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The role of environmental temperature in immunocompetence and disease susceptibility in bivalves

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The role of environmental temperature in immunocompetence and disease susceptibility in bivalves

By

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AUTHOR'S DECLARATION

At no point during the registration for the degree of *Research Masters* has the author been registered for any other University award, without prior agreement of the Doctoral College Quality SubCommittee.

No work submitted for this research degree has been used as part of any other degree at either the University of Plymouth or at any other establishment.

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George Liam Mason

Disease outbreaks and mass mortality events affecting marine species and ecosystems during periods of high temperature have increased in frequency and intensity over the past several decades. Accordingly, understanding the causes of such thermally-induced outbreaks is increasingly important, particularly in the context of global climate change and predictions of increased pathogen prevalence and disease incidence. Whilst our understanding of immune functions and their adequate energetic provisioning for disease resistance is well developed, little is known on how temperature affects immunological and energetic status in bivalves. Furthermore, even less is known on the potential combined effects between thermal and pathogenic stressors that may further alter host-pathogen dynamics. Consequently, the aim of this thesis was to investigate the individual effects of thermal stress as well as its combined effects with pathogenic challenge on the immunological and energetic condition of bivalves. A rise in temperature from 18 to 21°C caused a significant loss of haemocyte viability (p = < 0.001) in the Pacific oyster, *Crassostrea gigas* from an average of 80 % to 70 %. Oysters at 21 °C also had a significantly decreased ATP level (p = < 0.001) that fell from an average of 0.78 to 0.51 (µmol g^{-1}). The loss of this immune parameter may indicate stress-induced immunosuppression at higher temperatures, driven by the depletion of this vital cellular biochemical reserve. Exposure of *C. gigas* to the previously unstudied oyster pathogen, *Nocardia crassostreae* caused a significant rise in haemocyte counts from an average of 0.623 of to 0.863 (cells 1x10^6) compared to non-exposed groups. This

was associated with a significant loss of glycogen reserves in oysters kept at both 18 and 21 °C, where levels declined from an average of 232 to 105 (μmol g^{-1}) in challenged individuals. Exposure also caused a significant rise in glucose levels (p = 0.002) from an average of 0.34 to 1.42 (μmol g^{-1}) and the total adenylate nucleotides (p = 0.035) from an average of 12.0 to 14.3 (μmol g^{-1}). This suggests that as with other pathogens, oysters may combat *N. crassostreae* through the mobilisation of energetic reserves and increased glycolytic activity to support the increased metabolic demands of an upregulated immune defence. Overall, this study supports the hypotheses that the suppressive effects of temperature on key immune defences along with the effects of both temperature and pathogen exposure on energy availability may be a factor in influencing disease susceptibility and mass mortalities in oyster populations. In contrast to previous studies, no strong interactive effects between *N. crassostreae* and temperature were seen on the immunology or energetics of oysters, suggesting the mechanisms that may govern mass mortalities need to be considered individually, based on the host-pathogen model at play. Further, incorporating bioenergetics measurements may prove important in understanding the consequence for host immunity and disease resistance during periods of thermal stress.

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by the same letter are significantly different from one another based on pair-wise tests (p = <0.05).

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Abbreviations

GCC - Gobal Climate Change

SST – Sea Surface Temperatures

SMS – Summer Mortality Syndrome

Chapter 1: General introduction

Introduction

Current multi-model projections and agreed mitigation strategies suggest sea surface temperatures (SST) will increase by at least 3°C by 2100 and are expected to continue to rise over the next several hundred years (IPCC, 2018). One major concern of this anthropogenically induced global climate change (GCC) is the effect it will have on pathogen prevalence. Already, rises in SST over the past several decades (Pachauri and Mayer, 2015) have issued structural changes to the ocean's microbial community (Boyce et al., 2010; De Senerpont Domis et al., 2014; Godh et al., 2015). By facilitating the geographical expansion and proliferation of pathogenic agents (Baker-Austin et al., 2012; Lee et al., 2001; Petton et al., 2013; Vezzulli et al., 2013, 2015), rising SST have been linked to the emergence of large-scale disease outbreaks and mass mortalities effecting marine species and ecosystems at a historically unprecedented rate (Anderson, 1998; Burge et al., 2014; Epstein et al., 1998; Harvell et al., 2002, 2009; Lafferty et al., 2004, 2009; Morgan and Wall, 2009).

Bivalves are a group of marine organism threatened by the potential increases in infectious diseases under GCC predictions. Bivalves are sedentary filter feeders inhabiting shallow estuarine and coastal habitats. These environments regularly experience intense local warming events (Pernet et al., 2012) and nutrient run-off (Soletchnik et al., 2007) that further encourage microorganism proliferation and virulence (Lee et al., 2001; Petton et al., 2013). As such, bivalves are naturally exposed to large volumes of pathogens (Hernroth et al., 2002; Sinderman 1990; Thompson et al., 2004). Disease outbreaks and mass mortalities are extensively and increasingly reported in bivalves and have been associated with a growing range of bacterial (Friedman et al., 2001; Gomez-León

et al., 2005), viral (Repamo, 2009; Segarra et al., 2010) and parasitic species (Fernandez Robledo and Vasta, 2014; Kleeman and, Adlard 2000; Park and Choi, 2001).

Both wild and commercial populations have already suffered severe losses as a result of pathogen induced mass mortalities, including several species of oyster (Cheney et al., 2000; Ford and Borrero, 2001), mussel (Newell and Lutz, 1991), scallop (Xiao et al., 2005) and clam (Gomez-León et al., 2005; Jonsson and André, 1992). Larval (Garcia et al., 2011), juvenile (Petton et al., 2013) and adult (Pernet et al., 2012) life stages have been affected by mass mortalities, with losses amounting to entire populations in some instances (Barbosa-Solomieu et al., 2015). The emergence of novel bivalve pathogens and corresponding mass mortality events is considered one of the greatest challenges to the sustainability of bivalve populations (Cheney et al., 2000) as well as the socioeconomic (Godray et al., 2010; Herbert et al., 2012; Humphreys et al., 2014) and ecosystem services they provide (Calow and Forbs et al., 1998; Ermgassen et al., 2013; Leung and Bates, 2013).

The reasons behind mass mortality events are not fully understood and cannot be attributed to the singular effects of pathogenic or parasitic infections (Garcia et al., 2011; Myrand and Gaudreault, 1995; Newell and Lutz, 1991; Renault and Samain, 2005). Therefore, the prevailing hypothesis is that a multifactorial etiology is the cause, with the complex combination of extrinsic (environmental stressors and infectious agents) and intrinsic (immunological, physiological and genetic) factors leading to severe disease development and mortality (Samain and McCombie, 2008; Soletchnik et al., 2006). Numerous environmental factors have been associated with mass mortalities including phytoplankton blooms

(Goulletquer et al., 1998), salinity changes (Kristensen et al., 1958), pollutants (SEEEC, 1998) and high nutrient levels (Samain and McCombie, 2008). Most commonly, mass mortality events have been associated with high summer water temperature (Ford and Borrero, 2001; Garcia et al., 2011; Malham et al., 2009; Meyers and Short, 1990; Xiao et al., 2005). As such, the phenomenon is routinely termed "summer mortality syndrome" (SMS).

Survival under pathogenic challenge is determined by an organism's ability to resist disease (Fisher et al., 1999). Bivalves possess an innate immune system based on non-adaptive responses, characterised by non-self recognition, the stimulation of defensive responses and the elimination of infectious agents and diseased cells (Mydlarz et al., 2006; Roch, 1999). Through numerous phagocytic, cytotoxic and inflammatory mechanisms (Schmid-Hempel, 2003), bivalves can efficiently eradicate pathogens (Harris-Young et al., 1993, 1995; Venier et al., 2011), enabling infections to be maintained at a sub-lethal level (Carella et al., 2013; Longshaw et al., 2013; Paillard et al., 2008; Suffredini et al., 2014; Van Regenmortel et al., 2000). However, ambient environmental conditions are capable of altering bivalve immunocompetence (Mosca et al., 2013; Farcy et al., 2009; Hannam et al., 2009), defined as an organism's ability to mount a successful immune defence (Schmid-Hempel, 2003). Environmental temperature is especially important as it controls virtually all physiological processes in ectotherms (Pörtner et al., 2006). Although the response to temperature varies within and between species, immunological function is often supressed when species-specific thermal thresholds are exceeded (reviewed by Ellis et al., 2011; Matozzo and Marin, 2011) leading to a rise in disease prevalence and mortality (Li et al., 2009; Malham et al., 2009; Turner et al., 2016; Wang et al., 2012;

Wendling and Wegner, 2013). Therefore, along with increasing pathogen prevalence (Lee et al., 2001; Petton et al., 2013), the negative impacts of temperature on host immunocompetence is likely a contributing factor towards the emergence of SMS with rising SST.

The incorporation of climactic data, and physiological responses into predictive models is essential to manage the threat of SMS in commercial and natural bivalve stocks under GCC scenarios (Mydlarz et al., 2006; Viney et al., 2005; Wendling and Wegner, 2013). This requires an understanding of the key immunological functions involved in pathogen elimination and the responses of these same parameters to temperature elevations which may affect disease outcomes. Thus, the overall aim of this review is to explore the role of temperature in bivalve disease outbreaks and mass mortalities with a focus on its effects for immunocompetence. Firstly, I will review major bivalve pathogens and their relationship with temperature in bivalve mass mortality events. Through detailing the various immune functions providing protection against these same pathogens, I will then explore the capacity of environmental temperature to modify these responses and ultimately, host-pathogen interactions. Finally, the relevance of incorporating non-immunological physiological responses to further elucidate the factors underpinning immunosuppression and disease susceptibility during SMS will be outlined.

1.1 The association between pathogens and temperature in bivalve mass mortality events

Bivalve mass mortalities were first reported in 1945 within Japanese farms of the Pacific oyster, *Crassostrea gigas* (Koganezawa, 1975). Since then, this phenomenon has been reported globally in a range of bivalve species (Lynch et al., 2012; Sweet and Bateman, 2015; Zannella et al*.,* 2017). Epidemiological investigations prior to and following mortality events have regularly associated losses with a large variety of bacterial, viral and parasitic infections (Sweet andateman, 2015; Zannella et al*.,* 2017). However, relatively few have monitored disease incidence along with contemporaneous alterations in abiotic stressors, like temperature. This means we have limited understanding of how both pathogenic and thermal stress may be cofactors towards mass mortality events and therefore also the importance that thermally-induced alterations in immunocompetence may have towards these losses. Thus, this section will outline cases of mass mortalities associated with major bivalve pathogens that were either significantly triggered by temperatures exceeding a thermal threshold or that occurred exclusively during warmer summer seasons. Furthermore, it should be noted that whilst disease outbreaks and mass mortality have been reported in both commercially important and wild bivalve species globally, epidemiological investigation has prioritised commercially important bivalve species, primarily within rich nations. This is likely because of the more prominent issue of microbial disease within these intensive commercial set-ups (Farley 1992; Ganzhorn et al., 1992), the economic and social consequences associated with severe losses (FAO, 2008; Herbert et al., 2012) and the substantial costs associated with this research (Zannella et al., 2017). As such, our knowledge of

the causal mechanisms associated with these pathogen-induced mass mortalities is limited to commercial productions mainly within a European and American context (Sweet and Bateman, 2015).

Viral pathogens are particularly infectious and transmissible (Zannella et al., 2017) and have been associated regularly with disease outbreaks and high mortality rates in numerous bivalve taxa around the world. Within these cases, there is evidence of mortalities occuring suddenly and extensively during spring and summer seasons, following significant rises in temperature (Garcia et al., 2011; Renault et al., 1994) (**Table 1**). Most notable is Pacific oyster mortality syndrome caused by various strains of the herpes virus (OsHV-1 and OsHV-1 µVar). Recently, the herpes virus has been reported as one of the main etiological agents behind mass mortalities occurring in French Pacific oyster farms in 2008 (Segarra et al., 2010) and to a greater extent in 2009 (Repamo, 2009; Segarra et al., 2010) and 2010 (Segarra et al., 2010). Within these cases, the virus detection frequency increased with temperature and mortality events occurred significantly after a threshold of 16 °C was exceeded. Both viral strains have also been linked with mortalities of up to 90 % occurring significantly during warm summer periods in farmed stocks of *C. gigas* in Ireland (Cotter et al., 2010), Italy (Dundon et al., 2011), Sweden and Norway (Mortensen et al., 2016). Similar patterns have also been observed within farms of *C. gigas* in Korea (Choi et al., 1999), Australia (Paul-Pont et al., 2014), both East (Burge et al., 2006) and West coasts of the USA (Friedman et al., 2005) and within farmed stocks of the clam, *Scapharca broughtonii* in China (Bai et al., 2016). Another notable bivalve virus is Acute Viral Necrobiotic Virus (AVNV) whos association with 90 % mortalities of the Zhikong scallop, *Chlamys farreri* within farmed productions throughout China is

significantly linked to summer temperatures exceeding 25–27 °C (Wang et al., 2004).

Due to their filter feeding habits, bivalves concentrate a diverse bacterial community within their tissue, some of which may be pathogenic (Kueh and Chan, 1985; Thompson et al., 2004). Bacterial diseases in adults tend to be relatively uncommon and expressed facultatively, with severe infections and mortalities mostly occurring in immunosuppressed animals (Mchenery and Birkbeck, 1986) and juveniles (Plana et al., 1996). Despite this, bacterial pathogens are increasingly associated with mortal outbreaks in a number of bivalve species with evidence suggesting temperature as a causal factor in these losses (**Table 1**). Gram-negative bacterial disease such as Vibrosis have been associated with increased rates of mortality following seasonal rises in temperature within aquaculture productions of the oyster *C. gigas* in France (Garnier et al., 2007; Lacoste et al., 2001; Soletchnik et al., 1999) and the scallop *Argopecten purpuratus* in Chile (Riquelme et al., 1995, 1996). Conversely, gram-positive bacteria are rarely associated with disease and mortalities in bivalves, the exception being *Nocardia crassostreae*. Originally, summer mortalities removing up to 50 % of stocks within US Pacific oyster fisheries once temperatures exceeded 20 °C were found to coincide temporally and spatially with the presence of *N. crassostreae* (Elston et al., 1987). Infection experiments by Friedman and Hendrick (1991) confirmed that the bacteria was infectious to oysters and a likely contributor towards these losses when in combination with high temperature. More recently, the same species has been associated with summer mass mortalities of farmed bivalves under similar water temperatures. This includes the oysters *C. gigas* in the Netherlands (Englesma et al., 2008) as

well as the oyster *Ostrea edulis* and the mussel *Mytilus galloprovincialis* in the Mediterranean (Carella et al., 2013).

Protozoan parasites are also commonly associated with disease and summer mass mortalities in bivalves (**Table 1**). The most prominent example comes from the haplosporidian parasites belonging to the genus *Bonamia,* which encompass 63% of European protozoan diseases (Fernandez et al., 2014). *Bonamia* species were largely responsible for the 85% reduction in wild and farmed stocks of *O. edulis* worldwide (Beck et al., 2011), the collapse of a historical fishery (Vanstaen and Palmer, 2009) and the loss of considerable ecological services (Cranfield et al., 1999; Carbines et al., 2004) from the 1970's onwards. Whilst *Bonamia* species cause infection throughout the year (Culloty and Mulcahy, 1999), recently the importance of high temperatures in mass mortalities associated with these parastites has been elucidated (Engelsma et al., 2010). For example, infection intensity and mortalities of up to 100 % were found to occur concommitently with the progression towards maximum summer temperatures in oyster species throughout the Netherlands (Englesma et al., 2010), New Zealand (Hine et al., 2002) and the West coast of the US (Carnegie et al., 2008). Another notable protozoan parasite is *perkinus marinus*, associated with losses of up to 90 %, primarily during summer months in the cultured clam *Ruditapes philippinarum* in Korea (Park et al., 1999; Park and Choi, 2001) and the oyster *Crassotrea virginica* in Mexico (Soniet, 1996).

