Antiviral immune responses in abalone and influence of potential abiotic and biotic factors

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Contents

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Abbreviations

AbHV	Abalone herpesvirus
AVG	Abalone viral ganglioneuritis
DMSO	Dimethyl sulfoxide
EMEM	Eagle's minimal essential medium
HSV-1	Herpes simplex virus type 1
NBT	Nitroblue tetrazolium
OD	Optical density
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PFU	Plaque-forming unit
qPCR	Quantitative real time polymerase chain reaction
RT	Room temperature
SO	Superoxide anion
THC	Total haemocyte count

Declaration

I certify that this thesis does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university; and that to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text.

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Abstract

Abalone (Haliotidae) are marine gastropod mollusks and important aquaculture species worldwide. Unfortunately, severe mortality of abalone caused by a herpesvirus (AbHV) has been reported in Australia. The manifestation of disease involves an interaction between virus, environment and abalone immunity (Chapter 1). Therefore, this research aimed to investigate the presence of antiviral activity in abalone *Haliotis laevigata* (greenlip), *H. rubra* (blacklip) and their hybrid. Due to the lack of molluscan cell lines for culturing AbHV, antiviral activity of abalone was assessed against a similar neurotropic herpesvirus, herpes simplex virus type 1 (HSV-1) using the plaque assay. Assessment of antiviral activity was complemented with other immune assays, measuring total haemocyte count (THC), phagocytosis, phenoloxidase activity, respiratory burst and antibacterial activity against *Vibrio* spp. to provide an overall view of immune status in abalone after exposure to various biotic and environmental factors.

A number of abalone organs were screened for anti-HSV-1 activity, but only the haemolymph (20%, v/v) and the lipophilic extract of digestive gland (3,000 μg ml^{-1}) were found to substantially decrease the number and size of virus plaques (Chapter 2). Haemolymph inhibits viral infection at an early stage (e.g. viral entry) whereas the antiviral effect of the lipophilic extract is greatest when added one hour after infection (e.g. the intracellular stage of viral infection).

There was considerable variation in the levels of antiviral and antibacterial activity in the haemolymph among abalone within the same aquaculture family lines and natural populations in different geographic locations (Chapter 3). Antiviral and antibacterial

activity increased slightly with an increase in shell length. However, there was no significant effect of gender or spawning status on antiviral or antibacterial status.

Concomitant with strong antiviral activity against HSV-1 in a lipophilic extract of *Ulva lactuca* and *Spyridia filamentosa*, higher antiviral activity was detected in the digestive gland lipid extract of abalone fed *Ulva lactuca* (64.2% at 650µg ml^{-1}) or *Spyridia filamentosa* (69.51%) compared to abalone fed pellets (47.42%) or pellets supplemented with *Arthrospira maxima* (46.3%) or *Dunaliella salina* (46%) (Chapter 4). There was no influence of diet on the humoral antiviral activity, indicating antiviral factors in the haemolymph are likely to be innately biosynthesized by the abalone.

Sampling of wild-caught *H. rubra* showed a significant correlation between temperature and antiviral or antibacterial activity, with higher activity in summer than in winter months (Chapter 5). However, antibacterial activity was compromised in favour of antiviral activity as the water temperatures peaked in summer. A controlled laboratory experiment with water temperature raised from 18 to 21 or 24 °C showed that THC and SO increased at day 1 and then dropped back to control levels by days 3 and 7. By comparison, the humoral immune parameters showed a delayed response with antibacterial and antiviral activity significantly increasing on days 3 and 7, respectively. Consistent with the field study, antibacterial activity became significantly depressed after prolonged exposure to elevated temperatures. Consequently, abalone may have more resilience to viruses than bacterial pathogens under conditions of elevated temperature.

Experimental transmission trials were undertaken using an immersion model to study the abalone immune response to infection with AbHV (Chapter 6). The infection

status of abalone was confirmed by real-time PCR. THC decreased by 38.8% in moribund abalone, but increased by 42.6 and 13.6% in apparently healthy abalone that were PCR-negative and PCR-positive for AbHV, respectively, in comparison to the non-infected group. The level of SO decreased in abalone confirmed as PCR-positive for AbHV, by 30.8% in moribund abalone and by 7.2% in apparently healthy abalone. However, for apparently healthy abalone that were PCR-negative after viral challenge, SO significantly increased, by 59.3%, in comparison to uninfected controls. These results suggest that THC and SO provide potential immune markers for AbHV infection status.

In conclusion, abalone have at least two antiviral compounds with different modes of action against viral infection. Humoral antiviral factors appear to be constitutively produced and are influenced by high temperature but not by diet or infection status. Further investigation is required to establish whether the individual variability in antimicrobial activity is heritable in breeding programs and whether higher activity confers greater resistance to disease.

Acknowledgement

Thesis Structure

This thesis encompasses five discrete experiments, each of which is presented in an independent manuscript format. Thus, some repetition of background and methods may be found between chapters. Each chapter has independent hypotheses and aims, and these complement the overall aims mentioned in the introductory chapter. For chapters that have been published or have been accepted for publication in peer-reviewed journals, the literature citation is given on the title page of that chapter. Within each manuscript, the "study" refers to the particular experiment associated with only that chapter. References to other experiments are made by formal citation of published or submitted work. Although the nominative form "we" is used in journal submitted manuscripts of Chapter 2-6, the work presented was undertaken by myself under supervision of the coauthors.

My supervisors, Peter Speck and Kirsten Benkendorff, are listed on all papers due to intellectual input in experimental design, interpretation and feedback on manuscript drafts. Contributions of other co-authors are listed for chapters below.

- Chapter 3: Prof. Mehdi Doroudi is a named investigator on the original Seafood CRC grant that funded this project and he had intellectual input into the idea of screening different geographic populations and family lines. Ben Smith is my industry mentor and he facilitated access to farmed family lines and helped set up the spawning experiment.

- Chapter 4: Yan Li facilitated bacterial culture for antibacterial assay and helped set up the phagocytosis assay.

- Chapter 6: Dr Mark Crane is a co-investigator on the Seafood CRC grant that funded this research and he facilitated the experiments at AAHL, arranged animal ethics approval for the infection experiment and had intellectual input into the experimental design and interpretation. Dr Serge Corbeil and Lynette Williams helped set up the infection experiments and facilitated the PCR and interpretation. John Hoad facilitated cell culture work at AAHL.

Chapter 1 is a general introduction to the concepts involved in this research, as well as a presentation of the aims and significance of this work. Chapter 2 contains a manuscript, published in *Journal of General Virology*, that describes the *in vitro* antiviral activity against herpes simplex virus in abalone *Haliotis laevigata*. Chapter 3 contains a paper published in *Aquaculture* that describes variation in the antiviral and antibacterial activity of abalone *H. laevigata*, *H. rubra* and their hybrid in South Australia. Chapter 4 contains a manuscript, published in *Aquaculture,* which identified the effects of micro- and macro-algal diet supplementations on growth and immunity of greenlip abalone, *H. laevigata*. Chapter 5 contains a manuscript published in *Fish and Shellfish Immunology* that documents the influence of elevated temperatures on immune responses of abalone, *H. rubra*. Chapter 6 contains a manuscript, submitted to *Fish and Shellfish Immunology* (3rd July 2012), which describes immunological changes in response to herpesvirus infection in abalone *Haliotis laevigata* and *Haliotis rubra* hybrids. Chapter 7 represents a general conclusion and summary of the entire study, drawing together the results and implication from all papers.

CHAPTER 1: General introduction

1.1. Major economic abalone species in Australia

Abalone belong to the Gastropoda, the most diversified class in the phylum Mollusca, and they are major economic and revenue species worldwide (Fleming *et al.* 1996; Cook 1998; Godoy *et al.* 1998; Gordon *et al.* 2001; 2004). Australia contributes more than half of the world fishery production of abalone and is becoming a major contributor to the world aquaculture production of this species (Brown *et al.* 1997; Gordon 2000). In Australia, the blacklip abalone *Haliotis rubra,* the greenlip *Haliotis laevigata* and their hybrid cross are the most commercially important abalone species (Freeman 2001). These abalone are extensively farmed and/or fished from the wild in Tasmania, Victoria, South Australia and Western Australia, generating revenue of approximately $217 million in 2007-2008 (ABARE 2009). Other abalone species of considerable interest for aquaculture are Roe's *Haliotis roei* and brownlip *Haliotis conicopora* abalone in Western Australia and donkey-ear *Haliotis asinina* in Queensland and Western Australia.

The blacklip *H. rubra* and the greenlip *H. laevigata* are widely found in southern waters, living on encrusting coralline algae or substrata of granite and limestone (Hone *et al.* 1997). Most of them inhabit intertidal or inshore areas at depths of up to 20m. *H. rubra* is a cold-water species and the most common abalone in Tasmania, with a preferred water temperature of 8-17 °C (Prince *et al.* 1992; Gilroy *et al.* 1998). *H. laevigata* is more dominant in the warmer waters in South and Western Australia, with mean preferred temperature of 18.9 °C (Prince *et al.* 1992; Gilroy *et al.* 1998).

Optimal growth rates depend on food abundance and nutritional quality. In Australia, feeding abalone with commercial formulated pellets has been widely applied on large-scale farms due to the constant supply of formulated pellets and their stability in water (Sales *et al.* 2004). Meanwhile, algae cultivation is also commercialized and well-developed for potential use as a supplementary feed in abalone aquaculture. Some macroalgae, such as red algae *Gracilaria heteroclada* and *Gracilaria gracilis*, green alga *Ulva lactuca*, and kelp *Ecklonia maxima* have been found suitable for abalone aquaculture since they promote the growth rate of abalone better than commercially-formulated diets (Capinpin *et al.* 1996; Naidoo *et al.* 2006). Since microalgae (e.g. *Arthrospira maxima, Dunaliella salina, Navicula* sp., *Cylindrotheca closterium*) contain a significant amount of protein and polyunsaturated fatty acids, chlorophyll, carotenoids, vitamins, minerals, and unique pigments (Kay 1991; Spolaore *et al.* 2006; Becker 2007), diets supplemented with microalgae also enhance growth and survival rates of abalone *H. rubra* and *H. laevigata* (Daume *et al.* 2003; Daume *et al.* 2007).

H. laevigata and *H. rubra* reach maturity in 3 years at sizes 90-100 and 70-110 mm in shell length, respectively (Shepherd *et al.* 1974). The sexes are distinctive in abalone with white creamy gonads in males and dark brown gonads in females, during the reproductive stage. Fertilization is external and sexually mature individuals often aggregate before spawning, which is likely to increase synchrony of spawning (e.g. males and females spawn on the same night and within hours of each other) and external fertilization success (McShane 1992; Fallu 1994). Aggregations of the greenlip *H. laevigata* can be up to 20-25 individuals (Shepherd *et al.* 1995) with the size of an aggregation dependent on habitat type, density and movement (Shepherd 1973). On the other hand, the blacklip *H. rubra* has a low level of

spawning synchrony (Shepherd *et al.* 1974). Wild greenlip abalone spawn in summer months (October-March), while wild blacklip abalone spawn in early summer and late spring seasons (October-January and March-June). The spawning cycles of abalone can be triggered by a number of environmental factors, including temperature, photo-period and food abundance (Shepherd *et al.* 1985; Fleming 2000).

1.2. Effects of microbial infection and environmental stress on abalone production

1.2.1. Virus infections

A major threat for the Australian abalone industry is abalone viral ganglioneuritis (Table 1.1), which has been reported in *H. laevigata, H. rubra* and their hybrid cross (Hooper *et al.* 2007a). Abalone ganglioneuritis is caused by a herpesvirus (AbHV) (Savin *et al.* 2010), which damages the cerebral nerves, resulting in up to 95% mortality within 14 days from the onset of clinical signs (Hooper *et al.* 2007a). Due to herpesvirus outbreaks in Victoria in recent years, wild and farmed production of abalone in Australia has been reduced by about 14 per cent (799 tonnes) (ABARE 2009). Prior to the abalone viral ganglioneuritis, a herpesvirus was found during disease outbreaks in *Haliotis diversicolor supertexta* in Taiwan (Chang *et al.* 2005; Chen *et al.* 2012). This viral infection was considered responsible for high mortality of abalone from land-based and ocean-based farms in north-eastern Taiwan and the loss of US$11.5 million to the abalone industry (Chang *et al.* 2005) (Table 1.1). Disease outbreaks due to other unknown viruses have been also reported in *Haliotis*

diversicolor reeve and *Haliotis discus hannai* in China (Wang *et al.* 2004; Zhuang *et al.* 2010).

Electron microscopy and histopathology of AbHV-infected abalone have revealed the hexagonal double-stranded DNA viruses with a single coat, very similar to a herpesvirus described from the oyster *Crassostrea virginica* (Farley *et al.* 1972; Tan *et al.* 2008). The abalone herpesvirus genome has been sequenced (Fegan *et al.* 2009; Savin *et al.* 2010) with a 59682 bp portion of the sequence deposited in the GenBank database (accession number HM 63198.1) and the complete genome is estimated to be in the range of 200-210kb in length. Phylogenetic analysis of DNA polymerase proteins suggests that the abalone and oyster herpesviruses are within the one family *Malacoherpesviridae* and are distantly related to other members of the *Herpesviridae* (Fegan *et al.* 2009; Savin *et al.* 2010; Chen *et al.* 2012). Herpesviruses in the *Malacoherpesviridae* have been found to infect other molluscan species, including oyster *C. gigas* (Renault *et al.* 1994a; Friedman *et al.* 2005; Burge *et al.* 2006; Nicolas *et al.* 2008), scallop *Pecten maximus* (Arzul *et al.* 2001) and clam *Ruditapes philippinarum* (Renault *et al.* 2001b).

1.2.2. Bacterial infections

Bacterial infections have been reported in the grow-out stock of *H. rubra*, *H. laevigata* and their hybrids in land-based farms (Table 1.1). The most common cause of infection is *Vibrio* species, which have been isolated from moribund abalone during disease outbreaks in Australia (Handlinger *et al.* 2005). The outbreaks have been mainly associated with two species, *Vibrio harveyi* and *Vibrio splendidus I* (Handlinger *et al.* 2005). *V. harveyi* is the most common bacterial pathogen of marine invertebrates (Austin *et al.* 2006) and it can cause septicaemia in abalone,

resulting in up to 80% mortality (Nicolas *et al.* 2002; Handlinger *et al.* 2005). *V. harveyi* (syn *Vibrio carchariae)* has been linked to summer mortality of *H. rubra*, *H. laevigata* and their hybrids in Australia, as well as *Sulculus (Haliotis) diversicolor supratexta* in Japan and *Haliotis tuberculata* in France (Nishimori *et al.* 1998; Nicolas *et al.* 2002; Handlinger *et al.* 2005; Travers *et al.* 2008; Travers *et al.* 2009). *Vibrio alginolyticus* and *Vibrio parahaemolyticus* have also been described as pathogens causing disease outbreaks and mass mortality in abalone *Haliotis rufescens* (Elston *et al.* 1983; Anguiano-Beltran *et al.* 1998), *Haliotis diversicolor supertexta* (Liu *et al.* 2000; Cai *et al.* 2006; Cai *et al.* 2007; Cheng *et al.* 2008) and *Haliotis midae* (Dixon *et al.* 1991). Abalone can also be infected by bacterial pathogens other than *Vibrio* spp. (Table 1.1), for example *Clostridium lituseberense* in south African abalone *Haliotis midae* (Dixon *et al.* 1991) and *Francisella* sp. in giant abalone *Haliotis gigantean* (Kamaishi *et al.* 2010; Brevik *et al.* 2011).

Table 1.1. The reported infectious bacteria and viruses in abalone (Haliotidae, Gastropoda)

Microorganisms	Host species	Mortality/Industry loss	References
Viruses			
Herpesvirus	*H. diversicolor supertexta, H. laevigata, H. rubra*	Up to 95% mortality; loss of US$11.5 million to the abalone industry of Taiwan; abalone production reduced by about 14% (~799 tonnes) in Australia	(Chang *et al.* 2005; Hooper *et al.* 2007a; ABARE 2009; Chen *et al.* 2012)
Spherical unknown virus	*H. diversicolor reeve*	Up to 100% mortality in commercial farms in China	(Wang *et al.* 2004)
Shrivelling syndrome-associated virus	*H. discus hannai*	High mortality (most infected abalone died during disease outbreak in China)	(Zhuang *et al.* 2010)
Bacteria			
Vibrio harveyi	*H. rubra, H. laevigata, S. diversicolor supratexta, H. tuberculata*	Up to 80% mortality	(Nishimori *et al.* 1998; Nicolas *et al.* 2002; Handlinger *et al.* 2005; Travers *et al.* 2008; Travers *et al.* 2009)
Vibrio parahaemolyticus	*Haliotis diversicolor supertexta*	Mass mortality among cultured abalone in Taiwan and China	(Liu *et al.* 2000; Cai *et al.* 2007; Cheng *et al.* 2008)
Vibrio alginolyticus	*H. rufescens, H. diversicolor supertexta, H. midae*	Occasional peak in mortality of *H. rufescens* with persistent routine morbidity and mortality in juvenile abalone in intensive culture systems in the United States Massive mortality of farmed abalone resulting in loss of several million yuan in Southern China	(Elston *et al.* 1983; Dixon *et al.* 1991; Anguiano-Beltran *et al.* 1998; Cai *et al.* 2006)
Clostridium lituseberense	*Haliotis midae*	40% mortality in juvenile abalone from feeding trials	(Dixon *et al.* 1991)
Francisella sp.	*Haliotis gigantea*	Cumulative mortality rate reached about 84% on an abalone farm in Japan	(Kamaishi *et al.* 2010; Brevik *et al.* 2011)

1.2.3. Environmental stress factors

Many pathogens are opportunistic and cause disease in abalone when their immune system is suppressed as a result of environmental stressors (Hooper *et al.* 2007b, Figure 1.1), such as high water temperature (Lee *et al.* 2001; Cheng *et al.* 2004a; Handlinger *et al.* 2005; Travers *et al.* 2008; Travers *et al.* 2009), poor water quality (i.e. high level of ammonia and nitrite or low level of dissolved oxygen) (Cheng *et al.* 2004b; Cheng *et al.* 2004d; Cheng *et al.* 2004a; Cheng *et al.* 2004e; Cheng *et al.* 2004c; Hooper *et al.* 2007b; Day *et al.* 2010) or manual handling (Malham *et al.* 2003; Hooper *et al.* 2011). High temperature, population density, limited water exchange, food availability and oxygen content, and high water pollution level are also thought to contribute to mass-mortality disease outbreaks (e.g. herpes or herpes-like virus infection, viral gametocytic hypertrophy) in oyster (Renault *et al.* 1994a; Hine *et al.* 1997; Hine *et al.* 1998; Watermann *et al.* 2008).
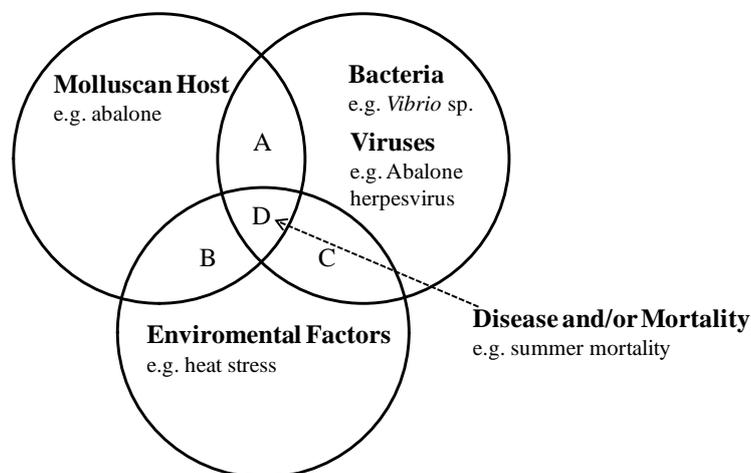
Figure 1.1. The "holy triad" of disease causality: interactions between the molluscan host, environmental factors and pathogens: A) Subclinical stage of infection where the molluscan host does not show disease signs; B) Immune suppression by environmental stressors; C) Increased abundance and virulence of pathogens driven by environmental factors; D) Disease and/or mortality due to immune-suppression by environmental stressors in the presence of pathogen(s) (adapted from Snieszko 1974).

High water temperature associated with climate change is often regarded as the most significant threat to the economic and ecological sustainability of marine fisheries and aquaculture. The average sea surface temperatures have increased by 0.6°C in the last 100 years (Trenberth *et al.* 2007; Hoegh-Guldberg *et al.* 2010) and the waters around Australia have been predicted to warm 1-3 ºC by 2070, with greatest warming in southern and south-eastern Australia (Hobday *et al.* 2006; Poloczanska *et al.* 2007; Ridgway *et al.* 2009). Outbreaks associated with *Vibrio* species in farmed *H. laevigata* and *H. rubra* typically occur as peaks of mortality in summer, after a rapid increase in water temperature (Handlinger *et al.* 2005). In addition, high temperature could contribute to the occurrence of herpesvirus outbreaks, which are typically reported in summer months in abalone (Hooper *et al.* 2007a) and other mollusc species such as oyster *C. gigas* (Renault *et al.* 1994a; Friedman *et al.* 2005; Burge *et al.* 2006; Nicolas *et al.* 2008), scallop *Pecten maximus* (Arzul *et al.* 2001) and clam *Ruditapes philippinarum* (Renault *et al.* 2001b). Increased water temperature, especially in summer months, to above the temperature tolerance of *H. laevigata* and *H. rubra,* could cause immune depression, favouring prevalence of disease outbreaks (Handlinger *et al.* 2005). Other abalone species, such as *H. diversicolor supertexta* and *H. tuberculata*, when exposed to elevated temperatures outside their preferred temperature range, have shown increased susceptibility to infection by *Vibrio parahaemolyticus* or *V. harveyi* (Huang *et al.* 2001; Cheng *et al.* 2004a; Travers *et al.* 2008; Travers *et al.* 2009). Indeed, some cell-mediated immune parameters (i.e. total haemocyte count, superoxide anion level) have been found to be significantly increased in high water temperature treatment groups (Cheng *et al.* 2004a), indicating a stress response. However, to date, no studies have investigated the effects of temperature on humoral antimicrobial defense mechanisms in abalone.

Apart from the immune depression effect on the host abalone, elevated temperature can increase the growth rate and virulence of microbial pathogens. For example, it has been demonstrated that growth of *V. harveyi* is triggered by temperature, resulting in 90% mortality of the European abalone *H. tuberculata* at 19 °C in comparison to no mortalities at 17 °C (Travers *et al.* 2008). Viral abundance has also been observed to increase with water temperature in different oceanic regions. Increase of water temperature by only a few degrees was associated with a doubling of viral abundance (Danovaro *et al.* 2010). Increasing temperature leads to higher host metabolism, which has been linked to higher rates of virus production (Proctor *et al.* 1993; Hadas *et al.* 1997; Danovaro *et al.* 2010).

1.3. Immune responses against microbial infections

Like other marine invertebrates species, abalone have innate immunity as a critical line of defence against pathogens, consisting of physical barriers (e.g. shell, skin, epithelium) and cell-mediated and humoral immune responses (Tiscar *et al.* 2004; Hooper *et al.* 2007b). Cellular responses including encapsulation, phagocytosis, respiratory burst, melanisation, cytotoxicity, cell-to-cell communication and pro-phenoloxidase activation are mediated by haemocytes, while humoral responses including production of lectins, defensive enzymes, extracellular reactive oxygen intermediates and antimicrobial peptides are released by granulocytes into the haemolymph plasma. While cellular and humoral immunity has been shown to be effective against bacterial infections (Hooper *et al.* 2007b), the mechanisms directed against viral infections are still mostly unknown.

1.3.1. Cell-mediated immunity

A well-known pathway in the innate immune system of invertebrates is recognition of pathogen-associated molecular patterns by pattern recognition receptors, which then interact with serine proteinases to initiate encapsulation, phagocytosis and the phenoloxidase enzyme system (Ratner *et al.* 1983; Aladaileh *et al.* 2007). Bacteria have several conserved molecular patterns including carbohydrates (e.g. lipopolysaccharide, mannose or glucans), nucleic acids (e.g. bacterial DNA or RNA), peptides (flagellin), peptidoglycans, lipoteichoic acids, N-formylmethionine and lipoproteins. These conserved molecular patterns can bind to different types of pattern recognition receptors, which are transmembrane (receptor kinases, Toll-like receptors) or cytoplasmic (NOD-like receptors, RNA helicases, non-RD kinases) proteins on/in haemocytes or secreted proteins in the plasma (complement receptors, collectins, pentraxin) (Beutler *et al.* 2006; Dommett *et al.* 2006; Boller *et al.* 2009). Although viruses lack many conserved pattern molecules found in bacteria, double-stranded RNA and envelop proteins both in RNA and DNA viruses could bind to pattern recognition receptor to induce antiviral responses. Indeed, resistance against white spot syndrome virus (WSSV) in shrimp *Penaeus monodon* and crayfish *Procambarus clarkii* was obtained by pre-exposing them to specific viral dsRNA and envelop proteins (Witteveldt *et al.* 2004; Jha *et al.* 2006; Kumar *et al.* 2008).

In previous studies, invertebrate immunity against microbial pathogens has been indirectly indicated by a range of immune parameters including phagocytosis, phenoloxidase activity, and superoxide level (Itami *et al.* 1998; Takahashi *et al.* 2000; Chotigeat *et al.* 2004; Deachamag *et al.* 2006; Balasubramanian *et al.* 2008; Kumar *et al.* 2008). Increases in phagocytosis, phenoloxidase activity and superoxide

anion level have been observed in crustacean species, for example shrimp, with no clinical signs, after being exposed to WSSV (Itami *et al.* 1998; Takahashi *et al.* 2000; Chotigeat *et al.* 2004; Deachamag *et al.* 2006; Balasubramanian *et al.* 2008; Kumar *et al.* 2008).

1.3.2. Humoral immunity defense

Antimicrobial activity has been found in abalone extracts as described in several *in vitro* studies. For example, a fraction isolated from abalone juice by ion exchange chromatography was found to inhibit *Staphylococcus aureus, Streptococcus pyogenes*, *Salmonella typhi*, and *Salmonella paratyphi* (Prescott et al. 1960)**.** Several antibacterial compounds isolated from abalone are peptides, for example abhisin, defensin, Nod1 and Nod2 (De Zoysa *et al.* 2009a; De Zoysa *et al.* 2010; Park *et al.* 2012). Nod1 and Nod2 show antibacterial activity due to cell membrane lysis, as demonstrated by SYTOX-green uptake (Park *et al.* 2012). Transcription of the abalone defensin gene was significantly up-regulated in haemocytes, gills and digestive tract after challenge with the mixture of pathogenic bacteria containing *Vibrio alginolyticus*, *Vibrio parahemolyticus* and *Lysteria monocytogenes* (De Zoysa *et al.* 2010). These experiments suggest that antimicrobial peptides play a major role in immune response of abalone against pathogenic bacteria. However, preliminary studies by Grant (2002) and Vakalia (2005) indicate that abalone haemolymph retains antibacterial activity after heat treatment and protease digestion, thus suggesting that there may also be non-peptide based antibacterial factors in abalone haemolymph.

Another fraction (designated Paolin 2) from canned abalone did not show antibacterial activity, but inhibited replication of polyoma, influenza A, and polio

viruses *in vitro* and protected mice from infection with polio and influenza viruses (Li *et al.* 1962b). The location of the antiviral compounds within abalone, their cytotoxicity, mode of action and chemical nature all remains to be investigated. The antiviral substances could be gene-encoded peptides (Table 1.2). Indeed, peptide extracts from three bivalve molluscs *Cerastoderma edule*, *Ruditapes philippinarum* and *Ostrea edulis* and two gastropods *Buccinum undatum* and *Crepidula fornicate* have shown antiviral activity against herpes simplex virus type 1 (Defer *et al.* 2009b). Two peptides defensin and mytilin from bivalve mollusc *Mytilus galloprovincialis* (Mediterranean mussel) can interfere with virus infection (Roch *et al.* 2004; Roch *et al.* 2008). The recombinant expression of myticin class C peptide of the Mediterranean mussel *Mytilus galloprovincialis* in Chinook salmon embryo cells has been found to protect against viral hemorrhagic septicaemia virus and infectious pancreatic necrosis virus (Balseiro *et al.* 2011). A range of antiviral peptides, for example tachyplesin I, protein-pmAVP, hemocyanin, PmRab7, LvCTL1 (C-type lectine) have been successfully characterized from other invertebrate species, for example horseshoe crabs and *penaeid* shrimp (Morimoto *et al.* 1991; Murakami *et al.* 1991; Pan *et al.* 2000; Luo *et al.* 2003; Zhang *et al.* 2004; Sritunyalucksana *et al.* 2006; Zhao *et al.* 2009). Antiviral compounds can induce an antiviral state in the host cell, inactivate virus (i.e. by destroying viral envelop subunits), prevent attachment and entry of virus into cell (i.e. binding directly onto virus envelop), or inhibit viral transcription and DNA/RNA synthesis (i.e. inhibiting virus DNA polymerase α and reverse transcriptases) (Table 1.2).

Table 1.2. Proposed mode of actions for antiviral compounds

Virus and cell interaction models	Activity of antiviral compounds	Evidence from marine invertebrate species
	Induce antiviral state in the cell	- Lipophilic extract of blue crab *Callinectes sapidus* against Sindbis and vaccinia virus (Pan *et al.* 2000).
	Inactivate virus (e.g. destroy viral envelop subunits)	- Tachyplesin I and related isopeptides from horseshoe crab *Tachypleus tridentatus* and *Limulus polyphemus* against influenza A (H1N1) and vesicular stomatitis virus (Murakami *et al.* 1991).
	Prevent attachment and entry of virus into cell (e.g. bind directly onto virus envelop)	- Tachyplesin I from horseshoe crab *Tachypleus tridentatus* against human immunodeficiency virus (Morimoto *et al.* 1991). - Lipophilic extract of blue crab *Callinectes sapidus* against Sindbis, vaccinia, vesicular stomatitis, mengo, banzi and poliomyelitis viruses (Pan *et al.* 2000). - Lectins from mussel *Crenomytilus grayanus* against human immunodeficiency virus (Luk'yanov *et al.* 2007).
	Inhibit viral transcription and DNA/RNA synthesis (e.g. inhibit virus DNA polymerase α and reverse transcriptases)	- Kelletinin A from marine gastropod *Buccinulum corneum*, against human T-cell leukemia virus type-1 (Silvestri *et al.* 1995). - Mytilins from mussel *Mytilus galloprovincialis* against white spot syndrome virus (Dupuy *et al.* 2004). - Defensin from mussel *Mytilus galloprovincialis* against human immunodeficiency virus (Roch *et al.* 2004).
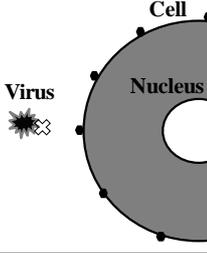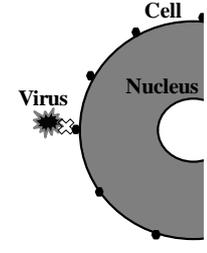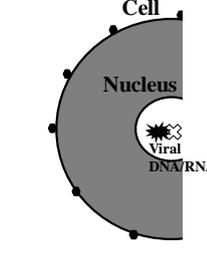
1.3.3. Dietary derived anti-microbial factors

Antimicrobial compounds in marine invertebrates could also be obtained from external source such as micro- and macroalgae foods. Indeed, antiviral and antibacterial activities, or bioactive compounds have been widely reported in microalgae (Ohta *et al.* 1998; Singh *et al.* 2005; Gastineau *et al.* 2012), as well as brown, green and red macroalgae (Ponce *et al.* 2003; Lee *et al.* 2004; Talarico *et al.*

2004; Puglisi *et al.* 2007; Salvador *et al.* 2007; Stirk *et al.* 2007; Mandal *et al.* 2008; Kamenarska *et al.* 2009). Sulfated polysaccharides such as fucoidans, calcium spirulan, carrageenan, galactan sulfate and xylomannan, isolated from marine algae,can inhibit a broad range of bacteria (*Vibrio harveyi*, *Staphylococcus aureus* and *Escherichia coli*, *Enterococcus faecalis* and *Klebsiella pneumoniae* (Chotigeat *et al.* 2004; Rhimou *et al.* 2010) and viruses (e.g. herpes simplex virus, poliovirus, adenovirus, human immunodeficiency virus, influenza virus and respiratory syncytial virus) (Hasui *et al.* 1995; Hayashi *et al.* 1996a; Witvrouw *et al.* 1997; Damonte *et al.* 2004; Li *et al.* 2008). Consequently, after consuming bioactive algae, antimicrobial compounds could be released and potentially stored in the digestive system of abalone offering a form of protection from oral pathogens.

Dietary derived compounds and microalgae could also act to stimulate the immune system of abalone. Feeding crude fucoidan extracted from brown seaweed *Sargassum polycystum* or green algae *Acrosiphonia orientalis* to shrimp *Penaeus monodon* resulted in increased cellular immune responses (e.g. total haemocyte count, phagocytosis, phenoloxidase activity) and survival rate of up to 88-93% after infection by WSSV (Chotigeat *et al.* 2004; Manilal *et al.* 2009). Similarly, it is conceived that these useful compounds in algae could be passed on to abalone through diet to enhance their pathogen resistance.

1.4. Research Aims

Herpesviruses and *Vibrio* bacteria have caused mass-mortality disease outbreaks that seriously impact the abalone industry, not only in Australia but also in many other countries. Therefore, it is important to investigate appropriate immune markers for infection status and the resistance potential of abalone. A small proportion of abalone

typically survive the disease outbreaks, indicating the potential for genetic variability in the immune responses. The aims of this research are to determine:

1. The antiviral activity of different extracts from abalone including haemolymph and the peptide, lipid, and polar extracts of different tissues of abalone;

2. Variation in antimicrobial activity of abalone across individuals of different reproductive stages, sexes (male and female), farmed family lines, and wild populations;

3. The effect of diet on abalone antimicrobial activity and other immune parameters (total haemocyte count, phagocytosis, phenoloxidase activity, level of intracellular superoxide anions);

4. The effect of high water temperature on abalone antimicrobial activity and other immune parameters (total haemocyte counts and level of intracellular superoxide anion); and

5. Immunological changes of abalone in response to herpesvirus infection.

CHAPTER 2: *In vitro* antiviral activity against herpes simplex virus in abalone *Haliotis laevigata*

Abstract

Because viruses are extremely abundant in oceans, marine organisms may have evolved novel metabolites to protect themselves from viral infection. This research examines a well-known commercial gastropod, abalone (Haliotidae), which have recently in Australia experienced disease due to a neurotropic infection, abalone viral ganglioneuritis (AVG), caused by an abalone herpesvirus (AbHV). Due to the lack of molluscan cell lines for culturing AbHV, antiviral activity of abalone *Haliotis laevigata* was assessed against another neurotropic herpesvirus, herpes simplex virus type 1 (HSV-1) using plaque assay. The concentration range at which abalone extract was used for antiviral testing caused minimal (<10%) mortality in Vero cells. Haemolymph (20%, v/v) and the lipophilic extract of digestive gland (3,000 µg ml^{-1}) both substantially decrease the number and size of plaques. By adding haemolymph or lipophilic extract at different times during the plaque assay, we show that haemolymph inhibits viral infection at an early stage. In contrast, the antiviral effect of the lipophilic extract is greatest when added one hour after infection, suggesting that it may act at an intracellular stage of infection. These results suggest that abalone have at least two antiviral compounds with different modes of action against viral infection and provides a novel lead for marine antiviral drug discovery.

