGCTA
AGAAAAAAAAAAAAAAAA

201-37

AAGGTTCTCGTGATGCTTACCGGCGAGCTCCATTTGGTATTGATAAGACT
GGTGATGCTGGTGCAGGATCAGCTCCCATGGAATTCCGTGGGGGATTTGG
TCGTGGCACCAAGAGGCCTTAGTAAAGTGTAAATAAACGGGAGAAAAAAA
GGAAAAAAAAAAAAAAAAAAAAAAAAAAAA

201-38

CTACTTGAAGGAAGGACACTTAATGAATCTTGGCGTGAGTTCAAGGAGAA
GTTCCCAACAGTCTATGCTTTTGATTGGTTCATATGGCCGCCCAGCCAAG
CCATCAACTTTTACTTCGTTCCTACACAGTATAGAGTCTTGTATATAAAC
GGTGTCACAGTTATATGGGACATTTTTCTCTCTCATATGAAACACATGAA

TCAAGTCACTAAGAAGACACAAGTAACAGCTGTCAAGGCGTAAGAGAAAC
TGCTACTTGGAATCTTCAGTCTGAAACTCACAGGACAAATATAATCCATA
ACAATAGCTATACTGGTAAGTGGAGATAAGAAGCTATATTATATTGTACA
GTTTACACAAGAAATTCAATAGATTGTGAGATGAAAAAAAAAAAAAAAAA
AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA

201-40

AGTCACACCTAAAACAATTATCATATAAACAACGTCAATACGAGCACTCG
GCAGCCAGACCTTCCTCCCCACCCAGTAATGCAGTTCTCGAGAAAGTTCT
TTTGGGTCAGAAGGTCGAGACAAGATGGAAGGAACACTTTAGTTGGCTCA
AAATAGAACTTGGAACGTGATCCACTCGAGTACCACCAAATGTTACACAT
GATGAACTGTGTATTATTATTGCTGTTATTATGTTCTTCAGGTTTGTTGT
ATCTGCTTTATGTTCATTAGAGAGTATTGTTTCTTACAATRAAGTTGTTG
TCATCAGTTACAAGGTTTTACAATAAACTTNGTCATCAGNNNNAAAAAAA
AAAAAAAAAAA

### C.2.2 *Hematodinium* sp.

H-23

AGCATGACACGGTTATCGAGGACCTGAAACTGAAATTCGAAGAGAAACAG
AAGACAAACCGCACTGACAGGCAGCACATACTCGAAGAGAAGGAGGGAGC
ATTCGCGGACTATCAGGACACGCAGAGGCAACTCGAGGAGGATGCAGACC
GGGAAATTGAAGAGCTGAAAGAATTGTACGAAGCAAAACTGGCGCAGGAG
AAGGATGAAAAGGTGCGCCTTCGTGGCCAGGCTGGTATTCACCGAAAACA
CCACGAAGATTTGAAGCGGCAAATGANNNAAAAAAAAAAAAAA

H-34

AGTCACACCTAAAACAATTATCATATAAACAACGTCAATACGAGCACTCG
GCAGCCAGACCTTCCTCCCCACCCAGTAATGCAGTTCTCGAGAAAGTTCT
TTTGGGTCAGAAGGTCGAGACAAGATGGAAGGAACACTTTAGTTGGCTCA
AAATAGAACTTGGAACGTGATCCACTCGAGTACCACCAAATGTTACACAT
GATGAACTGTGTATTATTATTGCTGTTATTATGTTCTTCAGGTTTGTTGT
ATCTGCTTTATGTTCATTAGAGAGTATTGTTTCTTACAATGAAGTTGTTG
TCATCAGTTACAAGGTTTTACAATAAACTTGTCATCAGTTAAAAAAAAAA
AAAAAAAA

H-35

AAATGTATTTAGGAGTGGACWCTCCATTATCCCCTGGTAAAGAGTCRCCT
GACCGAGCGCATGCAGTGAAGGCAAGCGACGCACCGCAAGCCAAATCGCC
TGAAGAACTTCCGCCAGTTCGGCCACCAAGCCGGAAAAAGTCGCCTACAG
CCGGGCATTCAGAACTCCCAAGCGACGAAGGGCCCGCTGTGCCGTACGCA
GGTAGAGTGATCGATGGTAAATTTGTCAGGAAACACGTGGTATCATTGGA
AAGATTGTCCAAGGAGGAGAAAATACAGTTGCTCAAGGATATGGAGAAAG
AGCGAACGGAGAAGGCGAAATTACTAGCAGAACGCCAACATAAACACGCA
CTATGGAAGAAAGAACAAGAAAAAAAAAAAAAAAAA

# References


Acharya, S., J. Mohanty and P. K. Sahoo (2004). 'Humoral defence factors in Indian river prawn, *Macrobrachium malcolmsonii*'. *Fish & Shellfish Immunology* **17**, 137–147.

Adachi, K., T. Hirata, T. Nishioka and M. Sakaguchi (2003). 'Hemocyte components in crustaceans convert hemocyanin into a phenoloxidase-like enzyme'. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology* **134**, 135–141.

Aflalo, E. D., A. Bakhrat, S. Raviv, D. Harari, A. Sagi and U. Abdu (2007). 'Characterization of a Vasa-like gene from the Pacific white shrimp *Litopenaeus vannamei* and its expression during oogenesis'. *Molecular Reproduction and Development* **74**, 172–177.

Ai, H. S., Y. C. Huang, S. D. Li, S. P. Weng, X. Q. Yu and J. G. He (2008). 'Characterization of a prophenoloxidase from hemocytes of the shrimp *Litopenaeus vannamei* that is down-regulated by white spot syndrome virus'. *Fish & Shellfish Immunology* **25**, 28–39.

Alberts, B., A. Johnson, J. Lewis, M. Raff, K. Roberts and P. Walter (2008). *Molecular Biology of the Cell*. 5 edn. Garland Science, Taylor & Francis Group. New York.

Alcaide, P. and M. Fresno (2004). 'The *Trypanosoma cruzi* membrane mucin AgC10 inhibits T cell activation and IL-2 transcription through L-selectin'. *International Immunology* **16**, 1365–1375.

Alderman, D. J. (1996). 'Geographical spread of bacterial and fungal diseases of crustaceans'. *Revue Scientifique Et Technique De L Office International Des Epizooties* **15**, 603–632.

Altschul, S. F., W. Gish, W. Miller, E. W. Myers and D. J. Lipman (1990). 'Basic Local Alignment Search Tool'. *Journal of Molecular Biology* **215**, 403–410.

Amparyup, P., H. Kondo, I. Hirono, T. Aoki and A. Tassanakajon (2008). 'Molecular cloning, genomic organization and recombinant expression of a crustin-like

antimicrobial peptide from black tiger shrimp *Penaeus monodon*'. *Molecular Immunology* **45**, 1085–1093.

Amparyup, P., W. Charoensapsri and A. Tassanakajon (2009). 'Two prophenoloxidases are important for the survival of *Vibrio harveyi* challenged shrimp *Penaeus monodon*'. *Developmental and Comparative Immunology* **33**, 247–256.

Anderson, R. S. (1999*a*). '*Perkinsus marinus* secretory products modulate superoxide anion production by oyster (*Crassostrea virginica*) haemocytes'. *Fish & Shellfish Immunology* **9**, 51–60.

Anderson, R. S. (1999*b*). 'Lack of hemocyte chemiluminescence stimulation by *Perkinsus marinas* in eastern oysters *Crassostrea virginica* with dermo disease'. *Journal of Aquatic Animal Health* **11**, 179–182.

Andreu, D. and L. Rivas (1998). 'Animal antimicrobial peptides: An overview'. *Biopolymers* **47**, 415–433.

Appleton, P. L. and K. Vickerman (1998). '*In vitro* cultivation and developmental cycle in culture of a parasitic dinoflagellate (*Hematodinium* sp.) associated with mortality of the Norway lobster (*Nephrops norvegicus*) in British waters'. *Parasitology* **116**, 115–130.

Arts, J. A. J., F. H. J. Cornelissen, T. Cijsouw, T. Hermsen, H. F. J. Savelkoul and R. J. M. Stet (2007). 'Molecular cloning and expression of a Toll receptor in the giant tiger shrimp, *Penaeus monodon*'. *Fish & Shellfish Immunology* **23**, 504–513.

Arya, M., I. S. Shergill, M. Williamson, L. Gommersall, N. Arya and H. R. H. Patel (2005). 'Basic principles of real-time quantitative PCR'. *Expert Review of Molecular Diagnostics* **5**, 209–219.

Aspan, A., J. Sturtevant, V. J. Smith and K. Soderhall (1990). 'Purification and characterization of a prophenoloxidase activating enzyme from crayfish blood cells'. *Insect Biochemistry* **20**, 709.

Aspan, A., T. S. Huang, L. Cerenius and K. Soderhall (1995). 'Cdna cloning of prophenoloxidase from the fresh-water crayfish *Pacifastacus leniusculus* and its activation'. *Proceedings of the National Academy of Sciences of the United States of America* **92**, 939–943.

Atkinson, R. J. A. and E. Naylor (1976). 'Endogenous activity rhythm and rhythmicity of catches of *Nephrops norvegicus* (L)'. *Journal of Experimental Marine Biology and Ecology* **25**, 95–108.

Avison, M. B. (2007). *Measuring Gene Expression: The Basics*. The Basics. Taylor & Francis Group. Abingdon.

Baca, O. G., M. J. Roman, R. H. Glew, R. F. Christner, J. E. Buhler and A. S. Aragon (1993). 'Acid phosphatase activity in *Coxiella burnetii* - a possible virulence factor'. *Infection and Immunity* **61**, 4232–4239.

Bachere, E., E. Mialhe and J. Rodriguez (1995). 'Identification of defense effectors in the hemolymph of crustaceans with particular reference to the shrimp *Penaeus japonicus* (Bate): Prospects and Applications'. *Fish & Shellfish Immunology* **5**, 597–612.

Barracco, M. A., B. Duvic and K. Soderhall (1991). 'The beta-1,3-glucan binding protein from the crayfish *Pacifastacus leniusculus*, when reacted with a beta-1,3-glucan, induces spreading and degranulation of crayfish granular cells'. *Cell and Tissue Research* **266**, 491–497.

Barreda, D. R. and M. Belosevic (2001). 'Transcriptional regulation of hemopoiesis'. *Developmental and Comparative Immunology* **25**, 763–789.

Bartlett, T. C., B. J. Cuthbertson, E. F. Shepard, R. W. Chapman, P. S. Gross and G. W. Warr (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.

Basu, S., R. J. Binder, T. Ramalingam and P. K. Srivastava (2001). 'CD91 is a common receptor for heat shock proteins gp96, hsp90, hsp70, and calreticulin'. *Immunity* **14**, 303–313.

Bateman, K. S. and G. D. Stentiford (2008). '*Cancer pagurus* bacilliform virus (CpBV) infecting juvenile European edible crabs *C. pagurus* from UK waters'. *Diseases of Aquatic Organisms* **79**, 147–151.

Battison, A. L., R. J. Cawthorn and B. Horney (2004). 'Response of American lobsters *Homarus americanus* to infection with a field isolate of *Aerococcus viridans* var. *homari* (gaffkemia): survival and haematology'. *Diseases of Aquatic Organisms* **61**, 263–268.

Battison, A. L., R. Summerfield and A. Patrzykat (2008). 'Isolation and characterisation of two antimicrobial peptides from haemocytes of the American lobster *Homarus americanus*'. *Fish & Shellfish Immunology* **25**, 181–187.

Battistella, S., P. Bonivento and G. A. Amirante (1996). 'Hemocytes and immunological reactions in crustaceans'. *Italian Journal of Zoology* **63**, 337–343.

Bauchau, A. G. (1981). Crustaceans. In N. Ratcliffe and A. F. Rowley (Eds.). 'Invertebrate Blood Cells'. Vol. 2. Academic Press Inc.. London. pp. 385–420.

Bayne, C. J. (1990). 'Phagocytosis and non-self recognition in invertebrates'. *Bioscience* **40**, 723–731.

Beckage, N. E. (1998). 'Modulation of immune responses to parasitoids by polydnaviruses'. *Parasitology* **116**, S57–S64.

Bell, K. L. and V. J. Smith (1993). '*In vitro* superoxide production by hyaline cells of the shore crab *Carcinus maenas* (L)'. *Developmental and Comparative Immunology* **17**, 211–219.

Belloncik, S., O. Petcharawan, M. Couillard, G. Charpentier, B. Larue, H. Guardado, S. Chareonsak and S. Imanishi (2007). 'Development and characterization of a continuous cell line, AFKM-On-H, from hemocytes of the European corn borer *Ostrinia nubilalis* (Hubner) (Lepidoptera, Pyralidae)'. *In vitro Cellular & Developmental Biology-Animal* **43**, 245–254.

Berg, J. M., J. L. Tymoczko and L. Stryer (2007). *Biochemistry*. 6 edn. W. H. Freeman and Company. New York.

Betts, J. C., P. T. Lukey, L. C. Robb, R. A. McAdam and K. Duncan (2002). 'Evaluation of a nutrient starvation model of *Mycobacterium tuberculosis* persistence by gene and protein expression profiling'. *Molecular Microbiology* **43**, 717–731.

Biosoft, P. (2008). 'PCR Primer Design Guidelines'.

Biosystems, A. (2010). 'TRI Reagent Solution Manual 9738M'.

Bower, S. M., G. R. Meyer, A. Phillips, G. Workman and D. Clark (2003). 'New host and range extension of bitter crab syndrome in *Chionoecetes* spp. caused by *Hematodinium* sp.'. *Bulletin of the European Association of Fish Pathologists* **23**, 86–91.

Breitbart, M., L. R. Thompson, C. A. Suttle and M. B. Sullivan (2007). 'Exploring the vast diversity of marine viruses'. *Oceanography* **20**, 135–139.

Brett, C. J., A. S. G. Curtis, J. A. T. Dow, J. G. Edwards, J. M. Lackie, A. J. Lawrence and G. R. Moores (1989). *The Dictionary of Cell Biology*. Academic Press. London.

Briggs, R. P. and M. McAliskey (2002). 'The prevalence of *Hematodinium* in *Nephrops norvegicus* from the western Irish Sea'. *Journal of the Marine Biological Association of the United Kingdom* **82**, 427–433.

Brisson, M., S. Hall, R. K. Hamby, R. Park and H. K. Srere (2004). Optimization of single and multiplex real-time PCR. In S. Bustin (Ed.). 'A-Z of quantitative PCR'. International University Line. La Jolla. pp. 619–642.

Brites, D., S. McTaggart, K. Morris, J. Anderson, K. Thomas, I. Colson, T. Fabbro, T. J. Little, D. Ebert and L. Du Pasquier (2008). 'The Dscam homologue of the crustacean Daphnia is diversified by alternative splicing like in insects'. *Molecular Biology and Evolution* **25**, 1429–1439.

Brivio, M. F., M. Mastore and A. J. Nappi (2010). 'A pathogenic parasite interferes with phagocytosis of insect immunocompetent cells'. *Developmental and Comparative Immunology* **34**, 991–998.

Brivio, M. F., M. Moro and M. Mastore (2006). 'Down-regulation of antibacterial peptide synthesis in an insect model induced by the body-surface of an entomoparasite (*Steinernema feltiae*)'. *Developmental and Comparative Immunology* **30**, 627–638.

Brivio, M. F., M. Pagani and S. Restelli (2002). 'Immune suppression of *Galleria mellonella* (Insecta, Lepidoptera) humoral defenses induced by *Steinernema feltiae* (Nematoda, Rhabditida): involvement of the parasite cuticle'. *Experimental Parasitology* **101**, 149–156.

Brockton, V. and V. J. Smith (2008). 'Crustin expression following bacterial injection and temperature change in the shore crab, *Carcinus maenas*'. *Developmental and Comparative Immunology* **32**, 1027–1033.

Brockton, V., J. A. Hammond and V. J. Smith (2007). 'Gene characterisation, isoforms and recombinant expression of carcinin, an antibacterial protein from the shore crab, Carcinus maenas'. *Molecular Immunology* **44**, 943–949.

Brody, M. D. and E. S. Chang (1989). 'Development and utilization of crustacean long-term primary cell cultures: Ecdysteroid effects *in vitro*'. *Invertebrate Reproduction and Development* **16**, 141–147.

Brown-Peterson, N. J., P. Larkin, N. Denslow, C. King, S. Manning and M. Brouwer (2005). 'Molecular indicators of hypoxia in the blue crab *Callinectes sapidus*'. *Marine Ecology-Progress Series* **286**, 203–215.

Buda, E. S. and T. H. Shafer (2005). 'Expression of a serine proteinase homolog prophenoloxidase-activating factor from the blue crab, *Callinectes sapidus*'. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology* **140**, 521–531.