Table 1 Main viral, bacterial and parasitic infections associated with thermally-induced mass mortalities in marine bivalves

Host species	Disease and associated pathogenic species	Geographical distribution	Season and/or temperatures associated with mortalities	References
		Viral		
Crassostrea gigas (mainly larvae and juvenile), *Scapharca broughtonii* and *Chlamys farreri*	Herpes virus infection (OsHV-1 and OsHV-1 µVar)	Europe (France, Ireland, Italy, Sweden and Norway), USA (Washington and California) and Asia (Korea and China)	Spring-Autumn Most commonly 16-20°C in Europe, although can be as low as 10°C. 22-25°C in USA, Asia and Australia	Bai et al., 2016; Burge et al., 2006; Cotter et al., 2010; Garcia et al., 2011; Park et al., 1999; Paul-Pont et al., 2014; Renault et al., 1994; Repamo, 2009; Segarra et al., 2010
1-2 year old *Chlamys farreri*	Acute Viral Necrosis Disease (Acute Viral Necrobiotic Virus)	China	Summer temp exceeding 25–27 °C	Wang et al., 2004
		Bacterial		
Crassostrea gigas, *Ostrea edulis* and *Mytilus galloprovincialis*	Nocardiosis (*Nocardia crassostreae*)	USA (mainly west coast of North America, British Columbia, Washington state and California) and Europe (Netherlands and Gulf of Naples)	Summer Temp exceeding 20°C in Europe and 20-27°C in USA	Carella et al., 2013; Elston et al., 1987, Englesma et al., 2008; Friedman and Hendrick, 1991
≤ 2 year old *Crassotrea gigas* and *Argopecten purpuratus* larvae	Vibriosis (*Vibrio aestuarianus*, *V. anguillarum*, *V. alginolyticus*,	France and Chile	Summer	Garnier et al., 2007; Lacoste et al., 2001; Riquelme et al., 1995, 1996;

	members of the *V. splendidus* group , *V.natriegens*, *V. parahaemolyticus*, and *Pseudoalteromonas* sp)		Temp exceeding 16°C in France and 21°C in Chile	Soletchnik et al., 1999
		Parasitic		
Osrea edulis, Ostrea chilensis and Crassostrea ariakensis (all sized and ages effected although prevalence and intensity increases with size)	Bonamiosis (mainly: *Bonamia exitiosa, B. ostreae, B. roughleyi and B. perspora*)	Netherlands, New Zealand and USA (North Carolina)	Spring-Autumn 12–20°C Netherlands. Temp exceeding 25°C degrees in NZ and USA	Carnegie et al., 2008, Englesma et al., 2010, Hine et al., 2002
Ruditapes philippinarum and *Crassostrea virginica*	Perkiniosis (*Perkinus marinus*)	Mexico and Korea	Summer	Park et al., 1999, Park and Choi, 2001, Soniet, 1996

In conclusion, evidence suggests that the development of sever disease outbreaks and mortality events involving major bivalve pathogens appears to be intrinsically linked to the complex combination of pathogenic and thermal stress. However, whilst rises in temperature are shown to influence pathogen transmission, infection intensity and disease incidence, these studies add little resolution to the internal mechanisms controlling vital host-pathogen dynamics. To help reveal the deterministic factors behind SMS, it is therefore important to investigate the effects of temperature on pathogenic activity along with key aspects of bivalve physiology as well as any potential interactions between these two stressors that further influence disease susceptibility in hosts.

1.3 The immune system of bivalves and its role in disease mitigation

Given the range of diseases impacting bivalves and the socioeconomic and ecological consequeces they bring, there is a growing interest in understanding bivalve immunity and the various mechanisms used to resist infectious agents (Allam and Raftos, 2015; Bachère et al., 2003, 2004; Roch, 1999; Zannella et al., 2017). Crucially, such knowledge also provides a framework to investigate the responses of key host defences to environmental alterations and therefore the factors driving disease susceptibility and mortalities during periods of high temperature (Galloway and Depledge, 2001).

Immune defences first appeared in the Protozoa around 2.5 billion years ago (Beck, 1996) and following phylogenetic diversification have since formed the foundation of physiological immunity in almost all living organisms (Hoffman and Riechhart, 2002). In response to the myriad of tactics employed by pathogenic species, intense selection on the immune system has subsequently modified it into a complex, highly organised system, which promotes host survival through a variety of physiological mechanisms (Medzhitov and Janeway, 1997; Lochmiller and Deerenberg, 2000). Beginning with the pioneered work of Leslie Stauber, who in the 1950's traced the elimination of intracardially injected ink within the oyster *Ostrea virginica*, the defence-related functions of the bivalve immune system have become widely described. From this, we know that bivalves possess a typical invertebrate innate and non-adaptive immune system that includes a variety of sensing and effector mechanisms (reviewed in: Allam and Raftos, 2015; Bachére et al., 2004; Roch, 1999; Zannella et al., 2017). Following pathogen

recognition, bivalves trigger a multifaceted defensive response that can be divided roughly into cellular and humoral systems. This section will firstly detail the key mechanisms involved in initiating host defences. I will then provide an overview of the major cellular and humoral components of the bivalve immune system and discuss how they may contribute to disease mitigation.

1.3.1 Pathogen recognition and immune stimulation

Outlined in this section are the complex recognition systems bivalve's use to detect pathogens and initiate antimicrobial processes. Due to their semi open circulatory system, external agents may quickly contaminate the internal environment of bivalve's (Hine, 1999). Foreign bodies contain distinctive molecular structures that hosts can associate with invaders known as pathogen associated molecular patterns or PAMPs (Janeway and Medzhitov, 2002). Recently developed -omic tools have allowed the characterisation of a broad suit of molecular structures that may contribute to the invertebrate immune response (reviewed in: Beutler, 2004; Matzinger, 2002; Schmitt et al., 2010). Bivalves respond to numerous forms of peptidogylcans, toxic macromolecular-complexes, viral RNA's, lipopolysacchacarides and lipoteichoic acids. These diverse molecular signatures are essential for the survival of microbes and therefore represent an evolutionarily stable strategy for bivalves to recognise bacterial (Cheng and Howland, 1979; Howland and Cheng, 1982), viral (Beutler, 2004) and parasitic invaders (Cheng et al., 1974). Other cues include the intracellular nucleic or cytoplasmic protein components of host cells released by the cytotoxic action of pathogens, which are known as damage associated molecular-patterns, or DAMPs (Rubartelli and Lotze, 2007).

Hosts detect these molecules through soluble pattern recognition proteins (PRPs) (Harris-Young et al., 1995) and Pattern Recognition Receptors (PRRs) (Canesi et al., 2002). Many structurally diverse classes of receptor molecules have been described across a range of mussel (Chen and Bayne, 1994; Renwrantz et al., 1985; Tunkijjanukij et al., 1998), oyster (Suzuki and Mori, 1990; Tamplin and Fisher, 1989) and clam species (Olafsen, 1995). Among these structures associated with innate pattern recognition are toll like receptors (TLRs), peptidoglycan recognition receptors, glucan-binding proteins (GNBP) and lipopolysaccharide-binding proteins (LBPs) (Jang et al., 2015). These receptors show broad specificity and can respond to the variable set of PAMPs, shared between infectious and parasitic agents. Transcriptome sequencing of the eastern oyster, *Crassostrea virginica* revealed 657 genes associated with innate immunity with many relating to pathogen pattern recognition (Zhang et al., 2014). Such molecular diversity points to the high recognition capacity of bivalve PRRs, suggested to be an adaptation to the microfauna rich environment they inhabit (Zhang et al., 2014).

Following recognition, proteins belonging to the IκB kinase family (Escoubas et al., 1999; Xiong et al., 2008) initiate signalling cascades; inducing the rapid expression of numerous immune-genes (He et al., 2015), with the type of response apparently specific to the pathogen bivalves are exposed to (Génard et al., 2013). With the development of genomic tools, this rapid induction upon pathogen exposure has been linked to the amplification of pathogenic recognition mechanisms (De Lorgeril et al., 2011), cellular signalling cascades (Green and Montagnani, 2013; Renault et al., 2011) and the production of cytokines (Baeza-

Garcia et al., 2010), all acting to initiate and enhance the cellular and humoral components of bivalve anti-microbial defence (Beutler, 2004).

Given this evidence, bivalves demonstrate a comprehensive ability to recognise a broad suite of pathogenic and parasitic species and respond specifically and rapidly at the transcriptional level in order to mount cellular and humoral defences.

1.3.2 Cellular defences

Haemocyte-mediated immunity constitutes the primary form of defence in bivalves (Cheng and Combes, 1990). Haemocytes are functionally viable through their ability to degrade microorganisms via phagocytosis (Bachère et al., 1995), or the release of antimicrobial and hydrolytic factors (Allam and Raftos, 2015). Outlined below are the various cell types that assist towards host defence and their specific responses that promote host resistance. These defensive cells are highly heterogeneous (Fryer and Bayne, 1996) and are chategorised based on their predominant functions and morphological characteristics into two sub-groups: granulocytes (granular) or hyalinocytes (non-granular) (Lambert et al., 2003). Granulocytes constitute the most numerous cell type (Fryer and Bayne, 1996) and are typically more phagocytically (Carball et al., 1997) and cytotoxically (Lambert et al., 2003) active than hyalinocytes, suggesting they are more important to immune defence in bivalves (Canesi et al., 2002). However, due to the limited information on their various contributions towards immune defence across bivalve species, they are commonly grouped together when evaluating the cellular response to pathogenic threat to avoid misinterpretation of defensive capabilities (Ellis et al., 2011). Immune challenge upregulates the production, mobilisation and infiltration of haemocytes, corresponding with an increased

concentration of circulating cells within infected individuals. This response has been observed in the oyster *Crassostrea gigas* (Wendling and Wegner, 2013), the mussel *Mytilus galloprovincialis* (Carballal et al., 1998) and the clam *Chamelea gallina* (Monari et al., 2007), following pathogen exposure, suggesting that haemocytic activity is an important mechanism of antimicrobial defence across bivalve groups. As such, the measurement of haemocyte concentrations (Monari et al., 2007), the proportions of their subpopulations (Carballal et al., 1998) and their functional viability (Perrigault et al., 2011) is routinely incorporated in studies as a measurement of immunocompetence.

Phagocytosis is the key function of haemocytes, evolved from the phagosomes ancestral role in digestion and transport (Evariste et al., 2016). Haemocytes show chemoattraction towards PAMPs and concentrate at infection sites (Cheng and Howland, 1979), where they attach and engulf foreign bodies (Canesi et al., 2002). After a period of dormancy, infectious agents are intracellularly degraded through antimicrobial processes employing lytic enzymes (Monari et al., 2007), anti-microbial peptides (Schmitt et al., 2012) or the respiratory burst, involving the production of reactive oxygen species (ROS) (Lambert et al., 2003). Phagocytosis increases following pathogenic challenge, constituting an important form of cellular defence in bivalve species including the oysters *C. virginica* (Chu and La Peyre., 1993) and *C. gigas* (Li et al., 2009), the clams *Mercenaria mercenaria* (Perrigault et al., 2011), *Mactra veneriformis* (Yu et al., 2009) and *R. philippinarum* (Paillard et al., 2004), and the mussel, *Mytilus galloprovincialis* (Cansesi et al., 2013; Venier et al., 2011). However, phagocytic rate and subsequent degradation can vary depending on the bacterial species that haemocytes are exposed to (Feng, 1966; Hardy et al., 1977; Howling and Cheng,

1982). Specific variations in the peptidoglycan cell wall components of microbes may impede the recognition potential of haemocyte surface receptors, limiting their ability to attach and engulf some pathogens (Hannam et al., 2010). Further, this ability to recognise and phagocytose specific pathogens may also vary interspecifically, based on differences in the composition of host haemocyte surface receptors (Allam and Ford, 2006). Ultimately, these cellular interactions may alter the effectiveness of this mechanism in eradicating pathogens (Canesi et al., 2002) which may explain the persistence of particular pathogenic and parasitic species within hosts, and ultimately their virulent effects (Harris-Young et al., 1993).Nevertheless, given its central role in the bivalve immune response, phagocytosis is typically measured in immunological studies via a variety of methods. These involve either measuring phagocytic activity, that is the proportion of haemocytes capable of phagocytosing foreign particles (Cheng et al., 2004), or the phagocytic index, the quantity of foreign particles haemocytes are capable of phagocytosing (Monari et al., 2007).

1.3.3 Humoral defences

Outlined in this section are the components of the bivalve humoral defence system and the evidence of their contribution towards pathogen elimination. When phagocytosis is ineffective or when bivalve's are under considerable pathogenic infection, haemocytes secrete antimicrobial factors into haemolymph for extracellular degredation (Chu, 1988; Dove et al., 2004). This response markedly enhances disease resistence by attacking pathogens through: lysis, growth suppression, cell membrane and metabolic disruption leading to the opsonisation, agglutination and neutralization of foreign invaders (Smith et al., 1995). Haemocytes can interact indirectly with pathogens through soluble

recognition proteins known as opsonins. These humoral proteins attach and bind microbes, facilitating phagocytosis (opsonisation). When comparing the response of haemocytes belonging to the mussel, *Mytilus edulis,* in serum and artificial sea water, adhesion, association and intracellular killing increased by approximately double (Canesi et al., 2013). Furthermore, the expression of numerous opsonins groups is upregulated in the scallop, *Argopecten irradians* (Song et al., 2011), and the clams *Venerupis philippinarum* (Mu et al., 2014) and *R. philippinarum* (Kim et al., 2008), following immune challenge, suggesting that opsonins are important for antimicrobial activity across a range of bivalve species.

The synthesis and release of free radicals, including ROS (superoxides and peroxides) and reactive nitrogen species (RNS) (nitric oxides), is another important humoral mechanism of microbial degradation. ROS production increases within haemolymph following pathogenic challenge in numerous bivalve species including the clam *M. mercenaria* (Perrigault et al., 2011), the oyster *C. gigas* (Lambert et al., 2003), the mussels *M. edulis* and *M. galloprovincialis* (Pipe, 1992), and the scallop *Pecten maximus* (Le gall et al., 1991). A similar response for NO production has been found in the oysters *C. virginica* (Villamil et al., 2007) and *C. gigas* (Lambert et al., 2007), and the mussel *M. galloprovincialis* (Tafalla et al., 2002; Torreilles and Romestand 2001). Although these studies suggest a general role of free radicals in pathogen elimination, some bivalve species have an undetectable level of ROS production following immune challenge (Anderson, 1994; Lopez et al., 1994). Further, ROS production can be supressed by the action of some pathogens; including the parasite, *Perkinsus marinus* (Lopez et al., 1994) and some *Vibrio* species

(Lambert and Nicolas, 1998). This suggests that the contibution of this parameter towards host defence depends on the host and further the host-pathogen model being investigated. Significant free radical production can also lead to oxidative stress states that impact protein function and cellular structure, having deleterious consequences for tissues (Valko et al. 2006). This effect may be mediated by the production of antioxidant enzymes; mainly superoxide dismutase (SOD) and catalase (CAT) (Gonzalez et al., 2005; Michiels and Remacle, 1988). Following pathogen exposure, antioxidant activity is shown to increase in bivalves including the oyster, *C. gigas* (Génard et al., 2013; Meng et al., 2014) and the scallop *C. farreri* (Tang et al., 2010; Wang et al., 2012), suggesting that free radical production may offer benefits for bivalve health, when in conjunction with antioxidant activity.

Anti-microbial peptides (AMPs) are ancient defence factors conserved within invertebrate taxa that may also contribute to host defence as part of the humoral defence system (Costa et al., 2009). Characterisation of AMPs has mostly been performed in the mussel *M. edulis* and the oyster *C. gigas* (Allam and Raftos 2015; Schmitt et al., 2011). Within these species, a diverse range of AMPs are able to kill microbes through a variety of disruptive mechanisms (Gonzalez et al., 2007; Gueguen et al., 2005), such as via inhibition of bacterial cell wall biosynthesis (Schmitt et al., 2010). AMPs are upregulated following pathogenic challenge in several bivalve species including the clams *R. philippinarum*, (Adhya et al., 2012) and *M. mercenaria* (Perrigault el al., 2009), and the oyster *C. gigas* (Gonzalez et al., 2007), suggesting this defence mechanism offers protection across bivalve species.

Melanisation may also contribute towards humoral defence by encapsulating and neutralisation foreign invades. The role of melanisation in immunity is well understood in arthropods, but remains poorly eluded in molluscs. This process is regulated by phenoloxidase (PO) (Cerenius and Söderhäll, 2004) an enzyme shown to increase in activity in the clam, *Tapes decussatus* following challenge with the protist *Perkinsus atlanticus*, and is associated with a decline in the level of parasitism (Munoz et al., 2006). Similar induction of PO activity occurs in the oyster, *Saccostrea glomeratal,* in response to immune challenge (Kuchel et al., 2010; Aladaileh et al., 2007), suggesting PO plays an important role in host defence.

Complementing these other humoral defences is the activity of hydrolytic enzymes. These include a broad range of antimicrobials including: proteases (Gagnaire et al., 2003; Munoz et al., 2003), esterases (Gangnaire et al., 2003; 2006; Parisi et al., 2017), lysozymes (Cheng, 1983) and acid phosphatase (Chen et al., 2007). Lysozymes are the most commonly studied due to their broad range of defensive functions (Xue et al., 2004), their particularly destructive properties against microorganisms cell wall components (Cheng and Rodrick, 1974; Chu, 1988; McHenery and Birkbeck, 1982) and their ability to reduce pathogenic infection within invertebrates (Cheng et al., 1977). Whilst the degree of lysozyme activity in bivalves varies interspecifically (La Peyre et al., 1995) and between the specific host-pathogen model being investigated (Hauton et al., 2000; Rodrick and Cheng, 1974), this humoral factor is shown to upregulated following pathogenic exposure in a range of bivalve species including: the clams, *Chamelea gallina* (Monari et al., 2007), *Mactra veneriformis* (Yu et al., 2009) and *M. mercenaria* (Perrigault et al., 2011) and the oyster *crassostrea virginica* (Chu

and La Peyre, 1993; Oliver and Fisher, 1995). This suggest that lysozymes provide a “generic” form of immune defence against microorganism invaders across bivalve species. Therefore, the activity of these lytic enzymes is regularly used as a proxy for immunocompetence against pathogenic infection (Chu and La Peyre, 1989).

Cumulatively, evidence suggests that bivalve’s possess a diverse range of humoral defence mechanisms which may compliment cellular defences in the fight against pathogenic threats (Rinkevich and Muller, 1996). By developing our understanding of this component of host immunity, we are gaining a comprehensive view of the multifaceted defensive strategies employed by bivalves (Ellis et al., 2011).

