The impact of ocean acidification, increased seawater temperature and a bacterial challenge on the immune response and physiology of the blue mussel, *Mytilus edulis*.

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ABSTRACT

Anthropogenic activities are fundamentally altering the chemistry of the world's oceans. Many of these modifications could have a significant impact on the health of marine organisms. Yet, despite being proposed as one of the most significant threats that marine ecosystems face, to date very little is known about the impact of anthropogenic climate change, and ocean acidification in particular, on host defence. The aims of this thesis are to investigate the impact of environmental stressors on the invertebrate immune response, providing empirical data on how anthropogenically induced stressors will impact the invertebrate immune system and how this will impact organism condition and subsequent physiological trade-offs. Exposure to reduced seawater pH and increased temperature significantly reduced the immune response in the blue mussel, *Mytilus edulis*. This reduction in immune response could indicate stress-induced immune dysfunction. However, the immune system protects an organism from infectious disease, ensuring survival, and should therefore be evaluated functionally rather than immunologically. By subsequently exposing mussels to a bacterial challenge this study demonstrated that an earlier study which measured a reduction in host defence represented a trade-off of immune system maintenance costs, with mussels maintaining a capacity to up-regulate immune defence when required. However, whilst this immune plasticity ensures mussels are able to survive a pathogen exposure, such a strategy appears to be physiologically costly. This cost is seen as a reduction in

reproductive investment, an altered energy metabolism and an altered fatty acid composition in organisms exposed to low pH. Therefore the overarching picture that emerges is, without measuring physiological processes functionally, and in neglecting any physiological trade-offs, it is possible that many studies may misinterpret the complex physiological responses of marine organisms to ocean acidification.

The impact of ocean acidification, increased seawater temperature and a bacterial challenge on the immune response and physiology of the blue mussel, *Mytilus edulis*.

By

ROBERT PETER ELLIS

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"The scientist does not study nature because it is useful; he studies it because he delights in it, and he delights in it because it is beautiful. If nature were not beautiful, it would not be worth knowing, and if nature were not worth knowing, life would not be worth living."

Jules Henri Poincaré (1854-1912)

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* Oral presentation to the Kristineberg Marine Research Centre, Kristineberg, Sweden, Jun. 2010

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External Contacts:
Dr D.M. Lowe
Dr D. White
A. Beesley
Prof. Mark Viant
Dr. J. Byrne
Dr U. Sommer
Dr S. Dupont
Dr B. Hernroth

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CHAPTER 1. GENERAL INTRODUCTION

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1.1 INTRODUCTION

Evolutionarily, host defence appeared alongside the emergence of the Protozoa approximately 2.5 billion years ago (Beck and Hanbicht, 1996), with its successful development being a prerequisite for more than a billion years of separate phyla evolution (Hoffmann and Reichhart, 2002). The early appearance of host defence has ensured elements of immunity are detectable in almost all living things (Beck and Hanbicht, 1996), with the immune system forming a major physiological mechanism to ensure host survival (Lochmiller and Deerenberg, 2000), controlling or fighting any pathogenic or parasitic insult (Lochmiller, 1996; Lochmiller and Deerenberg, 2000; Sheldon and Verhulst, 1996; Zuk, 1996). This has led to immunity being of vital importance to all animals (Zuk et al., 2004), and in plants the selection for an improved host defence has led to the evolutionary acquisition of over 100,000 secondary metabolites (Dixon, 2001). Therefore the immune system offers an ideal model system with which to investigate the ecological and evolutionary significance of stressors.

The notion of immunity, or what it is now understood to have been acquired immunity, was first described in historical records in 430 BC through Thucydides' accounts of the plague of Athens during the Peloponnesian war (Seder and Hill, 2000). However, it took a further 2000 years before the physiological mechanisms underpinning this observation began to be properly understood. Firstly with Edward Jenner's historic discovery of vaccination in 1796, finding that an inoculation with the cowpox virus protected recipients against the often lethal smallpox virus (Seder and Hill, 2000), and later through the groundbreaking work of others including Louis Pasteur, Elie Metchnikoff, Robert Koch, Emil von Behring and Paul Ehrlich (Silverstein, 1989).

Traditionally, immunological investigation has focused on the molecular and physiological foundations of host-pathogen relationships under optimal conditions, often in the absence of pathogens (Hoffmann and Reichhart, 2002; Rolff and Siva-Jothy, 2003; Tirapé et al., 2007). However, through the renewed application of these traditional investigations to include ecological, evolutionary biology and population biology theories, one of the most rapidly increasing areas of Biology has appeared; namely ecological immunology (Rolff and Siva-Jothy, 2003; Schmid-Hempel, 2003; Sheldon and Verhulst, 1996). The inception of ecological immunology has led to an ever increasing body of literature that investigates the impact of environmental stressors on the immune response and in particular how these stresses act to create and maintain immune system variation, although the majority of studies to date are restricted to the study of vertebrates (Rolff and Siva-Jothy, 2003; Zuk et al., 2004). However, as innate immunity is the only immunological defence mechanism available for the five to ten million species of invertebrate metazoans, compared with around 45,000 extant vertebrate species that are able to rely on both innate and adaptive immunity (Hoffmann and Reichhart, 2002), there is growing realisation that invertebrate immunology provides an ideal model system with which to investigate the response, and subsequent evolution, of immune defences to environmental stressors.

The invertebrate immune response is a non-adaptive system, based on both cellular and humoral components (Schmid-Hempel, 2003; Fig 1.1), each being divided into two aspects: being the *afferent* (or sensing) and *efferent* (or effector) arms (Beutler, 2004). The afferent arm involves receptor-mediated recognition of pathogen-associated molecular patterns (PAMPs; Fig 1.1a), which are highly conserved within microbial species and generally absent in the host (Janeway and Medzhitov, 2002). Pathogen recognition receptors (PRRs), such as toll like receptors (TLRs), lipopolysaccharide-

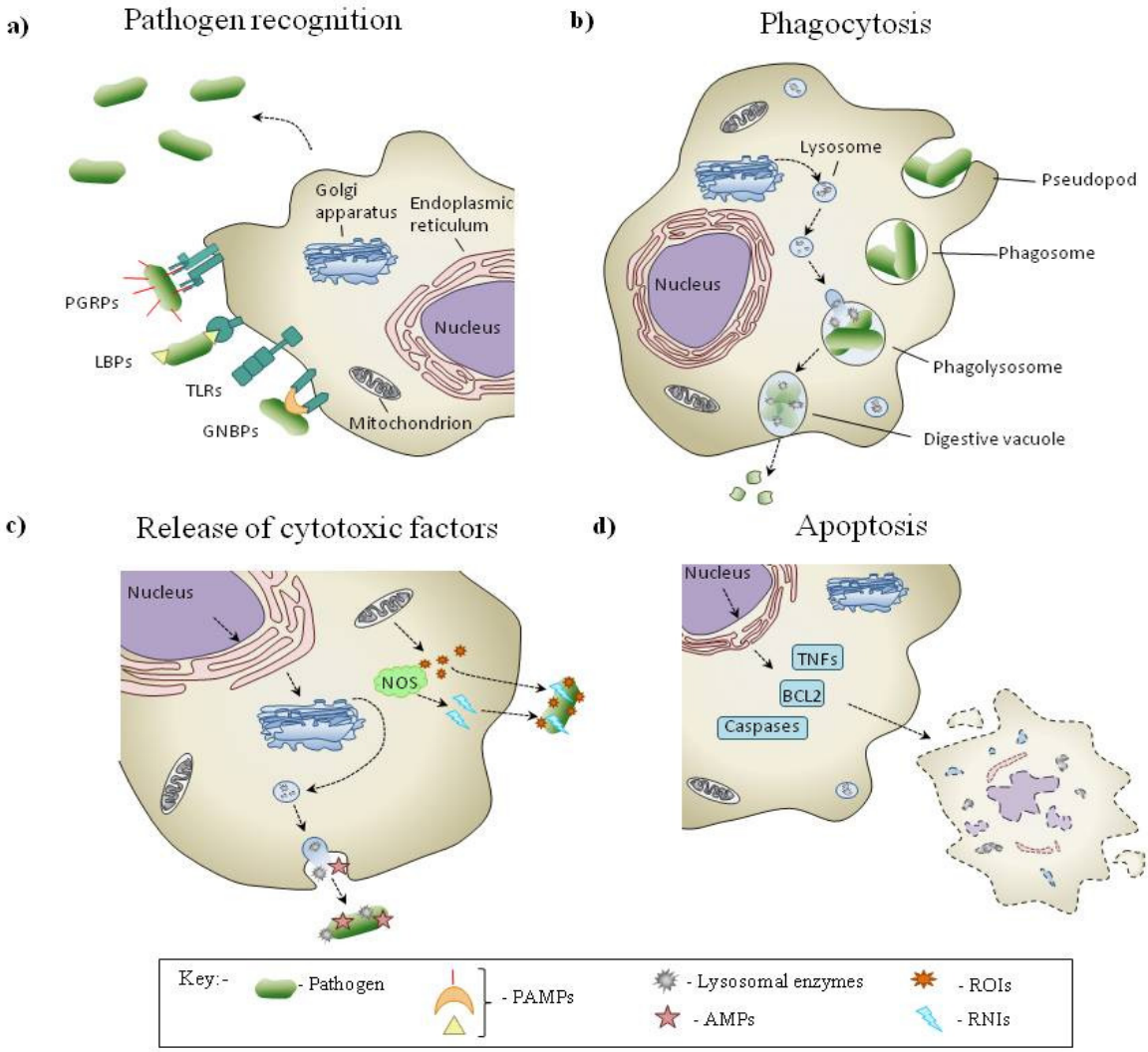

Figure 1.1 Schematic overview of the marine invertebrate immune response, using the bivalve mollusc, *Mytilus edulis*, as a model species (following Philipp et al. 2012). **a)** Pathogen recognition: recognition of non-self PAMPs is via PRRs, triggering a multifaceted immune response, **b)** Phagocytsis: PAMP recognition induces bacterial engulfment by haemocytes, phagosome then fuses with lysosome, releasing lysosomal enzymes and reactive oxygen intermediates (ROIs), leading to pathogen degradation. **c)** Release of cytotoxic factors: pathogen recognition also stimulates production of cytokines, chemokines, antimicrobial peptides (AMPs) and ROIs via a diverse array of transcriptional and regulatory pathways (see Philipp et al. 2012), as well as reactive nitrogen intermediates (RNIs) enzymatically catalysed by nitric oxide synthase (NOS). **d)** Apoptosis: programmed cell death is induced via complex regulatory pathways (which includes tumour necrosis factors (TNFs), BCL2 and caspase-like factors), leading to cell shrinkage, chromatin condensation, cell membrane blebbing, nuclear collapse and, finally, the production of apoptotic bodies, which are destroyed via phagocytosis.

binding proteins (LBPs), peptidoglycan recognition receptors (PGRPs) and glucan binding proteins (GNBPs), bind to a pathogen, triggering a multifaceted immune response, being the efferent arm (Beutler, 2004; Bosch, 2008). Through the employment of a variety of cells that are capable of performing phagocytic (Fig 1.1b), cytotoxic (Fig 1.1c) or inflammatory responses, alongside apoptosis (Fig 1.1d) and autophagy, a broad suite of possible innate immune responses are triggered when an organism is immunologically challenged (Roch, 1999). Understanding the exact complexity of the invertebrate immune response, and understanding how the immune system responds to changes in the environment is vital to help further our understanding of how host-pathogen interactions will be affected by such changes, which in turn will help us to understand and predict how changes in immunocompetence, caused by environmental variability, may impact at a population or community level (Morley, 2010).

In what follows I discuss the response of the immune system to environmental perturbation and the methodological advances made within marine invertebrate ecological immunology over the past decade. In focusing on the efferent, or effector, arm of the immune response, the environmental stressors investigated and the immune parameters these stressors affect will be highlighted. In critically reviewing the cellular and humoral immune parameters typically tested within this field, the potential for the advancement of ecological immunity through the incorporation of newly emerging molecular and genetic techniques will be emphasised. Finally, the necessity of incorporating additional stressors and the need to employ different stressor models to further elucidate the ecological and evolutionary impact of stressors will be outlined.

1.2. CELLULAR IMMUNITY

The predominant mechanism of marine invertebrate internal defence involves phagocytosis by immune cells (Coteur et al., 2005a; Pipe et al., 1995a). Active phagocytosis has even been demonstrated in animals such as the Cnidaria, which lack mobile phagocytes, haemolymph or an impermeable barrier to invading organisms, with phagocytosis being carried out by ectodermal as well as endodermal epithelial cells in *Hydra* (Bosch et al., 2009; Bosch and David, 1984, 1986). This led Bosch et al. (2009) to hypothesise that the epithelium is an ancient line of host defence. Immune cells, or phagocytes, can be classified into sub-populations based upon separate functional and staining characteristics (Noël et al., 1994; Pipe, 1990a; Pipe et al., 1995a), and they are particularly abundant in haemolymph, reaching a concentration between 2 to 4 x 10^6 cells ml^{-1} in bivalves (Mitta et al., 2000a) and 2 to 9 x 10^6 cells ml^{-1} in starfish (Pinsino et al., 2007). In phagocytising a pathogen, phagocytes are capable of; non-self recognition, *via* the use of lectins (Mitta et al., 2000a; Pipe, 1990a; Renwrantz et al., 1985), attaching to and endocytosing a pathogen (Mitta et al., 2000a) and killing an invader, *via* the production of cytotoxic factors which include reactive oxygen intermediates, nitric oxide and antimicrobial enzymes (Bachère et al., 1991; Carballal et al., 1997; Mitta et al., 2000a).

Considering its integral role in innate immune defence, it is not surprising that phagocytosis is the measure that has received the greatest amount of investigation when assessing the impact of changing environmental conditions on the marine invertebrate immune response. Typically the measure of phagocytosis investigates one of two components; the proportion of haemocytes that are phagocytically active in a haemocyte population (e.g. Gagnaire et al., 2006a) or the phagocytic index, being the number of

bacteria engulfed by each haemocyte, (e.g. Duchemin et al., 2007). Indeed, when measuring phagocytosis there are many separate methodologies used, which include conventional methods of microscopic assessment or agarose plate assays (Pipe et al., 1999), measuring the activity *in vivo via* endocytosis of ferritin (e.g. de Faria and da Silva, 2008) or fluorescently labelled bacteria (e.g. Coteur et al., 2005a), measuring the uptake of neutral red stained zymosan, *via* a change in optical density using microplate analysis (e.g. Parry and Pipe, 2004) or measuring the uptake of fluorescent latex beads by flow cytometric analysis (e.g. Duchemin et al., 2007). However, irrespective of the measure investigated or the method used, each approach aims to measure the same end point, which is the ability of haemocytes to phagocytose bacteria, and therefore these measures will be considered as one entity, phagocytic activity, and results will be compared irrespective of the method the study used.

In demonstrating an alteration in phagocytic activity, a number of authors have shown this immune system component to be sensitive to environmental perturbation. Changes in environmental parameters such as temperature (Chen et al., 2007a, b; Cheng et al., 2004c; Hégaret et al., 2003; Monari et al., 2007; Parry and Pipe, 2004), salinity (Cheng et al., 2004d; Gagnaire et al., 2006a; Martello et al., 2000; Matozzo et al., 2007), air exposure (Chen et al., 2007b; Malagoli et al., 2007; Malham et al., 2002), seawater pH (Bibby et al., 2008), hypoxia (Cheng et al., 2004e) and anoxia (Matozzo et al., 2005; Pampanin et al., 2002), as well as changes in concentrations of ammonia (Cheng et al., 2004a) and nitrite (Cheng et al., 2004b) have been shown to reduce phagocytic activity significantly. Additionally, phagocytic activity is significantly reduced by a number of anthropogenically-induced stressors, such as mechanical disturbance related to aquaculture (Ballarin et al., 2003; Lacoste et al., 2002; Malagoli et al., 2007) and pollution, *via* contaminants such as butyltins (Bouchard et al., 1999),

polyaromatic hydrocarbons (Wootton et al., 2003), pesticides (Gagnaire et al., 2006b) and metals including; lead (Coteur et al., 2005a), cadmium (Bouilly et al., 2006), copper (Parry and Pipe, 2004; Pipe et al., 1999) mercuric chloride ($HgCl_2$) and methylmercury chloride (CH_3HgCl) (Fournier et al., 2001).

Whilst phagocytic activity is shown to be sensitive to both natural and anthropogenically induced environmental change, this immune parameter also demonstrates natural seasonal variation caused by alterations in organism physiology. Duchemni et al. (2007) showed that the lowest phagocytic activity occurred during late spring spawning in the Pacific oyster, *Crassostrea gigas,* whilst maximum activity occurred in autumn. Furthermore, this study noted uneven variation in this immune parameter between males and females during ongoing gametogenesis and, when phagocytic activity was compared in diploid and triploid individuals, the immune response in triploids seemed to be less sensitive to environmental changes than in diploids. Therefore as highlighted by this study, understanding seasonal variability of the immune response, and variation in the response of males and females in a given population, is an area of invertebrate immunology that requires significant investigation (Li et al., 2009a; Nahrgang et al., 2012).

Studying the impact of environmental stressors on the general immune response, as has been done in many studies to date, offers a vital step in understanding how organisms will be impacted by changing environmental conditions. Yet when assessed alone, a change in the immune response at a single time point/season assumes that an organisms provisioning in the immune response is fixed throughout the reproductive cycle and that environmental conditions do not fluctuate temporally. However given many environmental parameters, such as temperature and salinity, vary seasonally and given the immune response of an organism is affected by factors such as nutritional

status (Moret and Schmid-Hempel, 2000) and different reproductive provisioning between sexes (Zuk et al., 2004), understanding exactly how an organism will respond to environmental stress at different times seasonally is crucial to predict how environmental stress will affect organism disease resistance and impact population dynamics.

Measuring phagocytic activity serves as a commonly used proxy for immunocompetence (Hooper et al., 2007), with immunocompetence being the general capacity of the individual to mount an immune response (Schmid-Hempel, 2003). However, demonstrating a change in phagocytic activity in isolation fails to show the mechanisms by which this immune parameter is impacted and indeed, this approach fails to account for any further possible cellular immune dysfunction that would go unnoticed if the apparent ability of haemocytes to engulf bacteria remained unaffected. Therefore, in measuring a number of additional haemocyte parameters, such as changes in the abundance (e.g. Parry and Pipe, 2004), morphology (e.g. Gagnaire et al., 2003) or viability (e.g. Gagnaire et al., 2004) of haemocytes, investigators are able to understand the processes involved in phagocytosis that are specifically affected by environmental stressors. Consequently, studies are able to demonstrate subtle differences in the response of phagocytic activity and overall cellular immunity that may go unnoticed if phagocytic activity was the only endpoint measured.

Whilst an increase in phagocytic activity might indicate an increase in the activity of the haemocytes themselves, it is also entirely possible that this increase could be caused by an alteration in haemocyte proliferation, with the increase just an indirect effect of increased haemocyte numbers. Therefore, in measuring total cell counts (THC), it is possible to demonstrate a change in the number of haemocytes in context with changes in other cellular immunological measures. In investigating the effect of

both a pathogen and temperature stress on the Caribbean fan coral, *Gorgonia ventalina*, Mydlarz et al. (2008) demonstrated that an *Aspergillus sydowii* infection led to a localised increase in the number of amoebocytes in tissue closest to the infection, amoebocytes being phagocytically active cells in gorgonian corals (Olano and Bigger, 2000), whereas an increase in temperature from 27°C – 29 °C to 31.5 °C for 8 days led to a systemic and spatially homogenous increase in amoebocyte numbers throughout undamaged coral tissue. Temperature was also shown to significantly affect THC in the Pacific whiteleg shrimp, *Litopenaeus (=Penaeus) vannamei* (Pan et al., 2008). Individuals maintained at lower temperatures (18 °C and 21 °C) compared to the controls (maintained at 24 °C) demonstrated a sustained reduction in THC for the entire 12 day experiment, whilst shrimp maintained in higher temperatures (27 °C and 30 °C) decreased THC during the first 3 days of the 12 day exposure, after which THC returned to levels comparable to those in the controls. The ability of the number of circulating haemocytes to recover after exposure to higher temperatures, as was noted by Pan et al. (2008), has also been demonstrated by a number of other investigators, where THC has been shown to recover either during or after an exposure to a number of environmental stressors. Lorenzon et al. (2001) demonstrated an exposure to trace metal contamination, including Hg^{2+}, Cd^{2+}, Cu^{2+}, Cr^{6+}, Zn^{2+} and Pb^{2+}, in the glass prawn, *Palaemon elegans* led to a decrease in THC during the first 8 h of contaminant exposure, however after a 16 h immersion haemocyte numbers returned to levels noted prior to the investigation. Furthermore, whilst THC failed to stabilise and return to levels noted in control individuals in the blue swimmer crab, *Portunus pelagicus*, during a 48 h sub-lethal exposure to elevated ammonia-N, Romano and Zeng (2010) showed that the number of haemocytes was able to recover to levels noted in control individuals after 84 h of recovery post exposure. In considering the ability of immune parameters to

recover either immediately after an exposure to an environmental stressor, or in some instances during the stressor exposure period, it is vital to account for the duration of an exposure to an environmental stressor and the stage of exposure at which the immune system is investigated.

Pipe et al. (1999) proposed migration of haemocytes from tissues to the haemolymph as the principle mechanism to increase haemolymph cell counts, and in doing so demonstrated the ability of copper concentration to alter total numbers of circulating haemocytes within the blue mussel, *Mytilus edulis*. Additionally, alongside measuring total number of circulating haemocytes, this study also measured the differential cell count (DHC), in this case being the proportion of eosinophilic and basophilic cells. After a 7-day exposure at copper concentrations of 0.02 and 0.05 mg l^{-1}, total haemocyte count significantly increased, with DHC remaining unaffected. However, at copper concentrations of 0.2 and 0.5 mg l^{-1} this result was reversed with no significant impact of the copper on total cell counts, compared with controls, yet the proportion of differential haemocytes was significantly affected; eosinophils decreasing and basophils increasing compared with the controls. Furthermore, phagocytic activity was only significantly affected by a copper concentration of 0.2 mg l^{-1}, with other concentrations (0.02, 0.05 and 0.5 mg l^{-1}) failing to impact mussel phagocytic activity significantly. This study therefore highlights the different possible effects of just one contaminant on various haemocyte parameters that would have been missed if phagocytic activity had been measured in isolation.

With different haemocyte subpopulations undertaking different functions with respect to immune defence, an alteration in the proportion of different haemocyte subgroups could potentially have significant effects on differential immune functions and the overall immunocompetence of the organism concerned. This is highlighted by

Mercier et al. (2009) who suggest a change in the proportion of hyalinocytes to be a possible mechanism for the increased clotting time demonstrated in the Pacific whiteleg shrimp, *Litopenaeus (=Penaeus) vannamei* 1 h after exposure to a handling stress.

To further elucidate the way in which environmental stressors affect haemocytes, Oweson et al. (2010) investigated the effect of manganese and hypoxia on the proliferation of haematopoietic cells and the number of circulating immune cells (coelomocytes in echinoderms) in the starfish, *Asterias rubens,* together with measuring the composition of coelomocyte sub-populations. Manganese contamination had previously been shown to reduce the number of circulating haemocytes in the Norway lobster, *Nephrops norvegicus* (Hernroth et al., 2004; Oweson et al., 2006) and the blue mussel, *Mytilus edulis* (Oweson and Hernroth, 2009), yet increase coelomocyte numbers in *Asterias rubens* (Oweson et al., 2008). Whilst Oweson et al. (2010) also showed manganese to increase number of coelomocytes, proliferation of haematopoietic cells and the number of dividing cells in the coelomic epithelium, believed to be the main tissue for renewal of coelomocytes in *Asterias rubens* (Holm et al., 2008), this study failed to show any effect of hypoxia on coelomocyte numbers or cell proliferation. This reflects results from previous studies on *Asterias rubens* that found the numbers of circulating coelomocytes to be unchanged by other physical stressors including temperature and salinity (Coteur et al., 2004).

Whilst Pipe et al. (1999) measured DHC by investigating the different staining characteristics of mussel haemocytes, separation of *Asterias* coelomocytes by conventional methods does not allow reliable identification of coelomocyte sub-populations (Oweson et al., 2010). Therefore to identify the impact of manganese and hypoxia on the DHC in *Asterias rubens* Oweson et al. (2010) additionally measured *Ar*Runt mRNA expression. *Ar*Runt is a runt-related transcription factor expressed in the

coelomocytes and coelomic epithelium of *Asterias rubens*, with runt-related transcription factors playing a major role in the differentiation of specific blood cell lineages in vertebrates and invertebrates (Oweson et al., 2010). Runt factors are evolutionarily conserved and have been shown to carry out various functions, which alongside cell differentiation also include regulating cell proliferation and maintenance of stem cells, with their roles often differing between developmental stages (Braun and Woollard, 2009). However, in finding no relation between elevated *Ar*Runt mRNA expression and either elevated coelomocyte numbers or cell proliferation in *Asterias rubens,* Oweson et al. (2010) suggest this transcription factor plays an important role in cell differentiation. Therefore in demonstrating that hypoxia but not manganese exposure stimulated *Ar*Runt expression, these authors propose that the adjustment in the composition of coelomocyte subpopulations possibly compensates for the increased demand on gas exchange under hypoxic conditions (Oweson et al., 2010).

In exposing the lagoon cockle, *Cerastoderma glaucum* to the xenoestrogen, 4-nonylphenol (NP), Matozzo et al. (2008) showed this endocrine-disrupting chemical to affect haemocyte population distribution, with a 7 day exposure to 0.1 mg l^{-1} NP increasing the THC of cockles compared to control organisms. Furthermore, in measuring the haemocyte size frequency distribution, through the use of a coulter counter, these authors showed that after a 7 day exposure to NP, at a concentration of 0.1 mg l^{-1}, the haemocyte fraction measuring 7-8 μm in diameter (250 femtolitres in volume) significantly increased in exposed cockles compared to controls (Matozzo et al., 2008). However, whilst NP is shown to significantly impact haemocyte numbers and size frequency distribution, there is a need for experimental studies that measure the impact of chemical pollutants to avoid the masking of mechanisms of specific immunotoxic versus non-specific systemic toxicity. For example, understanding the

maximum tolerated concentration of a toxicant will help distinguish between specific immunotoxic effects versus gross physiological stress or systemic toxicity (Hutchinson et al., 2009).

Brousseau et al. (2000) measured the impact of a number of different trace metal contaminants, including cadmium chloride ($CdCl_2$), zinc chloride ($ZnCl_2$), mercuric chloride ($HgCl_2$), methyl mercury chloride (CH_3HgCl) and silver nitrate ($AgNO_3$), on phagocytic disturbance in the soft shelled clam, *Mya arenaria* together with haemocyte viability, at a range of metal concentrations (10^{-9} – 10^{-3} M). In measuring phagocytic activity using flow cytometry, these authors showed that a low dose exposure (10^{-9} or 10^{-8} M for 18 h) of 4 out of the 5 trace metals tested, induced a stimulation of phagocytic activity, yet cell viability remained unaffected. At greater concentrations of three of the contaminants (methyl mercury at 10^{-6} and 10^{-5} M, mercuric chloride at 10^{-5} M and silver nitrate at 10^{-4} M), phagocytic activity was significantly impaired yet there was no cytotoxic impact of these metals and again no reduction of cell viability at these contaminant concentrations. However, exposure to 10^{-4} M of both cadmium and zinc resulted in a notable reduction in phagocytic activity attributed to a marked cytotoxic impact of these two contaminants, leading to a reduced cell viability of haemocytes. If Brousseau et al. (2000) had measured phagocytic activity in isolation, the contaminant dose at which trace metal contamination impaired phagocytic activity would still have been correctly presented. However, this approach may have resulted in the reduced phagocytic activity, noted with an exposure to methyl mercury at 10^{-6} and 10^{-5} M, mercuric chloride at 10^{-5} M and silver nitrate at 10^{-4} M, being attributed to a cytotoxicity of trace metal contamination, masking the true nature of the way in which these metals impact the immune system of the clams. Therefore, by measuring cell viability concurrently, the authors were able to attribute trace metal cytotoxicity

specifically to cadmium and zinc concentrations of 10^{-4} M.

In a study investigating the haemocyte condition of the Pacific whiteleg shrimp, *Litopenaeus (=Penaeus) vannamei*, Costa et al. (2009a) measured the percentage of apoptotic haemocytes in shrimp naturally infected with myonecrosis virus (IMNV), during different stages of the infection. Apoptosis plays an important role during viral infection in shrimp (Flegel, 2007), inducing early cell death of the host cell to limit or inhibit viral replication (Costa et al., 2009a). Therefore, in measuring the percentage of apoptotic cells, alongside THC and DHC, Costa et al. (2009a) were able to demonstrate the impact of IMNV on the cellular immune response of *Litopenaeus vannamei* during infection. In asymptomatic infected shrimp and shrimp showing initial signs of infection there appeared to be no impact of IMNV on the immune response, with the shrimp immune system seemingly failing to detect the virus. However, in shrimp at an advanced stage of infection, a reduction in THC of 30 % was demonstrated compared to the earlier stages of infection, alongside a reduction in the percentage of circulating granulocytes (7 %) and an increase in apoptotic haemocytes (8-fold). This failure of *Litopenaeus vannamei* to detect the IMNV before advanced stages of the virus, at which point shrimp recovery is unlikely due to the serious damage of shrimp tissues (Costa et al., 2009a), indicates the complex nature in which host-pathogen interactions manifest. Such studies indicate the vital need to investigate many aspects of the immune system simultaneously to fully understand the mechanisms by which stressors impact the immune system and affect host-pathogen relationships, and additionally account for the dynamic nature in which a host organism detects and interacts with a pathogen.

Parry and Pipe (2004) demonstrated the impact of a combination of stressors on phagocytic activity, total number of circulating haemocytes and differential haemocyte counts. The study exposed the blue mussel, *Mytilus edulis* to copper concentrations of

0.02 and 0.05 mg l^{-1} for 7 days. However, as well as investigating the impact of the metal contamination this study additionally investigated the interactive impact of temperature and pathogen stressors. Mussels were held at either 10 or 15 ºC and, after the initial 7 day copper exposure, were exposed to the pathogen, *Vibrio tubiashii* for a further 3 days. Temperature was shown to significantly impact phagocytic activity, having increased at 15 ºC compared to 10 ºC, a result also noted in THC with mussels at 10 ºC having lower numbers of circulating haemocytes than at 15 ºC, however temperature did not affect DHC. Mussels exposed to 0.02 mg l^{-1} copper also demonstrated increased levels of phagocytic activity and an increased THC compared to controls, yet the percentage of circulating basophils decreased compared to controls. Mussels exposed to a copper concentration of 0.05 mg l^{-1}, however, demonstrated a significantly reduced phagocytic activity when compared with controls matched by a decreased THC and an increase in the percentage of circulating basophils. Copper was also shown to interact with the pathogen stressor, with mussels exposed to 0.02 mg l^{-1} and *Vibrio* demonstrating the highest noted phagocytic activity and THC, and mussels exposed to 0.05 mg l^{-1} and *Vibrio* demonstrating the lowest noted phagocytic activity. Whilst both temperature and copper concentration were shown to impact phagocytic activity individually, the most striking result of this study is the interactive effect of all three stressors, highlighting the complex nature of the impact of environmental stressors on the immune response. Mussels exposed to copper at 0.02 mg l^{-1} and *Vibrio* at 15 ºC had a significantly higher level of phagocytosis and a higher THC than mussels exposed to any other treatment, at either temperature. In nature, stressors seldom occur in isolation and in highlighting the complex nature by which just three stressors can interact to impact just one aspect of an organism's immune response, Pipe and Parry (2004) emphasized the need to investigate a wider array of environmental stressors and

a greater number of stressors in combination.

In addition to phagocytosis of foreign particles, haemocytes are able to secrete soluble antimicrobial peptides (AMPs) and other cytotoxic substances into the haemolymph (Mitta et al., 2000a). Together with other non-specific humoral defence molecules, including agglutinins, opsonizing lectins, bactericidins, lysozymes and serine proteases (Roch, 1999), these comprise the humoral component of invertebrate immunity. Measuring humoral immunity offers a further insight into the impact of stressors on haemocyte functionality, associated with an organism's immunocompetence.

1.3. HUMORAL IMMUNITY

Humoral defence molecules effect bacterial killing by opsonising and/or agglutinating an invader and by neutralizing the pathogen, either causing lysis or causing the disruption of metabolism, cell wall binding, cell membrane permeability or growth inhibition (Smith et al., 1995). The suite of antimicrobial molecules, together with the site of both production and storage of these molecules is species, tissue and even cell specific (Mitta et al., 2000b). Invertebrate humoral investigation therefore investigates the production of these antimicrobial factors, which include superoxides, lectins, microbicidal pigments and AMPs, as well as their storage, secretion and release pathways.

Within humoral immunological investigation, over the past decade, there has been an increasing understanding of the genetic control of the humoral immune response. The development and application of novel genetic and proteomic techniques, which demonstrate changes in gene expression and function of innate immune response

components, has led to the production of a new and potentially distinct field of research when considered alongside traditional humoral techniques; the study of AMP expression and production.

1.3.1 Haemolymph cytotoxicity and the respiratory burst

Traditionally humoral immunological investigation has studied haemocyte functionality, measuring the ability of haemocytes to produce antimicrobial molecules, as well as the release of these molecules and their ability to kill bacteria. To this end studies investigating humoral immunity often adopt one of three methodologies; i) investigating the functionality of haemocytes themselves, ii) measuring the antimicrobial activity of cell-free haemolymph by separating the cellular and cell-free fractions and studying each independently or, iii) studying the haemolymph containing both the cellular and cell-free fractions simultaneously.

Measuring the cellular fraction of haemolymph independently allows investigation of any change in haemocyte functionality, and specifically the ability of haemocytes to produce antimicrobial factors, associated with an alteration in environmental conditions. In measuring haemocyte reactive oxygen intermediate (ROI) production and enzyme activity any stressor-induced change in haemocyte mediated bactericidal activity can be empirically investigated.

Measuring the production of ROIs, using either chemiluminescence or fluorescence, allows researchers to study the respiratory burst associated with phagocytosis and assess how the oxidative killing of bacteria is impacted by environmental stressors. In assessing the production of ROIs *via* luminol-enhanced chemiluminescence, a method which mainly measures peroxidases produced both

internally and externally by amoebocytes (Coteur et al., 2002), Coteur et al. (2004) demonstrated the ability of both an increase in temperature and salinity to decrease ROI production in the common starfish *Asterias rubens*. Conversely, Hégaret et al. (2003) measured the production of reactive oxidative species (ROS) in the American cup oyster, *Crassostrea virginica* by measuring a change in fluorescence. Fluorescence, in this case was induced by adding 2',7'-dichlorofluorescein diacetate (DCFH-DA) to the haemolymph sample. DCFH-DA diffuses into the cells where it is hydrolysed and subsequently oxidised by ROS production to form 2',7'-dichlorofluorescein (DCF), a highly fluorescent probe, which is then measured using flow cytometry. Whilst in this study Hégaret et al. (2003) showed that a sudden temperature elevation failed to significantly alter oyster ROS production, in *A. rubens* an exposure to cadmium has been shown to increase the production of ROS under laboratory and field conditions (Coteur et al., 2003, 2005b). By studying the impact of cadmium exposure under field conditions, Coteur et al. (2003) demonstrated the triphasic nature in which an environmental stressor impacts the immune response. Initially, stressors act *via* short-term direct inhibition of the immune response followed by immune recovery due to induction of protective mechanisms. After this, animal physiology is impacted globally due to the overwhelming of protective measures leading to the onset of durable and indirect stimulation of the immune response. The stage of impact of an environmental stressor will therefore depend on two factors, the duration of stressor exposure and the condition of the organism which ultimately dictates its ability to maintain successful protective mechanisms. Therefore, accounting for latent effects of natural stressors in organisms studied both in the wild, and wild-collected animals for laboratory study, is vital to fully understand possible impacts of experimentally applied stressors on immune function.

In measuring a change in chemiluminescence or fluorescence, investigators demonstrate a change in overall haemocyte ROI production. However, within marine invertebrate immunology the most widely used technique to investigate the ability of haemocytes to produce ROIs is to measure a specific reactive oxidative species, such as superoxide anion (O_2^-) production, rather than a suite of ROIs. Superoxide anion production can be measured both as an extracellular kinetic assay through the reduction of cytochrome-C or alternatively as an intracellular end point assay with the reduction of nitroblue tetrazolium, NBT, (Pipe et al., 1995a). Of the marine invertebrates investigated the bivalve molluscs have received the vast majority of attention with respect to ROI production with studies investigating this immune parameter in the American cup oyster, *Crassostrea virginica* (Anderson et al., 1998; Boyd and Burnett, 1999; Fisher et al., 2000; Hégaret et al., 2003; Oliver et al., 2001), the Pacific oyster, *Crassostrea gigas* (Gagnaire et al., 2006a, b; Lacoste et al., 2002), the blue mussel *Mytilus edulis* (Bibby et al., 2008; Parry and Pipe, 2004; Pipe et al., 1999; Wootton et al., 2003), the Zhinkong scallop, *Chlamys farreri* (Chen et al. 2007a, b), the Taiwan abalone *Haliotis diversicolor supertexta* (Cheng et al., 2004a, b, c, d, e), the surf clam, *Mactra veneriformis* (Yu et al., 2010), the striped venus clam, *Chamelea gallina* (Monari et al., 2005, 2007) and the lagoon cockle, *Cerastoderma glaucum* (Matozzo et al., 2008). In investigating the impact of anoxic stress induced by air exposure in the surf clam, *Mactra veneriformis*, Yu et al. (2010) noted a significant reduction in the production of superoxide anions as measured through a decreased reduction of NBT after a 24 h air exposure. Furthermore, after an initial 24 h recovery in seawater, clam ROS production returned to levels noted pre-exposure. However, after a 48 h or 72 h air exposure, superoxide anion production was unable to recover even when returned to seawater for 24 h.

Whilst the majority of studies that have specifically measured superoxide anion production do so in bivalve molluscs, a number of authors have also employed these techniques to investigate ROS production in other phyla. In the Indian spiny lobster, *Panulirus homarus*, superoxide anion production was significantly reduced by both a reduction and an increase in salinity compared to controls after a 7 day exposure (Verghese et al., 2007). Additionally a natural infection with IMNV was demonstrated to cause a 50 % increase in superoxide anion production in the Pacific whiteleg shrimp *Litopenaeus (=Penaeus) vannamei*, although only at an advanced stage of the infection when recovery was unlikely (Costa et al., 2009a). Exposure to a number of immunostimulants has also been shown to increase ROS production in the Japanese common sea cucumber, *Apostichopus japonicus* (Gu et al., 2010). Immunostimulants have been suggested as an effective mechanism to increase disease resistance and immunocompetence, and therefore reduce mortality, during aquaculture (Sakai, 1999). Therefore, investigating the ability of these compounds to stimulate the immune response offers an alternative method to the expensive use of antibiotics and other chemicals which often have an additional unwanted risk through environmental contamination (Gräslund and Bengtsson, 2001). In using pathogen-associated molecular patterns (PAMPs) as immunostimulants, such as β-glucan (a homopolysaccharide found in cell walls), CpG DNA (a nucleic acid motif) and Mannan oligosaccharides (MOS; yeast wall constituents), Gu et al. (2010) showed superoxide anion production to be increased. This increase in ROS production occurred 1 h and 3 h after exposure to β-glucan (5, 25 and 100 μg ml^{-1}), 1 h, 3 h and 6 h after exposure to CpG DNA (2.5 μM) and 6 h and 12 h after exposure to MOS (40 and 80 μg ml^{-1}), after which superoxide anion production returned to control levels despite a continued immunostimulant exposure for 24 h.

Nitric oxide (NO) is a short-lived radical, generated by nitric oxide synthases (NOS), which, like the superoxide anion, plays an important role in the elimination of pathogens as part of the innate immune response (Rodríguez-Ramos et al., 2010). NO is not toxic itself, playing an important role as a signal molecule throughout the animal kingdom (Colasanti et al., 2010), but together with superoxide anions it forms peroxynitrile anion (ONOO-) which is a highly toxic compound with antibacterial and antiviral activity (Beckman and Koppenol, 1996; Fang, 1997; Fuji et al., 1999; Roch, 1999). In demonstrating a significant increase in the production of NO in the Mediterranean mussel, *Mytilus galloprovincialis*, exposed to *Micrococcus lysodeikticus* and *Vibrio anguillarum*, Costa et al. (2009c) showed NO to be both an important, and inducible, factor in the invertebrate immune response. Additionally NO, through the bystander response, has been shown to be involved in the host defence in sponges challenged with xenobiotica and attacking microorganisms (Colasanti et al., 2010; Muller et al., 2006). An exposure to β-glucan has been shown to induce an up-regulation in the production of NO in the carpet shell clam, *Ruditapes decussates*, and in the Mediterranean mussel *Mytilus galloprovincialis* (Costa et al., 2008). Furthermore, as well as demonstrating the ability to induce NO production, Rodrígues-Ramos et al. (2010) show the activity of nitric oxide synthase (NOS) and the expression of this gene can also be upregulated. When exposed *in vitro* to *Escherichia coli* O55:B5 lipopolysaccharide (LPS), haemocytes of the Caribbean spiny lobster, *Panulirus argus*, increased both the activity of NOS and also NOS gene expression.

Alongside measuring ROI and NO production, by measuring the activity of a number of separate enzymes within haemocytes, such as NOS, the bactericidal activity of haemocytes can be further quantified. When studying enzyme activity, a number of investigators have concentrated on the activity of hydrolytic, lysosomal enzymes shown

to be involved with bactericidal killing including; acid phosphatase (e.g. Chen et al., 2007a), β-glucuronidase (e.g. Ballarin et al., 2003; Pampanin et al., 2002), esterase (e.g. Gagnaire et al., 2003, 2004, 2006a, b), peroxidase (Couch et al., 2008; Mydlarz and Harvell, 2007), amino peptidase (e.g. Gagnaire et al., 2003, 2004), and lysozyme (e.g. Matozzo et al., 2007 ; Monari et al., 2007 ; Wang et al., 2008a, b).

Wootton et al. (2003) investigated the impact of the polycyclic aromatic hydrocarbon, phenanthrene, on the immune response of three bivalve species; the blue mussel, *Mytilus edulis*, the razor shell, *Ensis siliqua*, and the common edible cockle, *Cerastoderma edule*. In demonstrating the differential effects of phenanthrene on the immune response of these three bivalve species, Wootton et al. (2003) concluded that one had to be cautious when using only one species as an indicator, or sentinel, for the likely immune response of an entire group or community. The razor shell, *Ensis siliqua,* showed no modulation in the percentage of haemocytes expressing non-specific esterase or acid phosphatase activity, while *Mytilus edulis* also demonstrated no significant change in non-specific esterase activity. However, acid phosphatase activity significantly increased after a 7-day incubation at contaminant concentrations of 50, 100 and 200 μg l^{-1} with a significant decrease in acid phosphatase activity after 14 days at higher contaminant concentrations of 100, 200 and 400 μg l^{-1}. Conversely, in *Cerastoderma edule* phenanthrene contamination significantly affected enzyme activity. Non-specific esterase activity decreased after a 7-day incubation at 400 μg l^{-1} with acid phosphatase activity significantly increasing at a concentration of 100 μg l^{-1} and significantly decreasing at a concentration of 400 μg l^{-1} after a 7-day incubation. In considering this result, and other immune parameter results, Wootton et al. (2003) highlighted the pressing need to investigate the impact of environmental stressors on the invertebrate immune system from within a wider group of organisms and from a

number of differing groups, families and phyla.

As well as measuring humoral immunity specifically within haemocytes and through haemocyte functioning, many authors have measured humoral immunity and more specifically the presence and activity of humoral antimicrobial components within the cell-free lysate, separating the cellular and cell free fractions of the haemolymph. It is possible to demonstrate the antimicrobial activity of invertebrate serum, as outlined by Smith et al. (1995), by measuring bacterial viability using a spread plate, scoring the percentage of colony forming units both before and after exposure to the test material. Yet this method does not elucidate the mechanism of bactericidal activity, merely showing a response due to direct killing (Smith et al., 1995). A method more commonly used investigates the lysozyme-like bactericidal activity of the cell-free haemolymph (Matozzo et al., 2005; Yu et al., 2010). Lysozyme is a lysosomal enzyme capable of hydrolyzing mucopolysaccharides (bacterial cell wall constituents) (Pipe, 1990b), therefore measuring its activity measures the ability of the cell-free haemolymph to undertake bactericidal killing, *via* the production and activity of hydrolytic enzymes. In exposing *Micrococcus lysodeikticus* to the mucus of the spiny starfish, *Marthasterias glacialis* using an agar diffusion lysozyme test, Stabili and Pagliara (2009) noted the ability of zinc exposure, at a concentration of 5 mg l^{-1} for 48 h, to lower the lysozyme-like activity of *Marthasterias glacialis*. Alterations in lysis diameter, being the diameter of cleared zones around wells of mucus on a Petri dish, were standardised against a known concentration of lysozyme from crystalline hen egg white (Stabili and Pagliara, 2009). Whilst this study does not measure the antibacterial activity of organism haemolymph, it does demonstrate the true nature of mucus as a first line of host defence in species such as *Marthasterias glacialis*.

In exposing a log growth phase broth culture of a known bacterium to the

invertebrate serum and subsequently measuring the change in optical density of this culture over time, it is also possible to demonstrate the inhibition of bacterial growth using turbidometry. Alterations in optical density can then be quantified by standardising these values against a known concentration of lysozyme from crystalline hen egg white, as was done by Mattozzo et al. (2007) where an increase in salinity was shown to decrease lysozyme-like activity of cell-free haemolymph in the striped venus clam, *Chamelea gallina*.