Bustin, S. A. (2000). 'Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays'. *Journal of Molecular Endocrinology* **25**, 169–193.

Bustin, S. A. (2002). 'Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems'. *Journal of Molecular Endocrinology* **29**, 23–39.

Bustin, S. A. (2010). 'Why the need for qPCR publication guidelines?-The case for MIQE'. *Methods* **50**, 217–226.

Bustin, S. A., J. F. Beaulieu, J. Huggett, R. Jaggi, F. S. B. Kibenge, P. A. Olsvik, L. C. Penning and S. Toegel (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., V. Benes, J. A. Garson, J. Hellemans, J. Huggett, M. Kubista, R. Mueller, T. Nolan, M. W. Pfaffl, G. L. Shipley, J. Vandesompele and C. T. Wittwer (2009). 'The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments'. *Clinical Chemistry* **55**, 611–622.

Bustin, S. A., V. Benes, T. Nolan and M. W. Pfaffl (2005). 'Quantitative real-time RT-PCR - a perspective'. *Journal of Molecular Endocrinology* **34**, 597–601.

Bustin, S. and T. Nolan (2004*a*). Basic RT-PCR considerations. In S. Bustin (Ed.). 'A-Z of Quantitative PCR'. International University Line. La Jolla. pp. 361–395.

Bustin, S. and T. Nolan (2004*b*). Chemistries. In S. Bustin (Ed.). 'A-Z of quantitative PCR'. International University Line. La Jolla. pp. 217–278.

Bustin, S. and T. Nolan (2004*c*). Data analysis and interpretation. In S. Bustin (Ed.). 'A-Z of Quantitative PCR'. International University Line. La Jolla. pp. 439–492.

Bustin, S. and T. Nolan (2004*d*). 'Pitfalls of quantitative real-time reverse-transcription polymerase chain reaction'. *Journal of Biomolecular Techniques* **15**, 155–166.

Bustin, S. and T. Nolan (2004*e*). Primers and Probes. In S. Bustin (Ed.). 'A-Z of quantitative PCR'. International University Line. La Jolla. pp. 281–328.

Bustin, S. and T. Nolan (2004*f*). Template handling, preparation, and quantification. In S. Bustin (Ed.). 'A-Z of quantitative PCR'. International University Line. La Jolla. pp. 141–214.

Caban, K., S. A. Kinzy and P. R. Copeland (2007). 'The L7Ae RNA binding motif is a multifunctional domain required for the ribosome-dependent Sec incorporation

activity of Sec insertion sequence binding protein 2'. *Molecular and Cellular Biology* **27**, 6350–6360.

Canon, J. and U. Banerjee (2000). 'Runt and Lozenge function in *Drosophila* development'. *Seminars in Cell & Developmental Biology* **11**, 327–336.

Cardenas, M. E., N. S. Cutler, M. C. Lorenz, C. J. Di Como and J. Heitman (1999). 'The TOR signaling cascade regulates gene expression in response to nutrients'. *Genes & Development* **13**, 3271–3279.

Cardenas, W. and J. R. Dankert (1997). 'Phenoloxidase specific activity in the red swamp crayfish *Procambarus clarkii*'. *Fish & Shellfish Immunology* **7**, 283–295.

Cardenas, W., J. R. Dankert and J. A. Jenkins (2004). 'Flow cytometric analysis of crayfish haemocytes activated by lipopolysaccharides'. *Fish & Shellfish Immunology* **17**, 223–233.

Carter, J. B., C. Griffiths and C. F. Edwards (1994). 'Establishment of a cell-line from *Tipula paludosa* (Diptera, Tipulidae) hemocytes and replication of *T. paludosa* baculovirus *in vitro*'. *Journal of Invertebrate Pathology* **63**, 119–122.

Cerenius, L. and K. Soderhall (1995). 'Crustacean immunity and complement - a premature comparison'. *American Zoologist* **35**, 60–67.

Cerenius, L. and K. Soderhall (2004). 'The prophenoloxidase-activating system in invertebrates'. *Immunological Reviews* **198**, 116–126.

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

Cerenius, L., E. Bangyeekhun, P. Keyser, I. Soderhall and K. Soderhall (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., R. Babu, K. Soderhall and P. Jiravanichpaisal (2010). '*In vitro* effects on bacterial growth of phenoloxidase reaction products'. *Journal of Invertebrate Pathology* **103**, 21–23.

Cerenius, L., Z. C. Liang, B. Duvic, P. Keyser, U. Hellman, E. T. Palva, S. Iwanaga and K. Soderhall (1994). 'Structure and biological activity of a 1,3-Beta-D-glucan-binding protein in crustacean blood'. *Journal of Biological Chemistry* **269**, 29462–29467.

Chang, B., E. Larson and C. Whitman-Gullaev (2010). 'The Experion System: microfluidics-based automated electrophoresis'. *BioRad Bulletin*.

Chang, E. S. (2005). 'Stressed-out lobsters: Crustacean hyperglycemic hormone and stress proteins'. *Integrative and Comparative Biology* **45**, 43–50.

Chapman, C. J., A. D. F. Johnstone and A. L. Rice (1975). The behaviour and ecology of the Norway lobster, *Nephrops norvegicus* (L.). In H. B. Barnes (Ed.). 'Proceedings of the 9th European Marine Biology Symposium'. pp. 59–74.

Charoensapsri, W., P. Amparyup, I. Hirono, T. Aoki and A. Tassanakajon (2009). 'Gene silencing of a prophenoloxidase activating enzyme in the shrimp, *Penaeus monodon*, increases susceptibility to *Vibrio harveyi* infection'. *Developmental and Comparative Immunology* **33**, 811–820.

Chatton, E. and R. Poisson (1931). 'Sur l'existence, dans le sang des crabs, de peridiniens parasites: *Hematodinium perezi* n.g., n.sp. (Syndinidae).'. *Comptes rendus des seances de la Societe de biologie et des ses filiales* **105**, 553–557.

Chen, F. W. and Y. A. Ioannou (1999). 'Ribosomal proteins in cell proliferation and apoptosis'. *International Reviews of Immunology* **18**, 429–448.

Chen, F. Y., H. P. Liu, J. Bo, H. L. Ren and K. J. Wang (2010*a*). 'Identification of genes differentially expressed in hemocytes of *Scylla paramamosain* in response to lipopolysaccharide'. *Fish & Shellfish Immunology* **28**, 167–177.

Chen, J. C. and W. Cheng (2001). 'Effects of intrinsic and extrinsic factors on the haemocyte profile of the prawn, *Macrobrachium rosenbergii*'. *Fish & Shellfish Immunology* **11**, 53–63.

Chen, P., J. T. Li, J. Li, P. Liu, B. Q. Gao and Q. Y. Wang (2010*b*). 'Molecular cloning and characterization of prophenoloxidase gene in swimming crab *Portunus trituberculatus*'. *Fish & Shellfish Immunology* **28**, 106–112.

Cheng, W., C. H. Liu, J. P. Hsu and J. C. Chen (2002). 'Effect of hypoxia on the immune response of giant freshwater prawn *Macrobrachium rosenbergii* and its susceptibility to pathogen *Enterococcus*'. *Fish & Shellfish Immunology* **13**, 351–365.

Cheng, W. T., C. H. Liu, C. H. Tsai and J. C. Chen (2005). 'Molecular cloning and characterisation of a pattern recognition molecule, lipopolysaccharide- and beta-1,3-glucan binding protein (LGBP) from the white shrimp *Litopenaeus vannamei*'. *Fish & Shellfish Immunology* **18**, 297–310.

Chisholm, J. R. S. and V. J. Smith (1995). 'Comparison of antibacterial activity in the hemocytes of different crustacean species'. *Comparative Biochemistry and Physiology a-Physiology* **110**, 39–45.

Chou, H. Y., C. Y. Huang, C. H. Wang, H. C. Chiang and C. F. Lo (1995). 'Pathogenicity of a baculovirus infection causing white spot syndrome in cultured penaeid shrimp in Taiwan'. *Diseases of Aquatic Organisms* **23**, 165–173.

Chou, P. H., H. S. Chang, I. T. Chen, C. W. Lee, H. Y. Hung and K. C. H. C. Wang (2011). '*Penaeus monodon* Dscam (PmDscam) has a highly diverse cytoplasmic tail and is the first membrane-bound shrimp Dscam to be reported'. *Fish & Shellfish Immunology* **30**, 1109–1123.

Chou, P. H., H. S. Chang, I. T. Chen, H. Y. Lin, Y. M. Chen, H. L. Yang and K. C. H. C. Wang (2009). 'The putative invertebrate adaptive immune protein *Litopenaeus vannamei* Dscam (LvDscam) is the first reported Dscam to lack a transmembrane domain and cytoplasmic tail'. *Developmental and Comparative Immunology* **33**, 1258–1267.

Christensen, B. M., J. Y. Li, C. C. Chen and A. J. Nappi (2005). 'Melanization immune responses in mosquito vectors'. *Trends in Parasitology* **21**, 192–199.

Christie, A. E., S. Rus, C. C. Goiney, C. M. Smith, D. W. Towle and P. S. Dickinson (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.

Christophides, G. K., E. Zdobnov, C. Barillas-Mury, E. Birney, S. Blandin, C. Blass, P. T. Brey, F. H. Collins, A. Danielli, G. Dimopoulos, C. Hetru, N. T. Hoa, J. A. Hoffmann, S. M. Kanzok, I. Letunic, E. A. Levashina, T. G. Loukeris, G. Lycett, S. Meister, K. Michel, L. F. Moita, H. M. Muller, M. A. Osta, S. M. Paskewitz, J. M. Reichhart, A. Rzhetsky, L. Troxler, K. D. Vernick, D. Vlachou, J. Volz, C. von Mering, J. N. Xu, L. B. Zheng, P. Bork and F. C. Kafatos (2002). 'Immunity-related genes and gene families in *Anopheles gambiae*'. *Science* **298**, 159–165.

Chualain, C. N. and M. Robinson (2011). 'Comparison of assessment methods used to diagnose *Hematodinium* sp. infections in *Cancer pagurus*'. *Ices Journal of Marine Science*.

Chualain, C. N., M. Hayes, B. Allen and M. Robinson (2009). '*Hematodinium* sp in Irish *Cancer pagurus* fisheries: infection intensity as a potential fisheries management tool'. *Diseases of Aquatic Organisms* **83**, 59–66.

Clare, A. S. and G. Lumb (1994). 'Identification of hemocytes and their role in clotting in the blue crab, *Callinectes sapidus*'. *Marine Biology* **118**, 601–610.

Clontech (2006). 'SMART RACE cDNA Amplification Kit User Manual'.

Coates, A. R. M. (1996). Immunological Aspects of Chaperonins. In R. J. Ellis (Ed.). 'The Chaperonins'. Cell Biology: A series of Monographs. Academic Press, Inc.. London. pp. 268–296.

Coats, D. W. (1999). 'Parasitic life styles of marine dinoflagellates'. *Journal of Eukaryotic Microbiology* **46**, 402–409.

Cordin, O., J. Banroques, N. K. Tanner and P. Linder (2006). 'The DEAD-box protein family of RNA helicases'. *Gene* **367**, 17–37.

Cornet, S. (2010). 'Density-dependent effects on parasite growth and parasite-induced host immunodepression in the larval helminth *Pomphorhynchus laevis*'. *Parasitology*.

Cornet, S., N. Franceschi, A. Bauer, T. Rigaud and Y. Moret (2009). 'Immune depression induced by acanthocephalan parasites in their intermediate crustacean host: Consequences for the risk of super-infection and links with host behavioural manipulation'. *International Journal for Parasitology* **39**, 221–229.

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

Crosson, L. M., C. S. Friedman and J. F. Morado (2009). 'Development and application of a quantitative PCR (qPCR) assay to assess the life history of *Hematodinium*, a parasitic dinoflagellate, and Its impact on Tanner crab populations in Alaska.'. *Journal of Shellfish Research* **28**, 690–690.

Crosson, L. M., L. Hauser, C. S. Friedman and J. F. Morado (2008). 'Development of a quantitative real-time polymerase chain reaction (QPCR) assay to assess the life history of *Hematodinium*, a parasitic dinoflagellate, and its impact on tanner crab populations in Alaska'. *Journal of Shellfish Research* **27**, 469–469.

Crozatier, M. and M. Meister (2007). 'Drosophila haematopoiesis'. *Cellular Microbiology* **9**, 1117–1126.

Cui, Z. X., Y. A. Liu, D. H. Wu, W. S. Luan, S. Y. Wang, Q. Q. Li and C. W. Song (2010). 'Molecular cloning and characterization of a serine proteinase homolog prophenoloxidase-activating factor in the swimming crab *Portunus trituberculatus*'. *Fish & Shellfish Immunology* **29**, 679–686.

Dawe, E. G. (2002). 'Trends in prevalence of bitter crab disease caused by *Hematodinium* sp in snow crab (*Chionoecetes opilio*) throughout the Newfoundland and Labrador Continental Shelf'. *Crabs in Cold Water Regions: Biology, Management, and Economics* **19**, 385–400 866.

de Lorgeril, J., D. Saulnier, M. G. Janech, Y. Gueguen and E. Bachere (2005). 'Identification of genes that are differentially expressed in hemocytes of the Pacific blue shrimp (*Litopenaeus stylirostris*) surviving an infection with *Vibrio penaeicida*'. *Physiological Genomics* **21**, 174–183.

de Lorgeril, J., R. Zenagui, R. D. Rosa, D. Piquemal and E. Bachere (2011). 'Whole transcriptome progiling of successful immune response to *Vibrio* infections in the oyster *Crassostrea gigas* by digital gene expression analysis'. *Plos One* **6**, e23142.

de Lorgeril, J., Y. Gueguen, C. Goarant, E. Goyard, C. Mugnier, J. Fievet, D. Piquemal and E. Bachere (2008). 'A relationship between antimicrobial peptide gene expression and capacity of a selected shrimp line to survive a *Vibrio* infection'. *Molecular Immunology* **45**, 3438–3445.

Deachamag, P., U. Intaraphad, A. Phongdara and W. Chotigeat (2006). 'Expression of a Phagocytosis Activating Protein (PAP) gene in immunized black tiger shrimp'. *Aquaculture* **255**, 165–172.

Decker, H. and E. Jaenicke (2004). 'Recent findings on phenoloxidase activity and antimicrobial activity of hemocyanins'. *Developmental and Comparative Immunology* **28**, 673–687.

Decker, H. and N. Terwilliger (2000). 'Cops and robbers: Putative evolution of copper oxygen-binding proteins'. *Journal of Experimental Biology* **203**, 1777–1782.

Denisov, V., W. Strong, M. Walder, J. Gingrich and H. Wintz (2008). 'Development and validation of RQI: an RNA quality indicator for the Experion automated electrophoresis system'. *BioRad Bulletin*.

Derveaux, S., J. Vandesompele and J. Hellemans (2010). 'How to do successful gene expression analysis using real-time PCR'. *Methods* **50**, 227–230.

Destoumieux, D., M. Muñnoz, P. Bulet and E. Bachere (2000). 'Penaeidins, a family of antimicrobial peptides from penaeid shrimp (Crustacea, Decapoda)'. *Cellular and Molecular Life Sciences* **57**, 1260–1271.

Destoumieux, D., P. Bulet, J. M. Strub, A. van Dorsselaer and E. Bachere (1999). 'Recombinant expression and range of activity of penaeidins, antimicrobial peptides from penaeid shrimp'. *European Journal of Biochemistry* **266**, 335–346.

Destoumieux-Garzon, D., D. Saulnier, J. Garnier, C. Jouffrey, P. Bulet and E. Bachere (2001). 'Crustacean immunity - Antifungal peptides are generated from the C terminus of shrimp hemocyanin in response to microbial challenge'. *Journal of Biological Chemistry* **276**, 47070–47077.

Dezfuli, B. S., E. Simoni, L. Duclos and E. Rossetti (2008). 'Crustacean-acanthocephalan interaction and host cell-mediated immunity: parasite encapsulation and melanization'. *Folia Parasitologica* **55**, 53–59.

Dheda, K., J. F. Huggett, S. A. Bustin, M. A. Johnson, G. Rook and A. Zumla (2004). 'Validation of housekeeping genes for normalizing RNA expression in real-time PCR'. *Biotechniques* **37**, 112–119.

Dimarcq, J. L., P. Bulet, C. Hetru and J. Hoffmann (1998). 'Cysteine-rich antimicrobial peptides in invertebrates'. *Biopolymers* **47**, 465–477.

Donaldson, J. R., A. Hanif, H. A. Bowers, J. Pitula, G. A. Messick and E. J. Schott (2009). 'Evidence for the blue crab parasite, *Hematodinium* sp in coastal bay benthos.'. *Journal of Shellfish Research* **28**, 693–693.