1.3.4 Mucosal defences

Much of the research on immunological processes has focused on defences involving circulating haemocytes and humoral factors. However, the initial host-microbe interactions that determine subsequent infection are found at mucosal interfaces, commonly within environmentally exposed pallial surfaces (Allam and Pales, 2015). An abundance of functionally active haemocytes are present in these peripheral sites in the clam *Ruditapes philippinarum* (Allam 1998, Allam et al., 2000), and the oysters *Crassostrea virginica* (Lau et al., 2013) and *Ostrea edulis* (Takatsuki, 1934). These cells display a number of unique cell surface features, which may categorise them into a specialised haemocyte group (Lau et al., 2013). These mucus layers of the clam, *R. philippinarum* also contain the hydrolytic enzyme lysozyme, shown to increase in activity following exposure to *Vibrio tapetis* (Allam et al., 2000a, 2000b). The detection of these responsive defences at mucosal interfaces may suggest the importance of these external

mechanisms as a first line of defence. Further investigation to characterise the various protective functions provided at these sites and their role in maintaining organism health may therefore provide a better assessment of the complete bivalve defence system involved in disease mitigation.

Overall, research on the bivalve immune system has begun to reveal the complex and multifaceted mechanisms used for pathogenic recognition and elimination. Although the expression and function of these defences are subject to variation dependent on the species and further the host-pathogen model being tested, bivalves appear to be highly capable of defending themselves under optimal conditions through efficient and effective cellular and humoral defence mechanisms. This may explain how how bivalves can normally persist in microbe rich environments (Guo et al., 2015) and are part of the second most diverse and widely distributed phylum on earth (Barnes et al., 1993).

1.4 The effects of temperature on bivalve immunity

As shown in the previous sections, our understanding of the adverse effects of thermal stress on bivalve disease and mortality and the significance of the immune system in host protection has been greatly improved in recent years. Inevitably, this has lead to the study of how temperature may alter host defence mechanisms and how this may explain patterns of disease outbreaks and mortality within natural environments. Subsequently, temperature has been shown to modulate virtually all aspects of bivalve immunity (reviewed in Ellis et al., 2011; Matozzo and Marin, 2011). Although the magnitude and type of response is subject to intraspecific (Dang et al., 2012; Duchemen et al., 2007; Green et al., 2014; Pernet et al., 2012; Mosca et al., 2011; Wendling and Wegner, 2013) and interspecific variation (Matozzo et al., 2012; Rahman et al., 2019); generally temperature leads to a number of negative consequences for immune function in bivalves. However, this type of immune investigation has traditionally been formulated without the presence of a pathogen (Chen et al., 2007; Gagnaire et al., 2006; Hégaret et al., 2003; Monari et al., 2007; Yu et al., 2009) meaning despite the relatively well characterised effects of temperature on immune properties, few studies have been able to link thermal stress with functional alterations in immunocompetence (Ellis et al., 2011). This design also fails to incorporate potential temperature-pathogen interactions on host immunity that may further alter pathogenesis within naturally heterogenous environments. Therefore, this section will outline the effects of temperature elevation on the various cellular and humoral components of bivalve immune defence, with a focus on studies that have incorporated simultaneous pathogen exposure within their experimental design.

1.4.1 The effects of temperature on cellular defences

Given the integral role of haemocytes within bivalve antimicrobial defence, they serve as an important biomarker to assess the likelihood of disease under environmental stress (Galloway and Depledge, 2001). The abundance of haemocytes responds positively to temperature in bivalves (Chu and la peyre, 1993; Monari et al., 2007; Parry and Pipe, 2004; Yu et al., 2009). In the natural environment, yearly trends in haemocyte abundance also correlate positively with water temperature in a range of bivalve species including the mussel *M. galloprovincialis* (Carballal et al., 1998; Matozzo et al., 2012), the oyster *C. virginica* (Fisher et al., 1996), and the Zhikong scallop *C. farreri* (Lin et al., 2012). A similar response has been noted in the proportion of granulocyte populations, which peak in spring and summer in *C. farreri*, associated with a rise in water temperature (Lin et al., 2012).

Conversely, haemocyte abundance in the clam *Chamelea gallina* declines with increasing temperature (Matozzo et al., 2012). *Chamelea gallina* is a subtidal species, which rarely experiences dramatic fluctuations in temperature, unlike the predominantly intertidal species mentioned so far in this section. As proposed by: Pernet et al. (2007), Pörtner et al. (2006) and Somero (2002), the resistance of this parameter to temperature elevation may therefore represent an adaptation to the typical lifestyles and geographical ranges bivalves occupy. Furthermore, the general observation of increased haemocyte abundance and the percentage of defensive cell types encourage one to predict that bivalve immune performance is improved at elevated temperatures (La Peyre et al., 1995). However, through measurements of viable bacterial counts (VBC) following haemolymph exposure *in vitro*, it was found that although haemocyte counts

increased following a 7-day exposure to elevated temperature in surf clam *M. veneriformis,* this response did not translate into improved antimicrobial activity (Yu et al., 2009). This suggests that rather than representing a proliferation or mobilisation type response to improve immune defence, increased haemocyte abundance at higher temperatures may be an indirect effect of an increased heart rate and pumping force (Feng, 1965).

Moreover, the defence capabilities of haemocytes depends on their functional viability (Paillard et al., 1996). By measuring the ability of haemocytes to either retain or prevent the uptake of certain dyes, it is possible to assess their structural integrity and therefore their viability under environmental stressors (Grundy et al., 1996; Lowe and Pipe, 1994). With this method, acute (≤ 24 hours) and mid-term (≤ 14 days) exposures to environmentally relevant levels of thermal stress, are shown to significantly reduce the viability of haemocytes in the oyster's *C. gigas* (Gagnaire et al., 2006), *C. virginica* (Hégaret et al., 2003) and the clam *Chamelea ganilla* (Monari et al., 2007). This suggests that haemocytes may offer little protection following acute or mid-term bouts of thermal stress, despite maintaining a high abundance. Beaudry et al. (2016) also found viability to decline following a 7-day exposure to thermal stress in the mussel *M. edulis.* However, in finding that viability returned to the highest level observed in this study after 35 days, this study suggests that over chronic exposure scales (≥ 14 days), haemocytes may show an acclimation type response to high temperatures, potentially restoring their immunoprotective function and disease resistance generally. Nevertheless, when bivalves are thermally challenged under combined pathogenic presence, thermal stress is capable of significantly reducing haemocyte viability over chronic exposure scales (Perrigault et al., 2011; Turner et al., 2016), especially when the temperature matches the optimum conditions

for pathogen metabolism and growth (Hauton et al., 2001). Ultimately, the combined effects pathogenic and thermal stress on the structural integrity of haemocytes may lead to complete cell mortality, represented in significant reductions in haemocyte abundance (Paillard et al., 2004; Perrigault et al., 2011) as well as the percentage of granulocytes (Chu and La Peyre, 1993). These effects on haemocyte parameters correlate with significantly increased rates of disease development and mortality (Chu and La Peyre 1993; Turner et al., 2016). Together, evidence suggests that within natural environments, pathogen presence may reduce the thermoresistant qualities of haemocytes. Ultimately, these interactive effects on viability, abundance and the proportion of defensive cell types may significantly compromise cellular defence and disease resistance in bivalves.

Whilst measuring haemocyte counts and viability provides an assessment of the structural capacity of bivalves to perform efficient immune functions, this approach adds little to our understanding of how temperature may impact the specific antimicrobial processes associated with haemocytes (Auffret, 2005). As phagocytosis is the primary cellular defensive mechanism, the response of this parameter to temperature has received much attention. Phagocytic activity correlates negatively with increased water temperature in the oyster *C. gigas* (Li et al., 2007; Malham et al., 2009). Through turbidometry analyses of growth inhibition in a log phase bacterial colony, Li et al. (2007) found that this response correlated with a compromised ability to prevent bacterial proliferation, highlighting the implications of a thermally-reduced phagocytic activity for disease resistance. In other bivalve species, phagocytosis shows resistance under moderate levels of thermal stress. However, activity is still supressed at

temperatures representative of species-specific summer maximums. For example, Chen et al. (2007) found that after a 1 h stress application from 17 °C to 11, 23 and 28 °C, the scallop, *Chlamys farreri* displayed no change in the percentage of phagocytic cells between 11 and 23 °C. Conversely, at 28 °C there was a significant decrease compared to the two other treatments which magnified from 13.9 % initially to 6.3 % after 72 h. A similar response is shown in the clams *Mactra veneriformis* (Yu et al., 2009) and *C. ganilla* (Monari et al., 2007), demonstrating that although the sensitivity of this parameter varies interspecifically, thermal stress generally compromises phagocytosis in bivalves past a thermal threshold. Further, pathogenic exposure may also exaggerate the suppressive effects of temperature on phagocytosis. For example, phagocytic activity was higher in the clam *M. mercenaria* at 21 °C compared to clams at either 13 or 27 °C. However, in treatments exposed to the Quahog parasite, QPX throughout the course of the experiment, phagocytic activity fell below that of their respective control at both 21 and 27 °C, although this loss was most significant at 21 °C (Perrigualt et al., 2011). A similar response was shown in the oyster *C. virginica* exposed to the bivalve parasite, *Perkinsus marinus*, with a loss at the two highest experimental temperatures of 20 and 25 °C; correlating with increased diseased prevalence and mortality at these two temperatures (Chu and La Peyre, 1993). Overall, simultaneous pathogen exposure may further compromise bivalve's ability to combat pathogen infection through phagocytosis at elevated temperatures. Further, these interactive effects may increase disease susceptibility at temperatures where bivalves normally show a degree of resistance. This potentially represents an important mechanism influencing disease outbreaks in bivalve populations during periods of periods of elevated water temperature and pathogenic exposure.Through measurements of

intracellular ROS and ROD activity, the ability of bivalves to intracellularly degrade pathogens through the respiratory burst is also shown to be effected by temperature. Although internal ROS and ROD production may be enhanced under acute exposure to elevated temperature (Chen et al., 2007); mid-term or chronic exposure to high temperatures supresses the intracellular production of ROS in the mussel *M. edulis* (Parry and Pipe, 2004) as well as ROD in the scallop *C. farreri* (Monari et al., 2007; Tang et al., 2010). Whilst this may suggest intracellular antimicrobial activity to be inhibited during prolonged periods of elevated temperature, within the natural environment *C. farreri* demonstrates a positive correlation between ROD activity and temperature. Levels are highest during the late summer months and are maintained at temperatures as high as 29 °C (Lin et al., 2012). However, further evidence from Perrigualt et al. (2011) suggests that this may not be a direct effect of temperature but as a response to increased pathogenic prevalence during summer months. Similar to the previous studies, intracellular ROS production in the clam *M. mercenaria* declined with temperature in unchallenged controls at 2 and 4 months. However, in clams exposed to the QPX for the duration of the experiment, ROS production was highest at 21 °C compared to levels at 13 or 27 °C at both sampling dates. Similarly, clams also demonstrated an increased ROS production at this temperature following zymosan stimulation, but only after 4 months. Overall, this suggests that pathogen pressure is capable of altering the response of this parameter to temperature. In contrast to the other immune parameters mentioned thus far, these interactions may allow bivalve's to maintain respiratory burst immune function at intermediate temperatures. Similarly, Wang et al. (2012) found that the scallop, *Chlamys farreri* had no significant suppression in ROS production under combined exposure to thermal stress and the pathogen, *Vibrio*

anguillarum. However, after 96h SOD activity began to fall under combined exposure relative to ROS levels, corresponding with a rise in oxidative damage and mortality. This suggests that whilst pathogen pressure may increase aspects of the respiratory burst, by promoting imbalances between antioxidant and super oxide production, these interactions may have negative consequences for organism health, potentially contributing towards bivalve morality outbreaks during periods of high temperature and pathogenic presence.

1.4.2 The effects of temperature on humoral defences

Apart from cellular parameters, the individual components of the humoral defence system are also shown to be altered by environmental temperature. Whilst the humoral activity of some hydrolytic enzymes such as acid phosphatase (ACP) (Chen et al., 2007) and leucine aminopeptidase (LAP) (Paillard et al., 2004) increase under intermediate levels of thermal stress, others correlated negatively with temperature. For example, lysozyme activity declined with increasing temperature in the haemolymph of the clam *R. philippinarum* (Paillard et al., 2004). Likewise, in the natural environment, lysozyme activity is lowest during summer months in the oysters *C. virginica* (Chu and La Peyre, 1989; Feng and Canzonier, 1970) and *C. gigas* (Li et al., 2009); associated with a rise in water temperature. Similarly, Parisi et al. (2017) reported the kinetics of the hydrolytic enzymes, esterase and alkaline phosphatase to decrease in the haemolymph of *M. galloprovinciallis* with increasing temperatures. In the same study, phenoloxidase activity was also supressed at higher temperatures. Being crucial to melanotonic encapsulation, this response alone likely has a significant impact on disease susceptibility.

Given the limited number of humoral defence components that have been investigated under thermal stress and the inconsistencies in the ones that have; it is difficult to interpret how temperature may impact humoral defence as a whole. One way to overcome this is by measuring cytotoxicity; interpreted generally as the capacity of the humoral peptide components within haemolymph to degrade foreign cells. When *M. galloprovinciallis* haemolymph was exposed to a heat treatment of 25 °C *in vitro*, the proportion of cytotoxic individuals (measured through the ability to cytolyse human-A-positive erythrocytes past a predetermined threshold) was reduced from 75 % in controls to 16 % (Malagoli and Ottaviani, 2005). This suggests that, through its effects on the individual humoral defence components, temperature elevations may ultimately compromise the contribution of humoral defence towards disease resistance.

In summary, evidence suggests that temperature has a complex and dynamic effect on the various cellular and humoral components of the bivalve innate immune system. Confusing the link between temperature and SMS is the fact that, within temperature ranges conductive to mass mortalities, many of these immune parameters remain unaffected, if not improved. Nevertheless, when pathogenic and thermal stressors are present simultaneously, a number of interactive effects on host defence are observed. Typically, this results in the significantly greater suppression of immune functions at elevated temperatures as well as lower temperatures being capable of inducing immune dysfunction. These responses often correlate with significantly greater disease prevalence and mortality when compared to single stressor models. As such, these interactive effects between pathogen and temperature on host immunity are suggested to be a major contributor towards SMS (Harvell, 2009; Pernet et al.,

2012; Tamayo et al., 2014, Wendling and Wegner, 2013). Ultimately, factoring in these interactive effects may provide the best assessment of the likely consequences to bivalve populations under GCC predictions.

1.5 Energetic trade-offs and immunosuppression under thermal stress

As demonstrated by the previous section, rises in environmental temperature issues a variety of complex effects depending on the experimental design, the species being investigated and the physiological parameters measured. Thus, a comprehensive assessment of immunocompetence and disease susceptibility under thermal stress is hampered by our limited understanding of what constitutes normality (Galloway and Depledge, 2001). To advance our current understanding of SMS, it is therefore necessary to incorporate additional measures of stress and capacity for immune investment. Evidence suggests immunocompetence and mass mortalities may not be influenced directly by thermal and pathogenic stressors, but by the indirect effects of a compromised energy balance (Berthelin et al., 2000; Pernet et al., 2012; Samain and McCombie, 2008; Wendling and Wegner, 2013). Like all physiological stress responses, immune defences require energy to mount and maintain (Buttgereit et al., 2000). Life history theory states that under diminishing energetic resources, life history traits, including host defence are traded-off to maintain higher priority physiological mechanisms directly related to organism homeostasis (Bibby et al., 2008; Sibly, 2002; Sokolova et al., 2012). Therefore, the extent to which an organism can invest in immune defences will depend on the relative energy investment in other, competing physiological stress responses (Moret and Schmid-Hempel, 2000) as well as the overall energetic condition of an organism

(quantities of energetic reserves and the efficiency of their acquisition and metabolic conversions) (Sokolova et al., 2012). Therefore, measuring aspects of bivalve bioenergetics offers a useful tool to realistically assess the extent to which temperature may compromise disease resistance and dictate the sustainability of a bivalve population under conditions of pathogenic presence (Rolff and Siva-Jothy, 2003; Sokolova et al., 2012). Thus, this section will outline both the individual effects of thermal stress and any potential interactions with pathogenic challenge on the bioenergetic activities of bivalves that may ultimately influence immunocompetence, disease and mortality.

To maintain physiological homeostasis under thermal stress, bivalves increase the production of thermoregulatory and cytoprotective mechanisms (Farcy et al., 2009; Hamdoun et al., 2003; Hamdoun and Cher, 2001; Piano et al., 2002; Yang et al., 2016). By increasing energetic demands, these physiological responses may incur significant energetic costs on bivalves. For example, following an increase in environmental temperature, metabolic activity has been found to increase significantly in the clam *Pisidium amnicum* (Heinonen et al., 2003), the mussel *M. edulis* (Ellis et al., 2014), the scallop *C. farreri* (Tang et al., 2010), the ark shell *Scaphorca subcrenata* (Jiang et al., 2020), and the surf clam *Paphia undulata* (Zhang et al., 2019), suggesting a temperature induced increase in energy demand. Elevated temperatures have also led to decreased glycogen concentrations in *C. farreri* (Wang et al., 2012), the mussel *P. viridis* (Turner et al., 2016) and *C. gigas* (Li et al., 2007). Overall, by limiting the energy availability for immune defences, the energetic costs associated with temperature elevation may explain the observed immunosuppression during periods of elevated environmental temperature.