2.1. Introduction

The greatest biodiversity on earth is found in oceans, with 32 of the 33 animal phyla, 14 of which are not found on land (Nybakken *et al.* 2005). Currently there are an estimated 230,000-275,000 taxonomically described marine species and with the new species inventory accruing 1,300-1,500 species per year, there is predicted to be between 1.4-1.6 million species of macrofauna and flora in the ocean (Bouchet 2006). Viruses are also abundant in oceans with about 10^9 or more viruses per litre of water, exceeding numbers of bacteria and Archaea by about 15-fold (Bergh *et al.* 1989; Fuhrman 1999; Suttle 2007). Viral infections are common in marine environments with an estimated 10^{23} infections occurring every second (Suttle 2007).

Due to natural selection, a range of antiviral agents may evolve in marine organisms to protect them from viruses. The innate defences available to marine organisms can include secondary metabolites, bioactive peptides and proteins, thus serving as models for development of new drugs for treating human disease. Indeed, several important antiviral compounds of marine origin have been reported, including didemnins, (Caribbean tunicate, *Trididemnum solidum*), eudistomins (shallow-water tunicates within the genus *Eudistoma*), mycalamide A and B (New Zealand sponge, *Mycale* sp), Papuamides A (*Theonella mirabilis* and *T. swinhoei* sponges), Avarone (*Dysidea avara* sponge), Gymnochrome D (fossil crinoid *Gymnocrinus richeri*), Microspinosamide (*Sidonops microspinosa* sponge), Solenolide A (Gorgonian of the genus *Solenopodium*), Hennoxazole A (*Polyfibrospongia* sp sponge), Thyrsiferol (Red alga *Laurencia venusta*), Spongiadiol (Deep-water *Spongia* sp) (see reviews by Donia *et al.* 2003; Dunlap *et al.* 2007). Other antiviral compounds such as vidarabine (adenine arabinoside or ara-A), acyclovir and zidovudine (azidothymidine) have

been commercially synthesized with semisynthetic modifications from, or are structural analogs, of the arabinosyl nucleosides isolated from the sponge *Cryptotethia crypta* (Bergmann *et al.* 1951; De Clerq 2002).

Mollusca is the second largest animal phylum with an estimated diversity up to 200,000 extant species (Pechenik 2000), mainly occurring in marine habitats. Like all invertebrates, molluscs have innate immunity as a critical line of defence against pathogens (Hooper *et al.* 2007b). Molluscs represent a great resource for discovery of antiviral compounds. Although over 900 molluscan secondary metabolites have been described (Benkendorff 2010), few have been tested for antiviral activity. Nevertheless, antiviral activity has been reported in some molluscs, including unidentified bioactive macromolecules in abalone *Haliotis rufescens*, oyster *Crassostrea virginica*, clam *Mercenaria mercenaria* and *Mya arenaria*, queen conch *Strombus gigas*, squid *Loligo pealii*, and the sea snail *Tegula gallina* (Li 1960; Li *et al.* 1962b; Li *et al.* 1962a; Prescott *et al.* 1964; Li *et al.* 1965; Marderosian 1969). Many of these molluscs are currently used to provide traditional or alternative medicines (Benkendorff 2010).

Abalone are a major economic species in many countries (Fleming *et al.* 1996; Cook 1998; Godoy *et al.* 1998; Gordon *et al.* 2001; 2004). An antiviral fraction (designated Paolin 2) from canned abalone inhibited replication of polyoma, influenza A, and polio viruses *in vitro*, and protected mice from infection with polio and influenza viruses (Li *et al.* 1962b). However, the location of these antiviral compounds within the abalone, their cytotoxicity, mode of action and chemical nature, remain obscure. The need to understand abalone antiviral defence has become urgent, with epidemics of herpesvirus infection seriously affecting *H. diversicolor supertexta* in Taiwan and

blacklip *H. rubra* and greenlip *H. laevigata* in Australia, causing high rates of mortality up to 95% within 14 days of onset of clinical signs (Chang *et al.* 2005; Hooper *et al.* 2007a). On histopathological examination, infected abalone have severe nervous system damage, with ganglioneuritis and hemocytic infiltration (Chang *et al.* 2005; Hooper *et al.* 2007a). Electron microscopic examination of ganglia of affected abalone (Tan *et al.* 2008) revealed an icosahedral virus of about 150 nm in diameter, with an electron-dense core, that is, with the distinctive symmetry and appearance of a herpesvirus, and which is similar to the virus described from the oyster *Crassostrea virginica* (Farley *et al.* 1972). Initial characterization of the genome of oyster herpesvirus (named ostreid herpes virus 1) and the comparison of gene sequence and morphological structure of herpes viruses of mammals and birds, fish and amphibians and invertebrates including molluscs (e.g. oyster, clam), support the view that three major lineages of the herpes viruses have evolved from a common ancestor (Davison 2002; Renault 2008). The abalone herpesvirus genome has been sequenced (Fegan *et al.* 2009; Savin *et al.* 2010) and a 59682 bp portion of the sequence, deposited in the GenBank database (accession number HM 63198.1), shows sequence similarity to ostreid herpesvirus 1. The complete genome is estimated to be in the range of 200-210kb in length (S Warner, personal communication). Phylogenetic analysis of DNA polymerase proteins suggests that the abalone and oyster herpes viruses are within the one family *Malacoherpesviridae* and distantly related to other members of the Herpesviridae (Fegan *et al.* 2009).

The lack of a suitable molluscan cell line presents an obstacle to culturing AbHV. Therefore, in view of the innate immunity in abalone, we chose to investigate their activity against herpes simplex virus, which is readily measured using plaque assay

(Russell 1962). This technique has been used to measure antiviral effect of various natural products including peptides secreted by the African clawed frog (*Xenopus laevis*) (Egal *et al.* 1999) and recently for detecting anti-HSV activity in oyster (*C. gigas*) haemolymph (Olicard *et al.* 2005a). We report here that abalone haemolymph and the lipophilic extract of abalone digestive gland contain significant activity against HSV-1.

2.2. Materials and methods

2.2.1. Abalone

Greenlip abalone, 8 to 12 cm in shell size, from Southern Australian Seafoods farm, Port Lincoln, South Australia, were maintained in filtered seawater at 16 °C with continuous aeration in separate tanks at Flinders University (maximum of 6 abalone in each 20 L glass tank) and fed three times/week with formulated pellets (Adam and Amos Abalone Foods Pty Ltd, Mount Barker, South Australia).

2.2.2. Cell culture and virus

African green monkey kidney cells (Vero) were grown in EMEM (Sigma) supplemented with 10% newborn calf serum (NCS; Sigma) and 1% antibiotics (PCS; 10,000 IU ml^{-1} penicillin, 25,000 IU ml^{-1} colymicin, 10 mg ml^{-1} streptomycin; Sigma) at 37 °C in a humidified atmosphere of 5% CO_2. A well-characterized strain, SC16 (Speck *et al.* 1991; Speck *et al.* 1992) of wild-type herpes simplex virus type 1 (HSV-1) was obtained from Dr Tony Simmons at the Institute of Medical and Veterinary Science, Adelaide. Virus titer was calculated from plaque numbers according to the Reed & Muench method (Reed *et al.* 1938).

2.2.3. Tissue and haemolymph collection

To minimise inter-individual variability of antiviral activity, preparations of each of crude haemolymph, head, gill, mantle, muscle, and digestive gland were pooled from the respective tissues from 15 greenlip abalone for antiviral assay. Crude haemolymph was collected using a sterile syringe (10 ml, 25 G, Terumo) from the anterior sinus (Chen 1996) and kept in sterile tubes at 4 °C. Haemolymph plasma was obtained by centrifuging crude haemolymph (3,000 rpm, 10 min, 4 °C). Crude haemolymph and plasma were stored at -80 °C until assayed. Head, gill, mantle, muscle, and digestive gland were dissected according to Bevelander (1988), lyophilised, and stored at -80 °C until required.

2.2.4. Lipophilic and non-lipophilic extraction

Lipophilic extracts were purified according to Folch *et al.* (1957), with modifications. In particular, lyophilized tissues were finely ground and homogenized in methanol/chloroform (1:1, v/v; 20 ml g^{-1}) twice, for 2 h at room temperature. Solvent extract was filtered through Whatman paper #1. The crude extract was then mixed with 25% its volume of distilled water. The mixture was allowed to separate into two phases by standing for 30 min. The chloroform lower phase was separated in a separating funnel from the upper aqueous layer and dried on a rotary evaporator (Büchi, rotavapor R114, 40 °C at 474-72 mbar) to produce the crude lipid extracts. Lipophilic extracts were reconstituted in DMSO (0.5%) in Eagle's minimal essential medium (DMSO/EMEM; Sigma, Australia). The upper aqueous layer was also dried on a rotary evaporator (40 °C at 474-72 mbar) to produce the non-lipophilic extract, which was reconstituted in distilled water.

Haemolymph was separated into lipophilic and non-lipophilic fractions using hydrophobic interaction chromatography as described by Einbond *et al.* (2004). Briefly, dianion HP-20SS resin (Supelco, USA) (4 g) was placed into a 50 ml glass column, conditioned with methanol (50 ml, 15 min), and washed three times with H_2O (50 ml, 10 min). The non-lipophilic fraction was obtained by applying abalone haemolymph (5 ml) to the column and then allowed to absorb onto the resin for 20 min before draining the unbound extracted haemolymph from the column. Water and lipophilic fractions were obtained by eluting with 5 ml of H_2O, H_2O:methanol (1:1), methanol, methanol:acetone (1:1), or acetone at 15 ml h^{-1}. All fractions were dried on a rotary evaporator (Büchi, rotavapor R114, 40 $^{\circ}$C at 72 mbar), reconstituted in 5 ml of DMSO/EMEM.

2.2.5. Peptide extraction

Lyophilized tissues were homogenized in ethanol/hydrochloric acid 0.7 M (HCl) (3:1, v/v) and centrifuged (3,000 rpm, 30 min, 4 °C). Ethanol and HCl from supernatant were removed under reduced pressure (175 mbar, 40 °C, Büchi, rotavapor R114). Extracts were equilibrated with acetonitrile/water/trifluoroacetic acid (ACN/water/TFA, 80:20:0.05) before loading onto solid phase extraction Sep-pack C-18 cartridges (6 ml, 1,000 mg, Waters). After washes in 5 ml acidified water (TFA, 0.05%), bound material was eluted with an acetonitrile gradient (10, 40, 80, and 100%), at 2 ml min^{-1} (Matutte *et al.* 2000). Fractions were lyophilized and reconstituted in distilled water.

2.2.6. Protein determination

Determination of total protein in haemolymph samples was by the Bradford method (Bradford 1976) using Bio-Rad reagents (Gladesville Australia). Haemolymph samples were incubated with proteinase K (100 µg ml^{-1}, Qiagen) and/or trypsin (5 mg ml^{-1}, Sigma) overnight at 37 °C. Proteinase K and trypsin were inactivated by heating to 95 °C for 15 min. EMEM provided a negative control. Crude haemolymph was autoclaved at 120 °C for 30 min or fractionated using ultra-centrifuge speeds (28,000, 60,000 rpm, optima^{TM} L-100 XP Ultracentrifuge, Beckman Coulter) and each supernatant tested for antiviral activity and protein content.

2.2.7. Cytotoxicity assays

Cytotoxicity of abalone extracts was measured using trypan blue exclusion assay to determine the percentage of dead cells on the cell monolayer (George *et al.* 1996). Vero cells were seeded at a concentration of 2 x 10^5 cells per well in 24-well plates and grown at 37 °C for 1 day until monolayer was 95% confluent. The culture medium was replaced with fresh medium containing abalone extracts and cells were grown for 2 days. As 0.5% DMSO was used for diluting lipophilic extract, the cytotoxicity test was performed with DMSO controls. The cells were detached with trypsin (5 mg ml^{-1}, Sigma), stained with 4% trypan blue (Sigma) in phosphate buffered saline solution (PBS, 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na$_2$HPO$_4$, 1.47 mM KH$_2$PO$_4$) for 30 min, and counted for blue dead cells after dilution to 10^5 cells ml^{-1}, using a Neubauer counting chamber. The maximum concentration of haemolymph and other tissue extracts used for antiviral screening assay was that which caused 10% of cell death (CD$_{10}$) relative to solvent and media negative controls.

2.2.8. Anti-HSV assay

Antiviral activity of abalone extract against HSV-1 was determined by plaque reduction assay, as described by Russell (1962), with minor modifications. Briefly, Vero cell monolayers were infected in quadruplicate with about 30-40 plaque-forming units (PFU) in 0.3 ml of HSV-1 for 1 h in 24 well-plates. During incubation, plates were gently shaken every 15 min. After 1 h incubation, medium containing abalone extract and unabsorbed virus was removed. Cells were then washed twice with sterile PBS, and overlaid with fresh medium with the same concentration of abalone extract and 1% methylcellulose. Cells were incubated for 2 days at 37 °C. Acyclovir (Sigma) was used as positive control for antiviral activity. Monolayers were fixed with 5% formaldehyde and stained with 4% toluidine blue in PBS, and plaques were counted using a light microscope. Antiviral activity was expressed as percentage reduction of plaque numbers. The size of plaque was taken to be its longest diameter (mm).

2.2.9. Timing of antiviral activity

Differing addition and residency times of abalone extracts relative to virus-cell incubation were employed in order to address the question of at which point in infection antiviral activity is exerted, similar to the procedure described by Olicard (Olicard *et al.* 2005b). For varying the timing of contact of abalone substances with virus and/or cells, five different protocols were employed (Fig. 2.1). Briefly, abalone extract samples were included in the plaque assay for one of the following times: (i) preincubated with cells for 2 h before infection and removed prior to infection, to identify if abalone extracts induce an antiviral state on the cells; (ii) preincubated with virus for 1 h (on ice) prior to infection and included with the viral infection for duration of the assay; (iii) added simultaneously with virus and included for duration

of the assay; (iv) added simultaneously with virus and removed 1 h after infection; (v) added 1 h after infection and included for duration of the assay (Fig. 2.1). Thus, in protocol (i) above, abalone extracts are present only prior to mixing virus and cells. In protocol (iv) above, abalone extracts are present only for 1 hr; however in protocols (ii), (iii) and (v), the extracts are present in the culture medium from the time they are added to the end of the assay.
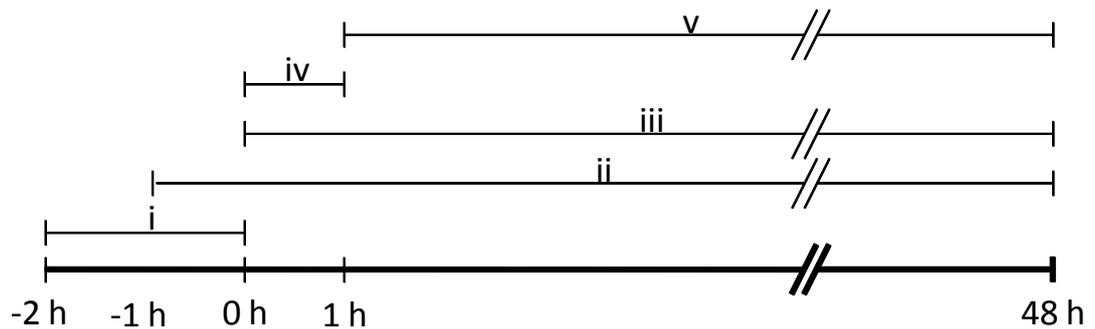
Figure 2.1. Differing infection protocols for addition and residency times of abalone extracts relative to virus-cell incubation. Abalone extracts were assayed at the maximum active concentration, causing less than 10% of cell cytotoxicity. (i) preincubated with cells for 2 hrs before infection and removed prior to infection, to identify if abalone extracts induce an antiviral state on the cells; (ii) preincubated with virus for 1 h (on ice) prior to infection and included with the viral infection for duration of the assay; (iii) added simultaneously with virus and included for duration of the assay; (iv) added simultaneously with virus and removed 1 h after infection; (v) added 1 h after infection and included for duration of the assay.

2.2.10. Attachment assay

The effects of abalone extracts on HSV-1 attachment were investigated using a modification of the assay described by MacLean (1998). Briefly, 30-40 PFU of virus and abalone extract at the maximum active concentration were added in quadruplicate to 4 °C prechilled Vero cell monolayers and incubated at 4 °C for 2

hrs. The negative control contained EMEM (with or without 0.5% DMSO). Cells were then washed with sterile PBS twice to remove abalone extract and unattached virus, overlaid with medium containing 1% methylcellulose, and incubated at 37 °C for 2 days before plaque count. To confirm that incubation at 4 °C allowed only viral attachment and not entry, cells to which virus had been preattached at 4 °C were treated with citric acid buffer: (40 mM citric acid, 10 mM KCl, 135 mM NaCl, pH 3.0) for 1 min to inactivate any particles that remained on the surface. The washing procedure resulted in 100% inhibition of plaque formation (data not shown).

2.2.11. Entry assay

To investigate the effect of abalone extracts on HSV-1 entry, a modification of a previously described assay (MacLean 1998) was used. Briefly, 30-40 PFU of virus was added in quadruplicate to 4 °C prechilled Vero cell monolayer and incubated at 4 °C for 2 hrs to allow for viral attachment. Abalone extract at the maximum active concentration were added to the cells, and the temperature was shifted to 37 °C for 1 h prior to inactivation of extracellular virus with citric acid buffer. The negative control contained EMEM (with or without 0.5% DMSO). Cells were overlaid with medium containing 1% methylcellulose, and incubated at 37 °C for 2 days before plaque count.

2.2.12. Virucidal assay

Virus was diluted with PBS to a concentration of 300-400 PFU ml^{-1} in centrifuge tubes. The maximum active concentration of abalone extract were then added and incubated at 4 °C for 2 hrs. The negative control contained EMEM (with or without 0.5% DMSO). The incubated samples were diluted with medium 10 fold then titrated

on Vero cells at 37 °C for 1 h prior to inactivation of extracellular virus with citric acid buffer. Cells were overlaid with medium containing 1% methylcellulose, and incubated at 37 °C for 2 days before plaque count. Another control was carried out to determine whether abalone extract at 10 fold dilution of its maximum active concentration induced any effect on viral attachment or entry.

2.2.13. Statistical analysis

All data are presented as means and standard error from at least three repeat experiments. For comparison of antiviral activity between treatment and control groups (e.g. for the same concentration of acyclovir, abalone haemolymph, or lipophilic extract of digestive gland), independent-samples t-tests were used (PASW/SPSS statistics 18). No statistical tests were made at 10^5 and 10^7 dilution of viral stock due to too many plaques in the control or two few plaques in treatment plates, respectively. Plaque numbers were compared between haemolymph concentration and addition times using a two-way ANOVA, with Tukey post hoc test. When equal variance is not assumed (Levene's test, $p<0.05$), Dunnett T3 post hoc test was used. $\alpha=0.05$ was set as the limit of significance (lowered to 0.01 if unequal variance was assumed). Correlation between protein concentration and antiviral activity of different haemolymph samples was identified using Pearson's correlation coefficient (PASW/SPSS statistics 18).

2.3. Results

Cytotoxicity assays were carried out to determine the concentration range of abalone haemolymph and peptide, lipophilic and non-lipophilic extracts of different tissues from adult abalone, greenlip *H. laevigata*, for antiviral screening against HSV-1 in

the non-toxic range for Vero cells. We considered an acceptable level of cytotoxicity to be when the abalone extract caused less than 10% of cell death (CD_{10}), as determined by trypan blue exclusion assay. Dimethyl sulphoxide (DMSO, 0.5% in distilled water) caused no cell death and no change in cell morphology, thus was used for dissolving the lipophilic extract before antiviral assay. The maximal concentrations of crude haemolymph and non-lipophilic and lipophilic extract from digestive gland of abalone that were not cytotoxic were 20% (v/v), 2,000 µg ml^{-1}, and 3,000 µg ml^{-1}, respectively (Table 2.1). Other abalone extracts appeared non-cytotoxic within the examined concentration range (Table 2.1, CD_{10}>1,000 µg ml^{-1} for all peptide extracts, >4,000 µg ml^{-1} for lipophilic and non-lipophilic extracts).

Within the non-cytotoxic range, antiviral activity was detected in haemolymph and the lipophilic extract of digestive gland of abalone using plaque reduction assay (Table 2.1). A series of two-fold dilutions of haemolymph and lipophilic extract of digestive gland were further examined for antiviral activity. Eagle's minimum essential medium (EMEM; Sigma) and 0.5% DMSO (in EMEM) were used as negative controls for antiviral assay of haemolymph and lipophilic extract of digestive gland, respectively. As there was no significant difference in number of plaques in the negative control plates (t=0.52, df=6, p=0.624), DMSO (0.5%, v/v) did not interfere with HSV-1 plaque formation. Crude haemolymph (20%, v/v) and lipophilic extract of digestive gland (3,000 µg ml^{-1}) induced up to approximately 96% and 100% protection of Vero cells from HSV-1 infection, respectively (Fig. 2.2). The positive control, acyclovir, induced 50% protection of Vero cells from HSV-1 infection at 0.48 µg ml^{-1} (Table 1) and at 2 µg ml^{-1} totally blocked HSV-1 replication (Fig. 2.2).

Table 2.1. Cytotoxicity and antiviral activity of haemolymph and lipophilic, non-lipophilic, and peptide extracts of different tissues from abalone *H. laevigata* in comparison to acyclovir (see protocol iii). Maximum concentration of abalone extracts tested caused death in less than 10% of Vero cells. DMSO for dissolving lipophilic extracts was also assessed for cytotoxicity and antiviral activity. The EC_{50} for abalone extracts and positive control acyclovir were determined by non-linear regression of sigmoidal dose-response curves using Graphpad Prism (Version 5, GraphPad Software, Inc.).

Abalone extracts and acyclovir control	Virus-plaque reduction assay		Cytotoxicity assay
	Range assay concentration	EC_{50}	CD_{10}
Crude haemolymph	1-40% (v/v)	6.23% (v/v)	20% (v/v)
Non-lipophilic extract of digestive gland	125-4,000 µg ml^{-1}	No	2,000 µg ml^{-1}
Other non-lipophilic extracts (head, gill, mantle, foot muscle)	125-4,000 µg ml^{-1}	No	No
DMSO	0.125-4% (v/v)	>4% (v/v)	1%[a] (v/v)
Lipophilic extract of digestive gland (crude lipid extract in 0.5% DMSO)	375-6,000 µg ml^{-1}	667 µg ml^{-1}	3,000 µg ml^{-1}
Other lipophilic extract (head, gill, mantle, muscle in 0.5% DMSO)	125-4,000 µg ml^{-1}	No	No
All peptide fractions (from a gradient of acetonitrile for different tissues including digestive gland, head, gill, mantle and foot muscle)	125-1,000 µg ml^{-1}	No	No
Acyclovir	0.1-2 µg ml^{-1}	0.48 µg ml^{-1}	No

EC_{50} Effective concentration required to inhibit HSV-1 plaque formation by about 50%

CD_{10} Concentration that causes 10% Vero cell death

[a] Concentrations equal to and above 1% caused changes in the morphology of Vero cells

No: Not active at the maximum test concentration
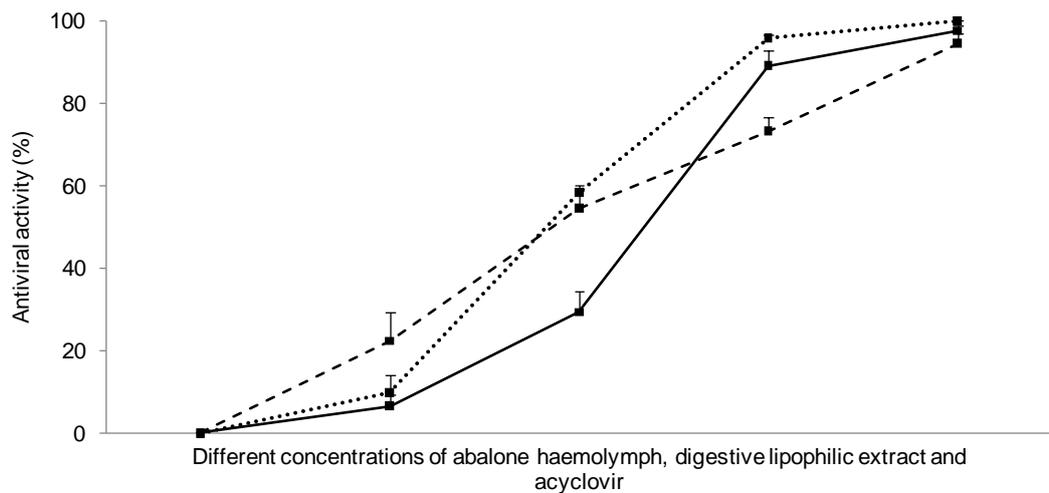
Figure 2.2. Activity against HSV-1, as % reduction in plaque numbers, for abalone haemolymph, lipophilic extract of digestive gland and acyclovir. Straight line, abalone haemolymph at different concentrations including 2.5, 5, 10 and 20%, v/v; dashed line, lipophilic extract of abalone digestive gland at 375, 750, 1500 and 3000 µg ml^{-1}; dotted line, acyclovir at 0.1, 0.5, 1 and 2 µg ml^{-1}. Abalone haemolymph, lipophilic extract of digestive gland and acyclovir were present during the course of viral infection (protocol iii). Means and standard error were derived from anti-HSV-1 assay in triplicate.

Acyclovir (2 µg ml^{-1}), abalone haemolymph (20%, v/v) and lipophilic extract of digestive gland (3,000 µg ml^{-1}) were tested for antiviral activity. There was a reduction in the number of plaques (>82%) and plaque size (>32%) in all test samples in comparison to relevant controls (EMEM for acyclovir and haemolymph; 0.5% DMSO in EMEM for the lipophilic extract of digestive gland, Table 2.2). Significant decreases in plaque numbers were confirmed for the positive control acyclovir (t=15.99, df=6, p<0.0001), abalone haemolymph (t=19.97, df=3.78, p<0.0001), and lipophilic extract of digestive gland (t=15.91, df=6, p<0.0001). Similarly, a reduction in plaque size was detected for acyclovir (t=5.75, df=14.57,

p<0.0001), abalone haemolymph (t=2.17, df=15, p=0.047) and lipophilic extract of
digestive gland (t=14.38, df=10, p<0.0001).

Table 2.2. Antiviral activity of acyclovir, abalone haemolymph and lipophilic extract
of digestive gland (used in protocol iii). The viral stock had a working concentration
of about 3 x 10^8 PFU per ml and was tested at three serial ten-fold dilutions (10^6-10^8
viral dilutions). The average number in each treatment and control was determined
from four replicate plates.

		Virus dilution		
		10^6	10^7	10^8
Negative control (EMEM)	Average number of plaques (s.e.)	TNC, ≥300	29.8 (±1.38)	2.25 (±0.63)
	Average plaque size (mm)		0.43 (±0.04)	
Acyclovir (1 µg ml^{-1})	Average number of plaques	6.75 (±1.11)	2 (±1.03)	0 (±0)
	Reduction in number of plaques (%)	97.8	93.3*	100
	Average plaque size		0.17 (±0.02)	
	Reduction in plaque size (%)		60.5*	
Haemolymph (20%, v/v)	Average number of plaques	2 (±0.71)	0.5 (±0.5)	0 (±0)
	Reduction in number of plaques (%)	99.3	98.3*	100
	Average plaque size		0.29 (±0.05)	
	Reduction in plaque size (%)		32.6#	
Negative control (5% DMSO in EMEM)	Average number of plaques	TNC, ≥300	28.8 (±1.31)	2 (±0.41)
	Average plaque size		0.44 (±0.01)	
Digestive lipid extract (3,000 µg ml^{-1})	Average number of plaques	30.8 (±2.87)	5 (±0.71)	0.25 (±0.25)
	Reduction in number of plaques (%)	89.8	82.6	87.5
	Average plaque size		0.1 (±0.02)	
	Reduction in plaque size (%)		77.3*	

TNC Too numerous to count

* Significant reduction in number plaques or plaque size in comparison to relevant control plates (one-way ANOVA, p<0.0001)

Insignificant reduction in plaque size (one-way ANOVA, p=0.07)

By adding haemolymph or lipophilic extract of digestive gland at differing times
(before, during and after infection), and varying the length of time the abalone
substances were in contact with the virus/cell culture, we tried to address the

question of at what time in infection do these abalone substances exert their antiviral activity (Fig. 2.1). The five differing protocols describing the times of contact of abalone substances with virus/cells are listed in "methods: timing of antiviral activity". Haemolymph acts at around the time of viral entry, while lipophilic extract of digestive gland blocks viral activity subsequent to entry (Fig. 2.3). The antiviral activity of haemolymph and lipophilic extract at different concentrations depend on at which time during the assay these substances are present (two-way ANOVA interaction between time and concentration of haemolymph, $F=21.69$, $df=16$, $P<0.0001$; lipophilic extract of digestive gland, $F=36.56$, $df=16$, $P<0.0001$). Pre-incubation of haemolymph with cells for 2 h prior to infection, with removal of haemolymph immediately before addition of virus to cells, as in timing protocol (i), did not reduce plaque number or size. Likewise, addition of haemolymph to the assay 1 h after virus is added to cells, in protocol (v), did not reduce plaque size or number (Tukeys HSD test, $p>0.5$). However the presence of haemolymph (at concentrations of 20%, v/v) during the time of viral entry into cells, whether pre-incubated with virus for 1 h before viral infection, or added simultaneously with virus (protocols (ii) and (iii), in each case left in the culture for the duration of the assay), or present during the first hour of virus-Vero cell incubation (protocols (iv)), gave rise to significant antiviral activity (Fig. 2.3A, $p<0.05$).

Additional assays were carried out to determine whether haemolymph affects virus binding or entry, or whether it has a direct virucidal effect. Because lipophilic extract was not active during the first hour of viral infection, it was tested for virucidal effect but not in the binding/entry assay. The assay for virus attachment showed that abalone haemolymph at the maximum active concentration of 20%, v/v,

reduces attachment of HSV-1 onto Vero cells (73.9% reduction in plaque formation*)*. The assay for virus entry showed that haemolymph did not significantly interfere with viral entry (14.6% reduction in plaque numbers). Haemolymph did not have a virucidal effect (<1% reduction in plaque numbers).
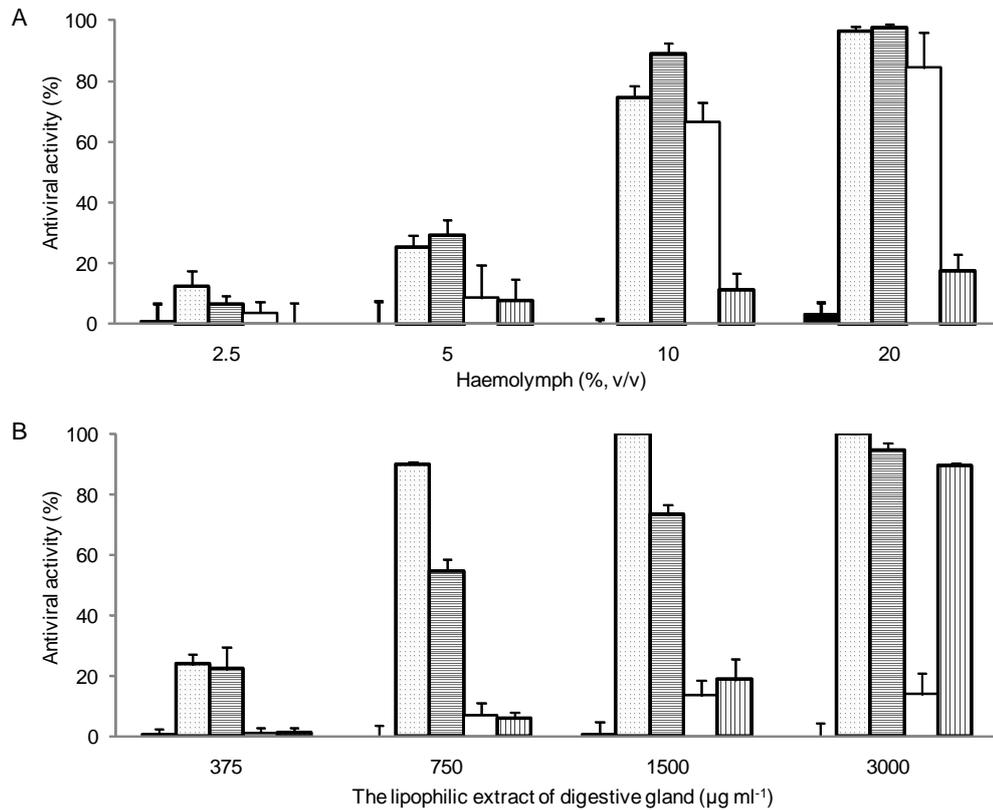
Figure 2.3. Antiviral activity of abalone A) haemolymph and B) lipophilic extract of digestive gland, at different concentrations and addition times in relation to HSV-1 infection of Vero cells. Black bars, preincubated with Vero cells for 2 h before infection (see protocol i); dot-filled bars, preincubated with virus for 1 h before infection and included with the viral infection for duration of the assay (protocol ii); horizontal line-filled bars, added simultaneously with virus and included for duration of the assay (protocol iii); blank bars, present only during the first hour of viral infection (protocol iv); and vertical line-filled bars, added 1 h after viral infection (protocol v). Means and standard error were derived from anti-HSV-1 assay in triplicate.