Dong, Y. M., H. E. Taylor and G. Dimopoulos (2006). '*AgDscam*, a hypervariable immunoglobulin domain-containing receptor of the *Anopheles gambiae* innate immune system'. *Plos Biology* **4**, 1137–1146.

Dowd, P. F. (1999). 'Relative inhibition of insect phenoloxidase by cyclic fungal metabolites from insect and plant pathogens'. *Natural Toxins* **7**, 337–341.

Du, X. J., X. F. Zhao and J. X. Wang (2007). 'Molecular cloning and characterization of a lipopolysaccharide and beta-1,3-glucan binding protein from fleshy prawn (*Fenneropenaeus chinensis*)'. *Molecular Immunology* **44**, 1085–1094.

Duvic, B. and K. Soderhall (1990). 'Purification and characterization of a Beta-1,3-glucan binding-protein from plasma of the crayfish *Pacifastacus leniusculus*'. *Journal of Biological Chemistry* **265**, 9327–9332.

Duvic, B. and K. Soderhall (1993). 'Beta-1,3-glucan binding proteins from plasma of the freshwater crayfishes *Astacus astacus* and *Procambarus clarkii*'. *Journal of Crustacean Biology* **13**, 403–408.

Dziarski, R. (2004). 'Peptidoglycan recognition proteins (PGRPs)'. *Molecular Immunology* **40**, 877–886.

Eaton, W. D., D. C. Love, C. Botelho, T. R. Meyers, K. Imamura and T. Koeneman (1991). 'Preliminary results on the seasonality and life-cycle of the parasitic dinoflagellate causing Bitter Crab Disease in Alaskan Tanner crabs (*Chionoecetes bairdi*)'. *Journal of Invertebrate Pathology* **57**, 426–434.

Edward, E. (1979). *The Edible crab and its fishery in British waters*. Fishing News Books Ltd. Farnham.

Edward, E. (1989). Crab fisheries and their management in the British Isles. In J. F. Caddy (Ed.). 'Marine Invertebrate Fisheries: Their Assessment and Management'. John Wiley & Sons. Chichester. p. 752.

Eigemann, F., A. Burmeister and A. Skovgaard (2010). '*Hematodinium* sp (Alveolata, Syndinea) detected in marine decapod crustaceans from waters of Denmark and Greenland'. *Diseases of Aquatic Organisms* **92**, 59–68.

Ellender, R. D., A. K. Najafabadi and B. L. Middlebrooks (1992). 'Observations on the primary culture of hemocytes of Penaeus'. *Journal of Crustacean Biology* **12**, 178–185.

Ellis, R. J. (1996). Chaperonins: Introductory Perspective. In R. J. Ellis (Ed.). 'The Chaperonins'. Cell Biology: A series of Monographs. Academic Press, Inc. London. pp. 1–26.

Epand, R. M. and H. J. Vogel (1999). 'Diversity of antimicrobial peptides and their mechanisms of action'. *Biochimica Et Biophysica Acta-Biomembranes* **1462**, 11–28.

Eslin, P. and G. Prevost (1998). 'Hemocyte load and immune resistance to *Asobara tabida* are correlated in species of the *Drosophila melanogaster* subgroup'. *Journal of Insect Physiology* **44**, 807–816.

Fagutao, F. F., M. Yasuike, C. M. Caipang, H. Kondo, I. Hirono, Y. Takahashi and T. Aoki (2008). 'Gene expression profile of hemocytes of Kuruma shrimp, *Marsupenaeus japonicus* following peptidoglycan stimulation'. *Marine Biotechnology* **10**, 731–740.

Fanjul-Moles, M. L. (2006). 'Biochemical and functional aspects of crustacean hyperglycemic hormone in decapod crustaceans: Review and update'. *Comparative Biochemistry and Physiology C-Toxicology & Pharmacology* **142**, 390–400.

Field, R. H. (1992). The control of escape behaviour in, and the histopathology of, the Norway lobster, *Nephrops norvegicus (L.)*. PhD thesis.

Field, R. H. and P. L. Appleton (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.

Field, R. H. and P. L. Appleton (1996). 'An indirect fluorescent antibody technique for the diagnosis of *Hematodinium* sp infection of the Norway lobster *Nephrops norvegicus*'. *Diseases of Aquatic Organisms* **24**, 199–204.

Field, R. H., C. J. Chapman, A. C. Taylor, D. M. Neil and K. Vickerman (1992). 'Infection of the Norway lobster *Nephrops norvegicus* by a *Hematodinium*-like species of dinoflagellate on the west coast of Scotland'. *Diseases of Aquatic Organisms* **13**, 1–15.

Field, R. H., J. M. Hills, R. J. A. Atkinson, S. Magill and A. M. Shanks (1998). 'Distribution and seasonal prevalence of *Hematodinium* sp. infection of the Norway lobster (*Nephrops norvegicus*) around the west coast of Scotland'. *ICES Journal of Marine Science* **55**, 846–858.

Flegel, T. W. (1997). 'Major viral diseases of the black tiger prawn (*Penaeus monodon*) in Thailand'. *World Journal of Microbiology & Biotechnology* **13**, 433–442.

Flegel, T. W. (2001). 'The shrimp response to viral pathogens'. *New Wave Proceedings of the Special Session on Sustainable Shrimp Farming* pp. 254–278.

Flegel, T. W. (2007). 'Update on viral accommodation, a model for host-viral interaction in shrimp and other arthropods'. *Developmental and Comparative Immunology* **31**, 217–231.

Fleige, S. and M. Pfaffl (2006). 'RNA integrity and the effect on the real-time qRT-PCR performance'. *Molecular Aspects of Medicine* **27**, 126–139.

Ford, S. E., V. M. Bricelj, C. Lambert and C. Paillard (2008). 'Deleterious effects of a nonPST bioactive compound(s) from *Alexandrium tamarense* on bivalve hemocytes'. *Marine Biology* **154**, 241–253.

Fragkiadakis, G. A. and E. K. Stratakis (1997). 'The lectin from the crustacean *Liocarcinus depurator* recognizes O-acetylsialic acids'. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology* **117**, 545–552.

Franssens, V., G. Simonet, B. Breugelmans, S. Van Soest, V. Van Hoef and J. V. Broeck (2008). 'The role of hemocytes, serine protease inhibitors and pathogen-associated patterns in prophenoloxidase activation in the desert locust, *Schistocerca gregaria*'. *Peptides* **29**, 235–241.

Freshney, R. I. (2005). *Culture of animal cells: a manual of basic techniques*. John Wiley & Sons, Inc.. Hoboken.

Frischer, M. E., R. F. Lee, M. A. Sheppard, A. Mauer, F. Rambow, M. Neumann, J. E. Brofft, T. Wizenmann and J. M. Danforth (2006). 'Evidence for a free-living life stage of the blue crab parasitic dinoflagelate, *Hematodinium* sp.'. *Harmful Algae* **5**, 548–557.

Furutani, Y., A. Kato, R. Kawai, A. Fibriani, S. Kojima and S. Hirose (2004). 'Androgen-dependent expression, gene structure, and molecular evolution of guinea pig caltrin II, a WAP-motif protein'. *Biology of Reproduction* **71**, 1583–1590.

Gai, Y. C., J. M. Zhao, L. S. Song, C. H. Li, P. L. Zheng, L. M. Qiu and D. J. Ni (2008). 'A prophenoloxidase from the Chinese mitten crab *Eriocheir sinensis*: Gene cloning, expression and activity analysis'. *Fish & Shellfish Immunology* **24**, 156–167.

Gao, H. W., F. H. Li, B. Dong, Q. L. Zhang and J. H. Xiang (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-Vallejo, J. J., B. Van het Hof, J. Robben, J. A. E. Van Wijk, I. Van Die, D. H. Joziasse and W. Van Dijk (2004). 'Approach for defining endogenous reference genes in gene expression experiments'. *Analytical Biochemistry* **329**, 293–299.

Gargioni, R. and M. A. Barracco (1998). 'Hemocytes of the palaemonids *Macrobrachium rosenbergii* and *M. acanthurus*, and of the penaeid *Penaeus paulensis*'. *Journal of Morphology* **236**, 209–221.

George, S. K. and A. K. Dhar (2010). 'An improved method of cell culture system from eye stalk, hepatopancreas, muscle, ovary, and hemocytes of *Penaeus vannamei*'. *In vitro Cellular & Developmental Biology-Animal* **46**, 801–810.

Gey, G. O., W.D. Coffman and M. T. Kubicek (1952). 'Tissue culture studies of the proliferative capacity of cervical carcinoma and normal epithelium'. *Cancer Research* **12**, 264.

Ghiretti-Magaldi, A., C. Milanesi and G. Tognon (1977). 'Hematopoiesis in Crustacea Decapoda - origin and evolution of hemocytes and cyanocytes of *Carcinus maenas*'. *Cell Differentiation* **6**, 167–186.

Ghosh, J., C. M. Lun, A. J. Majeske, S. Sacchi, C. S. Schrankel and L. C. Smith (2011). 'Invertebrate immune diversity'. *Developmental and Comparative Immunology* **35**, 959–974.

Ginzinger, D. G. (2002). 'Gene quantification using real-time quantitative PCR: An emerging technology hits the mainstream'. *Experimental Hematology* **30**, 503–512.

Glare, E. M., E. H. Walters, M. Divjak and M. J. Bailey (2002). 'beta-actin and GAPDH housekeeping gene expression in asthmatic airways is variable and not suitable for normalising mRNA levels'. *Thorax* **57**, 765–770.

Gomes, S. A. O., D. Feder, N. E. S. Thomas, E. S. Garcia and P. Azambuja (1999). '*Rhodnius prolixus* infected with *Trypanosoma rangeli*: *in vivo* and *in vitro* experiments'. *Journal of Invertebrate Pathology* **73**, 289–293.

Grace, T. D. C. (1962). 'Establishment of four strains of cells from insect tissues grown *in vitro*'. *Nature* **195**, 788–789.

Graham, N. and R. S. T. Ferro (2004). The Nephrops fisheries of the Northeast Atlantic and Mediterranean - A review and assessment of fishing gear design. Technical report. International Council for the Exploration of the Sea.

Grantham, J., L. W. Ruddock, A. Roobol and M. J. Carden (2002). 'Eukaryotic chaperonin containing T-complex polypeptide 1 interacts with filamentous actin and reduces the initial rate of actin polymerization *in vitro*'. *Cell Stress & Chaperones* **7**, 235–242.

Gregorio, E. A. and N. A. Ratcliffe (1991). 'The prophenoloxidase system and *in vitro* interaction of *Trypanosoma rangeli* with *Rhodnius prolixus* and *Triatoma infestans* hemolymph'. *Parasite Immunology* **13**, 551–564.

Gruebl, T., M. E. Frischer, M. Sheppard, M. Neumann, A. N. Maurer and R. F. Lee (2002). 'Development of an 18S rRNA gene-targeted PCR-based diagnostic for the blue crab parasite *Hematodinium* sp.'. *Diseases of Aquatic Organisms* **49**, 61–70.

Gudnason, H., A. Wolff, M. Dufva and D. D. Bang (2007). 'Comparison of multiple DNA dyes for real-time PCR: effects of dye concentration and sequence composition on DNA amplification and melting temperature'. *Nucleic Acids Research* **35**, e127.

Gupta, A. P. (2001). 'Immunology of invertebrates: Humoral'. *Encyclopedia of Life Sciences*.

Gupta, R. S. (1990). 'Sequence and structural homology between a mouse T-complex protein Tcp-1 and the chaperonin family of bacterial (Groel, 60-65-kDa heat shock antigen) and eukaryotic proteins'. *Biochemistry International* **20**, 833–841.

Guzo, D. and D. B. Stoltz (1987). 'Observations on Cellular-Immunity and Parasitism in the Tussock Moth'. *Journal of Insect Physiology* **33**, 19–31.

Hagen, H. E., J. Grunewald and P. J. Ham (1994). 'Induction of the prophenoloxidase-activating system of Simulium (Diptera, Simuliidae) following *Onchocerca* (Nematoda, Filarioidea) infection'. *Parasitology* **109**, 649–655.

Hagger, J. A., T. S. Galloway, W. J. Langston and M. B. Jones (2009). 'Application of biomarkers to assess the condition of European Marine Sites'. *Environmental Pollution* **157**, 2003–2010.

Hagiwara, K., T. Kikuchi, Y. Endo, Huqun, K. Usui, M. Takahashi, N. Shibata, T. Kusakabe, H. Xin, S. Hoshi, M. Miki, N. Inooka, Y. Tokue and T. Nukiwa (2003). 'Mouse SWAM1 and SWAM2 are antibacterial proteins composed of a single whey acidic protein motif'. *Journal of Immunology* **170**, 1973–1979.

Hamilton, K. (2007). Infection of Clyde Sea crustaceans by the protist parasite *Hematodinium*. PhD thesis.

Hamilton, K., D. Morritt and P. Shaw (2007*a*). 'Is genetic variability of the alveolate parasite *Hematodinium* host-specific?.'. *Journal of Eukaryotic Microbiology* **54**, 41S–41S.

Hamilton, K. M., D. Morritt and P. W. Shaw (2007*b*). 'Molecular and histological identification of the crustacean parasite *Hematodinium* sp (Alveolata, Syndinea) in the shore crab *Carcinus maenas*'. *Acta Protozoologica* **46**, 183–192.

Hamilton, K. M., D. Morritt and P. W. Shaw (2010). 'Genetic diversity of the crustacean parasite *Hematodinium* (Alveolata, Syndinea)'. *European Journal of Protistology* **46**, 17–28.

Hamilton, K. M., I. F. Tew, R. J. A. Atkinson and E. C. Roberts (2011). 'Occurrence of the parasite genus *Hematodinium* (Alveolata: Syndinea) in the water column'. *Journal of Eukaryotic Microbiology* **58**, 446–451.

Hamilton, K. M., P. W. Shaw and D. Morritt (2009). 'Prevalence and seasonality of *Hematodinium* (Alveolata: Syndinea) in a Scottish crustacean community'. *Ices Journal of Marine Science* **66**, 1837–1845.

Hammond, J. A. and V. J. Smith (2002). 'Lipopolysaccharide induces DNA-synthesis in a sub-population of hemocytes from the swimming crab, *Liocarcinus depurator*'. *Developmental and Comparative Immunology* **26**, 227–236.

Hanafy, K. A., E. Martin and F. Murad (2004). 'CCT eta, a novel soluble guanylyl cyclase-interacting protein'. *Journal of Biological Chemistry* **279**, 46946–46953.

Harrison, R. G. (1907). 'Observations on the living developing nerve fiber'. *Proceedings of the Society for Experimental Biology and Medicine* **4**, 140–143.

Hauton, C. and V. J. Smith (2004). '*In vitro* cytotoxicity of crustacean immuno stimulants for lobster (*Homarus gammarus*) granulocytes demonstrated using the neutral red uptake assay'. *Fish & Shellfish Immunology* **17**, 65–73.

Hauton, C., J. A. Hammond and V. J. Smith (2005). 'Real-time PCR quantification of the *in vitro* effects of crustacean immunostimulants on gene expression in lobster

(*Homarus gammarus*) granular haemocytes'. *Developmental and Comparative Immunology* **29**, 33–42.

Hauton, C., J. A. Williams and L. E. Hawkins (1997*a*). '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.

Hauton, C., L. E. Hawkins and J. A. Williams (1995). 'Circatidal rhythmicity in the activity of the phenoloxidase enzyme in the common shore crab, *Carcinus maenas*'. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology* **111**, 347–352.

Hauton, C., L. E. Hawkins and J. A. Williams (1997*b*). '*In situ* variability in phenoloxidase activity in the shore crab, *Carcinus maenas* (L)'. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology* **117**, 267–271.

Hauton, C., V. Brockton and V. J. Smith (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., V. Brockton and V. J. Smith (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.

Hegaret, H. and G. H. Wikfors (2005). 'Time-dependent changes in hemocytes of eastern oysters, *Crassostrea virginica*, and northern bay scallops, *Argopecten irradians irradians*, exposed to a cultured strain of *Prorocentrum minimum*'. *Harmful Algae* **4**, 187–199.

Hegaret, H., P.M. da Silva, G.H. Wikfors, H. Haberkorn, S. E. Shumway and P. Soudant (2011). '*In vitro* interactions between several species of harmful algae and haemocytes of bivalve molluscs'. *Cell Biology and Toxicology* **27**, 249–266.

Hernandez-Lopez, J., T. Gollas-Galvan and F. Vargas-Albores (1996). 'Activation of the prophenoloxidase system of the brown shrimp *Penaeus californiensis*'. *Comparative Biochemistry and Physiology C: Pharmacology, Toxicology and Endocrinology* **113**, 61–66.