A number of studies have also successfully measured bacterial growth inhibition in corals, demonstrating the antimicrobial activity of coral extracts *via* a change in optical density of a bacteria culture. Mydlarz et al. (2009), in testing the impact of yellow band disease (YBD) and bleaching on coral immune response in the mountainous star coral, *Montastraea faveolata*, noted a significant elevation in the antibacterial activity of coral extracts from both healthy and diseased tissue in corals infected with YBD compared to tissue from healthy corals. Interestingly however, coral extracts from bleached corals had the lowest noted antibacterial activity, possibly implicating the above average temperature and/or bleaching event in the depleted immune defence and subsequent spread of YBD in *Montastraea favolata* colonies (Mydlarz et al., 2009). Temperature was also shown to affect antifungal activity of coral extracts in the Caribbean sea fan coral, *Gorgonia ventalina*, interacting with an *Aspergillus sydowii* infection (Ward et al., 2007). In this study, antifungal activity was increased in all colonies inoculated with *Aspergillus sydowii*, with the greatest increase in extracts of coral fragments maintained at the warmest temperature. Whilst an increase in temperature and a bleaching event are suggested to have played a key role in the variation noted within the coral immune response by Mydlarz et al. (2009), Couch et al. (2008) also correlate site-specific environmental factors to an altered immune defence

in healthy, uninfected, *Gorgonia ventalina*. Whilst there was no correlation between previous disease prevalence, or severity, and any measured immune parameter, there was a relationship between antifungal activity and the percentage of bare substrate cover, with a low coral density possibly activating a change in antifungal activity, SOD activity and a change in exochitinase expression (Couch et al., 2008).

In studying the cell-free haemolymph it is also possible to measure the activity of numerous enzymes with respect to environmental perturbation. Bouilly et al. (2006) showed an increase in phenoloxidase like (PO-like) activity of the Pacific oyster, *Crassostrea gigas* when exposed to a cadmium concentration of 0.5 μg l^{-1} after an exposure of 66 days. This study found no effect of cadmium on PO-like activity when compared with controls at lower concentrations or a shorter exposure duration. In contrast, Verghese et al. (2007) showed PO activity to decrease in the Indian spiny lobster, *Panulirus homarus* in response to hypoxia, ammonia-N concentration and a change in pH. Together with a reduction in PO activity, environmental stressors have also been shown to alter the activity of the antioxidant enzyme superoxide dismutase (SOD). Increasing anoxia was shown to cause a decrease in SOD activity (Monari et al., 2005), and an increase in temperature was shown to cause an increase in the activity of this enzyme (Monari et al., 2007), in cell-free haemolymph of the striped venus clam, *Chamelea gallina*. Conversely, Chen et al. (2007b) showed that while SOD activity was not affected by temperature stress in the Zhinkong scallop, *Chlamys farreri*, activity of the hydrolytic enzyme, acid phosphatase (ACP), decreased significantly with increasing temperature.

Whilst demonstrating a reduction in the activity of different hydrolytic enzymes shows a clear reduction in the bactericidal activity of the cell-free haemolymph, and a reduction in antioxidant enzyme activity shows a reduction in an organisms ability to

protect itself against the production of ROIs, the significance of a reduction in PO, or PO-like, activity for host defence remains uncertain for many invertebrate species (Coles and Pipe, 1994). Within many arthropod species PO is involved in melanisation and encapsulation of foreign bodies (Coles and Pipe, 1994), and also it is involved with the melanisation cascade (the prophenoloxidase or proPO activating system), recently shown to be intimately associated with the appearance of factors that aid phagocytosis by stimulating cellular defence (Cerenius et al., 2008). PO activity is shown to be involved in the susceptibility of the Sydney rock oyster, *Saccostrea glomerata*, to QX disease caused by the protozoan *Marteilia sydneyi* (Butt et al., 2006; Newton et al., 2004; Peters and Raftos, 2003) and a reduction in PO activity has been suggested to compromise disease resistance in other bivalve species (Muñoz et al., 2006; Yu et al., 2010). Furthermore, in the Caribbean sea fan coral, *Gorgonia ventalina*, PO activity is involved in melanisation and immune defence of the host in response to an *Aspergillus sydowii* infection (Mydlarz et al., 2008). However, much controversy and uncertainty still remains within the field as to the importance of PO for immune defence in non-arthropod systems (Cerenius et al., 2008), but despite this PO activity is still often used as a measure of the immune response in studies investigating the impact of environmental stressors on the invertebrate immune system.

Costa et al. (2009a) demonstrated that PO activity was reduced in farm-reared Pacific whiteleg shrimp, *Litopenaeus (=Penaeus) vannamei* during late stages of an IMNV infection, and temperature was also shown to decrease PO activity, serine protease activity and proteinase inhibitor activity, in the same species (Pan et al., 2008), demonstrating an impact of temperature on the prophenoloxidase (proPO) system. However, whilst solid evidence exists for the role of the proPO system in arthropod immune defence, Cornet et al. (2009) suggest that caution is still needed when

measuring immunocompetence using proPO activity in an arthropod system, as the activity of the proPO system was shown be an in-sufficient estimate of bacterial resistance at a population level in the freshwater amphipod, *Gammarus pulex*.

Whilst the studies described above investigate the activity of a number of separate enzymes with a range of functions within the cell-free haemolymph, a number of others have demonstrated the ability of environmental stressors to alter the enzymatic activity of total haemolymph, where both the cellular haemocyte fraction and the cell-free serum fractions of the invertebrate haemolymph are investigated simultaneously (e.g. Hauton et al., 2000). Soudant et al. (2004) showed that the lysozyme-like activity in total haemolymph of the Manila clam, *Venerupis* (=*Tapes* or *Ruditapes*) *philippinarum* was affected by season (decreasing from Oct to Feb in a population from Marennes, France), also finding that the lysozyme-like activity varied with rearing site. Wang et al. (2008a) also show season to affect lysozyme activity, along with the activity of SOD, catalase (CAT) and myeloperoxidase (MPO) (all of which are involved in the detoxification of ROIs), in the Japanese common sea cucumber, *Apostichopus japonics*. The activity of these enzymes was changed significantly from July to October, caused by the induction of aestivation which is an indispensible state in sea cucumber life history induced by high temperature, causing major physiological and morphological changes that increase organism survival (Liu et al., 1996; Wang et al., 2008a). However, alongside a physiological cause, a direct influence of temperature on *A. japonicus* immune enzyme activity should also be considered, as both acute temperature changes and changes in salinity have been shown to significantly alter lysozyme, SOD, CAT and MPO activity (Wang et al., 2008b). Hauton et al. (2000) also showed a decrease of lysozyme-like activity and demonstrated a decrease in hydrogen peroxide concentration (indicating a reduction in the respiratory burst and production of

this ROI), compared with controls, in total haemolymph of the European flat oyster, *Ostrea edulis* inoculated with *Listonella anguillarum*, and Paillard et al. (2004) showed that temperature and a pathogenic challenge altered the leucine aminopeptidase concentration in haemolymph from *Venerupis philippinarum*.

Although investigating the total haemolymph fraction allows the study of both intra- and extra- cellular presence of bactericidal activity simultaneously, providing a good overall measure of humoral immunity, it does not allow the distinction to be made between stressors acting on the haemocytes themselves or acting on the release of antimicrobial factors into the haemolymph. Therefore, understanding how differences in the sampling methodology may impact experimental results within humoral immunology will allow a greater understanding of the mechanisms by which stressors are impacting haemocyte and humoral functionality.

1.3.2 Antimicrobial peptides and immune genes– A change in expression and direction?

Antimicrobial peptides (AMPs) are ubiquitous antibiotic defence agents, highly conserved throughout evolution, being present in all phyla (Bachére et al., 2004). They comprise one of the main humoral components of the innate immune system (Costa et al., 2009b) and recently their study has received increasing attention with over 1,200 peptides having been characterized from within eukaryotes (Wang et al., 2009). These defence molecules have little or no functional specificity and possess a broad spectrum of antimicrobial activity, acting against Gram-positive and Gram-negative bacteria, fungi, yeast and in some instances viruses and protozoa (Bachére, 2003). These peptides possess an enormous sequence and structural diversity, with only a few shared characteristics which include their small size, cationic character and the presence of 30

– 50 % hydrophobic residues (Hancock et al., 2006), thus enabling different groups of AMPs to demonstrate significantly different antimicrobial activities and modes of action (Muňoz et al., 2002).

Since their discovery in the cecropia silkmoth, *Hyalophora cecropia* (Steiner et al., 1981), at least 50 % of the inducible AMPs reported have been identified within invertebrates, mainly within insects (Bulet et al., 1999). Within marine invertebrates the expression and action of these defence molecules has predominantly been investigated in bivalve molluscs (mussels and oysters) and crustaceans (Bachére, 2003). However, with an increasing understanding of the importance of AMPs for invertebrate innate immunity, alongside the potential for their use in drug development (Li et al., 2008a), AMPs have now been discovered and characterised in many marine invertebrate phyla including the Echinodermata, Porifera, Annelida, Chelicerata, Chordata (Urochordata) and Cnidaria (Li et al., 2008a; Ovchinnikova et al., 2006).

Despite the growing level of interest that the investigation of AMPs has received within marine invertebrates, there is still a scarcity of studies in which these genetic and proteomic tools have been used to investigate the impact of environmental variability on invertebrate immunology, with many studies to date having focused on the discovery, characterization and regulation of AMPs (e.g. Gonzalez et al., 2007; Jung et al., 2009; Li et al., 2008a; Ovchinnikova et al., 2006). Yet due to the integral role of AMPs in the innate immune system, investigating genomic and proteomic expression of AMPs provides a unique opportunity to vastly advance current understanding in the field of ecological immunology, and specifically the impact of environmental stressors.

In investigating the impact of a temperature, physical and pathogen stress on the expression of MGD2, an AMP in the defensin family, in both the blue mussel, *Mytilus edulis* and the Mediterranean mussel, *Mytilus galloprovincialis*, Mitta et al. (2000a)

demonstrated the ability of multiple environmental stressors to impact AMP regulation. This study showed the expression of MGD2 increased when mussels were exposed to a physical shock or a heat shock, yet in contrast, expression was reduced when exposed to a bacterial challenge. Cellura el al. (2007) also demonstrated a down-regulation of AMP gene expression when *Mytilus galloprovincialis* were exposed to a heat-shock and several bacterial challenges. An exposure to *Micrococcus lysodeikticu*s, was shown to significantly decrease defensin mRNA expression immediately after the challenge, however this decrease lasted less than 24 h. Exposure to *Vibrio splendidus* decreased the expression of mytilin and myticin mRNAs yet increased defensin expression, whilst exposure to *Vibrio anguillarum* increased mytilin expression. Heat-shock was also shown to increase myticin mRNA.

In undertaking a three year survey between 2005 and 2008, investigating the expression of AMPs in response to temperature, salinity and *Escherichia coli* tissue content, Li et al. (2009a) successfully demonstrated the seasonal variability of AMP expression. Temperature was shown to positively influence the regulation of defensin, and myticin B but failed to alter mytilin B, whereas salinity only affected defensin expression. Interestingly, given the role of AMPs in the innate immune response, *E. coli* tissue content failed to influence AMP expression in this study. In analysing these results in context with season therefore, defensin and myticin B appeared to be expressed to a greater extent in spring-summer compared to winter due to the decrease in temperature. However, these results need to be interpreted with caution due to the failure of this study to quantify the relationship between immune gene regulation and the measured environmental parameters despite their positive influence. Whilst the studies by Mitta et al. (2000a), Cellura el al. (2007) and Li et al. (2009a) offer only three examples in which AMP expression can be differentially altered by various

environmental stressors, they demonstrate how a variable environment can differentially change the genetic expression of an integral component of the innate immune system. Therefore, to increase our understanding of the extent to which environmental stressors alter the gene expression, structure and function of the immune response, a fundamental mechanism involved in organism survival (Lochmiller and Deerenberg, 2000), there is a need to investigate changes in AMP expression with response to a greater array of environmental stressors and across a wider range of organisms.

Furthering the investigation of AMP expression with respect to environmental perturbation, the investigation of the immune genome within marine invertebrates has received a rapidly increasing level of study. The development of new molecular methodologies has allowed the investigation of an enormous suite of immune genes, and more specifically enabled investigators to further elucidate which specific immune genes are important for the evolution of the immune response with respect to environmental variability and stress. Through the employment of novel molecular techniques such as quantitative real-time PCR (qRT-PCR) (e.g. Li et al., 2008b; Yang et al., 2010), cDNA microarray analysis (e.g. de la Vega et al., 2007b; Desalvo et al., 2008; Place et al., 2008), transciptomics (e.g. De Zoysa et al., 2009; Philipp et al., 2012) and suppression subtractive hybridisation (SSH) (e.g. de la Vega et al., 2007a), the influence of a range of environmental stressors and the induction and expression of a wide range of immune genes is possible.

Philipp et al. (2012) demonstrated a complex immune gene repertoire within the blue mussel, *Mytilus edulis*, providing an unprecedented in-depth analysis of the mussel transcriptome. In highlighting a high number of innate immune recognition receptors and downstream pathway members, these authors demonstrate the sophisticated nature of the invertebrate innate immune system repertoire, which provides a novel insight in

to the phylogeny of the immune system (Philipp et al., 2012). Tirapé et al. (2007) also demonstrated a complex expression of immune related gene, in this instance during ontogenesis of the Pacific oyster, *Crassostrea gigas*. In measuring the expression patterns of 18 selected genes in the developing oysters, they demonstrated differential gene expression at separate developmental stages, increasing our understanding of the ontogeny of the oyster immune response during very early life cycle stages. This potentially explains the variability of susceptibility to infections noted during oyster development (Tirapé et al., 2007). Additionally, by exposing the developing oysters to a bacterial challenge this study demonstrated the ability of gene expression to be altered not only by developmental stage but also by the timing of the bacterial challenge and also by the amount of bacteria used for the challenge. Yang et al. (2010) also investigated the expression of immune-related genes in embryos and larvae of a marine invertebrate. In studying the Japanese sea cucumber, *Apostichopus japonicus,* these authors demonstrated further evidence for the up-regulation of immune gene expression in larvae, exposing larval *A. japonicus* to a lipopolysaccharide challenge (Yang et al., 2010). By furthering the understanding of immune response ontogenesis within *Crassostrea gigas* and *Apostichopus japonicus* the studies by Tirapé et al. (2007) and Yang et al. (2010) demonstrated the importance of understanding the natural variation noted within marine invertebrate immune response during different life cycle stages. Whilst these studies only investigated the very early life cycle stages of these species, the changes in gene expression and differences noted in the ability of a bacterial challenge to alter gene expression at different developmental stages highlighted the importance of life cycle stage in understanding the invertebrate immune response. Therefore, investigating the impact of a variable environment together with the difference in gene expression during immune system ontogenesis may help to highlight

and explain any noted increase in disease susceptibility during the early life cycle stages of marine invertebrates. This will in turn help to outline tipping points and potential thresholds for the survival of developing marine invertebrates exposed to environmental stressors and anthropogenically induced climate change (Luna-Acosta et al., 2012).

1.4. DISEASE RESISTANCE AND MORTALITY

Our increased understanding of the immune system and of its separate components has fed a tendency to then use the separate components as a proxy for overall immunity or immunocompetence (Viney et al., 2005). However, as has been demonstrated above (Sect. 1.2 and 1.3), whilst this approach offers an accurate measure of the impact of stressors on the separate immune parameters, the perceived level of stress-induced immune dysfunction will be wholly dependent upon which parameters are chosen as the immunocompetence proxy. Consequently, this approach alone does not directly address an actual change in overall susceptibility to a pathogenic insult. By measuring a change in host susceptibility, disease prevalence and both the onset, and overall mortality caused by a pathogen during or after exposure to a stressor, the functional capacity of the immune system can be quantified. Such quantification is crucial to our understanding of the ecological impact of environmental stressors on the immune response of marine invertebrates (Morley, 2010; Viney et al., 2005). Linking an alteration in immunocompetence to an organism's susceptibility to a pathogen offers the greatest opportunity to measure the reduction in immune functional capacity, and a reduction in organism fitness (Viney et al., 2005). However, investigating the impact of stressors in the presence of a pathogen and measuring disease resistance has attracted significantly less attention than the measure of either cellular or humoral immune

parameters alone. This may be due, in part, to the practical challenges involved in developing reliable experimental models for infection studies (Le Moullac and Haffner, 2000).

Naturally in the marine environment, a host organisms' exposure to a pathogen will be wholly dependent upon a number of overriding factors that will influence the number of viable and virulent bacteria the host is exposed to. These include the heterogeneity of pathogen abundance within the environment, bacterial survival and virulence under changing environmental conditions, levels of other competing bacteria in the environment and physical variables in the marine environment such as tides and water currents which will affect bacterial distribution. Whilst all these influences combine to increase the variability of the level of bacteria a host organism is naturally exposed to, experimentally this natural variability is significantly reduced and an equal exposure level is desired to successfully investigate the effect of experimental conditions on the host immune defence. To achieve an equal bacteria exposure experimentally, a method commonly employed exposes a host organism to a known number of pathogenic bacteria directly, through an injection of a set volume and abundance of bacteria into the host's internal tissues (e.g. Hauton et al., 2007). Whilst this method ensures an even level of bacterial exposure, which is required for an experimental approach, it also highlights the limitations inherent in an experimental pathogen exposure. Bacterial exposure brought about by an injection removes the natural variability of pathogen distribution and survival in a natural environmental setting. Additionally, this method bypasses the first line of defence offered in a host organism, being the defence offered by an intact epithelium or exoskeleton. Whilst removing this natural variability is unavoidable, it is also necessary to truly elucidate the impact of environmental stressors on the host's immune defence, and ensure that it

is not a general change in host-pathogen interactions which could be due to a change in any number of physiological or behavioural mechanisms in the host, or the pathogen. Therefore, whilst there is a vital need to experimentally expose organisms to a realized pathogenic threat, it is crucial to understand the limitations that are inherent with current experimental infection models when comparing to a natural system.

In studying an alteration in overall immunocompetence and a reduction in disease resistance, one method currently used to demonstrate a change in pathogenic resistance is to measure an organism's clearance efficiency to a realized pathogenic challenge. In studying clearance efficiency, Oweson and Hernroth (2009) investigated the impact of trace metal contamination, in this case manganese exposure, on the bactericidal response of the starfish, *Asterias rubens*, the blue mussel, *Mytilus edulis*, and the Norway lobster, *Nephrops norvegicus*. In studying the antibacterial activity of these three species, measured *via* the clearance capacity when inoculated with *Vibrio parahaemolyticus* and subsequently counting the remaining viable bacteria, this study showed manganese contamination (15 mg l^{-1} for 5 d) had no effect on the bactericidal activity of *A. rubens* haemolymph or digestive tissue. In *M. edulis* however, the bactericidal activity of haemolymph was significantly lower in animals exposed to manganese contamination 8 h after inoculation with *V. parahaemolyticus*, yet 24 h after bacterial exposure, the number of bacteria remaining in the mussel haemolymph had reduced significantly and there was no difference between manganese exposed or control animals. In mussel digestive tissue, the mean number of remaining viable bacteria was significantly higher in manganese exposed mussels, 24 h after bacterial injection, compared to control organisms, and this significant difference remained 48 h after bacteria were injected. Similarly to *A. rubens*, there was no significant impact of manganese on the number of viable bacteria in the blood of *N. norvegicus*, however

manganese significantly reduced the bactericidal impact of *N. norvegicus* digestive tissue. There was a higher number of remaining bacteria in manganese contaminated individuals after just 8 h post-injection, compared to controls, and this significant difference lasted over 48 h after injection. Therefore, in measuring clearance efficiency there is again a need to account for species differences in the impact of stressors and how stressors impact immune dysfunction and overall disease resistance.

In transplanting the blue mussel, *Mytilus edulis* from what was perceived to be a contaminated site, where mussel general immunity was depressed, to what was perceived to be a relatively clean site, Mayrand et al. (2005) tested the impact of contamination on bactericidal activity of mussel haemolymph measured by clearance efficiency. Additionally however, this study investigated the recovery potential of the invertebrate immune system. In comparing a wide set of mussel immune parameters, the authors noted the ability of a number of these parameters, after the 9 day transplant, to recover in contaminated mussels towards a level seen in mussels originating from the clean site. Following this 9 day period mussels were then inoculated with a known pathogen, *Listonella anguillarum*. Mussel haemolymph was assessed 36 h later to quantify the remaining levels of viable bacteria. Those originating from the contaminated site expressed a higher bacterial count, thus meaning a reduction in their clearance efficiency and a suggestion that the functional recovery of the immune response in the transplanted mussels may not have been as complete as had been indicated by the measured parameters.

Whilst measuring clearance efficiency does successfully demonstrate a reduction in overall immunocompetence, the extent to which the population dynamics of the species may be impacted cannot be fully interpreted from this result, as an organism with higher levels of viable bacteria remaining in the haemolymph after a 36 h

inoculation may feasibly still survive and recover fully from the infection. St-Jean et al. (2002) found the clearance efficiency of *Mytilus edulis* exposed to tributyltin (TBT) and dibutyltin (DBT), whilst lower in a dose dependant fashion compared with control individuals, was still able to show recovery with a reduction of bacterial levels in the haemolymph from 4 days post challenge up to 14 days post-challenge in all treatment groups. Therefore, accounting for possible recovery of the immune system and understanding the time scale a recovery could still feasibly occur over is both an interesting and critically important measure when assessing the impact of stressors.

To further the current understanding on the link between immune system and population biology, Cheng and colleagues (Cheng et al., 2004a, b, c, d, e) tested the impact of various environmental stressors by measuring the clearance efficiency together with the onset of mortality and the overall increase in cumulative mortality, in the Taiwan abalone, *Haliotis diversicolor supertexta*, exposed to *Vibrio parahaemolyticus*. In studies investigating the impact of elevated ammonia (Cheng et al., 2004a) and nitrite (Cheng et al., 2004b) concentrations, a significant reduction in clearance efficiency and an increase in cumulative mortality was demonstrated when individuals were exposed to both stressors, increasing the susceptibility of this abalone to a *V. parahaemolyticus* infection. Additionally, together with an increase in cumulative mortality, an increase in water temperature was shown to shorten the onset of mortality, with individuals transferred to 28 ºC or 32 ºC beginning to die after just 6 h compared to 12 h for individuals transferred to 20 ºC or 24 ºC (Cheng et al., 2004c). Hypoxia (Cheng et al., 2004e) and salinity (Cheng et al., 2004d) have also been shown to negatively impact the pathogenic susceptibility and subsequent survival of *H. diversicolor supertexta*, decreasing the clearance efficiency and the onset of mortality together with increasing the cumulative mortality in this species.

In addition to measuring the impact of environmental stressors on the resistance of *Haliotis diversicolor supertexta* to a known pathogenic species, it is also possible to investigate whether a decrease in pathogenic resistance is attributable to an increased virulence in the bacteria. In measuring the impact of temperature on the susceptibility of the Taiwan abalone to either *Vibrio alginolyticus* H11 or to *V. parahaemolyticus* B4 challenges, Lee et al. (2001) showed that this bivalve, when kept at higher temperatures, was more susceptible to the bacteria. However, by also measuring the susceptibility of *H. diversicolor supertexta* to the extracellular products (ECP) of both *V. alginolyticus* H11 and *V. parahaemolyticus* B4, this study demonstrated a reduced LD_{50} of ECP, with a lower dosage able to kill the abalone at higher temperatures. The reduced dosage of both bacterial cells and ECP able to affect killing in the abalone indicated a difference in virulence of these bacterial species at different temperatures, and also highlighted the role of temperature in mass mortality outbreaks of vibriosis associated with warm water exposure in this bivalve.

In their ability to alter bacterial virulence, environmental stressors further complicate host-pathogen interactions and the impact of stressors on host population biology. However, in investigating and attributing an alteration in host disease resistance to an increase in pathogen virulence, Lee et al. (2001) demonstrated the value of elucidating bacterial performance in an experimental system and how this may influence the perceived outcome of a study. Therefore, understanding pathogen dynamics allows the true understanding of whether environmental stress is impacting the host or pathogenic organism.

In all the studies discussed above, the authors have simultaneously attributed changes in the susceptibility and survival of the study organisms to both an environmental and a pathogenic stressor. However, in exposing individuals of the

Pacific oyster, *Crassostrea gigas* to the pathogen *Vibrio splendidus*, followed by a mechanical stress three days later, Lacoste et al. (2001) demonstrated the ability of a stressor to modulate an organisms disease susceptibility to an already established pathogenic challenge. In showing an increase in the severity of infection, measured by an increase in both the pathogenic load and the cumulative mortality of the test oysters, compared with control organisms exposed to the pathogen and no mechanical stress, the authors demonstrated the ecological impact of an acute stressor on an already established infection. These results were supported by Anderson et al. (1998) in a study where the cumulative mortality of the American cup oyster, *Crassostrea virginica*, naturally infected by the protozoan parasite, *Perkinsus marinu*s, increased when exposed to hypoxia when compared with untreated control oysters. This study also showed that an exposure to TBT failed to alter the species' cumulative mortality yet when exposed simultaneously to hypoxia and TBT these stressors acted synergistically, significantly increasing the cumulative mortality of the oysters to a greater extent than the combined magnitude of either of the two stressors when tested in isolation. The results from Anderson et al. (1998) therefore highlight the need to study multiple stressors simultaneously, to allow the impact of complex stressor interactions to be elucidated.

Whilst a number of studies have shown the benefit and also demonstrated the potential pitfalls encountered when measuring disease resistance, the number of investigations that have actually studied a change in the susceptibility of a host to a pathogenic threat is still very low. Therefore, to fully understand the impact of environmental stress on organism disease resistance, and to understand what this will mean at an ecological scale, there is a need to investigate the response of a host organism to a realised pathogenic threat.

1.5. THE COST OF IMMUNITY – PHYSIOLOGICAL TRADE-OFFS

Our understanding of the marine invertebrate immune response, immune response effector systems and the impact of environmental stress on immune defence mechanisms has significantly advanced over the past decade following the development and employment of novel genetic techniques (see Philipp et al., 2012). However, the basic observation that immune defences are induced by infection rather than being constitutively active suggests immune activity is costly (Lazzaro and Little, 2009). It is these costs that are central to ecological immunology understanding (Rolff and Siva-Jothy, 2003; Sheldon and Verhulst, 1996). Owing to finite resources, the costs associated with immune system maintenance and activation divert resources away from other important physiological processes and life history traits, such as reproduction or growth (Bonneaud et al., 2003; Lochmiller and Deerenberg, 2000; Sokolova et al., 2012). A hosts ability to maintain an effective immune response is subsequently affected by its overall condition and energetic reserves (Kelly, 2011; Lazzaro and Little, 2009), and understanding the impact of environmental stress on host defence therefore requires an understanding of condition, energy homeostasis and physiological trade-offs within the host organism (Fig. 1.2; Sokolova et al., 2012).

Whilst the acquisition of energy, its allocation to different physiological processes and its expenditure are vital to an organism's fitness (Sokolova et al., 2012), very few studies to date have investigated the energetic trade-offs between host defence and other physiological processes in invertebrate organisms. Furthermore, the majority of invertebrate studies that have investigated the energetic trade-offs induced by host defence have focused on insects, and specifically the trade-off between immune defence and reproduction in this group (e.g. Kelly, 2011; Steiger et al., 2011; Zuk et al., 2004).

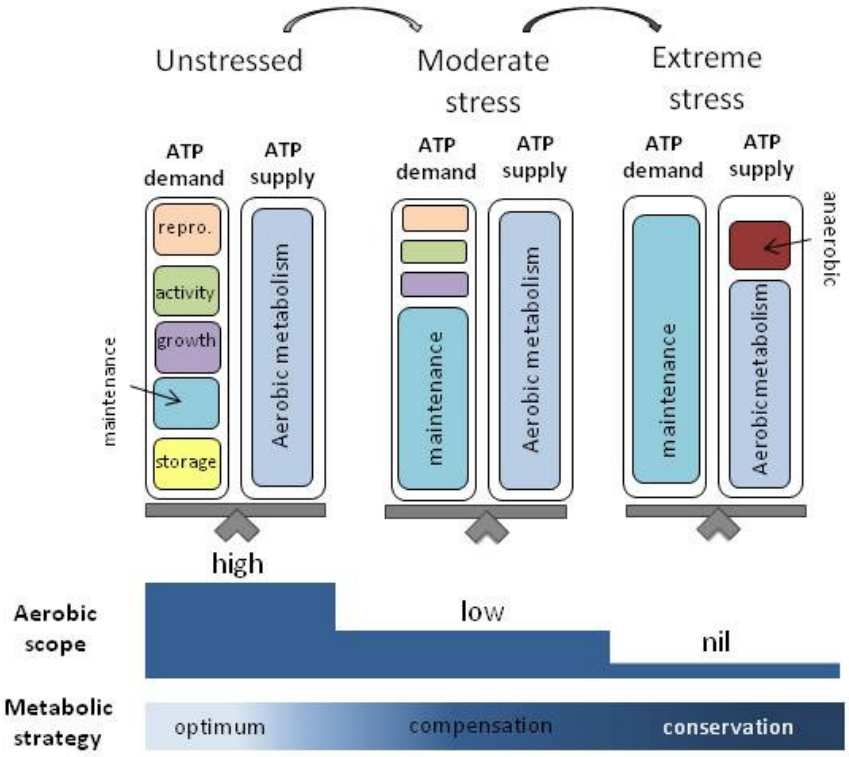

Figure 1.2 A bioenergetic framework of resource allocation, adapted from Sokolova et al. (2012), incorporating the general model of resource allocation from Pook et al. (2009), and the 'oxygen- and capacity- limited thermal tolerance concept (OCLTT)' from Pörtner (2010).

Within marine invertebrate research there is currently a dearth of studies that have investigated the trade-off between host defence and other life history traits. Petes et al. (2008), investigating the energetic trade-off between stress resistance and reproduction in the Californian mussel, *Mytilus californianus*, demonstrated organisms sampled from higher vertical edges of the intertidal zone invested less relative energy in reproduction in this stressful environment compared to lower-tidal edge organisms, suggested they experience less environmental stress. Furthermore, high-edge mussels released all their gametes in one spawning period early in the summer, whilst accumulating high concentrations of carotenoid pigments in mantle tissue. Conversely, organisms collected from the lower-tidal edge, invested more energy in reproduction,

spawning throughout the year, whilst accumulating less carotenoid pigments in mantle tissues. The higher concentration of caroteniod pigments in high-edge organisms is proposed to protect the host from oxidative stress induced from living in a higher stress environment at higher tidal levels. The authors suggest that reallocating energy from reproduction towards costly physiological processes such as stress resistance and host defence under environmental stress may improve survival but could impact population dynamics and ultimately species persistence (Petes et al., 2008). Similarly, Li et al. (2009b) investigated the effect of spawning activity on host defence in the Pacific oyster, *Crassostrea gigas*. Oysters that were studied following a spawning period were shown to suffer significantly higher mortality when exposed to extracellular products from *Vibrio harveyi*, compared to pre-spawning oysters. Furthermore, glycogen reserves were shown to be lower in post-spawned oysters, highlighting the energetic cost of spawning. This increased metabolic demand of spawning oysters is therefore shown to compromise immune defence and metabolic reserves in post-spawned organisms, ultimately influencing the susceptibility of these organisms to a pathogenic challenge.

Despite very few studies having investigated the trade-off between immune defence and other physiological processes in invertebrate systems, the research that has taken place to date indicates pathogen resistance involves the entire physiology of the host organism and is influenced by the demand of non-immunological physiological processes (Lazzaro and Little, 2009). Therefore, to fully understand the response of the immune system to environmental stress it is vital to include measures of organism condition, energy homeostasis and physiological trade-offs when assessing host defence and disease susceptibility (Pook et al., 2009; Sokolova et al., 2012).

1.6. THE IMMUNE RESPONSE AND CLIMATE CHANGE

Anthropogenically induced climate change poses a major threat to marine ecosystems and the organisms that reside within them (Harley et al., 2006). Increased atmospheric carbon dioxide (CO_2) levels have already led to an increase in global temperatures (IPCC, 2007) and a concurrent reduction in seawater pH (Caldeira and Wickett, 2003; discussed in Sect 2.2). Also, as anthropogenic CO_2 emissions continue to increase, global temperatures are projected to increase by a further 4ºC by the end of this century (IPCC, 2007), and over the same period seawater pH is projected to fall lower than it has been for 55 million years (Zachos et al., 2005). These environmental changes are predicted to have a significant impact on the health and functioning of marine organisms (Raven et al., 2005), potentially having significant implications for marine biodiversity and ecosystem functioning (Widdicombe and Spicer, 2008). Therefore to fully understand the impact of environmental stressors on marine invertebrate health and immune response it is vital to investigate the impact of climate change, and the environmental stressors associated with this global phenomenon, on immunological functioning.

1.6.1 The impact of temperature on the invertebrate immune response

Habitat temperature is a key environmental variable as it governs all physiological processes in ectothermal organisms (Pörtner et al., 2006). Due to its importance in regulating organismal performance, temperature is an often used stressor to investigate the impact of environmental stress on invertebrate immune functionality. Indeed it is one of the chief stressors, covered throughout this chapter, that is shown to affect all

aspects of the invertebrate immune system (e.g. Cellura et al., 2007; Lee et al., 2001; Li et al., 2009a; Pan et al., 2008; Parry and Pipe, 2004). However, whilst temperature is shown to affect the invertebrate immune response in a number of different organisms, the majority of studies to date have focused on an acute (< 48 h), or a short term (less than 10 days), temperature exposure (e.g. Chen et al., 2007b; Cheng et al., 2004c; Hégaret et al., 2003; Wang et al., 2008a). These studies offer important insight into the impact of temperature on immune system functioning, and yet paradoxically may not contribute as much to our understanding of increased seawater temperatures in the context of global climate change. Anthropogenically induced climate change is projected to cause shifts in environmental temperature that are often much lower than those used in acute exposures (e.g. Wang et al., 2008b) and which are occurring over a much longer time scale. Therefore to understand the potential impact of long term exposure to altered seawater temperatures and to investigate the possibility for organism acclimation or adaptation, it is vital to undertake longer term experimental perturbations under realistic future warming scenarios.

One area of ecological immunology that is currently addressing the impact of increased temperature stress in the context of climate change is the study of coral immunology, and specifically the impact of temperature on coral bleaching and disease resistance (e.g. Mydlarz et al., 2009; Ward et al., 2007). With increasing global temperatures it is projected organisms, such as corals, will be exposed to prolonged periods of abnormally high sea surface temperatures. One such warming event occurred in 2005, when annual sea surface temperatures rose 1 °C above the mean monthly maximum for a continuous 14 week period in the Caribbean. This temperature rise created conditions that were significantly warmer than previous years and also warmer than the following summer period (Clark et al., 2009). Using this higher temperature

year as a temperature stress proxy, Mydlarz and colleagues (2009) investigated the impact of increased temperature on bleaching and resistance to yellow band disease (YBD) in the mountainous star coral, *Montastraea faveolata*. The study found that the coral immune system was significantly impacted by temperature with prophenoloxidase (PO) activity increasing in bleached or diseased corals during the warming event, compared to healthy corals, whereas lysozyme-like activity and antibacterial activity was reduced in bleached corals. Despite this noted increase in PO activity in bleached or diseased corals during the warming event, this stimulation of the immune response did not confer increased disease resistance as 20 of the 21 bleached or diseased corals originally sampled had died by the end of the study in 2007. Whilst temperature was shown to initially increase apparent immunocompetence in the coral host during a temperature stress event, this study showed that increased temperature led to deleterious impacts on overall coral health, outbreaks of bleaching and YBD disease, and thus significantly increased coral mortality over a longer time scale. The complex nature by which temperature interacts with bleaching, host defence and pathogen resistance in corals highlights the potential catastrophic impact of increased temperature on coral reef ecosystems, within a realistic climate change scenario, and emphasizes the need to understand the impact of prolonged temperature increases on host-pathogen interactions.

Whilst temperature can have a significant impact on organism immunological function, it is also important to understand the potential implication of climate change on host-pathogen interactions. Ward et al. (2007) exposed the Caribbean sea fan coral, *Gorgonia ventalina*, to an *Aspergillus sydowii* infection and increased seawater temperatures. There was an increased in antifungal activity in sea fans maintained at higher temperatures following the bacterial infection, thus highlighting a temperature-

induced stimulation of the immune response. Critically, however, this study also noted an increased growth rate of the pathogen at increased temperatures. If the pathogen could establish an infection before host defence systems are able to act, due to this increased growth rate, any immune system stimulation induced by increased temperature could be rendered inconsequential. Thus increased pathogen virulence noted in warmer conditions could have significant implications for host pathogen interactions and thus host survival. Therefore to understand the impact of increased temperature, due to climate change, on the invertebrate immune response it is also vital to investigate the impact of increase temperature on pathogen dynamics.

1.6.2 The impact of ocean acidification on the invertebrate immune response

Unlike temperature, it is only over the past decade that the scientific community has begun to understand the potential impact of increasing atmospheric CO_2 on seawater carbonate chemistry (see Caldeira and Wickett, 2003). This phenomenon, referred to as 'ocean acidification', is now understood to significantly impact a number of key seawater carbonate chemistry parameters (outlined in Sect 2.2), and therefore potentially impact marine invertebrate health. These findings have sparked considerable interest, with the scientific community struggling to meet the knowledge and understanding deficit surrounding ocean acidification (Widdicombe and Spicer, 2008).

To date only a handful of studies have been published investigating the impact of ocean acidification on the invertebrate immune response. Bibby et al. (2008) exposed the blue mussel, *Mytilus edulis*, to reduced seawater pH for 32 days, and measured the response in a number of key immune parameters over the course of the experiment. Mussels maintained in seawater with reduced pH (7.7, 7.5 or 6.7) displayed

reduced phagocytic activity, compared with controls maintained at pH 7.8, after the 32 day exposure. Concluding that reduced phagocytic activity is likely caused by a reduction in the physiological condition, and thus the subsequent functionality, of haemocytes, these authors stress the potential impact of ocean acidification on invertebrate disease resistance and therefore organism survival. Matozzo et al. (2012) demonstrated a similar reduction in host defence in mussels exposed to reduced seawater pH, with the lysozyme-like activity of cell free haemolymph reduced in *Mytilus galloprovincialis* exposed to pH 7.7 and 7.4. Finally, Hernroth et al. (2011) demonstrated a suppression of phagocytic activity, a reduction in coelomocyte counts and an inhibition of p38 MAP-kinase activity, in *Asterias rubens* exposed to pH 7.7.

Ocean acidification (OA) is highlighted as one of the greatest threats the marine environment faces (Harley et al., 2006). Yet despite the potential impact of this global stressor, to date very little is known about how a reduced seawater pH will impact organism immune function, disease resistance, and consequently host survival. The few studies that have investigated the impact of OA on invertebrate immune function to date, by Bibby et al. (2008), Hernroth et al. (2011) and Matozzo et al. (2012), highlight the potential negative impact of reduced seawater pH on host defence mechanisms and therefore emphasize the importance of studying the impact of this environmental stressor on a wide range of marine invertebrates, in combination with other climate change stressors and in the context of host-pathogen interactions. Therefore to fully understand the potential implications of ocean acidification for invertebrate immunological functioning further study is urgently required.

1.7. CONCLUSIONS

Despite an increase in the number of studies addressing the impact of environmental stressors on the marine invertebrate immune system over the past decade, there are still many key areas where the understanding of marine invertebrate ecological immunology remains deficient. This incomplete understanding hampers our ability to predict how population biology, and therefore whole ecosystems, may respond to an ever changing and anthropogenically modified climate. This in turn limits our ability to predict possible impacts on species and ecosystem evolution. To advance our current understanding of the ecological and evolutionary significance of environmental stressor related immune dysfunction, and to allow policy makers and environmental managers to make informed decisions on how to mitigate any anthropogenic impact on Earth's climate, we therefore need to employ new stressor models and incorporate newly emerging techniques.

Life history theory dictates that physiological trade-offs exist in all organisms (Roff, 1992; Sibly and Calow, 1986; Stearns, 1992). Owing to finite resources, immune system maintenance and upregulation therefore requires resources that would otherwise be used to maintain other fitness related traits such as metabolism, growth or reproduction (Lochmiller and Deerenberg, 2000; Sheldon and Verhulst, 1996). Like many fitness-related traits, immunocompetence is condition dependant (Lochmiller and Deerenberg, 2000; Thomkins et al., 2004; Zuk and Stoehr, 2002), being affected by an organisms nutritional status (Moret and Schmid-Hempel, 2000) and reproduction (Kelly, 2011; Zuk et al., 2004). Therefore the extent to which an organism must trade-off resources allocated to physiological processes such as metabolism or reproduction, in an attempt to successfully defend itself from a pathogenic challenge, will depend on

its energy reserves and physiological state. Consequently, to fully the understand the impact of environmental stress on an organism's immune response, and ultimately its fitness, it is vital to also measure organism condition. By measuring an organisms metabolic functioning, energetic reserves and reproductive investment, alongside host defence, it will be possible to fully understand the fitness implications of any physiological trade-offs induced by an alteration in immunocompetence.

A major drawback of many of the studies that have investigated the impact of environmental stressors on the invertebrate immune response to date is their failure to account for seasonal variability. Environmental parameters naturally fluctuate, changing dramatically over a wide range of spatial and temporal scales. Therefore whilst it is key to understand organism's condition and subsequently the impact of any physiological trade-offs, to fully understand the dynamic interaction between environmental stressors and the invertebrate immune response, and to be able to predict how environmental stress will impact population dynamics, it is also vital for future studies to investigate the impact of season (Duchemin et al., 2007).

As demonstrated throughout this chapter, many recent studies investigating the impact of environmental stressors on the invertebrate immune response have demonstrated significant interspecific differences in response to a single environmental stressor. Perhaps more importantly still, many studies have also demonstrated a wide range of intraspecific responses to different environmental stressors, or to different exposure levels of single stressor. Therefore, to understand the impact of a variable environment at a species, population or even at an ecosystem level, there is a need within the field of ecological immunology to investigate responses across a wider group of species. Studying species from more families and from a greater range of phyla, as well as investigating these responses to a wider range of stressors, will build on our

current knowledge of the immune system and help further understand the impact of a variable environment.

Together with the need to investigate a greater range of stressors, there is also a requirement to investigate the impact of different stressors in combination. To date many ecological immunology studies have investigated a single stressor model which, whilst a necessary first step in elucidating stressor impacts, does not account for stressor interactions. However, as highlighted by Anderson et al. (1998) and Parry & Pipe (2004), when the invertebrate immune response is tested to demonstrate the impact of multiple environmental stressors, the impact varies significantly depending on whether the stressors were tested in isolation or in combination. In a heterogeneous and naturally variable world, environmental stressors seldom occur in isolation, therefore investigating a greater array of environmental stressors, and more importantly a greater number of stressors in combination, is crucial to further our understanding of the impact of environmental stressors on the immune response.

Whilst many of the studies cited in this review successfully employ a multi-assay approach to assess stressor induced immune dysfunction, crucial if we are to fully understand the impact of environmental stress on the invertebrate immune response, many do not investigate how organism disease resistance and subsequent survival are impacted. Measuring a number of cellular and humoral immune parameters does demonstrate how environmental stressors impact the invertebrate immune system, yet understanding how these changes affect the disease resistance and survival of the host organisms will enable the implications of any immune dysfunction to be understood at an ecological scale. The cost of maintaining the immune system may render the trade-off of immune defence, to ensure physiological homeostasis, advantageous in the absence of any realized pathogenic threat. However, it is equally likely that the host

organism may maintain the ability to up-regulate the immune response when encountering a realized pathogenic threat. Therefore to fully understand how changes in an organism's immune response will impact host-pathogen interactions, to understand how these changes will impact host organism survival and finally to predict any possible ecological and evolutionary implications of any change in survival induced by environmental stressors, immunocompetence should be measured functionally (Viney et al. 2005), measuring direct host-pathogen interactions and the survival of a host in the presence of a pathogen.

Alongside measuring host-pathogen interactions, there is also a need to investigate the experimental infection of marine invertebrates with a pathogen. The current lack of understanding surrounding invertebrate infection models is one of the major limitations in our ability to fully comprehend host-pathogen interactions. It is also a significant factor in contributing to the dearth of studies that have investigated the relationship between environmental stressors and host-pathogen interactions to date, probably the most important aspect of invertebrate immunology. Therefore improving the current understanding of infection models would enable a better prediction of how environmental change will impact host survival. Producing experimental results that are representative of a change in immunocompetence noted with a natural infection would then enable a better prediction of how the population dynamics of a particular species would change when faced with a pathogenic challenge.

Being able to demonstrate the possible impact of environmental stressors, from the level of gene expression to a population or an ecosystem level, will further the current understanding of the impact environmental stressors have on ecosystem dynamics and how these effects may drive evolution. Therefore, through the development and application of novel genomic and proteomic techniques, and

subsequently applying these alongside traditional cellular, humoral and disease susceptibility methodologies, invertebrate ecological immunology is uniquely placed in its ability to investigate the impact of anthropogenically induced environmental stressors. However, the number of studies that have employed these novel genetic techniques alongside traditional cellular and humoral techniques, as well as measuring disease resistance, to date remains very low. Therefore, to maximise the ability of ecological immunity to predict the ecological and evolutionary significance of environmental stressors, more studies need to employ traditional immunological methodologies alongside novel genetic and disease resistance techniques. Furthermore, in demonstrating differential expression of separate defence related genes during ontogenesis, Tirapé et al. (2007) demonstrate the importance of studying stressor impacts at different life cycle stages, with it being crucial to understand immune function from fertilization to reproduction and beyond, incorporating the entire life history of an organism.