In contrast to haemolymph, lipophilic extract of digestive gland at 3,000 µg ml^{-1} has significant antiviral activity (Dunnett's T3 test p<0.05) when employed in infection protocols (ii), (iii) and (v), in which it is in contact with virus and cells from 1 h after infection (Fig. 2.3B). However, as seen in Fig. 2.3B, this abalone lipophilic extract has insignificant antiviral activity when present during the first hour of virus-Vero cell incubation (p=0.67). The virucidal assay showed that the lipophilic extract at the maximum active concentration of 3000 µg ml^{-1} has virucidal effect, reducing plaque numbers by 37.4% in comparison to the 0.5% DMSO control.

Experiments were carried out to characterize antiviral substances within the extracts. Three haemolymph plasma fractions were obtained by centrifuging crude haemolymph at 3,000, 28,000 and 60,000 rpm. Crude haemolymph was treated with heat (120 °C, 30 min), or proteinase K and/or trypsin. There was no difference in antiviral activity against HSV-1 between crude haemolymph and each of the treated haemolymphs (one-way ANOVA, F=0.377, df=7, P=0.91). Treatment of haemolymph with proteinase K or trypsin reduced protein content (OD$_{595}$ from 2.49 to 0.85 and 1.64, respectively) (Table 2.3). Gel electrophoresis confirmed that after overnight incubation of abalone haemolymph or bovine serum albumin with proteinase K and/or trypsin, degradation of protein occurred, producing protein fragments smaller than 5 kDa (data not shown). Nevertheless, antiviral activity of haemolymph was not affected by digestion with proteinase K, trypsin or the combined protein digestion. Inactivated proteinase K or trypsin did not interfere with HSV-1 plaque formation in controls (Table 2.1). Protein concentration in haemolymph samples was not significantly correlated with antiviral activity (Pearson correlation, r²=0.44, p=0.07).

Table 2.3. Characterisation of antiviral activity in abalone haemolymph (see protocol iii). Haemolymph samples, treated as described, were tested for protein concentration (absorbance measured at 595 nm) and antiviral activity (in quadruplicate, mean ± standard error).

Abalone haemolymph	Protein concentration ($\mu g \ ml^{-1}$)	Antiviral activity: % reduction in plaques
Crude haemolymph	2,318±25	86±1
Haemolymph plasma - supernatant fraction I (3,000 rpm/ 10 min)	1,987±22	89±6
Haemolymph plasma - supernatant fraction II (28,000 rpm/1 h)	1,768±12	83±6
Haemolymph plasma – supernatant fraction III (60,000 rpm/1 h)	1,280±22	82±7
Haemolymph treated with heat (120 ºC for 30 min, 3,000 rpm/10 min)	201±5	81±6
Haemolymph treated with proteinase K (100 $\mu g \ ml^{-1}$ at 37 ºC overnight) and heat (95 ºC for 15 min, 3,000 rpm /10 min)	793±34	82±5
Proteinase K control (100 $\mu g \ ml^{-1}$ in EMEM at 37 ºC overnight) and heat (95 ºC /15 min)	N/A	0
Haemolymph treated with trypsin (5 mg ml^{-1}, 37 ºC overnight) and heat (95 ºC/15 min, 3,000 rpm/10 min)	1,527±6	82±2
Trypsin control (5 mg ml^{-1} in EMEM, 37 ºC overnight) and heat (95 ºC, 15 min)	N/A	0
Haemolymph treated with proteinase K (100 $\mu g \ ml^{-1}$ at 37 ºC, overnight), trypsin (5 mg ml^{-1}, 37 ºC overnight), and heat (95 ºC for 15 min, 3,000 rpm for 10 min)	650±24	84±6

NA – Not applicable

Separation of abalone haemolymph by hydrophobic interaction chromatography revealed that antiviral activity was mostly in the fraction that passed straight through the dianion column (Table 2.4). No antiviral activity was detected in water and subsequent lipophilic fractions extracted from the column using water, methanol and/or acetone (Table 2.4).

Table 2.4. Characterisation of lipophilic or non-lipophilic component in abalone haemolymph for antiviral activity. Separation of fractions is described in the text.

Abalone haemolymph	Antiviral activity: % reduction in plaques
Haemolymph plasma control (freeze dried and reconstituted in the same volume of EMEM)	78±5
Non-lipophilic fraction of haemolymph (passed through the dianion HP-20SS resin column)	67±4
Water fraction of haemolymph	3±0.16
Lipophilic fraction 1 of haemolymph (eluted with H_2O/MeOH, 1:1)	0±0.09
Lipophilic fraction 2 of haemolymph (eluted with MeOH)	0±0.14
Lipophilic fraction 3 of haemolymph (eluted with MeOH/acetone, 1:1)	0±0.12
Lipophilic fraction 4 of haemolymph (eluted with acetone)	0±0.12

2.4. Discussion

This study demonstrates that abalone (*H. laevigata*) have activity against HSV-1 in their crude haemolymph (EC_{50}=6.23%, v/v) and lipophilic extract of the digestive gland (EC_{50}=667 µg ml^{-1}), but not in other tissues. This expands on previous studies that found activity in *H. rufescens* against polyoma, influenza A, and polio viruses (Li 1960; Li *et al.* 1962b). Consequently, abalone provide a source of antiviral

compounds. Further experiments will be required to determine whether activity against HSV-1 provides a suitable model for screening abalone for potential resistance to AVG. Studies are underway to test abalone extracts against marine herpesviruses such as koi herpes virus, salmonid herpes virus type 1 and salmonid herpes virus type 2.

Trypan blue assay revealed that incubation of haemolymph, the lipophilic or non-lipophilic extract of digestive gland of abalone at high concentrations for 2 days can cause Vero cell death. Since this cytotoxicity could interfere with HSV-1 replication, we used a maximum test concentration of 20% (v/v) for haemolymph, and 3,000 μg ml^{-1} and 2,000 μg ml^{-1} for the lipophilic and non-lipophilic extract of digestive gland respectively, for the antiviral assays. Within the non-cytotoxic range, antiviral activity was detected in the haemolymph and lipophilic extract of the digestive gland of greenlip abalone in a concentration-dependent manner. Indeed, increasing concentration of crude haemolymph to 20% (v/v) and the lipophilic extract of digestive gland to 3,000 μg ml^{-1} in the antiviral assay resulted in a reduction in number of HSV-1 plaques by about 98% and 95%, respectively. Similar activity against HSV-1 is also found in *C. gigas* haemolymph (protein concentration 600 μg ml^{-1}) (Olicard *et al.* 2005a) and a protein extract, L_{40} fraction, of periwinkle *Littorina littorea* haemolymph (protein concentration of 530 μg ml^{-1}) (Defer *et al.* 2009a). However, at these concentrations, *C. gigas* haemolymph and the L_{40} fraction of periwinkle *L. littorea* haemolymph cause much higher cytotoxicity (approximately 20 and 34%, respectively) than *H. laevigata* haemolymph and lipophilic extract of the digestive gland (approximately 10%).

To enter a cell, HSV-1 binds cellular glycosaminoglycan chains (WuDunn *et al.* 1989) such as heparin sulfate (Lindahl *et al.* 1994) or chondroitin sulfate (Banfield *et al.* 1995; Stringer *et al.* 1997; Mardberg *et al.* 2002) via the viral glycoproteins gC and gB, (Tal-Singer *et al.* 1995; Qie *et al.* 1999). HSV-1 entry is mediated by fusion of the viral envelope with the cell membrane, involving viral glycoproteins gB, gD, gH, gL and specific cellular receptors (Montgomery *et al.* 1996; Spear 2004; Browne 2009).

Fusion of HSV-1 into Vero cells is a relatively fast process, mostly taking place within the first 10-15 minutes of virus-cell incubation at 37 ℃ (Koyama *et al.* 1987; Andersen *et al.* 2004). Thus, different addition times of abalone extracts, relative to the time of addition of virus to cells, have been used here to identify the stage of infection at which their antiviral activity is exerted. Antiviral activity of haemolymph was significant if it was present during the first hour of virus-cell incubation (Fig. 3A), suggesting that its action may be due to inhibiting an early stage of infection. The attachment and entry assay results are consistent with haemolymph reducing HSV-1 binding to Vero cells, although at this point we cannot absolutely preclude the possibility that a haemolymph component is internalized simultaneously with the virus to exert its effect on a post-entry event in infection. Antiviral action resembling that described here has been found in the milk protein lactoferrin, its pepsin cleavage product lactoferricin and a number of other highly cationic α-helical peptides, which mediate their activity by binding heparin sulfate on the cellular surface and blocking attachment or entry of HSV-1 (Marchetti *et al.* 1996; Andersen *et al.* 2003; Andersen *et al.* 2004; Jenssen *et al.* 2004b; Jenssen *et al.* 2004a; Jenssen 2005). Prevention of viral entry could also be due to sugar or other compounds with a high affinity to viral attachment/entry glycoproteins, competing

with cellular receptors, for example, chondroitin sulfate type E derived from squid cartilage (Bergefall *et al.* 2005).

In contrast, antiviral activity of lipophilic extract of digestive gland was most notable when added 1 h after viral infection. This suggests that the extract may be acting at a stage of infection subsequent to cellular entry (e.g blocking the uncoating of viral genomes or the trafficking of virion components). Recently, bovine lactoferrin, apart from inhibiting viral attachment and/or entry, has been shown to inhibit cell-to-cell viral spread, possibly by interacting with the structural viral proteins ICP5 (major capsid protein) and VP16 (viral tegument protein) (Jenssen *et al.* 2008). Lactoferrin, and its cleavage product lactoferricin, also interfere with trafficking of HSV-1 capsids along microtubules towards the nucleus (Marr *et al.* 2009). Being components of innate immunity, it is possible that the mechanism of the antiviral activity in the lipophilic extract of abalone digestive gland resembles that of lactoferrin. In contrast to lactoferrin, the lipophilic extract showed no effect on viral attachment or entry but had a virucidal effect when incubated with free virus. The antiviral compound in lipophilic extract could be internalized simultaneously with the virus to exert its effect at early stage of post-entry events such as transport, capsid uncoating or transcription. Similar to abalone haemolymph, the lipophilic extract does not give rise to an antiviral state in Vero cells. We are unable to exclude the lipophilic extract having an antiviral effect later in infection.

The digestive gland is the largest organ in gastropods and is a relatively complex organ linked by ducts to the stomach. It is a site for intracellular and extracellular digestion, absorption of digestive products, excretion and storage of reserves (Kay *et al.* 1998). A range of enzymes is excreted by the digestive gland, including lipases,

proteases and carbohydrases. However, these enzymes are not lipophilic and thus are unlikely to be responsible for the antiviral activity observed in the digestive gland. On the other hand, there is potential for dietary derived secondary metabolites to contribute activity in the digestive gland. Grazing gastropods are well known to acquire bioactive compounds from their algal diets for use in their own defence (e.g. (Faulkner 1984a; 1992). Antiviral activity (or compounds) has also been previously reported from a range of red and green algae (Ivanova *et al.* 1994; Hayashi *et al.* 1996b; Hayashi *et al.* 1996a; Ohta *et al.* 1998; Smit 2004; Park *et al.* 2005), which are consumed by abalone. However, it should be noted that the abalone used in this study were sourced from an aquaculture farm and fed an artificial pellet diet, with no known antiviral properties. Thus, it seems likely that the antiviral factors in the digestive gland are intrinsic to abalone.

A range of antiviral proteins and peptides are found in many marine species such as littorein, produced by the common periwinkle *L. littorea* (Defer *et al.* 2009a); defensins, produced by the Mediterranean mussel, *Mytilus galloprovincialis* (Roch *et al.* 2004), and hemocycanin, produced by the shrimp *Penaeus monodon* (Zhang *et al.* 2004). However, we have shown that anti-HSV-1 activity was retained after most protein in abalone haemolymph was destroyed by heat and proteinase treatment. In addition, no correlation exists between antiviral activity and protein concentration of crude haemolymph, three different plasma fractions (I, II and III), and heat or proteinases treated haemolymph samples. A possible interpretation is that small heat-resistant peptides, remaining after proteinase K and trypsin digestion, rather than large peptides, are responsible for the abalone anti-HSV-1 activity. Indeed, small peptides including lactoferricin (25-49 amino acids), defensins (29–33), indolicidin (13), brevenin-1 (24), protegrins (18), tachyplesin-1 (17), melittin (26), clavalin (23)

and megainin (23) have anti-HSV activity (Yasin *et al.* 2000; Andersen *et al.* 2003; Albiol Matanic *et al.* 2004; Marr *et al.* 2009). Separation of haemolymph on a hydrophobic dianion resin column further revealed that antiviral activity is not due to lipophilic active compounds (e.g. fatty acids and aliphatic or aromatic alkaloids), but more likely due to sugars, acids or small polar peptides/proteins, which would pass through the column or elute in the water fraction (Einbond *et al.* 2004). Further investigation is needed to elucidate the chemical structure of the antiviral compounds and the details of their mechanism of action.

In summary, two types of antiviral activity have been detected in abalone *H. laevigata*, with different modes of action against HSV-1. Many antiviral compounds produced by marine invertebrates are part of their innate defense against viruses (Pan *et al.* 2000; Zhang *et al.* 2004), however it remains to be determined whether the compounds with activity against HSV-1 in this study could also be effective against AbHV and other viruses. As phlebotomy of abalone is quick and non-lethal, their haemolymph can also be used in future studies aimed at understanding molluscan defence systems.

Acknowledgments

CHAPTER 3: Variation in the antiviral and antibacterial activity of abalone *Haliotis laevigata*, *H. rubra* and their hybrid in South Australia

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Abstract

Abalone (Haliotidae), well-known commercial gastropods, have experienced large scale disease outbreaks such as abalone viral ganglioneuritis caused by a herpesvirus and summer mortality typically caused by bacteria such as *Vibrio harveyi*. Identification of the factors that influence antimicrobial activity could assist future management of disease in the abalone industry. A proportion of abalone naturally survive these outbreaks (5-40%) raising the possibility that some abalone are relatively resistant. Identifying such abalone could enable breeding of resistant populations. This study applied *in vitro* assays to investigate antiviral and antibacterial activity of abalone haemolymph. Comparisons were made among *Haliotis laevigata* (greenlip), *H. rubra* (blacklip) and their hybrid. Intraspecific variation was examined at the individual scale, as well as between commercial aquaculture family lines and natural populations. Abalone sourced from the wild showed higher antiviral and antibacterial activities than those from a land-based farm. We found no significant difference in antiviral activity between greenlip, blacklip and hybrid abalone (p>0.05). The antibacterial activity of greenlip abalone was also similar to blacklip, but significantly lower than hybrid (p=0.001). There was substantial individual variation among abalone (maximum range of 31-69% for antiviral activity and 4-46% for antibacterial activity) within the same family line or geographic location. Antiviral and antibacterial activity increased slightly with an increase in shell length, and a 2yr old family line had lower activity than 3yr old family lines. There was no significant effect of gender or reproductive activity on antiviral or antibacterial status (p>0.05). Further investigation is required to establish

whether the individual variability in antimicrobial activity is heritable in breeding programs and whether higher activity confers greater resistance to disease.

3.1. Introduction

Abalone are herbivorous marine molluscs and important economic species worldwide (Fleming *et al.* 1996; Cook 1998; Godoy *et al.* 1998; Gordon *et al.* 2001; 2004). Among more than 11 species found along the Australian coast, greenlip *Haliotis laevigata* and blacklip *H. rubra* are the two most commercially important (Freeman 2001). A significant threat for the Australian abalone industry is the emerging disease, abalone viral ganglioneuritis, reported in *H. laevigata, H. rubra* and their hybrid cross (Hooper *et al.* 2007a). Similar viral infections have been reported in *H. diversicolor supertexta* in Taiwan (Chang *et al.* 2005) and *H. diversicolor reeve* in China (Wang *et al.* 2004). In Australia, abalone ganglioneuritis is caused by a herpesvirus (Savin *et al.* 2010), impairing the cerebral nerves and resulting in up to 95% mortality within 14 days from the onset of clinical signs (Chang *et al.* 2005; Hooper *et al.* 2007a).

Another major threat for the abalone industry is associated with *Vibrio* bacteria, some of which are common marine pathogens (Austin *et al.* 2006). *V. harveyi* and *V. splendidus I* have been isolated from moribund abalone during disease outbreaks in Tasmania, Australia (Handlinger *et al.* 2005). *V. harveyi* has been linked to summer mortality in *H. tuberculata* populations in France (Travers *et al.* 2008; Travers *et al.* 2009) and is also thought to contribute to summer mortality on aquaculture farms in South Australia (Vakalia *et al.* 2005). *V. harveyi*, (syn *V. carchariae)*, is a cause of high mortality of abalone in Japan (Nishimori *et al.* 1998) and France (Nicolas *et al.* 2002). These Gram-negative bacteria can cause septicaemia, resulting in up to 80% mortality (Nicolas *et al.* 2002; Handlinger *et al.* 2005). Typically *Vibrio* spp. are opportunistic and lead to disease when immunity is suppressed as a result of stressors

such as high temperature, poor water quality or handling (Malham *et al.* 2003; Cheng *et al.* 2004b; Cheng *et al.* 2004d; Cheng *et al.* 2004a; Cheng *et al.* 2004e; Cheng *et al.* 2004c; Hooper *et al.* 2007b; Day *et al.* 2010).

Reproduction is an important stressor that can impact the immunity of molluscs (Taskinen *et al.* 1999; Duchemin *et al.* 2007; Li *et al.* 2007; Matozzo *et al.* 2010), including abalone (Travers *et al.* 2009). In oysters, it has been shown that the high energetic cost of reproduction diverts energy from other biological processes, resulting in immune depression (Pouvreau *et al.* 2003; Soletchnik *et al.* 2006; Li *et al.* 2007; Li *et al.* 2009b). Antiviral activity of oyster haemolymph against herpes simplex virus type 1 (HSV-1) was found to increase from winter/spring to summer/autumn, when the spawning typically occurs (Olicard *et al.* 2005a). Ripe abalone (*H. tuberculata*) were found to be more susceptible to bacterial infection than immature abalone and mortality increased in conjunction with reproductive stress in the field (Travers *et al.* 2009). Furthermore, during gametogenesis there was evidence of immune-depression, such as reduced phagocytosis and phenoloxidase activity (Travers *et al.* 2008). Thus, reproductive status and activity both appear to be factors influencing the intraspecific variability of immune defense in bivalve molluscs.

Abalone immunity is principally based on their circulating haemocytes and secreted effectors (Hooper *et al.* 2007b). Haemocytes are involved in numerous innate defenses such as chemotaxis, lectin-mediated pathogen recognition, phagocytosis, encapsulation and elimination of pathogens via enzymatic destruction and/or by production of antimicrobial compounds (Cheng 1981; Mitta *et al.* 2000; Bachère *et al.* 2004; Hooper *et al.* 2007b). Antimicrobial compounds are a major component of

innate immunity and they can be constitutively expressed or rapidly induced to provide a prompt response to invading microorganisms (Tincu *et al.* 2004; Otero-Gonzalez *et al.* 2010). The antibacterial activity of abalone haemolymph has been demonstrated against marine *Vibrios* using *in vitro* assays (Vakalia *et al.* 2005; Day *et al.* 2010). Recently, we have shown that plasma of abalone haemolymph also contains antiviral compounds using the plaque assay for HSV-1 (Dang *et al.* 2011b). In the absence of mollusc cell lines for culturing abalone herpesvirus, the use of another herpesvirus on a compatible cell line (Vero) facilitates antiviral screening. Genome sequence and phylogenetic analysis of DNA polymerase proteins suggest that the abalone herpesvirus of the family *Malacoherpesviridae* is related to other members of the *Herpesviridae* including HSV-1 (Fegan *et al.* 2009; Savin *et al.* 2010). These *in vitro* antibacterial and antiviral assays have been broadly applied to investigating immunity of molluscs (Defer *et al.* 2009b; a), particularly the Pacific oyster *Crassostrea gigas* (Olicard *et al.* 2005a; Olicard *et al.* 2005b; Li *et al.* 2007; Li *et al.* 2009e; d; Li *et al.* 2009b) and thus are useful tools for screening antimicrobial defense in abalone.

The small proportion of abalone that survive microbial disease outbreaks may provide insights into their resistance mechanisms. It is possible that survivors have better adapted immune mechanisms than animals that succumb. For AVG infection, some healthy abalone have been found to contain viral DNA using real time polymerase chain reaction (PCR) (Crane *et al.* 2009), suggesting these animals had a subclinical infection. In the oyster *C. gigas*, larvae and juveniles are more sensitive to herpesvirus infections than adults (Renault *et al.* 2001a; Arzul *et al.* 2002b) and viral DNA was found in adults of normal appearance (Arzul *et al.* 2002a). Regarding *Vibrio* infection, a low level of mixed *Vibrio* species has been isolated from

clinically normal abalone held for experimental trials (Handlinger *et al.* 2005) and from healthy abalone on aquaculture farms (Vakalia *et al.* 2005). Thus the notion that some abalone possess an enhanced ability to resist infections relative to others is plausible. Identification of abalone with stronger immunity could be used to develop disease resistant populations. The first step towards investigating this is to examine intra and inter-specific variability in abalone immunity. We used *in vitro* assays to screen for antiviral activity against HSV-1, as well as antibacterial activity against *V. harveyi* in the haemolymph of *H. laevigata, H. rubra*, and their commercial hybrid. We also investigated variability between individuals, sexes, farmed family lines and wild populations from different geographic locations, as well as for farmed greenlip abalone before and after spawning.

3.2. Materials and methods

3.2.1. Abalone

Nine male and nine female abalone were obtained from each of four farmed family lines: greenlip FL-1 and FL-2 bred from different sets of commercial parents in 2007 and 2006, respectively; greenlip FL-3 from wild Elliston parents in 2006; and hybrid FL-4 from commercial greenlip and blacklip in 2006. These family lines were grown separately in concrete raceways on a land-based aquaculture farm in Port Lincoln, South Australia under the same diet and water conditions, including temperature, oxygen saturation and salinity. Wild greenlip abalone were collected in July 2009 from South Australian coastal sites including Cowell (n = 4), Elliston (n = 11), Farm Beach (n = 25) and Blackfellows Caves (n = 8). Wild blacklip abalone were collected from Elliston (n = 11), O'Sullivan Beach (n = 9) and Blackfellows Caves (n = 11) (Appendix 3.1). All collected abalone appeared healthy and firmly attached

to the substrate. They were transported to holding facilities on the farm in Port Lincoln or Flinders University in Adelaide and acclimatized prior to testing. Abalone were tagged on arrival using 3mm spring tags (Mollusc Pty Ltd, Victoria) and fed a standard 5mm commercial abalone diet (EP Aquafeeds, South Australia).

3.2.2. Spawning induction

Haemolymph samples were collected from 26 abalone of the FL-2 family line (day 1). Sixteen of these abalone were then induced to spawn (spawned group, 8 males and 8 females) using standard commercial protocols, while the remaining ten (unspawned/control group, 5 males and 5 females) were kept in a commercial broodstock tank. To achieve spawning, water temperature was raised by 5 °C using portable 300w heaters (Aqua One) with ultraviolet light passing through the reproduction tanks for 3 hrs (Hahn 1989a; Uki 1989). Spawning occurred between 12 and 24 hrs post-induction (day 2). Additional haemolymph samples were then taken from all abalone of spawned and control groups (day 3).

3.2.3. Haemolymph collection

All abalone were measured for maximum shell length with calipers (accurate to 0.1 cm) before collection of haemolymph samples. The maximum volume of haemolymph collected from each abalone was 50 µl g^{-1} (Chen *et al.* 1996). This non-lethal bleeding procedure was performed from the anterior sinus of abalone using a pre-cooled sterile syringe and 25G needle. Haemolymph samples were frozen at -80 °C until required.

3.2.4. Cell culture and virus

African green monkey kidney cells (Vero) were grown in EMEM (Sigma) supplemented with 10% newborn calf serum (NCS; Sigma) and 1% antibiotics (PCS; 10,000 IU ml^{-1} penicillin, 25,000 IU ml^{-1} colymicin, 10 mg ml^{-1} streptomycin; Sigma) at 37 °C in a humidified atmosphere of 5% CO_2. A well-characterized strain, SC16 (Speck *et al.* 1991; Speck *et al.* 1992) of wild type HSV-1 was obtained from Dr Tony Simmons at the Institute of Medical and Veterinary Science, Adelaide. Virus titer was calculated using the standard limiting dilution method (Reed and Muench 1938, Defer *et al*. 2009; Dang *et al*. 2011).

3.2.5. Anti-HSV assay

Antiviral activity of abalone extract against HSV-1 was determined by plaque reduction assay, as described by Russell (1962), with minor modifications. Briefly, Vero cell monolayers were infected in triplicate with about 40-50 plaque-forming units (PFU), in a volume of 0.3 ml, in presence of haemolymph (6%, v/v) for 1 h in 24 well-plates. Haemolymph (6%) was used throughout to compare antiviral activity of abalone (EC_{50} = 6.23%, v/v) (Dang *et al.* 2011b). During incubation, plates were gently shaken every 15 min. After 1 h incubation, medium containing abalone extract and unabsorbed virus was removed. Cells were then washed twice with sterile PBS, and overlaid with fresh medium with the same concentration of abalone haemolymph and 1% methylcellulose. Cells were incubated for 2 days at 37 °C. Monolayers were fixed with 5% formaldehyde and stained with 4% toluidine blue in PBS, and plaques were counted using a light microscope. Antiviral activity was expressed as percentage reduction of plaque numbers.

3.2.6. Bacteria

Stock cultures of *V. harveyi* TCFB 1477 were obtained from the Fish Aquatic Health Unit, Department of Primary Industries, Tasmania, and cultured directly into broth for use in the antibacterial assays. The bacterial isolate was identified using MicroSys V36 identification kit for the *Vibrios* (Carson *et al.* 2006). For antibacterial assays, *V. harveyi* was cultured in sterile nutrient broth (1 g NaCl, 2 g yeast extract and 1 g peptone per 100 ml distilled H_2O) overnight at 37 ºC on an orbital mixer shaker (Ratek) at 200 rpm. The cultures were diluted to an optical density at 600 nm (OD600nm) of 0.1 with a spectrophotometer (Metertech, UV/VIS SP8001) and returned to exponential growth phase (OD600nm=0.18-0.2) prior to use in antimicrobial assays.

3.2.7. Antibacterial assay

Antibacterial activity in the haemolymph plasma against *V. harveyi* was measured using MTS assay as described by Li et al. (2007). The MTS assay was based on reduction of MTS tetrazolium to a red formazan product by dehydrogenase enzymes from live cells (Cory *et al.* 1991). 90 μl of haemolymph plasma and 10 μl of *V. harveyi* in exponential growth culture were added into a 96-well plate in triplicate. Negative controls had 90 μl haemolymph in 10 μl nutrient broth and positive controls had 10 μl of *V. harveyi* in 90 μl of nutrient broth. After 30 min incubation, 20 μl of CellTitre 96® Aqueous One Solution (Promega, NSW, AUS) was added to each well. The plates were then incubated for a further 2 hrs or until development of the red formazan product in positive controls. Absorbance was measured at 492 nm using a 96-well plate reader (FluoStar Omega). Antibacterial activity (%) was

calculated from 100 – [(treatment absorbance-negative control absorbance)/positive control absorbance] x 100.

3.2.8. Statistical analysis

All data are presented as means and standard error from at least three repeat experiments. Mixed model univariate analyses were undertaken using Primer V6 with PERMANOVA add on. A minimum of 999 permutations of the residuals were undertaken using Euclidean distance resemblance matrices from the antiviral and antibacterial activity data. A three-factor PERMANOVA was performed with fixed factors for source (farmed vs. wild), species (*H. laevigata*, *H. rubra* and the hybrid) nested in source and population (family line or geographic location of wild populations) nested in species and source. To investigate the influence of gender and size on the antiviral and antibacterial properties, a three-factor ANCOVA was performed on the *H. laevigata* data. Size was used as a covariate, with gender, source (farm vs. wild) and populations (nested in source) used as fixed factors in the analysis. Pairwise tests were undertaken when a significant difference among species or populations was detected. The limit of significance was lowered to $\alpha=0.025$ according to Bonferroni adjustment, for repeated use of the same data in the two analyses (Hochberg 1988; Abdi 2007).

The correlation between antiviral activity and antibacterial activity of all abalone haemolymph samples was tested using Pearson's correlation coefficient (PASW/SPSS statistics 18). A two-way repeated-measures ANOVA on PASW/SPSS statistics version 18 was used to compare the same spawned and non-spawned abalone, at day 1 (prior to spawning) and day 3 (after spawning).

3.3. Results

Using plaque reduction assay for screening antiviral activity of abalone haemolymph against HSV-1, we found a large variation (maximum range of 31-69%) across individuals within the same farmed family line or wild population (Fig. 3.1A). The coefficient of variation within populations (33.6%) was higher than between populations (12.6%), demonstrating that most of the variation is at the individual level. MTS assay also revealed a large variation in antibacterial activity of haemolymph against *V. harveyi* (maximum range of 6-46%) across individuals within the same farmed family line or wild population (Fig. 3.1B). The coefficient of variation within populations (26.4%) was higher than between populations (18.1%). There was a weak correlation in antimicrobial activity of abalone haemolymph samples when tested across all individuals (n=190), with only 3% of the variation in antiviral activity being explained by variation in antibacterial activity (Pearson correlation, r = 0.17, p=0.02).
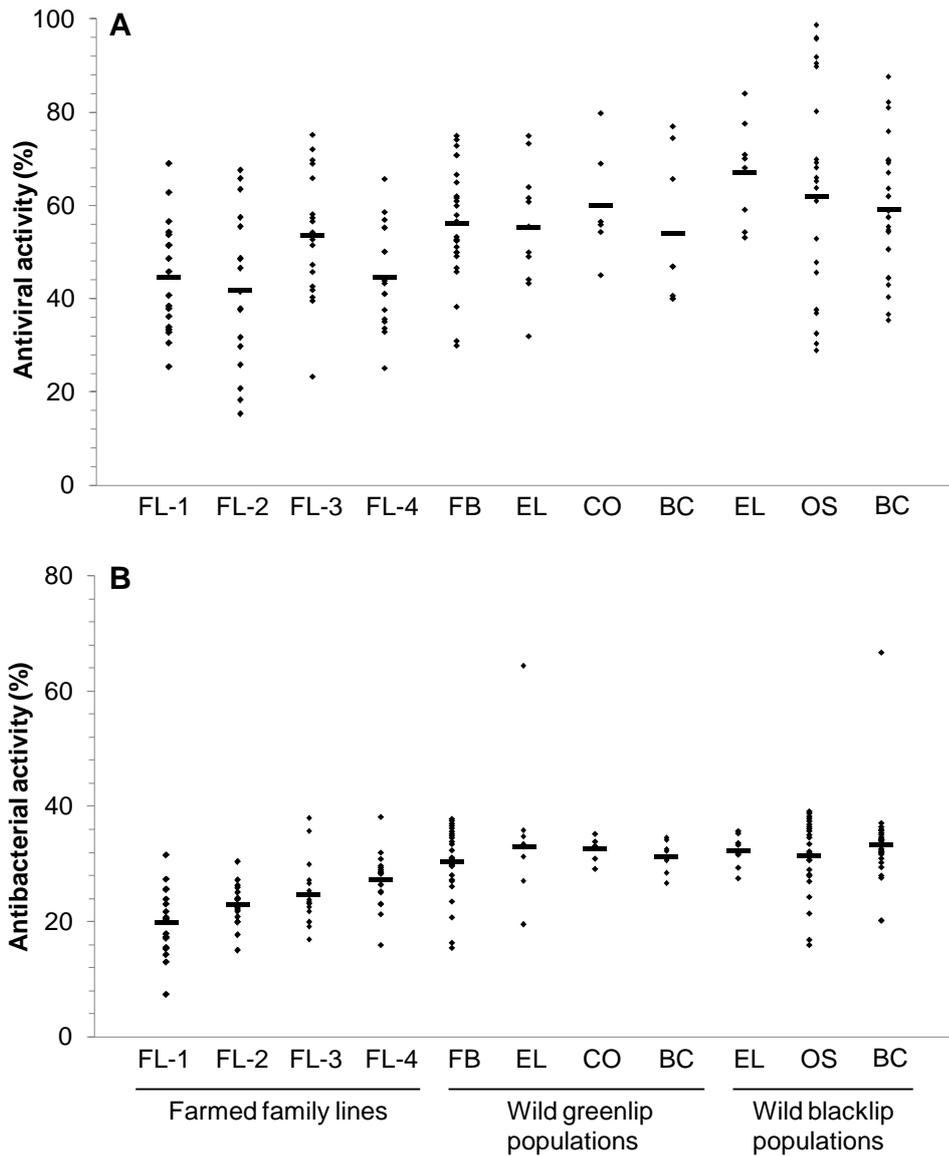
Figure 3.1. Variation in A) antiviral activity (%) and B) antibacterial activity (%) at individual, family line, population and species scale. All haemolymph plasma samples were tested in triplicate at the same concentration against HSV-1 (6%, v/v) using plaque reduction assay and *V. haveyi* using MTS assay. Antiviral and antibacterial activity was compared for three greenlip family lines (FL-1, FL-2 and FL-3) and one greenlip and blacklip hybrid (FL-4), as well as four wild populations of greenlip abalone *H. laevigata* (FB-Farm Beach, EL-Elliston, CO-Cowell, BC-Blackfellows Caves) and three wild populations of blacklip abalone *H. rubra* (EL, OS-O'Sullivan Beach, BC). Bars represent means of activity for each abalone group.

Overall, haemolymph from farmed abalone was found to have lower antiviral activity than that from wild-sourced abalone (Fig. 3.2A) and the three factor PERMANOVA revealed a significant difference according to source (Pseudo F=27.87, p=0.001). There was no difference in antiviral activity of haemolymph of the different species (Pseudo F=1.98, p=0.14), encompassing comparison of *H. laevigata* and *H. rubra* from wild populations (Fig 3.2A), as well as *H. laevigata* family lines and the hybrid cross (FL-4, Fig 3.1A). There was also no significant difference in antiviral activity between the different geographic populations of wild abalone or the farmed greenlip family lines (Pseudo F=1.18, p=0.3).