Hernroth, B., S. P. Baden, K. Holm, T. Andre and I. Soderhall (2004). 'Manganese induced immune suppression of the lobster, *Nephrops norvegicus*'. *Aquatic Toxicology* **70**, 223–231.

Hervio, D., D. Chagot, P. Godin, H. Grizel and E. Mialhe (1991). 'Localization and characterization of acid-phosphatase-activity in *Bonamia ostreae* (Ascetospora), an intrahemocytic protozoan parasite of the flat oyster *Ostrea edulis* (Bivalvia)'. *Diseases of Aquatic Organisms* **12**, 67–70.

Hill, J. and J. E. Samuel (2011). '*Coxiella burnetii* acid phosphatase inhibits the release of reactive oxygen intermediates in polymorphonuclear leukocytes'. *Infection and Immunity* **79**, 414–420.

Hochachka, P. W. and P. L. Lutz (2001). 'Mechanism, origin, and evolution of anoxia tolerance in animals'. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology* **130**, 435–459.

Holmblad, T. and K. Soderhall (1999). 'Cell adhesion molecules and antioxidative enzymes in a crustacean, possible role in immunity'. *Aquaculture* **172**, 111–123.

Hose, J. E., G. G. Martin, S. Tiu and N. Mckrell (1992). 'Patterns of hemocyte production and release throughout the molt cycle in the Penaeid shrimp *Sicyonia ingentis*'. *Biological Bulletin* **183**, 185–199.

Hose, J. E., G. G. Martin, V. Nguyen, J. Lucas and T. Rosenstein (1987). 'Cytochemical features of shrimp hemocytes'. *Biological Bulletin* **173**, 178–187.

Hsu, J. P., C. Huang, C. M. Liao, S. L. Hsuan, H. H. Hung and M. S. Chien (2005). 'Engulfed pathogen-induced apoptosis in haemocytes of giant freshwater prawn, *Macrobrachium rosenbergii*'. *Journal of Fish Diseases* **28**, 729–735.

Hu, F., L. Pan and F. Jing (2009). 'Effects of hypoxia on dopamine concentration and the immune response of white shrimp (*Litopenaeus vannamei*)'. *Journal of Ocean University China* **8**, 77–82.

Huang, T. S., H. Y. Wang, S. Y. Lee, M. W. Johansson, K. Soderhall and L. Cerenius (2000). 'A cell adhesion protein from the crayfish *Pacifastacus leniusculus*, a serine proteinase homologue similar to *Drosophila* masquerade'. *Journal of Biological Chemistry* **275**, 9996–10001.

Hudson, D. A. (1995). 'Biochemical parameters of the serum of the sand crab, *Portunus pelagicus*, with reference to the parasitic dinoflagellate, *Hematodinium australis*'. *Bulletin of European Association of Fish Pathology* **15**, 202–205.

Hudson, D. A. and J. D. Shields (1994). '*Hematodinium australis* N-Sp, a parasitic dinoflagellate of the sand crab *Portunus pelagicus* from Moreton Bay, Australia'. *Diseases of Aquatic Organisms* **19**, 109–119.

Hudson, D. A. and R. D. Adlard (1994). 'PCR techniques applied to *Hematodinium* spp. and *Hematodinium*-like dinoflagellates in decapod crustaceans'. *Diseases of Aquatic Organisms* **20**, 203–206.

Hudson, D. A. and R. D. Adlard (1996). 'Nucleotide sequence determination of the partial SSU rDNA gene and ITS1 region of *Hematodinium cf perezi* and *Hematodinium*-like dinoflagellates'. *Diseases of Aquatic Organisms* **24**, 55–60.

Hudson, D. A. and R. J. G. Lester (1994). 'Parasites and symbionts of wild mud crabs *Scylla serrata* (Forskal) of potential significance in aquaculture'. *Aquaculture* **120**, 183–199.

Hudson, D. A., N. B. Hudson and J. D. Shields (1993). 'Infection of *Trapezia* Spp (Decapoda, Xanthidae) by *Hematodinium* Sp (Duboscquodinida, Syndinidae) - a new family record of infection'. *Journal of Fish Diseases* **16**, 273–276.

Huggett, J., K. Dheda, S. Bustin and A. Zumla (2005). 'Real-time RT-PCR normalisation; strategies and considerations'. *Genes and Immunity* **6**, 279–284.

Humbert, E. and C. Coustau (2001). 'Refractoriness of host haemocytes to parasite immunosuppressive factors as a putative resistance mechanism in the *Biomphalaria glabrata-Echinostoma caproni* system'. *Parasitology* **122**, 651–660.

Hwang, I. T., Y. J. Kim, S. H. Kim, C. I. Kwak, Y. Y. Gu and J. Y. Chun (2003). 'Annealing control primer system for improving specificity of PCR amplification'. *Biotechniques* **35**, 1180–1184.

Invitrogen (2008). 'Real time PCR: from theory to practice'.

Iwanaga, S. (2002). 'The molecular basis of innate immunity in the horseshoe crab'. *Current Opinion in Immunology* **14**, 87–95.

Iwanaga, S. and B. L. Lee (2005). 'Recent advances in the innate immunity of invertebrate animals'. *Journal of Biochemistry and Molecular Biology* **38**, 128–150.

Jalal, C., H. Uhlmann-Schiffler and H. Stahl (2007). 'Redundant role of DEAD box proteins p68 (Ddx5) and p72/p82 (Ddx17) in ribosome biogenesis and cell proliferation'. *Nucleic Acids Research* **35**, 3590–3601.

Janknecht, R. (2010). 'Mutli-talented DEAD-box proteins and potential tumor promoters: p68 RNA helicase (DDX5) and its paralog, p72 RNA helicase (DDX17)'. *American Journal of Translational Research* **2**, 223–234.

Jayasree, S. (2009). 'Identification of immune cells interacting with *Vibrio* spp. and its *in vitro* post-phagocytic killing mechanism of haemocytes in the penaeid shrimp, *Penaeus indicus* H. Milne Edwards'. *Journal of Fish Diseases* **32**, 359–365.

Jensen, P. C., K. Califf, V. Lowe, L. Hauser and J. F. Morado (2010). 'Molecular detection of *Hematodinium* sp in Northeast Pacific *Chionoecetes* spp. and evidence of two species in the Northern Hemisphere'. *Diseases of Aquatic Organisms* **89**, 155–166.

Ji, P.-F., C-L Yao and Z-Y Wang (2011). 'Reactive oxygen system plays an important role in shrimp *Litopenaeus vannamei* defense against *Vibrio parahaemolyticus* and WSSV infection'. *Diseases of Aquatic Organisms* **96**, 9–20.

Jiravanichpaisal, P., B. L. Lee and K. Soderhall (2006*a*). 'Cell-mediated immunity in arthropods: Hematopoiesis, coagulation, melanization and opsonization'. *Immunobiology* **211**, 213–236.

Jiravanichpaisal, P., K. Soderhall and I. Soderhall (2006*b*). 'Characterization of white spot syndrome virus replication in *in vitro*-cultured haematopoietic stem cells of freshwater crayfish, *Pacifastacus leniusculus*'. *Journal of General Virology* **87**, 847–854.

Jiravanichpaisal, P., S. Y. Lee, Y. A. Kim, T. Andren and I. Soderhall (2007). 'Antibacterial peptides in hemocytes and hematopoietic tissue from freshwater crayfish *Pacifastacus leniusculus*: characterization and expression pattern'. *Developmental and Comparative Immunology* **31**, 441–455.

Jitvaropas, R., A. Tassanakajon, P. Amparyup and P. S. Gross (2009). 'Functional characterization of a masquerade-like serine proteinase homologue from the black tiger shrimp *Penaeus monodon*'. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology* **153**, 236–243.

Johansson, M. W. and K. Soderhall (1985). 'Exocytosis of the prophenoloxidase activating system from crayfish hemocytes'. *Journal of Comparative Physiology B-Biochemical Systemic and Environmental Physiology* **156**, 175–181.

Johansson, M. W. and K. Soderhall (1989). 'Cellular-immunity in crustaceans and the propo system'. *Parasitology Today* **5**, 171–176.

Johansson, M. W. and K. Soderhall (1995). Separation of crustacean blood cells by continuous density gradient centrifugation. In J. S. Stolen, T. C. Fletcher, S. A. Smith, J. T. Zelikoff, S. L. Kaattari, R. S. Anderson, K. Soderhall and B. A. Weeks-Perkins (Eds.). 'Techniques in Fish Immunology'. SOS Publications. New Haven, NJ. pp. 141–145.

Johansson, M. W., P. Keyser, K. Sritunyalucksana and K. Soderhall (2000). 'Crustacean haemocytes and haematopoiesis'. *Aquaculture* **191**, 45–52.

Johansson, M. W., T. Holmblad, P. O. Thornqvist, M. Cammarata, N. Parrinello and K. Soderhall (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.

Johnson, P. T. (1980). *Histology of the Blue crab,* Callinectes sapidus*: a model for the Decapoda*. Praeger Publishers. New York.

Khanobdee, K., C. Soowannayan, T. W. Flegel, S. Ubol and B. Withyachumnarnkul (2002). 'Evidence for apoptosis correlated with mortality in the giant black tiger shrimp *Penaeus monodon* infected with yellow head virus'. *Diseases of Aquatic Organisms* **48**, 79–90.

Kharlamenko, V. I., T. V. Ryazanova, M. G. Eliseikina and A. D. Kukhlevsky (2010). '*Hematodinium* sp. infection of red *Paralithodes camtschaticus* and blue *Paralithodes platypus* king crabs from the Sea of Okhotsk, Russia'. *Journal of Invertebrate Pathology* **105**, 329–334.

Kim, Y. J., C. I. Kwak, Y. Y. Gu, I. T. Hwang and J. Y. Chun (2004). 'Annealing control primer system for identification of differentially expressed genes on agarose gels'. *Biotechniques* **36**, 424–+.

Ko, C. F., T. T. Chiou, B. Vaseeharan, J. K. Lu and J. C. Chen (2007). 'Cloning and characterisation of a prophenoloxidase from the haemocytes of mud crab *Scylla serrata*'. *Developmental and Comparative Immunology* **31**, 12–22.

Kobayashi, M., M. W. Johansson and K. Soderhall (1990). 'The 76 kd cell adhesion factor from crayfish hemocytes promotes encapsulation *in vitro*'. *Cell and Tissue Research* **260**, 13–18.

Krusong, K., P. Poolpipat, P. Supungul and A. Tassanakajon (2012). 'A comparative study of antimicrobial properties of crustinPm1 and crustinPm7 from the black tiger shrimp *Penaeus monodon*'. *Developmental and Comparative Immunology* **36**, 208–215.

Kuballa, A. V., T. A. Holton, B. Paterson and A. Elizur (2011). 'Moult cycle specific differential gene expression profiling of the crab *Portunus pelagicus*'. *Bmc Genomics*.

Kubota, H., G. Hynes and K. Willison (1995). 'The chaperonin containing T-complex polypeptide-1 (Tcp-1) - multisubunit machinery assisting in protein-folding and assembly in the eukaryotic cytosol'. *European Journal of Biochemistry* **230**, 3–16.

Kwon, T. H., M. S. Kim, H. W. Choi, C. H. Joo, M. Y. Cho and B. L. Lee (2000). 'A masquerade-like serine proteinase homologue is necessary for phenoloxidase activity

in the coleopteran insect, *Holotrichia diomphalia* larvae'. *European Journal of Biochemistry* **267**, 6188–6196.

La Peyre, J. F., F. L. E. Chu and W. K. Vogelbein (1995). '*In vitro* interaction of *Perkinsus marinus* merozoites with Eastern and Pacific oyster hemocytes'. *Developmental and Comparative Immunology* **19**, 291–304.

Labbe, P. and T. J. Little (2009). 'ProPhenolOxidase in *Daphnia magna*: cDNA sequencing and expression in relation to resistance to pathogens'. *Developmental and Comparative Immunology* **33**, 674–680.

Lai, C. Y., W. Cheng and C. M. Kuo (2005). 'Molecular cloning and characterisation of prophenoloxidase from haemocytes of the white shrimp, *Litopenaeus vannamei*'. *Fish & Shellfish Immunology* **18**, 417–430.

Lang, G. H., N. Nomura and M. Matsumura (2002). 'Growth by cell division in shrimp (*Penaeus japonicus*) cell culture'. *Aquaculture* **213**, 73–83.

Latrouite, D., Y. Morizur, P. Noel, D. Chagot and G. Wilhelm (1988). 'Mortalite du tourteau *Cancer pagurus* progoquee par le dinoflagelle parasite: *Hematodinium* sp.'.

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

Le Moullac, G., C. Soyez, D. Saulnier, D. Ansquer, J. C. Avarre and P. Levy (1998). 'Effect of hypoxic stress on the immune response and the resistance to vibriosis of the shrimp *Penaeus stylirostris*'. *Fish & Shellfish Immunology* **8**, 621–629.

Lebestky, T., T. Chang, V. Hartenstein and U. Banerjee (2000). 'Specification of *Drosophila* hematopoietic lineage by conserved transcription factors'. *Science* **288**, 146–149.

Ledakis, P., H. Tanimura and T. Fojo (1998). 'Limitations of differential display'. *Biochemical and Biophysical Research Communications* **251**, 653–656.

Lee, K. Y., R. Zhang, M. S. Kim, J. W. Park, H. Y. Park, S. Kawabata and B. L. Lee (2002). 'A zymogen form of masquerade-like serine proteinase homologue is cleaved during pro-phenoloxidase activation by Ca2+ in coleopteran and *Tenebrio molitor* larvae'. *European Journal of Biochemistry* **269**, 4375–4383.

Lee, S. Y. and K. Soderhall (2001). 'Characterization of a pattern recognition protein, a masquerade-like protein, in the freshwater crayfish *Pacifastacus leniusculus*'. *Journal of Immunology* **166**, 7319–7326.

Lee, S. Y. and K. Soderhall (2002). 'Early events in crustacean innate immunity'. *Fish & Shellfish Immunology* **12**, 421–437.

Lee, S. Y., B. L. Lee and K. Soderhall (2003). 'Processing of an antibacterial peptide from hemocyanin of the freshwater crayfish *Pacifastacus leniusculus*'. *Journal of Biological Chemistry* **278**, 7927–7933.

Lee, S. Y., M. Y. Cho, J. H. Hyun, K. M. Lee, K. I. Homma, S. Natori, S. I. Kawabata, S. Iwanaga and B. L. Lee (1998*a*). 'Molecular cloning of cDNA for pro-phenol-oxidase-activating factor I, a serine protease is induced by lipopolysaccharide or 1,3-beta-glucan in coleopteran insect, *Holotrichia diomphalia* larvae'. *European Journal of Biochemistry* **257**, 615–621.

Lee, S. Y., R. G. Wang and K. Soderhall (2000). 'A lipopolysaccharide- and beta-1,3-glucan-binding protein from hemocytes of the freshwater crayfish *Pacifastacus leniusculus* - Purification, characterization, and cDNA cloning'. *Journal of Biological Chemistry* **275**, 1337–1343.

Lee, S. Y., T. H. Kwon, J. H. Hyun, J. S. Choi, S. Kawabata, S. Iwanaga and B. L. Lee (1998*b*). '*In vitro* activation of prophenoloxidase by two kinds of prophenoloxidase-activating factors isolated from hemolymph of coleopteran, *Holotrichia diomphalia* larvae'. *European Journal of Biochemistry* **254**, 50–57.

Lee, Y., K. Chae, J. Ha, J. S. Lee, I. C. Jang, S. Jeong, M. Kim and M. Yoon (2009). 'A novel stress-related gene in developing pepper anthers'. *Russian Journal of Plant Physiology* **56**, 654–662.

Lemaitre, B., E. Nicolas, L. Michaut, J. M. Reichhart and J. A. Hoffmann (1996). 'The dorsoventral regulatory gene cassette spätzle/Toll/cactus controls the potent antifungal response in *Drosophila* adults'. *Cell* **86**, 973–983.

Letunic, I., T. Doerks and P. Bork (2009). 'SMART 6: recent updates and new developments'. *Nucleic Acids Research* **37**, D229–D232.

Leu, J. H., S. H. Chen, Y. B. Wang, Y. C. Chen, S. Y. Su, C. Y. Lin, J. M. Ho and C. F. Lo (2011). 'A Review of the Major Penaeid Shrimp EST Studies and the Construction of a Shrimp Transcriptome Database Based on the ESTs from Four Penaeid Shrimp'. *Marine Biotechnology* **13**, 608–621.