With a fully integrated, multi-assay, experimental design incorporating cellular, humoral, molecular and organism disease resistance techniques, marine invertebrate immunology may be able to demonstrate the full impact of an anthropogenically altered climate on this physiological function. Understanding how a variable environment impacts upon an organism's physiological functioning is vital if we are to predict the possible ecosystem patterns, and understand possible evolutionary implications, any anthropogenically induced environmental change may generate.

1.8 AIMS AND OBJECTIVES

The aims of this thesis are to investigate the impact of environmental stressors on the

invertebrate immune response, providing empirical data on how anthropogenically-induced stressors will impact the invertebrate immune system and how this will impact organism condition and subsequent physiological trade-offs. By furthering the current understanding in the fields of invertebrate immunology, invertebrate physiology and climate change research, this thesis will then provide data to inform policy makers and environmental managers about the impact of anthropogenic stressors on the marine environment and the need to mitigate increasing levels of atmospheric carbon dioxide.

- Chapter 2 outlines the anthropogenic stressors used throughout this thesis to test the impact of environmental stress on the invertebrate immune response. In outlining the impact of increasing seawater temperature and reducing seawater pH on the marine environment, this chapter then highlights the marine organisms that are at particular risk to changing environmental conditions, before introducing *Mytilus edulis* as the model species which was chosen to study the invertebrate immune response. The Biology of this species is outlined and the current understanding of the impact of climate change stressors on mussel physiology discussed.

- Chapter 3 details an experiment used to investigate the impact of ocean acidification, temperature and a bacterial challenge on the immune response of adult *Mytilus edulis*. In studying cellular and humoral aspects of mussel immunity this experiment provides information on the impact of climate change on overall immune system functionality and how any immune dysfunction affects organism disease resistance and survival of a pathogenic challenge.

- Chapter 4 describes an experiment used to study the impact of environmental and pathogenic stressors on the physiological condition of adult mussels. By measuring the total lipid stores and fatty acid composition in mussel mantle tissue, alongside the investment in reproduction in response to ocean acidification, temperature and an

exposure to *Vibrio tubiashii*, this study investigated the impact of environmental stress on *Mytilus edulis* energy reserves and how this may impact potential physiological trade-offs used to manage stress.

- Chapter 5 outlines an experiment that used metabolomics to investigate the impact of ocean acidification, temperature and a bacterial challenge on the metabolic status in mussels. By investigating the impact of environmental stress on the metabolism of mussels, this study highlights the physiological impact of different environmental and pathogenic stressors at a cellular level. Thus providing a snapshot of the overall physiological conditioning of immunologically challenged organisms maintained under varying levels of environmental stress.

- Chapter 6 brings together all the previous experiments to discuss the impact of climate change stressors on overall mussel physiology, how future climate change scenarios may impact mussel fitness and ultimately what this means for mussel survival at a population, a community or an ecosystem level.

CHAPTER 2. EXPERIMENTAL RATIONALE

Anthropogenic climate change and the blue mussel (*Mytilus edulis*) – A model system to study the impact of environmental stress on the invertebrate immune system.

2.1. INTRODUCTION

The coastal marine ecosystem is one of the most ecologically and socio-economically diverse systems on the planet (Harley et al., 2006), providing US$ 14 trillion in goods and services per year (Costanza et al., 1997). However in exploiting these resources; through activities such as overfishing, coastal urbanisation, pollution and the introduction of alien species, man has altered the marine environment through both direct and indirect means (Halpern et al., 2008). One of the greatest threats the marine environment faces is anthropogenic climate change (Harley et al., 2006). Increased CO_2 emissions are predicted to significantly impact marine organism health and functioning (Raven et al., 2005), which could in turn lead to a reduction in marine biodiversity (Widdicombe and Spicer, 2008). Ultimately such changes to marine ecosystems could have far reaching consequences for human health and welfare (Harley et al., 2006).

The presence of CO_2 in the atmosphere is vital for the support of life on Earth, with this greenhouse gas helping to regulate the warm temperature of the Earth's atmosphere by trapping solar radiation (Thomson, 1997; Tuckett, 2009). However, since the industrial revolution (*circa* 1750), the levels of carbon dioxide (CO_2) in the atmosphere have risen from 280 to 385 ppm (IPCC, 2007). This increase is at least 100 times faster than has occurred during previous natural events (Blackford and Gilbert, 2007), and is due to increased fossil fuel burning, increased cement production and changes in land-use, such as deforestation and agriculture (Raupach et al., 2007). Based on a range of projected anthropogenic CO_2 emissions, the Intergovernmental Panel on Climate Change (IPCC) has predicted atmospheric CO_2 concentrations will continue to rise, and could reach as much as 970 ppm by 2100 (IPCC, 2007).

Being closely linked to global temperature, alterations in atmospheric CO_2 concentration represent a significant human driver of climate change (Canadell et al., 2007). Any temperature change impacts the entire Earth's system (atmosphere, continents, cryosphere and oceans), with 84 % of the total heating of the Earth's system having gone into warming the world's oceans over the last 40 years (Barnett et al., 2005). Therefore, since the industrial revolution annual mean sea surface temperatures have increased by 0.76 °C (IPCC, 2007).

Whilst increasing temperature marks a significant shift in the Earth's climate; this increase could have been far greater, were it not for oceanic and terrestrial sinks removing CO_2 from the atmosphere (Gattuso and Hansson, 2011). Since 1800, oceanic surface waters have removed 118 Pg C, or 25 % of the carbon, generated by human activities (Sabine et al., 2004). Yet, evidence suggests that the airborne fraction of anthropogenic emissions has increased yearly over the past 50 years from about 40 % to 45% (Le Quéré et al., 2009), suggesting a reduced efficiency of these CO_2 sinks (Canadell et al., 2007). With the projected rise in anthropogenic CO_2 emissions and a reduced efficiency of CO_2 sinks, it is predicted global temperatures could increase by as much as 1.5 °C – 4.5 °C by the end of the current century (IPCC, 2007).

2.2 THE IMPACT OF INCREASING ATMOSPHERIC CO_2 ON OCEAN CHEMISTRY

A large amount of CO_2 is naturally exchanged between the atmosphere and the ocean. Prior to the industrial revolution there was a natural net influx of around 0.6 Gt C yr^{-1} from the ocean to the atmosphere (IPCC, 2007). However, increasing atmospheric CO_2 concentrations have reversed this flux, with 2 Gt C now passing from the atmosphere

into the ocean annually. Initially it was thought, due to the oceans perceived ability to buffer changes brought about by increasing CO_2 concentration (Brewer, 1978), that seawater carbonate chemistry would not be affected by this increased flux of CO_2. However, in 2003 Caldeira and Wickett (2003) highlighted that although equally large changes in atmospheric CO_2 concentrations have occurred in the Earth's geological history, the current rate of change is reducing the ocean's buffering capacity, thus making it more sensitive to alterations in seawater carbonate chemistry than initially thought.

2.2.1 Ocean acidification

Seawater is unique in that it has a well-defined composition compared to other natural waters (Dickson, 2011), therefore alterations in its carbonate chemistry brought about by the addition of carbon dioxide are relatively well understood (Fig. 2.1). CO_2 is extremely soluble and exchanges with its dissolved form in surface seawater readily, here the aqueous form firstly reacts with water to form carbonic acid (H_2CO_3), which then rapidly dissociates to produce hydrogen ions (H^+) (Orr, 2011). Most of these hydrogen ions are then neutralised by reacting with carbonate ions (CO_3^{2-}), to form bicarbonate ions (HCO_3^-), however some of the hydrogen ions remain which in turn reduces the pH of the seawater (Orr, 2011). It is this reduction in seawater pH which has resulted in this phenomenon being termed 'ocean acidification'. However, this does not mean the oceans will become acidic (below pH 7.0) anytime in the near future, merely implying that the oceans are currently becoming more acidic than they were in the past (Gattuso and Hansson, 2011).

Since 1750, ocean acidification (OA) has led to a reduction in global surface seawater pH of 0.1 units (Caldeira and Wickett, 2003), representing a 30 % increase in $[H^+]$ (Caldeira and Wickett, 2003; Gattuso and Lavigne, 2009). With increasing atmospheric CO_2 concentrations, ocean pH is projected to reduce by a further 0.3-0.5 units by 2100 (Caldeira and Wickett, 2005; Gattuso and Lavigne, 2009). Moreover,

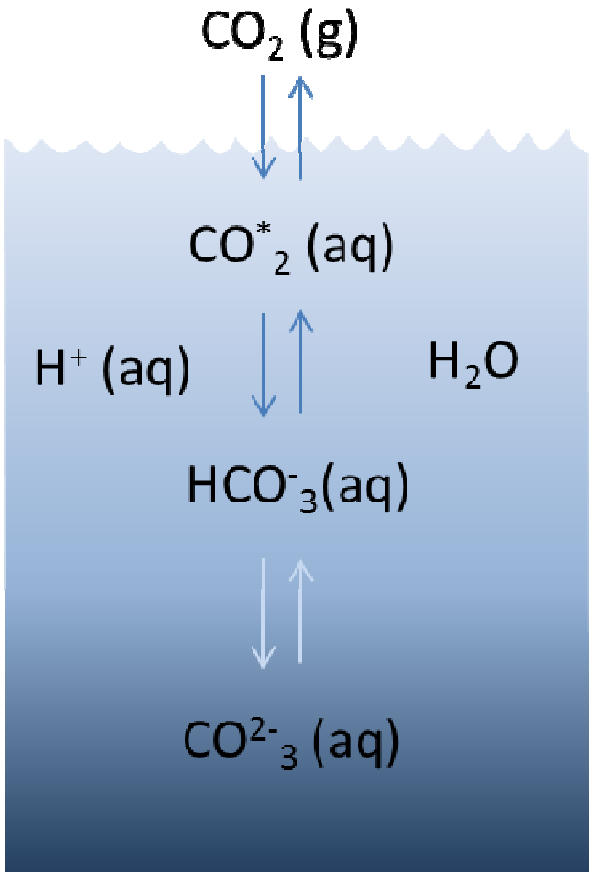

Figure 2.1 The chemical equilibrium of carbon dioxide in seawater. The notations (g) and (aq) refer to the state of the species, as a gas or in an aqueous solution respectively. CO^*_2 is the hypothetical aqueous species of carbon dioxide which ionises to form bicarbonate and carbonate ions (however CO^*_2 dominates in acid solutions (pH < 5)). Redrawn from (Dickson, 2011).

this OA signal is currently being superimposed upon natural seasonal events (Thomsen and Melzner, 2010). Having already been shown to reduce seawater pH through coastal upwelling of hypercapnic and hypoxic seawater, such seasonal events are therefore

likely to be exacerbated by the impact of OA, intermittently producing conditions that go way beyond any projected worst case acidification scenarios for surface oceans (Feely et al., 2010; IPCC, 2007; Thomsen et al., 2010).

Alongside the overall increase in $[H^+]$ and $[HCO_3^-]$, and the concurrent decrease in pH and $[CO_3^{2-}]$, the addition of CO_2 to seawater affects the saturation of the water with respect to calcium carbonate ($CaCO_3$) minerals (Orr et al., 2005). The saturation state (Ω) of $CaCO_3$ in seawater is affected by $[CO_3^{2-}]$, and therefore the addition of CO_2 results in reduced Ω. Seawater $CaCO_3$ saturation (Ω) is said to be in equilibrium when $\Omega = 1$ (Dickson, 2011). Therefore when $\Omega > 1$ seawater is said to be supersaturated with respect to that mineral and biogenic calcification is favoured, yet when $\Omega < 1$ the seawater is undersaturated and corrosive to $CaCO_3$ structures, possibly causing problems for organisms that form calcium carbonate shells or skeletons (Feely et al., 2008; Langdon and Atkinson, 2005).

Three main biogenic calcium carbonate minerals occur in seawater, being calcite, aragonite and high magnesium calcite, listed here in order of increasing solubility. Whilst the ocean is at present supersaturated with respect to calcium carbonate (Orr et al., 2005), an increasing partial pressure of CO_2 (pCO_2) in seawater will lead to undersaturation. The Arctic Ocean is projected to become undersaturated with respect to aragonite within the next 20 years (Steinacher et al., 2009). Furthermore, OA is already exacerbating the extent to which aragonite undersaturated seawater is naturally upwelling off the western north American coastline (Feely et al., 2010). Together with increased seawater temperatures, increasing $[H^+]$ and $[HCO_3^-]$, and a reduced pH and $[CO_3^{2-}]$, a reduction of Ω could pose a significant threat to the health of marine organisms and thus impact marine biodiversity (Widdicombe and Spicer, 2008).

2.2.2. Carbon capture and storage

With anthropogenic CO_2 emissions projected to have a catastrophic impact on the global climate, there is significant international pressure to reduce future emissions and mitigate any further human impact on the environment. However, given the continued development of emerging economies globally, and given our continued reliance on fossil fuels, the prospect of a rapid reduction or a complete end to CO_2 emissions in the near future is highly unlikely (Ohsumi, 2004). Therefore, whilst efforts are being made to exploit alternative energy sources, additional technologies are also being developed to reduce atmospheric CO_2 concentrations (Berge et al., 2006).

The principle method proposed to reduce atmospheric CO_2 is through carbon capture and storage, or "CCS" (Holloway, 2005). CCS is a technique that captures CO_2 emission from large point sources, such as power stations, and then injects this captured CO_2 into large underground saltwater aquifers, reducing the effect of this green house gas on global ecosystems (Berge et al., 2006). CCS is currently being investigated at number of sites globally (e.g. Goldberg et al., 2008), such as the Sleipner West gas field in Norway, where CO_2 is already being stored in large sub-seabed reservoirs (Holloway, 2005). A technique such as CCS would therefore seem a practical bridging technology to reduce CO_2 emissions during the global transition from fossil fuel burning to low carbon energies (Holloway, 2005). However, such a technology does not come without risk, and there is a significant threat of storage leaks over time (Hawkins, 2004). The Earth's geological system is extremely variable (Holloway, 2005) and any shift in seabed geology would potentially lead to a leak of stored CO_2 into the overlaying water column. The impact of such leaks would depend on the duration and spatial extent of any release, but it has been suggested that such an event could lead to a reduction in

seawater pH of 1.0 unit in the body of water immediately adjacent to the leak (Blackford et al., 2009). Any such leak event would then also be superimposed upon any OA scenario, producing extremely low pH conditions that could have a significant impact for the marine organisms residing within close proximity to a storage site habitat.

2.3. THE IMPACT OF ANTHROPOGENIC CLIMATE CHANGE ON MARINE ORGANISMS

Whilst the impact increasing atmospheric CO_2 concentrations will have on seawater temperature and carbonate chemistry are well understood, and whilst the magnitude of these changes are not widely debated within the scientific community (Orr, 2011), the impact anthropogenic climate change (namely increasing sea temperature and OA) will have on marine organisms is unclear. It is therefore vital for the scientific community to address the current dearth of empirical data and investigate the impact of increasing seawater temperatures, the impact of OA and the ecological impact of any possible mitigation technology, such as CCS, on marine organisms and on subsequent ecosystem function.

2.3.1 The impact of temperature on marine organisms

Environmental temperature plays an important role in determining body temperature in ectothermic organisms and thus directly impacts all physiological processes (Pörtner et al., 2006; Young et al., 2011). Metabolic rate, for example, generally increases with increasing environmental temperature (Ede and Krogh, 1914; Pörtner et al., 2006).

Furthermore, the rate of chemical and enzyme reactions, the fluidity of membranes, the structure of proteins and the rate of diffusion are all affected by temperature (Rayssac et al., 2010). Most biological processes that are temperature-sensitive are only able to operate optimally within a narrow thermal window, outside of which trait performance is seen to decline significantly (Angilletta, 2009; Kearney and Porter, 2009; Young et al., 2011). Such a temperature deviation will ultimately result in an organism re-allocating resources that would otherwise be used for growth or reproduction to maximise fitness (Pörtner et al., 2001; Sibly and Calow, 1986). Temperature is therefore a major determinant of the large scale geographical distribution of marine species (Jones et al., 2009; Pörtner, 2008), and the sensitivity of a species to changes in environmental temperature will thus largely be governed by how close an organism is to its thermal limits (both mean habitat temperature and extreme habitat temperatures) (Bosonovic et al., 2011; Pörtner et al., 2006).

With temperature shaping species' geographical distributions, global warming has already been shown to affect the geographical distribution of aquatic and terrestrial organisms (Bosonovic et al., 2011; Hoegh-Guldberg, 2005; Parmesan and Yohe, 2003; Perry et al., 2005). This trend for species movement from lower to higher latitudes under warming scenarios could result in local extinctions and major shifts in ecosystem functioning (Rayssac et al., 2010; Thomas et al., 2004). To predict the ecological consequence of global warming, a better understanding of thermal tolerance driven biogeographic patterns is therefore needed (Bosonovic et al., 2011; Jones et al., 2009). For example, Sagarin et al. (1999) compared the recent species distribution of intertidal communities along the Californian coastline with a historical dataset from the 1930's, and found that a general increase in habitat temperature of 0.79 °C was accompanied by a significant northward shift in species distribution. The successful shift of an organism

to a more favourable habitat will depend on the mobility of the species, its mode of life and its reproductive status, as well as on the speed at which habitats are being altered by climate change (Pörtner, 2008).

As well as impacting organism physiology directly, temperature is also shown to interact with a number of other biotic and abiotic factors to impact organism performance including: salinity (e.g. Strasser et al., 2008), oxygen (e.g. Niklitschek and Secor, 2009), ultraviolet radiation (e.g. Przeslawski et al., 2005), food availability and trophic interactions (e.g. Morelissen and Harley, 2007), and pollution and pathogen exposure (e.g. Parry and Pipe, 2004). Furthermore, in many of these studies temperature is shown to increase the sensitivity of the organism to the additional stressors even when it is not shown affect organism performance directly. Such interactions are therefore likely to determine the impact of climate change stressors on ecosystem functioning, and the physiological principles that dictate performance may be far more intertwined with climate dependant ecological patterns than traditionally thought (Pörtner, 2008; Pörtner and Farrell, 2008).

2.3.2 The impact of OA on marine organisms

Like temperature, OA has been shown to significantly impact the physiological functioning and survival of marine organisms. Reduced seawater pH has been shown to impact calcification (e.g. Gazeau et al., 2007; Kurihara and Shirayama, 2004; Orr et al., 2005; Wood et al., 2010), photosynthesis (e.g. Langdon and Atkinson, 2005; Schneider and Erez, 2006), acid-base balance (e.g. Miles et al., 2007; Spicer et al., 2007), metabolism (e.g. Michaelidis et al., 2005b; Small et al., 2010; Thomsen and Melzner, 2010), growth (e.g. Berge et al., 2006; Michaelidis et al., 2005b; Thomsen et al., 2010),

organism health (e.g. Beesley et al., 2008), immune response (e.g. Bibby et al., 2008; Hernroth et al., 2011) and behaviour (e.g. Bibby et al., 2007; Nilsson et al., 2012). Furthermore OA is also shown to affect different life cycle stages in a number of marine organisms; impacting embryonic development (e.g. Egilsdottir et al., 2009; Ellis et al., 2009), larval development (e.g. Arnold et al., 2009; Dupont et al., 2010a; Munday et al., 2010) and reproduction (e.g. Havenhand et al., 2008).

However, whilst there is a growing body of literature investigating the impact of OA on marine organisms, increasing from an average of 9 peer-reviewed papers published per year between 1989 and 2003, to 213 articles in 2010 (a 43-fold increase) (Gattuso and Hansson, 2011), there is often a lack of any "significant mean effect" reported, with the prevalence of apparently contradictory results in the literature growing (e.g. Iglesias-Rodriguez et al., 2008; Riebesell et al., 2000). Much of this uncertainty may stem from the fact that reduced seawater pH affects a variety of organismal processes (Kroeker et al., 2010), with each of these processes likely interacting and competing for energetic resources at the level of the whole organism (Wood et al., 2008). The high maintenance costs experienced under the stressful conditions associated with OA indicate that the impact of climate change on marine organisms may be far more complex than initially anticipated (Hendriks et al., 2010; Kroeker et al., 2010). Such complexity makes it difficult to predict the response of even a single organism to these environmental changes, with the prediction of ecosystem level responses harder still.

2.3.3. Combined impact of climate change stressors

The combined study of OA and temperature has received very little attention with most

OA studies to date focusing on a single stressor model, despite the fact that changes to these two environmental stressors are predicted to occur in concert with each other, alongside other biotic and abiotic stressors (Kroeker et al., 2010). From the handful of studies that have investigated temperature and OA in combination (Anthony et al., 2008; Byrne et al., 2009; Martin and Gattuso, 2009; Munday et al., 2009; Reynaud et al., 2003; Rodolfo-Metalpha et al., 2010), it is clear that at present we are unable to make any generalizations surrounding the overall ecological impact of these climate change stressors, with temperature having been shown to both increase and decrease the sensitivity of organisms to OA (Kroeker et al., 2010). Anthony et al. (2008) showed pCO_2 to act synergistically with temperature to lower the thermal bleaching threshold in coral species, with high temperature increasing bleaching by up to 50 % at high pCO_2 compared to high pCO_2 and low temperature. Similarly, Reynaud et al. (2003) showed that calcification remained unaffected by reduced seawater pH at control temperatures, however when additionally exposed to a concomitant increase in temperature calcification was seen to decrease by 50 % compared to ambient temperature and control pCO_2 in exposed corals. Conversely, Gooding et al. (2009) exposed the sea star, *Piaster ochraceus* to elevated pCO_2 and increased temperature, demonstrating that increasing both temperature and pCO_2 enhanced growth in this keystone echinoderm. Whilst likewise Connell and Russell (2009) demonstrated that temperature and pCO_2 acted synergistically, leading to a phase shift in algal communities from a kelp dominated ecosystem to an algal turf dominated system under a future climate compared to current environmental conditions, with turf communities doubling in biomass and covering 4 times as much available space (Connell and Russell, 2009). In finding such a confounding impact of temperature on the sensitivity of marine organisms to OA, these studies may go some way to explaining the variability reported

in much of the literature concerning calcification rates and increasing pCO_2.

The importance of studying the combined impact of temperature and CO_2 is also highlighted by a number of recent discussions, where these two stressors are proposed to have been influential during mass extinction events in the Earth's history; including the end-Permian mass extinction where marine ecosystems experienced a species loss estimated to be as high as 90% (Knoll et al., 1996, 2007; Knoll and Fischer, 2011). With temperature, alongside atmospheric and aquatic CO_2, appearing to have been influential in the course of the Earth's evolutionary history (Pörtner, 2008), understanding the impact of these stressors in combination will be vital to accurately predict the likely impact of climate change on ecosystem function and biodiversity.

2.4 MUSSELS AS A MODEL SYSTEM TO STUDY THE IMPACT OF CLIMATE CHANGE

Whilst a comprehensive review of the response of all marine organisms to a suite of environmental stressors, including OA and temperature, would offer the most accurate understanding of future ecosystem change, it is not possible to take this approach in the real world. Therefore selecting keystone species, or model organisms, to offer the most valuable scientific insight is crucial. In a recent meta-analysis investigating the vulnerability of marine organisms to OA, one group of organisms shown to be particularly vulnerable to changes in ambient pH were the bivalves (Hendriks et al., 2010). The class Bivalvia, comprises of groups such as mussels, oysters, scallops and clams, and numbers around 7,500 species (Gosling, 2003). As dominant members of coastal and estuarine communities, bivalves form an important component of intertidal

marine fauna, having an extensive geographical distribution and being of increasing importance economically (Widdows and Donkin, 1992).

The family Mytilidae, to which mussels of the genus *Mytilus* belong, is a dominant component of rocky shore communities in cooler waters of northern and southern hemispheres (Gosling, 1992; Seed and Suchanek, 1992). Of the genus *Mytilus*, the blue mussel, *Mytilus edulis* is the species with the widest recorded distribution, extending from the Arctic to mild sub-tropical regions (Gosling, 2003). It occurs from the White Sea to southern France in the north east Atlantic, from the Canadian Maritimes to North Carolina in the west Atlantic, along the coasts of Chile, Argentina, the Falkland Islands and along the west coast of North America (Beesley et al., 2008; Berge et al., 2006). Furthermore, in each of these regions it extends from the high intertidal to the shallow sublittoral, from sheltered to extremely wave-exposed shores and from fully marine to estuarine conditions (Gosling, 2003). Like many intertidal organisms the upper range limit of intertidal mussel populations is determined by a physiological intolerance of temperature extremes and desiccation, as well as a reduction in the time available for feeding, whereas its lower limits are governed by biological factors such as competition and predation (Seed and Suchanek, 1992).

Mussels are sedentary filter feeders, settling on a variety of hard or semi-consolidated substrates that are firm enough to provide a strong anchorage (Gosling, 2003; Seed and Suchanek, 1992). They attach themselves to other objects using byssus thread (Gosling, 2003) and aggregate to form large beds which are considered to be one of the most diverse temperate systems (Fig. 2.2) (Suchanek, 1994). In providing a structurally complex microhabitat, mussel beds can support up to 300 species at any one location, and up to 750 species regionally (Kanter, 1980; Suchanek, 1979), providing associated species with a large surface area for settlement, as well as a refuge from

Figure 2.2 *Mytilus edulis* growing on a wild mussel bed which creates a complex microhabitat for associated fauna, Exmouth, Exe Estuary, Devon, UK. Scale bar = 100 mm. (Picture taken by R. Ellis).

harsh environmental conditions and predation (Gutiérrez et al., 2003; Smith et al., 2006). By maintaining biodiversity and sediment stability in coastal and estuarine habitats, mussels carry out a vital role in temperate marine and estuarine ecosystems worldwide, making them a key ecosystem engineer (Beesley et al., 2008).

As well as their importance ecologically, mussels are also of huge importance economically. Bivalve culture dates back over 2,000 years, with Aristotle mentioning the cultivation of oysters in Greece in 350 BC (Gosling, 2003), and their importance to the aquaculture sector continues to steadily grow (Gestal et al., 2008). The worldwide harvest of mussels exceeded 1.7 million tonnes in 2008, with an estimated value of US $ 1.6 billion, of which approximately 95 % was from aquaculture (FAO, 2010).

However, an increasing distribution and intensity of bivalve culture worldwide has also led to an increase in the prevalence and severity of disease outbreaks, causing mass mortality events which significantly impact bivalve fisheries and natural bivalve populations alike (Elston et al., 2008; Gestal et al., 2008).

Due to their high abundance, cosmopolitan spread and filter feeding lifestyle, mussels have become established as one of the most widely used indicator species for environmental monitoring (Dondero et al., 2006; Rittschof and McClellan-Green, 2005). Previous studies investigating the impact of OA on mussels have shown a reduction in seawater pH is shown to have a significant and often negative impact on these bivalves. Michaelidis et al. (2005b) demonstrated that adult mussels reduced their metabolic rate and increased protein degradation when exposed to reduced seawater pH. In contrast Thomsen and Melzner (2010) demonstrated that during a long-term exposure to increased pCO_2 mussels did not undergo any global metabolic depression, however these authors did measure an increased protein metabolism, reduced shell length and reduced shell mass. Furthermore OA has also been shown to reduce growth (Berge et al., 2006), calcification (Gazeau et al., 2007), immune function (Bibby et al., 2008) and organism health (Beesley et al., 2008) in *Mytilus* species.

Early life cycle stages are generally considered to be more vulnerable than adults to environmental disturbance (Raven et al., 2005). Gazeau et al. (2010) demonstrated that mussel larvae developed under increasing pCO_2 had smaller shells at hatching, with these shells also being thinner. The population also demonstrated a reduced hatching rate under future OA conditions (Gazeau et al., 2010). Such changes at early life stages could have considerable knock on effects for overall population dynamics, and thus ecosystem function.

To date very little is known about what impact a concurrent exposure to temperature and OA will have on marine mussels, with only one study having measured mussel bed community dynamics in response to climate change. Carried out by Smith et al. (2006), this study measured a large decline in species diversity on Californian mussel beds between the 1960's/1970's and 2002, with 58.9 % of this mean diversity loss measured being attributed to climate change (Smith et al., 2006). Considering this result and the understanding of projected climatic change, future increases in global temperatures and reductions in seawater pH could potentially have a catastrophic effect on community biodiversity and ecosystem function.

Negative impacts of climate change stressors on mussels would not only impact coastal biodiversity and ecosystem functioning, but additionally would result in significant economic loss (Cooley and Doney, 2009; Gazeau et al., 2007). The importance of mussels, both economically and ecologically, thus highlights a need to investigate the impact of climate change stressors on these ecosystem engineers. In elucidating the interactive impact of climate changes stressors on disease prevalence and disease resistance in marine mussels and by understanding the ecological processes that control populations, communities and ecosystems, we will be better able to project which stressors will cause serious ecosystem alterations, in turn helping us avoid or alleviate these impacts (Suchanek, 1994).

CHAPTER 3. MUSSEL IMMUNE RESPONSE

Effect of CO_2-induced seawater acidification, increased temperature and a bacterial challenge on the immune response of the blue mussel, *Mytilus edulis*.

3.1 INTRODUCTION

Anthropogenic activities are fundamentally altering the chemistry of the world's oceans, through an excess input of nutrients, pollution, increased temperature and altered carbonate chemistry (Doney, 2010). As outlined in Chapter 1, these perturbations are in turn significantly impacting marine organism immune function. However, despite being proposed as one of the greatest threats that marine ecosystems face (Harley et al., 2006), to date very little is known about the impact of anthropogenic climate change, and ocean acidification (OA) in particular, on the immune response of marine organisms.

In demonstrating a reduced phagocytic activity at reduced seawater pH in the blue mussel, Bibby et al. (2008) highlight the potential impact of altered seawater carbonate chemistry on invertebrate immune function. However, the study by Bibby et al. (2008) is amongst only a handful of studies to date to have investigated the impact of OA on the invertebrate immune response. Furthermore, each of these previous studies (Bibby et al., 2008; Hernroth et al., 2011; Matozzo et al., 2012) were carried out in the absence of any pathogenic insult, and thus were not designed to quantify the potential impact of any immune suppression on organism fitness. The immune system protects an organism from infectious disease in order to maximise fitness (Viney et al., 2005). It is possible that when exposed to stressful environmental conditions, and in the absence of any pathogenic threat, an organism may reduce the energy it allocates to immune system maintenance, instead reallocating these resources to other physiological functions in an attempt to optimise fitness (Lochmiller and Deerenberg, 2000; Sheldon and Verhulst, 1996). However, whilst an organism may trade-off the cost of immune system maintenance, it is possible that it may maintain an ability to up-regulate its immune response when required. Immunocompetence should therefore be measured and

defined functionally, in the presence of a pathogen, to accurately assess any possible immune suppression (Morley, 2010; Viney et al., 2005).

Therefore the aim of this chapter is to, for the first time, assess the impact of climate change stressors on invertebrate immune system functionality in the presence of a pathogen. By investigating the impact of reduced seawater pH and increased seawater temperature on the total and differential cell counts, as well as the antibacterial activity of cell-free haemolymph, in the blue mussel, *Mytilus edulis*, this study will elucidate the impact of anthropogenic climate change on the maintenance of cellular and humoral host defence. Furthermore, in subsequently exposing mussels to a bacterium shown to be pathogenic to marine bivalves, namely *Vibrio tubiashii*, (Elston et al., 2008), this study will then be able to demonstrate the impact of any possible immune suppression on organism disease resistance.

3.2 MATERIALS AND METHODS

3.2.1 Study organisms and experimental setup

Adult *Mytilus edulis* (50 to 70 mm shell length) were collected by hand from an intertidal estuarine mussel bed, Exmouth, Devon, UK (50º 37.09'N, 03º 25.42'W) on 17th Dec 2009. This site was chosen as the mussel population at Exmouth are shown to comprise of pure *M. edulis,* despite previous research having demonstrated a complex speciation in the genus *Mytilus* across south-west England (Gilg and Hilbish, 2003; Hilbish et al., 2002). Following collection mussels were transported to a mesocosm at Plymouth Marine Laboratory (PML) within 2 h of harvest. Upon arrival mussels were

cleared of all epibionts using a fixed blade scalpel. Once cleaned, 4 individuals were placed into each of the 60 experimental chambers described below.

Experimental chambers (vol. = 250 ml) were evenly distributed between 10 re-circulating water baths (vol. = 75 l; 125 cm x 60 cm x 10 cm). Each chamber was haphazardly assigned to one of ten experimental treatments, consisting of five pH levels crossed with two different temperatures. Each treatment combination contained 6 replicate chambers. The nominal pH values used in this experiment were pH 8.05 (present day ambient seawater pH), pH 7.80 (reduced seawater pH predicted to occur by 2100; IS92 emissions scenario; IPCC, 2007), pH 7.60 (reduced seawater pH predicted to occur by 2100; A2 scenario; Caldeira and Wickett, 2005), pH 7.35 (reduced seawater pH predicted to occur by 2300; IS92 emissions scenario; IPCC, 2007) and pH 6.50 (low pH conditions expected from a Carbon Capture Storage CO2 leak under already acidified conditions; Blackford et al., 2009). The mesocosm was maintained at 12.5 ± 1 °C (surface seawater temperature recorded by the western channel observatory at station L4, 1st Dec 2009), with mussels in the elevated temperature treatment being exposed to a temperature of 17.0 ± 0.5°C (representing a temperature increase predicted to occur by 2100; IPCC, 2007; Sokolov et al., 2009). The increased temperature was achieved by placing heaters in the five water baths which had been haphazardly assigned to the elevated temperature treatment.

Each water bath contained six experimental chambers that were continuously supplied with seawater from one of ten header tanks (vol. = 450 l), *via* a peristaltic pump (13 ± 0.5 ml min^{-1}; Watson Marlow 2058). Overflow water from experimental chambers was allowed to run off, creating a flow-through experimental system that prevented the build up of any metabolic waste products (Fig. 3.1). Header tank pH was adjusted through the bubbling of CO_2 gas (Fig 3.2), using the system described by

Widdicombe and Needham (2007). The pH in header tanks was monitored using combination pH electrodes (Walchem S650CD), calibrated using NIST standardised buffers and connected to a computerised feedback system (Walchem Webmaster-GI controller USA), which regulated the addition of CO_2. During the experiment mussels

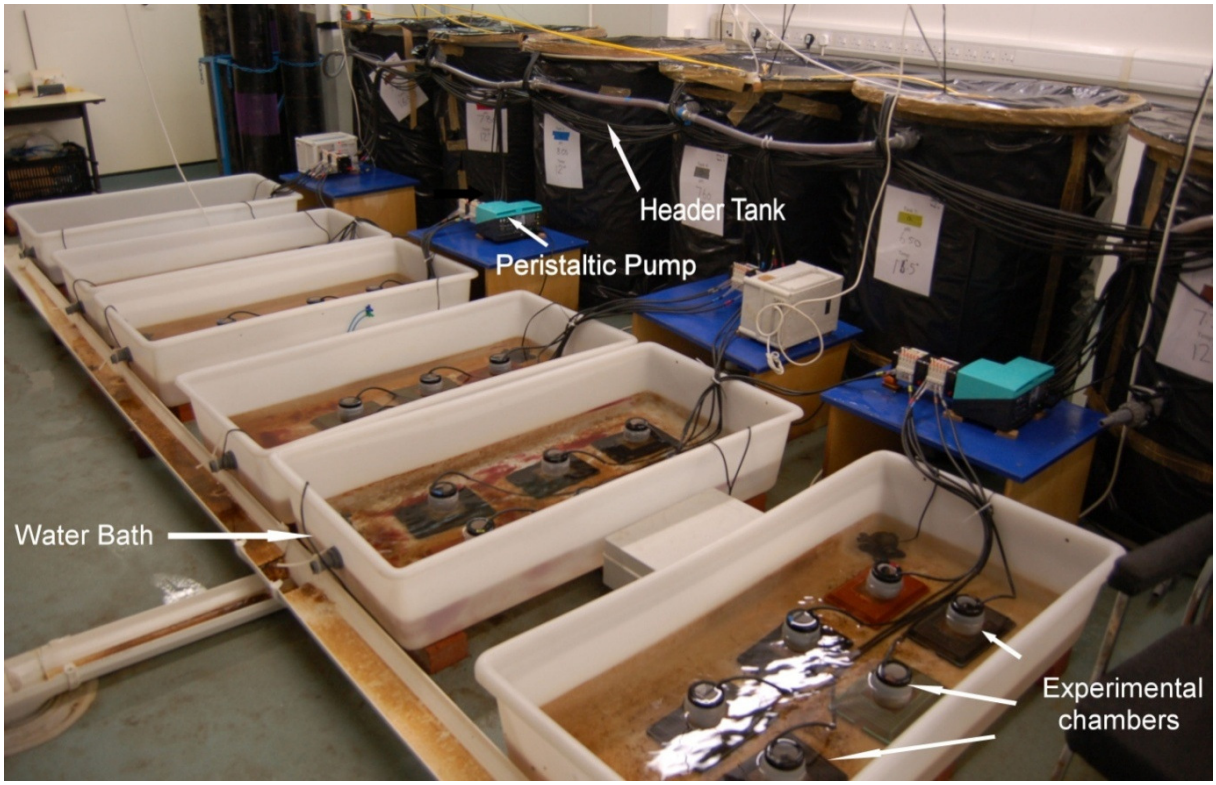

Figure 3.1 Experimental setup showing header tanks supplying seawater to experimental chambers *via* a peristaltic pump. Experimental chambers are housed in water baths that flow to waste, creating flow-through system. Photo taken by R.Ellis.

were fed with *Isochrysis galbana* (30 mg dry mass mussel^{-1} day^{-1}; Isochrysis 1800: Reed Mariculture Inc.), suspended in seawater and added to each header tank daily. Using daylight simulation lights, the mesocosm was subject to a 9 h light: 15 h dark regime. This closely replicated the natural day light hours for the collection site, measured at the time of mussel collection.

The pH, temperature and salinity were monitored 3 times a week in header tanks and experimental chambers. The pH was measured using a pH meter (Mettler Toledo InLab 413 SG) calibrated with NIST standardized buffers. Temperature (°C) and salinity were measured using a combined temperature and salinity probe (Tetra con 325). Total alkalinity (A_T) was measured in header tanks and experimental chambers

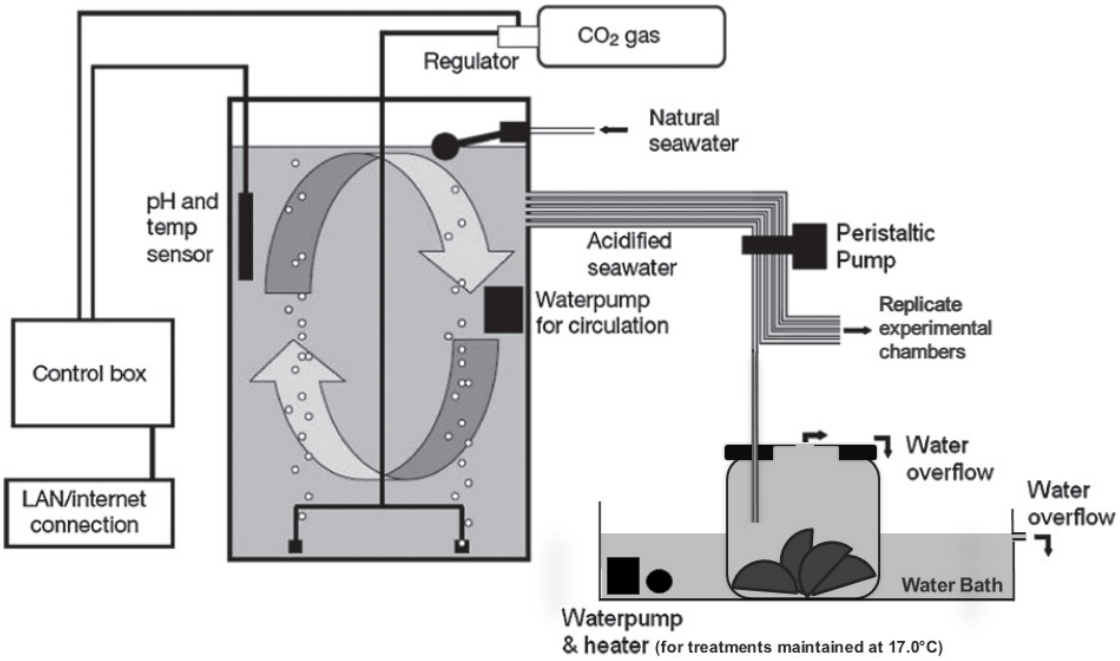

Figure 3.2 Schematic diagram of the experimental setup used during the mesocosm experiment (adapted from Hale et al., 2011).

at the beginning of the experiment and then every 7 days until completion. To measure A_T a 125 ml sample of seawater was taken from each experimental chamber and header tank, poisoned with a saturated solution of mercuric chloride (50 µl equating to 0.04 % of final volume) and stored in amber glass bottles (125 ml) until later analysis. Stored alkalinity samples were subsequently analyzed using an open-cell potentiometric titration technique (Apollo Sci Tech model AS-ALK2). Carbonate system variables

(pCO_2, TCO_2, $\Omega_{calcite}$, $\Omega_{aragonite}$, HCO_3^-, and CO_3^{2-}) were calculated from the measured pH (NBS scale) and A_T values using the CO2sys program (Pierrot et al., 2006), using the constants from Mehrbach et al. (1973), refitted by Dickson and Millero (1987), and using the KSO_4 dissociation constants from Dickson (1990).

3.2.2 Sampling protocol and bacterial exposure

Mussels were maintained in the experimental setup, as described above (Sect. 3.2.1), for a total of 98 days. On day 90, one mussel was sampled from each experimental chamber to measure the immune response (as described in Sect. 3.2.3). Following this initial sampling the remaining mussels were exposed to a bacterial challenge. *Vibrio tubashii* NCIMB 1337 (ATCC19106) was chosen as it is pathogenic to bivalves and due to its recent re-emergence having been linked to a reduction in hatchery bivalve populations (Elston et al., 2008). *V. tubiashii* was grown under thermo-stable conditions (24 °) in marine broth (sterile marine saline + 1 g l^{-1} yeast extract and 0.5 g l^{-1} tryptone). Log phase broth culture was harvested into a sterile centrifuge tube (vol = 15 ml; Sarstedt®), centrifuged (10 min, 2000 x rpm; 15 °C; Centrifuge 5810R, Eppendorf) and re-suspended in sterile marine saline. Bacteria were rinsed a further two times before being re-suspended in fresh marine saline at a concentration of ca. 2 x 10^8 ml^{-1} (OD_{600nm} ca. 2). Bacteria were then further diluted by two serial tenfold dilutions in marine saline to give a final working concentration of 2 x 10^6 ml^{-1} (Parry and Pipe, 2004). On day 91 these mussels were removed from the flow-through system and injected with 1.0 ml of a live bacterial suspension (*Vibrio tubiashii* at 2 x 10^6 bacterial cells ml^{-1}) directly into the posterior adductor muscle, using a 1.0 ml syringe fitted with a 21g needle. Mussels were then left immersed at their respective experimental temperatures (either 12.5 °C or 17.0

°C) for 2 h, held shut with an elastic band, after which time the elastic band was removed and they were returned to the experimental system. The mussel immune response was then measured again in one randomly chosen individual from each experimental chamber on day 92 (1 day post inoculation) and day 98 (7 days post inoculation).

3.2.3 Immunological assays

Haemolymph (0.5 ml) was extracted from the large sinus within the posterior adductor muscle, using a 1.0 ml syringe fitted with a 21g needle. Haemolymph was transferred to a siliconised microcentrifuge tube (vol. = 1.6 ml, Eppendorf®) stored on ice, to minimise cell aggregation.

Total and differential cell counts:

An aliquot of haemolymph (vol. = 150 µl) was added to an equal volume of Baker's formal calcium (10 % formalin, 1 % calcium chloride and 2.5 % sodium chloride). The total number of fixed haemocytes per ml of haemolymph was then quantified using an improved Neubauer haemocytometer on a Leitz (Leitz Wetzlar) compound microscope. Differential haemocyte counts were prepared using a cytocentrifuge (Shandon, UK); 100 µl of fixed haemolymph was spun (1,000 rpm) onto glass microscope slides. Cells were then post fixed in methanol (100 %; Sigma-Aldrich) for 3 min, stained with Wrights stain (diluted 1:4 with 0.05 M Tris-buffered saline (TBS), pH 7.6) for 5 min, rinsed with deionised water, air dried and mounted in Canada Balsam. The Wrights stain enables eosinophilic (granular, dark pink/purple) and basophilic (non-granular, light blue) blood cells to be differentiated (Fig 3.3) (Pipe, 1990a). Relative numbers of

eosinophils and basophils were calculated by counting 200 haemocytes from each animal (Parry and Pipe, 2004).