Similar to antiviral activity, the antibacterial activity of abalone haemolymph collected from the farm was significantly lower than that of abalone from wild populations (Fig 3.2B, Pseudo F=42.7, P=0.001). A significant difference in antibacterial activity was detected according to species (nested in source, Pseudo F=3.92, p=0.026). Pairwise tests revealed that the *H. laevigata* x *H. rubra* hybrid (FL-4) had higher antibacterial activity than the other *H. laevigata* family lines (Fig. 3.1B, t=3.51, p=0.001). However, there was no significant difference between wild populations of *H. laevigata* and *H. rubra* (Fig.3.2B, t=0.31, p=0.75).
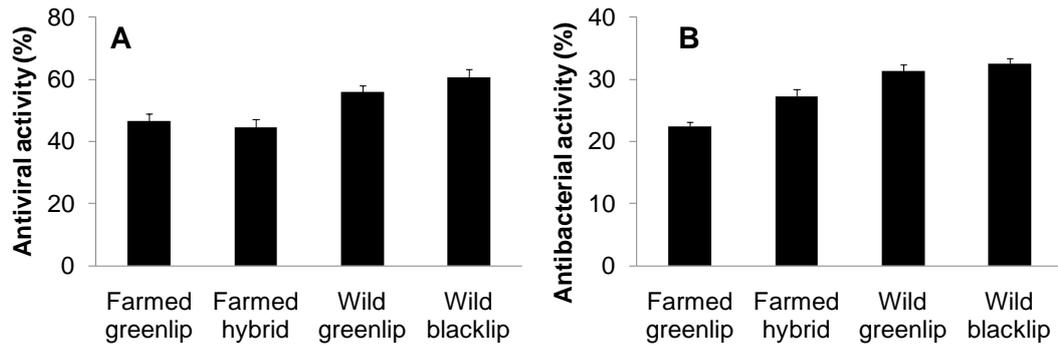
Figure 3.2. Comparison of A) antiviral activity (%) and B) antibacterial activity (%) between farmed greenlip *H. laevigata* (n=54), farmed hybrid *H. laevigata* x *H. rubra* (n=18), wild greenlip *H. laevigata* (n=51) and wild blacklip *H. rubra* (n=59) abalone. Means and standard error were derived from antiviral or antibacterial assay for haemolymph samples from the same abalone group.

Further investigation of the factors influencing antimicrobial activity was undertaken using populations of the green lip abalone *H. laevigata.* ANCOVA revealed that the shell length of abalone significantly influenced their antiviral activity (Pseudo F=11.12, p=0.001). Antiviral activity was found to increase slightly with an increase in shell length, although shell length explains less than 9% of the total variation in activity (Fig. 3.3A). Overall, abalone sourced from the wild populations were larger (Fig. 3.3) and antiviral activity was again found to vary according to source (farm vs. wild, Pseudo F=7.46, p=0.009). There was no significant difference in antiviral activity between populations nested within source (Pseudo F=1.27, p=0.29). However, farmed greenlip abalone from line FL-2 were larger (mean 9.61±0.14 cm), than the other lines (Fig. 3.3), including two in the same age cohort (3yrs, FL-4 hybrid mean 8.39±0.28; FL-3 mean 7.69±0.22 cm). There was no significant difference in antiviral activity according to gender (Pseudo F=0.003, p=0.96) and no interactions between gender and/or size with source or family lines (p>0.2). This implies that the effect of size was consistent across all populations, whereas gender did not affect the activity in any of the populations.
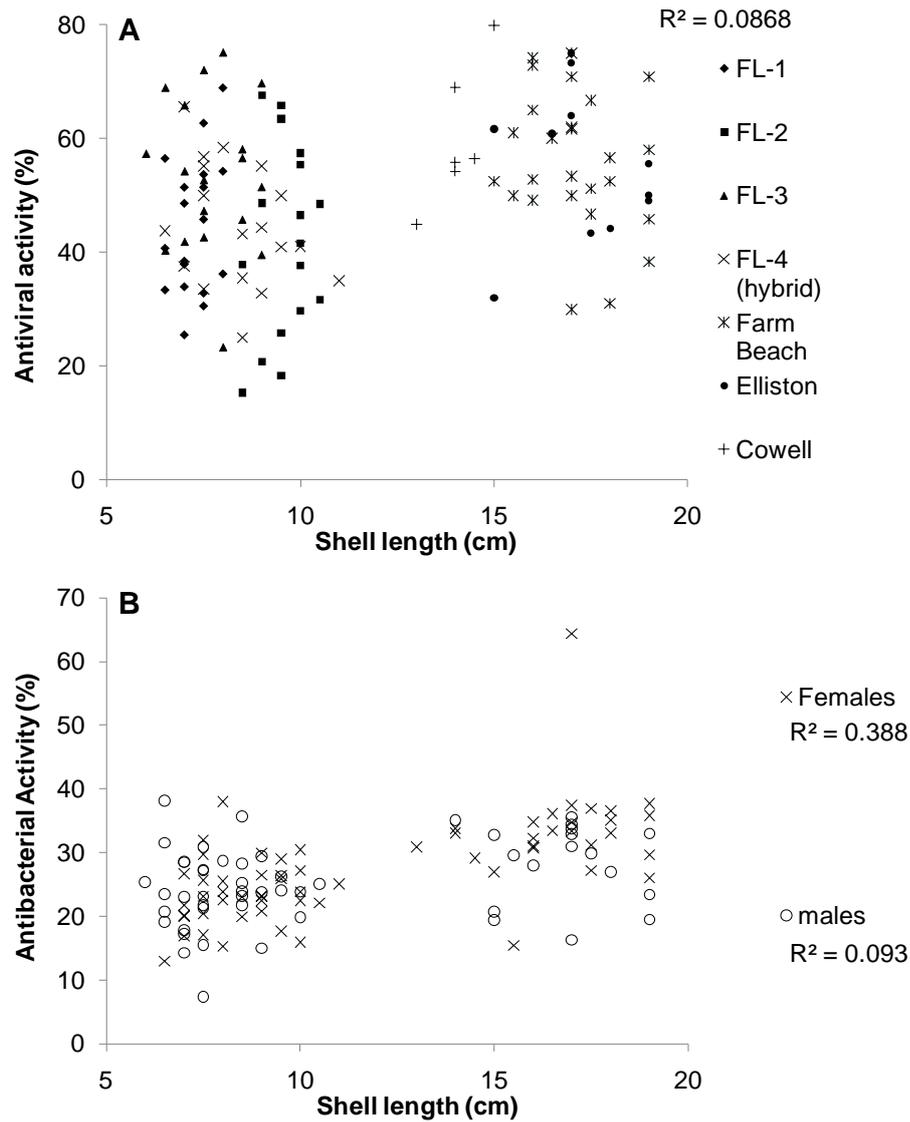
Figure 3.3. Correlation between the shell length (cm) and A) antiviral activity (%) and B) antibacterial activity (%). In A, data is grouped according to farmed family lines (FL 1-4) and geographic location for wild populations to illustrate the differences in shell length between populations and farmed family. In B, data is grouped according to sex, due to a significant interaction between size and sex in the PERMANOVA for antibacterial activity (P = 0.045).

Regarding antibacterial activity, ANCOVA again revealed a significant effect of shell length (Pseudo F=41.8, p=0.001). However, there was also a significant interaction between the shell length and gender (Pseudo F=4.9, p=0.045), implying

that the nature of correlation between antibacterial activity and shell length depends on gender. In both sexes, antibacterial activity increased with shell length (Fig 3.3B), however shell length explains 38.8% the total variation in antibacterial activity of females and less than 10% in males. Nevertheless, there was no overall significant effect of gender on antibacterial activity (Pseudo F=1.8, p=0.17). When size was taken into consideration, there was no significant difference in antibacterial activity according to source (Pseudo F=1.83, p=0.2) and no interactions between source and size (Pseudo F=1.92, p=0.19) or source and sex (Pseudo F=0.15, p=0.72). However, there was a significant difference in antibacterial activity between populations nested within source (Pseudo F=3.03, p=0.022). Pairwise tests on the farmed family lines revealed that the 3yr hybrid FL 4 had significantly greater antimicrobial activity than the 2yr greenlip family FL-1 (Fig. 3.1B, t=4.69, p=0.001). There was a tendency for FL-1 to have lower activity than the 3yr greenlip family line FL-3, but this was not significant (t=2.08, p=0.051). There was no significant different in the antibacterial activity between the hybrid FL-4 and the two 3 yr greenlip family lines (FL-2 & FL-3; p>0.05). There were no significant differences between the wild populations from different geographical locations (p>0.27). There were also no significant interactions between population and gender or size (p>0.3)

Reproductive status of the abalone did not appear to impact their antiviral activity (two-way repeated-measures ANOVA, F=0.18, p=0.67) or antibacterial activity (two-way repeated-measures ANOVA, F=0.13, p=0.72), as no significant difference was found in prespawning vs. spawned animals from family line FL-2 (Fig. 3.4A&B). There was no significant interaction between day and spawning for antiviral activity (F=0.19, p=0.67) or antibacterial activity (F=0.29, p=0.61). The lack of significant difference between days both in the control and spawned groups

illustrates that repeated sampling of the haemolymph did not affect their antiviral ($F=0.83$, $p=0.37$) or antibacterial ($F=0.08$ $p=0.78$) activity.
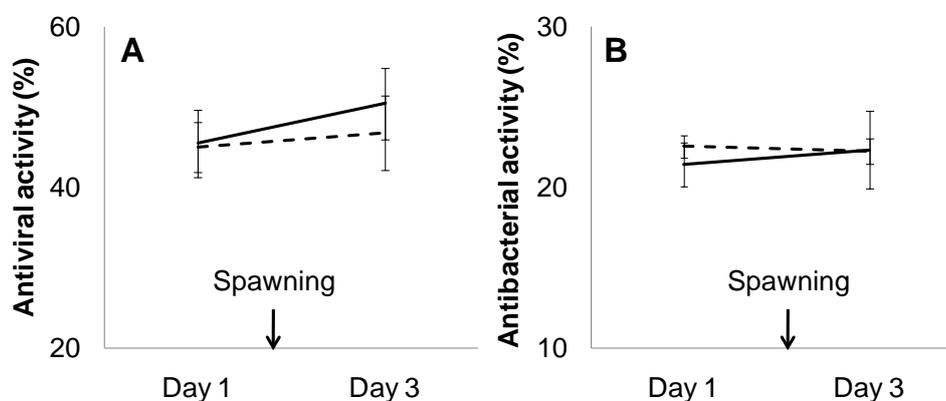
Figure 3.4. Comparison of A) antiviral activity (%) and B) antibacterial activity (%) between pre- and post-spawning. Abalone for spawning induction was from family line FL-2 Control or unspawned group (n=10) was shown in straight line while spawned group (n=16) was shown in dashed line. Means and standard error were derived from antiviral or antibacterial assay for haemolymph samples from control or unspawned abalone abalone group.

3.4. Discussion

Abalone haemolymph is involved in innate defense (Cheng 1981; Mitta *et al.* 2000; Bachère *et al.* 2004; Hooper *et al.* 2007b) and represents a valuable resource for studying antiviral and antibacterial activity at individual, family line, population or species level. Bleeding of abalone is a fast and non-lethal procedure, and offers an advantage over other commercial molluscs (e.g. snails, cuttlefish, octopuses, squids, clams, mussels, oysters and scallops), which typically require anesthetisation to obtain the haemolymph (Gunkel *et al.* 2008) if they are not killed. Non-lethal sampling with minimal handling allows for detection of immune responses from relatively unstressed animals and facilitates identification of individuals with enhanced immunity for future breeding and heritability studies. Our study has

applied *in vitro* antibacterial and antiviral assays, which have been broadly used for the investigation of immune defense in molluscs (Yasin *et al.* 2000; Maier *et al.* 2001; Olicard *et al.* 2005a; Olicard *et al.* 2005b; Vakalia *et al.* 2005; Li *et al.* 2007; Li *et al.* 2009b; Li *et al.* 2009d; Dang *et al.* 2011b) and confirms antiviral activity against HSV-1 and antibacterial activity against *V. harveyi* in the haemolymph of greenlip *H. laevigata,* blacklip *H. rubra* and their hybrid cross.

Antimicrobial activities have been previously reported in the haemolymph and organic extracts from a range of other molluscan species (Mitta *et al.* 2000; Li *et al.* 2009a; Benkendorff 2010). In particular, four classes of antibacterial peptides have been isolated from bivalves (Mitta *et al.* 2000; Li *et al.* 2009a). More recently, abhisin and a defensin peptide have been reported from the abalone *H. discus discus* with antibacterial activity against marine *Vibrio*s (De Zoysa *et al.* 2009a; De Zoysa *et al.* 2010). In general, marine invertebrate antimicrobial peptides appear to be amphiphilic and cationic to interact with bacterial membranes (Tincu *et al.* 2004; Li *et al.* 2009a; Otero-Gonzalez *et al.* 2010). Our preliminary studies indicate that the haemolymph antibacterial compounds in *H. rubra* and *H. laevigata* could be small peptides, but they are not proteins, as the activity was retained after proteinase-K and heat treatment (121°C for 20min, Benkendorff unpublished data). Attempts to purify the active factors resulted in antibacterial activity spreading across a number of column fractions. Similar findings on molluscan antibacterial activity spreading across column fraction have been reported by Defer *et al*. (2009a) and indicate that there may be more than one antibacterial factor may be present. In addition to peptides, a wide range of lipophilic antibacterial compounds have been isolated from gastropod molluscs, including polyunsaturated fatty acids and alkaloids (Benkendorff *et al.* 2000; 2001; Benkendorff *et al.* 2005; Benkendorff 2010). We have found

antibacterial activity in lipophilic extracts after passing abalone haemolymph through a dianion resin HP20 column (Benkendorff unpublished data). Consequently, abalone haemolymph may include both ubiquitous antibacterial peptides, as well as potentially novel lipophylic antibacterial compounds.

Relatively less is known about the compounds responsible for antiviral activity in marine molluscs. An anti-HIV peptide has been reported from oyster protein hydrolysate (Lee *et al.* 1998) and anti-HSV-1 compounds have been reported in acidic extract (40-80% acetonitrile SPE-fraction) from a range of bivalves and gastropods (Defer *et al.* 2009a; b), suggestive of amphiphilic peptides. Synthetic analogues of mytilin appear to have both antibacterial and antiviral activity (Roch *et al.* 2008). However, the antiviral and antibacterial activities of abalone haemolymph appear to be attributed by different compounds, since we found no correlation between the level of activity detected in the antiviral and antibacterial assays across all abalone samples in this study. Preliminary studies on antiviral factors from abalone by Li (1960) and Li *et al* (1962b) indicated they were likely to be macromolecules such as glycoproteins. Subsequently, De Zoysa *et al.* (2007) report the presence of a gene for myxovirus resistance (Mx) protein in the abalone *Haliotis discus discus.* However, our studies on *H. laevigata* haemolymph indicate that the anti-herpes virus compounds are unlikely to be proteins, as no significant loss of activity was detected after proteinase-K, trypsin and/or high temperature treatment (Dang *et al.* 2011b). The antiviral activity of *H. laevigata* haemolymph was also lost by lipophylic extraction on SPE columns, suggesting the active compounds are mostly likely to be sugars, acids or small polar heat resistant peptides (Dang *et al.* 2011b). Ultimately, further research is required to elucidate the chemical structures of both the antiviral and antibacterial compounds from abalone haemolymph.

The lack of significant difference in antiviral and antibacterial activity between wild greenlip *H. laevigata* and blacklip *H. rubra* suggests that neither species has inherently stronger antimicrobial defense properties than the other. This is consistent with the fact that neither of the above species is more resistant to AVG or Vibriosis than the other (Handlinger *et al.* 2005; Hooper *et al.* 2007a). Since our study only assesses general antiviral activity against a model virus, HSV-1 in Vero cells, it does not address the possibility of species-specific adaptation to endemic diseases. The situation of abalone herpes-like viral infection in Taiwan provides an example, where resistant abalone *H. discus* share the same habitat with susceptible *H. diversicolor supertexta* (Chang *et al.* 2005). All abalone from both species used in this study were collected from South Australia, where *V. harveyi* is endemic but viral outbreaks have not been previously detected. The fact that some antiviral activity was detected in all haemolymph samples tested implies that *H. laevigata* and *H. rubra* both constitutively express antiviral defense factors, but it remains to be seen whether these are induced by infection and/or effective against abalone herpesvirus.

Though interspecies hybridization between blacklip *H. rubra* and greenlip *H. laevigata* has been applied in Australia, evidence for hybrid vigour or heterosis, including immune fitness or growth rate, has not been previously reported. Previous studies on marine invertebrate species indicate that interspecies hybrids can sometimes show decreased vigour, for example hybrid larvae from mussels *Mytilus edulis* and *M. galloprovincialis* grew slower than larvae of either pure species (Beaumont *et al.* 2004). However, increased vigour was reported in hybrids from Pacific sea urchins *Echinometra* sp. A and *Echinometra mathaei*, which showed the superiority of growth traits compared to their parents (Rahman *et al.* 2005); and no significant difference in growth rates was reported for *Penaeus monodon* and *P.*

esculentus hybrids (Benzie *et al.* 1995). In this study, the hybrid FL-4 had a similar mean shell length to the other farmed green lip (*H laevigata*) family lines in the same age cohort. There was also no significant different in antiviral activity of the hybrid compared to the farmed family lines. On average, antibacterial activity of the hybrid was higher than the farmed *H. laevigata*, however, when size was taken into consideration, pairwise tests revealed that the hybrid differed only from 2yr old family line FL-1 and was not different to the two family lines in the same 3yr age cohort. Thus, despite maintaining genetic variability by outbreeding, the *H. laevigata* x *H. rubra* hybrid does not appear to benefit from increased growth or immune fitness. Genetic variability at the species level does not appear to contribute to variation in the constitutively expressed antiviral or antibacterial activity. However, this does not rule out the possibility that variability at the individual level is genetically based and thus heritable.

Significant variation of *in vitro* antiviral and antibacterial activity in abalone was found at the individual level (up to 69% and 46% of the variation, respectively). This variability in antimicrobial defense may explain the observed differences in susceptibility of individual abalone towards the herpesvirus (Hooper *et al.* 2007a; Crane *et al.* 2009) and *Vibrio* bacteria infections (Handlinger *et al.* 2005; Vakalia *et al.* 2005) within abalone farms and in natural populations. The large variation in antimicrobial activity of abalone haemolymph may relate to history of exposure in individual abalone, as antimicrobial compounds are expressed not only constitutively, but can also be induced in response to a particular stimulus (Li *et al.* 2009a). Several antimicrobial peptides such as defensins and mytilins have been isolated from the haemolymph of immune-challenged mussel *M. edulis* (Charlet *et al.* 1996) and *M. galloprovincialis* (Mitta *et al.* 2000), clam *R. decussatus* (Gestal *et*

al. 2007) and scallop *Argopecten irradians* (Zhao *et al.* 2007). Antimicrobial peptides in marine molluscs or invertebrates have been thoroughly reviewed by Li *et al.* (2009a), Otero-Gonzalez *et al.* (2010) and Tincu *et al.* (2004) and they are continuously expressed or rapidly induced at different cellular levels to interact directly with infectious agents or pathogenic microorganisms. A study by Li et al. (2009e) demonstrates that antibacterial activity in oysters significantly dropped in the week following a simulated bacterial challenge, whilst haemolymph protein significantly increased. Thus the variation between abalone may result from a combination of their recent infection history and their overall immune fitness.

The main factor found to influence antiviral and antimicrobial activity of abalone was whether they were sourced from natural populations or a farm. Environmental factors that impact the immune response could explain the higher antimicrobial activity in wild greenlip abalone compared to farmed ones. Abalone in farms could experience immunodepression from stress resulting from a change in their living environment (e.g. handling, water conditions and density) (Malham *et al.* 2003; Cheng *et al.* 2004b; Cheng *et al.* 2004d; Cheng *et al.* 2004a; Cheng *et al.* 2004e; Cheng *et al.* 2004c; Hooper *et al.* 2007b). The artificial diets on farms may also result in lower antiviral activity than natural populations that feed on algae, as antiviral and antimicrobial activity have been widely reported from marine algae (Ohta *et al.* 1998; Ponce *et al.* 2003; Lee *et al.* 2004; Smit 2004; Talarico *et al.* 2004; Puglisi *et al.* 2007; Salvador *et al.* 2007; Stirk *et al.* 2007; Mandal *et al.* 2008; Kamenarska *et al.* 2009). Overall, a combination of biotic and abiotic environmental factors that impact the immune system and individual history of exposure to disease are all likely to contribute to the high levels of observed variability in antiviral and antibacterial activity of abalone haemolymph.

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The size or age of the abalone may also influence their antimicrobial properties. Shell length was significantly correlated to antiviral and antibacterial activity in greenlip abalone *H. laevigata*, although in both cases only a weak positive relationship with size was observed. For antibacterial activity, 2yr old abalone tended to have lower activity than 3yr old family lines. These results suggest that abalone may develop stronger antimicrobial defense compounds and/or synthesize higher levels of the active compounds as they grow. Juvenile Pacific oysters *C. gigas* experience much higher mortality due to Ostreid Herpesvirus 1 infection than adults and adults can carry the virus without displaying clinical signs (Arzul *et al.* 2002a), supporting the development of improved immunity in molluscs with age. However, previous field observations on the AVG outbreak in farmed abalone indicate that high mortality occurs both in juvenile abalone from one to four years old and the older brood stock (Hooper *et al.* 2007a). The gender of the abalone did not influence their antimicrobial activitity and surprisingly, we found no effect of spawning on antiviral and antibacterial activity in abalone haemolymph. Gametogenesis and spawning has been linked to decreased immune function (Travers *et al.* 2008) and increased mortality due to *Vibriosis* in *H. tuberculata* (Travers *et al.* 2009). These studies were conducted over a longer time frame and hence it is possible that the abalone antimicrobial reserves within the haemolymph have not been exhausted within the short time frame of our studies (one day post spawning). Spawning is known to cause an instant reduction in the energy reserves for 5-8 days (e.g. glycogen or protein) and results in reduced antibacterial activity in oysters from day 3 to 25 (Li *et al.* 2007; Li *et al.* 2010). Consequently, over a longer time frame, it would be expected that the antibacterial reserves in abalone also become depleted in

a few days post-spawning and may not be replenished until their condition improves (Travers *et al.* 2009).

In conclusion, we found considerable variability in the levels of antiviral and antibacterial activity in haemolymph of blacklip *H. rubra*, greenlip *H. laevigata* and their hybrid cross. This extends our previous reports of antiviral activity in greenlip abalone *H. laevigata* (Dang *et al.* 2011b). Plaque assay for antiviral activity against HSV-1 represents a useful tool for comparing antiviral activity within and between abalone species. Further study on antiviral activity against marine herpesvirus (i.e. salmon herpesvirus type 1 and 2) is underway to confirm the variation of antiviral activity against ecologically relevant pathogens. *V. harveyi* provides a useful model for screening *in vitro* antibacterial activity in haemolymph. The variation in antiviral and antibacterial activity of abalone within the same family line or geographic location may explain why some individuals are more resistant to AVG and *Vibrio* infections than others. Further research is required to investigate whether high antiviral and antibacterial activity translates to reduced susceptibility towards infections.

Acknowledgments

Appendix 3.1. Map of abalone collecting locations
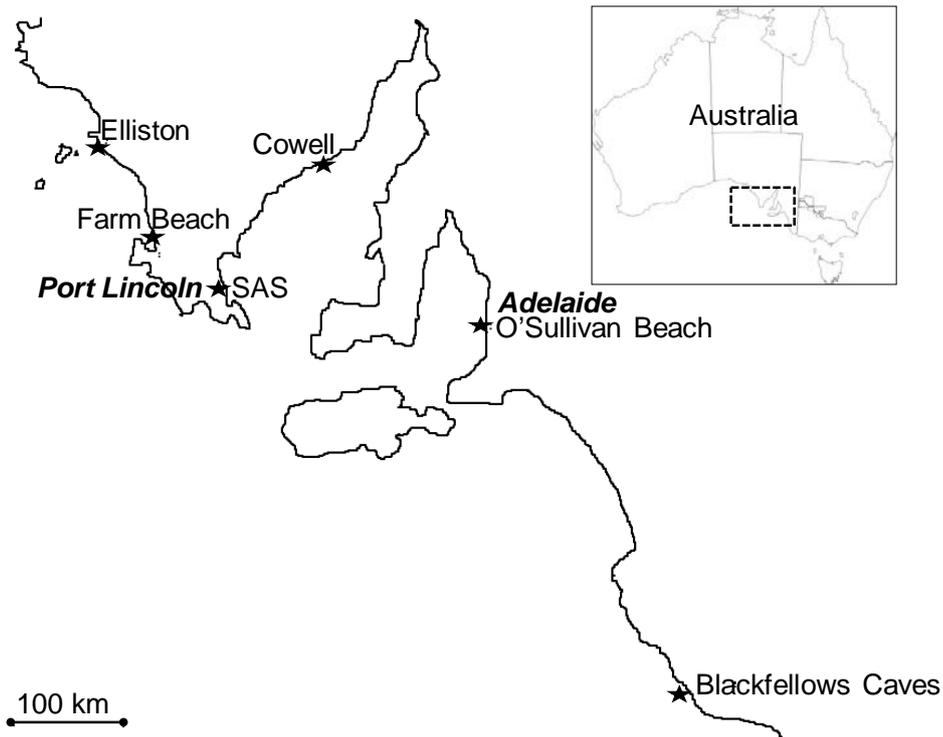
Figure 3.5. Map of locations within South Australia showing where wild abalone were collected. Greenlip abalone *Haliotis laevigata* were collected from Cowell, Elliston, Farm Beach and Blackfellows Caves and blacklip abalone *Haliotis rubra* were from Elliston, O'Sullivan Beach and Blackfellows Caves (drawn using Pant.NET v3.5.1).

CHAPTER 4: Effects of micro and macroalgal diet supplementations on growth and immunity of greenlip abalone, *Haliotis laevigata*

Abstract

Algae contain nutrients and bioactive compounds, which can have effects on the growth and immunity of aquatic organisms. This study examines the effect of macroalgae *Ulva lactuca* and *Spyridia filamentosa*, cyanobacteria *Arthrospira maxima,* and microalgae *Dunaliella salina* on the growth and immunity of *Haliotis laevigata*, in comparison to a commercially formulated pellet. Over 12 weeks, *U. lactuca* or *S. filamentosa* produced the lowest growth rate, indicating lack of nutrients for abalone growth in a single macroalgae diet. Pellets supplemented with 10% *A. maxima* or *D. salina* resulted in increased abalone shell length (13.3 and 30.7%, respectively) and body weight (19.9 and 33.4%) over the pellet control diet. The impact of algal supplementation on haemolymph immunity was mainly represented by a drop in total haemocyte counts (THC) and some diet specific effects on superoxide anion and antibacterial activity against *Vibrio anguillarum*. No effect of diet was observed on phagocytosis, phenoloxidase, or antiviral activity against herpes simplex virus type 1 (HSV-1). It is conceived that the humoral antimicrobial factors in abalone haemolymph are innately biosynthesized rather than dietary derived compounds. Nevertheless, concomitant with strong antiviral activity against HSV-1 in a lipophilic extract of *U. lactuca* and *S. filamentosa*, higher antiviral activity was detected in the digestive lipid extract of abalone fed *U. lactuca* (64.2% at 650 µg ml^{-1}) or *S. filamentosa* (69.51%) compared to abalone fed pellets (47.42%) or pellets supplemented with *A. maxima* (46.3%) or *D. salina* (46%). This suggests that abalone may also derive some protection against pathogens, which are transmitted orally or through gastrointestinal tract, by algal derived bioactive compounds. These results indicate that abalone growth rate could be optimized by *A.*

maxima and *D. salina* supplementations, whereas *U. lactuca* and *S. filamentosa* could be useful supplements for abalone aquaculture, especially in areas with high risk of herpesvirus infection.

4.1. Introduction

Abalone are marine gastropod mollusks and important aquaculture species worldwide (Gordon *et al.* 2001; 2004). In Australia, the abalone fishery generated annual revenue of approximately $217 million in 2007-2008 (ABARE 2009). The greenlip abalone *Haliotis laevigata* is one of the main commercial species, but unfortunately their productivity is threatened by infection with herpesvirus (Hooper *et al.* 2007a) and *Vibrio* bacteria (Handlinger *et al.* 2005; Vakalia *et al.* 2005; Dang *et al.* 2011c). As *H. laevigata* normally need 3-4 years to reach market size, it is essential to improve their growth and resistance to such pathogen infections to ensure that abalone aquaculture is economically viable. Rapid growth rates and effective immunity depend on an adequate supply and balance of nutrients. In recent years, there has been increasing interest in dietary administration of algal-derived micronutrients for proper modulation of the host immune system in aquaculture (Trichet 2010). Therefore, there is a desire to develop algal nutrition-based strategies that support growth and immune system development for abalone aquaculture.

Algae have received increased attention not only because of their rich nutrition (Borowitzka 1995; Li *et al.* 2009a), but also due to their biologically active substances (Li *et al.* 2009c). In fact, macroalgae have been extensively used for abalone aquaculture. For example, the red alga *Gracilaria heteroclada* was found to promote the growth rate of abalone *Haliotis asinina* better than a commercially formulated diet (Capinpin *et al.* 1996). Enriched *Ulva* was recommended as a suitable feed for *Haliotis tuberculata* (Shpigel *et al.* 1999), *Haliotis discus hannai* (Corazani *et al.* 1998; Shpigel *et al.* 1999) and *Haliotis roei* (Boarder *et al.* 2001). A mixture of fresh red alga *Gracilaria gracilis*, green alga *Ulva lactuca*, and kelp

Ecklonia maxima facilitated higher growth rates in *Haliotis midae* than a formulated food (Naidoo *et al.* 2006). Since microalgae contain a significant amount of proteins and polyunsaturated fatty acids, chlorophyll, carotenoids, vitamins, minerals, and unique pigments (Kay 1991; Singh *et al.* 2005; Spolaore *et al.* 2006; Becker 2007), application of microalgae feed has also been investigated for abalone growth. For instance, compared to the formulated food, feeding diatoms *Navicula* sp. and macroalgae *Ulvella lens* and *Ulva* sp. resulted in higher growth rates of juvenile *H. laevigata* (Daume *et al.* 2007). Further, *H. laevigata* juveniles fed a mixture of *Navicula* sp., *Cylindrotheca closterium* and *U. lens*, were found to have significantly better growth and survival rates than abalone fed a single microalgae diet (Daume *et al.* 2003). Whereas the investigation of using macro and microalgae is ongoing for abalone growth improvement, little attention has been paid to the effect of algal diets on the abalone immune system.

Antiviral and antibacterial activities, or bioactive compounds have been widely reported in microalgae (Ohta *et al.* 1998; Singh *et al.* 2005), as well as brown, green and red macroalgae (Ponce *et al.* 2003; Lee *et al.* 2004; Talarico *et al.* 2004; Puglisi *et al.* 2007; Salvador *et al.* 2007; Stirk *et al.* 2007; Mandal *et al.* 2008; Kamenarska *et al.* 2009). It has been reported that sulfated polysaccharides such as fucoidans, calcium spirulan, carrageenan, galactan sulfate, xylomannan in marine algae can inhibit a broad range of bacteria (e.g. *Vibrio harveyi*, *Staphylococcus aureus* and *Escherichia coli*, *Enterococcus faecalis* and *Klebsiella pneumoniae*) (Chotigeat *et al.* 2004; Rhimou *et al.* 2010) and viruses (e.g. herpes simplex virus, poliovirus, adenovirus, human immunodeficiency virus, influenza virus and respiratory syncytial virus) (Hasui *et al.* 1995; Hayashi *et al.* 1996a; Witvrouw *et al.* 1997; Damonte *et al.* 2004; Li *et al.* 2008). In shrimp *Penaeus monodon*, feeding with crude fucoidan

extracted from brown seaweed *Sargassum polycystum* or green algae *Acrosiphonia orientalis* resulted in an increased survival rate, of up to 88-93% after infection by White Spot Syndrome virus (Chotigeat *et al.* 2004; Manilal *et al.* 2009). Similarly, it is conceived that these useful compounds in algae could be passed on to abalone through diet and enhance their pathogen resistance.

To date, feeding abalone with commercial formulated pellets has been widely applied on large-scale farms due to their constant supply and water stability (Sales *et al.* 2004). Meanwhile, algae cultivation is also commercialized and well developed for potential use as a supplementary feed in abalone aquaculture. We hypothesize that a combination of formulated diet with algae could improve the growth rate and immune health of abalone. Since red macroalgae *Spyridia filamentosa* and green macroalgae *U. lactuca* are a natural food source for wild greenlip abalone and also abundant around the Australian coast, they were selected for macroalgae food supplementation of *H. laevigata* in this study. On the other hand, microalgae *Arthrospira maxima* (cyanobacteria) and *Dunaliella salina* were selected as microalgal additives in abalone diet, as they have been successively applied in feeding fish, shrimps and molluscs (Spolaore *et al.* 2006). This study compared the effect of *S. filamentosa*, *U. lactuca*, *A. maxima* and *D. salina* with commercially formulated pellets on growth rate (shell and weight gain) and immune status of *H. laevigata* (THC, phagocytosis, respiratory burst, phenoloxidase, antiviral and antibacterial activity).

4.2. Materials and methods

4.2.1. Experimental abalone and diets

A single cohort of *H. laevigata* (8-9 cm of shell length) was obtained from Southern Australian Seafoods, Port Lincoln, South Australia. They were tagged upon arrival at Flinders University using Hallprint glue-on tags (Hallprint Pty Ltd, South Australia). Prior to feeding experiments, abalone were acclimated at 16°C in flow-through seawater tanks (2 liter min^{-1}) with continuous aeration and also starved for 2 weeks. Fresh macroalgae *S. filamentosa* and *U. lactuca* were collected from O'Sullivan Beach, South Australia. They were stored at -20 °C and thawed before feeding abalone directly as whole algal fronds. Microalgae *A. maxima* and *D. salina* were purchased individually as dry powders from TAAU Australia Pty Ltd and Aqua Carotene Ltd, respectively. Microalgae were individually incorporated into abalone formulated pellets as a dry powder in 1: 9 ratio of dry weight by Adam and Amos Abalone Foods Pty Ltd (Mount Barker, South Australia). The same commercial pellets but without supplementation were used as the control pellet diet in both macro and microalgal feeding experiments.

4.2.2. Macro-and microalgae feeding trials

For macroalgae feeding, a total of 144 abalone were randomly divided into four feeding groups: commercial formulated pellets (control group), green algae *U. lactuca*, red algae *S. filamentosa*, and combinations of pellets and these two macroalgae (alternating between the two per feeding time). The 36 abalone in each group were further divided into six replicate tanks (with six abalone in each). For microalgae feeding, another 135 abalone were kept in a separate system with three

feeding groups: commercial formulated pellets (control group), *A. maxima* and *D. salina* supplemented formulated pellets. Each group was divided into replicates, with 15 abalone in each. Both feeding trials were conducted over a 12 week feeding period (Fig. 4.1).