Pathogenic exposure also causes significant energetic costs in bivalves (Plana et al., 1996, Tamayo et al., 2014, Turner et al., 2016) and may interact with temperature to increase the overall energetic demandson individuals. Compared to the single effects of high temperature and pathogen exposure, simultaneous exposure significantly increases the utilisation of glycogen reserves in bivalves (Pernet et al., 2012; Tamayo et al., 2014; Wang et al., 2012). In some cases, this reduction in energy stores is associated with an increase in metabolic activity, (Turner et al., 2016; Tang et al., 2010). Further, pathogenic stress may disrupt the normal utilisation of energy reserves in bivalves when in conjunction with thermal stress. Both Pernet et al. (2012) and Tamayo et al. (2014) found that combined exposure wasn't associated with any change in respiration rates or the activity of key enzymes related to gluconeogenesis and glycolysis in the oyster *C. gigas*; indicating no modification to the rate of energy supply. These responses corresponded with the maximum disease expression and mortality observed in these studies. Overall, by further reducing the energy available for immune defences, the increased depletion of energetic reserves along with the proliferation of metabolic disorders may factor in the enhanced immunosuppression, disease progression and mortality observed under combined thermal and pathogenic stressors.

Despite the fact optimum disease development and mortality often occur when energetic reserves are lowest in bivalves (Petton et al., 2010, 2012; Pernet et al., 2012, 2014, 2019; Plana et al., 1996), there is limited evidence of energetic trade-offs for immune defence in bivalves. Existing evidence comes mainly from a reproductive-immune context. Reproduction is an energetically costly process in bivalves (Berthelin et al., 2000). The added constraints reproduction has on energy balance have been associated with a reduction in key immune defences

including: phagocytic activity (Delporte et al., 2006; Duchemin et al., 2007; Wendling and Wegner, 2016), adhesive capacity (Delporte et al., 2006) and cytotoxicity (Malagoli et al., 2008). A similar response is seen in phenoloxidase activity (Lin et al., 2012) as well as haemocyte viability (Cho and Jeong, 2005; Hong et al., 2020) and abundance (Carballal et al., 1998; Fisher and Oliver, 1996). Further, SMS events occur at times of reproductive maturity in bivalves (Berthelin et al., 2000; Cho and Jeong, 2005; Delporte et al., 2006; Li et al., 2007, 2009; Perdue et al., 1981; Meo et al., 2006; Samain and McCombie, 2008; Solechnik et al., 1997, 2006), suggesting that disruptions in energy balance could indeed be a factor of immune performance and disease susceptibility in bivalves.

Few studies have incorporated bioenergetics when assessing the mechanisms controlling immunity and disease susceptibility in bivalves exposed to elevated temperature and pathogens. However from this design, the energetic costs associated with the combined presence of these stressors have been linked to the significant inhibition of immune parameters in bivalves, including the activities of antioxidants (Tang et al., 2010; Wang et al., 2012) and haemocytes (Turner et al., 2016). These responses correlate with the maximum disease expression and mortality observed in these studies, suggesting the combined effects of thermal and pathogenic stressors on energy availability may be a factor in immunocompetence, disease progression and mortality under times of SMS through trade-offs in immune defences.

1.6 Conclusion

Global climate change is causing significant alterations to ocean SST and to the organisms that inhabit the ocean. These alterations have been linked to increases in the frequency and severity of disease outbreaks and mass mortalities affecting marine species particularly, bivalves. Whilst this issue is considered one of the greatest challenges to the ecological and socioeconomic services bivalves provide, little is known on how increased temperature will affect important immunological defences and so disease resistance in bivalves.

The brief summary presented in this chapter demonstrates that rises in temperature can significantly impact bivalve immune defences. Currently, few of the defensive functions that comprise the bivalve immune system have been measured under thermal stress. Those that have, exhibit very variable responses compared with other immune parameters and those same parameters in individuals of different species. Overall, this highlights the difficulty of understanding the key immune responses to temperature that influence disease susceptibility in bivalves and their comparative importance in assessing the overall level of risk they face. Furthermore, few studies have investigated potential combined effects between thermal and pathogenic stress that may further alter host-pathogen dynamics. Ultimately, this means we have a limited understanding of the mechanisms driving mortalities within naturally heterogeneous environments and therefore a limited ability to design effective biomonitoring tools to assist in mitigating current and future losses. The cumulative evidence suggests that disease susceptibility is dependent on the interactions between pathogens and temperature on host defences. Often, the reduced effectiveness of the immune system, along with high densities and

virulence of pathogens, can result in enhanced disease development and mortality under elevated environmental temperature.

Evidence has also emerged that the energetic condition of bivalves plays a significant part in disease susceptibility. The complex effect of temperature and pathogenic challenge on energy availability is likely an important factor influencing summer mortalities by inducing trade-offs for immune functioning. The development of a multi-stressor approach incorporating a suit of immunological and biochemical parameters has been crucial in revealing the full impact of high temperature on the physiological condition of bivalves and the potential factors of SMS. However currently, these combined effects have only been studied within the context of few pathogenic or parasitic species associated with SMS, meaning the importance of these responses in controlling losses across a range of host-pathogen infection models remains poorly eluded.

Consequently, the aim of the experimental chapter of this thesis is to investigate the combined effects of temperature and the previously unstudied oyster pathogen, *N. crassostreae* on the Pacific oyster, *C. gigas*. In studying both cellular and humoral aspects of the immune response and metabolic activities relating to energy utilisation and physiological performance, this study provides evidence on some of the potential factors contributing towards disease and mortalities. It also explores the relevance of interactive effects in controlling losses across host-pathogen infection models generally.

Chapter 2: Effects of temperature and *Nocardia crassostreae* on the immune response of the Pacific oyster, *Crassostrea gigas*

Abstract

Summer mortality syndrome (SMS) is an ongoing problem within the Pacific oyster industry, having consequences for local economies and food security. Investigations into the potential causes strongly associate losses with a compromised immunological and energetic condition under times of high pathogen prevalence and environmental temperature. The disease nocardiosis, caused by the bacteria *Nocardia crassostreae* has been increasingly associated with oyster mass mortality, with evidence to suggest a role of temperature in these losses. To investigate the physiological mechanisms that potentially facilitate mass mortalities in oysters, a number of metabolic and immunological parameters were measured under *N. crassostreae* challenge, exposure to higher temperatures (21°C), and their combined treatment. No strong interactive effects were seen between these pathogenic and thermal stressors on any of the parameters measured in this study. Pathogenically challenged individuals had significantly decreased glycogen concentration and increased glucose and adenylate concentrations within their mantle tissue. This coincided with significantly higher haemocyte counts in these exposed groups. Within the higher temperature treatment, mantle ATP concentration, haemocyte viability and Na^+/K^+-ATPase activity were significantly reduced. Overall, this study presents some evidence that Pacific oysters combat *N. crassostreae* via the mobilisation and utilisation of energetic reserves, in order to meet the enhanced maintenance costs of immune defence. Temperature stress may increase the energetic costs on oysters and reduce the efficiency of immunobiological and metabolic functioning. Over longer time scales, the depletion of vital energy reserves and

the thermal suppression of homeostatic processes may be a key determinant of mass mortalities under *N. crassostreae* exposure and elevated environmental temperature. Furthermore, interactive effects between pathogenic and thermal stressors may not be important in controlling SMS in regards to every host-pathogen infection model.

Keywords: Mass mortalities, *Crassostrea gigas,* immunology*, Nocardia crassostreae,* energetics

2.1 Introduction

The Pacific oyster, *Crassostrea gigas* (Thunberg, 1793), is one of the most economically important molluscan species, with its aquaculture production accounting for 14 % of total marine production globally (2009-2014) (FAO, 2016). However, a major challenge to the Pacific oyster aquaculture industry is the regular occurrence of disease outbreaks and mass mortalities (Cheney, 2000).

Revealing the causal factors behind these mortality events has become a major research interest. Although there is a lack of consensus in the literature, it has been hypothesised that the multifactorial interaction between pathogenic exposure and environmental stressors on the physiological condition of hosts is a predominant factor behind these morality events (Garcia et al., 2011; Malham et al., 2009; Pernet et al., 2012, 2014). Numerous environmental stressors have been associated with mass mortalities (Goulletquer et al., 1998; Kristensen et al., 1958; Samain and McCombie, 2008; SEEEC, 1998). However, high summer temperatures exceeding 19 °C is by far the most commonly reported environmental factor associated with mass mortalities (Ford and Borrero, 2001; Garcia et al., 2011*;* Malham et al., 2009; Meyers and Short, 1990; Xiao et al.,

2005). As such, the phenomenon is regularly termed “Summer Mortality Syndrome” (SMS).

A diverse range of pathogenic species have been associated with SMS (Fernandez Robledo and Vasta, 2014; Kleeman and Adlard, 2000; Park and Choi, 2001; Gomez-León et al., 2005; Segarra et al., 2010), including the gram positive actinomycete bacteria, *Nocardia crassostreae*, the etiological agent behind the bivalve disease nocardiosis (Friedman and Hedrick, 1991). Originally reported within Japanese farmed stocks of *C. gigas* in the 1950’s, nocardiosis has subsequently become a worldwide issue, linked to mass mortality events during times of high environmental temperature in North America (Elston et al., 1987), the Netherlands (Englesma et al., 2008) and Italy (Carella et al., 2013).

A key determinant of the health and persistence of bivalves under pathogenic exposure is their ability to resist infection (Fisher et al., 1999). Oysters possess an innate immune system (Medzhitov and Janeway, 1997; Lochmiller and Deerenberg, 2000), which ensures survival against pathogenic assault through efficient cellular and humoral defensive mechanisms (Venier et al., 2011). These immune defences are energetically costly to mount (Buttgereit et al., 2000) and having sufficient energy reserves is essential to maintain resistance against pathogenic infection (Génard et al., 2013; Flye-Sainte-Marie et al., 2007; Pernet et al., 2014, 2019; Plana, 1996; Wang et al., 2012). Once energy reserves are depleted, immune function is quickly impaired, resulting in disease and mortality (Tang et al., 2010; Turner et al., 2016; Wang et al., 2012).

Temperature induces modifications to the oyster immune system, altering host-pathogen interactions. Exceeding a thermal threshold, a range of cellular and humoral immune functions are inhibited in oysters (Chu and La peyre, 1993;

Gagnaire et al., 2006; Hégaret et al., 2003; Li et al., 2007; Malham et al., 2009). Thermal stress can also impose a number of energetic costs on bivalves (Li et al., 2007; Tamayo et al., 2014; Yang et al., 2016), reducing energy allocation towards immune defence (Tang et al., 2010; Turner et al., 2016; Wang et al., 2012) as well as other physiological functions involved in environmental stress tolerance, such as ionic regulation (Bogdanova et al., 2016; Krauss et al., 2001; Pörtner, 2010). In addition, elevated temperature facilitates the proliferation and virulence of opportunistic bacteria (Harvell, 1999; Lopez-Joven et al., 2011; Sindermann, 1990). Overall, the direct and indirect effects of temperature on host physiology and pathogen prevalence increase the susceptibility of bivalves to disease and mortality within natural environments.

When combined, pathogenic and thermal stressors have significantly greater negative impacts on energy reserves (Pernet et al., 2012; Tamayo et al., 2014; Tang et al., 2010; Turner et al., 2016; Wang et al., 2012) and immune defences in bivalves (Chu and La Peyre, 1993; Perrigault et al., 2011; Hauton et al., 2001; Tang et al., 2010; Turner et al., 2016; Wang et al., 2012; Wendling and Wegner, 2013). Such interactive effects lead to higher rates of disease prevalence and mortality, when compared to single stressor models (Chu and La Peyre, 1993; Pernet et al., 2012; Turner et al., 2016; Wang et al., 2012). Therefore, the interactive effect between pathogens and temperature on host physiology have been suggested as a major cause of SMS in bivalve populations (Harvell, 2009; Pernet et al., 2012; Tamayo et al., 2014; Wendling and Wegner, 2013). Despite its potential importance, the combined effects of thermal and pathogenic stress on host physiology has only been investigated in few classes of bivalve pathogens, mainly involving *Vibrio*, Ostreid herpes virus, and protistan parasite

species. As such, the generality of these responses in controlling SMS associated with other important pathogenic species, including *N. crassostreae,* remains underexplored. Thus, the aim of this study was to investigate how the physiological mechanisms that underpin disease resistence and survival in the Pacific oyster, *C. gigas* are effected by combined exposure to both high temperature and *N. crassostreae* challenge. To address this aim, oysters were exposed to *N. crassostreae* at two experimental temperatures relating to the local summer mean and maxima where oysters were harvested for this study (Cefas, 2020). The extent of the immune response, ionic regulation and associated energetic costs were investigated by 1) measuring both cellular and humoral components of the oyster's immune system and Na^+/K^+-ATPase activity as well as 2) quantifying metabolic aspects of the oyster's energy utilisation. We predicted that 1) the immunocompetence of *C. gigas* would be enhanced following exposure to *N. crassosreae*, and would be associated with an increase in energy allocation towards immune defence; 2) exposure to elevated temperature would compromise oyster's immune and ionic regulation capacity due to energetic depletion at high temperatures ; 3) *Nocardia crassostreae* and temperature would interact significantly to ehance the immunological and metabolic costs on oysters.

2.2 Materials and methods

2.2.1 Oyster collection, depuration and acclimation

During June 2019, oysters (n = 300, average dry weight 1.54 ± 1.1 g) were harvested from natural soft bottom beds within the intertidal area of the Yealm estuary, Devon, UK (50°18'35.1"N, 4°03'12.8"W) and transported to the aquarium facilities at the University of Plymouth. Specimens were visually inspected and

displayed no obvious behavioural, morphological or pathological signs of disease (eg. shell gaping and tissue discoloration, pustules or lesions). They were cleared of epibionts and subjected to a depuration period in two 100 L tanks (n = 150 per tank). Tanks were provided with a continuous supply of UV treated, filtered sea water (temperature = 15 ˚C, salinity = 34, pH = 7.6 and dissolved oxygen = 95 % air saturation). Overflow water was allowed to run off from the base of the tanks creating a flow through system. During depuration, oysters were starved. Oysters were maintained under these conditions for 7 days, sufficient to reduce the level of contamination of *Vibrio* (Lopez-Joven et al., 2011), *E.coli* (Eyles and Davey, 1984), Ostreid herpes virus (Moreau et al., 2015) and *Salmonella* species (De Abreu Corrêa et al., 2007) in bivalves.

Immediately after the depuration period, oysters were randomly allocated to one of two temperatures, either 18 ˚C (representing the mean summer temperature in the Yealm estuary; Cefas 2020) or 21 ˚C (representing the maxima summer temperature for the Yealm estuary; Cefas, 2020). Each temperature treatment consisted of two 100 L holding tanks (n = 75 per tank) attached to independent biofilters (EHEIM, classic 250). Water temperature was gradually increased (1 ˚C d^{-1}) to the appropriate experimental temperature. Other environmental conditions were maintained as per the pre-exposure period (salinity = 34 ± 0.5, oxygen = 95 ± 5 % a.s, pH = 7.8 ± 0.2, 12:12 h light:dark regime). Total ammonia, nitrogen and nitrates were measured daily (API, saltwater master kit) and a 50 % water change was conducted every other day. Throughout acclimation, oysters were fed daily with Shellfish diet 1800 (Reed Mariculture®, California, USA) containing microalgae *Isochrysis, Parlora, Tetraseluis,*

Thallasoria and Thalassoria pseudodonana species and at a concentration of 23,000 cells mL^{-1} oyster $^{-1}$.

2.2.2 Bacteriological preparation

Cultures of *N. crassostreae* were sourced from DMSZ, Leibniz Institute, Germany. Cultures were incubated at 28 ˚C for 2 weeks, maintained on Brain heart infusion (BHI) agar containing 0.025 % Tyloxapol, selected to minimise clumping. Cultures were centrifuged (Sigma, 1-14) at 3,000 x G for 10 min and washed repeatedly in sterile seawater (SSW) before being resuspended in SSW. To avoid the clumping of cells, the culture was agitated five times by means of pipetting, and subsequently passed through a 70 µM cellular strainer. A 1:10 dilution in phosphate buffer (PBS) (66 mM, pH 6.2) was performed and the sample was read spectrophotometrically (Jenway ®, 7315) at λ= 595 nM to give an optical density of 0.1 µM. This gave an estimated working solution concentration of 1 x 10^8 cells mL^{-1}. The mucosaccharide secretions characteristic of *Nocardia spp.* resulted in colony aggregations. To ensure consistency on dosing, all treatment tanks were dosed from frozen aliquots of the same batch of *N. crassostreae* working solution.

2.2.3 Experimental set-up and design

Immediately after temperature acclimation, oysters were divided based on their respective acclimation temperatures into 20 x 29 L sealed tanks (n=14 oysters per tank), with 10 of these set to 18 ˚C and the other 10 to 21 ˚C. Temperature was maintained by means of an air conditioning system for the 18 ˚C treatments (± 0.4 ˚C), whilst 21 ˚C treatment tanks (± 0.5 ˚C) were individually supplied with 150 W aquarium heaters (EHEIM, thermocontrol). Within each temperature

treatment, tanks were subdivided (n= 5 tanks) into either infected or uninfected treatments. Following feeding (described in section *2.2.1*), infected tanks were each dosed with *N. crassostreae* by pipetting the solution directly over the water surface to achieve an in tank concentration of 7 x 10^3 cells mL^{-1} $oyster^{-1}$. Control treatments were dosed with an equal volume of PBS (66 mM, pH 6.2). Each tank contained a mesh tray to suspend oysters off the bottom, avoiding uptake of faecal matter and thus any potential false induction of immune response. Tanks were fitted with an aeration system and HEPA filters to allow air exchange, whilst preventing cross contamination between treatments. Environmental conditions and feeding were maintained as described in section *2.2.1*.