Levesque-Sergerie, J. P., N. Bissonnette, M. Duquette, C. Thibault and L. Delbecchi (2007). 'Detection limits of several commercial reverse transcriptase enzymes: impact on the low- and high-abundance transcript levels assessed by quantitative RT-PCR'. *Bmc Molecular Biology* **8**, 93.

Li, C. C., S. T. Yeh and J. C. Chen (2008*a*). 'The immune response of white shrimp Litopenaeus vannamei following Vibrio alginolyticus injection'. *Fish & Shellfish Immunology* **25**, 853–860.

Li, C. H., J. D. Shields, T. L. Miller, H. J. Small, K. Pagenkopp and K. S. Reece (2010). 'Detection and quantification of the free-living stage of the parasitic dinoflagellate *Hematodinium* sp. in laboratory and environmental samples'. *Harmful Algae* **9**, 515–521.

Li, C. W. and J. D. Shields (2007). 'Primary culture of hemocytes from the Caribbean spiny lobster, *Panulirus argus*, and their susceptibility to *Panulirus argus* Virus 1 (PaV1)'. *Journal of Invertebrate Pathology* **94**, 48–55.

Li, C. W., K. N. Wheeler and J. D. Shields (2011). 'Lack of transmission of *Hematodinium* sp in the blue crab *Callinectes sapidus* through cannibalism'. *Diseases of Aquatic Organisms* **96**, 249–258.

Li, Y. Y., X. A. Xia, Q. Y. Wu, W. H. Liu and Y. S. Lin (2008*b*). 'Infection with *Hematodinium* sp in mud crabs *Scylla serrata* cultured in low salinity water in southern China'. *Diseases of Aquatic Organisms* **82**, 145–150.

Liang, P. and T. H. MacRae (1997). 'Molecular chaperones and the cytoskeleton'. *Journal of Cell Science* **110**, 1431–1440.

Lightner, D. V. (2011). 'Virus diseases of farmed shrimp in the Western Hemisphere (the Americas): A review'. *Journal of Invertebrate Pathology* **106**, 110–130.

Lightner, D. V., L. B. Colvin, C. Brand and D. A. Danald (1977). 'Black death, a disease syndrome of penaeid shrimp related to a dietary deficiency of ascorbic acid'. *Proceedings of the annual meeting - World Mariculture Society* **8**, 611–524.

Lin, X. H., M. Novotny, K. Soderhall and I. Soderhall (2010). 'Ancient Cytokines, the Role of Astakines as Hematopoietic Growth Factors'. *Journal of Biological Chemistry* **285**, 28577–28586.

Lin, X., Kenneth Soderhall and Irene Soderhall (2008*a*). 'Transglutaminase activity in the hematopoietic tissue of a crustacean, *Pacifastacus leniusculus*, importance in hemocyte homeostasis'. *BMC Immunology* **9**, Article No.: 58.

Lin, Y. C., B. Vaseeharan and J. C. Chen (2008*b*). 'Identification of the extracellular copper-zinc superoxide dismutase (ecCuZnSOD) gene of the mud crab *Scylla serrata* and its expression following beta-glucan and peptidoglycan injections'. *Molecular Immunology* **45**, 1346–1355.

Linder, P., P. F. Lasko, M. Ashburner, P. Leroy, P. J. Nielsen, K. Nishi, J. Schnier and P. P. Slonimski (1989). 'Birth of the D-E-a-D Box'. *Nature* **337**, 121–122.

Liu, C. H., D. Y. Tseng, C. Y. Lai, W. T. Cheng and C. M. Kuo (2006). 'Molecular cloning and characterisation of prophenoloxidase cDNA from haemocytes of the giant freshwater prawn, *Macrobrachium rosenbergii*, and its transcription in relation with the moult stage'. *Fish & Shellfish Immunology* **21**, 60–69.

Liu, C. H., W. Cheng and J. C. Chen (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 & Shellfish Immunology* **18**, 431–444.

Liu, H., P. Jiravanichpaisal, L. Cerenius, B. L. Lee, I. Sorderhall and K. Soderhall (2007*a*). 'Phenoloxidase is an important component of the defense against *Aeromonas hydrophila* infection in a crustacean, *Pacifastacus leniusculus*'. *Journal of Biological Chemistry* **282**, –.

Liu, H. P., K. Soderhall and P. Jiravanichpaisal (2009). 'Antiviral immunity in crustaceans'. *Fish & Shellfish Immunology* **27**, 79–88.

Liu, H.-p., Rong-yuan Chen, Min Zhang and Ke-jian Wang (2010). 'Isolation, gene cloning and expression profile of a pathogen recognition protein: A serine proteinase homolog (Sp-SPH) involved in the antibacterial response in the crab *Scylla paramamosain*'. *Developmental and Comparative Immunology* **34**, 741–748.

Liu, W., D. Qian and X. J. Yan (2011). 'Proteomic analysis of differentially expressed proteins in hemolymph of *Scylla serrata* response to white spot syndrome virus infection'. *Aquaculture* **314**, 53–57.

Liu, Y. C., F. H. Li, B. Wang, B. Dong, Q. L. Zhang, W. Luan, X. J. Zhang and J. H. Xiang (2007*b*). 'A transglutaminase from Chinese shrimp (*Fenneropenaeus chinensis*), full-length cDNA cloning, tissue localization and expression profile after challenge'. *Fish & Shellfish Immunology* **22**, 576–588.

Loker, E. S. (1994). 'On being a parasite in an invertebrate host - a short survival course'. *Journal of Parasitology* **80**, 728–747.

Longshaw, M. (2011). 'Diseases of crayfish: A review'. *Journal of Invertebrate Pathology* **106**, 54–70.

Lorenzon, S., S. de Guarrini, V. J. Smith and E. A. Ferrero (1999). 'Effects of LPS injection on circulating haemocytes in crustaceans in vivo'. *Fish & Shellfish Immunology* **9**, 31–50.

Love, D. C., R. Thomas and D. A. Moles (1996). Bitter Crab hemolymph studies: indications of host physiological condition. In 'Proceedings of the International Symposium on Biology, Management, and Economics of Crabs from High Latitude Habitats'. University of Alaska. Fairbanks. pp. 549–562.

Love, D. C., S. D. Rice, D. A. Moles and W. D. Eaton (1993). 'Seasonal prevalence and intensity of Bitter Crab dinoflagellate infection and host mortality in Alaskan Tanner crabs *Chionoecetes bairdi* from Auke Bay, Alaska, USA'. *Diseases of Aquatic Organisms* **15**, 1–7.

Lu, K. Y., Y. T. Huang, H. H. Lee and H. H. Sung (2006). 'Cloning the prophenoloxidase cDNA and monitoring the expression of proPO mRNA in prawns (*Macrobrachium rosenbergii*) stimulated *in vivo* by CpG oligodeoxynucleotides'. *Fish & Shellfish Immunology* **20**, 274–284.

Luana, W., F. H. Li, B. Wang, X. J. Zhang, Y. C. Liu and J. H. Xiang (2007). 'Molecular characteristics and expression analysis of calreticulin in Chinese shrimp *Fenneropenaeus chinensis*'. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology* **147**, 482–491.

Luna, L. G. (1969). *Manual of Histological Staining Methods of the Armed Forces Institute of Pathology*. 3 edn. The Blakiston Division. New York.

Lynn, D. E. (1999). 'Development of insect cell lines: Virus susceptibility and applicability to prawn cell culture.'. *Methods in Cell Science* **21**, 173–181.

Lynn, D. E. (2001). 'Novel techniques to establish new insect cell lines'. *In vitro Cellular & Developmental Biology-Animal* **37**, 319–321.

Maclean, S. A. and C. L. Ruddell (1978). '3 new crustacean hosts for parasitic dinoflagellate *Hematodinium perezi* (Dinoflagellata Syndinidae)'. *Journal of Parasitology* **64**, 158–160.

Marchand, J., V. Leignel, B. Moreau and B. Chenais (2009). 'Characterization and sequence analysis of manganese superoxide dismutases from Brachyura (Crustacea: Decapoda): Hydrothermal Bythograeidae versus littoral crabs'. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology* **153**, 191–199.

Marion, R. M., A. Regev, E. Segal, Y. Barash, D. Koller, N. Friedman and E. K. O'shea (2004). 'Sfp1 is a stress- and nutrient-sensitive regulator of ribosomal protein gene expression'. *Proceedings of the National Academy of Sciences of the United States of America* **101**, 14315–14322.

Marques, M. R. F. and M. A. Barracco (2000). 'Lectins, as non-self-recognition factors, in crustaceans'. *Aquaculture* **191**, 23–44.

Martin, G. G. and J. E. Hose (1992). Vascular elements and blood (hemolymph). In F. W. Harrison and A. G. Humes (Eds.). 'Microscopic Anatomy of Invertebrates: Decapod Crustacea'. Vol. 10. Wiley-Liss, Inc. New York. pp. 117–146.

Martin, G. G., J. E. Hose and J. J. Kim (1987). 'Structure of hematopoietic nodules in the ridgeback prawn, *Sicyonia ingentis* - light and electron-microscopic observations'. *Journal of Morphology* **192**, 193–204.

Martin, G. G., J. E. Hose, M. Choi, R. Provost, G. Omori, N. Mckrell and G. Lam (1993). 'Organization of hematopoietic-tissue in the intermolt lobster, *Homarus americanus*'. *Journal of Morphology* **216**, 65–78.

Martin, J. W. and George E. Davis (2001). An updated classification of the recent crustacea. Technical report. Natural History Museum of Los Angeles County.

Matozzo, V. and M. G. Marin (2010). 'The role of haemocytes from the crab *Carcinus aestuarii* (Crustacea, Decapoda) in immune responses: A first survey'. *Fish & Shellfish Immunology* **28**, 534–541.

Matozzo, V., C. Gallo and M. G. Marin (2011*a*). 'Can starvation influence cellular and biochemical parameters in the crab *Carcinus aestuarii*?'. *Marine Environmental Research* **71**, 207–212.

Matozzo, V., C. Gallo, M. Monari and M. G. Marin (2011*b*). 'Cellular and biochemical parameters in the crab *Carcinus aestuarii* after experimentally-induced stress: Effects of bacterial injection, leg ablation and bacterial injection/leg ablation combination'. *Journal of Experimental Marine Biology and Ecology* **398**, 18–25.

Matozzo, V., L. Ballarin and M. G. Marin (2002). '*In vitro* effects of tributyltin on functional responses of haemocytes in the clam *Tapes philippinarum*'. *Applied Organometallic Chemistry*.

McPherson, M. J. and S. G. Moller (2000). *PCR: the basics*. The Basics. BIOS Scientific Publishers. Abingdon.

Medzhitov, R. (2007). 'Recognition of microorganisms and activation of the immune response'. *Nature* **449**, 819–826.

Mekata, T., T. Kono, T. Yoshida, M. Sakai and T. Itami (2008). 'Identification of cDNA encoding Toll receptor, MjToll gene from kuruma shrimp, *Marsupenaeus japonicus*'. *Fish & Shellfish Immunology* **24**, 122–133.

Menze, M. A., G. Fortner, S. Nag and S. C. Hand (2010). 'Mechanisms of apoptosis in Crustacea: what conditions induce versus suppress cell death?'. *Apoptosis* **15**, 293–312.

Messick, G. A. (1994). '*Hematodinium perezi* infections in adult and juvenile blue crabs *Callinectes sapidus* from coastal bays of Maryland and Virginia, USA'. *Diseases of Aquatic Organisms* **19**, 77–82.

Messick, G. A. and J. D. Shields (2000). 'Epizootiology of the parasitic dinoflagellate *Hematodinium* sp in the American blue crab *Callinectes sapidus*'. *Diseases of Aquatic Organisms* **43**, 139–152.

Meyers, T. R., C. Botelho, T. M. Koeneman, S. Short and K. Imamura (1990). 'Distribution of Bitter Crab Dinoflagellate syndrome in southeast Alaskan Tanner crabs *Chionoecetes bairdi*'. *Diseases of Aquatic Organisms* **9**, 37–43.

Meyers, T. R., T. M. Koeneman, C. Botelho and S. Short (1987). 'Bitter Crab Disease - a fatal dinoflagellate infection and marketing problem for Alaskan Tanner crabs *Chionoecetes bairdi*'. *Diseases of Aquatic Organisms* **3**, 195–216.

Mitsuhashi, J. and A. Shozawa (1985). 'Continuous cell-lines from larval hemocytes of the cabbage armyworm, *Mamestra brassicae*'. *Development Growth & Differentiation* **27**, 599–605.

MMO, M. M. O. (2011). UK Sea Fisheries Statistics 2010. Technical report. Marine Management Organisation.

Mohapatra, N. P., S. Soni, M. V. S. Rajaram, P. M. C. Dang, T. J. Reilly, J. El-Benna, C. D. Clay, L. S. Schlesinger and J. S. Gunn (2010). '*Francisella* acid phosphatases Inactivate the NADPH oxidase in human phagocytes'. *Journal of Immunology* **184**, 5141–5150.

Morga, B., I. Arzul, B. Chollet and T. Renault (2009). 'Infection with the protozoan parasite *Bonamia ostreae* modifies in vitro haemocyte activities of flat oyster *Ostrea edulis*'. *Fish & Shellfish Immunology* **26**, 836–842.

Mori, R., Q. C. Wang, K. D. Danenberg, J. K. Pinski and P. V. Danenberg (2008). 'Both beta-actin and GAPDH are useful reference genes for normalization of quantitative RT-PCR in human FFPE tissue samples of prostate cancer'. *Prostate* **68**, 1555–1560.

Morimoto, R. I. and M. G. Santoro (1998). 'Stress-inducible responses and heat shock proteins: New pharmacologic targets for cytoprotection'. *Nature Biotechnology* **16**, 833–838.

Mortensen, S. H. and J. Glette (1996). 'Phagocytic activity of scallop (*Pecten maximus*) haemocytes maintained *in vitro*'. *Fish & Shellfish Immunology* **6**, 111–121.

Mourton, C., V. Boulo, D. Chagot, D. Hervio, E. Bachere, E. Mialhe and H. Grizel (1992). 'Interactions between *Bonamia ostreae* (Protozoa, Ascetospora) and hemocytes of *Ostrea edulis* and *Crassostrea gigas* (Mollusca, Bivalvia) - *in vitro* system establishment'. *Journal of Invertebrate Pathology* **59**, 235–240.

Mu, C., Peilin Zheng, Jianmin Zhao, Lingling Wang, H. Zhang, Limei Qiu, Y. C. Gai and L. S. Song (2010). 'Molecular characterization and expression of a crustin-like gene from Chinese mitten crab, *Eriocheir sinensis*'. *Developmental and Comparative Immunology* **34**, 734–740.

Muñoz, M., F. Vandenbulcke, D. Saulnier and E. Bachere (2002). 'Expression and distribution of penaeidin antimicrobial peptides are regulated by haemocyte reactions in microbial challenged shrimp'. *European Journal of Biochemistry* **269**, 2678–2689.

Muñoz, M., R. Cedeno, J. Rodriguez, W. P. W. van der Knaap, E. Mialhe and E. Bachere (2000). 'Measurement of reactive oxygen intermediate production in haemocytes of the penaeid shrimp, *Penaeus vannamei*'. *Aquaculture* **191**, 89–107.

Mucklow, P. T. and D. Ebert (2003). 'Physiology of immunity in the water flea *Daphnia magna*: Environmental and genetic aspects of phenoloxidase activity'. *Physiological and Biochemical Zoology* **76**, 836–842.

Muller, G., G. Dimopoulos, C. Blass and F. C. Kafatos (1999). 'A hemocyte-like cell line established from the malaria vector *Anopheles gambiae* expresses six prophenoloxidase genes'. *Journal of Biological Chemistry* **274**, 11727–11735.

Murali, S., P. Mullainadhan and M. Arumugam (1994). 'A lipopolysaccharide-binding hemagglutinin with specificity for acetylated aminosugars in the serum of the hermit crab *Diogenes affinis* (Henderson)'. *Journal of Invertebrate Pathology* **64**, 221–227.

Murali, S., P. Mullainadhan and M. Arumugam (1999). 'Purification and characterization of a natural agglutinin from the serum of the hermit crab *Diogenes affinis*'. *Biochimica Et Biophysica Acta-General Subjects* **1472**, 13–24.

Nagle, L., A. R. Place, E. J. Schott, R. Jagus, G. Messick and J. S. Pitula (2009). 'Real-time PCR-based assay for quantitative detection of *Hematodinium* sp in the blue crab *Callinectes sapidus*'. *Diseases of Aquatic Organisms* **84**, 79–87.

Nakkrasae, L. I. and P. Damrongphol (2007). 'A vasa-like gene in the giant freshwater prawn, *Macrobrachium rosenbergii*'. *Molecular Reproduction and Development* **74**, 835–842.