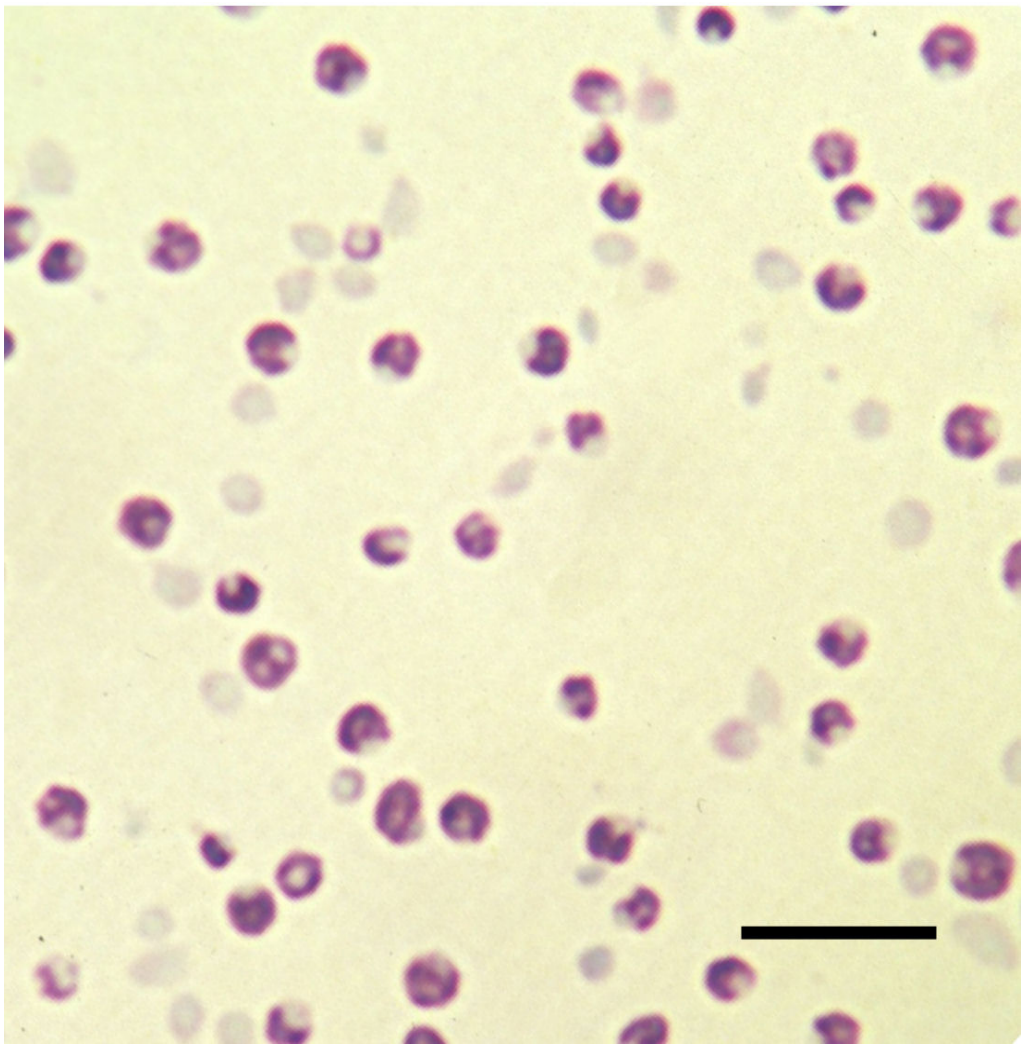

Figure 3.3 Light micrograph of *Mytilus edulis* haemocytes differentially stained with Wrights stain. Eosinophilic cells stained purple, basophilic cells stained light blue. Scale bar = 40µm.

Antibacterial activity of cell-free haemolymph:

An aliquot of haemolymph (vol. = 250 µl) was added to an equal volume of marine saline and centrifuged (2.5 min, 400 x *g*, 15 °C; Centrifuge 5810R, Eppendorf). The supernatant, cell-free haemolymph, was transferred to a microcentrifuge tube

(Eppendorf®) and stored at -20°C until analysed. To measure antibacterial activity of the cell-free haemolymph, a 100 µl aliquot of cell-free haemolymph was pipetted into 4 replicate wells of a microplate with an equal volume of *Vibrio tubiashii* suspension (2 x 10^6 bacterial cells per ml^{-1} suspended in marine saline). 50 µl of marine saline and 50 µl of marine broth were added to 100 µl of *Vibrio tubiashii* suspension in 4 replicate wells to determine bacterial growth over 22 h (bacterial controls). 150 µl of marine saline and 50 µl of marine broth was used as a blank. Plates were incubated at 20 °C and read using a microplate reader (Molecular Devices VersaMax Microplate Reader) at λ = 340 nm. Results were presented as bacterial growth inhibition after 22 h, measured as a change in optical density between haemolymph exposed samples and bacterial control growth.

3.2.4 Statistical analysis

All data were tested statistically using the PERMAONVA+ add in (beta version; Anderson et al., 2008) in PRIMER 6.1 (Clarke and Gorley, 2006). As PERMANOVA+ is sensitive to differences in multivariate dispersion between groups (Anderson, 2006), data were first tested for homogeneity of variance using PERMDISP. If data were seen to have heterogeneous variance an appropriate transformation, square root or log(x+1), was applied. Euclidean distance similarity matrices were then constructed for all data.

For carbonate chemistry parameters, P-values were calculated using an unrestricted permutation of raw data. When a statistically significant difference was shown, pair-wise comparisons between all levels of a given environmental parameter were undertaken, using PERMANOVA+. To measure the effect of reduced seawater pH, temperature and a bacterial exposure on immune system maintenance P-values were

calculated using 999 permutations of the residuals under a reduced model. Pair-wise comparisons were again undertaken where a significant P-value was encountered for a given factor with more than two levels, or where a significant interaction was demonstrated between two or more experimental factors. Furthermore, the effect of organism gender, determined using histology (as described in Sect. 4.2.1), on immune system maintenance was assessed using PERMANOVA. Again, P-values were calculated using 999 permutations of the residuals under a reduced model, and pair-wise comparisons undertaken where gender was shown to interact with another experimental factor.

To test the impact of OA, ocean warming and gender on the ability of mussels to up-regulate their immune response when encountering a bacterial challenge, the change in host defence following a pathogenic exposure was calculated. A change in the immune response was calculated as an increase or decrease in the ability of cell-free haemolymph to inhibit bacterial growth, an increase or decrease in total cell count or a change in the proportion of eosinophilic haemocytes in pathogen exposed mussels within each treatment group, compared to mussels at day 90. These data were then tested in PERMANOVA+ using the same procedure as used to test other immune system data.

3.3. RESULTS

3.3.1 Experimental conditions

The carbonate chemistry parameters within the header tanks and experimental chambers are summarised in Tables 3.1 and 3.2 respectively. The pH of seawater was significantly

lowered in all header tanks and experimental chambers assigned to the reduced seawater pH treatments, compared to the control treatment, and remained stable throughout the 98 d exposure. The slight difference recorded between the pH in header tanks and exposure chambers at pH 8.05, 7.80 and 7.60 is likely to be due to mussel respiration in experimental chambers, and bacterial respiration in the tubes supplying chambers.

Salinity did not differ significantly between treatments, whereas temperature was significantly lower in exposure chambers maintained at 12.5 °C, compared to 17.0 °C. However, there was no significant difference in the temperature of experimental chambers within treatments. Total alkalinity was not significantly different between treatment groups in header tanks; however in exposure chambers the A_T measured at pH 6.50 was significantly higher than at any other pH level, and this was the case at both 12.5 °C and 17.0 °C. Such an increase in A_T may be caused by increased dissolution of mussel shells at low pH, a phenomenon demonstrated by Melzner et al. (2011), and one which would not be unexpected due to the severe undersaturation of both calcite and aragonite at pH 6.50 (Table 3.1).

pCO_2 was shown to significantly increase with decreasing pH, as was total carbon dioxide (TCO_2) and [HCO_3^-]. Reduced pH was also shown to significantly decrease [CO_3^{2-}] and the saturation of both calcite and aragonite. Seawater was shown to become undersaturated with respect to calcite at pH 7.35 at 12.5 °C (Table 3.2a), and pH 7.60 at 17.0°C (Table 3.2b). Seawater was shown to become undersaturated with respect to aragonite at pH 7.80 at both 12.5 °C and 17.0 °C.

Table 3.1 Carbonate chemistry of seawater in header tanks for each pH exposure level.

Parameter	8.05	7.80	7.60	7.35	6.50
pH_{NBS}	8.09 ± 0.01^{a}	7.77 ± 0.01^{b}	7.60 ± 0.01^{c}	7.33 ± 0.01^{d}	6.46 ± 0.02^{e}
Temperature (°C)	13.83 ± 0.11	13.94 ± 0.10	13.85 ± 0.10	14.01 ± 0.10	13.87 ± 0.10
Salinity	34.17 ± 0.07	34.15 ± 0.07	34.17 ± 0.07	34.16 ± 0.07	34.15 ± 0.07
A_T (µmol kg^{-1} SW)	2402.32 ± 30.27	2396.70 ± 27.89	2412.67 ± 30.79	2392.42 ± 31.71	2420.65 ± 30.62
pCO_2 (µatm)†	518.02 ± 22.25^{a}	1177.85 ± 64.12^{b}	1940.40 ± 240.51^{c}	5075.37 ± 1402.88^{d}	25242.14 ± 1938.82^{e}
TCO_2 (µmol kg^{-1} SW)†	2229.22 ± 30.55^{a}	2344.67 ± 32.00^{b}	2422.27 ± 39.09^{b,c}	2558.20 ± 69.25^{c}	3396.81 ± 98.58
$\Omega_{Calcite}$†	3.201 ± 0.080^{a}	1.625 ± 0.058^{b}	1.098 ± 0.084^{c}	0.535 ± 0.091^{d}	0.091 ± 0.005^{e}
$\Omega_{Aragonite}$†	2.045 ± 0.051^{a}	1.038 ± 0.037^{b}	0.701 ± 0.053^{c}	0.342 ± 0.058^{d}	0.058 ± 0.003^{e}
HCO_3^- (µmol kg^{-1} SW)†	2075.62 ± 29.83^{a}	2230.62 ± 31.06^{b}	2300.70 ± 34.23^{b,c}	2337.81 ± 33.04^{c,d}	2411.68 ± 30.78^{d}
CO_3^{2-} (µmol kg^{-1} SW)†	133.31 ± 3.31^{a}	67.68 ± 2.34^{b}	45.70 ± 3.44^{c}	22.29 ± 3.79^{d}	3.80 ± 0.20^{e}

Data are represented as mean (± S.E.). Significant differences ($p \leq 0.05$) between treatment levels are indicated by different letters based on pair-wise tests.

†Calculated using CO2SYS software.

Table 3.2 Carbonate chemistry of seawater in experimental chambers maintained at **a)** 12.5 °C and **b)** 17.0 °C for each pH exposure level.

a)

Parameter	8.05	7.80	7.60	7.35	6.50
pH_{NBS}	8.02 ± 0.01[a]	7.68 ± 0.01[b]	7.51 ± 0.01[c]	7.30 ± 0.01[d]	6.55 ± 0.01[e]
Temperature (°C)	12.36 ± 0.09	12.27 ± 0.10	12.30 ± 0.10	12.32 ± 0.09	12.46 ± 0.10
Salinity	34.00 ± 0.06	34.01 ± 0.06	34.04 ± 0.06	34.00 ± 0.06	33.98 ± 0.06
A_T (µmol kg^{-1} SW)	2395.15 ± 20.60[a]	2394.45 ± 20.01[a]	2436.43 ± 19.58[a]	2434.78 ± 17.50[a]	2584.84 ± 44.15[b]
pCO_2 (µatm)†	787.87 ± 30.60[a]	1704.95 ± 46.67[b]	2413.11 ± 92.87[c]	3786.12 ± 174.12[d]	24751.24 ± 1109.09[e]
TCO_2 (µmol kg^{-1} SW)†	2266.87 ± 18.83[a]	2374.63 ± 18.16[b]	2459.72 ± 17.22[c]	2528.75 ± 21.03[d]	3457.39 ± 73.87[e]
$\Omega_{Calcite}$†	2.605 ± 0.079[a]	1.344 ± 0.044[b]	1.043 ± 0.057[c]	0.677 ± 0.028[d]	0.121 ± 0.004[e]
$\Omega_{Aragonite}$†	1.676 ± 0.051[a]	0.865 ± 0.029[b]	0.672 ± 0.037[c]	0.436 ± 0.018[d]	0.078 ± 0.003[e]
HCO_3^- (µmol kg^{-1} SW)†	2130.53 ± 17.57[a]	2258.08 ± 17.20[b]	2330.75 ± 16.48[c]	2366.17 ± 17.50[d]	2573.00 ± 44.04[e]
CO_3^{2-} (µmol kg^{-1} SW)†	108.33 ± 3.34[a]	55.86 ± 1.87[b]	43.41 ± 2.40[c]	28.18 ± 1.18[d]	5.04 ± 0.18[e]

b)

Parameter	8.05	7.80	7.60	7.35	6.50
pH_{NBS}	7.95 ± 0.01[a]	7.63 ± 0.01[b]	7.51 ± 0.01[c]	7.29 ± 0.01[d]	6.51 ± 0.01[e]
Temperature (°C)	17.12 ± 0.07	17.04 ± 0.07	17.11 ± 0.07	17.16 ± 0.06	17.07 ± 0.08
Salinity	34.07 ± 0.06	34.03 ± 0.06	34.06 ± 0.06	34.03 ± 0.06	34.03 ± 0.06
A_T (µmol kg^{-1} SW)	2407.38 ± 17.57[a]	2408.32 ± 18.32[a]	2424.32 ± 12.09[a]	2440.25 ± 17.63[a]	2630.13 ± 36.18[b]
pCO_2 (µatm)†	624.60 ± 21.85[a]	1535.21 ± 77.30[b]	2341.68 ± 104.76[c]	3494.13 ± 148.63[d]	21846.26 ± 1055.31[e]
TCO_2 (µmol kg^{-1} SW)†	2272.95 ± 14.85[a]	2396.34 ± 19.75[b]	2469.47 ± 12.96[c]	2549.17 ± 21.43[d]	3523.37 ± 67.34[e]
$\Omega_{Calcite}$†	2.640 ± 0.095[a]	1.242 ± 0.057[b]	0.849 ± 0.031[c]	0.587 ± 0.022[d]	0.116 ± 0.007[e]
$\Omega_{Aragonite}$†	1.682 ± 0.061[a]	0.791 ± 0.036[b]	0.541 ± 0.020[c]	0.374 ± 0.014[d]	0.074 ± 0.004[e]
HCO_3^- (µmol kg^{-1} SW)†	2137.02 ± 13.82[a]	2281.07 ± 18.20[b]	2337.58 ± 11.43[c]	2380.36 ± 17.67[d]	2618.70 ± 36.03[e]
CO_3^{2-} (µmol kg^{-1} SW)†	110.10 ± 4.04[a]	51.79 ± 2.40[b]	35.36 ± 1.30[c]	24.45 ± 0.91[d]	4.84 ± 0.28[e]

Data are represented as mean (± S.E.). Significant differences ($p \leq 0.05$) between treatment levels are indicated by different letters based on pair-wise tests. †Calculated using CO2SYS software.

3.3.2 Mortality

Mortalities were noted in all treatments during the course of the exposure; however cumulative mortality was higher at low pH. At pH 8.05 and pH 7.80 mussel survival was 93.75 % after the initial 90 d exposure, whereas at pH 7.60 survival fell to 89.58 %. Survival of mussels at pH 7.35 dropped to 81.25 % over the duration of the exposure. The most significant reduction in mussel survival was noted at pH 6.50, where survival was 33.33%. When the impact of temperature on mussel survival is considered, increased temperature appears to increase the sensitivity of mussels to low pH. At 12.5 °C mussel survival was maintained above 91 % at pH 8.05, pH 7.80, pH 7.60 and pH 7.35 (Fig 3.4a), however at pH 6.50 survival fell to 45.83%. Conversely, at 17.0 °C mussel survival was only maintained above 91 % at pH 8.05 (Fig 3.4b). At pH 7.80 and pH 7.60 survival was shown to drop to 87.50 %, whereas at pH 7.35 survival is shown to fall to 66.67%. The greatest reduction in survival however is shown at pH 6.50, in mussels maintained at 17.0 °C, where survival was shown to drop to just 20.85 % during the experimental exposure (Fig 3.4b). An inoculation with the pathogenic bacteria, *Vibrio tubiashii*, was not shown to further impact mussel mortality at any pH.

3.3.3 Antibacterial activity of cell-free haemolymph

As shown in Fig. 3.5a, an extreme reduction in seawater pH significantly reduced the antibacterial activity of cell-free haemolymph in mussels after an initial 90 day exposure (Pseudo-F = 8.68, d.f. = 4, p = 0.005). Pair-wise analyses showed that mussels maintained at pH 6.50 had a significantly lower antibacterial activity compared to mussels maintained at all other pH levels (Fig. 3.5a), with the ability of cell free

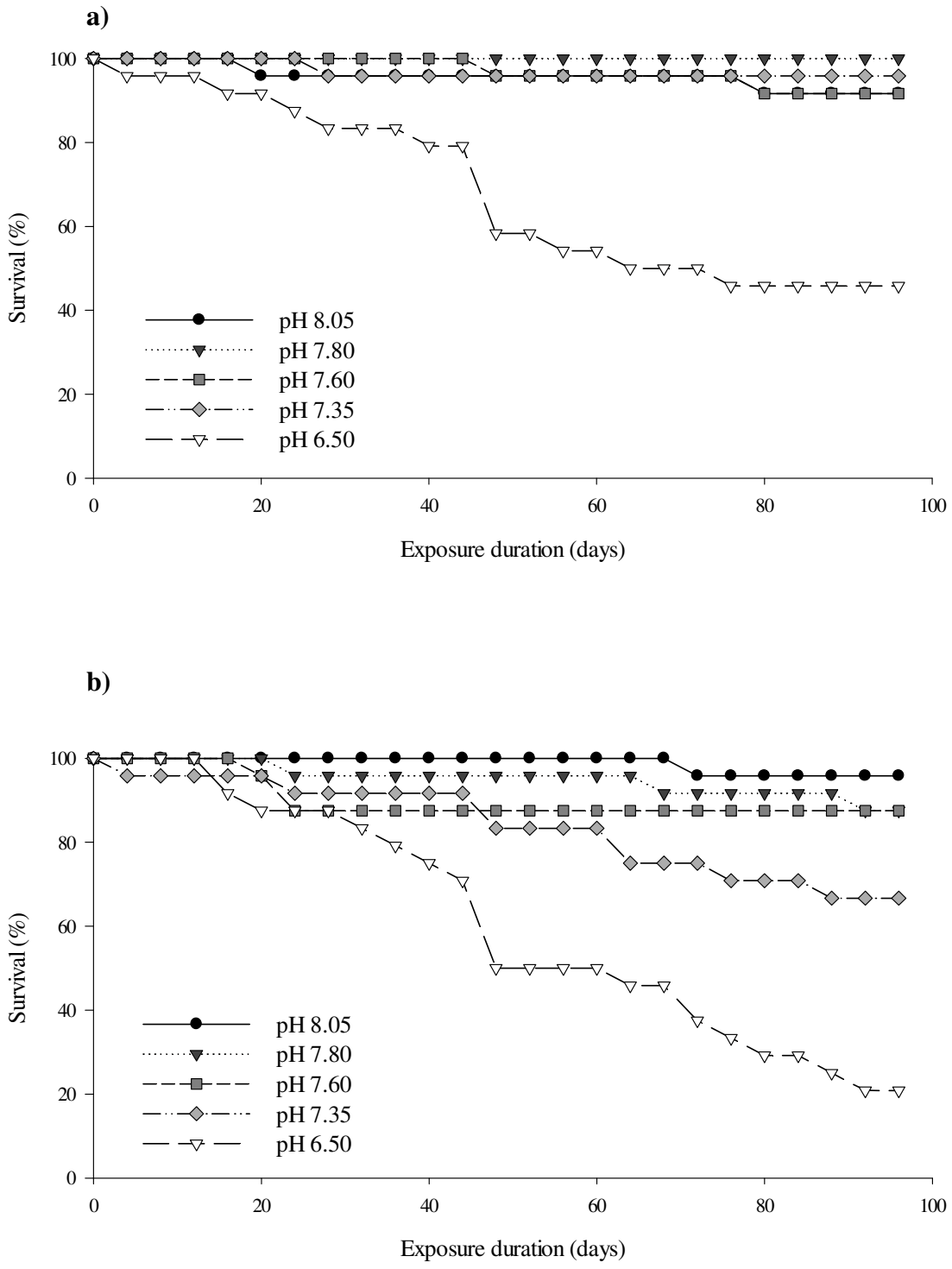

Figure 3.4 Percentage survival of *Mytilus edulis* maintained under control and acidified seawater conditions at **a)** 12.5 °C and **b)** 17.0 °C. Data show the percentage survival of the mussel population, pooled within treatment, over the duration of the experimental exposure.

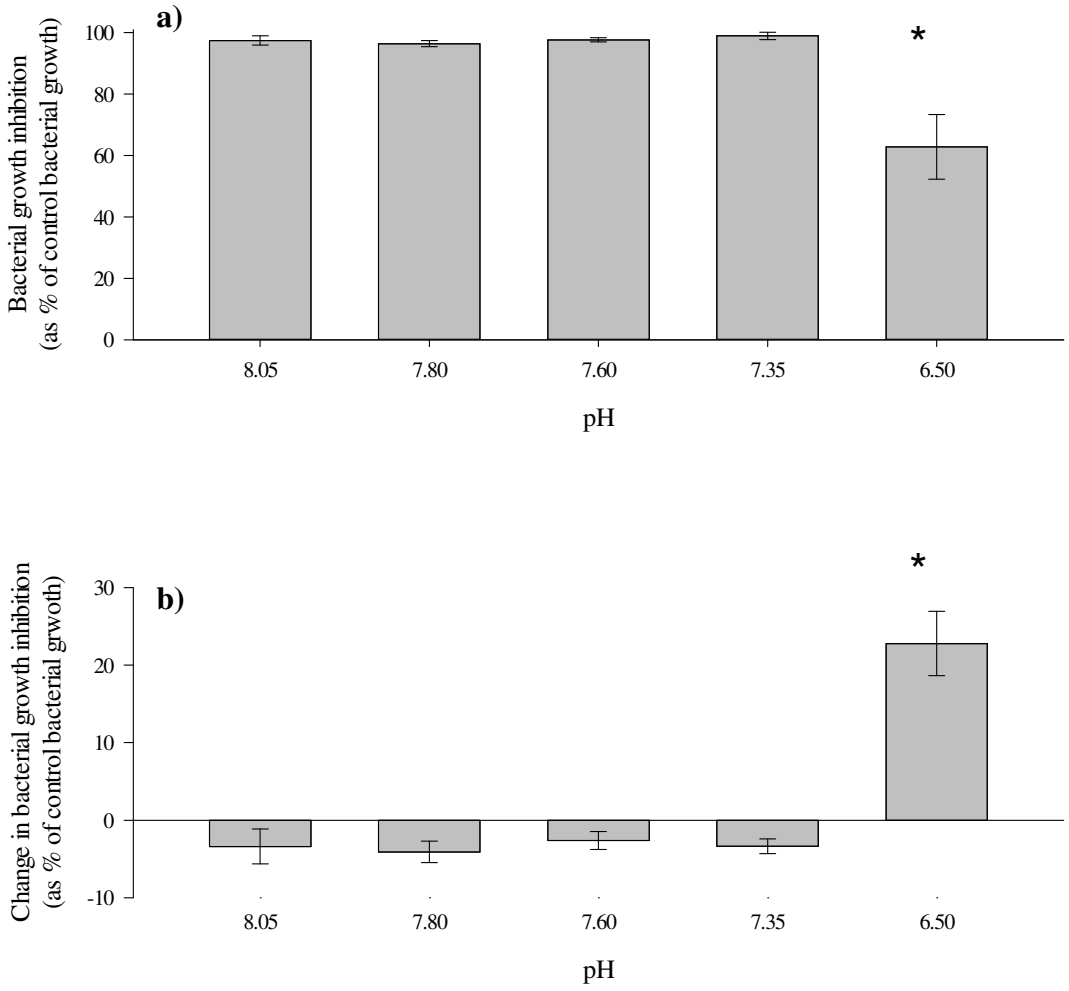

Figure 3.5 Antibacterial activity of mussel cell-free haemolymph, expressed as the inhibition of bacterial growth by cell-free haemolymph as a percentage of control bacterial growth, after exposure to low seawater pH. **a)** The bacterial growth inhibition of mussel haemolymph after an initial 90 day exposure (data are pooled for temperature and gender, N = 60 individuals). **b)** The change in antibacterial activity of mussel haemolymph after inoculation with *Vibrio tubiashii* (data are pooled from 1 d and 7 d post inoculation, as well as for temperature and gender, N = 84 individuals). Values are means (± S.E.). Significant differences ($p \leq 0.05$) between treatment levels are indicated by an asterisk and based on pair-wise tests.

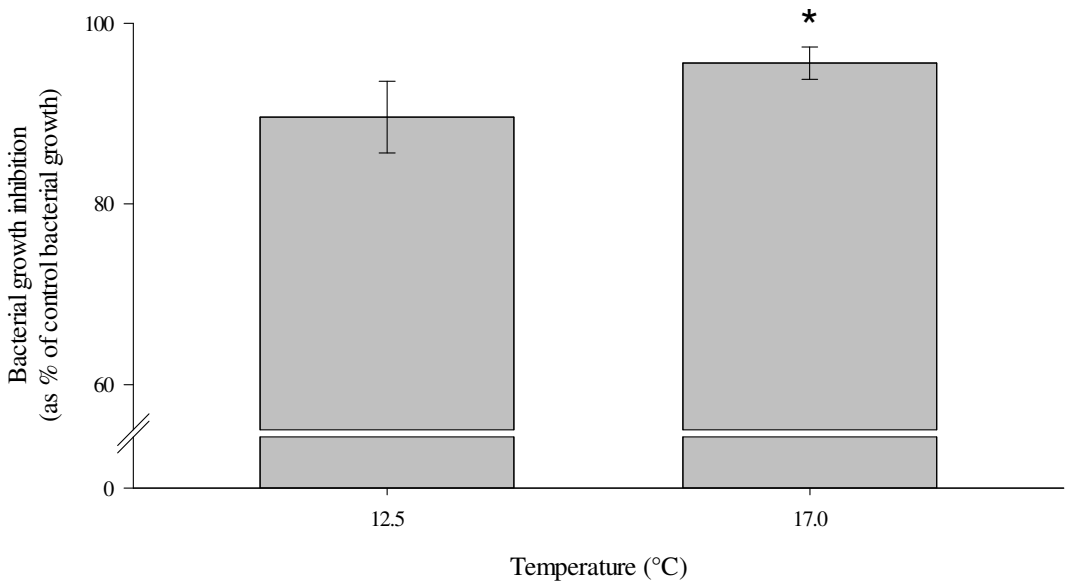

Figure 3.6 Antibacterial activity of cell-free haemolymph, expressed as the inhibition of bacterial growth by cell-free haemolymph as a percentage of control bacterial growth, following an initial 90 day exposure to increased seawater temperature. Values are means (± S.E.). Data are pooled for pH and gender. Significant differences ($p \leq 0.05$) between treatment levels are indicated by an asterisk and based on pair-wise tests. N = 60 individuals.

haemolymph to inhibit bacterial growth reduced by 35 % compared to controls. Temperature impacted antibacterial activity of mussel haemolymph in the absence of a pathogen (Pseudo-F = 4.78, d.f. = 1, p = 0.042), with mussels maintained at the elevated temperature of 17.0 °C having an enhanced ability to inhibit bacterial growth compared to mussels at 12.5 °C (Fig. 3.6).

Reduced seawater pH significantly impacted the response of the mussel immune system to a bacterial exposure (Pseudo-F = 26.13, d.f. = 4, p = <0.001). Interestingly, pair-wise analysis show mussels maintained at pH 6.50 significantly enhanced the antibacterial activity of cell-free haemolymph by 22.7 %, compared to the response

measured at day 90, whereas mussels maintained at pH 8.05, 7.80, 7.60 and 7.35 reduced the antibacterial activity of their haemolymph by around 4% (Fig. 3.5b).

Alongside reduced seawater pH, gender was also shown to affect the impact of a bacterial exposure on the antibacterial activity of mussel haemolymph (Pseudo-F = 9.15, d.f. = 1, p = 0.004). Inoculation with a pathogen was shown to increase the ability of cell-free haemolymph to inhibit bacterial growth in females, whereas in males antibacterial activity is reduced following a pathogenic exposure (Fig. 3.7). The duration of a bacterial exposure did not significantly affect the impact of reduced seawater pH, increased temperature or gender on the antibacterial activity of haemolymph, with no difference in the response measured 1 day and 7 days post inoculation.

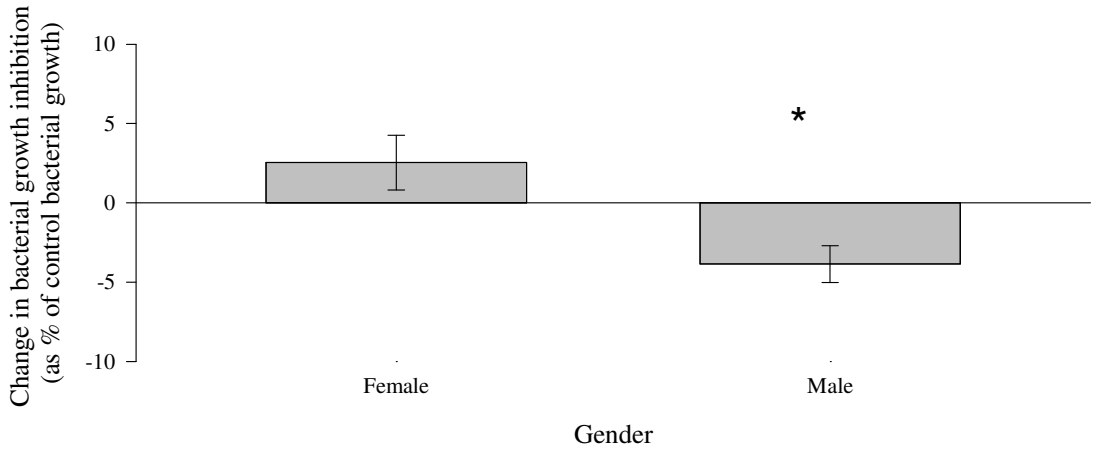

Figure 3.7 The change in antibacterial activity of cell-free haemolymph in male and female mussels, expressed as a change in the bacterial growth inhibition of cell-free haemolymph as a percentage of control bacterial growth, following an inoculation with *Vibrio tubiashii*. Values are means (± SEM). Data pooled from 1 d and 7 d post inoculation as well as for pH and temperature. Significant differences ($p \leq 0.05$) between treatment levels are indicated by an asterisk and based on pair-wise tests. N = 84 individuals.

3.3.4 Total cell count

Following an initial 90 day exposure, pH significantly affected total haemocyte numbers within mussel haemolymph (Pseudo-F = 3.10, d.f. = 4, p = 0.026). Pair-wise

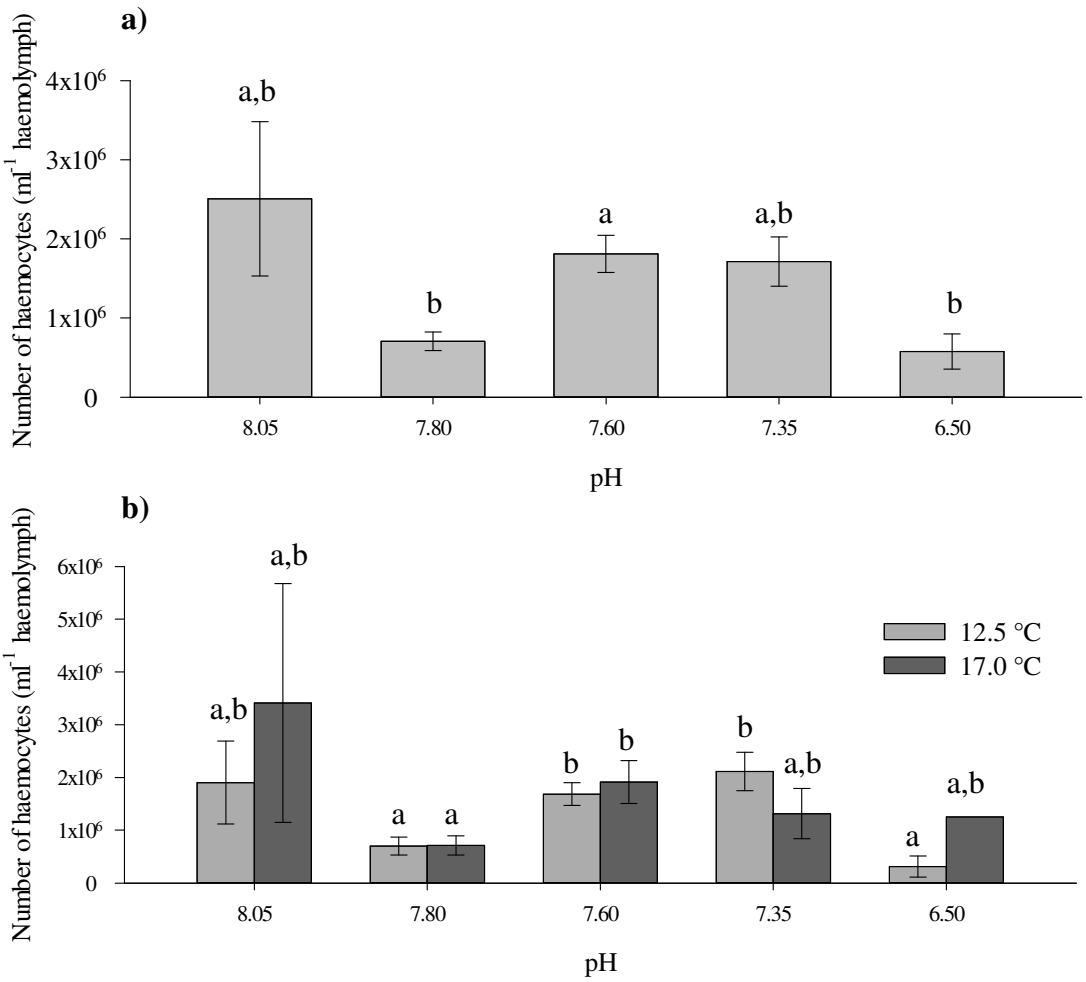

Figure 3.8 a) The effect of reduced seawater pH on the number of circulating haemocytes per ml of haemolymph (data pooled for temperature and gender) and **b)** the effects of reduced seawater pH and temperature on the number of circulating haemocytes per ml of haemolymph in mussels (data pooled for gender). Values are means (± S.E.). Significant differences ($p \leq 0.05$) between treatment levels are indicated by different letters and based on pair-wise tests. N = 60 individuals.

analyses indicated that total cell counts in mussels maintained at pH 7.60 were significantly higher than measured in mussels maintained at either pH 7.80 or 6.50 (Fig. 3.8a). However, as can be seen in Fig. 3.8a, total cell counts are naturally variable and the response shown at reduced seawater pH falls within the natural variability noted at pH 8.05.

Seawater pH was also shown to interact with temperature to affect total cell counts (Pseudo-F = 2.71, d.f. = 4, p = 0.04). At 12.5 °C mussels maintained at pH 7.80 and 6.50 had a significantly lower haemocyte count compared to mussels maintained at pH 7.60 or 7.35. Yet at 17.0 °C total cell counts were only lower in mussels maintained at pH 7.80 compared to those at pH 7.60. However, none of the total cell counts measured in mussels maintained at any of the reduced pH levels, or at either temperature, was shown to be significantly different to that measured at pH 8.05 (Fig. 3.8b).

Exposure to reduced seawater pH significantly affected the change in the number of circulating haemocytes in mussels exposed to *V. tubiashii* (Pseudo-F = 4.75, d.f. = 4, p = 0.003). However, contrary to the pattern shown after an initial 90 day exposure, pair-wise analyses indicate that control mussels reduce the number of circulating haemocytes in response to a bacterial challenge, whereas there was no significant change in total cell counts in mussels maintained at all other pH levels (Fig. 3.9a). Gender was also shown to affect the ability of mussels to alter the number of circulating haemocytes in response to a pathogenic challenge. Females significantly reduced the number of haemocytes in haemolymph when encountering *V. tubiashii*, while males slightly increased haemocyte numbers (Pseudo-F = 4.41, d.f. = 1, p = 0.042) (Fig. 3.9b). Again duration of a bacterial exposure did not affect total haemocyte counts.

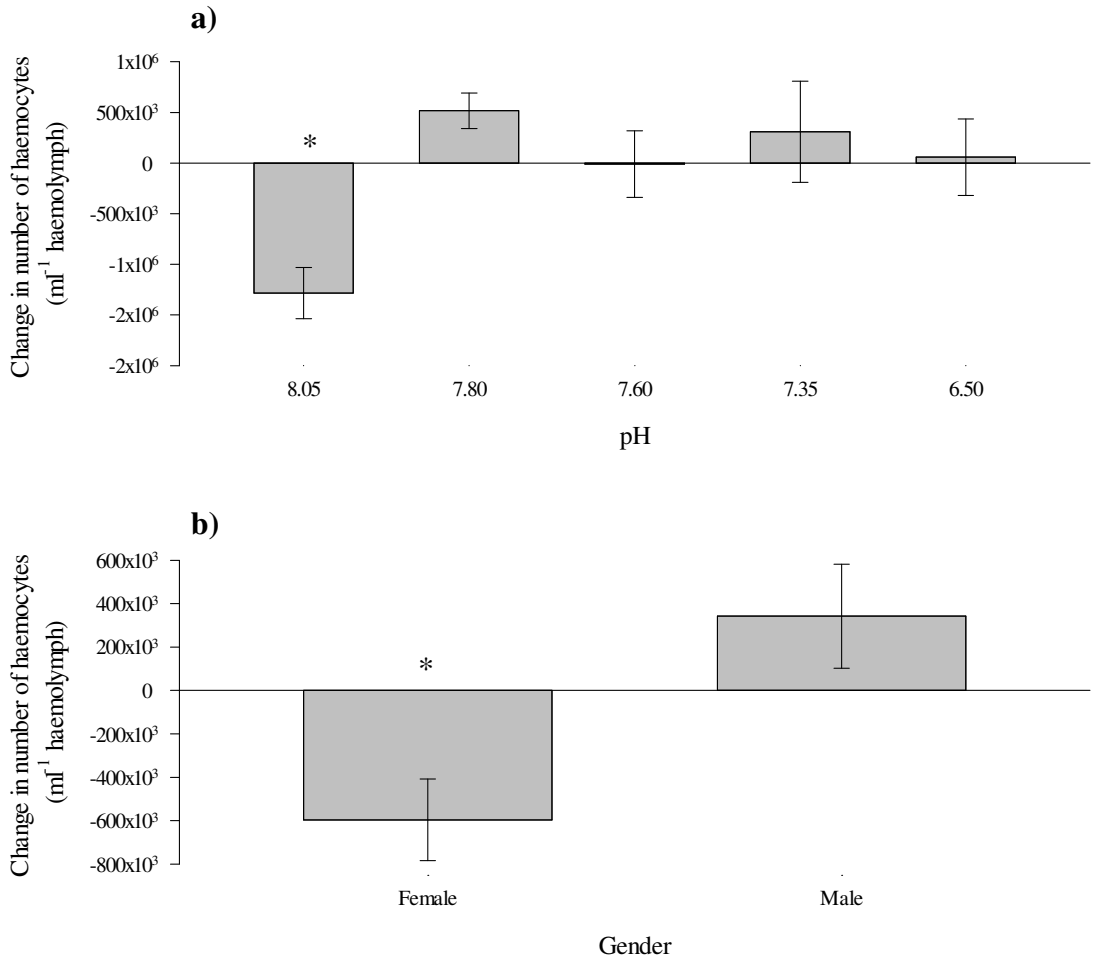

Figure 3.9 The change in the number of circulating haemocytes in mussels, expressed as the absolute change in cells per ml of haemolymph, following an inoculation with *Vibrio tubiashii.* **a)** The effect of reduced seawater pH on the change in the number of circulating haemocytes (data pooled from 1 d and 7 d post inoculation, and for temperature and gender). **b)** The effect of gender on the change in the number of circulating haemocytes (data pooled from 1 d and 7 d post inoculation as well as for pH and temperature). Values are means (± S.E.). Significant differences ($p \leq 0.05$) between treatment levels are indicated by an asterisk and based on pair-wise tests. N = 84 individuals.

3.3.5. Differential cell counts

After the initial 90 day exposure to experimental conditions, the only factor shown to significantly affect the proportion of circulating eosinophilic haemocytes was gender, with males shown to have a significantly greater proportion of eosinophils in their haemolymph compared to females (Pseudo-F = 4.37, d.f. = 1, p = 0.039) (Fig. 3.10).

Gender also significantly affected the impact of a pathogen exposure on differential cell counts (Pseudo-F = 4.98, d.f. = 1, p = 0.029). Females were shown to increase the proportion of circulating eosinophils within their haemolymph following a pathogen exposure, whilst males were shown to decrease eosinophils (Fig. 3.11a). Furthermore, gender was shown to affect how the proportion of circulating haemocytes changed over the seven days following a bacterial exposure (Pseudo-F = 6.70, d.f. = 1, p = 0.006) (Fig. 3.11b). Initially, one day after a pathogen exposure, females were shown to decrease the proportion of eosinophils in their haemolymph compared to female mussels measured pre-inoculation, whilst males were shown to subtly increase the proportion of eosinophils compared to pre-inoculation males (Fig. 3.11b). Conversely, seven days after a pathogen exposure, the proportion of eosinophils in female haemolymph was shown to have increased significantly compared to the level measured in females both pre- and one day post-inoculation. Furthermore, whilst not shown to be statistically significant, the proportion of eosinophils in male haemolymph was shown to have reduced seven days after inoculation compared to mussels measured pre- and one day post-inoculation (Fig. 3.11b).

Finally, exposure to reduced seawater pH was shown to affect the proportion of circulating eosinophilic haemocytes, following a pathogen exposure (Pseudo-F = 7.00, d.f. = 1, p = 0.001). Mussels maintained at pH 7.60 significantly increased the

proportion of eosinophils within their haemolymph when exposed to *V. tubiashii* compared to mussels at maintained at this pH sampled prior to a bacterial inoculation. Conversely, no significant difference was measured between mussels maintained at all other pH levels and sampled pre and post-inoculation (Fig. 3.12).

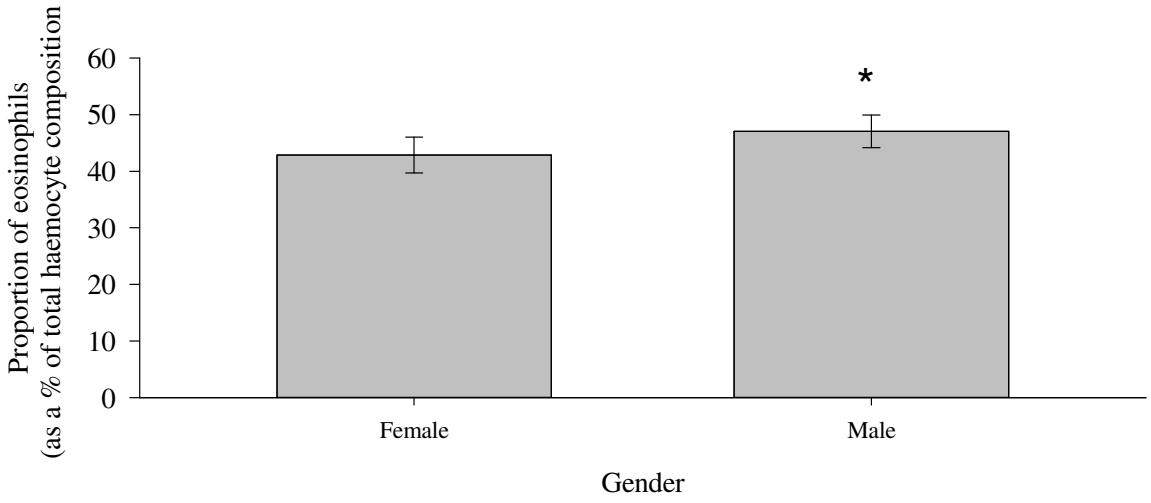

Figure 3.10 The effect of gender on the proportion of circulating eosinophilic haemocytes, expressed as the relative percentage of eosinophils in the total haemocyte fraction, following an initial 90 day exposure to experimental conditions. Values are means (± S.E.). Data are pooled for pH, temperature and bacterial exposure. Significant differences ($p \leq 0.05$) between treatment levels are indicated by an asterisk and based on pair-wise tests. N = 133 individuals.