Abalone were fed three times per week and a full water exchange was carried between feedings. In each diet treatment, six tagged abalone were randomly selected for measuring shell length ($\pm$ 0.1 cm) and body weight ($\pm$ 0.1 g) on weeks 0 and 12. Additionally, 6 abalone were sampled for haemolymph and digestive glands on the same day of weeks 2, 4, 8 and 12. The abalone haemolymph samples were used immediately for cell-mediated immune assays and the remaining samples were stored at -20°C until humoral immune assays.
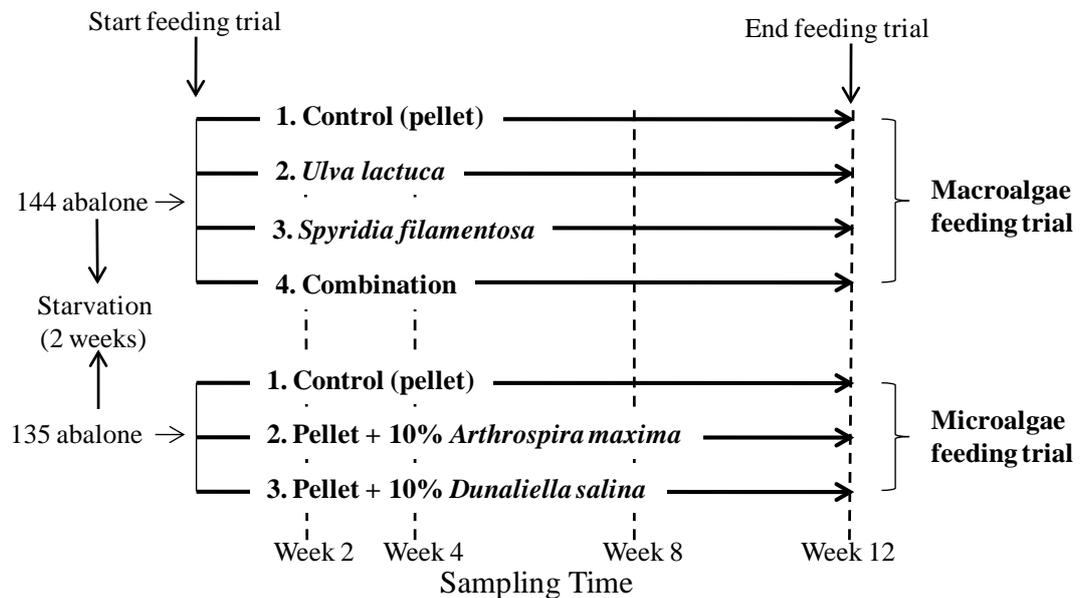
Figure 4.1. Summary of macroalgae and microalgae feeding experiments. Abalone were measured for shell length and body weight at the start (week 0) and at the end of experiment (week 12). At weeks 2, 4, 8 and 12, six abalone were sampled from each diet group for haemolymph and digestive glands to measure immune response.

4.2.3. Haemolymph analyses

4.2.3.1. Total haemocyte count (THC)

Fresh haemolymph (50 μl) was fixed in 10% formalin in 100 μl phosphate buffered saline solution (PBS, 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.47 mM KH_2PO_4) in a pre-cooled eppendorff tube and kept on ice to prevent haemocytes from clumping. After vortex, each haemolymph sample was loaded on a hemocytometer for haemocyte counting in duplicate under a microscope (Olympus CX40) as number of cells per ml.

4.2.3.2. Phagocytic rate

The phagocytic rate of abalone haemocytes was measured using the method of Chen *et al.* (2005) with some modifications. Briefly, a yeast solution for phagocytosis assay was prepared by autoclaving 2.5% baker's yeast (*Saccharomyces cerevisiae*) (Tandaco, Cerebos Foods, NSW) in 4% Congo red (Sigma) in PBS. The stained yeast cells were centrifuged at 1500 xg for 10 min, washed three times with PBS and resuspended in filtered sea water (FSW, 0.2μm) at 10^7 cells ml^{-1}. Fresh haemolymph (50 μl) was spread on a glass slide for 10 min at room temperature (RT). Afterwards, excess haemolymph was gently poured off and adhered haemocytes were incubated with 50 μl yeast suspension at RT for 30 min. Phagocytosis was ceased by washing the slide twice with FSW and fixing slides with 10% formaldehyde in FSW for 20 min. Phagocytic rate was determined in duplicates as percentage of phagocytic haemocytes (with yeast particles inside the cell) in 100 haemocytes under microscope (Olympus CX40).

4.2.3.3. Intracellular superoxide anion

Superoxide anion level (or the respiratory burst activity) of haemocytes was quantified by reduction of nitroblue tetrazolium (NBT) to formazan (Cheng *et al.* 2004a). Fresh haemolymph (150 µl) was placed in triplicate into wells of a 96-well microtitre plate and incubated at room temperature for 20–25 min to obtain a cell monolayer. PBS was used as negative control (NBT background). After serum was pipetted off, 100 µl of sodium alginate (0.2 mg ml^{-1} in PBS) was added to the cell monolayer. After 30-min incubation at 26 °C, sodium alginate was discarded and the haemocyte monolayer was stained with 100 µl NBT solution (0.3%) at 26 °C for another 30 min. After the NBT solution was poured off, the haemocytes were fixed with 100 µl methanol (100%), washed three times with 100 µl methanol (70% in PBS) and air-dried. Finally, the formazan was dissolved by adding 120 µl KOH (2 M) and 140 µl dimethyl sulfoxide (DMSO). The absorbance was measured at 630 nm on a microplate reader (FLUOstar Omega). Intracellular superoxide anion was expressed as sample absorbance - negative control absorbance.

4.2.3.4. Phenoloxidase activity

Phenoloxidase activity of haemocytes was quantified using dopachrome formation (Chen *et al.* 2005). Fresh haemolymph (150 µl) was placed in triplicate into the wells of a 96-well microtitre plate and incubated at room temperature for 20–25 min to obtain a cell monolayer. The supernatant fluid was then discarded, and 100 µl of sodium alginate (0.5 mg ml^{-1} in PBS) was added to help activate phenoloxidase activity. After incubation at 26-27 °C for 30 min, 50 µl of L-DOPA (3 mg ml^{-1} in PBS) was added. The optical density (OD$_{490nm}$) was measured 10 min later using a microplate reader (FLUOstar Omega). In controls, sodium alginate was replaced by

an equal volume of PBS to measure the background of phenoloxidase activity. The phenoloxidase activity was calculated as sample absorbance - control absorbance.

4.2.3.5. Antiviral assay

Phylogenetic analyses of DNA polymerase proteins from abalone and oyster herpesviruses have suggested that the abalone and oyster herpes viruses belong to one family *Malacoherpesviridae* and are distantly related to other members of the *Herpesviridae* (Fegan *et al.* 2009; Savin *et al.* 2010). Due to a lack of a suitable cell line for culturing abalone herpes virus, a heterologous model using Vero cells and HSV-1 was chosen to investigate the effect of diet on abalone antiviral activity. The antiviral activity against HSV-1 was determined by a plaque reduction assay as described by our previous studies (Dang *et al.* 2011b; Dang *et al.* 2011c). Haemolymph at 6% (v/v) was used throughout the antiviral assay (EC_{50} = 6.23%, v/v).

4.2.3.6. Antibacterial assay

Marine vibrio *V. anguillarum* were obtained from the Fish Health Unit, Department of Primary Industries, Tasmania and maintained at -80 ℃ in 10% glycerol. This bacteria was cultured in sterile nutrient broth (1 g NaCl, 2 g yeast extract and 1 g peptone per 100 ml distilled H_2O) overnight at 37 ℃ on an orbital mixer shaker (Ratek) at 200 rpm. The cultures were diluted to an optical density at 600 nm (OD600nm) of 0.1 with a spectrophotometer (Metertech, UV/VIS SP8001) and returned to exponential growth phase (OD600nm = 0.18-0.2) prior to antimicrobial assays (Li *et al.* 2007). Antibacterial activity of abalone haemolymph against *V. anguillarum* was measured using MTS assay (Dang *et al.* 2011c). The MTS assay

was based on reduction of MTS tetrazolium to a red formazan product by dehydrogenase enzymes from live cells. 90 µl of haemolymph plasma and 10 µl of *V. anguillarum* in exponential growth culture were added into a 96-well plate in triplicate. Negative controls had 90 µl haemolymph in 10 µl nutrient broth and positive controls had 10 µl of *V. anguillarum* in 90 µl of nutrient broth. After 30 min incubation, 20 µl of CellTitre 96® Aqueous One Solution (Promega, NSW, AUS) was added to each well. The plates were then incubated for a further 2 hrs or until development of the red formazan product in positive controls. Absorbance was measured at 492 nm using a 96-well plate reader (FluoStar Omega). Antibacterial activity (%) was calculated from 100 - [(treatment absorbance - negative control absorbance) / positive control absorbance] x 100.

4.2.4. Lipophilic extraction and antiviral assay

Lipophilic extracts were obtained from each abalone diet and the dissected digestive glands of the fed abalone. To minimize inter-animal variability, the digestive glands from 6 abalone were pooled in each treatment. The lipophilic extraction was prepared as outlined in our previous study using chloroform and methanol (1:1, v/v) (Dang *et al.* 2011b). Lipophilic extracts were reconstituted in DMSO (0.5%) in Eagle's minimal essential medium (DMSO/EMEM; Sigma, Australia) and subjected to antiviral assay against HSV-1. Lipophilic extracts at 650 µg ml^{-1} were used throughout in antiviral assay (EC$_{50}$ = 667µg ml^{-1}).

4.2.5. Statistical analysis

The effect of diet on shell length and body weight of abalone was detected by one-way ANOVA on PASW/SPSS statistics version 18. Tukey post hoc test was

undertaken to determine which of the treatment diets differed from the controls. The effect of diet and feeding period (fixed factors) on the overall immune status of *H. laevigata* was assessed by PERMANOVA (Permutational Multivariate Analysis of Variance) using Primer V6 + PERMANVOA add-on. The six immune parameters were normalized to the same scale. A minimum of 999 permutations of the residuals was undertaken on the standardized data using the Euclidean distance resemblance matrix. Pair-wise analyses were conducted to investigate the significant differences, focusing on comparisons between the control and treatment diets. Permutational univariate ANOVA analyses were performed to identify the effects of diet and/or feeding period on each individual haemolymph immune parameter and antiviral activity of digestive lipid extract. The level of significant difference was set at $p < 0.05$.

4.3. Results

4.3.1. Growth rates

There was no mortality obtained in both feeding trials. In the macroalgae feeding trial, the lowest growth rate was found in abalone fed on macroalgae alone (Table 4.1). One-way ANOVA revealed a significant difference in the rate of shell length and body weight increases according to diet (df = 3, F = 4.69, p = 0.008 and df = 3, F = 4.63, p = 0.008, respectively). Pairwise tests revealed that the abalone fed on macroalgae *U. lactuca* or *S. filamentosa* had significantly lower growth rates than those fed on the pellet control diet (shell length p = 0.03 and p = 0.01; weight gain p = 0.04 and p = 0.02, respectively). Over the 12 week feeding period, shell length and body weight gain in controls were nearly double that of *U. lactuca* or *S. filamentosa* fed abalone (Table 4.1). There was no significant difference between control and

combination diets (p > 0.05), although the mean value of abalone growth was slightly higher in the control, by 0.012 mm day^{-1} of shell length and 0.004 mm day^{-1} of body weight.

Table 4.1. The impact of macro- and microalgae supplementations on shell gain (mm day^{-1}) and body weight gain (g day^{-1}) of greenlip abalone *H. laevigata* over a 12 week period.

		Shell length gain	Weight gain
		Daily gain (mm day^{-1})	Daily gain (g day^{-1})
Macroalgae	pellet control	0.048 ± 0.008	0.037 ± 0.004
	U. lactuca	0.026 ± 0.004[a]	0.022 ± 0.004[a]
	S. filamentosa	0.022 ± 0.003[a]	0.020 ± 0.004[a]
	combination	0.036 ± 0.006	0.033 ± 0.003
Microalgae	pellet control	0.056 ± 0.007	0.049 ± 0.006
	pellet+10% *A. maxima*	0.063 ± 0.007	0.058 ± 0.009
	pellet+10% *D. salina*	0.073 ± 0.005[a]	0.065 ± 0.005[a]

[a] Significant difference in shell or weight gain in comparison to relevant pellet control (one-way ANOVA, p < 0.05)

In the microalgae feeding experiment, abalone fed on microalgae supplements showed an increase in shell length of 0.007-0.017 mm day^{-1} and body weight of 0.009-0.016 g day^{-1} over 12 weeks, compared to pellet diet (Table 4.1). One-way ANOVA detected a significant difference in growth rate between these diets (shell length df = 2, F = 5.47, p = 0.011; for weight gain, df = 2, F = 5.1, p = 0.014). Pairwise tests revealed a significant difference in growth rate between abalone fed control pellets and *D. salina* (p < 0.05), but not between the control diet and *A. maxima* supplemented pellets (p > 0.05, Table 4.1).

4.3.2. Haemolymph immune parameters

For overall immune status of abalone, multivariate PERMANOVA revealed that there was no interaction between diet and feeding period ($p > 0.05$, Table 4.2). The impact of diet was significant in both macro- and microalgae feeding trials ($p < 0.05$). Pairwise comparisons in the macroalgae feeding trial revealed that the immune status was different between abalone fed on pellets vs. *S. filamentosa* ($t = 1.66$, $p = 0.03$), *U. lactuca* vs. *S. filamentosa* ($t = 1.63$, $p = 0.03$) and *U. lactuca* vs. the combination diet ($t = 1.96$, $p = 0.006$). In the microalgae feeding trial, a significant difference was obtained in the overall immune status of abalone fed pellet vs. *D. salina* supplemented pellet ($t = 1.93$, $p = 0.01$) and *A. maxima* vs. *D. salina* supplemented pellet ($t = 1.64$, $p = 0.04$). The immune status of abalone did not change over time in the macroalgal feeding trial ($p > 0.05$, Table 4.2), however, significant variation in immune status was observed according to the feeding period in the microalgal feeding trial (Table 4.2).

Table 4.2. The effect of diet and feeding period (fixed factors) on the overall immune status (multivariate PERMANOVA) and specific immune parameters (univariate PERMAONVA) of *H. laevigata*. Different immune parameters including total haemocyte count, superoxide anion, phagocytosis, superoxide anion, phenoloxidase activity, antiviral and antibacterial activity were measured at weeks 2, 4, 8 and 12 for macroalgae and microalgae feeding experiments. The level of significant differences was set at p = 0.05.

Parameter	Factor	Macroalgae			Microalgae		
		df	Pseudo-F	p	df	Pseudo-F	p
Overall Immune status	Diet	3	2.41	0.003[a]	2	2.30	0.02[a]
	Feeding period (FP)	3	1.21	0.23	3	2.27	0.01[a]
	Diet x FP	9	0.87	0.70	6	0.67	0.89
Total haemocyte count	Diet	3	6.95	0.001[a]	2	4.43	0.01[a]
	Feeding period (FP)	3	1.27	0.28	3	0.33	0.81
	Diet x FP	9	0.22	0.99	6	0.70	0.65
Phagocytosis	Diet	3	1.39	0.26	2	1.74	0.19
	Feeding period (FP)	3	3.38	0.02[a]	3	1.83	0.14
	Diet x FP	9	1.76	0.09	6	0.81	0.56
Superoxide anion	Diet	3	1.02	0.42	2	3.29	0.04[a]
	Feeding period (FP)	3	1.69	0.16	3	2.57	0.08
	Diet x FP	9	0.80	0.61	6	0.61	0.73
Phenoloxidase activity	Diet	3	1.15	0.35	2	1.93	0.18
	Feeding period (FP)	3	0.28	0.84	3	11.9	0.001[a]
	Diet x FP	9	0.52	0.84	6	0.26	0.96
Antiviral activity	Diet	3	0.47	0.70	2	0.004	0.97
	Feeding period (FP)	3	1.87	0.16	3	0.31	0.82
	Diet x FP	9	1.36	0.25	6	0.002	1.00
Antibacterial activity	Diet	3	3.98	0.01[a]	2	2.92	0.06
	Feeding period (FP)	3	0.38	0.77	3	1.18	0.33
	Diet x FP	9	0.68	0.75	6	1.12	0.38

[a] Significant effect of diet, feeding period or their interaction on overall immune status or individual immune parameter of abalone

In univariate permutational ANOVA, there were no significant interactions between diet and feeding period for any of the individual immune parameters in both feeding

trials ($p > 0.05$, Table 4.2). Among the six immune parameters, only THC and antibacterial activity against *V. anguillarum* were significantly affected by macroalgae diet (Table 4.2, Fig. 4.2). THC in abalone fed control pellet or combination diets was significantly higher than abalone fed *U. lactuca* or *S. filamentosa* (Fig. 4.2a). Antibacterial activity in abalone fed *S. filamentosa* was significantly lower than abalone fed pellets and *U. lactuca* (Fig. 4.2f). On the other hand, phagocytosis significantly varied according to feeding period (Table 4.2, Fig. 4.2d).

In the microalgae feeding trial, only THC and superoxide anion were affected by diet ($p < 0.05$, Table 4.2). THC in abalone fed *A. maxima* supplemented pellet was significantly higher than abalone fed *D. salina* supplemented pellet (Fig. 4.3a). Similarly, superoxide anion was significantly higher in abalone fed formulated pellet or *A. maxima* supplemented pellet than in abalone fed *D. salina* supplemented pellet (Fig. 4.3c). Phenoloxidase activity was significantly different between sampling periods ($p < 0.05$, Table 4.2), with the highest values in three treatments all obtained in week 2 (Fig. 4.3d).
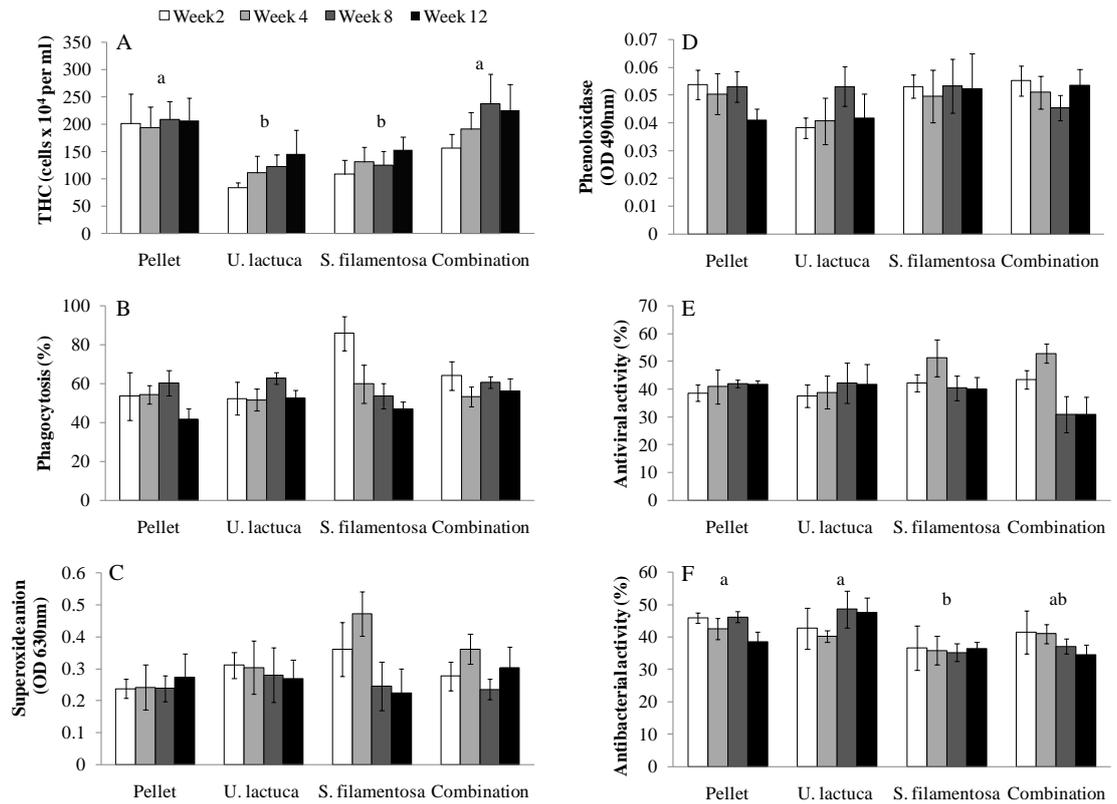
Figure 4.2. Effect of macroalgal diets on immune status of *H. laevigata*. A) Total haemocyte counts (THC) (cells x 10^4 per ml); B) phagocytosis (%); C) superoxide anion (OD 630 nm); D) phenoloxidase activity (OD 490 nm); E) antiviral activity against HSV-1 (%); F) antibacterial activity against *V. anguillarum* (%). Different small letters indicate significant differences between feeding treatments of pellet, *U. lactuca*, *S. filamentosa*, and their combination ($p < 0.05$). Means and standard error were derived from assay for six abalone haemolymph samples per sampling point.
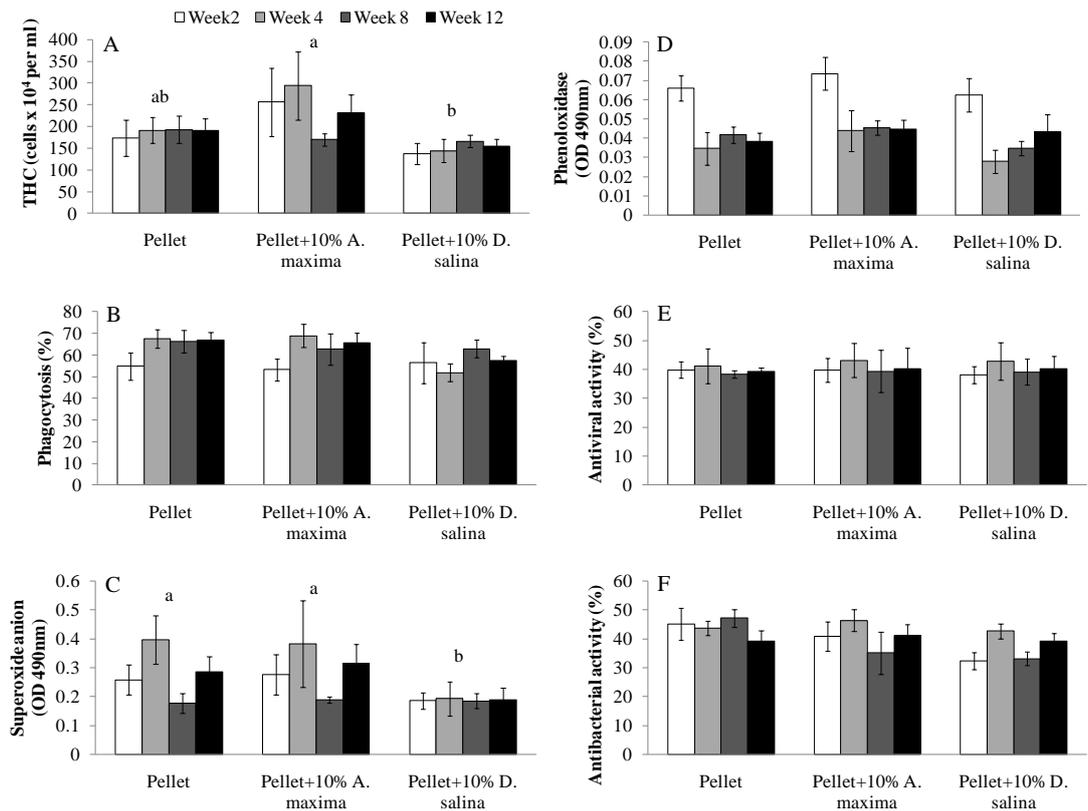
Figure 4.3. Effect of microalgae supplementations on immune status of *H. laevigata*. A) Total haemocyte counts (THC) (cells x 10^4 per ml); B) phagocytosis (%); C) superoxide anion (OD 630 nm); D) phenoloxidase activity (OD 490 nm); E) antiviral activity against HSV-1 (%); F) antibacterial activity against *V. anguillarum* (%). Different small letters indicate significant differences between feeding treatments of pellet, *A. maxima* and *D. salina* formulated pellets ($p < 0.05$). Means and standard error were derived from assay for six abalone haemolymph samples per sampling point.

4.3.3. Antiviral activity of lipophilic extracts

Besides abalone haemolymph, antiviral activity was also found in the lipophilic extracts of *U. lactuca, S. filamentosa* and abalone digestive glands (Table 4.3). At a concentration of 650 µg ml^{-1}, lipophilic extracts of *U. lactuca* and *S. filamentosa* did not cause cytotoxicity of Vero cells using trypan blue assay (<10% dead cells) and

showed strong antiviral activity against HSV-1 (inhibited 100% and 96% of plaque formation, respectively).

Table 4.3. Effect of different diets on percent antiviral activity of lipophilic extract of abalone digestive gland against HSV-1.

	Antiviral activity (%) of lipophilic extract of abalone diet (650 µg ml^{-1})	Antiviral activity (%) of lipophilic extract of abalone digestive gland (650 µg ml^{-1})			
		Week 2	Week 4	Week 8	Week 12
pellet control	2.56±1.03	45.91±1.62	47.77±5.74	46.54±3.76	49.44±5.91
U. lactuca	100±0[a]	63.52±0.73[a]	56.05±2.63[a]	69.81±2.05[a]	67.42±0.65[a]
S. filamentosa	95.9±1.67[a]	67.92±1.89[a]	61.78±1.04[a]	76.97 ± 3.6[a]	71.35±1.69[a]
pellet+10% *A. maxima*	0.51±1.32	44.65±3.08	43.95±4.65	47.17±6.89	49.44±5.91
pellet+10% *D. salina*	3.08±1.75	44.65±2.30	44.59±10.58	45.91±2.99	48.88±5.97

[a] Significant difference in antiviral activity in comparison to pellet control group (one-way ANOVA, p < 0.05)

In the macroalgae feeding trial, univariate analyses revealed that antiviral activity of the digestive gland lipid extracts was significantly influenced by diet (df = 2, pseudo-F = 54.48, p = 0.001) and feeding period (df = 3, pseudo-F = 5.16, p = 0.003), while there was no significant interaction between these two factors (df = 6, pseudo-F = 1.47, p = 0.23). Abalone fed *S. filamentosa* or *U. lactuca* had higher antiviral activity in their digestive gland than abalone fed control pellets. This difference in antiviral activity varied somewhat overtime, peaking after 8 weeks of feeding (Table 4.3). However, antiviral activity of abalone digestive gland was not changed by microalgae supplements, corresponding with little or no antiviral activity detected in *A. maxima* or *D. salina* (Table 4.3).

4.4. Discussion

This study shows that the growth and immune system of *Haliotis laevigata* are significantly affected by macro- and microalgae diets. Abalone fed by either *U. lactuca* or *S. filamentosa* had the lowest growth rate and total haemocyte counts. *A. maxima* or *D. salina* supplements produced better abalone growth, as shown by shell length and body weight, compared with the control pellet diet. However, antiviral activity against HSV-1 was detected in lipophilic extract of *U. lactuca* and *S. filamentosa*, but not in the pellet-formed *A. maxima* and *D. salina*. This was coincident with strong antiviral activity detected in digestive gland lipid extract of abalone fed on the two macroalgae. The antiviral activity of haemolymph was not altered by any of the algal diets, however abalone antibacterial activity and superoxide anion level were reduced by certain macro- and microalgae diets, respectively. This study highlights a complex relationship between abalone diet, growth and immunity, with no single diet providing the optimal outcome across all the parameters tested.

A few previous studies have shown that the quality of the algal diet plays an important role in the growth of molluscs, such as scallops *Pecten maximus* (Delaunay et al. 1993), oysters *Crassostrea gigas* (Knauer *et al.* 1997) and *Ostrea edulis* (Berntsson *et al.* 1997) and clams *Ruditapes decussatus* (Albentosa *et al.* 1996; Fernández-Reiriz *et al.* 1999) and *Tapes philippinarum* (Caers *et al.* 1999). A similar effect of algal diet quality is demonstrated by the poor growth of abalone fed exclusively on macroalgae *U. lactuca* or *S. filamentosa* in this study. This is likely to be due to the insufficient nutrition derived from a single macroalgae diet, thus not meeting the nutritional demand for abalone growth (Naidoo *et al.* 2006). Abalone

growth is known to be enhanced by increased protein content in their macroalgal diets (Shpigel *et al.* 1999). However, the protein content in macroalgae is typically less than 5% (Hahn 1989b). Most macroalgae are limited in the amino acids arginine, methionine, threonine or histidine and among them *U. lactuca* has the lowest essential amino acids (Mai *et al.* 1994). Thus low protein content and essential amino acid availability in macroalgae are likely to be the major reason for low growth rates in abalone fed these diets. Furthermore, the experimental abalone in this study were sourced from farms and thus they may be acclimatized to the formulated diet, resulting in lower feeding preference for the macroalgae. Two weeks of starvation might not change this feeding preference, resulting in high feeding rates and consequently higher growth rates with formulated pellets compared to macroalgae alone.

In contrast to the macroalgae diets, supplementation with the cyanobacteria *Arthrospira* or microalgae *Dunaliella* in abalone formulated diets improves the growth rate significantly. Over the 12 week feeding period, pellets supplemented with 10% *A. maxima* or 10% *D. salina* produced 13.3 and 30.7% higher shell length and 19.9 and 33.4% heavier body weight than the control pellet diet, respectively. Similar microalgae-induced growth has been also observed in fish and shrimp (Belay *et al.* 1996; Supamattaya *et al.* 2005; Badawy 2009). *D. salina* has more essential nutrients than *Arthrospira*, such as minerals, lipids, carbohydrates and *β*-carotene (Spolaore *et al.* 2006), explaining the higher abalone growth with the *D. salina* supplemented diet. In comparison to *Isochrysis galbana* and *Phaeodactylum tricornutum,* higher carbohydrate content in *Tetraselmis suecica* resulted in the highest growth rate in clams *R. decussatus* (Albentosa *et al.* 1996). Lipid supplements were found to significantly promote growth of clams *T. philippinarum*

and oysters *O. edulis* (Berntsson *et al.* 1997; Caers *et al.* 1999). It was also found that lipids such as phospholipids and triacylglycerols, in addition to carbohydrates contribute to high growth in clams *R. decussatus* (Fernández-Reiriz *et al.* 1999). Therefore, the lipid and carbohydrate content, in addition to essential amino acid availability could be important for choosing the optimal algal supplement for abalone growth improvement. Nevertheless, both *D. salina* and *A. maxima* appear to be promising diet supplements and could be further investigated in a larger scale on-farm feeding program over a longer period.

Meanwhile, these microalgal diets contain bioactive compounds that may be associated with immunocompetence in aquatic animals. For example, high levels of 20:5(n-3) and 20:4(n-6) fatty acids in *Chaetoceros calcitrans* have a positive effect on total haemocyte count, granulocyte percentage, phagocytic rate and oxidative activity of haemocytes in the clam *Ruditapes philippinarum* (Delaporte *et al.* 2003). High 22:6(n-3) content of *Isochrysis* sp. is correlated to phagocytic activity of haemocytes in the oyster *C. gigas* (Delaporte *et al.* 2003). Even in rainbow trout *Oncorhynchus mykiss Walbaum*, dietary carotenoids from *D. salina* significantly increased the phagocytic rate of their leucocytes (Amar *et al.* 2004). Although microalgal bioactive compounds are supposed to enhance immune health, this may require substantial research to optimize the dose and timing, because no effects on abalone immunity were found in this study. Abalone feeding with *D. salina* supplement did not result in any obvious immune enhancement, including antiviral activity in digestive gland. Indeed, superoxide anions and THC decreased when abalone were fed 10% *D. salina* formulated pellets. A negative effect of *D. salina* as a diet supplement has been previously reported on the total haemocyte count (THC) of black tiger shrimp *P. monodon* (Supamattaya *et al.* 2005). Nevertheless, shrimp

fed *D. salina* diet showed higher resistance to WSSV infection and were more tolerant to low dissolved oxygen conditions (Supamattaya *et al.* 2005). Thus, specific immune responses in aquatic invertebrates may not reflect their overall immunity in response to microalgal supplementation.

The antibacterial and antiviral factors in abalone haemolymph appear to be synthesized independently of their diet. Both *U. lactuca* and *S. filamentosa* exhibit antibacterial activity towards several Gram-positive and Gram-negative bacteria (Robles-Centeno *et al.* 1996; Zamora Tovar *et al.* 2000; Abd El-Baky *et al.* 2009). However, abalone fed on these diets did not have significantly improved humoral antibacterial activity against the marine pathogen *V. anguillarum*. Similarly, we found antiviral activity in extracts from *U. lactuca* and *S. filamentosa*, and yet abalone fed on these macroalgae did not have enhanced antiviral activity in their haemolymph. This suggests that the humoral antimicrobial factors in abalone haemolymph are innately biosynthesized rather than dietary derived compounds. Indeed, several antimicrobial peptides have been identified to be synthesized by abalone (e.g. defensin and abhisin) (Hong *et al.* 2008; De Zoysa *et al.* 2009a; De Zoysa *et al.* 2010) and other marine molluscs (e.g. defensins, mytilins, myticins, mytimycin, big defensin) (Li *et al.* 2009a).

Different from abalone haemolymph immunity, this study revealed enhanced antiviral activity in abalone digestive gland extracts in the macroalgae feeding trial. This suggests that abalone may derive some protection against pathogens, which are transmitted orally or through gastrointestinal tract, by dietary derived bioactive compounds. It has been shown that there was strong antiviral activity against HSV-1 in lipophilic extracts of *S. filamentosa* (100% inhibition of viral infection at 0.65mg

ml^{-1}) and *U. lactuca* (96% inhibition of viral infection at 0.65mg ml^{-1}). Interestingly, antiviral activity in *U. lactuca* has also been reported to be effective against human and avian influenza viruses (Ivanova *et al.* 1994), so it is conceivable that more than one type of antiviral compound is present in these macroalgae. So despite the fact that feeding abalone macroalgae did not increase abalone growth rate, dietary supplementation with bioactive macroalgae may still be promising in abalone aquaculture by boosting the first line of defense against oral pathogens and promoting abalone resistance to virus infection. On the other hand, antiviral activity was not detected in lipophilic extracts of microalgae pellets and there was no impact of microalgae additives on antiviral activity in abalone digestive glands detected in this study. Nevertheless, antiviral activity in extracts of microalgae cannot be excluded, since hot water extracts from *A. maxima* have reported activity against herpes simplex virus type 2, pseudorabies virus, human cytomegalovirus, and HSV-1 (Hernández-Corona *et al.* 2002). In addition, antiviral activity in *A. maxima* and *D. salina* could be lost through drying and pellet preparation process.