Nappi, A. J. and E. Ottaviani (2000). 'Cytotoxicity and cytotoxic molecules in invertebrates'. *Bioessays* **22**, 469–480.

Newman, M. W. and C. A. Johnson (1975). 'Disease of blue crabs (*Callinectes sapidus*) caused by a parasitic dinoflagellate, *Hematodinium* sp.'. *Journal of Parasitology* **61**, 554–557.

Niedbala, W., B. Cai and F. Y. Liew (2006). 'Role of nitric oxide in the regulation of T cell functions'. *Annals of the Rheumatic Diseases* **65**, 37–40.

Nolan, T. (2004). Getting started - the basics of setting up a qPCR assay. In S. Bustin (Ed.). 'A-Z of Quantitative PCR'. International University Line Press. La Jolla. pp. 529–543.

Nolan, T., R. E. Hands and S. A. Bustin (2006). 'Quantification of mRNA using real-time RT-PCR'. *Nature Protocols* **1**, 1559–1582.

Ofek, I. and N. Sharon (1988). 'Lectin phagocytosis: a molecular mechanism of recognition between cell surface sugars and lectins in the phagocytosis of bacteria'. *Infection and Immunity* **56**, 539–547.

Okumura, T. (2007). 'Effects of lipopolysaccharide on gene expression of antimicrobial peptides (penaeidins and crustin), serine proteinase and prophenoloxidase in haemocytes of the Pacific white shrimp, *Litopenaeus vannamei*'. *Fish & Shellfish Immunology* **22**, 68–76.

Oliveira, G. T., I. C. Rossi and R. S. M. da Silva (2001). 'Carbohydrate metabolism during anoxia and post-anoxia recovery in *Chasmagnathus granulata* crabs maintained on high-protein or carbohydrate-rich diets'. *Marine Biology* **139**, 335–342.

Oliver, L. M. and W. S. Fisher (1999). 'Appraisal of prospective bivalve immunomarkers'. *Biomarkers* **4**, 510–530.

Ordas, M. C., B. Novoa and A. Figueras (1999). 'Phagocytosis inhibition of clam and mussel haemocytes by *Perkinsus atlanticus* secretion products.'. *Fish & Shellfish Immunology* **9**, 491–503.

Ozhan-Kizil, G., J. Havemann and M. Gerberding (2009). 'Germ cells in the crustacean *Parhyale hawaiensis* depend on Vasa protein for their maintenance but not for their formation'. *Developmental Biology* **327**, 230–239.

Padhi, A. and B. Verghese (2008). 'Detecting molecular adaptation at individual codons in the pattern recognition protein, lipopolysaccharicle- and

beta-1,3-glucan-binding protein of decapods'. *Fish & Shellfish Immunology* **24**, 638–648.

Pagenkopp, K. M., K. S. Reece, T. L. Miller, K. N. Wheeler, H. J. Small and J. D. Shields (2011). 'The role of alternate hosts in the ecology and life history of *Hematodinium* sp., a parasitic dinoflagellate of the blue crab (*Callinectes Sapidus*)'. *Journal of Shellfish Research* **30**, 541–541.

Pais, R., M. Shekar and I. Karunasagar (2007). 'Hemagglutinating activity and electrophoretic pattern of hemolymph serum proteins of *Penaeus monodon* and *Macrobrachium rosenbergii* to white spot syndrome virus injections'. *Aquaculture* **270**, 529–534.

Pascual, C., A. Sanchez, E. Zenteno, G. Cuzon, G. Gabriela, R. Brito, R. Gelabert, E. Hidalgo and C. Rosas (2006). 'Biochemical, physiological, and immunological changes during starvation in juveniles of *Litopenaeus vannamei*'. *Aquaculture* **251**, 416–429.

Perazzolo, L. M. and M. A. Barracco (1997). 'The prophenoloxidase activating system of the shrimp *Penaeus paulensis* and associated factors'. *Developmental and Comparative Immunology* **21**, 385–395.

Perdomo-Morales, R., V. Montero-Alejo, E. Perera, Z. Pardo-Ruiz and E. Alonso-Jimenez (2008). 'Hemocyanin-derived phenoloxidase activity in the spiny lobster *Panulirus argus* (Latreille, 1804)'. *Biochimica Et Biophysica Acta-General Subjects* **1780**, 652–658.

Persson, M., L. Cerenius and K. Soderhall (1987). 'The influence of hemocyte number on the reistance of the freshwater crayfish, *Pacifastacus leniusculus* Dana, to the parasitic fungus *Aphanomyces astaci*'. *Journal of Fish Diseases* **10**, 471–477.

Pestal, G. P., D. M. Taylor, J. M. Hoenig, J. D. Shields and R. Pickavance (2003). 'Monitoring the prevalence of the parasitic dinoflagellate *Hematodinium* sp in snow crabs *Chionoecetes opilio* from Conception Bay, Newfoundland'. *Diseases of Aquatic Organisms* **53**, 67–75.

Pfaffl, M. W. (2010). 'The ongoing evolution of qPCR Introduction'. *Methods* **50**, 215–216.

Pfaffl, M. W., G. W. Horgan and L. Dempfle (2002). 'Relative expression software tool (REST (c)) for group-wise comparison and statistical analysis of relative expression results in real-time PCR'. *Nucleic Acids Research* **30**, e36.

Philip, R., S. P. Antony, I. S. B. Singh, N. S. Sudheer, S. Vrinda and P. Priyaja (2011). '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.

Pipe, R. K., J. A. Coles and S. R. Farley (1995). Assays for measuring immune response in the mussel *Mytilus edulis*. In J. S. Stolen and T. C. Fletcher (Eds.). 'Techniques in Fish Immunology'. Vol. 4. p. 303.

Py, B., C. F. Higgins, H. M. Krisch and A. J. Carpousis (1996). 'A DEAD-box RNA helicase in the *Escherichia coli* RNA degradosome'. *Nature* **381**, 169–172.

Qin, C. J., L. Q. Chen, J. G. Qin, D. X. Zhao, H. Zhang, P. Wu and E. C. Li (2010). 'Characterization of a serine proteinase homologous (SPH) in Chinese mitten crab *Eriocheir sinensis*'. *Developmental and Comparative Immunology* **34**, 14–18.

Raff, M. (1998). 'Cell suicide for beginners'. *Nature* **396**, 119–122.

Rajeevan, M. S., D. G. Ranamukhaarachchi, S. D. Vernon and E. R. Unger (2001). 'Use of real-time quantitative PCR to validate the results of cDNA array and differential display PCR technologies'. *Methods* **25**, 443–451.

Raman, T., M. Arumugam and P. Mullainadhan (2008). 'Agglutinin-mediated phagocytosis-associated generation of superoxide anion and nitric oxide by the hemocytes of the giant freshwater prawn *Macrobrachium rosenbergii*'. *Fish & Shellfish Immunology* **24**, 337–345.

Ranford, J. C. and B. Henderson (2002). 'Chaperonins in disease: mechanisms, models, and treatments'. *Journal of Clinical Pathology-Molecular Pathology* **55**, 209–213.

Ranganathan, S., K. J. Simpson, D. C. Shaw and K. R. Nicholas (1999). 'The whey acidic protein family: A new signature motif and three-dimensional structure by comparative modeling'. *Journal of Molecular Graphics & Modelling* **17**, 106–+.

Ratcliffe, N. A. and A. F. Rowley (1979). 'A comparative synopsis of the structure and function of the blood cells of insects and other invertebrates'. *Developmental and Comparative Immunology* **3**, 189–243.

Ratcliffe, N. A., Keith N. White, A. F. Rowley and Julia B. Walters (1982). Cellular defense systems of the Arthropoda. In N. Cohen and M. M. Sigel (Eds.). 'The Reticuloendothelial System: a Comprehensive Treatise (Phylogeny and Ontogeny)'. Vol. 3. Plenum Press. New York. pp. 167–256.

Reece, K. S., C. W. Li, J. D. Shields, T. L. Miller, H. J. Small and K. M. Pagenkopp (2010). 'Detection and quantification of the free-living stage of the parasitic dinoflagellate *Hematodinium* sp in laboratory and environmental samples'. *Harmful Algae* **9**, 515–521.

Reeson, A. F., K. Wilson, A. Gunn, R. S. Hails and D. Goulson (1998). 'Baculovirus resistance in the noctuid *Spodoptera exempta* is phenotypically plastic and responds to population density'. *Proceedings of the Royal Society of London Series B-Biological Sciences* **265**, 1787–1791.

Relf, J. M., J. R. S. Chisholm, G. D. Kemp and V. J. Smith (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.

Remaley, A. T., D. B. Kuhns, R. E. Basford, R. H. Glew and S. S. Kaplan (1984). 'Leishmanial phosphatase blocks neutrophil O2 production'. *Journal of Biological Chemistry* **259**, 1173–1175.

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

Rigaud, T. and Y. Moret (2003). 'Differential phenoloxidase activity between native and invasive gammarids infected by local acanthocephalans: differential immunosuppression?'. *Parasitology* **127**, 571–577.

Rocak, S. and P. Linder (2004). 'Dead-box proteins: The driving forces behind RNA metabolism'. *Nature Reviews Molecular Cell Biology* **5**, 232–241.

Roche (2000). 'Roche Molecular Biochemicals Technical Note No. LC 11/2000'.

Roche (2001). 'Roche Applied Science Technical Note No. LC 13/2001: Relative Quantification'.

Rodriguez-Ramos, T., G. Espinosa, J. Hernandez-Lopez, T. Gollas-Galvan, J. Marrero, Y. Borrell, M. E. Alonso, U. Becquer and M. Alonso (2008). 'Effects of *Echerichia coli* lipopolysaccharides and dissolved ammonia on immune response in southern white shrimp *Litopenaeus schmitti*'. *Aquaculture* **274**, 118–125.

Rodriguez-Ramos, T., Y. Carpio, J. Bolivar, G. Espinosa, J. Hernandez-Lopez, T. Gollas-Galvan, L. Ramos, C. Pendon and M. P. Estrada (2010). 'An inducible nitric oxide synthase (NOS) is expressed in hemocytes of the spiny lobster *Panulirus*

*argus*: Cloning, characterization and expression analysis'. *Fish & Shellfish Immunology* **29**, 469–479.

Rosen, G. M., S. Pou, C. L. Ramos, M. S. Cohen and B. E. Britigan (1995). 'Free-Radicals and Phagocytic-Cells'. *Faseb Journal* **9**, 200–209.

Ross, J., H. Jiang, M. R. Kanost and Y. Wang (2003). 'Serine proteases and their homologs in the *Drosophila melanogaster* genome: an initial analysis of sequence conservation and phylogenetic relationships'. *Gene* **304**, 117–131.

Roulston, C. and V.J. Smith (2011). 'Isolation and *in vitro* characterisation of prohaemocytes from the spider crab, Hyas araneus (L.)'. *Developmental and Comparative Immunology* **35**, 537–544.

Roux, M. M., A. Pain, K. R. Klimpel and A. K. Dhar (2002). 'The lipopolysaccharide and beta-1,3-glucan binding protein gene is upregulated in white spot virus-infected shrimp (*Penaeus stylirostris*)'. *Journal of Virology* **76**, 7140–7149.

Rungrassamee, W., R. Leelatanawit, P. Jiravanichpaisal, S. Klinbunga and N. Karoonuthaisiri (2010). 'Expression and distribution of three heat shock protein genes under heat shock stress and under exposure to *Vibrio harveyi* in *Penaeus monodon*'. *Developmental and Comparative Immunology* **34**, 1082–1089.

Ryazanova, T. V. (2008). 'Bitter crab syndrome in two species of king crabs from the Sea of Okhotsk'. *Russian Journal of Marine Biology* **34**, 411–414.

Ryazanova, T. V., M. G. Eliseikina, A. D. Kukhlevsky and V. I. Kharlamenko (2010). '*Hematodinium* sp. infection of red *Paralithodes camtschaticus* and blue *Paralithodes platypus* king crabs from the Sea of Okhotsk, Russia'. *Journal of Invertebrate Pathology* **105**, 329–334.

Sagrista, E. and M. Durfort (1990). 'Ultrastructural study of hemocytes and phagocytes associated with hemolymphatic vessels in the hepatopancreas of *Palaemonetes zariquieyi* (Crustacea, Decapoda)'. *Journal of Morphology* **206**, 173–180.

Sakai, T., T. Hirae, K. Yuasa, T. Kamaishi, T. Matsuyama, S. Miwa, N. Oseko and T. Iida (2007). 'Mass mortality of cultured kuruma prawn *Penaeus japonicus* caused by *Vibrio nigripulchritudo*'. *Fish Pathology* **42**, 141–147.

Saldarriaga, J. F., F. J. R. M. Taylor, T. Cavalier-Smith, S. Menden-Deuer and P. J. Keeling (2004). 'Molecular data and the evolutionary history of dinoflagellates'. *European Journal of Protistology* **40**, 85–111.

Santiago-Vazquez, L. Z., L. K. Ranzer and R. G. Kerr (2006). 'Comparison of two total RNA extraction protocols using the marine gorgonian coral *Pseudopterogorgia elisabethae* and its symbiont *Symbiodinium* sp.'. *Electronic Journal of Biotechnology* **9**, 598–603.

Sarathi, M., V. P. I. Ahmed, C. Venkatesan, G. Balasubramanian, J. Prabavathy and A. S. S. Hameed (2007). 'Comparative study on immune response of *Fenneropenaeus indicus* to *Vibrio alginolyticus* and white spot syndrome virus'. *Aquaculture* **271**, 8–20.

Satoh, D., A. Horii, M. Ochiai and M. Ashida (1999). 'Prophenoloxidase-activating enzyme of the silkworm, *Bombyx mori* - Purification, characterization, and cDNA cloning'. *Journal of Biological Chemistry* **274**, 7441–7453.

Scharlaken, B., D. C. de Graaf, K. Goossens, M. Brunain, L. J. Peelman and F. J. Jacobs (2008). 'Reference gene selection for insect expression studies using quantitative real-time PCR: The head of the honeybee, *Apis mellifera*, after a bacterial challenge'. *Journal of Insect Science*.

Schmucker, D., J. C. Clemens, H. Shu, C. A. Worby, J. Xiao, M. Muda, J. E. Dixon and S. L. Zipursky (2000). '*Drosophila* Dscam is an axon guidance receptor exhibiting extraordinary molecular diversity'. *Cell* **101**, 671–684.

Schnapp, D., G. D. Kemp and V. J. Smith (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.

Schneider, I. (1972). 'Cell lines derived from late embryonic stages of *Drosophila melanogaster*'. *Journal of Embryology and Experimental Morphology* **27**, 353–365.

Schroder, M., M. Baran and A. G. Bowie (2008). 'Viral targeting of DEAD box protein 3 reveals its role in TBK1/IKK epsilon-mediated IRF activation'. *Embo Journal* **27**, 2147–2157.

Schupbach, T. and E. Wischaus (1986). 'Maternal-effect mutations altering the anterior-posterior pattern of the *Drosophila* embryo'. *Development Genes and Evolution* **195**, 302–317.

Scientific, T. (2008). 260/280 and 260/230 Ratios (NanoDrop 1000). Technical report. Thermo Fisher Scientific.

Sequeira, T., D. Tavares and M. AralaChaves (1996). 'Evidence for circulating hemocyte proliferation in the shrimp *Penaeus japonicus*'. *Developmental and Comparative Immunology* **20**, 97–104.

Shelby, K. S. and B. A. Webb (1997). 'Polydnavirus infection inhibits translation of specific growth-associated host proteins'. *Insect Biochemistry and Molecular Biology* **27**, 263–270.

Shelby, K. S., O. A. Adeyeye, B. M. Okot-Kotber and B. A. Webb (2000). 'Parasitism-linked block of host plasma melanization'. *Journal of Invertebrate Pathology* **75**, 218–225.

Shen, X. Y., A. M. Belcher, P. K. Hansma, G. D. Stucky and D. E. Morse (1997). 'Molecular cloning and characterization of lustrin A, a matrix protein from shell and pearl nacre of *Haliotis rufescens*'. *Journal of Biological Chemistry* **272**, 32472–32481.

Sheppard, M., A. Walker, M. E. Frischer and R. F. Lee (2003). 'Histopathology and prevalence of the parasitic dinoflagellate, *Hematodinium* sp, in crabs (*Callinectes sapidus*, *Callinectes similis*, *Neopanope sayi*, *Libinia emarginata*, *Menippe mercenaria*) from a Georgia estuary'. *Journal of Shellfish Research* **22**, 873–880.

Shields, J. D. (1994). 'The parasitic dinoflagellates of marine crustaceans'. *Annual Review of Fish Diseases* **4**, 241–271.

Shields, J. D. (1999). 'Partial culture and cryopreservation of the parasitic dinoflagellate *Hematodinium perezi* from the blue crab'. *Journal of Shellfish Research* **18**, 334.