To ensure water quality, trays were moved into replica tanks containing clean, UV-filtered sea water set to the appropriate treatment temperature every 3 d. Original tanks were then disinfected with hypochlorite (14 %) for 24 h, rinsed and filled with UV treated filtered sea water maintained at the required treatment temperature until the next water change. Trays were circulated between treatment tanks to avoid potential tank effects. After every water change, oysters were fed and infected treatment tanks were re-dosed with *N. crassostreae* (7 x 10^3 cells mL^{-1} $oyster^{-1}$), whilst control treatments were re-dosed with an equal volume of PBS (66 mM, pH 6.2).

2.2.4 Response variables

Oysters were maintained in experimental conditions, as described in section *2.2.3* for a total of 35 days. Sampling for immunological parameters and condition index was carried out at 14, 28 and 35 d of exposure (n = 15 per time point per treatment). To reduce processing times, oysters were sampled in two batches, with both containing oysters sampled from every treatment tank. Batch effects

were later incorporated into the analyses. Haemolymph (≥ 400 µL) was withdrawn from the posterior adductor muscle using a 1 mL syringe, fitted with a 25 G needle, through a small hole drilled in the ventral edge of the upper shell valve. Heamolymph was placed in a 1.5 mL microcentrifuge tube cooled on ice to prevent aggregation (Gagnaire et al., 2006), vortexed for 30 s and aliquoted for the various immune assays. All immunological assays were carried out on haemolymph from each individual. For haemocyte counts and cell viability measurements, 20 µL haemolymph was added to an equal volume of cooled anticoagulant TBS buffer (pH 7.6 Tris-HCl buffer; 0.1 M, containing 2 % NaCl, 2 % glucose and 0.5 mmol L^{-1} EDTA). For protein analysis, 40 µL was flash frozen in liquid nitrogen and stored at -80 °C until further use. The remainder was then centrifuged at 700 x G for 10 min and 170 µL of cell free haemolymph (CFH) was separated and flash frozen for lysozyme activity. The resultant pellet was resuspended in any remaining supernatant (≥170 µL) by vortexing, before 90 µL was added to an equal volume of cooled TBS for phagocytic activity analysis. Following the final immunological sampling point at 35 d, all remaining oysters were sacrificed and the gill and mantle tissue was exercised, flash frozen and stored at -80 °C for biochemical analysis. Again, oysters were sampled in two batches.

2.2.4.1 Condition index

Following Widdows and Johnson (1988), condition, based on the dry weight of tissues (g) was calculated according to the equation:

Condition = 1000 (Dry tissue wt (g)) / (Shell cavity volume (mL)) **Eq 1**

Before immunological processing, the closed volume for each individual was calculated by displacement. Following haemolymph withdrawal, whole body tissue was dissected out of the shell and dried at 60 ˚C for 4 d and weighed on an analytical balance (Mettler Toledo ®, ME54TE/00) to obtain dry tissue weight. Volume by displacement was then calculated for each shell valve and shell cavity volume subsequently calculated by subtracting shell valve volume from the closed volume.

2.2.4.2 Haemocyte counts and cell viability

Haemolymph suspension was combined with 40 µL of chilled Eosin Y solution (0.02 %) to stain dead/damaged cells, and allowed to stand for 3 min to enable uptake of the dye. After a 10 s vortex, 10 µL was loaded into the top and bottom chamber of a haemocytometer (Neubauer ®, 5971C10). Cell counts and viability was quantified simultaneously under a compound light microscope (Olympus, CHT-G) (x 40) from an average of four corner squares. Results are expressed as total haemocyte counts (1 x 10^6 mL^{-1}) and cellular viability (%).

2.2.4.3 Lysozyme activity

CFH (50 µL) was pipetted into three replicate multiplate wells. Into each well, 230 µL of a *Micrococcus lysodeikticus* (0.2 mg L^{-1}) suspension in PBS (66 mM, pH 6.2), was added (Monari et al., 2007). *M. lysodeikticus* solution was added to PBS alone as a control and into standard solutions of crystalline hen egg white lysozyme (Sigma) in TBS buffer (6.2 pH) at 1, 2.5, 5, and 10 µg lysozyme mL^{-1}. Plates were mixed by shaking and the decrease in absorbance (DA min^{-1}) was recorded spectrophotometrically in a VersaMax™ microplate reader (Molecular Devices LLC., California, U.S) for 4 h at 25 °C (shown by preliminary analysis to

cause the maximum decrease in absorbance). An average decrease in absorbance (DA) was calculated from each sample and divided by the recorded time. Average (DA min^{-1}) was plotted against the standard enzyme concentrations. Values were expressed as lysozyme activity (µg mL^{-1} haemolymph protein).

2.2.4.4 Haemolymph protein content

Haemolymph protein concentrations were determined using the Pierce™ BCA Protein Assay Kit adapted to use in microplate format. Bovine serum albumin was used as a standard. Haemolymph diluted 1:10 with distilled water and 200 µL of dye reagent was mixed with 10 µL of each sample and incubated at 37 °C for 30 min. Absorbance was read in a VersaMax™ microplate reader (Molecular Devices LLC., California, U.S) at λ = 562 nm. An average from three replicates was generated for each sample and the values were expressed as mg protein mL^{-1} of haemolymph.

2.2.4.5 Tissue processing for biochemical analysis.

Biochemical extraction and analysis was conducted on samples following the protocols of Bergmeyer (1985a, 1985b), adapted to a microplate format (see Appendix 1). Mantle samples were homogenised in 0.9 mol L^{-1} perchloric acid (PCA) (1:2 w/v ratio) with a bullet blender (Thistle Scientific), centrifuged at 3,000 rpm for 3 min and the supernatant extracted. The solution was neutralised with 3.75 mol L^{-1} K_2CO_3 (1:5 parts of PCA originally used), centrifuged and the supernatant extracted to be frozen at -80 °C. For gill tissue extraction, frozen tissue were homogenised with a 1:10 w/v ratio of 7.3 pH SEHD buffer (Sucrose; 0.17 mol L^{-1}, EDTA; 0.19 mol L^{-1}, HEPES; 0.03 mol L^{-1}, sodium deoxycholate;

0.21 mol L^{-1}). Homogenate was centrifuged at 13,000 rpm for 60 s and the supernatant collected and frozen at -80 °C. Tissue samples and solutions were stored on ice and sample processing was conducted in a 4 °C cold room.

2.2.4.6 ATP Concentration

Homgenised mantle tissue samples were added to microplate wells in duplicate along with the reaction buffer (pH 7.6 Triethanolamine hydrochloride; 0.1 mmol L^{-1} added to: 3-phosphoglyceric acid; 18 mmol L^{-1}, K_2CO_3; 4.5 mmol L^{-1}, $MgSO_4$; 4 mmol L^{-}1, EDTA; 1.1 mmol L^{-1}, Triethanolamine hydrochloride; 100 mmol L^{-1}). This reaction buffer was used as a blank and 1 mmol L^{-1} ATP (untreated) was used as a control. The initial absorbance was recorded in a VersaMax™ microplate reader (Molecular Devices LLC., California, U.S) at λ = 339 nm for 6 min (Abs1) before the reaction was started with the addition of a GAPDH (320 U mL^{-1}) / PGK (180 U mL^{-1}) enzyme mixture. Decrease in absorbance was then recorded for a further 10 min (Abs2). Results are expressed as µmol g^{-1} using the following equation:

$$[ATP] = \frac{\Delta As - \Delta b}{6.3} \times \frac{\text{Sample Vol}}{\text{well Vol}} \qquad \textbf{Eq 2}$$

Where:

Δs = the change in absorbance of the sample (Abs1-Abs2)

Δb = the change in absorbance of the blank (Abs1-Abs2)

6.3 = the extinction coefficient of NADH (L x mmol L^{-1} x cm^{-1})

2.2.4.7 AMP and ADP concentration

Homogenised mantle tissue samples were added to microplate wells in duplicate along with the reaction buffer (K_2CO_3; 430 mmol L^{-1}, NADH; 17 mmol L^{-1} in 0.6 mol L^{-1} $NaHCO_3$). Equal measures of buffer was used as a blank and wells containing both ADP (0.5 mmol L^{-1}) and AMP (0.25 mmol L^{-1}) were used as a control. The initial absorbance was recorded in a VersaMax™ microplate reader (Molecular Devices LLC., California, U.S) at λ = 340 nm for 6 min (Abs 1) before the reaction was started with the addition of the enzyme pyruvate kinase (17.9 U mL^{-1}). The fall in absorbance was recorded for 10 min (Abs2), indicating the concentration of ADP. Following this the enzyme myokinase (15.9 U mL^{-1}) was added and the subsequent fall in absorbance was recorded for a further 10 min (Abs 3), representing the concentration of AMP in each sample. The concentrations of ADP and AMP were obtained and presented as µmol g^{-1} using the following equations:

$$[ADP] = \frac{\Delta AS - \Delta Ab}{6.3} \qquad \textbf{Eq 3}$$

Where:

∆As = change in absorbance of the sample (Abs1–Abs1)

∆Ab = change in absorbance of the contol (Abs1–Abs1)

6.3 = the extinction coefficient of NADPH (L x mmol L^{-1} x cm^{-1})

$$AMP = \frac{\Delta Ass - \Delta bb}{6.3} \quad \textbf{Eq 4}$$

Where:

ΔAss = change in absorbance of the sample (Abs2 – Abs3)

ΔAbb = change in absorbance of the control (Abs2 – Abs3)

6.3 = the extinction coefficient of NADPH (L x mmol L^{-1} x cm^{-1})

From these respective values, the total adenylate nucleotide pool (TAN) and the adenylate energy charge (AEC) were calculated using the equations:

$$[TAN] = [ATP] + [ADP+ [AMP] \quad \textbf{Eq 5}$$

$$AEC = \frac{[ATP]+ ½[ADP]}{[AMP]+[ADP]+[ATP]} \quad \textbf{Eq 6}$$

2.2.4.8 Glucose and glycogen concentration

In order to quantify the glycogen concentration of homogenised mantle samples, glycogen was hydrolysed into glucose. Homogenate was added to 134 µL of glucoamylase and incubated at 40 °C with routine mixing every 15 min. After 2 h, 67 µL of $HClO_4$ (0.6 mol·L^{-1}) was added to stop this reaction. The digested sample was centrifuged (Sigma, 1-14) at 10, 000 x g for 5 min and the supernatant was removed for analysis. Samples (hydrolysed or unhydrolysed) (12 µL) were added to microplate wells in duplicate followed by 200 µL of buffer (ATP; 1mmol L^{-1}, NADP; 0.9 mmol L^{-1}, G-6-P DH; 140 U mL^{-1}). Wells containing buffer only

(212 µL) were used as a blank and glucose (2.78 mmol L^{-1}) was added as a control. The absorbance was recorded at λ = 340 nm in a VersaMax™ microplate reader (Molecular Devices LLC., California, U.S) for 10 min to give an initial absorbance value (As1). The reaction was then started with the addition of the enzyme hexokinase (280 kU L^{-1}) and the increase in absorbance was recorded for 5 min to give a final absorbance value (As2). The glucose content of mantle was calculated using the equation below. Glycogen content of mantle samples were calculated by subtracting the concentration of glucose in the samples from the glucose concentration obtained from glycogen digestion. Both are expressed as µmol g^{-1}.

$$[\text{Glucose}] = \frac{\Delta As - \Delta Ab}{6.3} \times \frac{\text{Well}}{\text{Sample}} \qquad \textbf{Eq 7}$$

Where:

ΔAs = the change in absorbance of the sample (Ab2 - Ab1)

ΔAb = the change in absorbance of the blank (Ab2 - Ab1)

6.3 = the extinction coefficient of NADPH (L x mmol L^{-1} x cm^{-1})

Well = the total volume of the solution in the well

Sample = the volume of the sample in the well

2.2.4.9 Na^+/K^+- ATPase activity

Na^+/K^+-ATPase activity was measured following the protocol described by McCormick (1993), recently adapted for bivalves (Giacomin et al., 2013). Briefly, this method calculates the difference in ADP production in gill samples by measuring a control (no inhibition) and in an inhibited reaction, thus enabling the correction for the activity of other ATPases in the tissue. Quabain (Sigma) was used as an inhibitor of Na^+/K^+-ATPase activity. Absorbance was continuously recorded in a VersaMax™ microplate reader (Molecular Devices LLC., California, U.S) at λ = 340 nm for 20 min. The linear rate was calculated for each sample and corrected against a blank of SEHD buffer and the activity of other present ATPases. Protein content of samples was determined as described in section *2.2.4.4.* Results were expressed as μmol ADP min^1 mg $protein^{-1}$ using the equation:

$$\text{ATPase activity} = \frac{\Delta A \cdot V_T}{\varepsilon \cdot L \cdot V_S \cdot [\text{protein}]} \qquad \textbf{Eq 8}$$

Where:

ΔA = difference between linear rate A and linear rate B (min^{-1}).

VT = total assay volume (mL)

ε = molar extinction coefficient of NADH @340 nm (6.22 mM^{-1} cm^{-1})

L = light path length (0.6251 cm)

V_S = sample volume (mL)

[protein] = concentration of total soluble proteins (mg mL^{-1})

2.2.5 Statistical analysis

All statistical analysis was conducted using R studio v1.2.1335 (RStudio team 2019). Data were plotted to visually inspect for homogeneity of variance and normality. Data that did not satisfy these assumptions were either log_{10} or square root transformed before analysis. Data in figures are non-transformed values. For data pertaining to each assay, linear mixed effect model tests were used to investigate the effects of the temperature and infection, incorporating the terms 'tank' and 'batch' as random factors and individual 'dry mass' as a covariate. Terms were removed if they produced an insignificant P-value and if Akaike information criterion produced a smaller sample correction for the simplified model (AIC) (Akaike, 1974). The main and interactive effects of fixed factors on immunological and energetic parameters was tested on the simplest model using ANOVA. Pair-wise comparisons using Tukey's honest significant difference test were performed where a significant effect was found involving more than two levels or where significant interactions involving two or more factors were found. Significance was accepted at $p < 0.05$.

2.3 Results

2.3.1 Effects of *N. crassostreae* and temperature on the energetics and ionic regulation of *C. gigas*

Exposure length and *N. crassostreae* challenge were both shown to significantly affect condition index in oysters (**Table 2**). Generally, oyster condition declined temporally across all treatments. *Nocardia crassostreae* exposure also caused a significant decline in the condition of oysters, when compared to uninfected controls (**Fig 1.a)**

Whilst individually temperature and *N. crassostreae* exposure had no significant main effect on mantle glycogen content of oysters, the two stressors were shown to interact (**Table 3**). Pair-wise analysis indicated that compared to the unchallenged control at 18°C, glycogen levels were significantly reduced within *N. crassostreae* exposed treatments at both 18 °C and 21 °C, causing a 55 % and 59 % reduction respectively (**Fig 1.b**). Whilst not significant, unchallenged oysters maintained at 21 °C also showed a comparable 53 % decline in this parameter, compared to unchallenged oysters maintained at 18 °C (**Fig 1.b**). No significant differences were found between the effects of high temperature, *N. crassostreae* exposure or their combined treatment on this parameter (**Fig 1.b**)

Nocardia crassostreae exposure also caused significant alterations to the mantle glucose levels detected within oysters (**Table 3**). Compared to unexposed controls, groups challenged with *N. crassostreae* had on average, 295 % higher glucose concentrations within their mantle tissue (**Fig 1.c**).

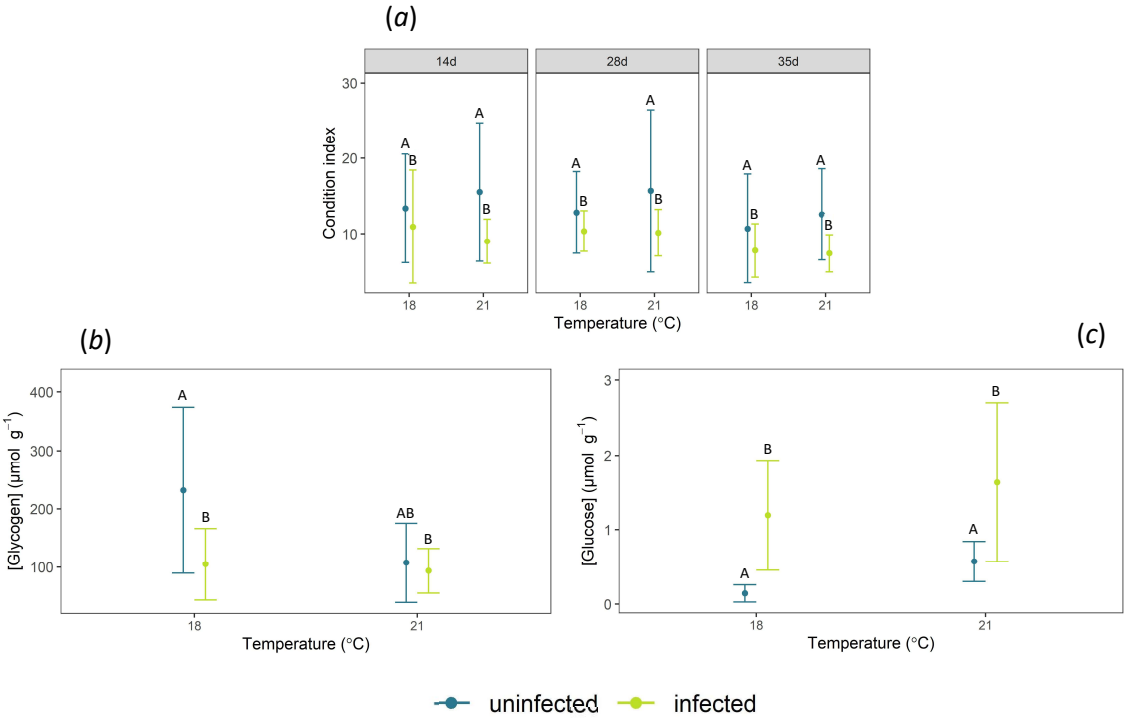

Fig 1 Average values (± se) for energetic parameters at 18 °C and 21 °C sampled following a 35d exposure to *N. crassostreae* or control conditions. (a) Condition index, (b) Glycogen concentration (µmol g^{-1}) and (c) Glucose concentration (µmol g^{-1}). Treatment groups not connected by the same letter are significantly different from one another based on pair-wise tests (p = <0.05).