In conclusion, *A. maxima* or *D. salina* can be used as supplements in abalone formulated diet to enhance the growth of *H. laevigata*. Although *U. lactuca* and *S. filamentosa* are not suitable to replace the current formulated diet, some proportion of these two macroalgae in abalone diet could be a potential strategy for boosting the antimicrobial defense systems of abalone in aquaculture, especially in areas with high risk of abalone herpesvirus infection. These two macroalgae have been shown to increase the antiviral activity in the abalone digestive system. In addition, macroalgal supplementation may help reduce the feeding cost while still maintaining competent growth. Further studies are needed to elucidate which nutrients and

bioactive compounds in algae are important for abalone growth and immune enhancement.

Acknowledgments

CHAPTER 5: Influence of elevated temperatures on the immune response of abalone, *Haliotis rubra*

Abstract

Elevated water temperature can act as a stressor impacting the immune responses of molluscs, potentially increasing their susceptibility to microbial infections. Abalone are commercially important marine molluscs that have recently experienced disease outbreaks caused by a herpesvirus and *Vibrio* bacteria. Sampling of wild-caught *Haliotis rubra* showed a significant correlation between water temperature and both antiviral and antibacterial activity, with higher activity in summer than in winter months. However, antibacterial activity was compromised in favour of antiviral activity as the water temperatures peaked in summer. A controlled laboratory experiment was then used to investigate several immune responses of *H. rubra*, including total haemocyte count (THC), stimulated superoxide anion production (SO), antiviral activity against a model herpesvirus, herpes simplex virus type 1 and antibacterial activity against a representative pathogenic bacterium, *Vibrio anguillarum*, over one week after raising water temperature from 18 to 21 or 24 °C. THC and SO increased at day 1 and then dropped back to control levels by days 3 and 7. By comparison, the humoral immune parameters showed a delayed response with antibacterial and antiviral activity significantly increasing on days 3 and 7, respectively. Consistent with the field study, antibacterial activity became significantly depressed after prolonged exposure to elevated temperatures. A principal components analysis on the combined immune parameters showed a negative correlation between antiviral and antibacterial activity. SO was positively correlated to THC and neither of these cellular parameters were correlated to the humoral antimicrobial activity. Overall, this study indicates that abalone may have

more resilience to viruses than bacterial pathogens under conditions of elevated temperature, such as those predicted under future climate change scenarios.

5.1. Introduction

Climate change is a major threat to the economic and ecological sustainability of marine fisheries and aquaculture. The average sea surface temperatures have increased by 0.6°C in the last 100 years and these changes are ongoing (Trenberth *et al.* 2007; Hoegh-Guldberg *et al.* 2010). Recently, climate change has been implicated in the increasing frequency and severity of disease outbreaks in marine environments (Hayes *et al.* 2001; Lafferty *et al.* 2004; Marcogliese 2008; Harvell *et al.* 2009; Lejeusne *et al.* 2010). Notable examples of recent disease epidemics in marine molluscs include the northward expansion of oyster diseases (Ford 1996; Cook *et al.* 1998), escalating summer mortality in European and Australian bivalve and abalone aquaculture (Soletchnik *et al.* 1999; Li *et al.* 2007; Samain *et al.* 2007; Li *et al.* 2009d; Li *et al.* 2009b) and the recent detection of emerging oyster and abalone herpesviruses (Savin *et al.* 2010). Climate change associated with El Niño and La Niña events has also been implicated in the prevalence of *Perkinsus* infections in oysters (Powell *et al.* 1996) and in withering syndrome in abalone (Moore *et al.* 2000; Raimondi *et al.* 2002). Increased incidence and prevalence of marine disease is likely to have substantial ecological and economic costs, providing a compelling need to understand the complex interactions leading to disease outbreaks (Hoegh-Guldberg *et al.* 2010).

Disease outbreaks in marine molluscan populations are often associated with increases in water temperature (Lee *et al.* 2001; Cheng *et al.* 2004a; Handlinger *et al.* 2005; Travers *et al.* 2008; Travers *et al.* 2009). The manifestation of disease involves an interaction between pathogen, environment and the physiological status of the molluscan host (Fig. 5.1). The incidence of disease increases under environmental

conditions that cause stress to the host and/or increase pathogenicity and pathogen prevalence. Elevated temperature can increase the growth rate and virulence of microbial pathogens, including *Vibrio* spp. (Harvell *et al.* 1999; Marcogliese 2008). Environmental stressors, such as elevated temperature, are also known to decrease molluscan host resistance to bacterial pathogens (Harvell *et al.* 2002; Hooper *et al.* 2007b). For example, when exposed to elevated temperatures outside their preferred temperature range, abalone *Haliotis diversicolor supertexta* and *Haliotis tuberculata* have shown increased total haemocyte counts (THC), superoxide anion levels (SO) and susceptibility to infection by *Vibrio parahaemolyticus* or *Vibrio harveyi* (Huang *et al.* 2001; Cheng *et al.* 2004a; Travers *et al.* 2008; Travers *et al.* 2009). Viral abundance and virulence also appear to increase with rising water temperature (Danovaro *et al.* 2010). However, to date, no studies have investigated the effects of temperature on antiviral defense mechanisms in marine molluscs.
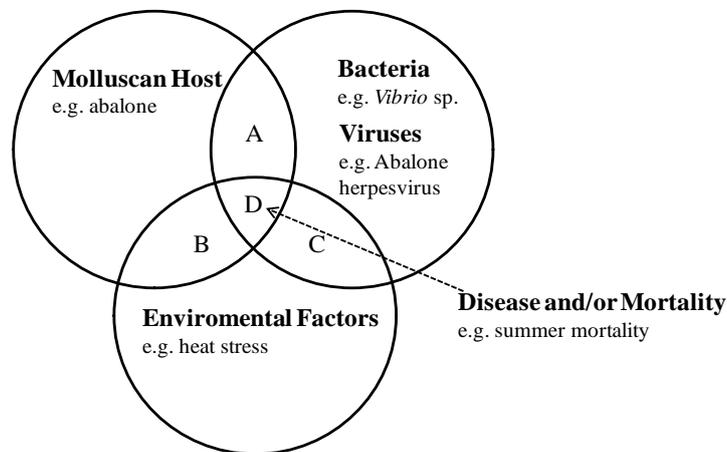
Figure 5.1. The "holy triad" of disease causality: interactions between molluscan host, environmental factors and pathogens. A) Subclinical stage of infection where the molluscan host does not show disease signs. B) Immune suppression by environmental stressors. C) Increased abundance and virulence of pathogens driven by environmental factors. D) Disease and/or mortality due to immune-suppression by environmental stressors in the presence of pathogen(s) (adapted from Snieszko 1974).

The abalone innate immune system consists of cellular and humoral components (Hooper *et al.* 2007b). Antimicrobial compounds acting as humoral effectors of molluscan immunity can be constitutively expressed and rapidly induced to provide an immediate response to invading microorganisms (Tincu *et al.* 2004; Otero-Gonzalez *et al.* 2010). Humoral immunity of abalone has been demonstrated using antibacterial activity assays against marine pathogenic bacteria, *V. harveyi* and *Vibrio anguillarum* (Dang *et al.* 2011a; Dang *et al.* 2011c) and an antiviral assay against herpes simplex virus type 1 (HSV-1) (Dang *et al.* 2011b; Dang *et al.* 2011c) on cell-free haemolymph. Because antimicrobial factors in the haemolymph are often synthesized by haemocytes (Hooper *et al.* 2007b), humoral immunity could be partly dependent on the concentration and activity of haemocytes. Cellular immunity is centered on the activity of haemocytes, including the elimination of infectious agents by release of superoxide anion, phagocytosis of microbial pathogens and the recognition and elimination of infected cells (Loker *et al.* 2004; Hooper *et al.* 2007b). Antibacterial and antiviral levels in the haemolymph plasma, total haemocyte count and superoxide anion production in haemocytes have been used as representative humoral and cellular immune parameters of abalone in the current study.

Abalone are major economic species in many countries including the United States, Mexico, South Africa, Australia, New Zealand, Japan, China, Taiwan, Ireland, and Iceland (Fleming *et al.* 1996; Cook 1998; Godoy *et al.* 1998; Gordon *et al.* 2001; 2004). *Haliotis rubra* is a common cold water abalone species in south-eastern Australia, with a preferred temperature range of 8 - 17 °C (Prince *et al.* 1992; Gilroy *et al.* 1998) and a critical thermal maximum reported at 26.9 °C (Gilroy *et al.* 1998). With elevated temperatures, animals need to increase their metabolism to acquire an

adequate energy supply for respiration and survival (Head 1962; Brown *et al.* 1984). We therefore hypothesize that less energy is available for an immediate immune response and the synthesis and release of antibacterial and antiviral factors will be compromised after temperature stress. Field sampling across seasons was used to correlate changes in antiviral activity against HSV-1 and antibacterial activity against *V. anguillarum* to natural changes in water temperature. A manipulative laboratory experiment was used to further investigate THC, SO, antiviral and antibacterial activity in response to short-term elevated temperature. These experiments provide an insight into the potential resilience of abalone to viral and bacterial pathogens under realistic seasonal and rapid (within a low tide cycle) temperature fluctuations and provide a model for predicting the longer term impacts of oceanic climate change.

5.2. Materials and methods

5.2.1. Field-sampled abalone

H. rubra were sampled for haemolymph at O'Sullivan Beach, South Australia, every month from August 2009 (austral winter) to February 2010 (summer). For each time point, at least 20 abalone were collected for haemolymph sampling. Water temperature was also recorded at each sampling time point using a hand-held thermometer.

5.2.2. Laboratory temperature challenge

The laboratory experiment complements the field study by examining rapid changes in water temperature, which occur on a day-to-day basis in natural habitats and in shallow water tanks on abalone farms in South Australia. Wild abalone can

experience significant changes in temperature over diurnal tidal cycles, especially for those in shallow intertidal rock pools (exceeding 6 °C increases during summer low tides). Abalone, *H. rubra* (n = 90), were collected in May 2010 (post-spawning) from the intertidal zone of O'Sullivan Beach, South Australia, then acclimated in aquaria at Flinders University for two weeks in filtered seawater with continuous aeration at 18 °C (to match the mean temperature in the field at the time of collection). The abalone were determined to be healthy, with no visible lesions, and they maintained a firm grip on the tank surface. The abalone of similar size, 8-9 cm in shell length, were kept in 9 PVC tanks (50 liter capacity, 10 abalone in each) containing 45 L per tank of sea water. Abalone holding tanks were connected to a 50 liter sump tank in groups of three, allowing water to flow continuously at the same rate of about 2 L min^{-1}. During the acclimation period, abalone were fed three times per week with fresh *Ulva lactuca*, collected from O'Sullivan Beach.

H. rubra were subjected to indoor temperature challenge in triplicate tanks (Fig. 5.2). Water temperature was kept at 18 °C in one sump tank (unchallenged group), and raised to 21 °C or 24 °C in the other sump tanks (temperature-challenged groups) at the rate of 1 degree h^{-1} using portable 300 watt glass heaters (Aqua One). Haemolymph was sampled from six replicate abalone from the unchallenged and challenged groups (two abalone from each of three tanks per treatment) at days 1, 3 and 7 (Fig. 5.2). Haemolymph (3 ml) was withdrawn from the anterior sinus of abalone using a pre-cooled sterile syringe and 25G needle, and kept on ice.
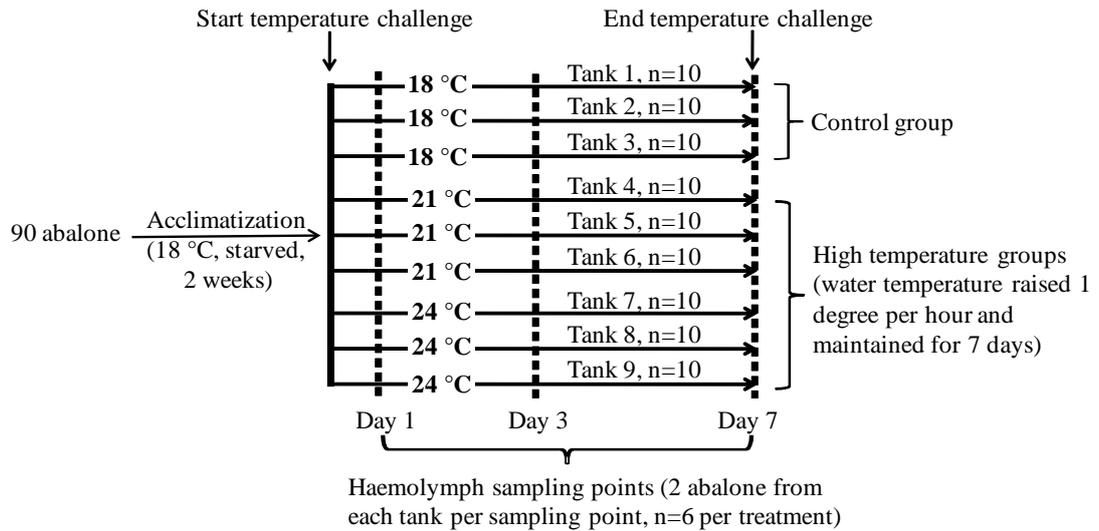
Figure 5.2. Summary of laboratory temperature challenge experiment. Abalone (90) were randomly divided into 9 tanks (n = 10 per tank) and three replicate tanks were allocated to each temperature treatment group (18, 21 and 24 °C). From each tank, 2 abalone were sampled for haemolymph at days 1, 3 and 7 (total of 6 individual samples per temperature treatment per day). Fresh haemolymph was measured for total haemocyte count and intracellular superoxide anion. The remaining haemolymph was centrifuged (3,000 rpm, 10 min, 4 °C) to obtain cell-free plasma, which was then stored at -80 °C for antibacterial and antiviral assay.

5.2.3. Haemolymph parameter measurements

H. rubra haemolymph collected from the field from August 2009 to February 2010 was assessed for antiviral and antimicrobial activity. *H. rubra* haemolymph from the laboratory temperature challenge experiment was measured for total haemocyte count, intracellular superoxide anion, antiviral activity against HSV-1 and antibacterial activity against *V. anguillarum*.

5.2.3.1. Total haemocyte count (THC)

Fresh haemolymph (50 μl) was fixed in 10% formalin in PBS solution (100 μl) in pre-cooled centrifuge tubes on ice to prevent haemocytes from aggregating or

clumping. All haemolymph samples were briefly vortexed before being placed on an improved Neubauer haemocytometer (Weber, England) in duplicate for counting the number of haemocytes under a microscope (Olympus CX40). THC is expressed as cells x 10^4 per ml.

5.2.3.2. Superoxide anion production

Superoxide anion level (SO) of haemocytes was quantified using reduction of nitroblue tetrazolium (NBT) to formazan, as described by Cheng et al. (2004a). Fresh haemolymph (150 µl) was kept on ice before being placed in triplicate into wells of a 96-well microtitre plate for 20–25 min at room temperature to obtain a cell monolayer. PBS was used as negative control to measure the background breakdown of NBT. After attachment, the supernatant was discarded, and 100 µl of sodium alginate (0.2 mg ml^{-1} in PBS) was added to activate SO production within the haemocytes (e.g. Cheng *et al.* 2004d; Cheng *et al.* 2004a; Cheng *et al.* 2004c; Dang *et al.* 2011a). After incubation at 26 °C for 30 min, sodium alginate was discarded and haemocytes were stained with 100 ml NBT solution (0.3 %) for 30 min at 26 °C. The NBT solution was removed and haemocytes were fixed with 100 µl methanol (100 %), washed three times with 100 µl methanol (70 %, in PBS) and air dried. The formazan was dissolved by addition of 120 µl KOH (2 M) and 140 µl dimethyl sulphoxide (DMSO). Absorbance was measured at 630 nm using a microplate reader (FLUOstar Omega). Superoxide anion level was expressed as treatment absorbance - negative control absorbance.

5.2.3.3. Antiviral assay

Phylogenetic analysis of DNA polymerase genes from abalone and oyster herpesviruses suggests that the abalone and oyster herpesviruses are within the family *Malacoherpesviridae* and are distantly related to other members of the *Herpesviridae* (Davison 2002; Davison *et al.* 2009; Savin *et al.* 2010). Due to the lack of a cell line for culturing abalone herpesvirus, a heterologous model using Vero cells and HSV-1 was chosen for investigating the effect of increased temperature on abalone antiviral activity, as this has been successfully used to assess antiviral activity in previous studies on molluscs (Olicard *et al.* 2005a; Olicard *et al.* 2005b; Dang *et al.* 2011b; Dang *et al.* 2011a; Dang *et al.* 2011c). A well-characterized strain, SC16 (Speck *et al.* 1991; Speck *et al.* 1992) of wild-type HSV-1 was obtained from the Institute of Medical and Veterinary Science, Adelaide. Culture of Vero cells and HSV-1 and the plaque reduction assay to measure antiviral activity of abalone haemolymph against HSV-1, were carried out as in our previous studies (Dang *et al.* 2011b; Dang *et al.* 2011c). Haemolymph plasma was obtained by centrifuging crude haemolymph (3,000 rpm, 10 min, 4 °C) then the cell free plasma layer was pipetted off, leaving behind the cell pellet. Cell free plasma was stored at -80 °C until assayed. Haemolymph plasma at 6 % (v/v) was used throughout to compare antiviral activity (EC_{50} = 6.23 %, v/v i.e. the effective concentration required to inhibit HSV-1 plaque formation by 50%) (Dang *et al.* 2011b).

5.2.3.4. Antibacterial assay

V. anguillarum is a common pathogen of marine molluscs including abalone. Stock cultures of *V. anguillarum* were obtained from the Fish Health Unit, Department of Primary Industries, Tasmania, and held at -80 ºC in 10% glycerol, nutrient broth until

use. This bacterium was cultured in sterile nutrient broth (1 g NaCl, 2 g yeast extract and 1 g peptone per 100 ml distilled H_2O) overnight at 37 ºC on an orbital mixer shaker (Ratek) at 200 rpm. The cultures were diluted to an optical density at 600 nm (OD600nm) of 0.1 with a spectrophotometer (Metertech, UV/VIS SP8001) and returned to exponential growth phase (OD600nm = 0.18 - 0.2) prior to use in antimicrobial assays. Antibacterial activity in the cell-free haemolymph plasma was measured using MTS assay against *V. anguillarum*, as described (Dang *et al.* 2011a; Dang *et al.* 2011c). The MTS assay is based on reduction of MTS tetrazolium to a red formazan product by cellular dehydrogenase. Haemolymph plasma (90 μl) and 10 μl of *V. anguillarum* in exponential growth culture were added into a 96-well plate in triplicate. Negative controls had 90 μl haemolymph in 10 μl nutrient broth and positive controls had 10 μl of *V. anguillarum* in 90 μl of nutrient broth. After 30 min incubation, 20 μl of CellTitre 96® Aqueous One Solution (Promega) was added to each well. The plates were then incubated at 37 °C for a further 2 hrs or until development of red formazan product in positive controls. Different incubation temperatures including 18, 24, 30 and 37 °C were tested and showed a similar trend in antibacterial activity (Appendix 5.1). Absorbance was measured at 492 nm using a 96-well plate reader (FluoStar Omega). Antibacterial activity (%) was calculated from 100 – [(treatment absorbance - negative control absorbance) / positive control absorbance] x 100.

5.2.4. Statistical analysis

To investigate how abalone antimicrobial activity varies in the field in relation to water temperature, a correlation between water temperature and antiviral or antibacterial activity was tested using Pearson Correlation in PASW/SPSS statistics

18. Univariate PERMANOVA analyses using Primer V6 + PERMANOVA (Plymouth Marine Lab) were performed to identify how each immune parameter (THC, superoxide anion level, antiviral activity and antibacterial activity) was individually influenced by temperature and/or length of exposure in laboratory challenge experiment. The four immune parameters were normalized to the same scale (Anderson *et al.* 2008) before conducting multivariate analysis for all four immune parameters combined. A principal components analysis was run using the multivariate data with vector overlay to investigate how each immune parameter influenced the grouping of the data.

5.3. Results

5.3.1. Field survey

Water temperature was lowest in August and September, (13 and 12.5 °C respectively), and reached 26.5 °C in February (Fig. 5.3). Generally, antiviral activity increased across months consistently with an increase in water temperature (Fig. 5.3). There was a strong correlation between water temperature and antiviral activity, with 85% of the variation in antiviral activity across the seven months explained by water temperature (Pearson correlation, r=0.92, p=0.003). Antiviral activity was lowest in September 2009 (mean of 46.04 %) and highest (63.76 %) in February 2010 (Fig. 5.3). At each monthly sampling point, there was high variation in antiviral activity (up to 42-86 %) with a trend towards more variation in higher temperature months (November - February, Fig. 5.3).

Antibacterial activity against *V. anguillarum* showed more variation across months, which was not directly explained by increase in water temperature (Fig. 5.3).

Nevertheless, 61% of the variation in antibacterial activity was still explained by water temperature (Pearson correlation, r=0.78, p=0.04). Antibacterial activity peaked in December 2009 (42.31 %) then decreased slightly in January (38.12 %) and February 2010 (36.54 %), despite increases in water temperature (Fig. 5.3). Antiviral and antibacterial activities across monthly sampling points were not significantly correlated (Pearson correlation, r=0.62, p=0.14).
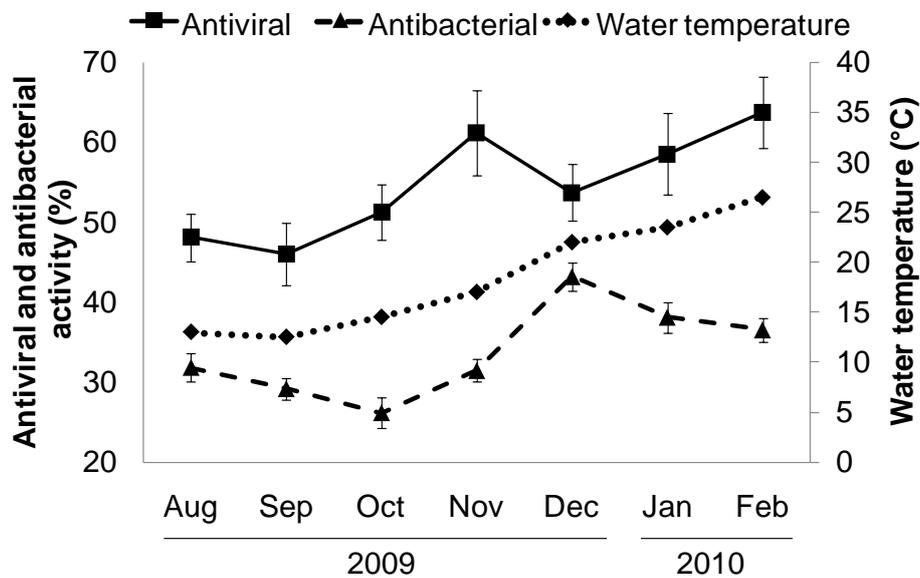
Figure 5.3. The relationship between antiviral and antibacterial activity and water temperature. Abalone *H. rubra* were collected from the same site, O'Sullivan Beach, South Australia from August 2009 (winter) to February 2010 (summer) (n ≥ 20 per month). Error bars showed the variability of antiviral and antibacterial activity from ≥ 20 abalone haemolymph samples in each month.

5.3.2. Laboratory temperature challenge experiment

There was no mortality of abalone in the temperature challenge experiment over 7 days. The effect of temperature across days was tested on each of the individual immune parameters. By day 1, THC was elevated in the temperature-challenged

groups, and these haemocyte numbers remained variable with prolonged exposure at 21 °C, but appeared to drop back to the level of controls by day 7 in the 24 °C group (Fig. 5.4a). Univariate analysis revealed a significant difference in the THC of *H. rubra* according to temperature, but there was no significant effect of day or an interaction between these factors (Table 5.1). Pair-wise tests between temperatures across all days revealed that THC in both of the temperature-challenged groups was significantly higher than in control groups (18 vs 21 °C, t=3.79, p=0.003; 18 vs 24 °C, t=2.18, p=0.037). Intracellular superoxide anion levels in haemocytes were elevated in temperature treated groups compared to controls. In the 24 °C group, SO anions peaked on day 1 then dropped to the level of controls by day 7, whereas at 21 °C, SO anion levels appeared to rise more slowly across the 7 days (Fig. 5.4b). However, univariate PERMANOVA found no significant difference in superoxide anion levels according to temperature or day (Table 5.1).
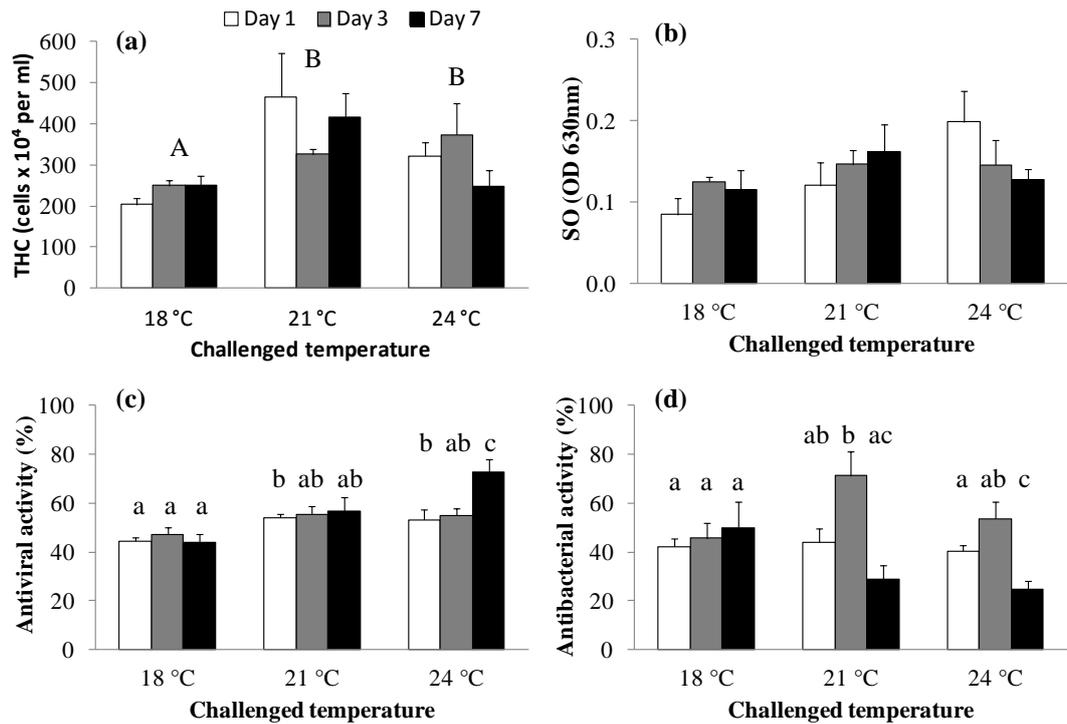
Figure 5.4. Effect of challenge temperature and length of exposure on (a) total haemocyte count (THC, cells x 10^4 per ml), (b) superoxide anion (SO, OD 630nm), (c) antiviral activity (%) against HSV-1 and (d) antibacterial activity (%) against *Vibrio anguillarium*. Each immune parameter was measured from six replicate abalone. Different capital letters indicate significant differences ($p<0.05$) between temperature groups across all days for THC (a) where there was no significant interaction. However, for antiviral (c) and antibacterial activity (d), univariate PERMANOVA revealed a significant interaction between day and temperature. Therefore, different small letters indicate significant differences between temperatures within the relevant days and between days within temperature groups. Means and standard error were derived from assay for six abalone haemolymph samples per sampling point.

Table 5.1. The effect of temperature and length of exposure (fixed factors) on individual immune parameter (univariate PERMAONVA) or combined four different immune parameters of *H. rubra*. The level of significant differences was set at p=0.05.

Parameter	Factor	PERMANOVA		
		df	Pseudo-F	p
Total haemocyte count	Temperature	2	7.04	0.002*
	Length of exposure (LE)	2	0.16	0.85
	Temperature x LE	4	1.6	0.21
Superoxide anion	Temperature	2	2.93	0.06
	Length of exposure (LE)	2	0.03	0.97
	Temperature x LE	4	1.76	0.13
Antiviral activity	Temperature	2	12.8	0.001*
	Length of exposure (LE)	2	2.84	0.07
	Temperature x LE	4	2.92	0.03*
Antibacterial activity	Temperature	2	1.3	0.28
	Length of exposure (LE)	2	8.63	0.002*
	Temperature x LE	4	3.38	0.02*
Combined four different immune parameters	Temperature	2	5.39	0.001*
	Length of exposure (LE)	2	3.12	0.007*
	Temperature x LE	4	1.92	0.022*

* Significant effect of temperature, length of exposure or their interaction on individual immune parameter or combined different immune parameters of abalone.

On average, antiviral activity was higher in both temperature-challenged groups compared to control groups and reached a maximum of 72.5% inhibition of HSV-1 plaque formation at day 7 in the 24 °C group (Fig. 5.4c). There was a significant interaction between temperature and day (Table 5.1), however, most of the variation was due to temperature (coefficients of variation for temperature and day were 54.4 and 8.5, respectively) (Fig. 5.4c). Pair-wise analyses for temperature across days revealed a significant difference in antiviral activity on day 1 between 18 vs 21 °C (t=4.54, p=0.001) and 18 vs 24 °C (t=1.96, p=0.05) and on day 7 between 18 vs 24 °C (t=4.37, p=0.01). There was no significant difference between different days within the controls or 21 °C challenged abalone. The antiviral activity of abalone in the 24 °C group was significantly higher on day 7 than on day 1 (t=2.78, p=0.025).

On day 3, antibacterial activity tended to be elevated in the temperature-treated groups, whilst by day 7, antibacterial activity was depressed relative to 18 °C

controls (Fig. 5.4d). Antibacterial activity of *H. rubra* haemolymph against *V. anguillarum* showed more variability between days than temperature treatments (estimates of variation for temperature and day were 4.4 and 113.5, respectively), and the interaction between temperature and day was significant (Table 5.1). Pair-wise analyses for temperature across days revealed significantly higher antibacterial activity in the 21 °C challenged group than in the control group on day 3 (t=2.17, p=0.048) and lower activity in the 24 °C challenged group than in the control group on day 7 (t=2.27, p=0.043). According to pair-wise analyses for day within the control group at 18 °C, antibacterial activity was similar across all days (p>0.05). At 21 °C, antibacterial activity was higher on day 3 than on day 7 (t=3.63, p=0.01), but was not significantly different between day 1 vs 7 (t=1.84, p=0.1) and day 1 vs 3 (t=2.35, p=0.079). At 24 °C, antibacterial activity was higher on day 1 and 3 than on day 7 (t=3.7, p=0.02 and t=3.59, p=0.002, respectively).

Principal coordinates analysis revealed that when the four immune parameters are combined, data from the control abalone at 18 °C clusters much more tightly than 21 and 24 °C groups, with the greatest variability in the abalone treated at 24°C (Fig. 5.5). The first two eigenvectors explain 64.6 % of the variability in the immune data (Fig. 5.5). Vector overlay of immune parameters (r>0.2) reveals that the data separates along the first eigenvector (X axis) primarily due to differences in THC and SO, whereas antibacterial and antiviral activity drive the separation of data points along the second eigenvector (Y axis). Antibacterial and antiviral activity appears to be inversely related, and these humoral parameters show no correlation to the cell-mediated immune parameters of THC and SO. By using multivariate PERMANOVA, we found that the combined immune responses of abalone were

significantly affected by both temperature and length of exposure and there was an interaction between these two factors (Table 5.1).
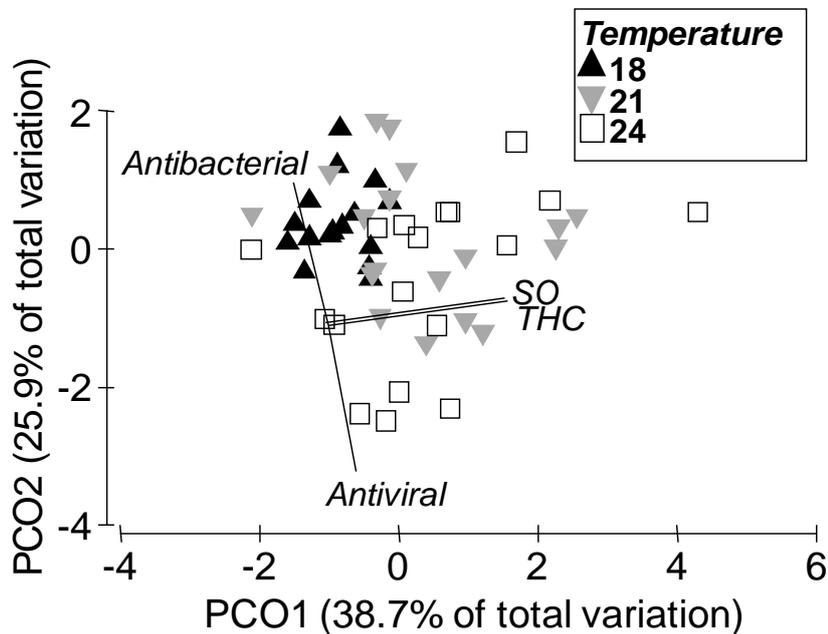
Figure 5.5. Principal coordinates plot showing the grouping of abalone according to temperature based on the first two eigenvectors that contribute the most to the variability in abalone immune parameters. Vectors overlayed for the four immune parameters show that the data points separate according to THC and SO along the first eigenvector (X axis), whereas antibacterial and antiviral activity drive the separation of data points along the second eigenvector (Y axis). THC and SO vary in the same way, whereas antibacterial and antiviral activity are inversely related.