Shields, J. D. (2003). 'Research priorities for diseases of the blue crab *Callinectes sapidus*'. *Bulletin of Marine Science* **72**, 505–517.

Shields, J. D. and C. M. Squyars (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., C. Scanlon and A. Volety (2003). 'Aspects of the pathophysiology of blue crabs, *Callinectes sapidus*, infected with the parasitic dinoflagellate *Hematodinium perezi*'. *Bulletin of Marine Science* **72**, 519–535.

Shields, J. D., D. M. Taylor, P. G. O'Keefe, E. Colbourne and E. Hynick (2007). 'Epidemiological determinants in outbreaks of bitter crab disease (*Hematodinium* sp.) in snow crabs *Chionoecetes opilio* from Conception Bay, Newfoundland, Canada'. *Diseases of Aquatic Organisms* **77**, 61–72.

Shields, J. D., D. M. Taylor, S. G. Sutton, P. G. O'Keefe, D. W. Ings and A. L. Pardy (2005). 'Epidemiology of bitter crab disease (*Hematodinium* sp.) in snow crabs *Chionoecetes opilio* from Newfoundland, Canada'. *Diseases of Aquatic Organisms* **64**, 253–264.

Shukalyuk, A. I., K. A. Golovnina, S. I. Baiborodin, K. V. Gunbin, A. G. Blinov and V. V. Isaeva (2007). 'vasa-related genes and their expression in stem cells of colonial parasitic rhizocephalan barnacle *Polyascus polygenea* (Arthropoda : Crustacea : Cirripedia : Rhizocephala)'. *Cell Biology International* **31**, 97–108.

Siemsen, D. W., L. N. Kirpotina, M. A. Jutila and M. T. Quinn (2009). 'Inhibition of the human neutrophil NADPH oxidase by *Coxiella burnetii*'. *Microbes and Infection* **11**, 671–679.

Silacci, P., L. Mazzolai, C. Gauci, N. Stergiopulos, H. L. Yin and D. Hayoz (2004). 'Gelsolin superfamily proteins: key regulators of cellular functions'. *Cellular and Molecular Life Sciences* **61**, 2614–2623.

Sindermann, C. J. (1970). *Principle Disease of Marine Fish and Shellfish*. 1 edn. Academic Press. New York.

Sindermann, C. J. and A. Rosenfield (1967). 'Principal diseases of commercially important marine bivalve mollusca and crustacea'. *Fishery Bulletin* **66**, 335–385.

Skovgaard, A., R. Massana, V. Balague and E. Saiz (2005). 'Phylogenetic position of the copepod-infesting parasite *Syndinium turbo* (Dinoflagellata, Syndinea)'. *Protist* **156**, 413–423.

Small, H. J. (2004). Infections of the Norway lobster, *Nephrops norvegicus* (L.) by dinoflagellate and ciliate parasites. PhD thesis. University of Glasgow.

Small, H. J., D. M. Neil, A. C. Taylor, R. J. A. Atkinson and G. H. Coombs (2006). 'Molecular detection of *Hematodinium* spp. in Norway lobster *Nephrops norvegicus* and other crustaceans'. *Diseases of Aquatic Organisms* **69**, 185–195.

Small, H. J., J. D. Shields, D. M. Neil, A. C. Taylor and G. H. Coombs (2007*a*). 'Differences in enzyme activities between two species of *Hematodinium*, parasitic dinoflagellates of crustaceans'. *Journal of Invertebrate Pathology* **94**, 175–183.

Small, H. J., J. D. Shields, J. A. Moss and K. S. Reece (2007*b*). 'Conservation in the first internal transcribed spacer region (ITS1) in *Hematodinium* species infecting crustacean hosts found in the UK and Newfoundland'. *Diseases of Aquatic Organisms* **75**, 251–258.

Small, H. J., J. D. Shields, K. L. Hudson and K. S. Reece (2007*c*). 'Molecular detection of *Hematodinium* sp infecting the blue crab, *Callinectes sapidus*'. *Journal of Shellfish Research* **26**, 131–139.

Small, H. J., S. Wilson, D. M. Neil, P. Hagan and G. H. Coombs (2002). 'Detection of the parasitic dinoflagellate *Hematodinium* in the Norway lobster *Nephrops norvegicus* by ELISA'. *Diseases of Aquatic Organisms* **52**, 175–177.

Smith, V. and J. R. S. Chisholm (1992). 'Non-cellular immunity in crustaceans'. *Fish & Shellfish Immunology* **2**, 1–31.

Smith, V. J. and C. M. Peddie (1992). 'Cell cooperation during host defense in the solitary tunicate *Ciona intestinalis* (L)'. *Biological Bulletin* **183**, 211–219.

Smith, V. J. and K. Soderhall (1983). 'Beta-1,3 glucan activation of crustacean hemocytes *in vitro* and *in vivo*'. *Biological Bulletin* **164**, 299–314.

Smith, V. J. and K. Soderhall (1986). 'Cellular immune mechanisms in the Crustacea'. *Symposium of the Zoological Society of London* **56**, 59–79.

Smith, V. J. and K. Soderhall (1991). 'A comparison of phenoloxidase activity in the blood of marine invertebrates'. *Developmental and Comparative Immunology* **15**, 251–261.

Smith, V. J. and N. A. Ratcliffe (1978). 'Host defence reactions of shore crab, *Carcinus maenas* (L,), *in vitro*'. *Journal of the Marine Biological Association of the United Kingdom* **58**, 367.

Smith, V. J. and N. A. Ratcliffe (1980*a*). 'Cellular defense reactions of the shore crab, *Carcinus maenas* - *in vivo* hemocytic and histopathological responses to injected bacteria'. *Journal of Invertebrate Pathology* **35**, 65–74.

Smith, V. J. and N. A. Ratcliffe (1980*b*). 'Host defense reactions of the shore crab, *Carcinus maenas* (L) - clearance and distribution of injected test particles'. *Journal of the Marine Biological Association of the United Kingdom* **60**, 89–102.

Smith, V. J., J. M. O. Fernandes, G. D. Kemp and C. Hauton (2008). 'Crustins: Enigmatic WAP domain-containing antibacterial proteins from crustaceans'. *Developmental and Comparative Immunology* **32**, 758–772.

Soderhall, I. and X. H. Lin (2011). 'Crustacean hematopoiesis and the astakine cytokines'. *Blood* **117**, 6417–6424.

Soderhall, I., E. Bangyeekhun, S. Mayo and K. Soderhall (2003). 'Hemocyte production and maturation in an invertebrate animal; proliferation and gene expression in hematopoietic stem cells of *Pacifastacus leniusculus*'. *Developmental and Comparative Immunology* **27**, 661–672.

Soderhall, I., X. H. Lin and K. Soderhall (2011). 'Invertebrate hematopoiesis: an astakine-dependent novel hematopoietic factor'. *Journal of Immunology* **186**, 2073–2079.

Soderhall, I., Y. A. Kim, P. Jiravanichpaisal, S. Y. Lee and K. Soderhall (2005). 'An ancient role for a prokineticin domain in invertebrate hematopoiesis'. *Journal of Immunology* **174**, 6153–6160.

Soderhall, K. (1981). 'Fungal cell wall beta-1,3-glucans induce clotting and phenoloxidase attachment to foreign surfaces of crayfish hemocyte lysate'. *Developmental and Comparative Immunology* **5**(4), 565–573.

Soderhall, K. (1982). 'Prophenoloxidase activating system and melanization - a recognition mechanism of arthropods - a review'. *Developmental and Comparative Immunology* **6**, 601–611.

Soderhall, K., A. Aspan and B. Duvic (1990). 'The Propo system and associated proteins - role in cellular communication in arthropods'. *Research in Immunology* **141**, 896–907.

Soderhall, K. and L. Cerenius (1992). 'Crustacean Immunity'. *Annual Review of Fish Diseases* pp. 3–23.

Soderhall, K. and L. Cerenius (1998). 'Role of the prophenoloxidase-activating system in invertebrate immunity'. *Current Opinion in Immunology* **10**, 23–28.

Soderhall, K. and V. J. Smith (1983). 'Separation of the hemocyte populations of *Carcinus maenas* and other marine decapods, and prophenoloxidase distribution'. *Developmental and Comparative Immunology* **7**, 229–239.

Soderhall, K., H. G. Hergenhahn, M. W. Johansson and A. Aspan (1986). 'The regulation of the prophenoloxidase activating system in crustaceans'. *Developmental and Comparative Immunology* **10**, 622–622.

Song, Y. L., C. I. Yu, T. W. Lien, C. C. Huang and M. N. Lin (2003). 'Haemolymph parameters of Pacific white shrimp (*Litopenaeus vannamei*) infected with Taura syndrome virus'. *Fish & Shellfish Immunology* **14**, 317–331.

Sperstad, S. V., V. J. Smith and K. Stensvag (2010). 'Expression of antimicrobial peptides from *Hyas araneus* haemocytes following bacterial challenge *in vitro*'. *Developmental and Comparative Immunology* **34**, 618–624.

Sricharoen, S., J. J. Kim, S. Tunkijjanukij and I. Soderhall (2005). 'Exocytosis and proteomic analysis of the vesicle content of granular hemocytes from a crayfish'. *Developmental and Comparative Immunology* **29**, 1017–1031.

Sriton, A., N. Kanthong, W. Gangnonngiw, S. Sriurairatana, S. Ubol and T. W. Flegel (2009). 'Persistent expression of shrimp-virus antigens in two insect cell lines challenged with two shrimp viruses'. *Fish Pathology* **44**, 86–93.

Sritunyalucksana, K. (2001). Characterisation of some immune genes in the Black Tiger Shrimp, *Penaeus monodon*. PhD thesis.

Sritunyalucksana, K. and K. Soderhall (2000). 'The proPO and clotting system in crustaceans'. *Aquaculture* **191**, 53–69.

Sritunyalucksana, K., P. Sithisarn, B. Withayachumnarnkul and T. W. Flegel (1999). 'Activation of prophenoloxidase, agglutinin and antibacterial activity in haemolymph of the black tiger prawn, *Penaeus monodon*, by immunostimulants'. *Fish & Shellfish Immunology* **9**, 21–30.

Stahlberg, A., J. Hakansson, X. J. Xian, H. Semb and M. Kubista (2004*a*). 'Properties of the reverse transcription reaction in mRNA quantification'. *Clinical Chemistry* **50**, 509–515.

Stahlberg, A., M. Kubista and M. Pfafffl (2004*b*). 'Comparison of reverse transcriptases in gene expression analysis'. *Clinical Chemistry* **50**, 1678–1680.

Stein, D., S. Roth, E. Vogelsang and C. Nussleinvolhard (1991). 'The polarity of the dorsoventral axis in the *Drosophila* embryo is defined by an extracellular signal'. *Cell* **65**, 725–735.

Stentiford, G. D. (2008). 'Diseases of the European edible crab (*Cancer pagurus*): a review'. *Ices Journal of Marine Science*.

Stentiford, G. D. and D. M. Neil (2000). 'A rapid onset, post-capture muscle necrosis in the Norway lobster, *Nephrops norvegicus* (L.), from the West coast of Scotland'. *Journal of Fish Diseases* **23**, 251–263.

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

Stentiford, G. D. and J. D. Shields (2005). 'A review of the parasitic dinoflagellates *Hematodinium* species and *Hematodinium*-like infections in marine crustaceans'. *Diseases of Aquatic Organisms* **66**, 47–70.

Stentiford, G. D. and K. S. Bateman (2007). '*Enterospora* sp., an intranuclear microsporidian infection of hermit crab *Eupagurus bernhardus*'. *Diseases of Aquatic Organisms* **75**, 73–78.

Stentiford, G. D. and S. W. Feist (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**, 136–146.

Stentiford, G. D., D. M. Neil and G. H. Coombs (1999). 'Changes in the plasma free amino acid profile of the Norway lobster *Nephrops norvegicus* at different stages of infection by a parasitic dinoflagellate (genus Hematodinium)'. *Diseases of Aquatic Organisms* **38**, 151–157.

Stentiford, G. D., D. M. Neil and G. H. Coombs (2000). 'Alterations in the biochemistry and ultrastructure of the deep abdominal flexor muscle of the Norway lobster *Nephrops norvegicus* during infection by a parasitic dinoflagellate of the genus *Hematodinium*'. *Diseases of Aquatic Organisms* **42**, 133–141.

Stentiford, G. D., D. M. Neil and G. H. Coombs (2001*a*). 'Development and application of an immunoassay diagnostic technique for studying *Hematodinium* infections in *Nephrops norvegicus* populations'. *Diseases of Aquatic Organisms* **46**, 223–229.

Stentiford, G. D., D. M. Neil and R. J. A. Atkinson (2001*b*). 'The relationship of *Hematodinium* infection prevalence in a Scottish *Nephrops norvegicus* population to season, moulting and sex'. *Ices Journal of Marine Science* **58**, 814–823.

Stentiford, G. D., E. S. Chang, S. A. Chang and D. M. Neil (2001*c*). 'Carbohydrate dynamics and the crustacean hyperglycemic hormone (CHH): Effects of parasitic infection in Norway lobsters (*Nephrops norvegicus*)'. *General and Comparative Endocrinology* **121**, 13–22.

Stentiford, G. D., K. S. Bateman, M. Longshaw and S. W. Feist (2007). '*Enterospora canceri* n. gen., n. sp., intranuclear within the hepatopancreatocytes of the European edible crab *Cancer pagurus*'. *Diseases of Aquatic Organisms* **75**, 61–72.

Stentiford, G. D., M. Evans, K. Bateman and S. W. Feist (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., M. Green, K. Bateman, H. J. Small, D. M. Neil and S. W. Feist (2002). 'Infection by a *Hematodinium*-like parasitic dinoflagellate causes Pink Crab Disease (PCD) in the edible crab *Cancer pagurus*'. *Journal of Invertebrate Pathology* **79**, 179–191.

Strand, M. R. (1994). '*Microplitis demolitor* polydnavirus infects and expresses in specific morphotypes of *Pseudoplusia includens* hemocytes'. *Journal of General Virology* **75**, 3007–3020.

Strand, M. R. and L. L. Pech (1995). 'Microplitis demolitor polydnavirus induces apoptosis of a specific hemocyte morphotype in *Pseudoplusia includens*'. *Journal of General Virology* **76**, 283–291.

Strand, M. R. and T. Noda (1991). 'Alterations in the hemocytes of *Pseudoplusia includens* after parasitism by *Microplitis demolitor*'. *Journal of Insect Physiology* **37**, 839–850.

Stricker, S., A. J. Poustka, U. Wiecha, A. Stiege, J. Hecht, G. Panopoulou, A. Vilcinskas, S. Mundlos and V. Seitz (2003). 'A single amphioxus and sea urchin runt-gene suggests that runt-gene duplications occurred in early chordate evolution'. *Developmental and Comparative Immunology* **27**, 673–684.

Sung, H. H., W. Y. Kao and Y. J. Su (2003). 'Effects and toxicity of phthalate esters to hemocytes of giant freshwater prawn, *Macrobrachium rosenbergii*'. *Aquatic Toxicology* **64**, 25–37.

Sung, H. H., Y. L. Yang and Y. L. Song (1996). 'Enhancement of microbicidal activity in the tiger shrimp *Penaeus monodon* via immunostimulation'. *Journal of Crustacean Biology* **16**, 278–284.

Supungul, P., S. Klinbunga, R. Pichyangkura, I. Hirono, T. Aoki and A. Tassanakajon (2004). 'Antimicrobial peptides discovered in the black tiger shrimp *Penaeus monodon* using the EST approach'. *Diseases of Aquatic Organisms* **61**, 123–135.

Takle, G. B. (1986). 'Chemokinetic behaviour of insect haemocytes *in vitro*'. *Journal of Cell Science* **85**, 85–94.

Tassanakajon, A., P. Amparyup, K. Somboonwiwat and P. Supungul (2010). 'Cationic Antimicrobial Peptides in Penaeid Shrimp'. *Marine Biotechnology* **12**, 487–505.

Tassanakajon, A., S. Klinbunga, N. Paunglarp, V. Rimphanitchayakit, A. Udomkit, S. Jitrapakdee, K. Sritunyalucksana, A. Phongdara, S. Pongsomboon, P. Supungul, S. Tang, K. Kuphanumart, R. Pichyangkura and C. Lursinsap (2006). '*Penaeus monodon* gene discovery project: The generation of an EST collection and establishment of a database'. *Gene* **384**, 104–112.

Taylor, A. C., R. H. Field and P. J. ParslowWilliams (1996). 'The effects of *Hematodinium* sp infection on aspects of the respiratory physiology of the Norway lobster, *Nephrops norvegicus* (L)'. *Journal of Experimental Marine Biology and Ecology* **207**, 217–228.