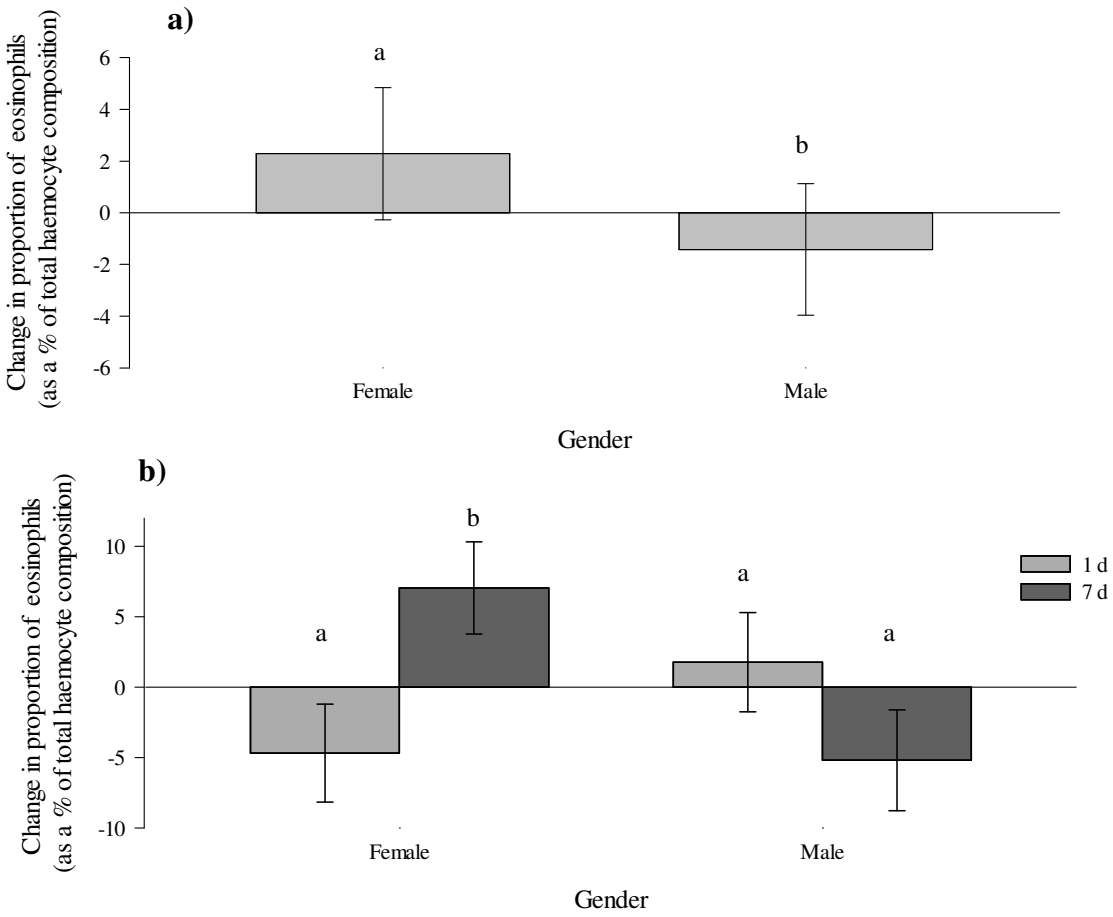

Figure 3.11 The change in the proportion of circulating eosinophils in mussel haemolymph following an inoculation with *Vibrio tubiashii*, expressed as the change in the relative percentage of eosinophils compared to the response measured pre-inoculation. The effect of: **a)** gender on the proportion of eosinophils (data are pooled from mussels sampled 1 d and 7 d post inoculation as well as for pH and temperature), **b)** gender and the duration of a bacterial exposure on the proportion of eosinophils (data are pooled for pH and temperature). Values are means (± S.E.). Significant differences ($p \leq 0.05$) between treatment levels are indicated by different letters and based on pair-wise tests. N = 83 individuals.

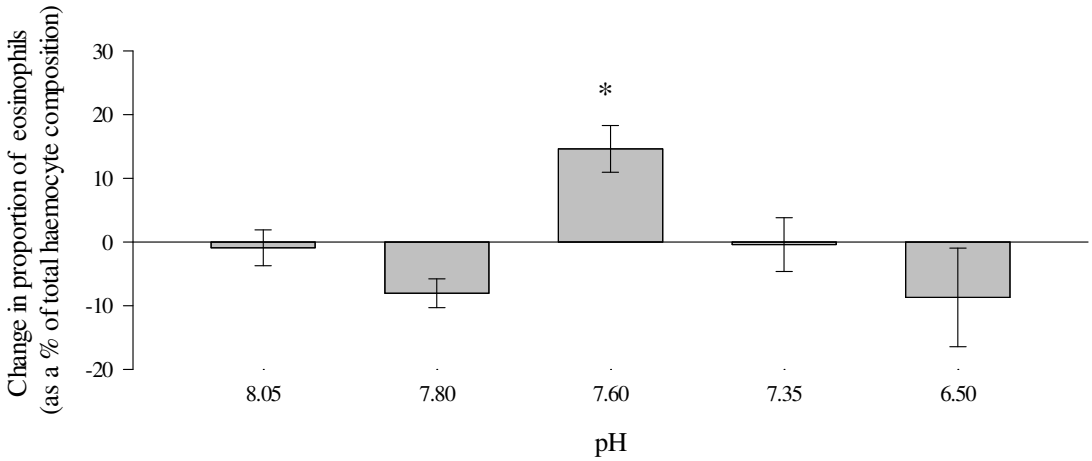

Figure 3.12 The effect of reduced seawater pH on the change in circulating eosinophil proportion in mussel haemolymph following an inoculation with *Vibrio tubiashii*, expressed as the change in the relative percentage of eosinophils compared to the response measured pre-inoculation. Values are means (± S.E.). Data are pooled from mussels measured 1 d and 7 d post inoculation as well as for temperature and gender. Significant differences ($p \leq 0.05$) between treatment levels are indicated by an asterisk and based on pair-wise tests. N = 83 individuals.

3.4 DISCUSSION

Exposure to reduced seawater pH and increased temperature is shown to reduce mussel survival and immune system maintenance. This result largely supports the findings of Bibby et al. (2008), Hernroth et al. (2011) and Matozzo et al. (2012), which are the only published studies to date to have investigated the impact of OA on the immune response of marine invertebrates. However, in contrast to these previous studies, in the present study reduced seawater pH is only shown to impact mussels at a level expected to occur in conjunction with a catastrophic leak from a CCS storage site, with the mussels used in this study seemingly tolerant of seawater acidification predicted to occur within the next 100 years (IPCC, 2007). Whilst mussels seem tolerant to the levels of reduced

seawater pH predicted to occur by the end of the current century, a concurrent exposure to elevated temperature increases the sensitivity of these mussels, further reducing survival. A reduction in immune system maintenance at low pH could indicate reduced immunocompetence, and thus an increased susceptibility to disease, in mussels. However in the current study, a subsequent exposure to a pathogenic bacterium led to an up-regulation in the immune response, suggesting that the initial reduction was a physiological trade-off, rather than a negative impact of acidification on immune system functionality, as has been suggested previously (Bibby et al., 2008). This result could have significant consequences for the perceived susceptibility of mussels to anthropogenic climate change.

3.4.1 The impact of anthropogenic climate change on mussel mortality

Reduced seawater pH is shown to significantly reduce mussel survival over a 90 day exposure to experimental conditions in mussels maintained at 12.5 °C. However, survival is only impacted in mussels maintained at pH 6.50. In showing no impact of OA on mussel mortality, at a level predicted to occur within the next 100-300 years (Caldeira and Wickett, 2005; IPCC, 2007), this study supports previous research that has shown mussels are relatively resilient to changes in seawater carbonate chemistry (e.g. Ries et al., 2009; Thomsen et al., 2010; Thomsen and Melzner, 2010). The mussels used in the current study were collected from an intertidal estuarine mussel bed. Such habitats frequently experience CO_2 concentrations significantly higher than expected from equilibrium with the atmosphere (Feely et al., 2010; Frankignoulle et al., 1998), with estuarine pH ranges of 6.7-8.9 reported (Attrill et al., 1999). Therefore in maintaining survival at reduced seawater pH, the mussels used in this experiment may

be pre-adapted to coping with the impact of low pH. Such resistance to naturally acidified seawater has been demonstrated by Thomsen et al. (2010), where a population of mussels from Kiel fjord, naturally exposed to pH values <7.5 during summer and autumn periods, maintained calcification, somatic growth and juvenile recruitment.

In showing a reduced survival of mussels maintained at pH 6.50, these findings support Bamber (1990) who demonstrated an exposure to a pH of ≤ 6.5 significantly increased mortality in these ecosystem engineers. Such low pH conditions may be experienced during a catastrophic leak from sub-seabed CO_2 storage (Blackford et al., 2009). This means any proposed mitigation project would need to carefully consider the impact of a leak on the local ecosystem and its resident fauna. However, with carbonate chemistry already shown to fluctuate drastically in near shore coastal areas (Andersson and Mackenzie, 2011; Frankignoulle et al., 1998), and with pH ranges in these habitats already shown to far exceed those projected to occur in the open ocean (Attrill et al., 1999; Feely et al., 2010; Morris and Taylor, 1983), OA may also result in these extreme pH conditions (i.e. pH 6.50) being prevalent in near shore coastal areas within the next 100-300 years (Feely et al., 2008; Pelejero et al., 2010). If this were to occur it could have a devastating impact on the population dynamics of the organisms residing in these coastal zones, significantly impacting the aquaculture practices which occur in these productivity 'hot spots' (Turley and Boot, 2010).

Whilst this present study appears to support the findings of Bamber (1990), care should be exercised when comparing studies using different methods of seawater acidification. In the current study seawater was acidified *via* the addition of CO_2 gas which is suggested as the most applicable method for understanding the impact of OA (Gattuso and Lavigne, 2009). However, Bamber (1990) used the addition of a strong acid to lower seawater pH, which does not alter seawater carbonate chemistry

comparably to CO_2 bubbling (Gattuso and Lavigne, 2009). Therefore the extent to which the data generated by Bamber (1990) can be used to understand the ecological implications of anthropogenic climate change is questionable.

The only other study to date to have measured mussel survival in response to OA using the addition of CO_2, was carried out by Beesley et al. (2008). In contrast to the current study however, these authors reported mussel survival to be unaffected by reduced seawater pH at any level, including pH 6.5. However, whilst this result appears to directly contradict the current findings, it is again crucial to carefully consider the experimental protocol used in each study, and consequently the experimental conditions each study reports. Beesley et al. (2008) measured a significant increase in seawater pH between header tank and exposure tanks in the pH 6.5 treatment (measuring an increase from pH 6.5 to 7.36). These authors suggest that this increase in seawater pH was caused by the dissolution of mussel shells, a response that has subsequently been measured in mussels exposed to OA (Melzner et al., 2011). However, whilst in the present study it is likely that reduced seawater pH led to the dissolution of mussel shells, the flow of seawater was maintained at a sufficient rate to ensure that this dissolution did not subsequently affect the pH of seawater in the exposure chambers. As a result no significant difference was measured between the pH of header tanks and exposure chambers at this treatment level. Consequently, in showing no mortality in the pH 6.5 treatment (analogous to the pH 7.35 exposure in the current study), the study by Beesley et al. (2008) supports the current research that suggests mussels are resilient to seawater acidification predicted to occur by 2300 (IPCC, 2007).

As well as showing an impact of reduced seawater pH on mussel mortality, this is the first study to date to have investigated the combined impact of OA and elevated temperature on adult mussel survival, with temperature shown to compound the effect

of reduced seawater pH. Anthropogenic climate change is causing a gradual decrease in seawater pH and a concomitant increase in seawater temperature (IPCC, 2007). Therefore, to fully understand how climate change will impact marine organism survival it is essential to study how these two stressors interact. The increased sensitivity of mussels to reduced seawater pH, when concurrently exposed to increased temperature, highlights the fact that organisms may be more vulnerable to climate change over the next 100 - 300 years than suggested by single stressor studies.

3.4.2. The impact of reduced seawater pH, increased temperature and gender on immune system maintenance

Adult mussels exposed to reduced seawater pH demonstrated a significant reduction in their immune response following an initial 90 day exposure, with antibacterial activity and total cell counts being impacted at low seawater pH. In showing immune system maintenance to be compromised by seawater acidification, this study therefore supports previous research where a reduction in seawater pH was shown to reduce phagocytic activity (Bibby et al., 2008), as well as lysozyme-like activity of cell free haemolymph (Matozzo et al., 2012), in mussels. Bibby et al. (2008) found that a 32 day exposure to OA reduced the ability of impacted mussels to increase phagocytic activity when compared to controls, and suggest that exposure to acidified seawater may therefore impact the ability of stressed mussels to express an immune response. Similarly, Matozzo et al. (2012) demonstrated that mussels reduced lysozyme-like activity of cell-free haemolymph following a 7 day exposure to pH 7.7 or 7.4. However, whilst the studies by Bibby et al. (2008) and Matozzo et al. (2012) noted a reduction in phagocytic activity as a decreasing function of seawater pH, in the current study antibacterial

activity of cell-free haemolymph was only impacted at a seawater pH of 6.50.

It is possible that the difference noted in the immune response of mussels exposed to reduced seawater pH in the present study, and those of Matozzo et al. (2012) and Bibby et al. (2008), may be as a result of seasonal differences in the resource allocation of organisms studied. Previous research has shown the immune system of bivalve molluscs is significantly affected by season, with organisms demonstrating a reduced immune response during summer spawning, compared to spring and autumn, as a result of a reallocation of energetic resources from host defence to reproduction (Matozzo et al., 2003; Pipe et al., 1995b). In the present study organisms were collected in December and maintained in the laboratory until March, a period of energy reconstitution that avoids studying organisms during spawning in the population studied (Lowe et al., 1982). Conversely, Bibby et al. (2008) sampled organisms in June, during a summer spawning period. It is therefore possible that the reduction in phagocytic activity measured in organisms exposed to pH 7.7 and 7.4 seawater in the Bibby study may be as a result of a reduction in the energy available for host defence, as a result of the increased cost of maintaining homeostasis, as well as the energetically costly process of spawning. However, whilst a seasonal difference in the energy allocated to host defence may explain the contrasting results noted by Bibby et al. (2008) and this thesis, Matozzo et al. (2012) also studied mussels during winter. Thus a seasonal difference in immune system maintenance cannot fully explain the apparently contrasting results noted in studies investigating the impact of reduced seawater pH on mussel host defence.

Alternatively, as discussed in Section 3.4.1, it is possible the tolerance to low seawater pH noted in the mussels used in the current study is an adaptation to the natural conditions experienced in the intertidal estuarine habitat from which they were

collected. The mussels used in the study by Bibby et al. (2008) were collected from Trebarwith Strand, which is an exposed rocky shore population that is likely to experience different local conditions to the mussels from Exmouth. Similarly, Matozzo et al. (2012) collected organisms from along the west coast of the North Adriatic sea, which will again likely experience different environmental conditions from those noted in an intertidal estuarine habitat (Borges and Gypens, 2010; Borges et al., 2006). It is therefore possible that the difference in immune system tolerance between the populations studied in each of the three studies is due local adaptation. Parker et al. (2011) also demonstrated the response in two populations of bivalve, in this case the Sydney rock oyster, *Saccostrea glomerata*, to vary significantly when exposed to OA. These authors suggest that variability noted in the sensitivity of different populations to climate change may have a vital role to play in the protection of important aquaculture practices under future climate change scenarios, as selective breeding of resistant organisms may help to produce more tolerant populations. This in turn may therefore safeguard economic activities from climate change associated loss (Parker et al., 2011). However, local adaptation could also have significant consequences for the perceived vulnerability of a species to climate change, with any population specific sensitivity needing to be accounted for when predicting the response of a species to climate change at a global level.

In addition to seasonal differences in host defence and local adaptation of populations, it is possible that the contrasting results noted by Bibby et al. (2008), Matozzo et al. (2012) and this thesis may be a result of the complex mussel speciation in the north-west Atlantic. Matozzo et al. (2012) studied the response in *Mytilus galloprovincialis*, whilst the present study measured the response of mussels collected from a site confirmed to comprise of pure *M. edulis* (Hilbish et al., 2002). Conversely,

Bibby et al. (2008) studied mussels from a population proposed to fall within a *Mytilus* hybrid zone. Therefore, it is possible that, in predominantly occupying sheltered habitats under freshwater influence (Bierne et al., 2003), the reduced sensitivity noted in *M. edulis* in the present study may be due to a genetic adaptation of this species to reduced seawater pH.

Whilst the apparent difference in immune system tolerance between the mussels used in this study and those used by Bibby et al. (2008) may be due to local adaptation, the complex mussel speciation in south-west England or seasonal factors, it is also possible that this varying response is due to the different sensitivity of immune system parameters measured. It is widely accepted that the perceived sensitivity of the immune system is often dictated by the immune parameters chosen as an immunocompetence proxy (Ellis et al. 2011). Bibby et al. (2008) measured a reduction in the capacity of mussels to up-regulate phagocytic activity, with phagocytosis being carried out by the haemocytes in bivalves (Pipe et al., 1995a). Therefore in measuring a disruption in phagocytic activity, Bibby and colleagues (2008) suggest that altered seawater carbonate chemistry disrupts haemocyte function, and thus cellular immunity. However, in measuring a reduction in antibacterial activity the present study measured an impact of reduced seawater pH on humoral immunity.

Phagocytic activity was not measured in the current study, and therefore it is not possible to directly compare the impact of reduced seawater pH on cellular immunity between this study and that of Bibby et al. (2008). Nonetheless, reduced seawater pH was shown to impact total haemocyte counts in the present study, with mussels exposed to pH 7.60 and 7.35 increasing the number of circulating haemocytes in their haemolymph compared to mussels maintained at pH 7.80. An increased total blood cell count has been shown in mussels under increasing levels of stress, with an exposure to

an increasing temperature (Renwrantz, 1990), cadmium concentration (Coles et al., 1994a) and fluoranthene concentration (Coles et al., 1994b) leading to an increase in the number of circulating haemocytes. Furthermore, a higher number of circulating haemocytes has been shown in mussels inhabiting contaminated sites within the Mediterranean, compared to individuals from uncontaminated reference sites (Auffret et al., 2006). Thus, moderate seawater acidification predicted to occur within the next 100 - 300 years (IPCC, 2007) would appear to impact the cellular immune response of mussels from Exmouth, supporting the findings of Bibby et al. (2008).

Conversely, at pH 6.50 haemocyte number was not shown to increase compared to mussels maintained at pH 7.80. A similar stress response was shown by Pipe et al. (1999), where exposure to increasing copper concentrations (0.02 mg l^{-1} and 0.05 mg l^{-1}) were shown to increase haemolymph cell counts these bivalves, however beyond a concentration of 0.2 mg l^{-1} no increase in haemolymph cell counts was measured. These authors suggest the inability of mussels exposed to the very high copper concentrations to increase haemolymph cell counts may be due to the toxic impact of the metal on haemocytes themselves, or due to the movement of haemocytes out of circulation and into tissues. Therefore it is possible in the current study mussels maintained at pH 6.50 may be moving haemolymph cells out of circulation in order to minimise the pathological impacts of an exposure to reduced pH, indicating a significant level of stress.

Whilst the number of circulating haemocytes is seemingly impacted by reduced seawater pH in mussels from Exmouth, this result needs to be interpreted with caution. Haemocyte numbers are typically variable and shown to fluctuate greatly (Parry and Pipe, 2004), in the current study the haemocyte counts in mussels maintained at reduced seawater pH, despite being different between acidification treatments, all fell within the

natural variability measured within the control group. Thus the response of supposedly impacted mussels may merely be a result of natural variability, and immunocompetence may remain unaffected.

As with mortality, elevated temperature is also shown to significantly impact the mussel immune response, significantly increasing the antibacterial activity of cell-free haemolymph. This supports previous research, where Matozzo et al. (2012) noted an increase in lysozyme-like activity of cell-free haemolymph in *Mytilus galloprovincialis*, whilst Monari et al. (2007) demonstrated an increase in the antibacterial activity of the striped venus clam, *Chamelea gallina,* when exposed to increased seawater temperatures. It is widely accepted that temperature is shown to affect enzymatic activity and metabolism in ectothermic organisms (Somero, 2002). Therefore the increase in antibacterial activity measured in the current study, and the increase in lysozyme-like activity measured by Monari et al. (2007) and Matozzo et al. (2012), may represent an increased activity of hydrolytic enzymes at increasing temperature. Such an increase in antimicrobial activity with increased temperature was also shown the in the green shore crab, *Carcinus maenas*, where the activity of antimicrobial proteins was shown to be highest at high temperatures (Chrisholm and Smith, 1994). In measuring an elevated antimicrobial activity at increased temperature it is possible that the rise in seawater temperature predicted to occur within the next 100 - 300 years (IPCC, 2007) may counteract any reduction in immune system maintenance caused by a concomitant reduction in seawater pH.

Alongside measuring a significant impact of reduced seawater pH and increased temperature on immune system maintenance in the mussel, *M. edulis*, this is also the first study to demonstrate a gender difference in the immune response of this bivalve species. Females are shown to have a higher proportion of eosinophils within the

haemolymph compared to males. Haemocytes can be separated into different functional groups based on morphological and staining characteristics (Pipe et al., 1999), with different subpopulations undertaking separate functions with respect to an organisms immune response. In bivalves granular eosinophils are thought to be more phagocytic (Foley and Cheng, 1975) and account for most peroxidase and phenoloxidase activity (Pipe et al., 1997). Therefore, in measuring a higher proportion of eosinophils, females appear to maintain a higher immunocompetence than males when exposed to stressful environmental conditions. This would appear to support the dogma of sexual selection, with males, who are limited in their reproductive success by the number of females they can inseminate taking a more risk-prone strategy compared to females, who are limited by the far less variable number of offspring they are able to produce (Andersson, 1994; Trivers, 1972; Zuk et al., 2004). In appearing to invest less energy in immune system maintenance, males may therefore enable a greater allocation of resources to reproduction. However, any reduced allocation of energy to immune response could have significant consequences for the survival of the organism.

3.4.3. Response of the immune system to a bacterial challenge

In measuring a reduced immune response in mussels exposed to OA the current study supports the findings of Bibby et al. (2008) and Matozzo et al. (2012). However, whilst a reduced immune response may suggest reduced immune system functionality, when measured in the absence of a pathogen it is impossible to accurately assess the impact of any immune suppression on organism fitness. The evolved function of the immune system is to protect a host from any pathogenic challenge, ensuring survival. Its magnitude is therefore contingent on the presence and identity of any parasite, alongside

the fitness priorities of the host (Martin et al., 2010). With the resources an organism allocates to different life history traits, such as defence, reproduction or metabolism, coming from a finite energetic pool, such processes must trade-off against each other in order for an organism to maintain optimal fitness (Roff, 1992; Sibly and Calow, 1986). Under stressful environmental conditions, and in the absence of a pathogen, an organism may therefore trade-off the costs of immune system maintenance, reallocating these resources instead to other costly physiological processes in an attempt to maximise fitness (Lochmiller and Deerenberg, 2000; Sheldon and Verhulst, 1996; Zuk and Stoehr, 2002). However, an organism that has down-regulated immune system maintenance may maintain the ability to up-regulate its immune response when required, ensuring immunocompetence and the ability of an organism to survive a pathogenic challenge remains unaffected. Immune system regulation should therefore be measured functionally, rather than immunologically, in the presence of a pathogen (Viney et al., 2005).

In exposing mussels to the pathogenic bacterium, *V. tubiashii*, the current study was able to demonstrate that the initial reduction in immune system maintenance was in fact a physiological trade-off induced by environmental stress. Mussels maintained at pH 6.50, which had initially reduced the antibacterial activity of their haemolymph, reversed this trade-off when a pathogen was encountered. Whilst an exposure to a pathogenic challenge has been demonstrated to alter immune system regulation in a number of previous studies (e.g. Cellura et al., 2006, 2007; Ciacci et al., 2009; Kim et al., 2008; Rodríguez-Ramos et al., 2010), this is the first study to demonstrate a reversible trade-off of in immune maintenance costs in an invertebrate exposed to stressful environmental conditions. Such plasticity in immune system maintenance, and an apparent ability to control resource allocation between different physiological

processes, could have major implications for the interpretation of an organism's response to anthropogenic climate change. However, to fully understand the impact of a physiological trade-off on an organism's fitness it is vital to study the concurrent impact on other vital life-history traits. Wood et al. (2008) demonstrated an exposure to reduced seawater pH led to an increase in calcification in the brittlestar *Amphiura filiformis*. However whilst increased calcification was shown to ameliorate the impact of OA, it came with a physiological cost measured as an increase in muscle wastage (Wood et al., 2008). Therefore whilst the reallocation of resources between different physiological processes may reduce the immediate impact of unfavourable environmental conditions, the extent to which this strategy will be sustainable in the long term will depend on an organism's condition and energetic reserves (Wood et al., 2008, 2010).

Alongside demonstrating an impact of seawater pH on the change in antibacterial activity, reduced pH is also shown to impact total and differential cell counts in mussels exposed to *V. tubiashii*. An exposure to a pathogen reduced the number of circulating haemocytes in mussel haemolymph, supporting previous research by Ciacci et al. (2009). However, this response was only demonstrated in mussels maintained at pH 8.05, with the total cell count in mussels maintained at all other pH levels remaining unchanged by a bacterial inoculation. Conversely, a bacterial exposure was only shown to impact the proportion of circulating eosinophils in mussels maintained at pH 7.60. Haemocytes are responsible for phagocytic activity (Pipe et al., 1995a), and phagocytosis is the principle mechanism of bacterial clearance in molluscs (Parry and Pipe, 2004). Therefore it is possible that a reduction in the number of circulating haemocytes in response to a pathogenic challenge could be due in part to an increase in phagocytosis. Thus, in only showing a reduced cell count in control mussels,

reduced seawater pH could be inhibiting an increase in phagocytic activity in response to a subsequent bacterial challenge. However, with granular eosinophils thought to be more phagocytic (Foley and Cheng, 1975), any change in phagocytosis would be expected to induce a concomitant change in the proportion of haemocyte sub-populations. This response was demonstrated by Ciacci et al. (2009) where in concert with a reduction in total cell counts, an inoculation with bacteria induced a large decrease in the proportion of large granulocytes. Yet, in showing no change in haemocyte sub-populations in mussels exposed to bacteria at pH 8.05, and in only showing a slight increase in the proportion of eosinophils in response to a bacterial challenge in mussels maintained at pH 7.60, the impact of seawater pH on differential cell counts would not appear to support this hypothesis in the current study. Nonetheless, in not having measured phagocytic activity directly, and with some uncertainty still surrounding the exact function of different haemocyte sub-populations (Pipe et al., 1999), the exact impact of reduced seawater pH on phagocytosis in the current study is not clear.

Whilst seawater pH is shown to impact the cellular and humoral immune response of mussels exposed to a pathogenic challenge, gender is also demonstrated to affect the response of mussels to *V. tubiashii*. Females are proposed to benefit from longevity, with a greater immune defence ensuring long-term survival, yet conversely males are proposed to invest more in immediate reproductive effort, often at the expense of immunity (Stoehr, 2007; Zuk and Stoehr, 2002). Therefore the balance of costs and benefits of defence is suggested to lead to sexual dimorphism in immunocompetence, with females proposed to have a greater immune defence (Stoehr, 2007). In showing females to significantly decrease the number of circulating haemocytes, significantly increase the antibacterial activity of cell-free haemolymph and significantly increase the

proportion of eosinophils to a greater extent than males, when exposed to a pathogen, this study would appear to support the suggestion of sexual dimorphism in immunocompetence. Furthermore, with females reducing the number of circulating haemocytes in response to a pathogen, a response that is comparable to organisms maintained at control pH, and with females increasing antibacterial activity of cell free haemolymph compared to males, it would also appear females invest more in defence than males. Such a differing strategy of immune investment between males and females, both in immune system maintenance and in response to a bacterial exposure, could have a significant consequence for the response of mussel populations to an outbreak of disease.

3.4.4. Conclusions

The current study demonstrates that the survival and immune response of mussels appears to be impacted by reduced seawater pH. However, this response only became apparent at a level of seawater acidification that is predicted to occur in conjunction with a catastrophic leak from a sub-seabed geological CO_2 storage site. This estuarine population of mussels is therefore seemingly tolerant to moderate fluctuations in carbonate chemistry, yet when concurrently exposed to increased temperatures the sensitivity of mussels to reduced pH is shown to increase. This result highlights the importance of studying multi-stressor exposures. As well as indicating an impact of anthropogenic climate change on mussel survival and immune defence, gender is also shown to impact mussel immunocompetence, with females shown to maintain a higher proportion of circulating eoisinophils compared to males.

Alongside investigating the impact of anthropogenic climate change on mussel immune system maintenance, this study also measured the impact of a subsequent pathogenic challenge on host immune defence. Exposure to *V. tubiashii* highlighted that an initial reduction in humoral immunity in mussels exposed to low seawater pH was in fact a trade-off of immune system maintenance costs. In reallocating the energy afforded to immune system maintenance under stressful conditions, yet maintaining the ability to up-regulate immune defence when required, mussels may be better able to cope with the energetic requirements of surviving in a sub-optimal environment. However, such physiological trade-offs likely come at a cost, which must be paid in terms of other life-history traits such as reproduction or growth (Roff, 1992; Sibly and Calow, 1986; Stearns, 1992). The sustainability of such a strategy therefore will depend on an organisms energy reserves and the detrimental impact of a reduction in energy allocated to other processes (Wood et al., 2008, 2010).

CHAPTER 4. REPRODUCTIVE PROVISION AND ORGANISM CONDITION

The cost of stress resistance in *Mytilus edulis*: The effect of CO_2-induced seawater acidification, increased temperature and a bacterial challenge on reproductive provisioning and organism condition.

4.1. INTRODUCTION

The immune response is a major physiological mechanism that protects against a pathogenic challenge and thus ensures host survival (discussed in Chapter 1; Ellis et al., 2011; Lochmiller and Deerenberg, 2000). Yet, mounting an immune response, or maintaining the immune response in a state of readiness, is costly (Schmid-Hempel, 2003; Sheldon and Verhulst, 1996). Owing to finite resources, such costs must therefore be traded off against other physiological processes such as reproduction or growth (Lochmiller and Deerenberg, 2000; Sheldon and Verhulst, 1996; Steiger et al., 2011). However, whilst successfully defending itself from a pathogenic challenge would ensure a host maintains itself in a given environment, any parallel suppression in reproduction could have large and negative consequences for the population dynamics of an organism, reducing its persistence within an environment (Petes et al., 2008). Therefore, to fully understand the implication of an immune challenge, and the subsequent up-regulation of immune defence, it is vital to understand the environmental, or life-history, circumstances that would render an increase or decrease in the allocation of resources towards other life-history traits, such as reproduction, as profitable.

Investment in reproduction has been shown to be accompanied by a reduction in immune function in a number of taxa (e.g. Adamo et al., 2001; Kerr et al., 2010; Siva-Jothy et al., 1998; Zuk et al., 2004). Furthermore, a number of studies have shown immune challenged individuals to have compromised reproduction and reduced fecundity (e.g. Ahmed et al., 2002; Bonneaud et al., 2003; Fevrier et al., 2009; Jacot et al., 2004; Kelly, 2011; Kerr et al., 2010). However the extent to which an organism must trade-off its resources between different physiological processes will ultimately

depend upon its condition, with many fitness-related traits, such as immunocompetence and reproduction, being condition dependant (Lochmiller and Deerenberg, 2000; Schulte-Hostedde et al., 2005; Zuk and Stoehr, 2002).

An organism's condition, defined nutritionally as its relative energy reserves, has important fitness consequences and is often considered as a sign of overall health (Moya-Laraño et al., 2008; Schulte-Hostedde et al., 2005; Thomkins et al., 2004). Whilst a number of body condition indices have been developed, such as body mass or the regression of body mass on an ordinary least squares regression index of body size (which attempts to determine the mass of an individual associated with energy reserves after correcting for structural body size) (Schulte-Hostedde et al., 2005), much recent debate has focused on the validity of these approaches (Green, 2001). However, lipids are a major source of metabolic energy (Bergé and Barnathan, 2005), with body fat often providing the energy to produce and maintain life-history traits (Kelly, 2011). Thus, an organism's lipid content provides an accurate measure of its condition (Rolff and Joop, 2002; Thomkins et al., 2004). In molluscs, lipids provide energy for growth when resources are limited and when carbohydrate levels are low (Beninger and Lucas, 1984; Beukeme and De Bruin, 1979; Pazos et al., 1996, 1997).They also provide an important energetic food reserve in bivalve oocytes (Gallager and Mann, 1986; Gallager et al., 1986; Helm et al., 1973). Therefore, measuring tissue lipid content arguably allows a better assessment of an organism's condition, its metabolic resource allocation and thus a greater understanding of any physiological trade-offs (Pazos et al., 1997).

As discussed in Section 2.3, anthropogenic climate change is projected to lead to a concurrent increase in seawater temperature and reduction in seawater pH. Yet whilst previous research has shown that exposure to future climate change scenarios has led to varying, but often negative impacts on marine organisms (Hendriks et al., 2010;

Kroeker et al., 2010), very little is currently known about the impact of anthropogenic climate change on reproductive provisioning. Any impact of climate change on gamete production could potentially have significant carry-over consequences for population biology, possibly affecting larval numbers and larval development, as well as the dispersal, distribution and abundance of adults (Kurihara, 2008; Parker et al., 2009, 2010; Pechenik, 2006). The aim of this chapter is therefore to investigate the impact of reduced seawater pH and increased temperature on the reproductive provisioning in the blue mussel, *Mytilus edulis*. By additionally measuring the impact of anthropogenic climate change on the lipid content and fatty acid composition of mussel mantle tissue, this study will also enable a greater understanding of the trade-off between reproductive provisioning and the investment in energy storage in organisms experiencing environmental stress. Furthermore, in exposing mussels to a subsequent pathogenic challenge, this study will also elucidate the impact of immune system activation on the allocation of resources to reproduction under future climate change scenarios, providing vital information on stressor induced physiological trade-offs.

4.2. MATERIALS AND METHODS

The adult mussels used in the present study were collected, maintained and sampled exactly as described previously in Sections 3.2.1 and 3.2.2 respectively. Briefly, mussels were collected during Dec 2009, from an intertidal estuarine mussel bed in Exmouth, east Devon, before being returned to the PML mesocosm. Mussels were immediately placed in the experimental system (described in Sect. 3.2.1) and maintained for 90 days. During the experiment mussels were fed with *Isochrysis galbana* (30 mg dry mass mussel^{-1} day^{-1}). The impact of reduced seawater pH and

increased seawater temperature on the mussel immune response, reproductive status and lipid content, was subsequently assessed following an initial 90 day exposure to experimental conditions. To test the impact of experimental conditions on the mussel immune response haemolymph was extracted from the posterior adductor muscle to measure the total cell count, differential cell count and the antibacterial activity of cell-free haemolymph (described in Sect. 3.2.3). The reproductive status of individuals exposed to experimental conditions was assessed histologically (described in Sect. 4.2.2), whilst tissue lipid content was assessed using GC-MS (described in Sect. 4.2.2 and 4.2.3). Following this initial sampling time point the remaining mussels in the experimental system were then exposed to a bacterial challenge exactly as described in Section 3.2.2, and the abovementioned parameters measured again 1 day and 7 days post inoculation.

4.2.1 Preparation of tissue sections for histological analysis

Mussel soft-tissues were collected for histological analysis following the protocol used by Beesley et al. (2008). To sample soft-tissues, mussel shells were held open using a fixed bladed scalpel. A single incision was made to sever the anterior adductor muscle to allow access to the mantle cavity. Tissues were completely removed from the shell and a transverse slice, containing the digestive gland and mantle tissue, was excised from the body mass. These sections were immediately placed in Bakers formal calcium (10 % formalin, 1 % calcium chloride and 2.5 % sodium chloride) and stored in fixative for a minimum of 24 h at 4 °C. Once fully fixed, specimens were removed from the fixative and excess tissue was removed.

Using an automatic programmable tissue processor (Pathcentre, Thermo

Shandon), samples were then dehydrated, being passed through a graded alcohol series (Bakers formal calcium [100 %, 10 min], Industrial Methylated Spirit [IMS; 70 %, 1 h], IMS [90 %, 1 h], IMS [100 %, 3 h], IMS / Xylene [50% / 50 %, 1.5 h]), cleared in xylene (100 %, 3.5 h) and finally impregnated in paraffin wax over a 5 h period. Once impregnated, samples were blocked up in fresh molten wax using a stainless steel mould and were allowed to cool at room temperature prior to cutting. Sections, 7 µm thick, were cut using a microtome (Microm HM340E, Thermo Scientific) and then floated out onto microscope slides, which were coated with (3-Aminopropyl) triethoxysilane (A3648, Sigma-Aldrich) to aid adhesion. Dried sections were stained using Papanicolaou's stain (Culling, 1963), which colours adipogranular (ADG) cells orange, male reproductive tissues mauve and female reproductive tissues blue, whereas vesicular connective tissue (VCT) cells remain unstained (Fig. 4.1) (Lowe et al., 1982). Stained slides were then cover slipped before being analysed.

The sex of mussels was assessed based on the presence of eggs or sperm in the mantle tissue. Where the gender of an individual could not be reliably identified, e.g. due to the absence of any gametes, individuals were classified as unsexed. To assess the condition of mussel mantle tissue, the volume fraction of the different tissue components within the mantle were determined using stereology. Stereology measures the relative quantity of different tissue components from within a thin tissue section by obtaining a point count, using a Weibel test grid (Lowe et al., 1982). This point count is then converted to a volume fraction, through the extrapolation of two dimensional to three dimensional space (Briarty, 1975). The stereological protocol outlined by Lowe and Moore (1985) was followed. Mantle sections were examined under high power magnification (x400) with a Leitz (Leitz Wetzlar) compound microscope fitted with a Weibel eyepiece graticule (Graticules Ltd). Point counts on ADG cells, VCT cells,

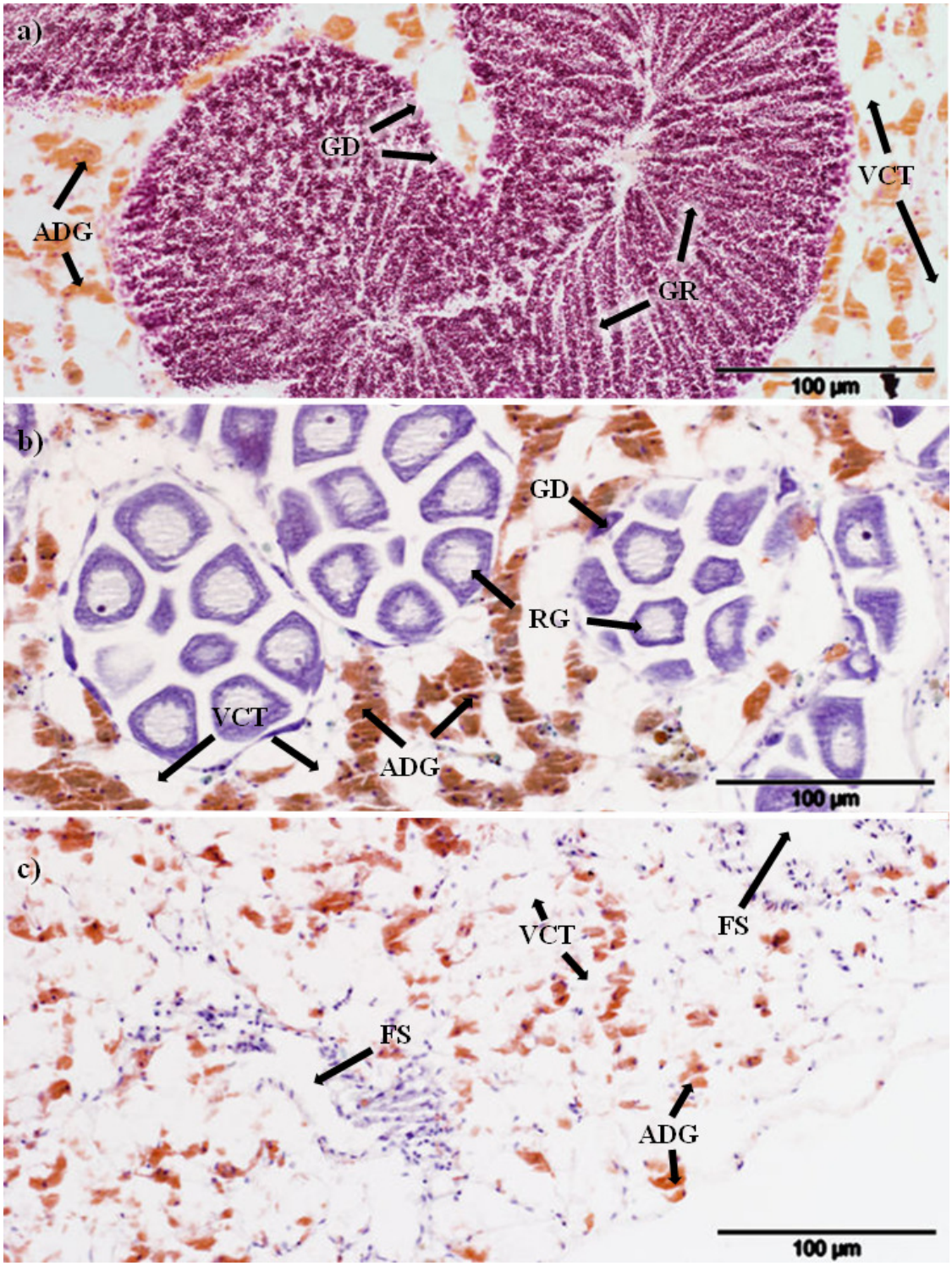

Figure 4.1 Section through the mantle tissue of **a)** male, **b)** female and **c)** unsexed *Mytilus edulis* showing adipogranular cells (ADG), vesicular connective tissue cells (VCT), developing gametes (GD), ripe gametes (GR) and spent follicles (FS). Magnification x 400.

developing gametes and morphologically ripe gametes were made on five fields per mussel, following a raster scanning pattern, to quantify the volume fraction of these different tissue components (Lowe and Moore, 1985; Weibel and Elias, 1967).

4.2.2 Lipid extraction

After dissecting mussel soft tissue for histological analysis, an additional transverse section of mantle tissue was excised and immediately snap frozen in liquid nitrogen. Samples were stored at -80 °C for further analysis. Lipids were extracted from the stored mantle tissue using the method of Bligh and Dyer (1959) modified as follows. Approximately 100 mg of mantle tissue was first transferred to a Precellys tube, stored on ice, and 4 µl mg^{-1} of methanol and 0.85 µl mg^{-1} of deionised water were added. Samples were then homogenised, being subjected to 2 cycles of 10 s (with a 5 s pause in between) homogenisation at 6400 rpm using a mechanical homogeniser (Precellys®24). Homogenised samples were transferred to a clean 1.8 ml glass vial, with Precellys tubes being rinsed using a further 2 µl mg^{-1} of deionised water. Chloroform (4 µl mg^{-1}) was then added to this mixture and the samples were vortexed for 30 s to ensure complete mixing. Samples were placed on ice for 10 min before being centrifuged (10 min, 1800 x *g*, 4 °C; Centrifuge 5810R, Eppendorf). After centrifugation, samples were allowed to acclimate to room temperature for 5 min before the non-polar lower chloroform phase was removed using a Hamilton syringe and transferred to a clean 1.8 ml glass vial. This sample was then stored at -80 °C prior to derivatisation for fatty acid methyl ester (FAME) analysis (see below).

4.2.3 Lipid derivatisation

Fatty acid concentrations and profiles in mussel mantle tissue were determined post conversion to fatty acid methyl esters (FAMEs) and analysis by GC-MS (Agilent 7890A GC and 5975C inert MSD, Agilent Technologies Ltd.). A 100 µl aliquot of each sample obtained from the lipid extraction (see above; Sect. 4.2.2) was transferred to a 15 ml glass tube, placed on a heating block at 45 °C and blown down under a continuous stream of N_2 until all the solvent had evaporated. A 20 µl aliquot of nonadecanoic acid (C19:0; 1 mg ml^{-1}) was added to each tube as an internal standard and fatty acids were then converted directly to fatty acid methyl esters (FAMEs) by adding 1ml of transesterification mix (95:5 v/v 3N methanolic HCl; 2,2-dimethoxypropane), flushing with N_2 and incubating at 90 ºC for 1 h. After cooling, FAMEs were recovered by addition of 1 % w/v NaCl solution (1 ml) and *n*-hexane (1 ml). Samples were then vortexed for 10 s before being centrifuged (30 s, 1,200 x *g*, 15 °C; Centrifuge 5810R, Eppendorf). The upper hexane layer was transferred to a clean 1.8 ml GC glass vial before being injected directly into the GC-MS system. FAMEs were separated on a fused silica capillary column (15 m x 0.1 mm x 0.1 µm; Omegawax™ 100, Supelco, Sigma-Aldrich) using an oven temperature gradient of 140 ºC to 280 ºC at 40 ºC min^{-1} followed by a 3 min hold time at 280 ºC. Helium was used as a carrier gas (0.4 ml min^{-1}) and the injector and detector inlet temperatures were maintained at 280 ºC and 230 ºC, respectively. FAMEs were identified using retention times and qualifier ion response, and quantified using respective target ion responses. All values were derived from calibration curves generated from a FAME standard mix (Supelco, Sigma-Aldrich, Gillingham, UK).

4.2.4 Statistical analysis

The effect of seawater pH, temperature, bacterial exposure and their interaction on reproductive histology and mantle lipid composition were analysed in Primer 6.1 (Clarke and Gorley, 2006) using the PERMANOVA+ add in (beta version, Anderson et al., 2008). As outlined in Section 3.2.5, data were tested for homogeneity of variance and transformed when necessary. Euclidean distance similarity matrices were constructed for all data, and P-values were calculated using 999 permutations of the residuals under a reduced model. Where a significant main effect or an interaction was shown, pair-wise comparisons between all levels of a given factor were undertaken. Where the percentage composition of saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) or polyunsaturated fatty acids (PUFA) in mantle tissue were shown to be significantly impacted by an experimental factor, SIMPER analyses were employed. SIMPER analysis, using a Bray-Curtis similarity matrix, determines the dissimilarity between treatment groups, indicating the percentage contribution of each individual fatty acid, within each class, to this dissimilarity.

4.3. RESULTS

The environmental conditions and organism mortality measured during this study are reported in Chapter 3 of this thesis (Sect. 3.3.1 and 3.3.2; Table 3.1, Table 3.2 & Fig 3.4 respectively).