5.4. Discussion

The innate immune system of abalone has evolved to cope with a wide range of microbial pathogens, including viruses and bacteria. Our field study indicates that antimicrobial activity in the haemolymph of abalone generally increases over summer as water temperatures increase. However, as temperatures peak in summer, antibacterial activity decreases and immune responses appears to favour antiviral activity. Viral abundance has been observed to increase with water temperature in

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different oceanic regions, where increase of water temperature by only a few degrees was associated with a doubling of viral abundance (Danovaro *et al.* 2010). Increase in temperature also leads to higher host metabolism, which has been linked to higher rates of virus production (Proctor *et al.* 1993; Hadas *et al.* 1997; Danovaro *et al.* 2010). The elevated antiviral activity with increasing temperature, observed in the field study and further supported by our laboratory study, is possibly an adaptation to higher viral abundances in warmer conditions. However, this effect of temperature on antiviral activity cannot be generalized between molluscan species. For example, antiviral activity against HSV-1 in Pacific Oysters, *Crassostrea gigas*, has been reported to show an inverse pattern, with low activity in summer (<40 %) compared to winter months (90-100 %) (Olicard *et al.* 2005a). Indeed high temperature is likely to be an important factor contributing to the occurrence of herpesvirus outbreaks, which are typically reported in summer months in abalone (Hooper *et al.* 2007a) and other molluscs species such as oyster *C. gigas* (Renault *et al.* 1994a; Friedman *et al.* 2005; Burge *et al.* 2006; Nicolas *et al.* 2008), scallop *Pecten maximus* (Arzul *et al.* 2001) and clam *Ruditapes philippinarum* (Renault *et al.* 2001b).

In addition to a greater threat of viruses at higher temperature, there is also greater risk of bacterial infection (Harvell *et al.* 1999; Marcogliese 2008). Although we observed some correlation between antibacterial activity and temperature in the field study, unlike antiviral activity, the antibacterial activity dropped off in January and February 2010 when the water temperature approached the critical thermal maximum of *H. rubra* (26.9 °C). Consistent with this, in the laboratory experiment, the antibacterial activity appears to decrease after prolonged (7 day) exposure to elevated temperatures. Such compromised antibacterial response under high temperature stress may explain the high "summer mortality" reported in abalone populations (Lee

et al. 2001; Cheng *et al.* 2004a; Handlinger *et al.* 2005; Travers *et al.* 2008; Travers *et al.* 2009). Summer mortality has a major economic impact on Australian and European abalone fisheries and has primarily been linked to pathogenic *Vibrio* species (Reuter *et al.* 1999; Handlinger *et al.* 2005; Vakalia *et al.* 2005; Travers *et al.* 2008; Travers *et al.* 2009). In the European abalone *H. tuberculata*, it has been demonstrated that growth of *V. harveyi* is triggered by temperature, resulting in 90% mortality at 19 °C in comparison to no mortalities at 17 °C (Travers *et al.* 2008). Travers *et al.* (2009) further showed that a 1 °C difference of temperature leads to an increased mortality rate after exposing abalone to pathogenic bacteria. *H. diversicolor supertexta* are also more susceptible to *V. parahaemolyticus* at high temperature, 28-32 °C, than at 20-24°C (Cheng *et al.* 2004a). Therefore, abalone in general appear to be more susceptible to bacterial infection at elevated temperatures, which could be due to exhaustion of humoral antibacterial factors with prolonged heat stress.

The water temperature at O'Sullivan Beach, SA, was measured above 17 °C, that is above the preferred temperature range of *H. rubra*, for three summer months, with the highest recorded temperature at 26.5 °C in February 2010, near the lethal thermal limit for this species. The waters around Australia have been predicted to warm 1-3 ºC by 2070, with greatest warming in southern and south-eastern Australia (Hobday *et al.* 2006; Poloczanska *et al.* 2007; Ridgway *et al.* 2009). As a result, there will be an increase in the amount of time that abalone spend above their optimal temperature. With the longer term chronic temperature stress predicted to accompany ocean warming, antibacterial activity is likely to be compromised, leaving abalone more vulnerable to infections. Since the optimal temperature for *H. rubra* survival has been reported to range from 8 to 17 °C (Prince *et al.* 1992; Gilroy *et al.* 1998),

the low antibacterial activity in winter months, with temperatures below 18 °C, does not appear to contribute to mortality in this species. It is possible that moderate expression of antibacterial factors in winter months is due to lower pathogen abundance in colder months (Harvell *et al.* 1999; Marcogliese 2008).

Immunity depends on a complex interaction between cell-mediated and humoral factors, all of which can be impacted by environmental stressors (Cheng 1981; Mitta *et al.* 2000; Bachère *et al.* 2004; Hooper *et al.* 2007b). Consequently in our manipulative laboratory study, THC and intracellular superoxide anion were selected as haemocyte responses and antibacterial activity against *V. anguillarum* and antiviral activity against HSV-1 were used as indicators of humoral immunity. All four immune parameters showed an initial elevation in response to elevated temperature, but the humoral antimicrobial activity varied according to length of exposure to high temperature. Consistent with the field study, antiviral activity increased over time at the higher temperatures, whereas antibacterial activity became depressed after seven days exposure to elevated heat stress. Using multivariate analysis, this study reveals significant differences in the combined immune parameters of abalone subjected to elevated temperature. Principle components analysis revealed a correlation between the two cellular parameters, whereas antibacterial and antiviral activities appear to be negatively correlated, indicating possible trade-offs in the humoral immune system of heat stressed abalone. Similar evidence for trade-offs in the immune system of abalone has been reported by Travers *et al* (2009), who used multivariate analysis to examine changes in the haemolymph profile of *H. tuberculata* over the summer reproductive season, using a large panel of cellular and humoral parameters. They observed that some parameters decreased, such as phagocytosis and phenoloxidase, whilst others such as

agglutination titre and basal reactive oxygen species concurrently increased. Furthermore, Travers *et al* (2008) established a correlation between the multi-factor haemolymph profile and susceptibility to *V. harveyi* infection. This confirms the benefits of investigating a range of cellular and humoral immune parameters to gain a good insight into the complex changes in basal immunity and how this relates to disease susceptibility.

Despite the fact that humoral antimicrobial compounds are primarily synthesized and released by haemocytes (Tincu *et al.* 2004; Otero-Gonzalez *et al.* 2010), there was no apparent correlation between total haemocyte count and antimicrobial activity in our study. Further, the lack of correlation between SO and antimicrobial activity in the PCO analysis confirms that humoral antimicrobial activity is not simply due to the presence of reactive oxygen species. In our study, there were no significant differences in SO anion levels, although similar to THC, SO peaked on day 1 in the 24 °C treatment. By comparison, in *H. diversicolor supertexta* SO was found to increase significantly after day 3 or 5 under elevated temperature stress (Cheng *et al.* 2004a), suggesting specific immune responses may vary according to species and the situation, despite a general elevation in haemocyte numbers. The effect of temperature was immediately evident on THC, with significant increases within the first day, followed by apparent recovery three days after temperature challenge. This may be due to influx of circulating haemocytes from peripheral tissues, as suggested for stressed abalone (Hooper *et al.* 2007b). Some of these haemocytes may then participate in extracellular killing resulting in elevated humoral activity, with a coincident drop in THC by day 3. Humoral antibacterial activity against *V. anguillarum* preceded antiviral activity against HSV-1, peaking at day 3 and day 7, respectively. Antibacterial activity subsequently showed a decrease on day 7,

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suggesting that antibacterial factors are not replenished within a few days, whereas, the abalone appeared to increase antiviral activity after prolonged heat stress. The lack of correlation between antiviral and antibacterial activity (against HSV-1 and *V. harveyi*, respectively) has been reported across a number of farmed and wild populations of *Haliotis laevigata* in South Australia (Dang *et al.* 2011c), suggesting different compounds are involved in defense against bacteria and viruses in abalone. Future studies aimed at investigating differential gene expression in haemocytes from abalone under elevated temperature conditions would provide further insight into the specific mechanisms responsible for these short-term changes in humoral antimicrobial activity.

THC is highly variable across individuals, both within and between the temperature treatment groups. Within the first day of heat stress, the number of circulating haemocytes increased, but then returned to baseline levels by day 7. These results are consistent with a study by Cheng *et al.* in *H. diversicolor supertexta*, where THC increased one day after transfer from 28 to 32 °C, followed by a small decrease in THC on day 3 and 5 (Cheng *et al.* 2004a). Increased THC as a result of increased water temperature has been observed in laboratory experiments for other molluscan species, for example the clams *Chamelea gallina*, *Mactra veneriformis* and *Ruditapes philippinarum* (Paillard *et al.* 2004; Monari *et al.* 2007; Flye-Sainte-Marie *et al.* 2009; Yu *et al.* 2009), as well as the oyster *Chamelea virginica* (Chu *et al.* 1995). Similar effects of temperature on THC has been also found in field studies on molluscan species, such as the clam *Venerupis philippinarum* (Soudant *et al.* 2004), the mussel *Mytilus galloprovincialis* (Carballal *et al.* 1998) and the oyster *C. virginica* (Fisher *et al.* 1996). Other invertebrate species, including the crustaceans *Panulirus interruptus* (lobster) and *Litopenaeus stylirostris* (blue shrimp), also

experience an increase in THC with rising water temperature (Gomez-Jimenez *et al.* 2000; Le Moullac *et al.* 2000), suggesting this may be a general invertebrate immune response to heat stress. Increased THC at higher temperatures could be, in part, attributed to metabolic activity, as higher respiration rates have been reported for a range of invertebrates at higher temperatures (Head 1962; Brown *et al.* 1984). Nevertheless, some exceptions have been reported for the effects of heat stress on THC in freshwater species (e.g. the prawn *Macrobrachium rosenbergii* (Cheng *et al.* 2000) and the snail *Lymnaea stagnalis* (Seppälä *et al.* 2010)), suggesting there are complex interactions between host immunity and water temperature in different environments. Since haemocytes are involved not only in immune responses, but also in non-immune functions such as tissue and shell repair, nutrition, transport and excretion (Cheng 1981; Sparks *et al.* 1988; Cheng *et al.* 2004e), variation in the number of haemocytes could be influenced by the physiological condition and metabolic activity of specific individuals.

Under prolonged heat stress, available energy is redirected to support essential metabolic functions such as respiration (Tomanek 2010), resulting in compromised abalone antibacterial activity. Reduced antibacterial activity under prolonged elevated temperature has also been reported from laboratory and field studies on the Pacific Oyster *C. gigas* (2009b). However, unlike in these studies on oysters, and in a previous investigation of summer immune depression in *H. tuberculata* (Travers *et al.* 2008), our results do not appear to be influenced by spawning. In South Australia, *H. rubra* is reported to spawn from October to December (PIRSA Fisheries 2011), during which time antibacterial activity and antiviral activity were both seen to increase. This is consistent with our previous on-farm experiment, which revealed no effect of spawning on abalone antimicrobial activity (Dang *et al.* 2011c).

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Nevertheless, it is likely that other cellular components of the immune system would be impacted by spawning (Travers *et al.* 2008). Furthermore, metabolic stressors, including reproduction and poor nutrition, would add to the overall impact of elevated temperature and further compromise the immune system of abalone. Previous studies on abalone and oyster immunity have reported synergistic effects of heat stress with reproduction (Li *et al.* 2007; Li *et al.* 2009b; Travers *et al.* 2009), starvation (Li *et al.* 2009d) and simulated bacterial challenge (Hooper *et al.* 2007b). Consequently, these studies highlight serious implications for the ability of molluscs to defend themselves against pathogenic bacteria under natural environmental conditions, where they simultaneously encounter multiple stressors. The combination of elevated temperature and other stressors that cause immune depression, along with changing oceanic conditions that favour pathogen growth (Harvell *et al.* 1999; Marcogliese 2008) is likely to explain the increasing frequency and intensity of disease in marine mollusc populations on a global scale (Lafferty *et al.* 2004; Ward *et al.* 2004).

In conclusion, this study provides the first evidence for an effect of high temperature on abalone antiviral activity and confirms the effects on other cellular and humoral immune effectors, as well as highlighting a time-lag difference in the responses of these immune components. Consequently, predictions about disease resilience in light of ocean warming cannot be generalized across all types of pathogens. Comparison with previous studies implies that different immune responses to elevated temperature can occur in different species, further complicating the ability to predict patterns of disease susceptibility. Nevertheless, a consensus appears to be that molluscs typically suffer some immune depression with prolonged exposure to elevated water temperatures, increasing the likelihood that epidemic disease due to

bacterial infection will occur with continued ocean warming. Further studies on antiviral activity and susceptibility of molluscs to viral infection under temperature stress would complement the current studies on *Vibriosis* in molluscs.

Acknowledgements

Appendix 5.1. Effect of incubation temperature on antibacterial activity
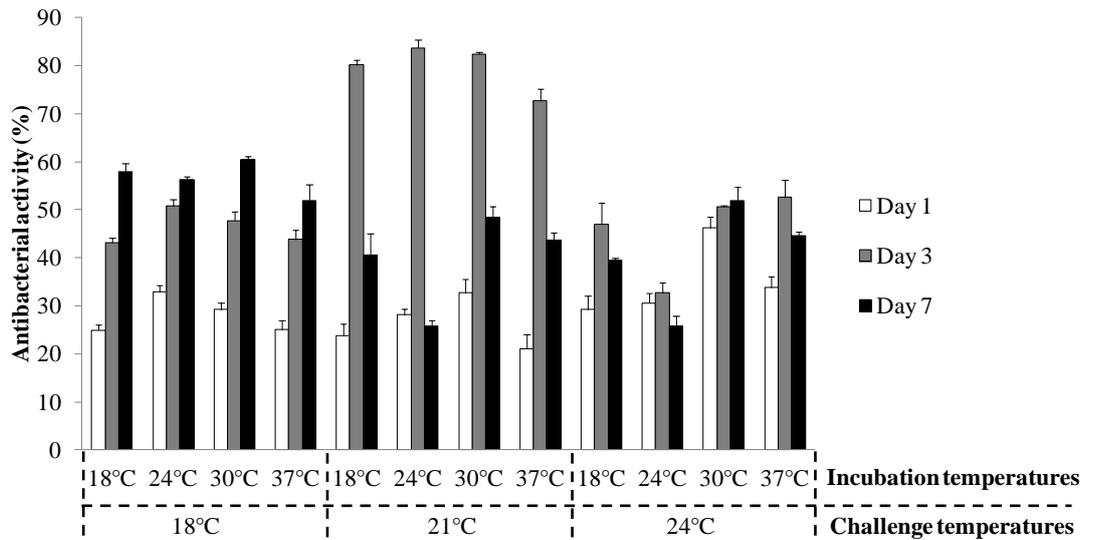
Figure 5.6. Effect of incubation temperature on antibacterial activity in antibacterial assay of abalone haemolymph against *Vibrio anguillarium*. Haemolymph pooled from 6 individuals of the same temperature treatment group and the same day were tested for antibacterial activity using 4 different incubations temperatures 18, 24, 30 and 37 °C. The overall trends in the results were the same irrespective of incubation temperature.

CHAPTER 6: Immunological changes in response to herpesvirus infection in abalone *Haliotis laevigata* and *Haliotis rubra* hybrids

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Abstract

Australian abalone production has been affected by outbreaks of abalone viral ganglioneuritis (AVG) caused by a herpesvirus (AbHV). The small proportion of abalone (~10%) that survives AVG outbreaks may provide insights into potential mechanisms of resistance. In this study, we undertook experimental transmission trials by immersion to study the abalone immune response to infection with AbHV. Representative cellular and humoral immune parameters of abalone, including total haemocyte count (THC), superoxide anion (SO) and antiviral activity against herpes simplex virus type 1 (HSV-1), were examined in apparently healthy (sub-clinical) and moribund abalone after challenge. In the early infection, sub-clinical stage (days 1-3), THC increased significantly in infected abalone on day 3, while SO and antiviral activity did not change significantly in challenged abalone. The number of viral gene copies in ganglion tissues was also determined by real time PCR (qPCR) to confirm infection status and examine any correlations with immune parameters. TaqMan qPCR confirmed 20.5% higher viral load in moribund abalone compared to apparently healthy abalone, indicating that the viral load within abalone is linked to their clinical signs. THC decreased by 38.8% in moribund abalone, but increased by 42.6 and 13.6% in apparently healthy (pre-clinical) abalone that were PCR-negative and PCR-positive for AbHV, respectively, in comparison to the non-infected control group. The level of SO decreased in abalone confirmed as PCR positive for AbHV; by 30.8% in moribund abalone and by 7.2% in apparently healthy abalone. However, for apparently healthy abalone that were PCR-negative after viral challenge, SO significantly increased by 59.3%, in comparison to uninfected controls. These results suggest that abalone mount an initial cellular immune

response to AbHV infection, but this response cannot be sustained under high viral

loads, leading to mortality.

6.1. Introduction

Abalone are marine gastropod molluscs of significant commercial importance worldwide (Gordon *et al.* 2001; 2004). In Australia, greenlip abalone *Haliotis laevigata,* blacklip abalone *H. rubra* and their hybrids are the main commercial species, generating an annual revenue from wild-capture and aquaculture of over AU$200 million (ABARE 2009). Unfortunately, the emergence of abalone viral ganglioneuritis (AVG) in south-eastern Australia (Victoria and Tasmania) threatens the sustainability of the abalone industries in the affected states (Hooper *et al.* 2007a). AVG is caused by infection with a herpesvirus (Savin *et al.* 2010), damaging neural tissues and resulting in up to 90% mortality of farmed abalone within 7-14 days from the onset of clinical signs (Hooper *et al.* 2007a). Similar viral infection has been reported in *H. diversicolor supertexta* in Taiwan (Chang *et al.* 2005). Phylogenetic analysis of DNA polymerase protein indicates that the abalone herpes virus (AbHV) belongs to the family *Malacoherpesviridae* and is distantly related to other members of the *Herpesviridae* (Savin *et al.* 2010).

Abalone defense against viral infection relies on their innate immune system, which consists of cellular and humoral components (Hooper *et al.* 2007b). Cellular immunity in molluscs, including abalone, is centered on the activity of haemocytes, including the elimination of infectious agents involving release of superoxide anion, phagocytosis of microbial pathogens and the recognition and elimination of infected cells (Loker *et al.* 2004; Hooper *et al.* 2007b). Antimicrobial compounds acting as humoral effectors of molluscan immunity can be constitutively expressed and rapidly induced to provide an immediate response to invading microorganisms (Tincu *et al.* 2004; Otero-Gonzalez *et al.* 2010). Humoral antiviral immunity of abalone has been

demonstrated using an assay measuring activity against herpes simplex virus type 1 (HSV-1) (Dang *et al.* 2011b; Dang *et al.* 2011a; Dang *et al.* 2011c; Dang *et al.* 2012) in cell-free haemolymph. Because antimicrobial factors in haemolymph are often synthesized by haemocytes (reviewed by Hooper *et al.* 2007b), humoral immunity could be partly dependent on the number and activity of haemocytes.

Of the AVG outbreaks reported by Hooper *et al*. (Hooper *et al.* 2007a), a small proportion of abalone survived (>10%). It is possible that survivors possessed an enhanced immunity. In this study, we performed *in vivo* infection trials (using abalone sourced from a farm with no previous history of AVG), by direct immersion in water in which AbHV is suspended (Corteel *et al.* 2009; Corbeil *et al.* 2012), to examine abalone immune responses during the first week after exposure to AbHV. Total haemocyte count, superoxide anion production in haemocytes and antiviral activity in the haemolymph plasma were used as representative cellular and humoral immune parameters of abalone to investigate differences in immune responses between apparently healthy and moribund abalone after challenge with AbHV. After immersion of abalone in AbHV-infectious water, their immunity was assessed at the early subclinical stage, and later, at the onset of disease.

6.2. Materials and methods

6.2.1. Abalone

H. laevigata x *H. rubra* hybrid abalone (n=150), obtained from Great Southern Waters Pty Ltd, Victoria, were placed in experimental aquaria and acclimated at 15-16 °C for a week in seawater with continuous aeration. The abalone were of similar size (7-8 cm in shell length) and kept in 150-liter PVC tanks containing 40 L of sea

water in separate infection and non-infection rooms. A complete water change was carried out in each tank daily.

Experimental infection trials were undertaken by immersing abalone in AbHV-infectious water, as well as by direct injection. Due to low infection rates in the injection experiment, only the results of the immersion trial are presented here and the injection trial is detailed in Appendix 6.1.

6.2.2. Infection of abalone by immersion

Virus stock was obtained from abalone previously infected with the Victorian isolate of AbHV (designated Vic-1) by homogenizing neural tissue in Eagle's Minimal Essential Medium containing 20% foetal bovine serum, with a Dounce homogeniser, followed by filtration (0.22µm) and the filtrate stored in liquid nitrogen, until use. AbHV-infectious water used for immersion challenge was produced by injecting six abalone intramuscularly in the foot with 100 uL of this stock virus (~1 x 10^5 viral gene copies (v.g.c.)/100uL). Inoculated animals were held in a tank containing 8L aerated sea water with daily 100% water exchange. Previous experiments have shown that water from day 4 post-inoculation contains high levels of infectious virus. Thus at 4 days post-inoculation, the water was harvested, titrated using the AbHV TaqMan assay (Corbeil *et al.* 2012) and diluted 1:5 to generate the challenge AbHV-infectious water. The amount of AbHV in this water was determined to be 15.6 x 10^6 v.g.c. ml^{-1}.

Abalone (n=72) were challenged by immersion for 20hr, in triplicate 40L tanks in the infection room (Fig. 6.1). Abalone (n=72) as negative controls were maintained in fresh seawater in triplicate tanks in a separate non-infection room. Haemolymph and

ganglia were sampled from nine abalone from the infected and non-infected (negative control) groups at days 1, 2 and 3. Haemolymph (~3 ml) was sampled from the anterior sinus using a sterile syringe and needle (5ml, 25G). The ganglion was then dissected from the head section using a sterile scalpel blade.
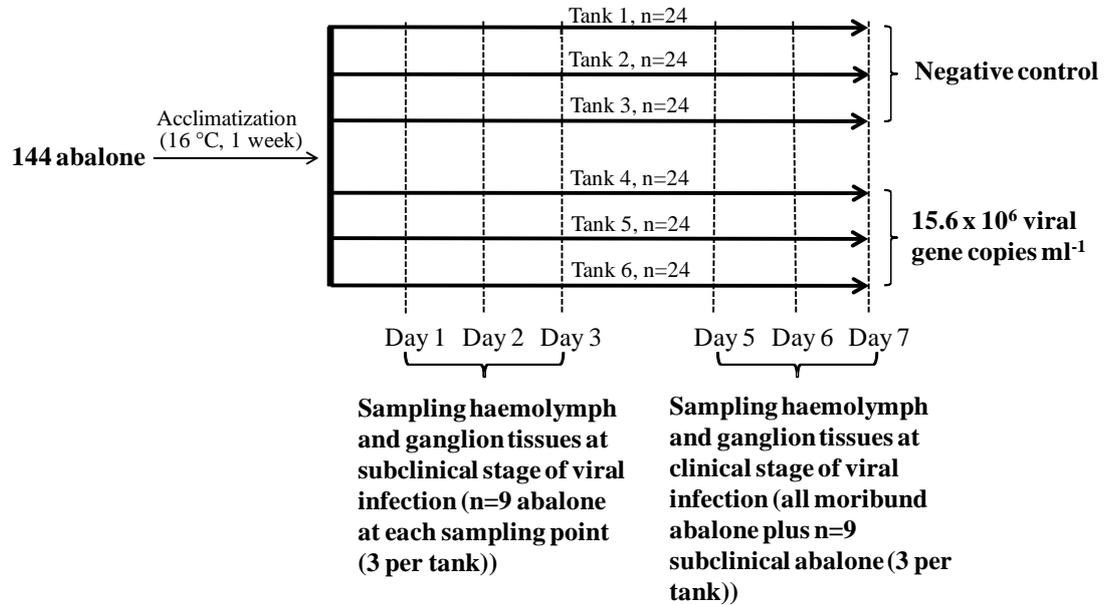
Figure 6.1. Summary of the abalone herpesvirus challenge trial. AbHV infectious water was prepared following intramuscular injection of six abalone. For the immersion challenge trial, abalone (n=72) were exposed to infectious water containing AbHV (15.6 x 10^6 v.g.c ml^{-1}) for 20h. Abalone were sampled for haemolymph at the subclinical stage of viral infection (days 1, 2 and 3; n=3 for each day from triplicate tanks). At the clinical stage of viral infection when abalone started to show morbidity signs, all moribund abalone, as well as healthy looking (subclinical abalone, n=9, three per tank) were sampled for haemolymph and the neural ganglia was dissected for qPCR to detect AbHV. Fresh haemolymph was assayed for total haemocyte count, intracellular superoxide anion and plasma antiviral activity against herpes simplex virus type 1 (HSV-1).

Moribund abalone, demonstrating loose attachment to the substrate, were collected on days 5, 6 and 7 for sampling their haemolymph and ganglion. On the same days, haemolymph and ganglion were also sampled from apparently healthy abalone in the infected room and negative control abalone from the non-infected room (n=9 per day, 3 from each tank).

6.2.3. TaqMan real-time PCR

Ganglion sections, including ganglion tissues and surrounding muscle from infected and uninfected abalone, were weighed and then nucleic acid was extracted using QIAamp DNA mini kit (QIAGEN). Nucleic acid extraction was also carried out on haemolymph from abalone injected with AbHV. Nucleic acid was eluted in 100 µl of buffer AE (10 mM Tris-Cl, 0.5 mM EDTA, pH 9.0). Samples were stored at -80°C until required.

ORF-49 qPCR (Corbeil *et al.* 2010) was carried out on DNA extracts from abalone tissues according to established protocols (Corbeil *et al.* 2010; Corbeil *et al.* 2012). All abalone samples were tested by real-time TaqMan PCR (7500 Fast Real-time PCR system, Applied Biosystems) in duplicate to obtain C_T values. AbHV-negative samples were identified as having C_T values >35.8 and AbHV-positive samples had C_T values <35.

To quantify the number of AbHV gene copies (v.g.c. μl^{-1}) in abalone samples from the C_T value, a plasmid DNA standard curve was prepared for plasmid Topo-ORF49 using serial 10-fold dilutions (Corbeil *et al.* 2010; Corbeil *et al.* 2012). The qPCR assay for plasmid Topo-ORF49 produced a linear correlation between recombinant

plasmid level and C_T value over 4 $\log_{10}$ dilutions (coefficient R^2=0.99, Appendix 6.2).

6.2.4. Haemolymph analyses

Fresh haemolymph from each abalone was assayed for THC and SO. The remaining haemolymph were centrifuged (1500 x g, 5 min) to obtain haemolymph plasma, which was frozen at -20°C until antiviral assays were undertaken against HSV-1.

6.2.4.1 Total haemocyte count (THC)

Fresh haemolymph (100 µl) was immediately fixed in 200 µl formalin (10%) in phosphate buffered saline solution (PBS: 137.93 mM NaCl, 2.67 mM KCl, 8.06 mM Na_2HPO_4, 1.47 mM KH_2PO_4) in pre-cooled Eppendorff tubes and kept on ice. Total haemocyte count for each haemolymph sample was obtained using an improved Neubauer hemocytometer.

6.2.4.2. Intracellular superoxide anion

Superoxide anion (SO) production by haemocytes was quantified using reduction of nitroblue tetrazolium (NBT) to formazan, as described by Cheng et al. (2004a). Fresh haemolymph (150 µl) was placed, in triplicate, into wells of a 96-well microtitre plate and incubated at room temperature (RT) for 20–25 min to obtain a cell monolayer. PBS was used as negative control to measure the background breakdown of NBT. After cell attachment, the supernatant was discarded, and 100 µl sodium alginate (0.2 mg ml^{-1} in PBS) was added to activate SO production within the haemocytes (e.g. Cheng *et al.* 2004d; Cheng *et al.* 2004a; Cheng *et al.* 2004c; Dang *et al.* 2011a). After incubation at RT for 30 min, sodium alginate was discarded and

haemocytes were stained with 100 ml NBT solution (0.3 %) for 30 min at RT. The NBT solution was removed and haemocytes were fixed with 100 µl methanol (100 %), washed three times with 100 µl methanol (70 %, in PBS) and air dried. The formazan was dissolved by addition of 120 µl potassium hydroxide (2 M) and 140 µl dimethyl sulphoxide (DMSO). Absorbance was measured at 620 nm on a microplate reader (Multiskan Ascent, Thermo Electron Corporation). Intracellular SO was expressed as sample absorbance - negative control absorbance.

6.2.4.3. Antiviral assay

Due to the lack of a cell line for culturing abalone herpesvirus, a heterologous model using Vero cells and HSV-1 was chosen for investigating antiviral activity in the haemolymph. A well-characterized strain, SC16 (Speck *et al.* 1991; Speck *et al.* 1992) of wild-type HSV-1 was obtained from the Institute of Medical and Veterinary Science, Adelaide. Culture of Vero cells and HSV-1, and the plaque reduction assay to measure antiviral activity of abalone haemolymph against HSV-1, were carried out as reported (Dang *et al.* 2011b; Dang *et al.* 2011c). Haemolymph plasma was obtained by centrifuging crude haemolymph (1500 x g, 10 min, 4 °C), then the cell-free plasma layer was removed. Cell-free plasma was stored at -80 °C. Haemolymph plasma (6 %; v/v) was used throughout to compare antiviral activity (EC_{50} = 6.23 %, v/v, i.e. the concentration required to inhibit HSV-1 plaque formation by 50%) (Dang *et al.* 2011b).

6.2.5. Statistical analysis

At the sub-clinical stage of infection (days 1-3), each abalone immune parameter (THC, SO level, antiviral activity) was compared between AbHV-exposed and non-

infected abalone using two-factor permutational analysis of variance in Primer V6 with PERMANOVA add-on (Anderson *et al.* 2008). There was no significant difference between tanks for abalone sampled from the same challenge group and on the same day (p>0.05). Post-hoc planned comparisons were undertaken between controls and treatments on each day using pair-wise tests. At the clinical stage of infection (days 5-7), four infected and non-infected abalone groups (1: negative control, 2: AbHV exposed PCR-negative and apparently healthy, 3: infected PCR-positive and apparently healthy, 4: PCR-positive and moribund) were compared for each immune parameter using one-factor univariate PERMANOVA. The correlation between v.g.c (log_{10}) in ganglion tissues and each immune parameter from AbHV exposed PCR-positive abalone in the immersion trial was tested using Pearson's correlation coefficient (PASW/SPSS statistics 18).

6.3. Results

6.3.1. Viral infection and TaqMan real-time PCR

There was no mortality, or clinical signs in abalone during the first three days after exposure to virus. All negative control abalone were PCR-negative for AbHV (Ct values > 35.8). Challenged abalone were all PCR-negative at days 1 and 2, and two out of the nine abalone were PCR-positive at day 3 (Ct values < 35.8). Viral DNA was detected in abalone ganglion tissues but not in their haemolymph. Abalone started showing clinical signs of ganglioneuritis (e.g. loss of pedal adhesion to tank surface, loss of righting reflex) on day 5 after exposure to virus. In total, 18 moribund and 29 healthy abalone from the infection room and 27 negative control abalone from non-infection room were sampled for haemolymph and ganglion tissues at days 5-7 post-infection. All moribund abalone were PCR-positive for

AbHV with a range in viral load from 41 x 10^4 to 14 x 10^7 v.g.c. per gram of tissue (Fig. 6.2). For abalone exposed to virus without clinical signs, 23 samples were PCR-positive with a range in viral load from $<10^2$ to 13 x 10^7 v.g.c. per gram of tissue (Fig. 6.2).
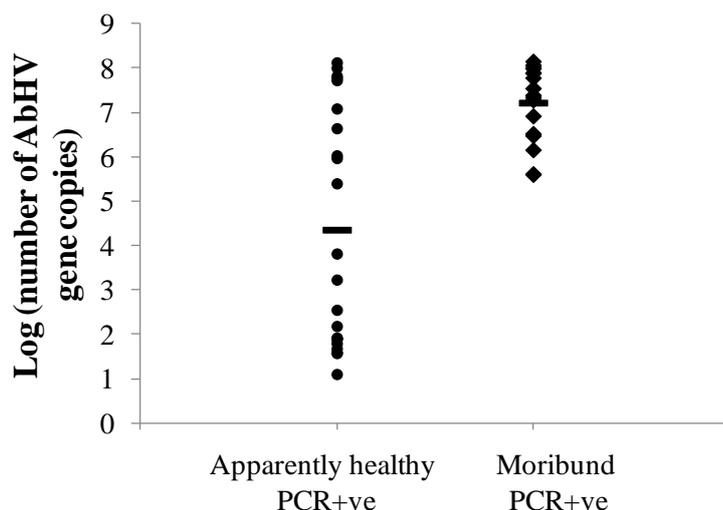
Figure 6.2. Quantification of AbHV gene copies ($\log_{10}$) based on ORF49 qPCR Ct values, in apparently healthy and moribund abalone from day 5 post-infection. Bars represent means of log values of number of AbHV gene copies.

6.3.2. Immune responses at subclinical stage of viral infection

In this trial, THC, SO and antiviral activity were highly variable across individuals within the negative control and AbHV exposed groups, within the same day (THC 138-588 x 10^4 cells ml^{-1}; level of SO 0.04-0.32 at OD620nm; antiviral activity 25.3-71.8%). The mean THC in AbHV-exposed abalone increased over time (day 1: 289 x 10^4 cells ml^{-1}; day 2: 347 x 10^4 cells ml^{-1}; and day 3: 382 x 10^4 cells ml^{-1}) (Fig. 6.3A). Pair-wise analysis detected significant difference ($p<0.05$) in THC between AbHV-exposed and control abalone on day 3 ($t=2.34$, $p=0.04$), but not on days 1 and 2 ($p>0.05$). The level of SO and antiviral activity against HSV-1 were not significantly different between AbHV-exposed and control groups on any day ($p>0.05$) (Fig. 6.3B & 6.3C).
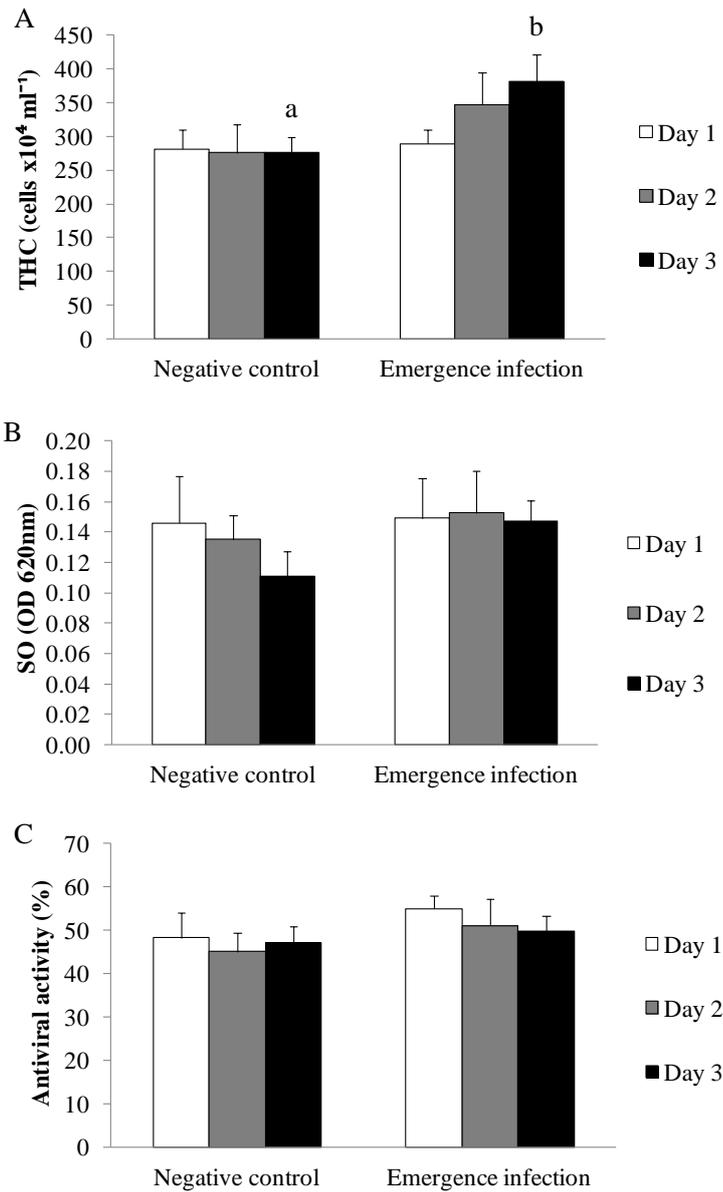
Figure 6.3. Effect of AbHV infection and time post-infection on A) total haemocyte count (THC, cells x 10^4 per ml), B) superoxide anion (SO, OD 620nm), C) antiviral activity (%) against HSV-1. Each immune parameter was measured from nine replicate abalone. Different small letters indicate significant differences ($p < 0.05$) between virus-exposed and negative control groups.