Taylor, D. M. and R. A. Kahn (1995). 'Observations on the occurrence 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, S., M. Wakem, G. Dijkman, M. Alsarraj and M. Nguyen (2010). 'A practical approach to RT-qPCR-Publishing data that conform to the MIQE guidelines'. *Methods* **50**, S1–S5.

Terwilliger, N. B. (2006). Hemocyanins and the immune response: defense against the dark arts. In '1st International Congress of Respiratory Biology'. Bonn, GERMANY. pp. 662–665.

Terwilliger, N. B. and M. C. Ryan (2006). 'Functional and phylogenetic analyses of phenoloxidases from brachyuran (*Cancer magister*) and branchiopod (*Artemia franciscana*, *Triops longicaudatus*) crustaceans'. *Biological Bulletin* **210**, 38–50.

Theaker, J. (2004). Genotyping using MBG-hydrolysis probes. In S. Bustin (Ed.). 'A-Z of quantitative PCR'. International University Line. La Jolla. pp. 735–765.

Thellin, O., W. Zorzi, B. Lakaye, B. De Borman, B. Coumans, G. Hennen, T. Grisar, A. Igout and E. Heinen (1999). 'Housekeeping genes as internal standards: use and limits'. *Journal of Biotechnology* **75**, 291–295.

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

Thornqvist, P. O., M. W. Johansson and K. Soderhall (1994). 'Opsonic activity of cell adhesion proteins and Beta-1,3-glucan binding-proteins from two crustaceans'. *Developmental and Comparative Immunology* **18**, 3–12.

Tsan, M. F. and B. C. Gao (2004). 'Cytokine function of heat shock proteins'. *American Journal of Physiology-Cell Physiology* **286**, C739–C744.

Tseng, S. S. L., P. L. Weaver, Y. Liu, M. Hitomi, A. M. Tartakoff and T. H. Chang (1998). 'Dbp5p, a cytosolic RNA helicase, is required for poly(A)(+) RNA export'. *Embo Journal* **17**, 2651–2662.

Vafopoulou, X. (2009). 'Mechanisms of wound repair in crayfish'. *Invertebrate Survival Journal* **6**, 125–137.

van de Braak, C. B. T., M. H. A. Botterblom, W. Liu, N. Taverne, W. P. W. van der Knaap and J. H. W. M. Rombout (2002). 'The role of the haematopoietic tissue in haemocyte production and maturation in the black tiger shrimp (*Penaeus monodon*)'. *Fish & Shellfish Immunology* **12**, 253–272.

van de Braak, C. B. T., N. Taverne, M. H. A. Botterblom, W. P. W. van der Knaap and J. H. W. M. Rombout (2000). 'Characterisation of different morphological features of black tiger shrimp (*Penaeus monodon*) haemocytes using monoclonal antibodies'. *Fish & Shellfish Immunology* **10**, 515–530.

van der Vies, S. M. and C. Georgopoulos (1996). Regulation of Chaperonin Gene Expression. In R. J. Ellis (Ed.). 'The Chaperonins'. Cell Biology: A series of Monographs. Academic Press, Inc. London. pp. 137–167.

Vargas-Albores, F. and G. Yepiz-Plascencia (2000). 'Beta glucan binding protein and its role in shrimp immune response'. *Aquaculture* **191**, 13–21.

Vargas-Albores, F., F. Jimenez-Vega and G. M. Yepiz-Plascencia (1997). 'Purification and comparison of beta-1,3-glucan binding protein from white shrimp (*Penaeus vannamei*)'. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology* **116**, 453–458.

Vargas-Albores, F., G. Yepiz-Plascencia, F. Jimenez-Vega and A. Avila-Villa (2004). 'Structural and functional differences of *Litopenaeus vannamei* crustins'. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology* **138**, 415–422.

Vargas-Albores, F., T. Gollas-Galvan and J. Hernandez-Lopez (2005). 'Functional characterization of *Farfantepenaeus californiensis*, *Litopenaeus vannamei* and *L. stylirostris* haemocyte separated using density gradient centrifugation'. *Aquaculture Research* **36**, 352–360.

Vatanavicharn, T., P. Supungul, N. Puanglarp, W. Yingvilasprasert and A. Tassanakajon (2009). 'Genomic structure, expression pattern and functional characterization of crustinPm5, a unique isoform of crustin from *Penaeus monodon*'. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology* **153**, 244–252.

Vaughn, J. L., R. H. Goodwin, G. J. Tompkins and P. McCawley (1977). 'The establishment of two cell lines from the insect *Spodoptera frugiperda* (Lepidoptera; Noctuidae)'. *In vitro Cellular & Developmental Biology-Plant* **13**, 213–217.

Vazquez, L., A. Perez, D. Millan, C. Agundis, G. Martin, E. L. Cooper, R. Lascurain and E. Zenteno (1997). 'Morphology of hemocytes from the freshwater prawn *Macrobrachium rosenbergii*'. *Journal of Morphology* **234**, 147–153.

Vazquez, L., J. Alpuche, G. Maldonado, C. Agundis, A. Pereyra-Morales and E. Zenteno (2009). 'Immunity mechanisms in crustaceans'. *Innate Immunity* **15**, 179–188.

Verghese, B., E. V. Radhakrishnan and A. Padhi (2008). 'Effect of moulting, eyestalk ablation, starvation and transportation on the immune response of the Indian spiny lobster, *Panulirus homarus*'. *Aquaculture Research* **39**, 1009–1013.

Vermeulen, J., J. Vandesompele, K. De Preter, S. Lefever, J. Nuytens, F. De Vloed, S. Derveaux, J. Hellemans and F. Speleman (2011). 'Measurable impact of RNA quality on gene expression results from quantitative PCR'. *Nucleic Acids Research* **39**, e63.

Vogan, C. L. and A. F. Rowley (2002). 'Effects of shell disease syndrome on the haemocytes and humoral defences of the edible crab, *Cancer pagurus*'. *Aquaculture* **205**, 237–252.

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

Volety, A. K. and F. L. E. Chu (1995). 'Suppression of chemiluminescence of Eastern oyster (*Crassostrea virginica*) hemocytes by the protozoan parasite *Perkinsus marinus*'. *Developmental and Comparative Immunology* **19**, 135–142.

Volety, A. K. and F. L. E. Chu (1997). 'Acid phosphatase activity in *Perkinsus marinus*, the protistan parasite of the American oyster, *Crassostrea virginica*'. *Journal of Parasitology* **83**, 1093–1098.

Volkov, R. A., I. I. Panchuk and F. Schoffl (2003). 'Heat-stress-dependency and developmental modulation of gene expression: the potential of house-keeping genes as internal standards in mRNA expression profiling using real-time RT-PCR'. *Journal of Experimental Botany* **54**, 2343–2349.

Walker, A. N., R. F. Lee and M. E. Frischer (2009). 'Transmission of the parasitic dinoflagellate *Hematodinium* sp infection in blue crabs *Callinectes sapidus* by cannibalism'. *Diseases of Aquatic Organisms* **85**, 193–197.

Walton, A. and V. J. Smith (1999). 'Primary culture of the hyaline haemocytes from marine decapods'. *Fish & Shellfish Immunology* **9**, 181–194.

Wang, C. and R. J. S. Leger (2006). 'A collagenous protective coat enables *Metarhizium anisopliae* to evade insect immune responses'. *Proceedings of the National Academy of Sciences of the United States of America* **103**, 6647–6652.

Wang, Y. C., P. S. Chang and H. Y. Chen (2007). 'Tissue expressions of nine genes important to immune defence of the Pacific white shrimp *Litopenaeus vannamei*'. *Fish & Shellfish Immunology* **23**, 1161–1177.

Wang, Y. C., P. S. Chang and H. Y. Chen (2008). 'Differential time-series expression of immune-related genes of Pacific white shrimp *Litopenaeus vannamei* in response to dietary inclusion of beta-1,3-glucan'. *Fish & Shellfish Immunology* **24**, 113–121.

Wang, Y., Y. Chen, K. Han, Z. Zou and Z. Zhang (2011). 'A vasa gene from green mud crab *Scylla paramamosain* and its expression during gonadal developmemt and gametogenesis'. *Molecular Biology Reports*.

Wang, Y. Z., X. X. Wang, Z. Q. Lu, L. N. Zhu, Y. F. Zhang and Y. Ren (2010). 'Innate immune response and related gene expression in red swamp crayfish [*Procambarus clarkii* (Girard)], induced by selenium-enriched exopolysaccharide produced by bacterium *Enterobacter cloacae* Z0206'. *Aquaculture Research* **41**, e819–e827.

Warner, J. R. (1999). 'The economics of ribosome biosynthesis in yeast'. *Trends in Biochemical Sciences* **24**, 437–440.

Watson, F. L., R. Puttmann-Holgado, F. Thomas, D. L. Lamar, M. Hughes, M. Kondo, V. I. Rebel and D. Schmucker (2005). 'Extensive diversity of Ig-superfamily proteins in the immune system of insects'. *Science* **309**, 1874–1878.

Watthanasurorot, A., P. Jiravanichpaisal, H. P. Liu, I. Soderhall and K. Soderhall (2011). 'Bacteria-induced Dscam isoforms of the crustacean, *Pacifastacus leniusculus*'. *Plos Pathogens*.

Webster, S. G. (1996). 'Measurement of crustacean hyperglycaemic hormone levels in the edible crab *Cancer pagurus* during emersion stress'. *Journal of Experimental Biology* **199**, 1579–1585.

Wheeler, K., J. D. Shields and D. M. Taylor (2007). 'Pathology of *Hematodinium* infections in snow crabs (*Chionoecetes opilio*) from Newfoundland, Canada'. *Journal of Invertebrate Pathology* **95**, 93–100.

White, T. J., T. Bruns, S. Lee and J. Taylor (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In M. A. Innis (Ed.). 'PCR Protocols: a guide to methods and applications'. Academic Press. San Diego, CA. pp. 315–322.

Wickins, J. F. and D. O'C. Lee (2002). *Crustacean Farming: ranching and culture*. 2 edn. Blackwell Science Ltd.. Oxford.

Wilhelm, G. and E. Mialhe (1996). 'Dinoflagellate infection associated with the decline of *Necora puber* crab populations in France'. *Diseases of Aquatic Organisms* **26**, 213–219.

Witke, W. (2004). 'The role of profilin complexes in cell motility and other cellular processes'. *Trends in Cell Biology* **14**, 461–469.

Wittwer, C. T., M. G. Herrmann, A. A. Moss and R. P. Rasmussen (1997). 'Continuous fluorescence monitoring of rapid cycle DNA amplification'. *Biotechniques* **22**, 130.

Woll, A. K. (2006). 'The Edible Crab: Biology - Grading - Handling live crabs'.

Wong, M. L. and J. F. Medrano (2005). 'Real-time PCR for mRNA quantitation'. *Biotechniques* **39**, 75–85.

Wonglapsuwan, M., A. Phongdara and W. Chotigeat (2009). 'Dynamic changes in gene expression during vitellogenic stages of the white shrimp: *Fenneropenaeus merguiensis* de Man'. *Aquaculture Research* **40**, 633–643.

Wongprasert, K., K. Khanobdee, S. S. Glunukarn, P. Meeratana and B. Withyachumnarnkul (2003). 'Time-course and levels of apoptosis in various tissues of black tiger shrimp *Penaeus monodon* infected with white-spot syndrome virus'. *Diseases of Aquatic Organisms* **55**, 3–10.

Woo, L. and W. Strong (2006). 'RNA Quantitation: A comparative analysis using the Experion automated electrophoresis system, RiboGreen reagent, and ultraviolet spectroscopy'. *BioRad Bulletin*.

Wu, C. L., I. Soderhall, Y. A. Kim, H. P. Liu and K. Soderhall (2008). 'Hemocyte-lineage marker proteins in a crustacean, the freshwater crayfish, *Pacifastacus leniusculus*'. *Proteomics* **8**, 4226–4235.

Xian, J. A., A. L. Wang, J. X. Tian, J. W. Huang, C. X. Ye, W. N. Wang and R. Y. Sun (2009). 'Morphologic, physiological and immunological changes of haemocytes from *Litopenaeus vannamei* treated by lipopolysaccharide'. *Aquaculture* **298**, 139–145.

Xu, W., J. Zie, H Shi and C. H. Li (2010). '*Hematodinium* infections in cultured ridgetail white prawns, *Exopalaemon carinicauda*, in eastern China'. *Aquaculture* **300**, 25–31.

Yamakawa, K., Y. K. Huo, M. A. Haendel, R. Hubert, X. N. Chen, G. E. Lyons and J. R. Korenberg (1998). 'DSCAM: a novel member of the immunoglobulin superfamily maps in a Down syndrome region and is involved in the development of the nervous system'. *Human Molecular Genetics* **7**, 227–237.

Yang, C. J., J. Q. Zhang, F. H. Li, H. M. Ma, Q. L. Zhang, T. A. J. Priya, X. J. Zhang and J. H. Xiang (2008). 'A toll receptor from Chinese shrimp *Fenneropenaeus*

*chinensis* is responsive to *Vibrio anguillarum* infection'. *Fish & Shellfish Immunology* **24**, 564–574.

Yang, L. S., Z. X. Yin, J. X. Liao, X. D. Huang, C. J. Guo, S. P. Weng, S. M. Chan, X. Q. Yu and J. G. He (2007). 'A Toll receptor in shrimp'. *Molecular Immunology* **44**, 1999–2008.

Yasunaga-Aoki, C., S. Imanishi, K. Iiyama and A. Kawarabata (2004). 'Establishment of phagocytic cell lines from larval hemocytes of the beet armyworm, *Spodoptera exigua*'. *In vitro Cellular & Developmental Biology-Animal* **40**, 183–186.

Yedavalli, V. S. R. K., C. Neuveut, Y. H. Chi, L. Kleiman and K. T. Jeang (2004). 'Requirement of DDX3 DEAD box RNA helicase for HIV-1 Rev-RRE export function'. *Cell* **119**, 381–392.

Yeh, M. S., C. H. Liu, C. W. Hung and W. Cheng (2009). 'cDNA cloning, identification, tissue localisation, and transcription profile of a transglutaminase from white shrimp, *Litopenaeus vannamei*, after infection by *Vibrio alginolyticus*'. *Fish & Shellfish Immunology* **27**, 748–756.

Yokota, S., H. Yanagi, T. Yura and H. Kubota (1999). 'Cytosolic chaperonin is up-regulated during cell growth - Preferential, expression and binding to tubulin at G(1)/S transition through early S phase'. *Journal of Biological Chemistry* **274**, 37070–37078.

Yokota, S., H. Yanagi, T. Yura and H. Kubota (2000). 'Upregulation of cytosolic chaperonin CCT subunits during recovery from chemical stress that causes accumulation of unfolded proteins'. *European Journal of Biochemistry* **267**, 1658–1664.

Yoshida, S., D. Ioka, H. Matsuoka, H. Endo and A. Ishii (2001). 'Bacteria expressing single-chain immunotoxin inhibit malaria parasite development in mosquitoes'. *Molecular and Biochemical Parasitology* **113**, 89–96.

Yu, X. Q., H. B. Jiang, Y. Wang and M. R. Kanost (2003). 'Nonproteolytic serine proteinase homologs are involved in prophenoloxidase activation in the tobacco hornworm, *Manduca sexta*'. *Insect Biochemistry and Molecular Biology* **33**, 197–208.

Zarnbrano-Villa, S., D. Rosales-Borjas, J. C. Carrero and L. Ortiz-Ortiz (2002). 'How protozoan parasites evade the immune response'. *Trends in Parasitology* **18**, 272–278.

Zasloff, M. (2002). 'Antimicrobial peptides of multicellular organisms'. *Nature* **415**, 389–395.

Zhang, Y., L. L. Wang, J. M. Zhao, Y. C. Gai, C. H. Dong, L. M. Qiu and L. S. Song (2010). 'The effects of CpG-C oligodeoxynucleotides on innate immune responses in *Eriocheir sinensis* (H. Milne-Edwards, 1853)'. *Aquaculture Research* **41**, e656–e666.

Zhu, J., P. Yang and G.X. Wu (2011). 'Prophenoloxidase from *Pieris rapae*: gene cloning, activity, and transcription in response to venom/calyx fluid from the endoparasitoid wasp *Cotesia glomerata*'. *Journal of Zhejiang University - Science B (Biomedicine & Biotechnology)* **12**, 103–115.

Zou, Z., S. W. Shin, K. S. Alvarez, G. Bian, V. Kokoza and A. S. Raikhel (2008). 'Mosquito RUNX4 in the immune regulation of PPO gene expression and its effect on avian malaria parasite infection'. *Proceedings of the National Academy of Sciences of the United States of America* **105**, 18454–18459.