4.3.1 Reproductive histology

Of the 153 mussels used for histology, 63 were females, 78 were males and it was not

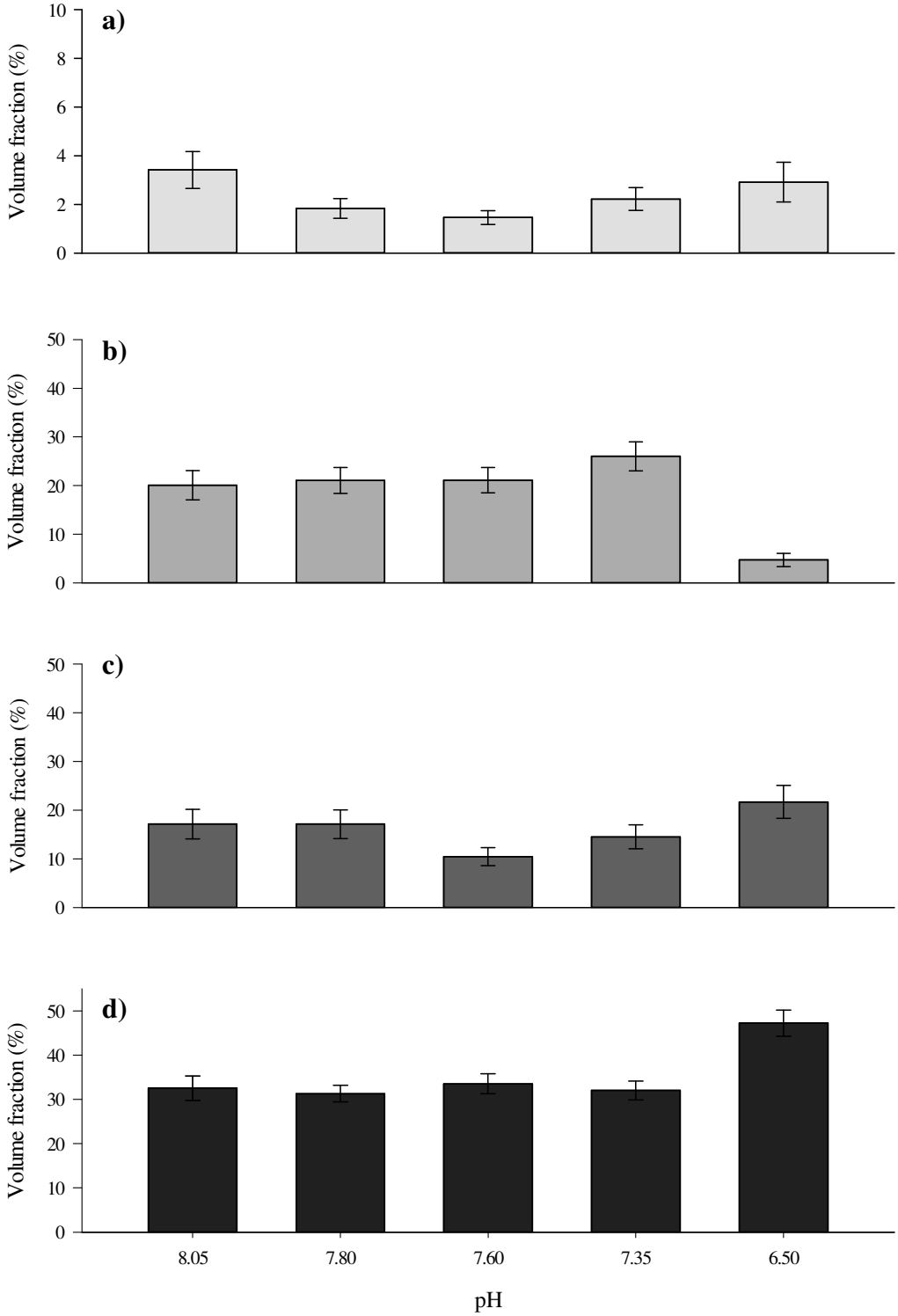

Figure 4.2 The effect of low seawater pH on the volume fractions (mean ± S.E.) of mussel mantle tissue: **a)** developing gametes, **b)** ripe gametes, **c)** ADG cells and **d)** VCT cells. Data are pooled for temperature, bacterial exposure and gender. Significant differences ($p \leq 0.05$) between treatment levels are indicated by an asterisk and based on pair-wise tests. N = 144 individuals.

possible to sex 11 individuals. Due to the relatively low number of unsexed individuals and the roughly equal level of males and females, it was decided that unsexed individuals would be omitted from the remaining statistical analysis to enable gender to be included as an additional experimental factor.

As shown in Figure 4.2, a severe reduction in seawater pH significantly reduced the percentage of ripe gametes within the mantle (Pseudo-F = 5.61, d.f. = 4, p = 0.002). Pair-wise analyses indicated that mussels exposed to a pH of 6.50 significantly reduced the percentage of ripe gametes compared to mussels maintained at all other pHs (Fig. 4.2b). When maintained at a pH of between 8.05 and 7.35 mussels had an average volume of 20.25 % ripe gametes within the mantle, whereas in mussels maintained at pH 6.50 this was reduced to 4.37 %. Reduced seawater pH was also shown to significantly affect the volume of VCT cells within the mantle (Pseudo-F = 3.14, d.f. = 4, p = 0.017), with mussels at pH 6.50 increasing volume of VCT cells compared to mussels maintained at all other pH levels (Fig. 4.2d). Reduced seawater pH did not significantly affect the volume fraction of developing gametes or ADG cells.

Similarly to pH, temperature was shown to have a significant impact on the volume fraction of different mantle tissue components. An increase in temperature was shown to significantly reduce the volume of developing gametes (Pseudo-F = 7.18, d.f. = 1, p = 0.014) (Fig. 4.3a) and ripe gametes (Pseudo-F = 16.94, d.f. = 1, p = < 0.001) (Fig. 4.3b). However this temperature induced reduction in gamete provisioning was marked by a concomitant increase in the volume of both ADG cells (Pseudo-F = 31.04, d.f. = 1, p = < 0.001) (Fig. 4.3c) and VCT cells (Pseudo-F = 6.27, d.f. = 1, p = 0.012) (Fig. 4.3d).

Gender was also shown to significantly impact the volume fraction of tissue components within the mantle (Fig. 4.4), with males having a significantly higher

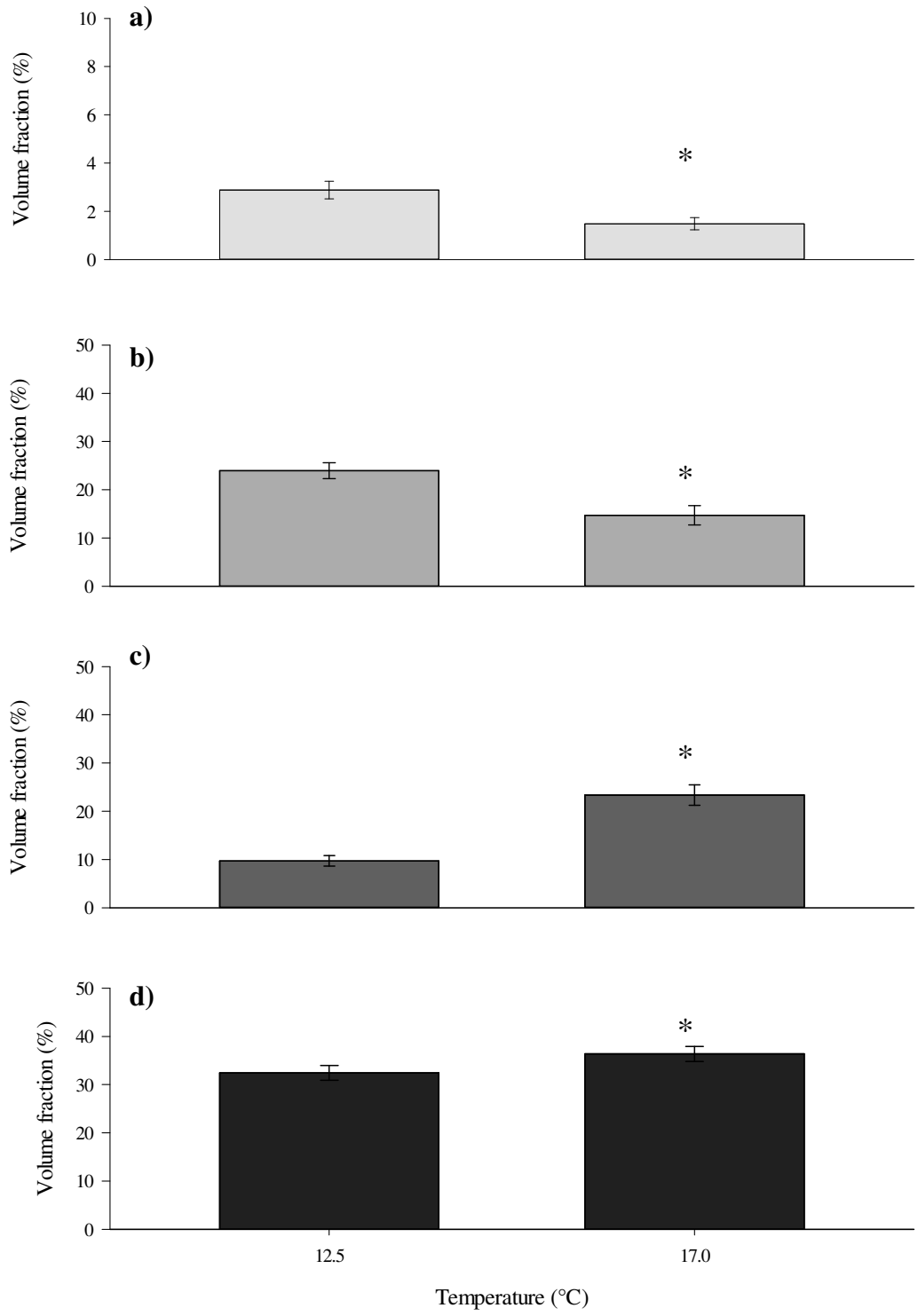

Figure 4.3 The effect of temperature on the volume fractions (mean ± S.E.) of mussel mantle tissue: **a)** developing gametes, **b)** ripe gametes, **c)** ADG cells and **d)** VCT cells. Data are pooled for pH, bacterial exposure and gender. Significant differences ($p \leq 0.05$) between treatment levels are indicated by an asterisk and based on pair-wise tests. N = 144 individuals.

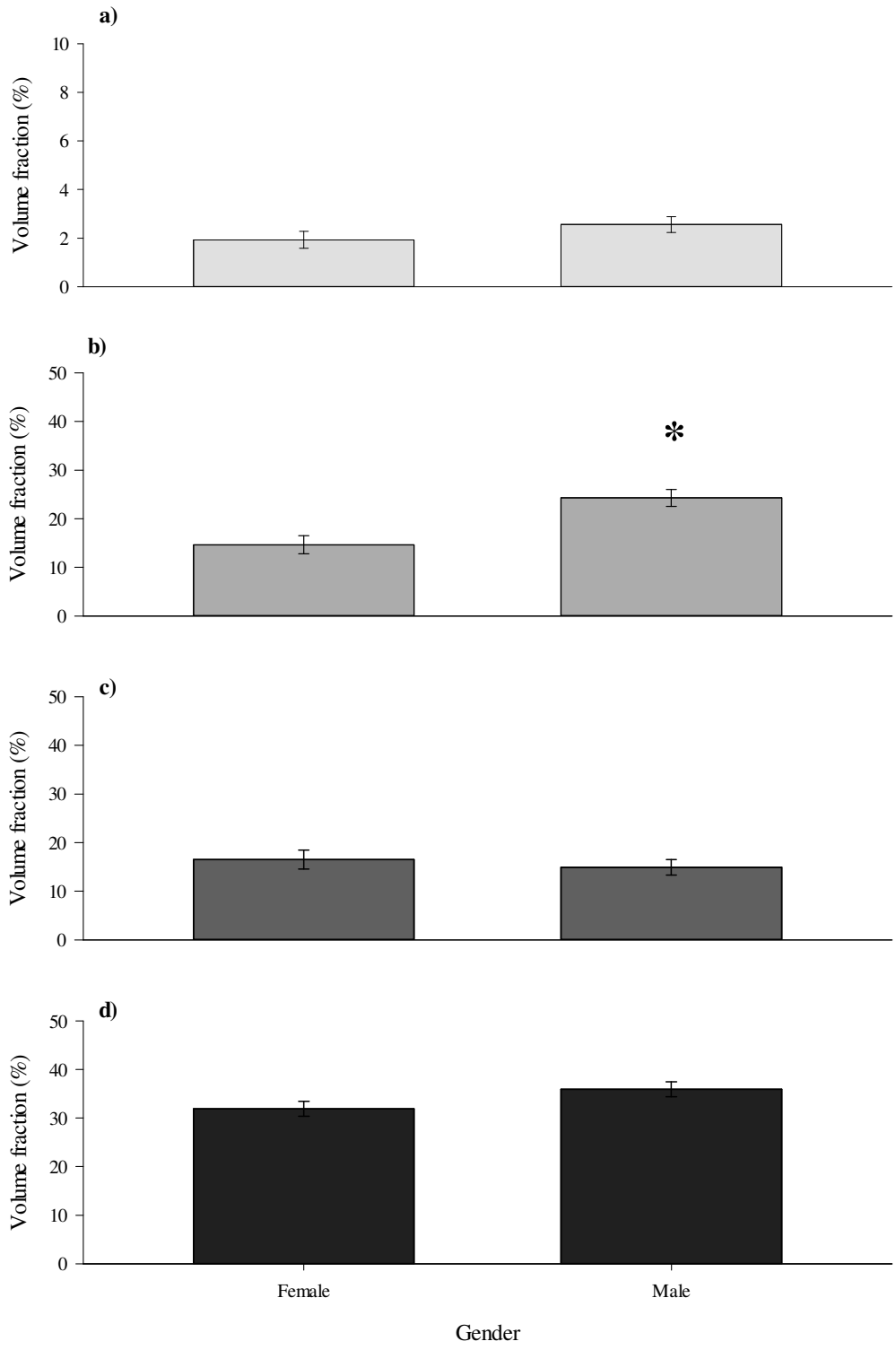

Figure 4.4 Volume fraction (mean ± S.E.) of mantle tissue components in male and female mussels: **a)** developing gametes, **b)** ripe gametes, **c)** ADG cells and **d)** VCT cells. Data are pooled for pH, temperature and bacterial exposure. Significant differences ($p \leq 0.05$) between treatment levels are indicated by an asterisk based on pair-wise tests. N = 133 individuals.

volume of both developing (Pseudo-F = 8.49, d.f. = 1, p = 0.008) (Fig. 4.4a) and ripe gametes (Pseudo- F = 19.38, d.f. = 1, p = < 0.001) (Fig. 4.4b) compared to females. Whilst the volume of ADG and VCT cells did not differ significantly between the two sexes, temperature was also shown to significantly interact with gender. Increased temperature was shown to increase the volume of VCT cells within the mantle tissue of females, however there was no significant difference in the volume of VCT cells in males maintained at 17.0 °C compared to those maintained at 12.5 °C (Pseudo-F = 4.64, d.f. = 1, p = 0.042) (Fig. 4.5). This difference may be due to the extent to which temperature reduced gamete provision in males and females.

Whilst temperature reduced gamete provision in both sexes, this effect was more pronounced in females with reproductive investment being reduced from 21.40 % at 12.5 °C to 6.70 % at 17.0 °C. However, in males, gamete investment was reduced from 25.88 % at 12.5 °C to 21.95 % of the mantle at 17.0 °C. The significant increase in VCT cells at 17.0 °C in females may therefore represent the larger decrease in gametes in females, compared to males.

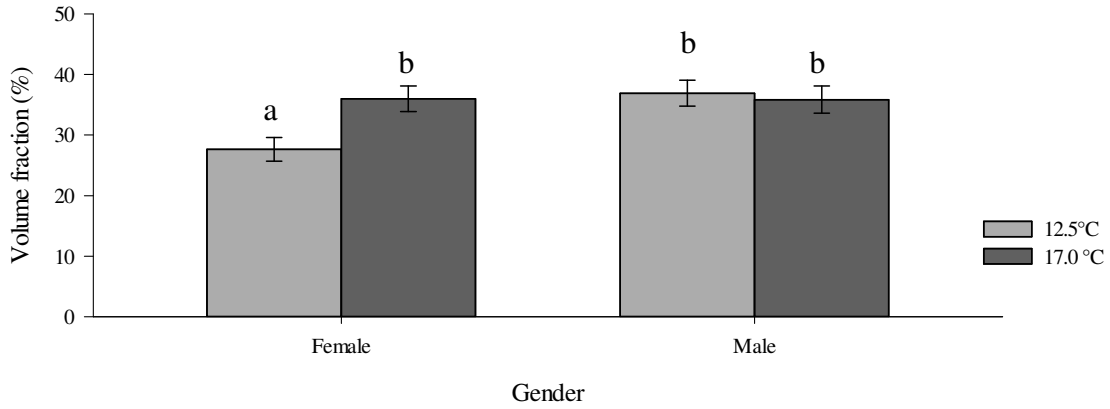

Figure 4.5 The effect of temperature (mean ± S.E.) on VCT cells within the mantle of male and female mussels. Data are pooled for pH and bacterial exposure. Significant differences ($p \leq 0.05$) between treatment levels are indicated by different letters based on pair-wise tests. N = 133 individuals.

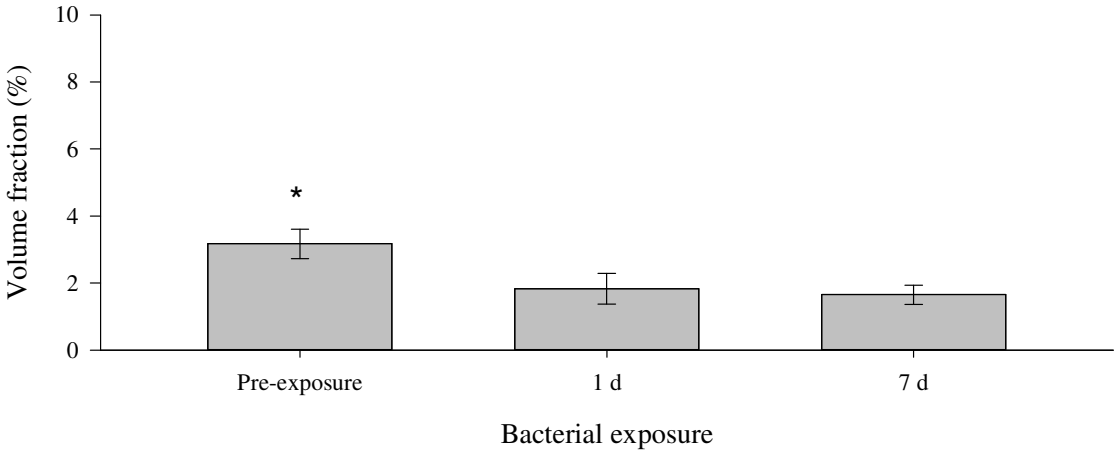

Figure 4.6 The effect of a bacterial exposure on the volume fraction (mean ± S.E.) of developing gametes within mantle tissue. Data are pooled for pH, temperature and gender. Significant differences ($p \leq 0.05$) between treatment levels are indicated by an asterisk and based on pair-wise tests. N = 133 individuals.

Alongside pH, temperature and gender, an exposure to *Vibrio tubiashii* was also shown to significantly impact the volume fraction of developing gametes, with inoculated mussels significantly reducing the volume of developing gametes compared to mussels sampled prior to a bacterial exposure (Pseudo-F = 6.58, d.f. = 2, p = < 0.001). Interestingly the duration of the exposure did not alter this effect, with no significant difference between mussels sampled 1 day and 7 days post inoculation (Fig. 4.6). Bacterial exposure was not shown to impact the volume of ripe gametes, ADG cells or VCT cells.

4.3.2 Fatty acid composition of mussel mantle tissue

The change in the total lipid content, measured as the total FAME content of mussel mantle tissue, and fatty acid composition of mussel mantle tissue were affected by

Table 4.1 Fatty acid profile (% of total fatty acids) and total lipid content (mean ± S.E.) of male and female *Mytilus edulis*. Data are pooled for pH, temperature and bacterial exposure. Significant differences ($p \leq 0.05$) are indicated by an asterisk, main contribution to group differences as measured by SIMPER are indicated by letters, with the three individual fatty acids that represent the greatest contribution marked in descending order (a-c). N = 133 individuals.

	Female	Male	Sign.
C14:0	2.13 ± 0.10	1.06 ± 0.11	
C15:0	0.37 ± 0.04	0.18 ± 0.02	
C16:0	18.60 ± 0.26	15.46 ± 0.25	a
C17:0	0.26 ± 0.03	0.38 ± 0.03	
C18:0	2.05 ± 0.09	3.15 ± 0.14	
C23:0	1.38 ± 0.28	1.98 ± 0.35	c
C24:0	1.76 ± 0.44	1.68 ± 0.41	b
Σ SFA	**26.54 ± 0.43**	**23.88 ± 0.54**	*
C16:1	13.59 ± 0.64	6.53 ± 0.41	a
C18:1	2.77 ± 0.20	1.30 ± 0.20	
C20:1	3.87 ± 0.16	3.33 ± 0.15	
C22:1	1.16 ± 0.27	1.47 ± 0.26	c
C24:1	0.73 ± 0.29	2.94 ± 0.53	b
Σ MUFA	**22.11 ± 0.78**	**15.57 ± 0.60**	*
C18:2	1.05 ± 0.07	0.85 ± 0.07	
C18:3 n3	1.86 ± 0.12	2.45 ± 0.23	
C18:3 n6	1.01 ± 0.07	0.87 ± 0.08	
C20:2	0.41 ± 0.05	0.46 ± 0.05	
C20:3	0.17 ± 0.03	0.22 ± 0.04	
C20:3 n3	ND	0.06 ± 0.06	
C20:4	3.14 ± 0.10	3.32 ± 0.12	
C20:5 n3 (EPA)	20.81 ± 0.34	23.14 ± 0.30	b
C22:2	0.55 ± 0.20	1.21 ± 0.26	c
C22:6 n3 (DHA)	22.35 ± 0.49	27.97 ± 0.43	a
Σ PUFA	**51.35 ± 0.79**	**60.54 ± 0.69**	*
Total lipid (µg/mg of dry weight)	120.88 ± 5.12	84.33 ± 3.1	*

SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids.

gender (Table 4.1). Females had a higher lipid content than males (Pseudo-F = 23.65, d.f. = 1, p = < 0.001), with a greater percentage of SFAs (Pseudo-F = 12.29, d.f. = 1, p = < 0.001) and MUFAs (Pseudo-F = 47.91, d.f. = 1, p = < 0.001), whereas males had a greater percentage of PUFAs (Pseudo-F = 81.91, d.f. = 1, p = < 0.001). SIMPER analysis identified three saturated fatty acids; 16:0, 24:0 and 23:0, that contributed over 70% of this gender dissimilarity in SFAs, with 16:0 contributing 30.27 % and having the largest contribution. With respect to the MUFAs, the three fatty acids shown to contribute most significantly to the difference between males and females were 16:1, 24:1 and 22:1, contributing 47.41 %, 19.33% and 12.65 % to the average dissimilarity respectively. Finally, of the PUFAs, the fatty acids shown to have the greatest contribution to the dissimilarity between males and females were two long-chain PUFAs; docosahexaenoic acid (DHA, 22:6n-3) and eicosapentaenoic acid (EPA, 20:5n-3). These essential fatty acids contributed 40.57 % and 22.47 % of the dissimilarity respectively, and alongside docosadienoic acid (22:2n-6), contributed over 70 % of the gender dissimilarity in PUFAs.

Whilst males and females had significantly different total FAME content in mantle tissue, they also responded differently to temperature. Temperature interacted with gender (Pseudo-F = 4.66, d.f. = 1, p = 0.036), with increased temperature shown to significantly increase the total lipid content in mantle tissues of males, yet in females temperature had no effect on lipid content (Table 4.2). Temperature also affected the percentage of MUFAs within mantle tissue (Pseudo-F = 5.75, d.f. = 1, p = 0.017), and interacted with gender to affect the percentage composition of PUFAs (Pseudo-F = 7.21, d.f. = 1, p = 0.009).

An increase from 12.5 °C to 17.0 °C decreased the percentage of MUFAs in both male and female mussels (Table 4.2), whereas an increase in temperature only

Table 4.2 Fatty acid profile (% of total fatty acids) and total lipid content (mean ± S.E.) of male and female *Mytilus edulis* maintained under control (12.5 °C) and increased (17.0 °C) seawater temperatures. Data are pooled for pH and bacterial exposure. Significant differences ($p \leq 0.05$) between mussels maintained at different temperatures, within gender, are indicated by an asterisk. N = 133 individuals.

	Female		Male		
	12.5	17.0	12.5	17.0	
C14:0	2.16 ± 0.15	2.08 ± 0.15	0.76 ± 0.12	1.48 ± 0.19	
C15:0	0.39 ± 0.05	0.35 ± 0.06	0.13 ± 0.02	0.25 ± 0.04	
C16:0	18.67 ± 0.33	18.52 ± 0.41	14.97 ± 0.36	16.18 ± 0.29	
C17:0	0.22 ± 0.03	0.30 ± 0.04	0.36 ± 0.05	0.41 ± 0.05	
C18:0	1.86 ± 0.12	2.26 ± 0.11	3.18 ± 0.19	3.12 ± 0.21	
C23:0	1.40 ± 0.40	1.35 ± 0.42	1.25 ± 0.37	3.05 ± 0.65	
C24:0	1.90 ± 0.60	1.59 ± 0.66	1.85 ± 0.57	1.43 ± 0.58	
Σ SFA	**26.61 ± 0.59**	**26.46 ± 0.66**	**22.50 ± 0.73**	**25.91 ± 0.65**	
C16:1	15.37 ± 0.96	11.47 ± 0.64	6.56 ± 0.60	6.48 ± 0.53	
C18:1	3.07 ± 0.28	2.41 ± 0.26	1.09 ± 0.31	1.62 ± 0.17	
C20:1	3.88 ± 0.18	3.85 ± 0.28	3.21 ± 0.20	3.50 ± 0.23	
C22:1	0.76 ± 0.35	1.62 ± 0.42	1.41 ± 0.35	1.56 ± 0.38	
C24:1	0.74 ± 0.42	0.71 ± 0.40	3.97 ± 0.75	1.44 ± 0.60	
Σ MUFA	**23.83 ± 1.19**	**20.07 ± 0.81** *	**16.23 ± 0.93**	**14.60 ± 0.56**	*
C18:2	1.04 ± 0.10	1.07 ± 0.11	0.70 ± 0.08	1.07 ± 0.11	
C18:3 n3	1.70 ± 0.18	2.05 ± 0.15	2.68 ± 0.36	2.11 ± 0.23	
C18:3 n6	0.96 ± 0.09	1.06 ± 0.10	0.77 ± 0.09	1.01 ± 0.13	
C20:2	0.42 ± 0.06	0.39 ± 0.08	0.48 ± 0.07	0.44 ± 0.07	
C20:3	0.16 ± 0.05	0.19 ± 0.05	0.17 ± 0.05	0.30 ± 0.08	
C20:3 n3	ND	ND	0.10 ± 0.10	ND	
C20:4	3.09 ± 0.13	3.20 ± 0.17	3.27 ± 0.16	3.40 ± 0.18	
C20:5 n3	20.45 ± 0.52	21.24 ± 0.42	23.12 ± 0.43	23.18 ± 0.38	
C22:2	0.21 ± 0.15	0.96 ± 0.39	1.18 ± 0.34	1.25 ± 0.41	
C22:6 n3	21.52 ± 0.76	23.32 ± 0.56	28.80 ± 0.58	26.73 ± 0.58	
Σ PUFA	**49.56 ± 1.17**	**53.47 ± 0.91** *	**61.27 ± 1.01**	**59.48 ± 0.83**	
Total lipid (µg/mg of dry weight)	124.05 ± 7.19	117.10 ± 7.58	77.63 ± 3.51	94.15 ± 5.05	*

SFA: saturated fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids.

increased PUFAs in females, with the proportion of PUFAs in males exposed to either 12.5 °C or 17.0°C not significantly different (Table 4.2). As with gender, SIMPER analysis indicated that the three main fatty acids that contributed to the temperature dissimilarity of the MUFAs were 16:1, 24:1 and 22:1, contributing 41.14 % 22.44 % and 15.01 % respectively, whilst in females the fatty acids that have the greatest contribution to the temperature dissimilarity in PUFAs were DHA, EPA and 22:2n-6.

Bacterial exposure also affected the FAME content of mussel mantle tissues (Pseudo-F = 3.61, d.f. = 2, p = 0.032). Pair-wise analysis showed that mussels which were sampled prior to the bacterial exposure had a higher lipid content compared to mussels sampled post inoculation, again there was no significant difference between mussels sampled 1 day and 7 days post inoculation (Fig 4.7).

Whilst pH did not have a significant main effect on the fatty acid profile or total lipid content of mussels, it did interact with gender to affect the percentage contribution of MUFAs to the overall fatty acid profile of mantle tissue (Pseudo-F = 2.82, d.f. = 4, p

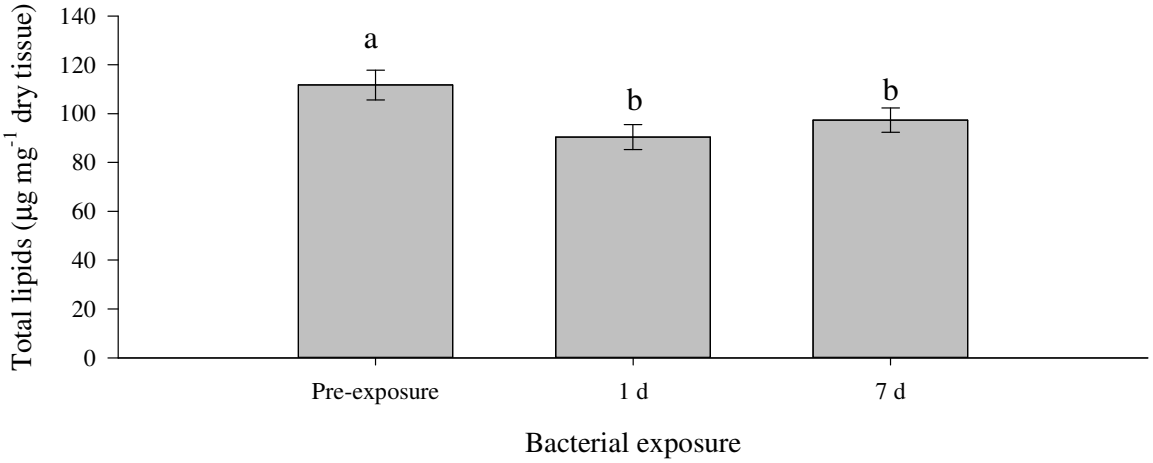

Figure 4.7 The effect of a bacterial exposure on total FAME content. Values are means (± S.E.), data are pooled for pH, temperature and gender. Significant differences ($p \leq 0.05$) are indicated by different letters based on pair-wise tests. N = 144 individuals.

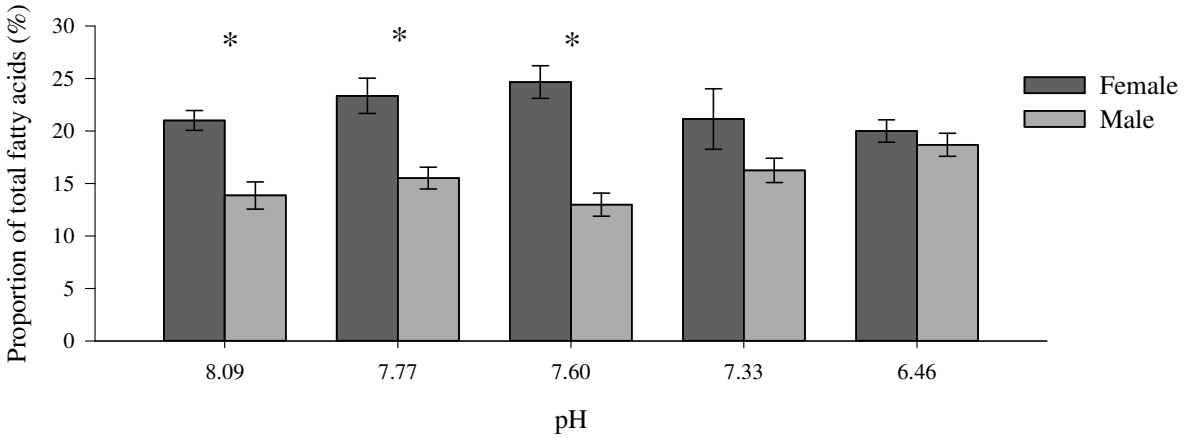

Figure 4.8 The effect of reduced seawater pH on the proportion of MUFA in the mantle tissue of male and female mussels. Values are means (± S.E.), data are pooled for both temperature and bacterial exposure. Significant differences ($p \leq 0.05$) between males and females at each pH are indicated by an asterisk based on pair-wise tests. N = 133 individuals.

= 0.019). At pH 8.05, 7.80 and 7.60 the percentage of MUFAs in mantle tissue of females was significantly higher than that noted in males, however a reduction in pH is shown to reduce the proportion of MUFAs in females yet increase these fatty acids in males (Fig 4.8). And although the increase in females and the decrease in males were not statistically significant within gender, it did mean that at pH 7.35 and 6.50 there was no significant difference between the sexes.

4.4. DISCUSSION

4.4.1. The effect of reduced seawater pH, increased temperature and gender on reproductive provisioning

When exposed to increased seawater temperature the energetic provision attributed to reproduction in the mussel, *Mytilus edulis*, is significantly reduced. This is marked by a

concurrent increase in both ADG cells and VCT cells at increased temperatures. These results support previous research where increasing seawater temperatures were shown to reduce the energy allocated to reproduction in marine mussels (e.g. Bayne et al., 1978; Fearman and Moltschaniwskyj, 2010). Furthermore, temperature was also shown to have a greater effect on the mantle tissues of females compared to that of males, a result that has not been shown previously but one that could have a significant impact on the population dynamics of this ecologically and commercially important species.

It is widely accepted that increasing temperature increases metabolism in ectothermic organisms (Somero, 2002). Therefore the reduced allocation of energy to reproduction in both males and females is likely due to the increased energetic demand of metabolism in mussels at increased temperature. However, in concurrently measuring an increase in ADG cells and VCT cells, which form the nutrient storage cells of mussel mantle tissue (Lowe et al. 1994), in addition to increasing the allocation of energy to metabolism, mussels also appear to maintain their ability to produce energy reserves. This pattern of reduced gamete investment, whilst maintaining a capacity for nutrient storage, has been suggested as a possible plastic response to prevailing environmental conditions in wild populations (Lowe et al., 1994). In measuring the composition of mussel mantle tissue monthly over three concurrent annual cycles from 1977 to 1980, Lowe et al. (1982) demonstrated a naturally occurring period of concurrent gamete production and nutrient storage after spring spawning in an estuarine mussel population, followed by a second period of spawning later in the year. Such a strategy may be a mechanism to maintain gamete production over an annual reproductive cycle, in this population sampled from Beggar's Island, at the intersection of the Rivers Tamar and Lynher, Plymouth, whilst reducing the energy required during any one period of gametogenesis. Reducing the energy required for any single spawning period would

then ensure that more residual energy was available for the increased metabolic requirements associated with unfavourable environmental conditions, whilst net gamete production would remain unaffected. However, the current study only lasted 3 months. Therefore to test this hypothesis fully, and to test the potential longevity of any such strategy under future conditions of oceanic warming, a longer term exposure to increased temperatures is required.

Whilst an increased proportion of nutrient storage cells may suggest mussels maintained the capacity for nutrient storage, it is also possible that such an increase merely reflects the proportional reduction in gamete investment. VCT cells are a rich store of glycogen within the mantle of mussels, however they are also the structural basis of mantle tissue, forming the interfollicular connective tissues (Lowe et al., 1982). In measuring an increase in nutrient storage tissues associated with a reduced gamete production in mussels exposed to increased temperatures, Fearman et al. (2010) also subsequently measured a reduction in glycogen levels. Therefore, whilst the fraction of the mantle that was attributed to nutrient storage appeared to increase, this did not provide an accurate assessment of the actual energy reserve in these tissues, merely reflecting a proportional increase in connective tissue. However, in grouping ADG and VCT cells as nutrient storage, the study by Fearman et al. (2010) was unable to account for any difference in the response of these two cell types. In the current study, measuring a significant increase in ADG cells, in addition to the increase in VCT cells, suggests mussels do maintain a capacity for increased nutrient storage, as ADG cells, which contain large reserves of protein and lipid as well as glycogen, have no structural function in mantle tissue (Lowe et al., 1994).

Unlike temperature, a reduction in seawater pH, at levels predicted to occur within the next 100 to 300 years (pH 7.80 and pH 7.35 respectively; IPCC, 2007), was

not shown to impact the reproductive investment in mussels. This supports previous research where the reproductive tissue of mussels was not impacted by reduced seawater pH, and where mussels maintained under acidification scenarios retained the ability to spawn (Beesley et al., 2008). Furthermore, in maintaining the proportion of ADG and VCT cells at a level comparable to those measured in control organisms, mussels in the current study maintained at a pH of 7.80 to 7.35 appeared capable of maintaining nutrient storage at reduced seawater pH. Such resilience to reduced seawater pH may be due to the naturally variable seawater carbonate chemistry that mussels experience in their natural habitat, as discussed in Chapter 3 (Sect. 3.4.1), leading to a natural pH tolerance in this organism.

In not detecting an impact of ocean acidification (OA) on reproductive provisioning, under seawater pH conditions predicted to occur within the next 100 - 300 years, the current study agreed with the findings of previous research (Beesley et al., 2008). However, in contrast to the research by Beesley et al. (2008), where there was no impact of very low pH seawater (pH 6.5) on reproductive processes, the current study demonstrated a significant reduction in the proportion of the mantle tissue attributed to reproduction in mussels maintained at pH 6.50. The apparent contradiction between this study and Beesley et al. (2008) could be due to the different seawater pH conditions experienced by mussels in the two studies (outlined in Sect. 3.4.1) or due to the different sampling methods employed. Beesley et al. (2008) qualitatively assessed the mantle tissue of mussels; however in using stereology the current study was able to gain a more objective assessment of the reproductive cycle (Morvan and Ansell, 1988). This enabled subtle differences in mantle composition to be assessed, which may have been missed by the qualitative approach used by Beesley et al. (2008).

In finding a reduced gamete provision within the mantle tissue of organisms maintained at pH 6.50, mussels exposed to very low pH were unable to maintain gamete production at a comparable level to organisms maintained under other pH conditions. However, in not being able to detect a significant change in the proportion of ADG cells at pH 6.50, this reduction does not appear to be accompanied by an increase in nutrient storage, as measured with increased temperature. Measuring a significant increase in VCT cells at pH 6.50 therefore likely represents a proportional increase in interfollicular connective tissues, proportional to the reduced gamete investment. The reduced energetic investment in reproduction is likely due to an increase in the energetic costs associated with maintaining cellular homeostasis or calcified structures at extremely low pH (e.g. Melzner et al., 2011; Wood et al., 2008, 2010), which in turn has led to a physiological trade-off. However the sustainability of this strategy will ultimately depend on the condition of impacted organisms, potentially impacting an organism's persistence.

Whilst temperature and low pH were shown to impact reproductive provisioning and nutrient storage in mussels, gender also significantly impacted the composition of mantle tissue. Males had a higher proportion of gametes in the mantle compared to females, irrespective of the exposure conditions. As discussed in Section 3.4.2., males are proposed to invest more energy in reproduction compared to females, being limited in their reproductive success by the number of females they can inseminate (Andersson, 1994; Trivers, 1972; Zuk et al., 2004). However, in not being able to detect a significant difference in the volume fraction of ADG cells between males and females, this increase in gamete production does not appear to be at the expense of nutrient storage cells within the mantle of males, and therefore must be at the expense of other physiological processes, or life-history traits. In measuring a reduction in immune defence (Sect.

3.4.2), males therefore appear to take a more risk prone strategy, reducing immunocompetence to maximise fecundity. The possible implications of such a strategy will be discussed in Chapter 6.

The interaction of temperature and gender affected the mantle composition of mussels. An increase in temperature significantly increased the proportion of VCT cells in females, but the proportion of VCTs in males was unaffected. When comparing the volume fraction of different mantle components in both sexes, it would appear this interaction between temperature and gender indicates a proportional reduction in gamete investment. An increase in temperature is shown to reduce gamete investment in both males and females, however this response is far more pronounced in females. The proportion of the female mantle attributed to reproduction is reduced by 14.7 % with increased temperature, whereas in males the same temperature increase reduces gamete investment by just 3.93 %. Therefore the significant increase in VCT cells with increased temperature in females represents the greater proportional decrease in reproductive tissues. Again, this would suggest males take a greater risk at high environmental temperatures by maintaining their energetic investment in reproduction to a greater extent than females, despite the unfavourable environmental conditions.

4.4.2 Effect of reduced seawater pH, temperature and gender on mantle lipid composition

Providing an important food reserve for larvae during the period of development until first feeding (Gallager and Mann, 1986; Gallager et al., 1986; Helm et al., 1973), it is the lipid content of the bivalve eggs that is a major determinant of larval fitness (Honkoop et al., 1999). With this lipid largely derived maternally, it is not surprising

that in the present study gender was shown to significantly affect both total lipid content and fatty acid composition of the mantle tissue in mussels. Despite being shown to have a higher proportion of mantle tissue attributed to gametes, the lipid content within the mantle of males was lower than that measured in females. This is likely due to the different strategies of reproduction between male and female mussels, and is a similar to the response measured in other bivalve species (e.g. Ansell, 1974; Caers et al., 1999; Napolitano and Ackman, 1992). Reproductive success in male sessile invertebrates is a function of total gamete production, with the number of gametes produced ultimately influencing the probability of successful fertilization (Yund and McCartney, 1994). In females however, reproductive success is governed to a greater extent by the quality of individual gametes (Levitan and Petersen, 1995), with a greater energetic investment in individual gametes increasing the probability of larvae surviving until first feeding. Therefore, measuring a higher lipid content in the mantle of females, despite having a lower number of individual gametes, represents the important role of females in providing energy reserves for the developing embryo (Blackmore, 1969).

The major fatty acids found in the mantle tissue of mussels were the SFAs 16:0, 18:0, the MUFAs 16:1, 20:1 and the PUFAs 20:4, DHA and EPA. This fatty acid profile was similar to that found in previous studies on mussels (e.g. Alkanani et al., 2007), with PUFAs contributing the greatest proportion of total fatty acids. PUFAs, and in particular EPA and DHA, are shown to be essential fatty acids in molluscs, particularly bivalves, forming an important tissue component which is crucial for growth and survival (Langdon and Waldock, 1981; Pettersen et al., 2010; Soudant et al., 1999). Whilst the fatty acid profile in mussels is essential for survival and growth, gender was also shown to affect the composition of major fatty acids in mantle tissue. Females were shown to have a higher percentage of SFAs and MUFAs, whereas males were shown to

have a higher proportion of PUFAs. This result supports previous research, where females of the prosobranch mollusc, *Patella depressa*, had a higher total lipid content as well as a higher proportion of SFAs and MUFAs (Brazão et al., 2003).

Fatty acids such as 16:0 (palmitic acid) are generally characterised as having an energetic-type function (Bergé and Barnathan, 2005), with SFAs and MUFAs forming an important energy source in the female gonad that is transferred to the developing embryo (Blackmore, 1969). With the energy from saturated fats released more efficiently than from unsaturated fats (Brown et al., 1997), the higher percentage of SFAs and MUFAs in the female gonad is likely due to their function in providing metabolic energy to developing embryos (Brazão et al., 2003). This is further supported by research that has shown larval mortality in mussels decreases proportionately with a higher SFA content (Pettersen et al., 2010). In contrast to female gametes whose function is the provision of energy, the gametes in male mussels are known to be composed largely of structural polar lipids, rich in PUFAs (Blackmore, 1969). Therefore, the higher percentage of PUFAs, yet lower SFA and MUFA composition, in males is likely due to the different role of gametes, and thus the different gamete composition, between genders.

Whilst PUFAs were greatest in males, they also formed the largest portion of the fatty acid profile in females. Female gametes require a large reserve of energy to ensure larval survival, however they also require a large proportion of structural polar lipids, rich in PUFAs, to provide the material required for the process of cell division (Napolitano and Ackman, 1992). Therefore a high proportion of PUFAs in female mantle tissue, such as DHA which is shown to have an important structural-type function (Bergé and Barnathan, 2005), highlights the additional role of females in also providing the structural material required for early larval development and cell division.

The importance of PUFAs for larval development in mussels has been further supported by Pettersen et al. (2010), who have shown the proportion of DHA to be positively correlated with larval survival, and the ratio of n-3 LC-PUFA (namely EPA and DHA) to the n-6 LC-PUFAs (20:4 n-6, arachidonic acid, ARA) to be positively correlated with settlement. Unfortunately, in the present study it was not possible to distinguish n-3 and n-6 ARA, and therefore the ratio of n-3 to n-6 LC-PUFA could not be compared between the two studies.

Alongside being affected by endogenous factors, such as sexual maturation and gender (Brazão et al., 2003; Galap et al., 1999), a fluctuation in environmental conditions is shown to impact the lipid composition in molluscs (Brazão et al., 2003; Pazos et al., 1997). Largely governed by the stage of gonad development and the quantity and quality of food available, the accumulation and depletion of lipid reserves in molluscs is also affected by the impact of environmental factors on metabolic activities (Pazos et al., 1996). In the present study temperature was shown to impact both the lipid content and the fatty acid composition of mussel mantle tissue, however interestingly the mantle tissue of male and female mussels was affected by temperature differently. An increase in temperature was shown to significantly increase the total lipid content of male mantle tissue, whereas in females the lipid content was shown to decrease slightly, although this decrease was not statistically significant. The difference in the response of the lipid content in males and females may be due to the impact of temperature on reproductive provisioning and the different composition of gametes between the genders. An increase in temperature was shown to slightly decrease the allocation of resources to reproduction in males, which was concurrently marked by a slight increase in ADG storage cells. This reallocation of resources from gametes, which in males have a lower provision of energy than in females, to ADG storage cells may

therefore result in the increased lipid content. In females, however, gametes are characterised by their large reserve of lipids with an energy-type function, which support larvae until first feeding. A decrease in resource allocation to gametogenesis in females would likely result in a large decrease in overall lipid content. Therefore, with male mussels increasing lipid content at increasing temperature, with females maintaining lipid reserves despite reduced gamete investment, this result appears to support the hypothesis that despite reducing gamete investment at increasing temperatures, mussels maintain an ability to lay down nutrient reserves, as discussed in Section 4.4.1.