Table 2 Three-way ANOVA results for the effects of temperature, *N. crassostreae* exposure, date and their interaction on condition index of *C. gigas*. Degrees of freedom (DF), F (F) and probability value (*p*). Significant values ($p < 0.05$) are highlighted in bold.

Source	DF	F	*p*
Temp	1	0.47	0.494
Noc	**1**	**17.7**	**<0.001**
Date	**2**	**3.84**	**0.024**
Temp + Noc	1	2.12	0.148
Temp + Date	2	0.06	0.939
Noc + Date	2	0.12	0.890
Temp + Noc + Date	2	0.04	0.961
Residuals	159		

Table 3 Two-way ANOVA results for the effects of temperature, *N. crassostreae* exposure and their interaction on energetic reserves, cellular energy status and ionic regulation in *C. gigas.* Significant values ($p < 0.05$) are highlighted in bold. See appendix B.1 for results of pair-wise tests for parameters with significant interactive effects (glycogen).

Parameter	Temperature	Nocardia exposure	Temperature+ Nocardia exposure
Glycogen	$F_{1, 25} = 2.59$, $p = 0.120$	$F_{1, 25} = 4.04$, $p = 0.055$	**$F_{1, 25} = 4.24$, $p = 0.050$**
Glucose	$F_{1, 25} = 11.6$, $p = 0.985$	**$F_{1, 25} = 11.6$, $p = 0.002$**	$F_{1, 25} = <0.001$, $p = 0.985$
ATP	**$F_{1, 97} = 19.4$, $p = <0.001$**	$F_{1, 97} = 0.33$, $p = 0.564$	$F_{1, 97} = 3.62$, $p = 0.060$
ADP	$F_{1, 97} = 0.06$, $p = 0.801$	**$F_{1, 97} = 6.85$, $p = 0.010$**	$F_{1, 97} = 0.41$, $p = 0.522$
AMP	$F_{1, 17} = 0.52$, $p = 0.479$	$F_{1, 60} = 1.84$, $p = 0.180$	$F_{1, 17} = 0.14$, $p = 0.708$
TAN	$F_{1, 97} = 1.15$, $p = 0.287$	**$F_{1, 97} = 4.56$, $p = 0.035$**	$F_{1, 97} = 0.37$, $p = 0.546$
AEC	$F_{1, 97} = 0.14$, $p = 0.711$	$F_{1, 97} = 1.62$, $p = 0.208$	$F_{1, 17} = 2.45$ $p = 0.136$
ATPase	**$F_{1, 57} = 5.00$, $p = 0.029$**	$F_{1, 57} = <0.001$, $p = 0.933$	$F_{1, 57} = 0.02$, $p = 0.227$

Temperature had no significant main or interactive effects with *N. crassostreae* exposure on any of the adenylate nucleotide parameters measured in this study apart from ATP, where a significant main effect was detected (**Table 3**). Within the mantle tissue of oysters maintained at 21 °C, ATP levels fell by an average of 34%, compared to controls groups kept at 18 °C (**Fig 2.a**). In terms of the effects of *N. crassostreae* exposure, significantly higher levels of ADP and total nucleotide pool (TAN) were detected within the mantle tissue of challenged oysters (**Table 3**), demonstrating an average increase of 12 and 19% respectively, when compared to unexposed controls (**Fig 2.b, Fig 3.a**).

Finally, ion regulation (measured through the activity of Na^+/K^+-ATPase within oyster gill tissue) showed a significant response to temperature (**Table 3**). The activity of this enzyme in oysters maintained at 21 °C declined by 25 %, when compared to non-thermally challenged controls (**Fig 3.c**).

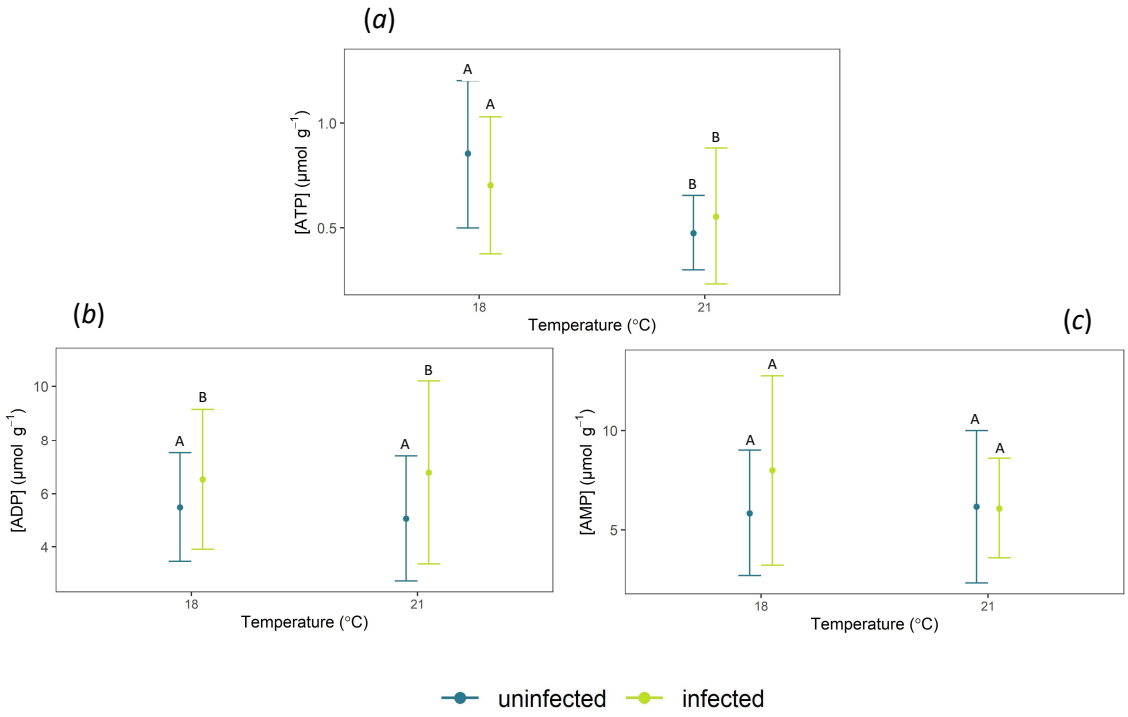

Fig 2 Average values (± se) for energetic parameters at 18 (left side) and 21 °C (right side) sampled following a 35d exposure to *N. crassostreae* or control conditions. (a) ATP concentration (µmol g^{-1}), (b) ADP concentration (µmol g^{-1}) and (c) AMP concentration (µmol g^{-1}). Treatment groups not connected by the same letter are significantly different from one another based on pair-wise tests ($p = <0.05$).

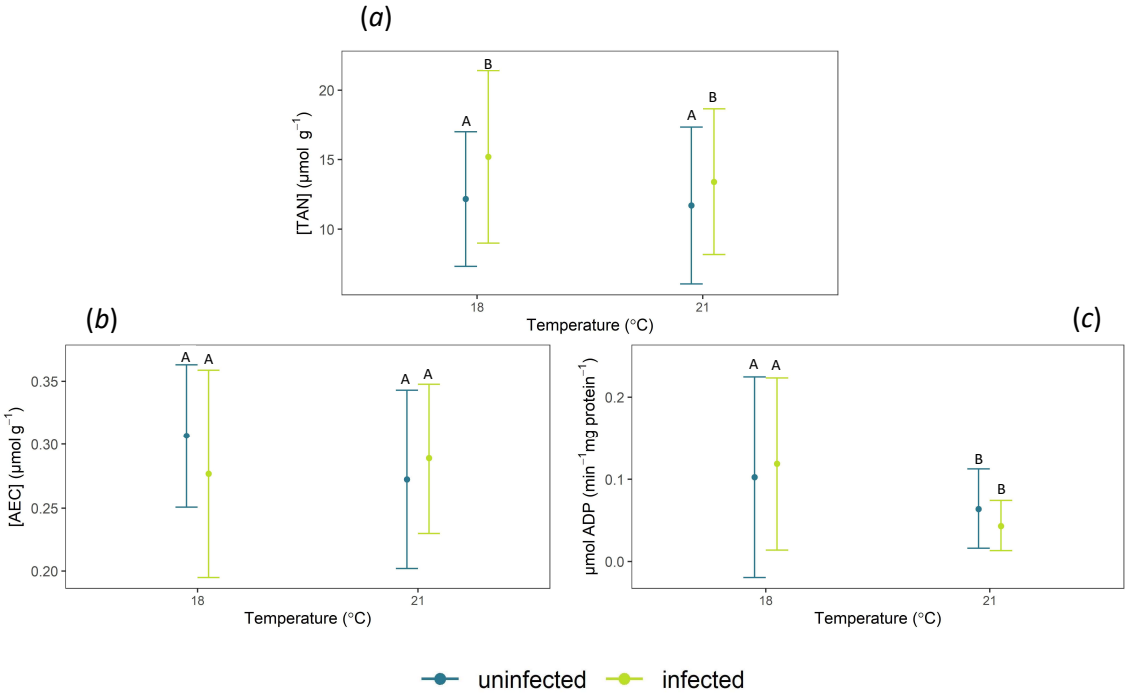

Fig 3 Average values (± *se*) for energetic parameters at 18 (left side) and 21 °C (right side) sampled following a 35d exposure to *N. crassostreae* or control conditions. (*a*) TAN concentration (µmol g^{-1}), (*b*) AEC concentration (µmol g^{-1}) and (c) Na+/K+-ATPase activity (µmole ADP (min^{-1} mg $protein^{-1}$)). Treatment groups not connected by the same letter are significantly different from one another based on pair-wise tests (p = <0.05).

*2.*3.2 Effects of *N. crassostreae* and temperature on the immunological activity of *C. gigas*

Nocardia crassostreae exposure had a significant main effect on the total number of circulating haemocytes within oyster haemolymph (**Table 4.a**). On average, pathogenically-challenged oysters had 39% higher haemocyte counts, when compared to unexposed controls (**Fig 4.a**).

The percentage viability of circulating haemocytes was significantly affected by exposure length and temperature (**Table 3.b).** Pair-wise analysis revealed that at 35 days post-exposure, the viability of non-pathogenically challenged oysters maintained at 21 °C was 20% higher and significantly different than the same treatment sampled at 28 days (**Fig 4.b**). When sampled at 14 days post-exposure, viability significantly declined by 20 % in pathogenically challenged oysters maintained at 21 °C, compared to both challenged and non-challenged oysters maintained at 18 °C (**Fig 4.b**). After 28 days, viability was also significantly reduced by 16 % in unchallenged oysters maintained at 21 °C, when compared to unchallenged oysters at 18 °C (**Fig 4.b**).

Lysozyme activity in the cell free haemolymph of oysters was effected by temperature, exposure length and the interactive effects of both date and infection (**Table 4. c**). Pair-wise analysis indicated that activity was significantly higher across all treatment groups at 28 and 35 days post-exposure, compared to their respective levels detected initially at 14 days (**Fig 4.c**). Conversely, when comparing lysozyme activitiy in oysters sampled later at 35 days with the same treatments at 28 days post-exposure, only oysters maintained at 18 °C

demonstrated significantly increased values. This was consistent between both infection groups however activity increased by 77% in challenged and by only 7% in unchallenged individuals at that temperature. Despite this, no significant differences were detected between these groups (**Fig 4.c**).

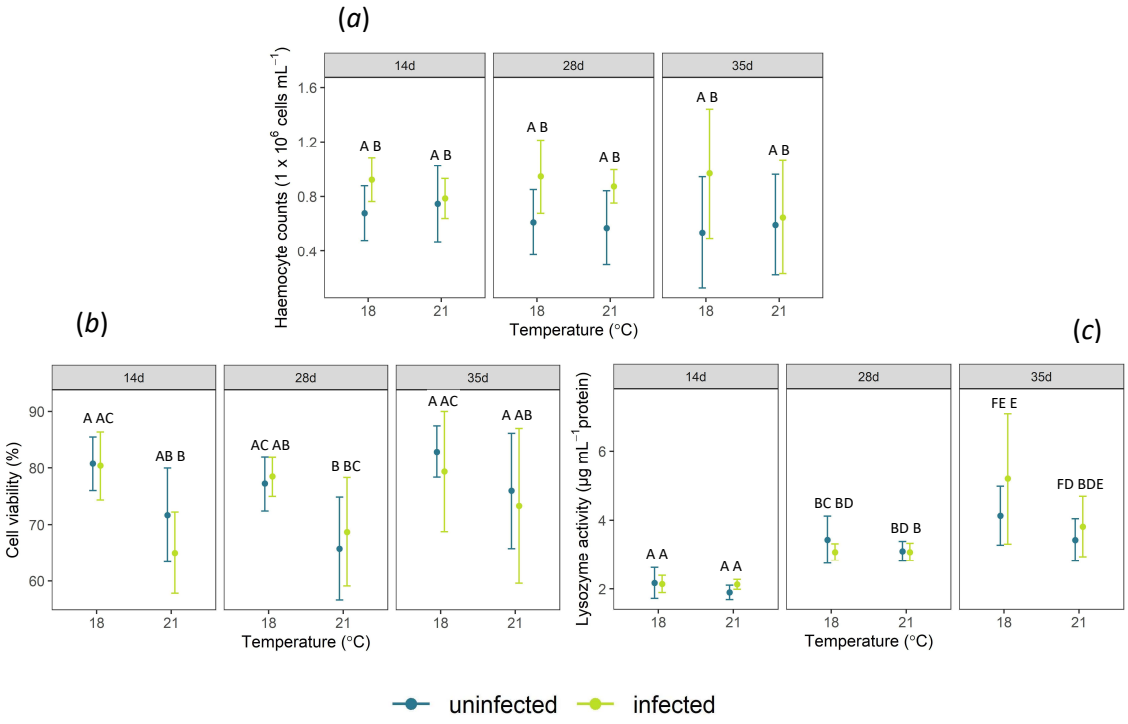

Fig 4 Average values (± *se*) for immunological parameters at 18 °C and 21 °C shown at 14, 28 and 35 day sampling points following exposure to *N. crassostreae* or control conditions (*a*) Haemocyte counts (1 x 10^6 cells mL^{-1}), (*b*) Cell viability (%) and (*c*) Lysozyme activity (µg mL^{-1} protein). Treatment groups not connected by the same letter are significantly different from one another based on pair-wise tests (p = <0.05).

Table 4 Three-way ANOVA results for the effects of temperature, *N. crassostreae* exposure, date and their interaction on (*a*) haemocyte counts, (*b*) cell viability and (*c*) lysozyme activity in *C. gigas*. Degrees of freedom (DF), F (F) and probability value (*p*). Significant values ($p < 0.05$) are highlighted in bold. See appendix B.1 for results of pair-wise tests for parameters with two or more significant main effects or significant interactive effects (percentage viability, lysozyme activity).

(*a*)

Source	DF	F	*p*
Temp	1	0.68	0.409
Noc	**1**	**19.6**	**<0.001**
Date	2	1.14	0.324
Temp + Noc	1	3.09	0.082
Temp + Date	2	0.19	0.828
Noc + Date	2	0.91	0.405
Temp + Noc + Date	2	0.84	0.434
Residuals	110		

(*b*)

Source	DF	F	*p*
Temp	**1**	**47.0**	**<0.001**
Noc	1	1.04	0.310
Date	**2**	**4.22**	**0.017**
Temp + Noc	1	0.17	0.678
Temp + Date	2	1.16	0.318
Noc + Date	2	1.62	0.202
Temp + Noc + Date	2	0.72	0.489
Residuals	102		

(*c*)

Source	DF	F	*p*
Temp	**1**	**47.0**	**0.023**
Noc	1	1.04	0.217
Date	**2**	**4.22**	**<0.001**
Temp + Noc	1	0.17	0.537
Temp + Date	2	1.16	0.051
Noc + Date	**2**	**1.62**	**0.045**
Temp + Noc + Date	2	0.72	0.362
Residuals	106		

2.4 Discussion

This study investigated the physiological changes in *C. gigas* that may influence disease susceptibility and mortality under exposure to *N. crassostreae* and temperature elevation. No strong interactive effects between *N. crassostreae* and temperature were seen on any of the parameters measured in this study. Nevertheless, oysters in this study still demonstrated a number of physiological responses to the effects of *N. crassostreae* and increased temperature which may provide information on some of the underlying mechanisms contributing towards the summer mortality events associated with this pathogen. Exposure to *N. crassostreae* caused significant reductions in glycogen concentrations and condition index as well as significant rises in glucose levels and the total adenylate pool. This corresponded with significantly increased concentrations of circulating haemocytes. This may suggest that *N. crassostreae* exposure can result in significant energetic costs to Pacific oysters, in part at least, to mount an effective immune response. Elevated environmental temperature may also reduce energy availability, indicated through the significant reduction of ATP concentrations in oysters maintained at 21 °C. Haemocyte viability and the activity of Na^+/K^+-ATPase was also significantly supressed at this temperature, suggesting the energetic constraints imposed by thermal stress may have negative consequences for disease resistance and stress tolerance. Overall, evidence is presented that within natural environments, these pathogenically and thermally induced negative effects on oyster metabolic and immunological functioning may be a contributing factor in mortalities of oysters exposed to *N. crassostreae* during periods of high temperature.