6.3.3. Immune responses at clinical stage of viral infection by immersion (days 5, 6, 7 post-challenge)

THC was elevated in AbHV exposed but apparently healthy abalone (for PCR-negative group 383 ± 55.1 x 10^4 cells ml^{-1}; for PCR-positive (sub-clinical) group 305 ± 30.21 x 10^4 cells ml^{-1}), whilst it was depressed in infected moribund abalone (165 ± 13.6 x 10^4 cells ml^{-1}), in comparison to negative controls (269 ± 18.1 x 10^4 cells ml^{-1}) (Fig. 6.4A). PERMANOVA revealed a significant difference (F=8.44, p=0.001) in THC depending on the infection status. Pair-wise tests confirmed significantly lower THC in moribund and PCR-positive abalone compared with all other groups, including apparently healthy and PCR-positive abalone (p=0.001), apparently healthy and PCR-negative abalone (p=0.001) and negative control abalone (p=001). Apparently healthy PCR-negative abalone had significantly higher THC than negative control animals (p=0.02). However, no significant difference in THC was found between PCR-positive and PCR-negative groups of apparently healthy AbHV exposed abalone or between apparently healthy PCR-positive abalone and negative control abalone (p>0.05). For PCR- positive abalone exposed to AbHV-infectious water, THC was negatively correlated with number of v.g.c. (log$_{10}$) from abalone tissues (n=40, Pearson's correlation coefficient r=-0.33, p=0.03).
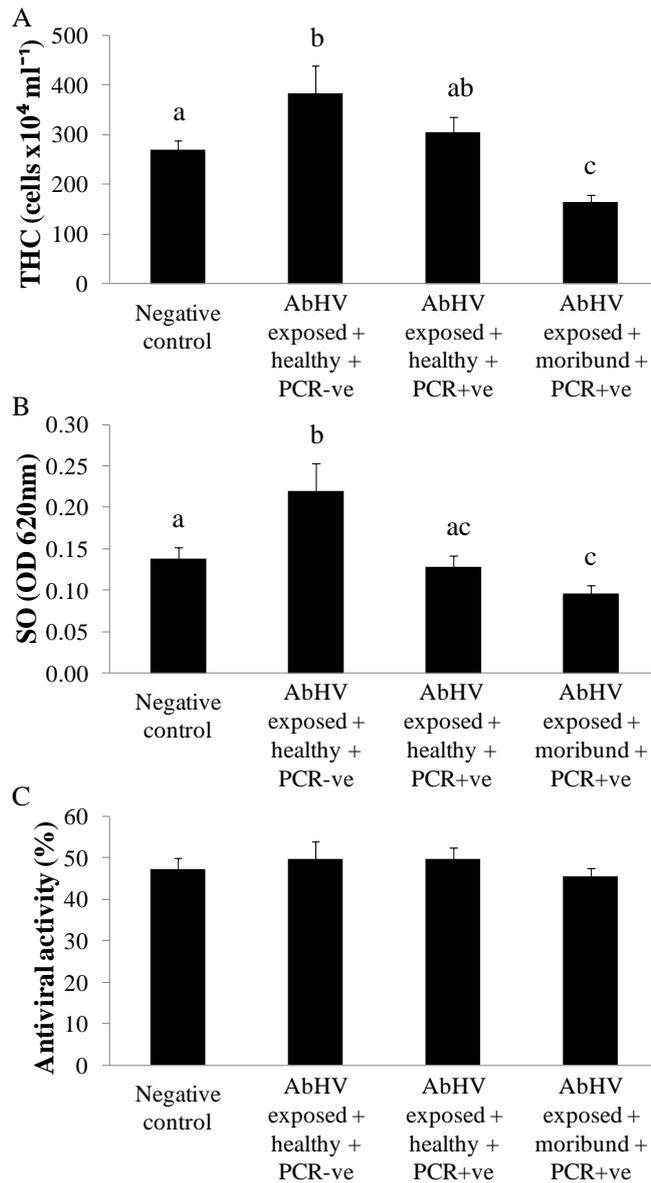
Figure 6.4. Effect of AbHV infection status at the clinical stage on abalone immune responses A) total haemocyte count (THC, cells x 10^4 per ml), B) superoxide anion (SO, OD 620nm), C) antiviral activity (%) against HSV-1. Comparison was made between non-infected negative control (n=27), AbHV exposed but apparently healthy and PCR-negative (n=6), apparently healthy and PCR-positive for AbHV (n=23), and moribund and PCR-positive (n=18) abalone. Different small letters indicate significant differences (p<0.05) between groups.

The SO level was highest in AbHV-exposed, healthy and PCR-negative abalone, followed by the negative control group, then infected healthy PCR-positive abalone, and then the moribund group (Fig. 6.4B). PERMANOVA revealed a significant difference in SO between abalone according to infection status (F=5.38, p=0.004). Pair-wise tests revealed a significantly higher level of SO in AbHV-exposed abalone, which were healthy and PCR-negative, compared with all other groups (p<0.05). In addition, the level of SO in moribund abalone was significantly lower than in the negative control group (p=0.04). No significant difference in SO was found between healthy and moribund abalone, which were confirmed to be PCR-positive (p = 0.1), or between healthy PCR-positive abalone and negative control abalone (p=0.63). For all abalone that were exposed to AbHV, the level of SO was negatively correlated to the number of v.g.c. ($\log_{10}$) in abalone tissues (n=40, r=-0.53, p<0.01).

Antiviral activity increased slightly in AbHV-exposed, healthy abalone, but decreased in the moribund group in comparison to the negative control (Fig. 6.4C). However, no significant difference was found between groups by PERMANOVA (F=0.44, p=0.7). No correlation was found between antiviral activity in haemolymph and number of v.g.c. ($\log_{10}$) in abalone tissues of abalone exposed to AbHV by immersion (n=40, r=0.13, p=0.4).

6.4. Discussion

Since haemolymph plays an essential role in the innate immune response of abalone (Cheng 1981; Mitta *et al.* 2000; Bachère *et al.* 2004; Hooper *et al.* 2007b), a range of immune parameters, including THC, SO and anti-HSV-1 activity in haemolymph was measured at the sub-clinical and clinical stages of AVG caused by exposure to

AbHV infected water. At the initial stages of infection, THC increased significantly in AbHV exposed abalone on day 3. Moribund abalone were evident from day 5 post-challenge on-wards, allowing comparison of their immune responses with AbHV-exposed abalone that remained sub-clinical. In comparison to non-infected controls, THC decreased by 38.8% in moribund abalone, but increased by 42.6 and 13.6% respectively in apparently healthy abalone groups that were PCR-negative and PCR-positive for AbHV. The level of SO decreased by 30.8% in the moribund and 7.2% in apparently healthy abalone, confirmed positive for AbHV by qPCR. However, for apparently healthy abalone with PCR-negative results, SO significantly increased by 59.3% compared with the negative control value. THC and SO were negatively correlated with number of viral genome copies within abalone. These results suggest that, at the early stage of infection, AbHV stimulates a typical stress response with increased circulating haemocytes, followed by cellular immune depression as the viral load accumulates and the disease progresses.

The qPCR assay provides high sensitivity for determining the intensity of AbHV in abalone compared with other diagnostic tests such as conventional PCR and histological examination (Corbeil *et al.* 2010). During the immersion infection trial, the earliest time that qPCR detected abalone positive for AbHV was day 3 post-challenge and this included some abalone that were not yet showing any clinical signs of disease. This confirms that qPCR can detect sub-clinical AbHV infections (Corbeil *et al.* 2012). As expected, qPCR confirmed higher viral load in moribund abalone than in apparently healthy abalone; moribund abalone had lower Ct values equating to a higher number of v.g.c (20.5%) in ganglion tissues than apparently healthy but PCR-positive abalone. Our results are similar to those found previously in Pacific oyster *Crassostrea gigas* infected with ostreid herpesvirus 1, where viral

DNA was not detected in subclinical individuals, but present in high amounts in moribund or dying individuals (Schikorski *et al.* 2011). However, there was great variability in the viral load of healthy-looking AbHV exposed abalone. Some healthy-looking PCR-positive animals had high viral loads, within the same range as moribund animals, thus suggesting some potential for these abalone to withstand or delay the impacts of AbHV infection. In addition, viral DNA was not detected in a small proportion of apparently healthy abalone (n=6) that were also exposed to AbHV by immersion. It is possible that these PCR-negative abalone, along with the PCR-positive abalone in the lower range of viral load, are in early stages of the infection cycle. These animals might have mounted a strong immune response in the early stages of exposure thus inhibiting viral replication *in vivo*. However, it is more likely that, due to the limitations in controlling viral dose in immersion trials, these abalone received lower doses of AbHV, and/or were exposed to the virus later during the 20hr exposure period, despite being in the same tanks as PCR-positive animals with high viral loads. Longer-term experiments would be required to confirm whether these healthy looking AbHV exposed abalone would ultimately succumb to the disease. Indeed, 100% cumulative mortality of abalone has been observed in previous AbHV infection trials after 10 days (Corbeil *et al.* 2012).

An increase in circulating haemocytes (THC) is a common stress response observed in abalone in response to a wide range of abiotic (e.g. handling, water temperature, salinity) and biotic stressors (e.g. *Vibrio* sp. infections) (Cheng *et al.* 2004e; Hooper *et al.* 2011; Dang *et al.* 2012). Increased THC has also been observed in response to biotic stressors in other mollusc species, such as simulated bacterial challenge in clams and oysters (Allam *et al.* 2006; Labreuche *et al.* 2006; Li *et al.* 2009e). Here we demonstrate that in the early stages of exposure to virus, abalone can also mount

an initial immune response by increasing the number of circulating haemocytes. Circulating haemocytes in abalone and other molluscan species are known to play prominent roles in defense against pathogenic microbes, by mechanisms such as phagocytosis and cytotoxic reactions, including the release of lysosomal enzymes and antimicrobial compounds, and respiratory burst which involves production of oxygen metabolites, such as SO and hydrogen peroxide (Pruzzo *et al.* 2005). Increase of SO by phagocytic haemocytes can accompany the release of active enzymes for the destruction of foreign particles (Pipe 1992; Noël *et al.* 1993) and triggers apoptotic cell death, which is an essential immunological pathway in molluscs to fight pathogens (Terahara *et al.* 2008). Therefore, abalone without capacity of producing and maintaining high levels of THC and SO could be vulnerable to viral infections.

At the early (sub-clinical) infection stage, the increase of circulating haemocytes could be linked to the prominent role of cell-mediated immunity in antiviral defense. For example, haemocyte recognition of viral components can induce subsequent immune responses, as shown in crustaceans (Witteveldt *et al.* 2004; Jha *et al.* 2006; Kumar *et al.* 2008). In addition, increased THC could be partly due to physiological functions of the haemocytes, such as wound repair and increased metabolism during the course of viral infection (Cheng 1981; Sparks *et al.* 1988; Cheng *et al.* 2004e). At the late infection stage (days 5-7), THC was still increased in apparently healthy abalone groups that were PCR-negative (42.6%) and PCR-positive (13.6%) for AbHV, but THC was significantly lower in moribund abalone (38.8%) in comparison to the negative controls. This result is consistent with previous reports of leucopenia in AbHV-infected abalone, where significantly decreased THC (37.8-77.8%) was observed in farmed abalone with mild and severe microscopic lesions of AVG, in

comparison to healthy abalone with no lesions of AVG (Hooper *et al.* 2012). The decrease in circulating haemocytes in moribund abalone is most likely due to the sequestration of haemocytes to the infected neural tissue (Hooper *et al.* 2012). The same situation has been observed in crustaceans, such as Pacific white shrimp *Litopenaeus vannamei* infected with white spot syndrome virus (Pan *et al.* 2008). Shrimp that were resistant to white spot syndrome were found to maintain THC at levels similar to uninfected shrimp, while a significant decrease in THC was observed for shrimp that were susceptible to the virus (Pan *et al.* 2008). Also, in *L. vannamei* with clinical signs of Taura syndrome virus infection, THC decreased to 21% of the level in uninfected controls (Song *et al.* 2003), thus emphasizing the central role of maintaining circulating haemocytes for immunity against viruses in marine invertebrates.

At the early infection stage, the SO level did not significantly change in challenged abalone. Induction of SO therefore appears to be a delayed response as the viral load increases by replication within the abalone. A significant change in SO level was observed when abalone started showing clinical signs of disease (from day 5 post-infection), with higher levels found in apparently healthy abalone that were exposed to the virus and a significant decrease in moribund abalone, compared to negative controls. Interestingly, significant change in intra-haemocytic SO level was also observed in shrimp *L. vannamei* that were infected with Taura syndrome virus in comparison to uninfected controls (Song *et al.* 2003). Thus the capacity for oxidative burst appears to be an integral aspect to the invertebrate immune response to viral challenge.

Consistent with our previous studies (Dang *et al.* 2011b; Dang *et al.* 2011a; Dang *et al.* 2011c; Dang *et al.* 2012), a high level of baseline antiviral activity against HSV-1 was observed in all treated and control abalone. Antiviral activity against HSV-1 was not significantly higher in apparently healthy infected abalone compared with moribund abalone. Furthermore, at the early infection stage, the antiviral activity did not increase significantly in abalone exposed to AbHV, in comparison to the negative controls. Consequently, there appears to be no ramping up of the constitutive levels of antiviral activity present in the unchallenged animal on exposure to AbHV, suggesting that there may not be an inducible component to the synthesis of humoral antiviral compounds in abalone. This is in contrast to the inducible synthesis of antibacterial factors in bivalves (Li *et al.* 2009a), some of which also appear to have antiviral activity (Roch *et al.* 2004; Roch *et al.* 2008; Balseiro *et al.* 2011).

In summary, changes to the abalone cellular immune response, but not the humoral antiviral activity, can be observed in to the early stages of infection with AbHV. Abalone with low viral load have a high number of circulating haemocytes and associated intracellular SO. Further research is required on the heritability of the ability to mount a cellular immune response to pathogen infection and specific antiviral factors in abalone that may help confer resistance to AbHV.

Acknowledgements

Appendix 6.1. Infection of abalone by injection

An additional *in vivo* infection trial was undertaken to assess the immediate immune response of abalone after exposure to known doses of AbHV.

Methods

The Victorian isolate of AbHV was diluted to 10^4 g.c. ml^{-1} (low dose) and 10^5 g.c. ml^{-1} (high dose) in PBS (viral titration by qPCR as described in Chapter 6). These virus doses were found to cause approximately 60 and 100% mortality respectively over 14 days post-infection, so they were called low and high viral dosage (M. Crane, unpublished data). All infections by injection used a total volume of 0.1 ml injected into the foot muscle of abalone (n=30) in duplicate tanks. In the non-infection room, abalone were injected with 0.1 ml PBS to control for the effect of PBS injection on abalone immunity. Negative control abalone (n=30) were not injected and held in duplicate tanks in the same non-infection room. Haemolymph was sampled randomly from nine abalone in each AbHV-injected, PBS-injected and negative control group at day 1 after infection.

One-way univariate PERMANOVA was undertaken on each immune parameter from the infection trial by injection with AbHV. Post-hoc pair-wise tests were performed, to identify how each immune parameter was influenced by injection (negative control vs. PBS injected control group) and viral doses (high and low) one day after injection.

Results

At day 1 post-injection, THC increased in the low viral dosage (381 ± 46.45 x 10^4 cells ml^{-1}) and high viral dosage groups (328.33 ± 48.44 x 10^4), in comparison to PBS-injected controls (251 ± 35.54 x 10^4) (Fig. 6.5A). However, no significant difference in THC was found according to viral dosage using PERMANOVA (F = 1.88, p = 0.17). On the other hand, the effect of viral injection on SO production was dependent on viral dosage (F = 4.26, p = 0.01). SO level was significantly higher in the high viral dosage group than in low viral dosage (Fig. 6.5B, pair-wise test p=0.03) and the negative control (p = 0.01). Although the high viral dosage group has, on average, higher SO production than the PBS-injected group (0.3 vs. 0.17 at OD620nm, Fig. 6.5B), no significant difference was found using pair-wise analysis (p=0.07). Similarly, there was no significant difference in SO between the negative control (non-injected) vs. PBS-injected control group (p=0.4), the negative control vs. low viral dosage group (p=0.24) and PBS-injected vs. low viral dosage group (p=0.82). Antiviral activity was not affected by infection with different AbHV injected doses (F=1, p=0.39, Fig. 6.5C).
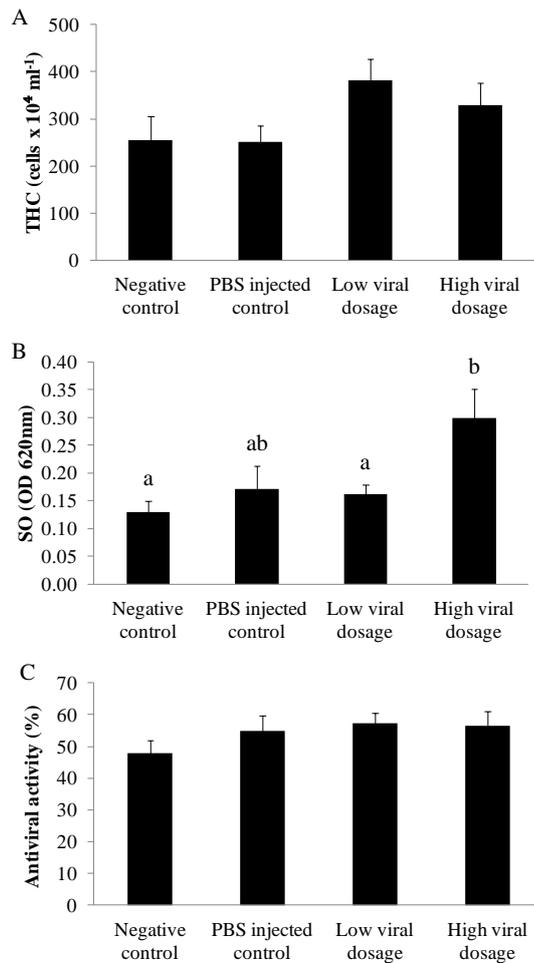
Figure 6.5. Effect of AbHV infection by injection with two different doses (10^4 g.c. ml^{-1} and 10^5 g.c. ml^{-1}) on A) total haemocyte count (THC, cells x 10^4 per ml), B) superoxide anion (SO, OD 620nm), C) antiviral activity (%) against HSV-1. Each immune parameter was measured from nine replicate abalone at day 1 after infection. Different small letters indicate significant differences ($p<0.05$) between negative control, PBS injection control, and two viral injection groups for THC and SO.

Appendix 6.2. Standard curve between plasmid Topo-ORF49 and C_T value
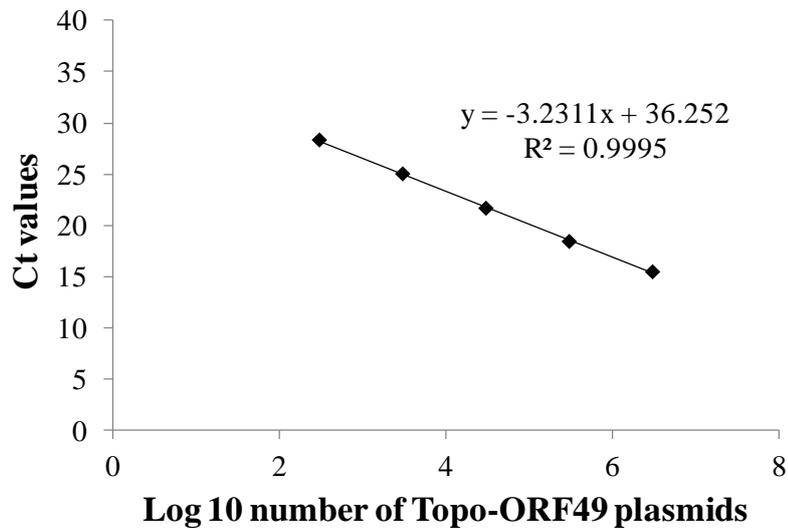
Figure 6.6. Standard curve between plasmid copy number and C_T value for $\log_{10}$ dilutions produced by TaqMan real-time PCR. The plasmid Topo-ORF49 was quantified calculated in triplicate by UV spectrophotometry (260 nm) and serially diluted 10-fold in DNA extract from known uninfected abalone tissue. There was a linear correlation between the recombinant plasmid and C_T value for over 4 $\log_{10}$ dilutions (coefficient $R^2 = 0.99$).

CHAPTER 7: General discussion

This study has demonstrated activity against herpes simplex virus type 1 (HSV-1) in the crude haemolymph and the lipophilic extract of digestive gland of abalone *Haliotis laevigata* (Chapter 2). Within the non-cytotoxic range to Vero cells, HSV-1 is inhibited in a concentration-dependent manner, as shown by the plaque assay. This expands on previous studies that found activity in *Haliotis rufescens* against polyoma, influenza A, and polio viruses (Li 1960; Li *et al.* 1962b). Antiviral activity in abalone is non-specific as it is effective against different DNA and RNA viruses. Similar activity against HSV-1 has been found in the haemolymph of other molluscan species, for example the oyster *Crassostrea gigas* (Olicard *et al.* 2005a) and the periwinkle *Littorina littorea* (Defer *et al.* 2009a). This implies molluscan antiviral factors could have been present in the ancestors of bivalves and gastropods and may be a conserved trait in all molluscs. However, further research is required to determine whether the same compounds are responsible for antiviral activity in different species.

By adding the haemolymph or the lipophilic extract at different times relative to the time of addition of virus to cells, we further showed that the antiviral activity is exerted at different stages of virus infection (Fig. 2.3, Chapter 2). Significant antiviral activity occurs when haemolymph was present during the first hour of virus-cell incubation, suggesting that its action may be due to inhibiting an early stage of infection. The attachment and entry assay results are consistent with haemolymph reducing HSV-1 binding to Vero cells. Prevention of viral attachment and entry into cells has been also found in antiviral factors in other invertebrates, such as tachyplesin I from horseshoe crab *Tachypleus tridentatus* against human

immunodeficiency virus (Morimoto *et al.* 1991), lipophilic extract of blue crab *Callinectes sapidus* against Sindbis, vaccinia, vesicular stomatitis, mengo, banzi and poliomyelitis viruses (Pan *et al.* 2000) and lectins from mussel *Crenomytilus grayanus* against human immunodeficiency virus (Luk'yanov *et al.* 2007) (Table 1.2, Chapter 1). Prevention of viral entry could be due to sugar, peptide or other compounds with a high affinity to viral attachment/entry glycoproteins, competing with cellular receptors (Morimoto *et al.* 1991; Marchetti *et al.* 1996; Pan *et al.* 2000; Andersen *et al.* 2004; Jenssen *et al.* 2004a; Bergefall *et al.* 2005; Jenssen 2005; Luk'yanov *et al.* 2007). In contrast, antiviral activity of the lipophilic extract of digestive gland was most notable when added 1 h after viral infection. This suggests that the extract may be acting at a stage of infection subsequent to cellular entry. Inhibition of viral transcription and DNA/RNA synthesis has been indicated in invertebrate antiviral compounds, such as Kelletinin A from the marine gastropod *Buccinulum corneum* against human T-cell leukemia virus type-1 (Silvestri *et al.* 1995), mytilins from mussel *Mytilus galloprovincialis* against white spot syndrome virus (Dupuy *et al.* 2004) and defensin from mussel *M. galloprovincialis* against human immunodeficiency virus (Roch *et al.* 2004). Recently, bovine lactoferrin, apart from inhibiting viral attachment and/or entry, has been shown to interfere with trafficking of HSV-1 capsids along microtubules towards the nucleus (Marr *et al.* 2009). Thus, the antiviral compound in the lipophilic extract of abalone digestive gland could be internalized simultaneously with the virus to exert its effect in the early stage of post-entry events, such as transport, capsid uncoating or transcription.

Although a range of antiviral proteins and peptides are found in many marine species (Roch *et al.* 2004; Zhang *et al.* 2004; Roch *et al.* 2008; Defer *et al.* 2009a), we have shown that anti-HSV-1 activity was retained after most protein in abalone

haemolymph was destroyed by heat, trypsin and proteinase treatment (Table 2.3, Chapter 2). A possible interpretation is that small heat-resistant peptides, remaining after proteinase K and trypsin digestion, rather than large peptides, are responsible for the abalone anti-HSV-1 activity. Indeed, small peptides including lactoferricin (25-49 amino acids), defensins (29–33), indolicidin (13), brevenin-1 (24), protegrins (18), tachyplesin-1 (17), melittin (26), clavalin (23) and megainin (23) have anti-HSV activity (Yasin *et al.* 2000; Andersen *et al.* 2003; Albiol Matanic *et al.* 2004; Marr *et al.* 2009). Separation of haemolymph on a hydrophobic dianion resin column further revealed that antiviral activity is not due to lipophilic active compounds (e.g. fatty acids and aliphatic or aromatic alkaloids), but more likely due to sugars, acids or small polar peptides/proteins (Table 2.4, Chapter 2). Antiviral compounds in abalone haemolymph are innately biosynthesized rather than dietary derived since antiviral activity is not influenced by diet (Table 4.2, Chapter 4). In contrast, antiviral activity in the digestive gland is due to lipophilic compounds (Table 2.1, Chapter 2), which are innately biosynthesized as well as dietary derived since antiviral activity in abalone digestive gland extracts increases in macroalgae feeding trial study (Table 4.3, Chapter 4).

In addition, antiviral activity in the abalone haemolymph is not influenced by AbHV infection *in vivo*. At the early infection stage, the antiviral activity did not increase significantly in abalone exposed to AbHV, in comparison to the negative controls (Fig. 6.3C, Chapter 6). Furthermore, antiviral activity against HSV-1 was not significantly higher in apparently healthy infected abalone compared with moribund abalone (Fig. 6.4C, Chapter 6). Therefore, there may not be an inducible component to the synthesis of humoral antiviral compounds in abalone. This is in contrast to the inducible synthesis of antibacterial factors in bivalves (Li *et al.* 2009a), some of

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which also appear to have antiviral activity (Roch *et al.* 2004; Roch *et al.* 2008; Balseiro *et al.* 2011).

Apart from antiviral activity, abalone haemolymph also possess antibacterial activity against *Vibrio harveyi* (Chapter 3) and *Vibrio anguillarum* (Chapter 4 & 5). Antibacterial compounds in abalone haemolymph could be small peptides, but not proteins, as the activity was retained after proteinase-K and heat treatment (121°C for 20min, (Grant 2002; Vakalia 2005). Also, Vakalia (2005) found antibacterial activity in lipophilic extracts after passing abalone haemolymph through a dianion resin HP20 column. However, the antiviral and antibacterial activities of abalone haemolymph appear to be attributed by different compounds, since we found no correlation between the level of activity detected in the antiviral and antibacterial assays across all abalone haemolymph samples (Chapter 3, 4 & 5). Under heat stress, antiviral activity increased whereas antibacterial activity decreases (Chapter 5), suggesting a trade-off in the biosynthesis of these antimicrobial components. The antiviral and antibacterial compounds in abalone could be different isoforms synthesized from the same prepro-peptide by various proteolytic events, as decribed for myticins A and B with antibacterial activity and myticin C with antiviral activity in the mussel, *M. galloprovincialis* (Mitta et al. 1999; Balseiro et al. 2011).

Haemolymph antimicrobial activity offers a useful screening tool for assessing abalone immune status. The main factor found to influence antiviral and antimicrobial activity of abalone was whether they were sourced from natural populations or a farm (Fig. 3.2, Chapter 3). Environmental factors that impact the immune response could explain the higher antimicrobial activity in wild greenlip abalone compared to farmed ones. Indeed, farmed abalone are subjected to handling

or movement stress (Hooper *et al.* 2011) and temperature stress in shallow land based tanks, where water temperature fluctuates a lot more on a daily basis, relative to the ocean. Antiviral and antibacterial activity was shown to be influenced by temperature in our field and laboratory studies, with an increase of antiviral activity in elevated water temperature (Chapter 5). Abalone on farms could also experience immunodepression from stress resulting from a change in their living environment (e.g. water conditions and density) (Malham *et al.* 2003; Cheng *et al.* 2004b; Cheng *et al.* 2004d; Cheng *et al.* 2004a; Cheng *et al.* 2004e; Cheng *et al.* 2004c; Hooper *et al.* 2007b; Hooper *et al.* 2011). Since antimicrobial activity has been widely reported from marine algae (Ohta *et al.* 1998; Ponce *et al.* 2003; Lee *et al.* 2004; Smit 2004; Talarico *et al.* 2004; Puglisi *et al.* 2007; Salvador *et al.* 2007; Stirk *et al.* 2007; Mandal *et al.* 2008; Kamenarska *et al.* 2009), it was originally proposed that the abalone from natural populations that feed on algae would have higher antimicrobial activity than farmed abalone on artificial diets. However, no significant effect of diet was found in the antimicrobial activity of abalone haemolymph from our feeding trial using macroalgae *Spyridia filamentosa* and *Ulva lactuca* and microalgae *Arthrospira maxima* and *Dunaliella salina* (Chapter 4). Both *U. lactuca* and *S. filamentosa* exhibit antibacterial activity towards several Gram-positive and Gram-negative bacteria (Robles-Centeno *et al.* 1996; Zamora Tovar *et al.* 2000; Abd El-Baky *et al.* 2009) and antiviral activity against HSV-1 (Table 4.3, Chapter 4). However, abalone fed on these macroalgae did not have significantly improved humoral antimicrobial activity against *V. anguillarum* and HSV-1. Thus, a decrease of humoral antimicrobial activity in farmed abalone in comparison to the wild abalone is likely due to factors other than diet.

Significant variation of *in vitro* antiviral activity against HSV-1 in abalone haemolymph was found among individuals of the same farmed family line or wild population (Chapter 2). However, the antiviral activity was not induced at subclinical and clinical stages of AbHV infection using the immersion method (Fig. 6.3 & 6.4, Chapter 6), or in the early stages of AbHV infection (day 1) using the injection method (Appendix 6.1). Hence the variability in antiviral activity against HSV-1 in abalone haemolymph does not appear to explain the small propotion of abalone reported to survive during AbHV outbreaks (Hooper *et al.* 2007a).

Cellular immune parameters THC and SO appear as immune indicators for the status of abalone under the stress of high temperature and AbHV infection. THC and SO increased within the first day then recovered on day 3 in the 24 °C treatment group in comparison the the 18 °C control group (Fig. 5.4, Chapter 5). Increases in THC may be due to influx of circulating haemocytes from peripheral tissues, as suggested for stressed abalone (Hooper *et al.* 2007b). At the early stages of exposure to AbHV, abalone showed initial immune responses by increasing the number of circulating haemocytes (Fig. 6.3, Chapter 6). At the later infection stage, THC was still increased in apparently healthy abalone groups that were PCR-negative and PCR-positive for AbHV, but THC was significantly lower in moribund abalone in comparison to the negative controls (Fig. 6.4, Chapter 6). The decrease in circulating haemocytes in moribund abalone is most likely due to the sequestration of haemocytes to the infected neural tissue (Hooper *et al.* 2012). On the other hand, induction of SO appears to be a delayed response as the viral load increases by replication within the abalone. A significant change in SO level was only observed after abalone started showing clinical signs of disease (from day 5 post-infection), with higher levels found in apparently healthy abalone that were exposed to the virus

and a significant decrease in moribund abalone, compared to negative controls (Fig. 6.4, Chapter 6). A previous study by De Zoysa *et al.* (De Zoysa *et al.* 2009b) found a novel Fas ligand mRNA in abalone to be significantly up-regulated after challenging abalone with viral haemorrhagic septicaemia virus. Since Fas ligand in other invertebrate species plays an important role in the immune defense system by inducing SO and other reactive oxygen species, the Fas ligand in abalone could have a similar immune function and it could be upregulated after AbHV exposure to increase SO level, as detected in our infection challenge study.

In summary, abalone provide a source of antiviral compounds against a human pathogenic virus, HSV-1. Antiviral activity in the haemolymph and the digestive lipophilic extract appear to have different modes of action since they are exerted at different stages of HSV-1 infection. Further investigation is needed to elucidate the chemical structure of the antiviral compounds in abalone haemolymph and their digestive lipophilic extract and the details of their mechanism of action against HSV-1. The antiviral activity in abalone haemolymph was significantly induced by high temperature challenge but not AbHV infection, suggesting that antiviral activity against HSV-1 may not be used as an immune indicator for AbHV infection. Antiviral activity against marine herpesviruses (e.g. koi herpes virus, salmonid herpes virus type 1 and type 2) should be further examined to provide immune indicators for AbHV infection status and resistant capability. On the other hand, cellular immune parameters including THC and level of SO appear to be indicators for high temperature stress, as well as AbHV infection. Indeed, generating and maintaining a high number of circulating haemocytes and intracellular SO by abalone is linked to low viral load and pre-clinical status for at least one week after exposure to AbHV. Further study is required on the heritability of SO in abalone to

establish if this immune response can potentially be used to select for AbHV resistance.

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