Whilst temperature affected lipid content differently in males and females, an increase in temperature also affected the fatty acid profile. The proportion of MUFAs was significantly reduced under increasing temperatures in both males and females, whereas temperature only increased the proportion of PUFAs in females. To maintain membrane fluidity under decreasing temperatures, the proportion of unsaturated fatty acids generally increases (Pazos et al., 1997). Therefore, in showing mussels maintained at 12.5 °C to have a higher proportion of MUFA compared to mussels maintained at 17.0 °C, the current study supports previous research where increased temperature corresponded to reduced unsaturation in bivalve molluscs (Piretti et al., 1988). Furthermore, whilst not statistically significant, increased temperature may have resulted in a slight decrease in the proportion of PUFAs and a slight increase in the proportion of SFAs in the mantle of males, which further supports the remodelling of membrane lipids under decreasing temperature, a process known as homeoviscous adaptation (HVA) (Hazel, 1995; Pernet et al., 2007).

Conversely, in females an increase in temperature was shown to lead to a significant increase in PUFA, alongside a slight decrease in SFA, although this decrease

in SFA was not significant. The response in females is not consistent with temperature induced remodelling of membranes due to HVA in mussels. As with total lipid content however, it is likely this difference in the response of the fatty acid profile of males and females is due to the strong influence of the gametogenic process on the lipid composition in mussel mantle tissues. With an increase in temperature shown to reduce the provision of energy to reproduction in females, the proportion of SFA and MUFA allocated to developing gametes would be reduced. Therefore, the reduction of SFA and MUFA allocated to gametes under increased temperature in females may mask the small fluctuations in fatty acid profile brought about by temperature induced HVA. Such a response was noted by Pazos et al. (1997), where the impact of temperature on the fatty acid profile of female gonads in the scallop, *Pecten maximus*, was masked by the strong influence of the gametogenic cycle. Furthermore, the increase in PUFAs in females at increasing temperature may also represent a proportional increase due to the decrease in both SFA and MUFA provision in female gametes.

Unlike temperature, reduced seawater pH did not have a significant main effect on the total lipid content or fatty acid composition of mussel mantle tissue. This is perhaps surprising, given the impact of OA on the provision of resources to reproduction in mussels, with organisms maintained at pH 6.50 reducing the proportion of the mantle attributed to reproduction yet not concurrently increasing the proportion of nutrient storage cells. However, whilst pH did not directly affect the lipid composition of mantle tissue directly, reduced pH was shown to interact with gender to affect the proportion of MUFA in mantle tissue of mussels. At pH 8.05 to 7.60 females are shown to have a higher proportion of the mantle attributed to MUFAs compared to males, whereas at pH 7.35 and pH 6.50 this gender difference is shown to disappear. With MUFAs representing an important energy source to developing gametes in

females, and with male gametes largely dominated by structural-type PUFAs (Blackmore, 1969), it is possible that the reduction in reproductive provisioning at low pH may affect the difference in mantle lipid composition between gender. However, whilst OA is shown to interact with gender, this is the first study to have investigated the impact of OA on the lipid composition in the mantle of male and female mussels. Therefore it is not possible to distinguish the exact mechanism by which pH may affect lipid composition differently between males and females.

4.4.3 Impact of a bacterial exposure on reproduction and the lipid profile of the mantle

In the current study, an immune challenge was shown to significantly reduce the total lipid content of mantle tissue in the blue mussel, *M. edulis*. This reduction in lipid content highlights the significant cost of a pathogenic challenge in mussels, and supports previous research where exposure to a pathogen was shown to reduce both organism condition and energetic reserves in a bivalve host (Dittman et al., 2001; Ford and Figueras, 1988). Similarly, in a study investigating the impact of *Vibrio tapetis* on the condition and energy budget of the Manila clam, *Ruditapes philippinarum*, Flye-Sainte-Marie et al. (2007) demonstrated that an exposure to a bacterial pathogen led to a significant weight loss in this clam species, when compared to uninfected hosts. These authors therefore proposed that the reduction in weight, alongside a reduced host condition, was due to the energetic burden of the pathogen on the host, with the energy mobilised for the development of an immune response depleting the hosts energy reserves (Flye-Sainte-Marie et al., 2007; Palliard, 2004).

Whilst it is possible that an increased immune response led to decreased energy reserves in mussel mantle tissue, it is also possible that this depletion was due to other

processes, such as a direct impact of bacterial respiration on the host's energy budget or as an indirect result of a host spawning. However, in calculating the energy consumption of a *Vibrio* population on a host's metabolism, Flye-Sainte-Marie et al. (2007) estimate that even in a bacterial population shown to have a high growth rate, bacterial respiration was calculated to use less than 1% of the host's total metabolised energy. Furthermore, in not measuring any reduction in the proportion of the mantle tissue attributed to ripe gametes, and in not measuring any increase in regression observed in mantle tissues, bacterial exposure was not shown to lead to a spawning event or to a significant increase in the reabsorption of gamete lipid reserves. Therefore, the reduction in the lipid content of mantle tissue in mussels exposed to a bacterial challenge is likely to be as a result of an enhanced immune response.

Reproduction, similarly to immune defence, is energetically expensive (Williams, 1966), therefore under stressful environmental conditions reproduction is likely to be compromised to ensure survival (Wingfield and Sapolsky, 2003). With lipids providing an important energy reserve in bivalves (Gallager and Mann, 1986; Gallager et al., 1986; Helm et al., 1973), any reduction in the lipid content of mantle tissues, as a result of a bacterial challenge, would therefore be expected to reduce the resources available for other life-history traits, leading to a physiological trade-off. Subsequently, in measuring a reduction in the energy allocated to reproduction when exposed to *V. tubiahsii*, measured as a reduced proportion of the mantle attributed to newly developing gametes, the current study demonstrated that increased immunocompetence was shown to lead to the physiological trade-off against reproduction, as predicted. This supports the findings of Kelly (2011), who measured the impact of a bacterial exposure in the insect, *Hemideina crassidens*. Exposure to this pathogen led to both a reduction in the body condition, measured as a reduced body fat

content, as well as a reduction in the number and quality of eggs in this terrestrial insect. This result led Kelly (2011) to suggest that both immunocompetence and reproduction are condition dependant, and furthermore that these two physiological processes trade-off against each other to maintain organism fitness, as was shown in the current study.

Whilst having predominantly been demonstrated in vertebrates and terrestrial insects, the up-regulation of host defence is shown to lead to a reduction in the energy allocated to reproduction in a number of species (Bonneaud et al., 2003; Jacot et al., 2004; Kelly, 2011; Kerr et al., 2010). However, being paid in terms of future reproductive success and survival, the costs associated with reproduction are the most prominent for life history (Roff, 1992; Sibly and Calow, 1986; Stearns, 1992). Impaired or suppressed reproduction has significant consequences for the population dynamics of a species and, in the most extreme cases, can lead to a reduction in species persistence (Petes et al., 2008). Therefore, in measuring a reduced investment in reproduction in response to a pathogenic exposure, the current study highlights the potential impact of emerging diseases, and of a pathogenic outbreak, on the population dynamics of the blue mussel, *M. edulis*.

Moreover, rising summer temperatures are shown to increase both the spread and severity of shell-fish pathogenic outbreaks (Elston et al., 2008), and an increase in global temperatures is shown to increase the occurrence of these outbreak events (Martin et al., 2010). Therefore, over the next 100 years anthropogenic climate change could have an additional indirect, but highly significant impact, on bivalve populations, through the mediation of host-pathogen interactions and through the increasing influence of disease outbreaks.

4.4.4 Conclusions

In the current study anthropogenic climate change predicted to occur within the next 100-300 years is shown to significantly impact reproductive provisioning in mussels. However, it is an increase in temperature, rather than a reduction in seawater pH that is shown to have the greatest impact on metabolic investment in gametes. Mussels exposed to increased seawater temperature significantly reduced gamete investment, yet mussels exposed to an OA scenario predicted to occur within the next 100 - 300 years appear tolerant of moderate changes in seawater carbonate chemistry, with both reproductive provisioning and mantle lipid composition remaining unaffected. When exposed to reduced seawater pH predicted to occur with a catastrophic CCS leak however, the reproductive provisioning in mussels is significantly reduced.

Whilst this response to temperature has been shown in previous bivalve studies, this is the first study to demonstrate a different response to environmental stress in male and female mussels. Males and females are known to employ a different reproductive strategy, which in the current study resulted in different level of reproductive investment and a different mantle fatty acid composition between the two sexes. However, when exposed to challenging environmental conditions males are shown to take a more risky strategy, maintaining gamete investment at a higher level than was shown in females. Such a discovery could have significant consequences for the understanding of anthropogenic climate change impacts on the population dynamics of mussels.

Alongside measuring a significant impact of gender and anthropogenic climate change on the reproductive investment in mussels, this study also highlighted a significant impact of a bacterial exposure on the total lipid content of mussel mantle

tissue, reducing the energetic reserve within mussels. This reduction in available energy led to a subsequent physiological trade-off, measured as a reduction in energy afforded to developing gametes. In showing a significant cost of a pathogenic exposure, this study demonstrated that both reproduction and immunocompetence are condition dependant and trade-off against each other in order to maintain host survival under stressful environmental conditions. Therefore to fully understand the impact of anthropogenic climate change on an organism's fitness, and to understand the impact of any additional environmental stressors, such as a pathogen exposure, it is crucial to account for any physiological trade-offs. This will then enable an understanding of the circumstances under which any increase or decrease in the allocation of resources to reproduction is profitable.

CHAPTER 5. MUSSEL METABOLOME

Metabolic responses in the mantle of the blue mussel, *Mytilus edulis* exposed to reduced seawater pH, increased temperature and *Vibrio tubiashii*: an NMR-based metabolomics approach

5.1. INTRODUCTION

Whilst traditional environmental monitoring programmes have attempted to develop biomarkers as indicators of ecosystem health, these studies have typically relied on the assessment of sentinel species and the measure of single test endpoints (Jones et al., 2008). Consequently, such approaches often fail to ascertain vital information concerning the mode of action of a particular stressor. This lack of information subsequently reduces our ability to understand which biological pathways are impacted, hampering our ability to predict the overall response of an organism to environmental stress (Lin et al., 2006). However, due to the technological development of molecular profiling techniques over the past two decades, our ability to investigate the composition of an organism's transcriptome, proteome and metabolome has vastly increased (Veldhoen et al., 2012). The application of these 'omics' techniques in environmental assessment has provided an additional level of understanding when monitoring ecosystem health, characterising the molecular signatures that signify an organism's ability to respond and/or adapt to changing conditions (Veldhoen et al., 2012). In displaying highly sensitive response profiles, molecular signatures have the potential to highlight the specific mode of action of an environmental stressor, and to provide the information required to understand which biological processes are being impacted and how an organism will respond to stress (Lin et al., 2006; Veldhoen et al., 2012). Whilst such techniques enable a previously unprecedented understanding of stressor impacts on an organisms physiological functioning, to interpret such information these 'omics' techniques require an accurate definition of the study species, collection site and season (Genard et al., 2012; Li et al., 2010; Philipp et al., 2012).

Metabolomics is the newest of these 'omic' approaches and is based on the study of low molecular weight endogenous metabolites within a biological sample (Lin et al., 2006; Tikunov et al., 2010; Viant, 2007). Enabling the cost-effective, unbiased and rapid analysis of a wide range of small-molecule metabolites simultaneously (Tikunov et al., 2010; Viant, 2007), metabolomics provides information on the functional status of an organism, which can in turn be related to its phenotype (Bundy et al., 2009; Spann et al., 2011). Metabolomics subsequently holds great promise as a research tool for environmental risk assessment (Ekman et al., 2008; Schock et al., 2010), for the development of environmental systems models (Bundy et al., 2008; Schock et al., 2010; Viant, 2008) and for the discovery of new biological insights (Bundy et al., 2002; Schock et al., 2010).

High-resolution proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy is a post-genomic metabolomic technique, combining the high throughput metabolic profiling capabilities of ^{1}H NMR with pattern recognition techniques to identify the metabolic differences between samples (Fiehn, 2002). Involving the rapid cessation of metabolic activity, following chemical exposure of a sample to liquid N_2, NMR-based metabolomics provides a holistic assessment of an organism's metabolic actions at the time of sampling (Tjeerdema, 2008). This method is therefore uniquely suited to detecting a large range of endogenous low molecular weight metabolites in a biological sample, due to the rapid processing of this technique, and the rich structural and quantitative information obtained (Wu and Wang, 2010). Yet, despite the advantages of applying metabolomics in environmental monitoring, to date much of this work has focused on vertebrate and terrestrial systems, with only a handful of studies having investigated the metabolome of aquatic species (Jones et al., 2008; Viant, 2007). Of the aquatic organisms studied it is perhaps not surprising that bivalves have received

particular attention. As outlined in Section 2.4, bivalves have traditionally been used for marine biomonitoring studies due to their life history and commercial importance (Dondero et al., 2006; Goldberg, 1986). The incorporation of metabolomic data into environmentally relevant results is therefore likely to be easier for this group than for many others (Jones et al., 2008).

With metabolomics providing a valuable technique for the investigation of environmental stressors, and with bivalves having received particular attention with respect to metabolomics in aquatic organisms, the application of metabolomics in bivalves holds great potential to increase the current understanding of anthropogenic climate change. To date only one study has investigated the impact of climate change stressors on the metabolic profile of a marine bivalve. Lannig et al. (2010) reported that exposure to reduced seawater pH led to an altered energy metabolism in the Pacific oyster, *Crassostrea gigas*, as measured by ^{1}H NMR spectroscopy. However, this study did not measure the impact of a concomitant increase in seawater temperature, instead assessing the ability of oysters to survive an acute temperature challenge following exposure to reduced pH. Anthropogenic climate change is projected to lead to a reduction in seawater pH and a simultaneous increase in seawater temperature (discussed in Sect. 2.1 and 2.2). Therefore studying the impact of these two stressors together, and in concert with additional stressors, arguably offers the most biologically relevant insight. Furthermore, the study by Lannig et al. (2010) did not take account of gender differences, and gender has been shown to significantly impact an organisms metabolic processes (Hines et al., 2007a). Consequently, to successfully detect a molecular signature of environmental stress, and to effectively decipher the information from such a technique, it is crucial to fully understand the phenotype of the organism concerned (Hines et al., 2007a, b, 2010).

In the present study, ^{1}H NMR-based metabolomics was applied to analyse the metabolomic response of the blue mussel, *Mytilus edulis*. The aim was to detect metabolic biomarkers that characterise exposure to reduced seawater pH and increased seawater temperature, to assess the impact of a pathogen exposure on the metabolism of blue mussels and finally to detect any difference in the metabolic response of male and female mussels exposed to these environmental stressors.

5.2. MATERIALS AND METHODS

The mussels used in this metabolomics study were collected, maintained and sampled exactly as described in Sections 3.2.1 and 3.2.2 respectively). Briefly, mussels were collected during Dec 2009, from an intertidal estuarine mussel bed in Exmouth, east Devon, before being returned to the PML mesocosm. Mussels were immediately placed in the experimental system (described in Sect. 3.2.1) and maintained for 90 days. During the experiment mussels were fed with *Isochrysis galbana* (30 mg dry mass mussel^{-1} day^{-1}). After an initial 90 day exposure to experimental conditions, one mussel was chosen at random from each replicate experimental chamber to sample the individual immune response, reproductive status, lipid content and metabolome. To study the mussel immune response haemolymph was extracted from the posterior adductor muscle to enable the antibacterial activity of cell-free haemolymph, total cell counts and differential cell counts to be measured (Sect. 3.2.3). In addition to measuring the immune response, tissue samples were taken to quantify reproductive status using histology (outlined in Sect. 4.2.1), GC-MS was used to quantify the fatty acid composition of mussel mantle tissue (described in Sect. 4.2.2. and 4.2.3) and the metabolite profile of mantle tissue was assessed using ^{1}H NMR spectroscopy (described

in Sect. 5.2.1, 5.2.2 and 5.2.3 below). After this initial sampling time point, the remaining mussels in the system were exposed to a pathogenic challenge, exactly as outlined in Section 3.2.2, with the aforementioned parameters being measured again 1 day and 7 days post inoculation.

5.2.1 Metabolite extraction.

Polar metabolites were extracted from the mantle tissue of mussels using the methanol/chloroform extraction method (Bligh and Dyer, 1959; Hines et al., 2007a, b, 2010), exactly as outlined previously in Section 4.2.2 for the extraction of lipids. Unlike the lipid extraction it was the methanol layer containing the polar metabolites that was collected for metabolomic analysis. Polar metabolites were collected using a Hamilton syringe and transferred to a 1.5 ml Eppendorf® centrifuge tube. Each metabolite sample was subsequently dried using a centrifugal concentrator (Thermo Savant, Holbrook, NY) and stored at -80 °C.

5.2.2 ^{1}H NMR Spectroscopy

Immediately prior to NMR analysis, dried polar extracts were resuspended in sodium phosphate buffer (0.1 M in 10 % D_2O and 90% H_2O, pH 7.0, containing 0.5 mM sodium 3-trimethylsilyl-2,2,3,3,-d_4-propionate (TMSP) chemical shift standard) (Hines et al. 2007a, b). Mantle tissue extracts were then analysed on a DRX-500 NMR spectrometer (Bruker Biospin, Coventry, UK; Fig. 5.1), equipped with a cryoprobe and operated at 500.18 MHz (at 300 K). One-dimensional (1-D) ^{1}H NMR spectra were obtained as described by Hines et al. (2007a). Spectra were obtained using excitation

Figure 5.1 Bruker DRX-500 MHz NMR spectrophotometer used to obtain ^{1}H NMR spectra. Image courtesy of J. Byrne, NERC metabolomics facility, University of Birmingham.

sculpting for water suppression (Hwang and Shaka, 1995) and using a 8.4 μs (60°) pulse, 6 kHz spectral width and a 2.5 s relaxation delay with water presaturation. A total of 64 transients were collected into 16 348 data points, requiring a 4.5 min acquisition time. Data sets were zero-filled to 32 768 points, before line-broadenings of 0.5 Hz were applied prior to Fourier transformation. To maximise metabolite discrimination two-dimensional (2-D) ^{1}H J-resolved (JRES) NMR spectra were also acquired (Viant, 2003), being processed according to Hines et al. (2007b). 2-D JRES spectra were acquired for each sample using 16 transients per increment, for 16 increments, which were collected into 16 000 data points with spectral widths of 6 kHz in F2 (chemical shift axis) and 50 kHz in F1 (spin-spin coupling constant axis). A 4.0 s relaxation delay

was employed resulting in a total acquisition time of 24 min. Data sets were zero-filled in F1, the F2 dimension was then multiplied by a SEM window function using 0.5 Hz line broadening while the F1 dimension was multiplied by a sine-bell window function, all prior to Fourier transformation. JRES spectra were tilted by 45°, symmetrised about F1 and calibrated using TopSpin (Bruker Biospin). Data were exported as the 1-D skyline projections of JRES spectra (pJRES) and converted to a format for multivariate analysis using custom-written ProMetab software in MATLAB (version 7.1; The MathsWorks, Natick, MA; Viant 2003).

5.2.3. Spectral pre-processing and statistical analysis

Each spectrum was segmented into 0.005 ppm bins between 0.6 and 10.0 ppm, with bins resulting from water and TMSP excluded from all spectra, and with data points between 7.988 and 8.016 ppm being compressed into a single point. Data were normalised using the Probabilistic Quotient approach and noise filtered, with the noise threshold set to 3 times the standard deviation of a region of known noise (9.5 – 10.0ppm). This produced a data matrix of 144 samples by 1000 bins. This matrix was then subject to a generalised log transformation using the lambda parameter 1.49 e^{-9}, which stabilised the technical variance across the bins (Parsons et al., 2007; Purohit et al., 2004).

To test the impact of reduced seawater pH, increased temperature, a bacterial exposure and organism gender, data were tested using the PERMANOVA+ add in (beta version; Anderson et al., 2008) in PRIMER 6.1 (Clarke and Gorley, 2006). Data were first tested for homogeneity of variance, as outlined in Section 3.2.5, and Euclidean distance similarity matrices constructed. P-values were calculated using 999

permutations of the residuals under a reduced model. Pair-wise comparisons were undertaken where a significant main effect, or an interaction between factors, was shown. Following pair-wise comparison, ordination of samples using non-metric multi-dimensional scaling (MDS) was performed to display the biological relationships between samples, highlighting sample relatedness, and subsequently sample grouping, in low-dimensional ordination space (Clarke and Warwick, 2001).

Where a significant main effect was shown using PERMANOVA, data were further tested using SIMPER analysis, using a Euclidean distance similarity matrix. SIMPER analysis determines the percentage contribution of individual variables, in this instance different shifts, to the overall group dissimilarity. Annotation of shifts shown to contribute over 50 % of the group dissimilarity was then undertaken, using the software FIMA (Unpublished), identifying those metabolites that were shown to be important in the separation of treatment groups, and furthermore indicating the direction of the changes within each treatment.

5.3. RESULTS

Experimental parameters and organism mortality recorded during this study are reported in Chapter 3 of this thesis (Sect. 3.3.1 and 3.3.2 respectively).

5.3.1. ^{1}H-NMR spectroscopy of mussel mantle tissue

A representative 1-D projection of a 2-D JRES NMR spectrum of mussel mantle tissue from *M. edulis* is presented in Figure 5.2. Several metabolite classes were identified, including amino acids (e.g. alanine), organic osmolytes (e.g. betaine, homarine), and

Krebs cycle intermediates (e.g. succinate). However, NMR spectra were found to be dominated by the key organic osmolytes betaine (3.27 and 3.91 ppm) and taurine (3.25 and 3.43 ppm), which were shown to have a 10 - 100 times higher intensity than all other metabolites.

5.3.2. Metabolic response to reduced seawater pH

Metabolic responses of mussels to environmental stress were investigated using PERMANOVA, which revealed a significant effect of reduced seawater pH on the mussel metabolome (Psuedo-F = 3.18, d.f. = 4, p = <0.001). As shown in Figure 5.3a exposure to reduced seawater pH resulted in significant separation of samples, with mussels exposed to pH 6.50 separating from all other pH treatments. This was further supported by pair-wise analysis, where the metabolome of mussels exposed to pH 6.50 was shown to be significantly different from all other samples. Additionally, whilst the MDS ordination of samples revealed minimal separation of mussels exposed to the other pH levels (Fig.5.3b), pair-wise comparison also highlighted a significant difference in the metabolic profile of mussels exposed to pH 7.60 and 7.35 compared to control individuals maintained at pH 8.05.

Of the successfully identified metabolites from within the mantle tissue of mussels, SIMPER analysis indicated 19 metabolites that were shown to contribute over 50 % of the dissimilarity between mussels exposed to pH 6.50, compared to all other pH levels. This group dissimilarity was largely caused by a decrease in metabolite concentration, with 14 out of the 19 identified metabolites shown to decrease (Table 5.1). Furthermore, of the five metabolites shown to contribute most significantly to the

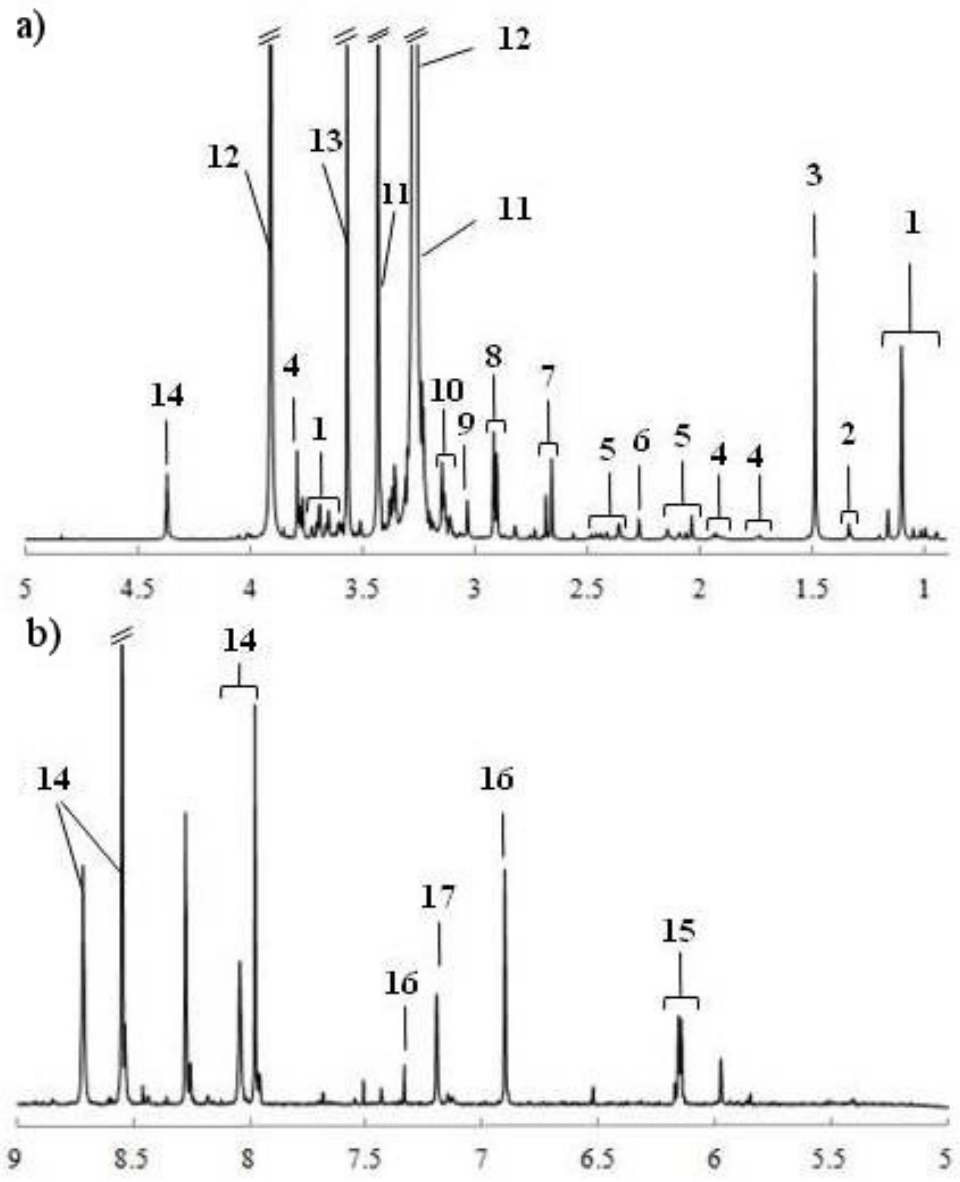

Figure 5.2 Representative one-dimensional 600 MHz ^{1}H NMR spectrum of mussel (*Mytilus edulis*) **a)** mantle issue extract and **b)** vertical expansion of the aromatic region. Keys: (1) Branched chain amino acids: isoleucine, leucine and valine, (2) lactate, (3) alanine, (4) arganine, (5) glutamate, (6) succinate, (7) aspartate, (8) asparagines, (9) lysine, (10) malonate, (11) taurine, (12) betaine, (13) glycine, (14) homarine, (15) ATP/ADP, (16) tyrosine and (17) histidine.

difference in mussels exposed to pH 6.50, valine (219.1 %), isoleucine (282.3 %), alanine (18.6 %), tyrosine (270.4 %) and succinic acid (219.5 %) were all shown to increase in concentration at this low pH.

Whilst exposure to pH 6.50 led to a general increase in metabolite concentration, interestingly it was a decrease in metabolite concentration in mussels exposed to pH 7.60 and 7.35 that was shown to contribute to the difference between these mussels and those in the control group. Of the 22 metabolites shown to contribute over 50 % of the group dissimilarity, 16 were shown to decrease in concentration compared to mussels at pH 8.05. The five metabolites shown to contribute most significantly to the difference between control mussels and those at pH 7.60 and 7.35, were alanine, shown to decrease by 27.9 %, succinic acid, shown to decrease by 44.7 %, and an unidentified peak at 1.10 ppm, which is shown to decrease by 2.2%, whilst glycine (2.6 %) and formic acid (24.4 %) were both shown to increase in concentration at pH 7.60 and 7.35.

5.3.3 Metabolic response to increased seawater temperature

As with reduced seawater pH, an increase in seawater temperature had a significant effect on the metabolome of mussel mantle tissue (Pseudo-F = 2.50, d.f. = 1, p = 0.01). However, minimal separation of samples was revealed in the MDS ordination based on seawater temperature (Fig. 5.3c). SIMPER analysis revealed that 23 metabolites were responsible for over 50 % of the group dissimilarity between mussels exposed to 12.5 °C and 17.0 °C. This group dissimilarity is predominantly caused by an increase in metabolite concentration, with 14 of the 23 metabolites shown to increase in mussels exposed to 17.0 °C, however a significant proportion of the metabolites were also shown to decrease in concentration, with 9 metabolites being reduced in mussels

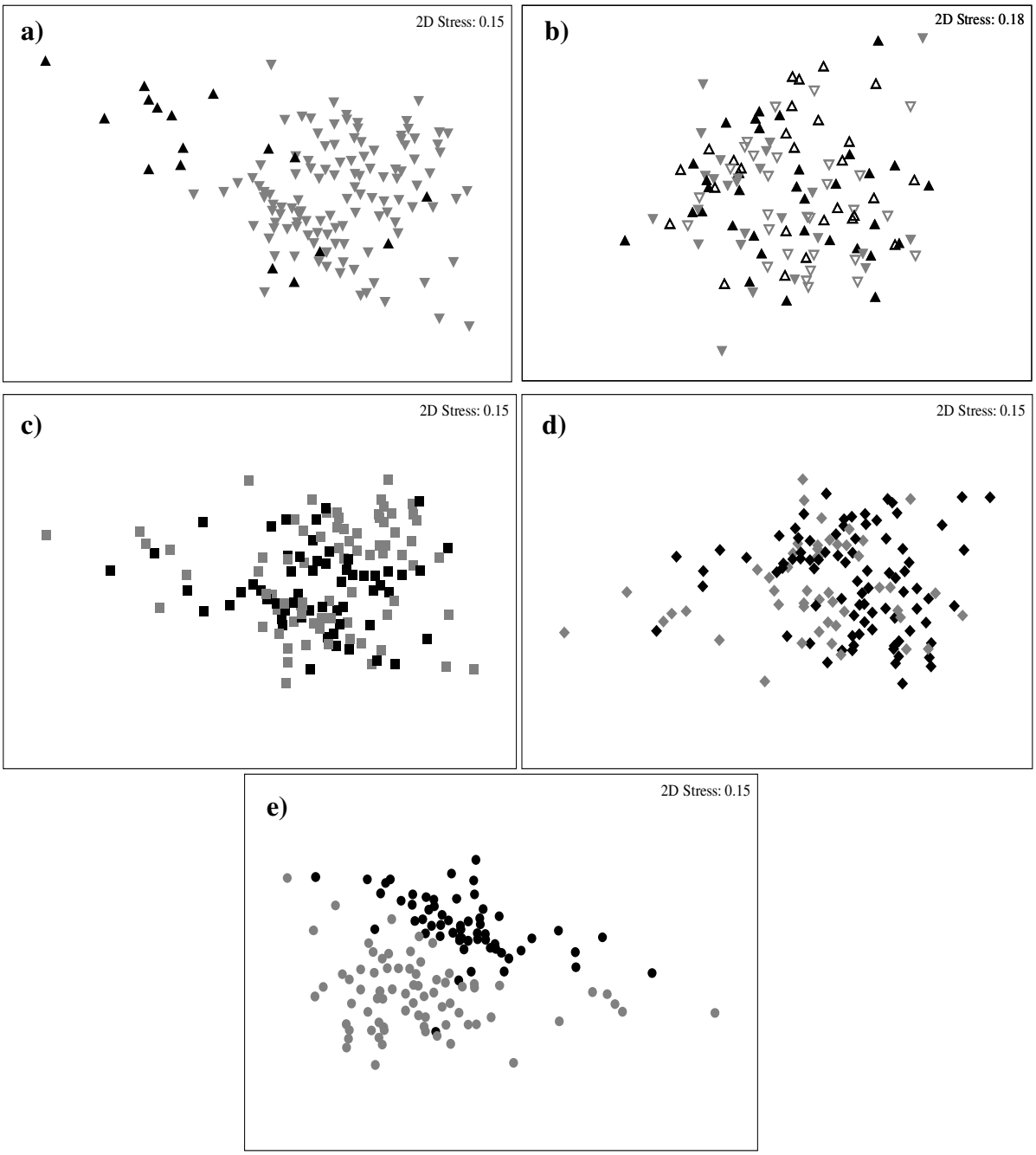

Figure 5.3 Non-metric multi-dimensional scaling ordination plots for the Euclidean distance similarity metabolite data for **a)** reduced seawater pH (▼= pH 8.05 – 7.35;▲= pH 6.5), **b)** reduced seawater pH (▼ = pH 8.05;▲ = pH 7.80,△ = pH 7.60; ▽ = pH 7.35), **c)** increased temperature (■ = 12.5°C; ■ = 17.0°C), **d)** a bacterial exposure (♦ = pre-bacterial exposure; ♦ = post inoculation) and **e)** gender (● = Female; ● = Male). N = 144 individuals.

Table 5.1 Relative changes in the metabolite concentration between treatment groups. Arrows represent the direction of change in metabolite levels. Relative metabolites have been selected as those which are shown to contribute over 50 % of the dissimilarity between different treatment groups as measured by SIMPER. Main contribution to group differences are indicated by letters, with the five individual metabolites shown to represent the greatest contribution marked in descending order (a-e). N = 144 individuals.

Metabolite	Effects of reduced seawater pH (pH 8.05-7.35 vs pH 6.5)	Effects of reduced seawater pH (pH 8.05 vs pH 7.60 + 7.35)	Effects of increased seawater temperature (12.5°C vs 17.0°C)	Effects of a bacterial exposure (Pre-exposure vs Post inoculation)	Effects of gender (Females vs Males)
3-Aminoisobutyric acid	54.7 % ↓	30.5 % ↑	100.1 % ↑	3.6 % ↑	902.2 % ↑
3-Methylhistidine		6.6 % ↓	12.7 % ↓		67.6 % ↑
Alanine	18.6 % ↑ c	27.9 % ↓ a	1.2 % ↑ a	27.2 % ↓ a	24.5 % ↓ a
AMP	18.6 % ↑	7.3 % ↓	9.2 % ↑	0.5 % ↓	51.9 % ↓
Arginine	9.9 % ↑	17.1 % ↓	1.4 % ↑	23.0 % ↓	8.6 % ↓
Asparagine	176.1 % ↑	19.0 % ↓	2.7 % ↑	23.5 % ↓	3.2 % ↑
Aspartic acid	53.7 % ↓		24.2 % ↑	17.1 % ↑	21.3 % ↓
Carnosine	114.1 % ↑	22.3 % ↑	5.9 % ↑	4.9 % ↑	42.3 % ↓
Dimethylamine		21.3 % ↑	20.6 % ↑	5.5 % ↑	27.3 % ↑
Formic acid	60.6 % ↓	24.4 % ↑ e	17.7 % ↓	18.1 % ↑	140.8 % ↑
Glutamine		27.1 % ↓	14.7 % ↑	2.7 % ↑	29.3 % ↓
Glutamine/Glutamate	217.3 % ↑	7.8 % ↓	1.9 % ↓		11.6 % ↑
Glycine	61.9 % ↓	2.6 % ↑ c	56.0 % ↓ e	23.2 % ↑ e	203.7 % ↑ c
Inosine 5 monophosphate	57.6 % ↓	0.5 % ↑	24.4 % ↓	13.8 % ↑	29.8 % ↑
Isoleucine	282.3 % ↑ b	17.4 % ↓	4.0 % ↓	43.8 % ↓ d	6.0 % ↑
Leucine	239.5 % ↑	5.9 % ↓	5.2 % ↓	40.7 % ↓	27.1 % ↓
Lysine	99.7 % ↑	5.2 % ↓	11.8 % ↑	20.5 % ↓	24.8 % ↑
Proline	97.9 % ↑	35.0 % ↓	25.7 % ↑	20.2 % ↓	23.3 % ↓
Succinic acid	219.5 % ↑ e	44.7 % ↓ b	26.6 % ↑ c	59.6 % ↓ c	28.9 % ↓ d
Threonine	140.4 % ↑	7.1 % ↓	8.7 % ↓	26.5 % ↓	11.8 % ↓
Tyrosine	270.4 % ↑ d	6.4 % ↓	17.9 % ↑	39.3 % ↓	4.4 % ↓
Unknown largae peak 1.098		2.2 % ↓ d	8.7 % ↓ d	12.3 % ↑	11.8 % ↑ e
Valine	219.1 % ↑ a	12.6 % ↓	0.7 % ↑ b	33.2 % ↓ b	4.7 % ↑ b

maintained at an elevated seawater temperature (Table 5.1). Of the five metabolites shown to contribute most significantly to group dissimilarity, an increase in temperature was shown to increase the concentration of alanine (1.2 %), valine (0.7 %) and succinic acid (26.6 %), yet decrease both glycine (8.7 %) and an unidentified peak at 1.10 ppm (8.7 %).

5.3.4 Metabolite response to a bacterial challenge

Whilst PERMANOVA demonstrated a significant impact of a bacterial challenge on the metabolite profile of mussel mantle tissue (Pseudo-F = 2.02, d.f. = 2, p = 0.009), MDS ordination of samples revealed little separation based on pathogen exposure (Fig. 5.3d). Subsequent pair-wise analysis indicated a significant impact of a pathogenic challenge but not exposure duration. Mussels measured prior to a bacterial exposure were shown to have a significantly different metabolome to those measured both 1 day and 7 days post inoculation. However, there was no significant difference between the metabolome of mussels sampled 1 d and 7 d post inoculation. SIMPER analysis revealed 21 metabolites that were responsible for over 50 % of this group dissimilarity. Of these, 9 metabolites were shown to increase in concentration following a pathogenic challenge, whilst 12 were shown to decrease (Table 5.1). The five metabolites shown to contribute most significantly to group dissimilarity between mussels measured pre- and post-bacterial exposure were alanine, shown to decrease by 27.2 %, valine, shown to decrease by 33.2 %, succinic acid, shown to decrease by 59.6 %, and isoleucine, shown to decrease by 43.8 %, whilst glycine was shown to increase by 23.2 %.

5.3.5 Metabolite composition of mantle tissue in male and female mussels

Whilst reduced seawater pH, increased temperature and a bacterial exposure were all shown to significantly impact the metabolome of mussels, PERMANOVA also demonstrated a significant impact of gender (Pseudo-F = 14.38, d.f. = 1, p = <0.001). Furthermore, this sample separation was also clearly demonstrated by MDS ordination (Fig 5.3e). SIMPER analysis indicated 21 metabolites that were responsible for over 50 % of the group dissimilarity between male and female mussels (Table 5.1). Males had a higher concentration of valine (4.7 %), glycine (203.7 %) and a compound that produced an unidentified peak at 1.10 ppm (11.8 %), whereas females had a higher concentration of alanine (24.5 %) and succinic acid (28.9%).

5.3.6 Metabolic response of mussels exposed to a combination of stressors

Whilst each of the experimental factors tested in this study were shown to impact the mussel metabolome independently, there was no significant interaction between these factors. Furthermore, whilst the metabolome of males and females was significantly different, there was no difference in the response of males and females to any of these experimental factors.

5.4. DISCUSSION

5.4.1. Effects of reduced seawater pH and increased temperature on the mussel metabolome

In showing a clear separation between the control and reduced seawater pH exposed groups, this study demonstrated that ocean acidification (OA) significantly affects the metabolic profile of mussel mantle tissue. Furthermore, as was shown for both immune system maintenance (Sect. 3.4.2) and for reproductive provisioning (Sect. 4.4.1), this effect became most apparent in mussels exposed to pH 6.50. Whilst an exposure to pH 7.60 and 7.35 was shown to impact the mussel metabolome, this was less pronounced, with no clear separation of these samples using MDS ordination.

Mussels exposed to pH 6.50 were characterised as having significantly higher levels of both alanine and succinic acid (also referred to as succinate), as well as valine, isoleucine and tyrosine. Constituting the major end-product in the anaerobic breakdown of glucose (Liu et al., 2011; Stokes and Awapara, 1968), alanine is an early indicator of acute anaerobiosis in marine bivalves (Grieshaber et al., 1994; Kurochkin et al., 2009), with its presence usually preceding an accumulation of succinate (De Zwaan et al., 1976; Michaelidis et al., 2005a). Therefore in demonstrating a significant elevation in both these metabolites in the mantle tissue of mussels exposed to pH 6.50, it would appear these mussels may have gone beyond their optimum range for aerobic performance, with a limited capacity for ventilation and cardiac performance at pH 6.50 leading to oxygen limitation and a reduced aerobic scope in this tissue (Frederich and Pörtner, 2000). Whilst metabolic rate and oxygen consumption were not measured in the present study, previous research showed that exposure to elevated CO_2 led to an

accumulation of succinate in both the gill and hepatopancreas of oysters, as measured by NMR-based metabolomics (Lannig et al., 2010). Furthermore, the study by Lannig et al. (2010) also measured a concomitant reduction in haemolymph oxygen concentration at a constant standard metabolic rate, which would suggest oysters exposed to reduced seawater pH experience anaerobiosis. Additionally, Michaelidis et al. (2005b) demonstrated that mussels exposed to reduced seawater pH experience a decreased rate of oxygen consumption and a marked decrease in their metabolic rate. Whilst Michaelidis et al. (2005b) did not measure alanine or succinate content in mussel tissues, these authors did measure a respiratory acidosis in the extracellular fluids of mussels, suggesting that the reduction in extracellular pH (pH_e) led to the reduced aerobic scope and the observed reduction in aerobic metabolism.

Extracellular pH has been shown to be an important determinant of metabolic rate in a number of organisms, with a reduction in metabolic rate occurring if pH_e drops below a certain threshold (Pörtner, 2008). Previous research has shown that mussels exposed to elevated pCO_2 have a poor capacity to control or compensate their extracellular pH (Thomsen et al., 2010). Subsequently, it is likely that the accumulation of both alanine and succinate in the mantle tissue of mussels exposed to pH 6.50 is therefore as a result of a drop in pH_e, reducing the scope for aerobic metabolism and thus increasing anaerobic metabolism.

In addition to measuring an increase in alanine and succinic acid in mussels exposed to pH 6.50, this study also measured an accumulation of valine, isoleucine and tyrosine in these organisms. Jones et al. (2008) measured an increase in the concentration of valine in mussels exposed to a combination of nickel, a trace metal, and chlorpyrifos, an insecticide, whilst Tuffnail et al. (2009) showed a hypoxia-related increase in valine levels in *M. edulis*. Furthermore, Liu et al. (2011) also measured an

elevated level of these branched-chain amino acids in the Manila clam exposed to mercury. These authors consequently proposed a disturbance in osmotic balance to be the cause of this metabolite accumulation, highlighting the role of the free amino acid pool in balancing intracellular osmolarity in molluscs (Viant et al., 2003). However, these oxidisable amino acids are also used extensively for energy metabolism (Tikunov et al., 2010). Therefore further study is required to confirm the mechanism by which these branched-chain amino acids increase in concentration in mussels exposed to pH 6.50.

Whilst exposure to pH 6.50 led to an increase in both alanine and succinic acid concentration, exposure to pH 7.60 and 7.35 resulted in a decrease in both amino acids. Furthermore, exposure to moderate seawater acidification led to a reduction in an unidentified metabolite (spectral peak 1.10 ppm), as well as an increase in the concentration of both isoleucine and glycine. Measuring a reduction in both alanine and succinic acid in mussels exposed to moderate seawater acidification would suggest they have not exceeded their aerobic scope at this pH. Indeed, as alanine can be transaminated to pyruvate as part of gluconeogenesis, a process that fuels an increased metabolic demand (Lannig et al., 2010), recording a decrease in alanine and succinic acid more likely suggests an increase in aerobic metabolism in these organisms. Such an increase in gluconeogenesis, and the subsequent decrease in alanine concentration as measured in the current study, has previously been recorded in fish and oysters exposed to moderate seawater acidification (Deigweiher, 2009; Lannig et al., 2010). Whilst gluconeogenesis was not measured in this present study it is interesting that Thomsen and Melzner (2010) also demonstrated an increase in metabolic rate in mussels exposed to pH 7.70, 7.38 and 7.14, compared to controls. These authors suggest that mussels exposed to moderate seawater acidification increase their metabolic rate to compensate

for the increased cellular energy demand and increased nitrogen loss experienced under these conditions.

As well as showing a decrease in alanine and succinic acid in mussels exposed to pH 7.60 and 7.35, mussels also show a reduced concentration of the unknown metabolite 1.10 ppm, whilst glycine and formic acid increased. The spectral peak at 1.10 ppm has been documented previously in NMR-based metabolomics, with this metabolite being important in the separation of control and cadmium exposed groups of the green mussel, *Perna viridis* (Wu and Wang, 2010). However, despite the impact of metal toxicity on this metabolite, its identity and therefore its mode of action remain unknown, as is also the case with formic acid at present. Conversely, glycine is one of the most commonly annotated metabolites in marine bivalves, being an important organic osmolyte, central to bivalve osmotic regulation. Previous research has demonstrated an increase in glycine in response to mercury (Liu et al., 2011), whilst an exposure to copper (Wu and Wang, 2010; Zhang et al., 2011), cadmium (Wu and Wang, 2010) and nickel (Jones et al., 2008) are all shown to decrease glycine concentration. As with valine and isoleucine, an alteration in glycine concentration is widely accepted as an indication of a disturbance to osmoregulation, therefore in measuring an increase in glycine at pH 7.60 and 7.35 likely indicates a disturbance to osmoregulation under moderate seawater acidification.