Temperature interacts significantly with a range of pathogenic species associated with SMS (Baker-Austin et al., 2010; Lopez-Joven et al., 2011; Oliver et al., 1998; Vezzulli et al., 2013). Under combination, these stressors result in magnified negative effects for the energetic and immunological condition of bivalves, compared to single stressor exposure (Chu and La Peyre, 1993; Pernet et al., 2012; Perrigault et al., 2011; Hauton et al., 2001; Tamayo et al., 2014; Tang et al., 2010; Turner et al., 2016; Wang et al., 2012; Wendling and Wegner, 2013). Such intercative effects can lead to significantly increased rates of disease development and mortality (Chu and La Peyre, 1993; Pernet et al., 2012; Turner et al., 2016; Wang et al., 2012). As such, they are increasingly suggested to be a predominant factor behind cases of SMS globally (Harvell, 2009; Pernet et al., 2012; Tamayo et al., 2014; Wendling and Wegner, 2013). Conversely, no strong interactive effects between *N. crassostreae* and temperature were seen on any of the parameters measured in this study. Interactive effects between temperature and pathogens on host physiology are most significant when temperature rises favour pathogen-specific requirements for proliferation and transmission (Hauton et al., 2001). Compared to the infectious agents typically associated with these interactive effects, *N. crassostreae* is relatively slow growing and has no proliferation type response following rises in temperature (Friedman and Hedrick, 1991). Therefore, whilst temperature effects on the activities of pathogens likely contributes towards disease progression and mortality in many cases, this study suggests that these effects may not be deterministic of SMS in regards to all pathogenic species, including *N. crassostreae.*

Exposure to *N. crassostreae* caused significant alterations to the immunology and energetics of oysters in this study. While no significant changes in lysozyme activity were detected in response to *N. crassostreae*, challenged oysters at both 18 and 21 °C were characterised by having consistently upregulated concentrations of circulating haemocytes. Oysters challenged with *N. crassosteae* at both 18 and 21 °C also had significantly reduced glycogen concentration, condition index and increased glucose, ADP and total adenylate nucleotide concentrations. Haemocytes are the most integral part of the molluscan immune system, providing protection predominantly through their phagocytic properties (Hooper et al., 2007; Gestal et al., 2008). Previously, histopathology has revealed high concentrations of haemocytes surrounding colonies of *N. crassostreae* in *C. gigas* that are capable of eliminating the bacteria *via* diapedensis (haemocyte encapsulation and ejection across epithelial borders) (Bower et al., 2005). Therefore, it has been speculated that these cells offer a protective role against the pathogen. Consistent with this, our findings suggest *C. gigas* accelerates the production and/or mobilisation of haemocytes from peripheral tissues to help combat infection, a similar reaction to that seen in response to other pathogenic species (Wendling and Wegner, 2013).

Haemocytes require large portions of energy to mount and maintain (Pernet et al. 2006) as well as to assist the action of their various defensive functions (Coyne, 2011). Glycogen is the predominant energy storage molecule in oysters (Giese, 1969). Following a significant increase in metabolic demands, oysters mobilise and convert these stores into more accessible compounds such as glucose (Caballero, 2009) and adenylate nucleotides (Gäde, 1981). Often, this correlates with a reduction in condition index (Walne, 1970). Previous reports have identified

these responses in oysters exposed to the parasite *Haplosporidium nelson* (Barber et al., 1988; Ford and Figueras, 1988) and the herpes virus (OsHV-1) (Pernet et al., 2012; Tamayo et al., 2014), which may represent the increased demand for immune defences (Génard et al., 2013). Therefore, the correlation between enhanced cellular defences and glycogenolytic/glycolytic activity observed in this study, supports the hypothesis that oysters produce an upregulated immune defence in response to *N. crassostreae* through the increased mobilisation and utilisation of energetic stores. Alteratively, other symptomatic factors of pathogenic infection such as lesions (Bower et al., 2005) and reduced feeding activity (Génard et al., 2011, 2013; Flye-SainteMarie et al., 2009) can induce significant energetic costs on bivalves (Freitak et al., 2003; Romanyukha et al., 2006) and may therefore have also contributed to the metabolic responses seen here. Further research is needed to quantify the impact that these behavioural and physiological responses may have on the energetic status of *C. gigas*, in turn giving a better assessment of the total costs of upregulated immune defence under *N. crassostreae* exposure.

Whilst this response can offer some immediate protection (Pernet et al., 2014, 2019), the continual loss of vital glycogen reserves under prolonged pathogenic pressure can ultimately jeopardise bivalve survival within natural environments (Goulletquer,1989; Pernet et al., 2010). Mortalities often occur in oysters, not as a direct effect of pathogenic assault, but as a consequence of an unfavourable energy balance, with times of high energy demand co-occurring during periods of limited glycogen availability (Berthelin et al., 2000; Pernet et al., 2012; Samain and McCombie, 2008). Within natural settings, fluctuations in food availability (Kang et al., 2006; Delaporte et al., 2006) or spawning events (Soletchnik et al.,

1997; Berthelin et al 2000; Delaporte et al., 2006; Soletchnik et al., 2006; Samain and McCombie, 2008) can have significant additive effects on the energetic demands on molluscs. Individuals in a previously reduced energetic condition may then enter a metabolically depressed state to preserve physiological functions more directly related to organism homeostasis (Guderley and Pörtner, 2010). Ultimately, this can lead to trade-offs and the significant loss of immune (Sokolva et al., 2012; Wendling and Wegner, 2013) and thermoregulatory defences (Clegg et al., 1998; Cruz-Rodriguez and Chu, 2002; Hamdoun and Cherr, 2001) in molluscs; increasing their vulnerability to opportunistic pathogens, severe disease expression and mortality (Li et al., 2007, 2009; Samain et al., 2007; Soletchnik et al., 2005; Wendling and Wegner, 2013). Similarly, Freidman et al (1991) reported the prevalence of Nocardiosis and related mortalities to rise throughout the summer to peak in September. This suggests, along with this data, that this pathogen may aggravate the risk of disease and mortality within naturally heterogeneous environments, through its long-term effects on vital energy stores and resulting life-history trade-offs.

Temperature elevation also caused alterations to the energetic and immunological status of *C. gigas* in this study. Both challenged and non-challenged oysters maintained at 21 °C demonstrated significant reductions in ATP levels compared to those at 18 °C. There was also evidence that increased temperature could reduce the functional viability of circulating haemocytes, though these differences were not significant at all sampling points. Following increases in temperature, oysters use cytoprotective and thermoregulatory mechanisms to maintain physiological homeostasis (Farcy et al., 2009; Hamdoun et al., 2003; Hamdoun and Cher 2001; Piano et al., 2002; Yang et al., 2016) which

are energetically costly to produce (Houlihua, 1991). ATP is the main biochemical energy reserve in cells where it is hydrolysed into the other adenosine nucleotides (ADP and then AMP) to provide energy for vital cellular functions (Krauss et al., 2001). While there is debate over using the relative concentrations of adenylate nucleotides as an effective measure of energy status within marine species generally (Ivanovici 1980; Baur and Bimbaum, 2014) and molluscs specifically (Rainer et al. 1979; Ivanovici, 1980), the approach has been used widely in the literature to assess the energetic costs associated with stress conditions (Nelson and Cox, 2008; Salgado-García et al., 2020; Sokolova, 2012; Yang et al., 2016). Therefore, the findings of this study may suggest that oysters at 21°C had enhanced rates of ATP turnover to support an upregulated physiological activity.

Haemocytes also require constant supplies of ATP for basic housekeeping (Coyne, 2011) and any physiological stress that interferes with ATP availability will likely impede haemocyte viability (Buttgereit et al., 2000). Previously, thermal stress has been found to reduce haemocyte viability in bivalves (Turner et al., 2016), including *C. gigas* (Gagnaire et al., 2006; Hégaret et al., 2004). By impeding immune performance (Buttgereit et al., 2000; Galloway and Depledge, 2001), this response is shown to correspond with the gradual rise in infection intensity and risk of disease and mortality over long-term exposure scales (Paillard et al., 1996). Along with the findings of this study, this support the hypothesis that elevated temperature would compromise the oyster's immune response, as a result of enhanced energetic depletion at high temperatures. Overall, by impeding cellular defence, this response to temperature may be an important mechanism affecting the long-term resistance of oysters against *N.*

crassostreae and be a contributing factor towards SMS, within natural environments.

Another potential mechanism influencing disease and mortality during periods of high temperature was demonstrated by the observed reduction of Na^+/K^+ activity in oysters, a similar response seen to that in fish (Schwarzbaum et al., 1991) and shrimp (Wang et al., 2006). Through the maintenance of Na^+, K^+ and Ca^{2+} gradients, the activity of these enzymes control optimal intracellular osmolality, pH and metabolic pathways (Bogdanova et al., 2016). In turn, they are essential for cellular and organ function as well as an organism's ability to maintain survival under stress conditions (Dietz, 1985; Pałecz et al., 2005). The activities of these enzymes are highly ATP demanding and are rapidly impaired under a situation of limited ATP availability (Bogdanova et al., 2016). This suggests that as well as the consequences for immunocompetence, the energetic constraints imposed by temperature may contribute towards SMS through its effects on this important class of enzymes and the potential loss of environmental stress tolerance.

2.5 Conclusion

Overall, this study provides evidence that exposure to *N. crassostreae* can cause a number of physiological alterations in Pacific oysters. Through the mobilisation and utilisation of energetic reserves, oysters are capable of mounting cellular defences which may be important for disease mitigation. However, the metabolic and immunological costs associated with simultaneous exposure to these pathogenic and thermal stressors may affect the long-term resistance of oysters against *N. crassostreae* as well as other environmental and biotic stressors affecting natural populations. Overall, this may help to explain the association

between these stressors and the increased incidence of disease and mortality in Pacific oyster populations. Future research should integrate additional metrics such as bacterial load, histopathology and mortality assessments over longer-term exposures. This will help quantify how these physiological responses translate into alterations for immunocompetence against the pathogen. Finally, by testing how this host-pathogen relationship may be modified by other exogenous (environmental) and endogenous factors (reproductive development), we may also get a more realistic assessment of the true factors driving mortalities.

Chapter 3: Conclusions

3.1 Conclusions

The overall aim of this thesis was firstly to outline the individual effects of thermal stress as well as its combined effects with pathogenic challenge on the immunological and energetic condition of bivalves (chapter 1). Subsequently, the aim for the experimental chapter (chapter 2) was to explore the relevance of these combined effects for immunocompetence and metabolic performance in controlling the susceptibility to disease caused by an understudied bivalve pathogen, namely *Nocardia crassostreae*.

3.2 Factors influencing disease susceptibility under *N. crassostreae* exposure and elevated temperature

To address the aim specified in chapter 2, I tested the hypotheses that *1) the immunocompentence of* C. gigas*, would be enhanced following exposure to* N. crassostreae*, associated with an increase in the energy allocation towards immune defence; 2) exposure to elevated temperature would compromise oyster's immune and ionic regulation capacity due to energetic depletion at high temperatures; 3) Nocardia crassostreae and temperature would interact significantly to ehance the immunological and metabolic costs on oysters*

To test these predictions, *oysters were subjected* to N. crassostreae *exposure at two experimental temperatures pertaining to the local summer mean and maxima where oysters were harvested for this study.* I then quantified *the extent of the oysters' immune response* using *both cellular and humoral* methods, their capacity for ionic regulation as well *as metabolic aspects of the oysters' energy utilisation.*

Exposure to *N. crassostreae* caused a significant upregulation in the abundance of circulating haemocytes compared to non-exposed treatments (p = <0.001). This matched with evidence of an increased glycogenolytic activity within pathogenically challenged treatments. Both condition (p = <0.001) and glycogen concentrations were significantly reduced (p = <0.05) whilst glucose concentrations increased (p = 0.002), compared to unchallenged treatments. Further evidence of an increased cellular energy allocation was demonstrated by a significant higher ADP (p = 0.010) and total anenylate nucleotide concentration (p = 0.035) when compared to non-challenged treatments. This supports the hypothesis that *C. gigas* will upregulate immune defences in response to *N. crassostreae* through an increased energy allocation.

Oysters at 21°C expressed reduced cellular viability (p = <0.001) compared to individuals at 18°C. A rise in temperature was also associated with a significant decline in the concentration of ATP (p = <0.001) and the activity of Na^+/K^+-ATPase (p = 0.03). Therefore, there is evidence to support the hypothesis that immune defence and ionic regulation will be supressed due to the energetic costs associated with elevated temperature.

There was no evidence of any strong interactive effects between temperature and *N. crassostreae* infection on either the energetics or the immunology of oysters. Therefore, the hypothesis that these stressors will interact to impose greater physiological consequences than the single effects of either treatment alone cannot be supported.

This study was the first to investigate the physiological mechanism employed by *C. gigas* to protect against *N. crasssosteae* and how temperature may impede

these same responses. Given the evidence presented here, it is likely that the direct and indirect effects of temperature and *N. crassostreae* exposure on energy availability and physiological performance are a contributing factor towards mass mortalities by compromising resistance against *N. crassostreae* as well as other abiotic and biotic stressors affecting natural populations.

3.3 Perspectives

This thesis investigated the effects of temperature and *N. crassostreae* exposure on the immunity and physiology of the oyster *C. gigas.* This has contributed to our understanding of some of the potential factors facilitating summer mortalities specifically in the context of oysters infected with *N. crassostrea*, but also in regards to other host-pathogen models.

Whilst there is extensive evidence of pathogen exposure modifying the energy balance of bivalves (Barber et al., 1988; Flye-Sainte-Marie et al., 2007; Ford and Figueras, 1988; Plana et al., 1996), few studies have explored the involvement of immune upregulation in this response and how this may influence host-pathogen interactions. In this study, an increased immune activity in response to pathogenic challenge coincided with the significant mobilisation and utilisation of energetic reserves. This supports the growing body of evidence to suggest the necessity of energetic allocation is supplying immune defences and therefore disease resistance in bivalves under pathogen exposure (Génard et al., 2011; 2013; Tang et al., 2010; Wang et al., 2012). The incidence of disease and the likelihood of mortality increases with the depletion of energy reserves in bivalves (Pernet et al., 2013, 2019). With this information, it is likely that the lack of investment towards immune defence is a factor influencing this pattern.

Generally, the severity of disease is positively correlated with temperature in a range of marine species (Baker-Austin et al., 2012; Burge, 2014; Harvell et al., 1999; Morgan and Wall, 2009). In this study, temperature elevation caused the suppression of a key aspect of the innate immune system. The innate immune system is the only form of defence available for all invertebrate species (Hoffmann and Reichhart, 2002), and is crucial for the elimination of pathogen infection (Harris-Young et al., 1993, 1995; Venier et al., 2011). Overall, this supports the hypothesis that the consequences for temperature elevation on host-defences may be a factor in the emergence of disease outbreaks and mass mortalities in marine invertebrates under GCC. Furthermore, the innate immune system is also important for higher vertebrates, providing a first line of defence and instructing the development of the acquired immune system and long lasting immune memory (Magnadottir, 2006). Whilst there is limited evidence on the subject, the obstructive effects of temperature on innate immunity may also be factor of host-pathogen interactions in vertebrates. This mechanism may therefore prove useful in understanding disease outbreaks and mass mortalities under GCC in marine species generally.

Furthermore, temperature elevation was associated with a reduction in the vital energy supply molecule, ATP. Whilst it is hypothesised that costs for energy balance associated with thermal stress may lead to trade-offs for immune defences of bivalves (Sokolova et al., 2012), few studies have measured both the immunological and energetic responses to temperature. In demonstrating these responses to coincide with the suppression of immune defence, this supports the growing body of evidence suggesting that the energetic costs associated with

temperature elevation may be important in deciding host-pathogen interactions and disease susceptibility by limiting the energy available for immune defence (Tang et al., 2010; Turner et al., 2016; Wang et al., 2012). Future investigations of thermal effects on immune responses in relation to the abundance of energetic resources as well as the viability of metabolic pathway may help elucidate the organism-level responses controlling mass mortalities under GCC.

In contrast to other pathogenic species, in combination with elevated temperature, *N. crassostreae* produced no significant interactive effects on the immunology or bioenergetics in oysters (Chu and La Peyre, 1993; Hauton et al., 2001; Perrigault et al., 2011; Tang et al., 2010; Turner et al., 2016; Wang et al., 2012; Wendling and Wegner, 2013). Interactive effects between temperature and pathogen on host physiology are most significant when temperature compliments the species-specific physiological requirements for proliferation and virulence (Hauton et al., 2001). Compared to the other bacterial or parasitic species that these interactive effects are normally demonstrated with (Baker-Austin et al., 2010; Lopez-Joven et al., 2011; Oliver et al., 1998; Vezzulli et al., 2013), *N. crassostreae* is relatively slow growing and has no proliferation type response to temperature increase (Friedman and Hedrick, 1991). Therefore, whilst temperature effects on pathogen proliferation and virulence likely contributes towards SMS in many cases, these effects may not be deterministic of mortality in regards to all pathogenic species, including *N crassostreae*. Overall, this suggests that the complex mechanisms driving mortalities during periods of summer mortalities should be considered in light of the specific host-pathogen model at play.