5.4.2 Effects of increased seawater temperature on the mussel metabolome

An increase in temperature is understood to lead to an increase in metabolism in ectothermic organisms (Somero, 2002). Subsequently, in showing alanine and succinic acid to be important factors in the separation of the control (12.5 °C) and increased

temperature (17.0 °C) mussel groups, the current study demonstrated such an impact of temperature on the energy metabolism in mussels. Interestingly however, as noted in mussels exposed to pH 6.50, individuals exposed to the increased temperature treatment in the present study were shown to have higher levels of both alanine and succinic acid in their mantle compared to mussels maintained at 12.5 °C. With an increase in alanine and succinate widely accepted as biomarkers of anaerobiosis (De Zwaan et al. 1976; Michaelidis et al. 2005a), this would suggest that at 17.0 °C the mussels used in the present study have reached their thermal tolerance limit and exceeded their aerobic scope. This is perhaps surprising, given the moderate level of warming used in the current study and the range of temperatures mussels would naturally be exposed to in their temperate intertidal estuarine habitat (Attrill et al., 1999; Morris and Taylor, 1983). One possible explanation for this response may be due to the difference between the temperature exposure used in the current study and the marked fluctuation of extreme temperatures mussels will naturally experience in the field. Estuaries are typically dynamic systems, with conditions fluctuating over a multitude of temporal scales, such as seasonal, diurnal and tidal cycles (Attrill et al., 1999). Therefore, whilst mussels will likely experience temperatures far in excess of 17.0 °C, often in concert with additional stressors such as air exposure, these exposures will often be of a much shorter duration (*circa* 6 hours) interspersed by periods of respite at cooler temperatures. Mussels may therefore have adapted to cope with such an exposure regime, with adaptation mechanisms having evolved in mussels to ensure survival of acute periods of stress exposure followed by periods in which organisms can restore physiological homeostasis. In the present study however, mussels were subjected to a prolonged exposure to increased seawater temperatures, as proposed to occur at the end of the current century (IPCC 2007), with no respite offered by periodic exposure to cooler

temperatures. Therefore, prolonged exposure to increased temperature may lead to a reduction in oxygen capacity, a reduction in aerobic scope and thus an increase in anaerobiosis. It is also possible however, that the increase in both alanine and succinic acid are as part of a tissue specific response, with mussels reducing metabolic demand and oxygen consumption in mantle tissues as a physiological trade-off to maintain homeostasis in other tissues, such as the gills or hepatopancreas. As a result, to fully understand the mechanism by which a moderate temperature increase impacts the overall mussel metabolic status, further study is required to investigate whole organism oxygen consumption and tissue specific metabolic profiles under stressful conditions. Nonetheless, in showing an altered energy metabolism in the mantle of mussels exposed to a warming scenario predicted to occur within the next century (IPCC, 2007), this study has emphasised the importance of this abiotic factor in determining organism performance even under a relatively moderate, yet stable, increase.

In addition to measuring an increase in alanine and succinic acid in mussels exposed to increased seawater temperatures, warming led to an increase in valine as well as a decrease in both glycine and an unidentified metabolite at 1.10 ppm. The increase in valine was also shown in mussels exposed to pH 6.50 (Sect. 5.4.1). It is likely that this increase in valine and reduction of glycine is due to a disturbance of osmoregulation or energy metabolism under increasing seawater temperatures, however further study is required to confirm this hypothesis. Additionally, the unidentified metabolite at 1.10 ppm was shown to be reduced by moderate seawater acidification, however with the identity of this metabolism remaining unknown, its mode of action cannot be understood at present.

5.4.3 Effects of a pathogen exposure on the mussel metabolome

Exposure to a pathogenic challenge is understood to be energetically costly (Flye-Sainte-Marie et al., 2007), therefore it is not surprising that in the current study a bacterial exposure was shown to have a significant effect on the mussel metabolome. As shown in mussels exposed to pH 7.60 and 7.35, an exposure to *Vibrio tubiashii* led to a decrease in both alanine and succinic acid concentration. As an increase in both alanine and succinic acid are clear biomarkers of anaerobic metabolism in marine molluscs (De Zwaan et al., 1976), this would suggest a pathogenic challenge does not lead to anaerobiosis in these organisms under the conditions of the current experiment. Furthermore, as was proposed with a moderate reduction in seawater pH (Sect. 5.4.1), it is possible that a reduction in both alanine and succinic acid indicates an increased energetic demand in infected mussels, and thus an increased metabolic rate. However, to confirm this hypothesis and to understand the full metabolic cost of a pathogen exposure further study is required taking into account seasonal factors and ideally a range of ecologically relevant pathogens for European mussel populations.

Alongside measuring a reduction in succinic acid and alanine, mussels exposed to a pathogenic challenge were shown to have lower levels of both valine and isoleucine in their mantle, compared to organisms studied prior to an inoculation, as well as increased levels of glycine. As discussed previously (Sect. 5.4.1), valine and isoleucine are branched-chain amino acids involved in both osmoregulation and energy metabolism in marine molluscs (Tikunov et al., 2010; Viant et al., 2003), whilst glycine is an important organic osmolyte (Liu et al., 2011). The accumulation of these metabolites is shown to be affected by trace metal contamination, hypoxia and an insecticide exposure (Jones et al., 2008; Liu et al., 2011; Tuffnail et al., 2009; Wu and

Wang, 2010; Zhang et al., 2011), indicating their importance as indicators of metabolic stress. However, whilst it is likely that it is a disturbance of energy metabolism or osmoregulation that has led to the altered levels of these metabolites in the present study, the exact mechanism resulting in altered accumulation remains unknown at present.

5.4.4 Effects of gender on the mussel metabolome

In demonstrating an effect of gender on the metabolome of mussel mantle tissue, this study supports previous research which has shown the metabolic profile in male and female mussels to differ significantly (Hines et al., 2007a, b). However, in contrast to previous research, in the present study the difference is predominantly caused by higher levels of alanine and succinic acid in females, and higher concentrations of valine, glycine and an unidentified metabolite at 1.10 ppm in males. Hines et al. (2007a) also showed that male mussels where characterised by a higher concentration of glycine. However these authors also demonstrated higher concentrations of phosphoarginine and glutamate in males, a difference that was not noted in the present study. Furthermore, in the study by Hines et al. (2007a), females were shown to have higher levels of acetoacetate, lysine, tyrosine and an unidentified metabolite at 3.69 ppm, whilst no significant difference in the levels of alanine or succinic acid were noted. The difference between the two studies may be due, in part, to the season in which the organisms were sampled. Hines et al. (2007a) studied the response of organisms collected during July, falling within the spawning period of the sample population. Conversely, in the present study the mussel metabolome was measured during winter. Therefore, the differences noted in the metabolome of male and female organisms between the current study and

that of Hines et al. (2007a) may be caused by a varying influence of reproductive processes on the metabolome at different times of the year. Conversely the different metabolic profile noted in the two studies may be due to species differences, caused by a slight difference in the reproductive strategy of the two study organisms. Whilst the present study used *M. edulis*, Hines et al. (2007a) investigated the metabolic profile of *M. galloprovincialis*. *M. edulis* is shown to have a shorter, more vigorous and concentrated spawning period compared to *M. galloprovincialis* (Bayne, 1976). Thus, a more distinct metabolic change might be predicted to occur in the ripe tissues of *M. edulis*, leading to a different metabolic profile and hence to different metabolites contributing to the gender differences. Such an impact of species on metabolite profile was subsequently demonstrated by Hines and colleagues in a subsequent study, where both the impact of gender and reproductive status were investigated (Hines et al., 2007b). In showing ripe *M. edulis* to differ in their metabolite profile compared to *M. galloprovincialis*, this second study by Hines et al. (2007b) outlined the subtle but significant differences in the metabolome of these two mussel species, highlighting that understanding an individual's species is crucial to accurately interpret any metabolomic data.

Whilst it is possible that the disparity between the two studies is due to species differences, it is also feasible this contrast is due to the time during the reproductive season at which mussels were sampled. In the present study mussels were collected in December and maintained until late March, a period during which mussels typically reconstitute energy reserves before undergoing gametogenesis (Lowe et al., 1982). However by sampling in July, typically a period in the middle of the mussel spawning season, the study by Hines and colleagues (2007a) investigated individuals later in their reproductive season, likely resulting in a different metabolic profile. Similarly, Hines et

al. (2007b) demonstrated an impact of season on the mussel metabolome. Whilst spawning was shown to lead to an increase in the glycine concentration of female *M. edulis*, spent males had a significantly lower glycine concentrations compared to ripe individuals. This impact of spawning led to a reversal of the gender difference in the concentration of glycine noticed in ripe mussels, leading the authors to suggest that investigators need to be cautious in using this metabolic marker as a determinant of gender assignment, and that as with species, understanding an individual's reproductive status will help in interpreting metabolomic data. Therefore in the present study, the fact that all the mussels included in the metabolomic study were ripe adults, or organisms still undergoing gametogenesis, this may have resulted in a different metabolic profile compared to mussels used by Hines et al. (2007a).

Interestingly, in showing both alanine and succinic acid to be important discriminates of gender, the present study highlights the importance of energy metabolism for differentiating between male and female mussels. Females are shown to have a higher level of both metabolites in their mantle tissue compared to males, suggesting anaerobic metabolism is more prevalent in female reproductive tissues. Given that males are widely understood to adopt a more risk prone strategy with respect to resource allocation and reproduction (as discussed in Sect. 3.4.2), this metabolic profile may therefore have been as a result of males investing more resources towards reproduction and thus reproductive tissues, potentially at the expense of other physiological processes under stressful environmental conditions. However, to test this hypothesis further study is again required to investigate the metabolic profile and oxygen consumption of other tissues within male mussels and to investigate the impact of anthropogenic climate change on the metabolic profile of mussels at different stages of the reproductive cycle.

5.4.5 Conclusions

In showing the metabolome of *M. edulis* to be impacted by reduced seawater pH, increased seawater temperature, a bacterial exposure and gender, the current study further highlights the potential of this post-genomic molecular technique for investigating the impact of environmental stress on marine invertebrate physiology. Unsurprisingly, exposure to reduced seawater pH, increased seawater temperature and a bacterial exposure were all shown to impact the energy metabolism in mussels, with both alanine and succinic acid contributing significantly to the group dissimilarity measured between impacted and control groups for each of these environmental stressors. Exposure to moderate seawater acidification is shown to lead to a slight increase in aerobic metabolism, whilst exposure to pH 6.50 led to anaerobiosis in the mantle tissues of mussels. Surprisingly, an increased seawater temperature expected to occur within the next century (IPCC 2007) was also shown to lead to an increase in metabolic biomarkers of anaerobic metabolism, despite this temperature being within the range of environmental temperatures mussels will be naturally exposed to in their estuarine intertidal habitat. In addition to measuring a cost of reduced seawater pH and increased temperature, a bacterial exposure was also shown to be metabolically costly, with a decrease in alanine suggesting increased aerobic metabolism. However, to fully understand the extent to which mussels increase aerobic respiration or the threshold at which they exceed their aerobic scope, under environmental stress requires the investigation of tissue specific oxygen consumption and metabolic profiles.

The free fatty acid pool, and specifically the metabolites valine, glycine and isoleucine are also shown to be important biomarkers of environmental stress in mussels in the current study. However, whilst these metabolites are the most predictive

parameters of environmental stress, the exact mechanism by which stress is impacting the mussel metabolism remains unknown. Branched-chain amino acids, such as glycine and valine, are important for both energy metabolism and osmoregulation in bivalves, and thus to understand how environmental stress is impacting mussel physiology again requires further study. Moreover, this study highlighted the importance of an unknown metabolite at 1.10 ppm for determining exposure to environmental stress, demonstrating its importance in the mussel stress response. Yet the identity of this metabolite is required to fully characterise and understand its mode of action.

Whilst environmental stressors are shown to significantly impact the mussel metabolome, as measured by ^{1}H-NMR based metabolomics; gender is also shown to be a key determinant of the metabolic profile in *M. edulis*. Furthermore, it is the same metabolites that are shown to be responsible for the group dissimilarity in organisms exposed to environmental stress that are also shown to be predictive of organism gender. This study therefore re-emphasises the importance of accounting for organism gender when investigating the impact of environmental stressors on marine invertebrate physiological functioning and when interpreting metabolomic data derived from perturbation experiments.

CHAPTER 6. OVERALL DISCUSSION AND CONCLUSIONS

6.1. INTRODUCTION

The aims of this thesis, as outlined in Chapter 1, were two-fold. To investigate the impact of environmental stressors on the mollusc immune response, providing empirical data on how anthropogenically induced stressors affect the invertebrate immune system, and how this in turn influences organism condition and physiological trade-offs. As discussed in chapter 2, this study focused on the impact of anthropogenic climate change, proposed as one of the greatest threats to marine ecosystems (Harley et al., 2006). Using a laboratory based experimental setup, adults of the blue mussel, *Mytilus edulis*, were exposed to a range of environmental temperatures and seawater pH levels predicted to occur over the next 100 – 300 years (IPCC, 2007). Assessing the impact of any stressor-induced immune suppression on organism disease resistance in the presence of the pathogenic bacterium, *Vibrio tubiashii,* this study investigated the impact of ocean acidification (OA) and ocean warming on mussel immunocompetence functionally (Chapter 3). Furthermore, in addition to measuring host defence, this study also investigated reproductive investment (Chapter 4), fatty acid composition of mantle tissue (Chapter 4) and the mussel metabolome (Chapter 5), with the aim of investigating the impact of anthropogenic climate change on organism condition as well as on the trade-off of resources allocated to different life-history traits.

The results obtained from this thesis have demonstrated that exposure to reduced seawater pH, increased temperature and a pathogen exposure induces a complex set of physiological responses in *M. edulis*. Mussels are seemingly tolerant of a moderate reduction in seawater pH, with immune response, reproduction and energetic resource sequestration remaining unaffected. However, when exposed to pH 6.50 mussel survival, immune defence, reproduction and metabolism are all significantly depressed.

Whilst exposure to OA had little impact on these organisms at a level predicted to occur by 2300, increased seawater temperature significantly affected reproduction and metabolism. Furthermore, increased temperature enhanced the sensitivity of mussels to low pH seawater, significantly reducing survival. However, perhaps the most notable result from this study is that an inoculation with the pathogenic bacterium *V. tubiashii* was shown to lead to an alteration in mussel energy allocation. Organisms exposed to pH 6.50 were shown to up-regulate host defence alongside a concurrent decrease in the energy allocated to reproduction. In exposing mussels to a pathogenic challenge, this study was able to demonstrate the complex physiological trade-offs employed by *M. edulis* exposed to environmental stress, yet failing to account for these trade-offs may lead to a misinterpretation of results and an inaccurate assessment of the sensitivity of mussels to anthropogenic climate change. Furthermore, as highlighted throughout this thesis it is crucial to account for the impact of gender, season and local adaptation when assessing the sensitivity of an organism to reduced seawater pH and increasing temperature.

Having above summarised the main experimental results obtained from this thesis, the remainder of this discussion chapter will first consider these results within the context of existing literature (Sect. 6.2). Section 6.3 will then outline the limitations of current research, highlighting the specific areas in which a concerted research effort is needed to help develop our understanding of environmental stressor impacts. Finally, any overall conclusions that can be drawn from this thesis will be presented in Section 6.4.

6.2. FROM METABOLITES TO META-ANALYSIS - THE RESPONSE OF MUSSELS TO ANTHROPOGENIC CLIMATE CHANGE

As calcifying marine organisms of significant commercial importance (FAO, 2010; Gestal et al., 2008; Gosling, 2003), mussels have received particular attention over the past decade with respect to OA and anthropogenic climate change. Yet despite being perceived as one of the most vulnerable groups to perturbations in seawater carbonate chemistry (Fabry et al., 2008; Kleypas et al., 2006; Orr et al., 2005), there is as yet no consensus on what the impact of anthropogenic climate change on marine mussels or other species of molluscs will be. The present study suggests that the inability to observe a significant mean effect in previous research may be due, in part, to the different experimental levels of acidification used in those studies or artefacts of the experimental design leading to a subsequent misinterpretation of results. Furthermore, as shown throughout this thesis, it is possible that failing to account for gender differences in the response of mussels to environmental stress may result in previous research having reported contrasting results. These contrasting results could in turn impede a conclusive meta-analysis of the sensitivity of mussels to OA. By considering the results of this thesis in light of previous research on *M. edulis*, the below subsections will discuss the likely impact of reduced seawater pH, increased seawater temperature and a pathogenic challenge on the survival of mussel populations in a future ocean.

6.2.1 Effects of reduced seawater pH on the immune response, reproduction and energy allocation in mussels

In showing the mussel immune response to be unaffected by a moderate reduction in

seawater pH, this present study appears to contradict previous research where mussels exposed to pH 7.7 and 7.4 were shown to significantly reduce phagocytic activity (Bibby et al., 2008) and lysozyme-like activity of cell-free haemolymph (Matozzo et al., 2012). Previous research has shown that the bivalve mollusc immune response is significantly affected by season, with organisms having a reduced immune response in summer, during their spawning period, compared to organisms studied in the spring or autumn (Matozzo et al., 2003; Pipe et al., 1995b). This reduced investment in immune system maintenance during summer could be as a result of a trade off between the cost of immunity and the energetically expensive process of spawning. As proposed in Chapter 3, it is possible that the difference between the current thesis and the studies carried out by Bibby et al. (2008) and Matozzo et al. (2012), may be due in part to the seasonality of the mussel immune response or to a difference in the sensitivity of the different populations used. In the present study, mussels were sampled during the winter, a period of energy reconstitution in these organisms (Lowe et al., 1982), whereas Bibby et al. (2008) sampled mussels during June, corresponding to the summer spawning period in the sampled population. However, whilst the seasonality of the immune system may partially explain this variation, a recent study by Matozzo et al. (2012) would appear to refute an overriding seasonal impact on organism response. These authors collected individuals during December, with organisms collected during winter still showing a reduction in host defence under OA scenarios (Matozzo et al. 2012).

Alternatively, it is possible that the difference between the present study, and that of Bibby et al. (2008) and Matozzo et al. (2012), is due to a different sensitivity of populations studied. The mussels used throughout this thesis were collected from an intertidal estuarine site, whereas the two previous studies collected individuals from

intertidal rocky shore locations. It is possible therefore that differences in the variability of carbonate chemistry parameters that exist naturally between these habitats (Borges and Gypens, 2010; Borges et al., 2006) may have led to local adaptation, and thus to different sensitivities of mussel populations to OA. Finally, it is possible that these differences are in part due to the complex speciation of the *Mytilus* genus in the north west Atlantic (Hilbish et al., 2002). In collecting mussels from Exmouth, east Devon, the current study investigated a population that is proposed to comprise entirely of pure *M. edulis* (Hilbish et al., 2002). Conversely, Matozzo et al. (2012) measured the response in *M. galloprovincialis*, whilst Bibby et al. (2008) investigated the response of a population of mussels from North Cornwall, proposed to fall within a *Mytilus* hybrid zone (Hilbish et al., 2002). Therefore, it is possible that the different responses noted in the three studies may be as a result of genotypic differences in the response of mussels to environmental stress.

As with host defence, this present study demonstrated that seawater acidification (pH 7.80 to 7.35) did not impact reproductive processes, or the lipid composition of mantle tissue (Chapter 4). In showing reproduction to be largely unaffected by OA, the present study supports the findings of Beesley et al. (2008). Thus it is concluded that the mussels used in the current study are seemingly tolerant of 'moderate' seawater acidification, at least at levels predicted to occur over the next 100 - 300 years. Thomsen et al. (2010) noted a similar tolerance to OA in *M. edulis* from a population in Kiel fjord, where seawater pH naturally falls below 7.5 during the summer and autumn months. The results from Thomsen et al. (2010), along with the findings from the present study, suggest mussels may be able to adapt to local carbonate chemistry conditions. Such increased tolerance, or local adaptation, may be critical in assessing the overall vulnerability of mussels to reduced seawater pH across their geographical

range. Indeed, Parker et al. (2011) argue that selective breeding of resistant populations may offer a vital mechanism to safeguard aquaculture brood stock, as well as natural populations, under future seawater conditions. Nevertheless, failing to account for variations in the response of different populations may hamper our ability to make accurate predictions on the likely fate of mussels in a future ocean.

Despite showing no impact of moderate seawater acidification (pH 7.80 to 7.35) on immune defence, reproductive provisioning or fatty acid composition, exposure to pH 7.60 and 7.35 seawater was shown to impact the mussel metabolome (Chapter 5), with a decrease in alanine levels indicating a possible increase in aerobic metabolism. This observation supports findings reported by Lannig et al. (2010), the only previous study to have use metabolomics to investigate the impact of OA on the bivalve metabolome. In showing a decreased level of alanine in oysters exposed to pH 7.7, Lannig et al. (2010) proposed that this increased metabolic flux is a mechanism to compensate for the increased energy demand during moderate stress. Similarly, Thomsen and Melzner (2010) also demonstrated an increase in routine metabolic rates, measured by an increase in rates of oxygen consumption, in mussels exposed to pH 7.70, 7.38 or 7.14. Previous research showed that exposure to reduced seawater pH significantly decreased calcification (Gazeau et al., 2007) and increased shell dissolution (Melzner et al., 2011) in mussels. It is therefore possible that the increased metabolic demand noted in the present study at pH 7.60 and 7.35 may be associated with the increased cost of maintaining net calcification under these conditions (Findlay et al., 2012; Melzner et al., 2011; Wood et al., 2008). In demonstrating that exposure to pH 7.60 and 7.35 led to a slight increase in the shell breaking strength in the mussels sampled in the present study, whilst shell composition remained unaffected, Pearce et al. (unpublished) highlight these organisms are indeed able to maintain net calcification.

Nonetheless, despite measuring an increased metabolic rate, mussels in the current study still appear able to maintain the energy allocated to other physiological processes, indicating a certain level of tolerance to reduced seawater pH. The extent to which this increase is sustainable longer term, and thus the perceived vulnerability of these mussels to OA, will ultimately depend on the energetic resources available to mussels, as well as organism condition (Melzner et al., 2011; Wood et al., 2008, 2010).

Exposure to extreme acidification (pH 6.50) resulted in a reduction in the mussel immune response, supporting the findings of Bibby et al. (2008) and Matozzo et al. (2012), albeit under a more severe acidification scenario. Furthermore, maintenance in pH 6.50 seawater resulted in a pronounced reduction in gamete investment, suggesting this low pH is exerting a significant energetic cost on these organisms. This hypothesis is further supported by the results from chapter 5, where mussels exposed to pH 6.50 were shown to switch from aerobic to anaerobic metabolism, indicating a disturbance of energy homeostasis. Whilst entering a metabolically depressed state may conserve energy, ensuring extended survival time under extreme stress (Guppy and Withers, 1999; Lannig et al., 2010), this strategy is unlikely to be sustainable in the long term. Prolonged exposure to pH 6.50 is therefore likely to impact a mussel's physiological performance and ultimately reduce survival.

Exposure to pH 6.5 is thought unlikely to occur in natural systems with the level of acidification projected to occur by the end of the current century, or indeed by 2300 (Caldeira and Wickett, 2003, 2005; IPCC, 2007). Importantly however, both the 0.5 unit decrease in seawater pH projected to occur by 2100 (Caldeira and Wickett, 2005) and the 0.7 unit decrease projected to occur by 2300 (IPCC, 2007) are largely derived from global models of surface seawater pH. These models have little resolution for regional variability and the complex influence of estuarine and upwelling systems (Feely et al.,

2010). With seawater pH already shown to fall below projected worst case OA scenarios in upwelling and estuarine ecosystems (Attrill et al., 1999; Feely et al., 2008; Thomsen et al., 2010), it is possible that mussels may in fact experience a reduced seawater pH of 6.50 due to OA. Furthermore, in many benthic (sessile) marine organisms the exchange of respiratory gases across the gills and/or general body surface results in the formation of a diffusive boundary layer, often resulting in a reduced seawater pH layer of up to 1 mm thick immediately surrounding the organism (Kühl et al., 1995). This can mean that the pH actually experienced by an organism may again differ greatly from that of the bulk water phase (Sand-Jensen et al., 1985). With recent research showing that OA exacerbates this boundary layer pH gradient (Flynn et al., 2012), it is again quite possible that benthic organisms may experience seawater pH levels far lower than models have predicted. In addition, extremely low levels of pH are projected to occur in conjunction with the possible leakage of CO_2 from a sub-seabed, geological storage reservoir (Blackford et al., 2009), which as reported here is likely to have a significant impact on the persistence of mussels in an impacted environment. The results obtained from this thesis at pH 6.50 are therefore pertinent to assessing the vulnerability of mussels under a variety of natural and anthropogenically induced scenarios of seawater acidification.

6.2.2 The response of host defence, reproduction and the mussel metabolome to increased seawater temperature

Unlike reduced seawater pH, in the present study increased seawater temperature was shown to have a significant impact on mussel host defence and reproductive provisioning at a level projected to occur in the next 100 years. Mussels maintained at

17.0 °C significantly increased the antibacterial activity of their cell-free haemolymph (Chapter 3), supporting previous research on the impact of temperature on the bivalve immune response (e.g. Chen et al., 2007a; Matozzo et al., 2012; Monari et al., 2007). This increase in immune system maintenance may represent an increase in the activity of hydrolytic enzymes at warmer temperatures, with mussels potentially experiencing an increase in immunocompetence due to increasing seawater temperature. Any increase in immune defence may prove to be a crucial factor in the survival of mussel populations under anthropogenic climate change conditions, with increased seawater temperatures also linked to an increase in the spread of pathogens (Elston et al., 2008; Martin et al., 2010) and to an increase in the occurrence of mass mortality events in bivalve populations (Li et al., 2009a).

In contrast to the response measured with host defence, an increase of 4.5 °C in seawater temperature was shown to significantly reduce the reproductive investment in mussels (Chapter 4). Individuals maintained at 17.0 °C reduced the proportion of the mantle attributed to gametes compared to the control. Any reduction in gamete production could have major a consequence for the persistence of mussels within an environment, with compromised larval production in turn jeopardising adult populations and marine communities (Byrne, 2011). Furthermore, when considered alongside previous research that showed larval bivalves to be particularly sensitive to anthropogenic climate change (e.g. Bechmann et al., 2011; Gaylord et al., 2011; Gazeau et al., 2010), any reduction in gamete investment in adults may compound the sensitivity of these calcified marine organisms during their early life cycle stages.

Although previous research demonstrated that mussels can reduce the energy allocated to reproduction under stressful environmental conditions (Petes et al., 2008), this present study is the first to demonstrate that males and females alter the investment

in reproduction differentially (Chapter 4). By reducing the investment in immune defence whilst maintaining reproductive investment, males may ensure that they are still able to successfully reproduce under stressful environmental conditions. Conversely, by significantly reducing the energetic investment in reproduction when exposed to stress, females may ensure maintained homeostasis and thus future fecundity.

In addition to gamete investment, gender also affected the fatty acid composition of mantle tissue and the impact of temperature on the fatty acid content (Chapter 4). Females had a greater fatty acid content, a greater proportion of SFAs and a greater proportion of MUFAs, compared to males. However, increased temperature was shown to increase the fatty acid content of mantle tissue in males, a response that was not found in females. Again this is the first study to have demonstrated a difference in the fatty acid composition of males and females exposed to increased temperature. With males and females responding differently to temperature, possibly as a result of different reproductive strategies under stressful environmental conditions, anthropogenic climate change could have significant consequences on the population dynamics of this species.

Perhaps the most unexpected result was that at 17.0 °C mussels appeared to have exceeded their aerobic threshold (Chapter 5), despite this temperature falling within the temperature range naturally experienced by this species. Furthermore, this response again appeared to be enhanced in females, with an increased level of alanine and succinate in females contributing to the difference between the male and female metabolome. In indicating a transition to anaerobic metabolism and a reduced reproductive investment at 17.0 °C, it is likely that seawater temperature, rather than a moderate reduction in seawater pH, will largely determine the impact of anthropogenic climate change on mussels over the next 100 years. This sensitivity to increased

temperature has already been shown to lead to a marked shift towards higher latitudes in wild mussel populations (Jones et al., 2009), as well as to a dramatic decline in the diversity of mussel bed communities over the past 50 years (Smith et al., 2006). Furthermore, in showing females to be particularly vulnerable to increased temperature, it is possible that rising seawater temperatures could lead to a significant shift in the dynamics of these populations.

Whilst increased temperature is proposed to lead to a general poleward shift in many organisms, recent research on mussel has shown this response, and the sensitivity of populations to increasing temperature, also varies both on a global and regional scale. Sorte et al. (2011) noted that populations of *M. edulis* in the Western Atlantic were more susceptible to increasing temperatures, compared to their Eastern Atlantic and Pacific counter-parts, indicated by a recent range contraction. Similarly Hilbish et al. (2012) noted a difference in the sensitivity of mussel populations to increasing temperature, as indicated by the *Mytilus* hybrid zone dynamics in the North West Atlantic. Whilst the hybrid zone in the English Channel was shown to shift approximately 100 km eastwards with continued warming over the past two decades, two similar hybrid zones along the Atlantic coast of France were not shown to change in position or shape over the same period. A temperature-induced shift in mussel speciation could affect the perceived susceptibility of mussel populations to additional environmental stressors if, as possibly indicated in this study, *M. edulis* is more tolerant to reduced seawater pH than *M. galloprovincialis,* or the hybrid of these two species. Alternatively, it is possible that the difference in susceptibility to anthropogenic climate change between these two species may reduce the distribution and spread of hybrid zones. As proposed by Bierne et al. (2003), habitat preference has a significant impact on the marine-speciation paradox, and whilst the hybrid zones of species such as *M.*

edulis and *M. galloprovincialis* could potentially spread over thousands of kilometres, in reality these hybrid zones are often characterised by a small-scale mosaic distribution influenced by the ecological barriers enforced by changing regional environmental conditions. Increasing temperatures could, therefore, potentially lead to an increase in the available habitat range of the more thermo-tolerant *M. galloprovincialis*, yet equally this could be counteracted by reducing seawater pH, and the possible enhanced vulnerability of *M. galloprovincialis* to moderate OA.

6.2.3 Impact of a bacterial challenge on Mytilus edulis *exposed to anthropogenic climate change*

Exposure to anthropogenic climate change is shown to have a significant impact on immune system maintenance in *M. edulis* (Sect. 6.2.2 and 6.2.3). Furthermore, the stress induced by altered environmental conditions also resulted in a reduction in the energy allocated to other important life-history traits, as well as to a shift in metabolism. However, whilst measuring an altered immune response could indicate immune dysfunction, as has been proposed previously (Bibby et al., 2008; Matozzo et al., 2012), the function of the immune system is to protect an organism from infection (Ellis et al., 2011) and arguably is best investigated in the presence of a pathogen (Viney et al., 2005). Following an inoculation with the pathogenic bacterium, *Vibrio tubiashii*, the present study noted that a pathogen exposure led to an increase in the mussel immune response. Furthermore, a bacterial exposure was also shown to lead to an increase in aerobic metabolism, as well as a reduction in the fatty acid content of mantle tissue and a reduction in the energetic investment in reproduction.

This is the first study to date to have investigated the trade-off between immune defence, reproduction and metabolism in organisms exposed to anthropogenic climate change. It is also the only study to date to have measured this response in the presence of a pathogen. Whilst exposure to *V. tubiashii* was shown to increase host defence in mussels, this was only noted in individuals maintained at pH 6.50. The immune system of mussels maintained at all other pH levels remained unaffected by this immune system challenge. Interestingly, when considered alongside the response of individuals sampled prior to a pathogenic challenge (as discussed in Sect. 6.2.2), it would appear that mussels exposed to pH 6.50 reduced the energy allocated to host defence. Crucially however, it appears mussels maintained the ability to increase host defence when required, in this instance when exposed to *V. tubiahsii*. Therefore, the initial reduction in the antibacterial activity of cell-free haemolymph measured in the absence of a pathogen likely indicates a physiological trade-off in the energy allocated to immune system maintenance, rather than a reduction in immune system functionality as previously suggested (Bibby et al., 2008; Matozzo et al., 2012). Consequently, without the context provided by a subsequent pathogen exposure, the complex response noted in the immune system of *M. edulis* exposed to reduced seawater pH and increased temperature could have been misinterpreted, altering the perceived susceptibility of this species to environmental stress.

In maintaining immune system plasticity, it appears that mussels in the current study were able to reduce the energy required to maintain host defence whilst not significantly reducing immunocompetence. However, a pathogenic challenge is known to be energetically expensive (Lochmiller and Deerenberg, 2000), with the resources required to mount an immune response being physiologically costly (Kelly, 2011). The cost of condition dependant life-history traits, such as reproduction and host defence,

must therefore trade-off against each other (Sheldon and Verhulst, 1996). In showing that mussels in the current study reduced their energetic investment in reproduction, as well as showing they have reduced fatty acid content in their mantle tissue, it appears immune-challenged mussels reallocate resources from energy storage and reproduction in order to overcome a bacterial exposure. Furthermore, as measured using ^{1}H NMR metabolomics, exposure to *V. tubiashii* was also shown to lead to an increased ATP demand, indicated by an increase in aerobic metabolism following a pathogen inoculation.

This is the first study to have demonstrated a physiological trade-off in mussels exposed to anthropogenic climate change and a pathogenic bacterium. However, in demonstrating a physiological trade-off in mussels exposed to reduced seawater pH, this thesis supports the research of Thomsen and Melzner (2010). These authors proposed the reduced shell growth measured under acidified conditions in *M. edulis* to be as a result of the increased cellular energy demand, and thus a reallocation of energetic resources under stressful environmental conditions. Furthermore, this response has since been shown to be closely linked to an organism's energy budget, with energy being re-allocated from shell conservation to more vital processes, such as somatic mass maintenance, under food limited conditions (Melzner et al., 2011). Seibel et al. (2012) also demonstrated food limited energetic plasticity in another mollusc, the pteropod *Limacina helicina* forma *antarctica*, with CO_2-induced metabolic depression being altered by changes in the abundance and composition of the phytoplankton community. These authors consequently argue that conflicting results on the ecological consequences of OA may reflect the true complexity of physiological responses, and physiological trade-offs, in marine organisms in response to a multi-faceted changing climate.

From the research carried out in the present study, it is clear that mussels exert significant biological control over the allocation of resources to competing physiological processes. Such plasticity, and the ability to modulate biological processes, could ensure *M. edulis* is able to survive in a future ocean. Nonetheless, the sustainability of this response will ultimately depend on organism condition and the availability of energetic resources (Wood et al., 2008, 2010). Therefore, understanding the capacity of different species to respond effectively to climate change thus requires investigation at the whole organism level, rather than the process level (Wood et al., 2008). This will ensure that the direct impacts of environmental stress on physiological functioning are considered alongside the indirect impacts on resource allocation and physiological trade-offs. Only when these responses are understood will we be able to accurately predict the likely impact of anthropogenic climate change on marine organisms.

6.2.4 The impact of reduced seawater pH, increased temperature and a bacterial exposure on mussel survival

Whilst measuring host defence or metabolism offers an understanding of how individual mussels may respond to a changing climate, ultimately to understand the impact of environmental stress at a population level it is important to measure mortality. In this thesis (Chapter 3) mortality was shown to increase when mussels were exposed to reduced seawater pH. It is therefore possible that OA could have a significant impact on mussel populations in a future ocean. Additionally, the magnitude of this mortality was shown to be temperature dependant. At 12.5 °C mussel mortality was only above 10% in mussels maintained at pH 6.50, yet at 17.0 °C mortality was greater than 10 % in

mussels maintained at pH 7.60, 7.35 and 6.50. In showing mussel mortality to be unaffected by moderate seawater acidification at 12.5 °C, this present study supports previous research where mussels were shown to be resilient to changes in seawater acidification (Beesley et al., 2008; Ries et al., 2009; Thomsen et al., 2010). Conversely, in showing mussel mortality to be drastically reduced when exposed to pH 6.50, this study highlights the potential negative impact of a carbon capture storage leak on mussel populations (Blackford et al., 2009).

Whilst previous research has shown mussels to be resilient to moderate changes in seawater pH, much of this literature has focused on single stressor 'OA' studies (e.g. Beesley et al., 2008; Gazeau et al., 2007; Thomsen and Melzner, 2010). In highlighting the possible implication of a combined increase in temperature and reduction in pH, this thesis further indicates the importance of multi-stressor studies. This is the only study to date to have investigated the combined impact of increased temperature and reduced seawater pH on mussel physiology, yet supports previous research where the sensitivity of marine invertebrates to OA is enhanced by a combined exposure to increased temperature (e.g. Findlay et al., 2010; Wood et al., 2010). In showing mussel mortality to increase when exposed to both increased temperature and reduced seawater pH, this present work demonstrates the important role environmental temperature will play in determining the sensitivity of this bivalve species to anthropogenic climate change over the next 100 - 300 years.

Increased mortality will ultimately reduce the persistence of mussel populations. However, it is critical to also consider what impact this will have on the perceived sensitivity of mussels to environmental stress. In the current study exposure to pH 6.50 seawater was shown to increase mussel mortality to 66.7 %. Consequently, all subsequent physiological measurements made in mussels exposed to pH 6.50 were

taken from the surviving 33.3 % of the population. This 'survivor response' could lead to a significant skew in the data, with the organisms that are able to survive representing the most resilient proportion of the population. When considered in the absence of any mortality data, this survivor response could thus significantly impact the perceived susceptibility of organisms to anthropogenic climate change. Alternatively, it is also possible that this surviving 33.3 % may represent the proportion of the population that genetically are naturally resilient to reduced seawater pH. Recent research has demonstrated such a genetic basis for the individual variation in response to OA (Pistevos et al., 2011; Sunday et al., 2011), and the extent to which this individual genetic variation is heritable within a population represents the potential of that population to evolve due to natural selection (Dupont et al., 2010b; Pistevos et al., 2011; Sunday et al., 2011; Widdicombe and Spicer, 2008). Therefore, whilst this survivor response could impede our ability to accurately assess the fate of mussels in a future ocean, it may also offer a vital mechanism with which to investigate the ability of mussel populations to adapt to changing conditions and to survive in the face of otherwise overwhelming stress.

6.3. TOWARDS AN IMPROVED UNDERSTANDING - KEY DIRECTIONS FOR FUTURE RESEACH

The work reported in this thesis has provided important data on the impact of environmental stress on invertebrate immune response. Furthermore, it provides a new insight into the impact of anthropogenic climate change on *Mytilus edulis* physiology. However, by considering the results of this study in light of previous research, this thesis has also highlighted a number of key areas in which a concerted research effort is

required to improve our understanding. This section will outline these research areas, highlighting where a focused effort will help to provide an accurate prediction of the ecological implications of a changing marine climate.

Many of the experiments that have investigated the impact of environmental stress on the invertebrate immune response have, to date, been carried out in the absence of a pathogenic challenge, investigating the immunological response of the separate immune system parameters rather than investigating host-pathogen interactions functionally (Ellis et al., 2011). However, as outlined in this study, it is possible such an approach could lead to an inaccurate interpretation of the impact environmental stress has on organism disease resistance. Moreover, it is clear from this thesis that to fully understand the impact of anthropogenic climate change future studies need to account for physiological trade-offs. In coming to such a conclusion, this thesis adds to a growing body of literature that has emerged in recent years (e.g. Melzner et al., 2011; Wood et al., 2008, 2010), showing organisms reallocate resources between important physiological processes, or different life-history traits, in order to maximise fitness. Whilst driven ultimately by organism condition and food availability, it is clear from the current study organisms also maintain a level of control over this energetic plasticity. Therefore, to fully understand the impact of environmental stress on organism physiology future studies should investigate processes functionally, accounting for the possibility that a reduction in function could be as a result of an energetic trade-off.

When accounting for physiological trade-offs, the extent to which an organism must trade-off its resources between different physiological processes, or life-history traits, is dependent upon an organism's condition (Kelly, 2011), as well as the resources that are available within its environment, such as food (Melzner et al., 2011). Furthermore, resource allocation will be dependent upon season, with gametogenesis

and spawning being two processes that will place a significant energetic burden on an organism's available resource pool (Lowe et al., 1982). Therefore, there is a need within ecological immunology, as well as within the field of OA research in general, to measure and account for the impact of seasonal cycles. Fully understanding the implications of variable food availability within a natural ecosystem, as well as the impact this has on an organism's resource sequestration, will help further explain sources of variability within current literature that to date have impeded our ability to predict species level responses.

As noted throughout this thesis, the mussels used in the current study were seemingly tolerant of a moderate reduction in seawater pH. This would appear to contradict previous research on this species (e.g. Bibby et al., 2008; Matozzo et al., 2012), impacting our ability to accurately predict the impact of OA on marine mussels. However, whilst this thesis has not been able to definitively explain the cause of this variation, a number of hypotheses have been proposed that require further investigation. Firstly, it is possible that the contrasting results noted from studies on *M. edulis* to date are due to local adaptation to environmental conditions. However, despite the understanding that seawater pH varies significantly both on a temporal and spatial scale (Attrill et al., 1999; Feely et al., 2010), to date no study has attempted to incorporate this pH variability when investigating the sensitivity of marine organisms. What's more, virtually all perturbation experiments to date have used the present day average surface seawater pH (nominally pH 8.05) as a control, using the mean values of projected change in seawater pH globally to represent impacted ecosystems (i.e. the IPCC projected reductions of 0.3 and 0.5 units by 2100 and 2300 respectively). It has been demonstrated that extreme events are likely to be far more important than average conditions, especially in the intertidal (e.g. Denny et al., 2006). Furthermore, OA is

likely to be superimposed upon this naturally variable pH profile noted in many marine habitats (Feely et al., 2010). Therefore, to fully understand the potential impact of reduced seawater pH on marine organisms, studies should ideally incorporate a measure of this natural variability. This will enable an understanding of the conditions marine organisms are actually likely to experience in their natural habitat, rather than investigating a global average change, which in reality very few benthic organisms will experience. Only then will we truly begin to understand the ability of marine organisms to acclimate and adapt to the climate conditions they will encounter in a future ocean.

Whilst local adaptation is indeed one possible explanation for the contrasting results achieved to date, it is important to investigate to what extent this adaptation is through phenotypic or genotypic variation. Many studies to date have, through necessity, investigated the response of marine organisms at a particular life-history stage, with very few studies having investigated the response of organisms across generational boundaries (Parker et al., 2012). Single generation studies inevitably incorporate a measure of intraspecific variation in response traits, often reported as a standard deviation or error of a mean, with this variation treated as 'noise' (Sunday et al., 2011). Yet it is this variation that could indeed hold the key to understanding the extent to which an organism may be able to evolve to a changing climate over a time-scale of multiple generations (Spicer and Gaston, 1999). If this noise is indeed attributed to genetic variation within a population, rendering a proportion of a population as more resilient to environmental change, then the heritability of this genotypic variation will determine the adaptability of a population through natural selection (Pistevos et al., 2011; Sunday et al., 2011). It is this lack of understanding, concerning the extent to which a species will be able to adapt or evolve in response to changing environmental conditions, which is one of the major limitations in our ability to accurately predict

species level responses. Future studies should therefore aim to investigate the response of different organisms to environmental stress, measuring the response across two or three generations. By elucidating the extent to which positive carry-over effects afford larvae genetically enhanced resistance will help explicate the true potential of a species to adapt and evolve in response to anthropogenically induced climate change.

It is becoming increasingly apparent, particularly with respect to studies investigating the metabolomic, proteomic or genomic response of an organism to environmental stress, phenotypic anchoring is key (Hines et al., 2007a). That is, knowing the true species, gender and reproductive status of the organism concerned. Whilst phenotypic anchoring is increasingly commonplace in these 'omics' based approaches, within other scientific disciplines this is not the case. However, in demonstrating that gender has a significant effect on the response of the blue mussel to anthropogenic climate change, particularly with respect to energy metabolism, this study highlights that the gender ratio of a sample population could significantly affect the result of a study. Additionally, as is particularly the case with the *Mytilus* genus in the north-west Atlantic (Hilbish et al., 2002; Riginos and Cunningham, 2005), speciation could significantly affect the response of an organism to environmental stress. Additionally, with reproductive processes shown to significantly affect an organism's resource allocation and condition (Lowe et al. 1982, Kelly 2011), as well as any subsequent physiological trade-offs (e.g. Petes et al. 2008), understanding the reproductive condition of a sample population is critical. Therefore, future studies should at least attempt to define the species composition, gender ratio and reproductive status of a sample population, offering a further insight that will help explain any variable results that will inevitably arise with continued species level investigation.

6.4. CONCLUSIONS

- *Mytilus edulis* appears to be resilient to moderate seawater acidification, at a level predicted to occur over the next 100 - 300 years; conversely exposure to pH 6.50 significantly increases mussel mortality, as well as additionally leading to a physiological trade-off, and a shift in energy metabolism, in surviving organisms.
- Host defence, like reproduction and energy metabolism, should be considered functionally, and in the context of the whole organism. Mussels are able to reduce the energy invested in host defence under stressful environmental conditions. However, these organisms maintain control over this plasticity, increasing immune response when required. Nonetheless, such plasticity is costly. To understand the implications of such costs it is crucial to measure the impact of a change in resource allocation for other physiological processes, or important life-history traits.
- Increased seawater temperature, projected to occur by 2100, has a significant impact on mussel physiology and energy metabolism. Furthermore, temperature acts antagonistically, increasing the sensitivity of mussels to reduced seawater pH by increasing mortality.
- Phenotypic anchoring is key to interpreting species level molecular 'omics' responses, with gender impacting the response of mussels to environmental stress.

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