A temperature of 21 °C is normally associated with mass mortalities in oysters within the natural environment (Ford and Borrero, 2001*;* Garcia et al., 2011; Malham et al., 2009*;* Meyers and Short, 1990). Whilst this temperature was sufficient to supress oyster immunocompetence in this study, the fact no significant mortality was observed suggests this level of immunity was still adequate to allow resistance against *N. crassostreae.* This suggests that the suppressive effects of temperature on the immunocompetence of oysters is likely not the sole cause of mortalities under *N. crassostreae* exposure. Furthermore, this temperature is shown to induce intense spawning in oysters (Berthelin et al., 2000). These spawning events severely deplete energetic resources in bivalves (Soletchnik et al., 1997, Berthelin et al., 2000, Delaporte et al., 2006, Soletchnik et al., 2006, Samain and McCombie, 2008), causing the suppression of key immune activities (Carballal et al., 1998; Lin et al., 2012; Malagoli et al., 2008; Wendling and Wegner, 2013). Whilst there is no evidence of *N. crassostreae* linked mortalities being associated with spawning, the additional energetic costs this may impose on oysters in a previously depleted energetic condition may supress host immunity past a threshold where mortality is likely to occur. Traditionally, temperatures induce mortalities have been considered as a result of either an increased spawning activity or a suppression of immune activity. This study therefore supports the growing body of evidence to suggest that in the case of some host-pathogen models, such as *C. gigas* and *N. crassostreae,* the two may be linked (Li et al., 2007, 2009; Lin et al., 2012; Wendling and Wegner, 2013). Furthermore, a complex etoilogy involving the combination of thermal stress and infectious agents effecting organisms during periods of heightened reproductive activity may prove to be a predominant cause of mass mortalities

more generally. However, further research incorporating all three factors is needed to substantiate the importance of this mechanism, especially in natural bivalve populations.

Appendix A

A.1 METABOLITIES and Na^+/K^+-ATPASE ACTIVITY.

A.1.1 Glycogen

$$\text{Glycogen or } (\alpha\text{-glycosyl}) + H_2O \xrightarrow{\text{Glycoamylase}} (\alpha\text{-glycosyl}) + \text{Glucose}$$

Fig. A.1 The determination of tissue [glycogen]. Glucoamylase from *Aspergillus niger* is a glycoprotein enzyme which hydrolyses glycogen into glucose the concentration of which is then measured. Background [glucose] was measured for each sample and this was subtracted from the glucose content obtained from the glycogen digestion (Bergmeyer, 1985a).

A.1.2 Glucose

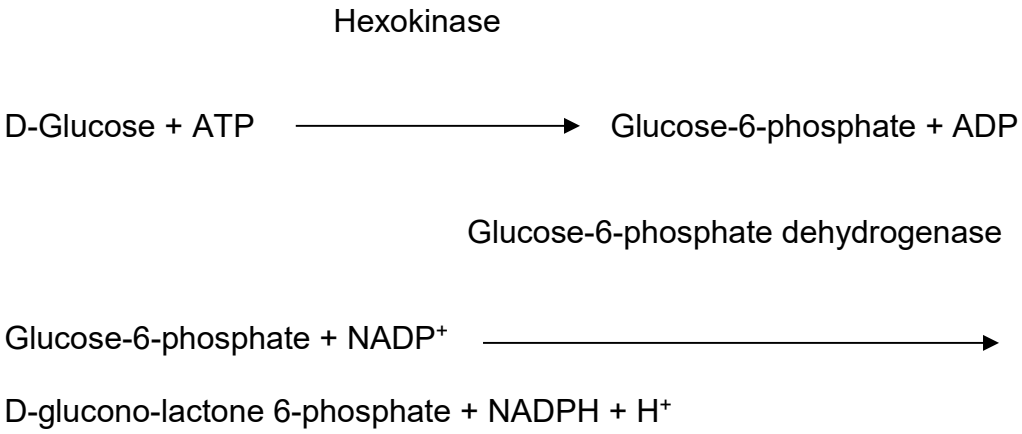

Fig. A.2 The hexokinase method for determination of mantle [glucose]. D-glucose is phosphorylated to glucose-6-phosphate (G-6-P) by ATP in the prsesnce of hexokinase. The G-6-P thus produced is converted into D-glucono-lactone 6-phosphate in the reaction catalysed by glucose-6-phosphate dehydrogenase (G-6-PDH). The formation of NADPH measured by the change in absorbance at 339 nm is proportional to the amount of glucose present (NADP: Nicotinamide adenine dinucleotide phosphate, NADPH: nicotinamide adenine dinucleotide phosphate-oxidase) (Bergmeyer, 1985a).

A.1.3 ATP

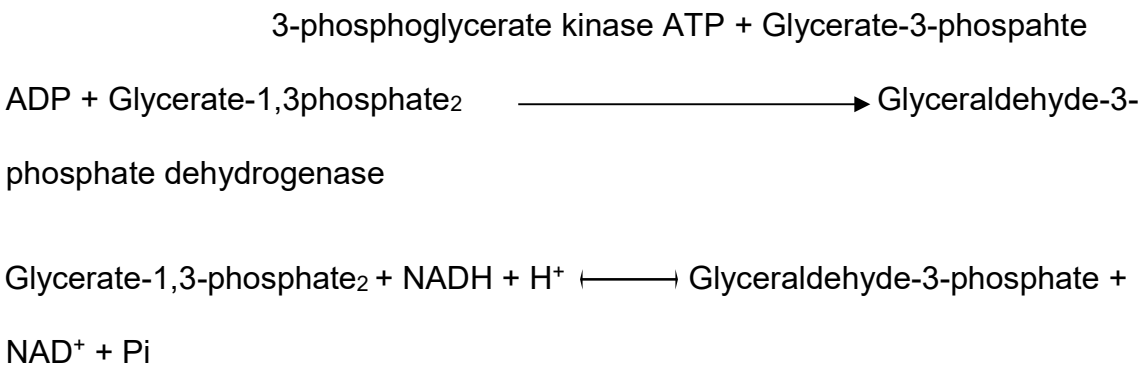

Fig. A.3 The determination of tissue [ATP]. The glycerate-1,3-phospahte formed in the first reaction is determined by the indicator reaction with glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The oxidation of NADH, measured by the change in absorbance at 339 nm is proportional to the amount of ATP present (Bergmeyer, 1985b)

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A.1.4 ADP and AMP

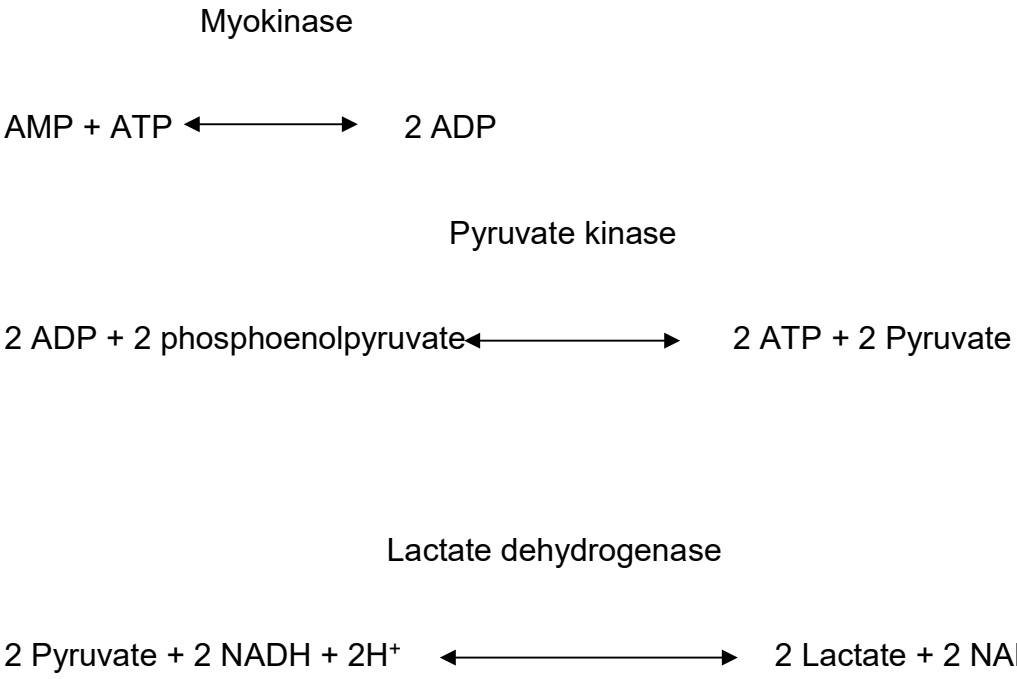

Fig. A.4 The determination of tissue [AMP and ADP]. The decrease of NADH, measured by the change in absorbance at 339 nm is proportional to the amount of AMP and ADP present (Bergmeyer, 1985b).

A.1.5 Na^+/K^+-ATPase activity.

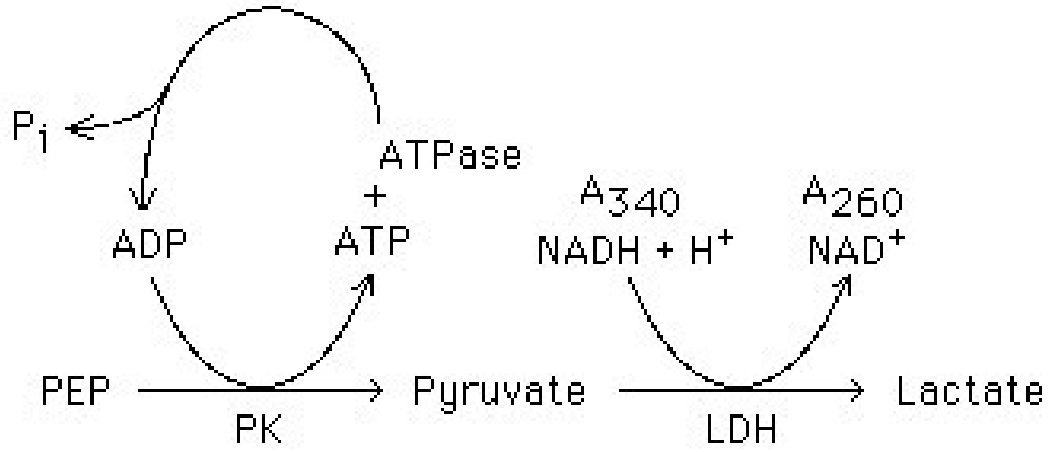

Fig. A.5 The determination of tissue Na^+/K^+-ATPase activity. The coupled enzyme ATPase assay is based on the conversion of phosphoenolpyruvate (PEP) to pyruvate by pyruvate kinase coupled to the conversion of pyruvate to lactate by lactate dehydrogenase. The latter step requires NADH which is oxidized to NAD+. Unlike the NAD+, the NADH absorbs strongly at 340 nm. The decrease in A340 can be converted into ATPase activity where 1 molecule of NADH oxidized to NAD+ corresponds to the production of 1 molecule of ADP by the ATPase. The use of ouabain, which inhibits the action of Na+/K+-ATPase, corrects for the activity of other ATPases present in the tissue. Deoxycholic acid is used in the buffer to solubilise any membrane bound ATPases (McCormick 1993).

Appendix B

B.1 Tables of pair-wise comparison tests

B.1.1 Glycogen

Table B.1 Results of pair-wise comparison test indicating interactions between temperature and *N. crassostreae* exposure on the glycogen concentration in *C. gigas*. Estimate, standard error (SE), degrees of freedom (df), t. ratio and probability value (*p*). Significant values ($p < 0.05$) are highlighted in bold.

Contrast		estimate	SE	df	t.ratio	p.value	
cold,infected	- hot,infected	11.39	30.6	25	0.373	0.9819	
cold,infected	**- cold,uninfected**	**-126.60**	**44.5**	**25**	**-2.843**	**0.0409**	**.**
cold,infected	- hot,uninfected	-2.61	34.0	25	-0.077	0.9998	
hot,infected	**- cold,uninfected**	**-137.98**	**42.8**	**25**	**-3.221**	**0.0174**	**.**
hot,infected	- hot,uninfected	-13.99	31.8	25	-0.440	0.9709	
cold,uninfected	- hot,uninfected	123.99	45.4	25	2.732	0.0520	.

B.1.2 Percentage viability

Table B.2 Results of pair-wise comparison test indicating interactions between temperature, *N. crassostreae* exposure and date on the percentage viability of haemocytes in *C. gigas*. Estimate, standard error (SE), degrees of freedom (df), t. ratio and probability value (*p*). Significant values ($p < 0.05$) are highlighted in bold.

Contrast		estimate	SE	df	t.ratio	p.value
cold,infected,time1	**- hot,infected,time1**	**15.368**	**3.88**	**102**	**3.956**	**0.0074**
cold,infected,time1	- cold,uninfected,time1	-0.375	3.81	102	-0.098	1.0000
cold,infected,time1	- hot,uninfected,time1	8.720	3.97	102	2.195	0.5569
cold,infected,time1	- cold,infected,time2	1.893	3.81	102	0.497	1.0000
cold,infected,time1	- hot,infected,time2	11.675	3.81	102	3.064	0.1059
cold,infected,time1	- cold,uninfected,time2	3.158	3.75	102	0.843	0.9995
cold,infected,time1	**- hot,uninfected,time2**	**14.634**	**3.88**	**102**	**3.767**	**0.0139**
cold,infected,time1	- cold,infected,time3	1.014	4.08	102	0.249	1.0000
cold,infected,time1	- hot,infected,time3	7.076	4.21	102	1.680	0.8733
cold,infected,time1	- cold,uninfected,time3	-2.536	3.88	102	-0.653	1.0000

cold,infected,time1	- hot,uninfected,time3	4.424	4.08	102	1.084	0.9947
hot,infected,time1	**- cold,uninfected,time1**	**-15.744**	**3.44**	**102**	**-4.571**	**0.0008**
hot,infected,time1	- hot,uninfected,time1	-6.648	3.62	102	-1.836	0.7944
hot,infected,time1	**- cold,infected,time2**	**-13.475**	**3.44**	**102**	**-3.913**	**0.0086**
hot,infected,time1	- hot,infected,time2	-3.693	3.44	102	-1.072	0.9952
hot,infected,time1	**- cold,uninfected,time2**	**-12.210**	**3.37**	**102**	**-3.618**	**0.0223**
hot,infected,time1	- hot,uninfected,time2	-0.734	3.52	102	-0.208	1.0000
hot,infected,time1	**- cold,infected,time3**	**-14.354**	**3.74**	**102**	**-3.839**	**0.0110**
hot,infected,time1	- hot,infected,time3	-8.292	3.88	102	-2.135	0.5995
hot,infected,time1	**- cold,uninfected,time3**	**-17.904**	**3.52**	**102**	**-5.079**	**0.0001**
hot,infected,time1	- hot,uninfected,time3	-10.944	3.74	102	-2.927	0.1471
cold,uninfected,time1	- hot,uninfected,time1	9.095	3.54	102	2.567	0.3121
cold,uninfected,time1	- cold,infected,time2	2.269	3.36	102	0.675	0.9999
cold,uninfected,time1	**- hot,infected,time2**	**12.050**	**3.36**	**102**	**3.585**	**0.0247**
cold,uninfected,time1	- cold,uninfected,time2	3.534	3.29	102	1.074	0.9952
cold,uninfected,time1	**- hot,uninfected,time2**	**15.009**	**3.44**	**102**	**4.358**	**0.0018**
cold,uninfected,time1	- cold,infected,time3	1.389	3.66	102	0.379	1.0000
cold,uninfected,time1	- hot,infected,time3	7.451	3.81	102	1.955	0.7216
cold,uninfected,time1	- cold,uninfected,time3	-2.161	3.44	102	-0.627	1.0000
cold,uninfected,time1	- hot,uninfected,time3	4.799	3.66	102	1.310	0.9760
hot,uninfected,time1	- cold,infected,time2	-6.827	3.54	102	-1.927	0.7397
hot,uninfected,time1	- hot,infected,time2	2.955	3.54	102	0.834	0.9995
hot,uninfected,time1	- cold,uninfected,time2	-5.562	3.48	102	-1.600	0.9051
hot,uninfected,time1	- hot,uninfected,time2	5.914	3.62	102	1.633	0.8927
hot,uninfected,time1	- cold,infected,time3	-7.706	3.83	102	-2.012	0.6842
hot,uninfected,time1	- hot,infected,time3	-1.644	3.97	102	-0.414	1.0000
hot,uninfected,time1	- cold,uninfected,time3	-11.256	3.62	102	-3.108	0.0946
hot,uninfected,time1	- hot,uninfected,time3	-4.296	3.83	102	-1.122	0.9930
cold,infected,time2	- hot,infected,time2	9.781	3.36	102	2.910	0.1530
cold,infected,time2	- cold,uninfected,time2	1.265	3.29	102	0.384	1.0000
cold,infected,time2	**- hot,uninfected,time2**	**12.740**	**3.44**	**102**	**3.699**	**0.0173**
cold,infected,time2	- cold,infected,time3	-0.880	3.66	102	-0.240	1.0000
cold,infected,time2	- hot,infected,time3	5.183	3.81	102	1.360	0.9684
cold,infected,time2	- cold,uninfected,time3	-4.429	3.44	102	-1.286	0.9791
cold,infected,time2	- hot,uninfected,time3	2.530	3.66	102	0.691	0.9999
hot,infected,time2	- cold,uninfected,time2	-8.517	3.29	102	-2.589	0.3001
hot,infected,time2	- hot,uninfected,time2	2.959	3.44	102	0.859	0.9993
hot,infected,time2	- cold,infected,time3	-10.661	3.66	102	-2.911	0.1528
hot,infected,time2	- hot,infected,time3	-4.599	3.81	102	-1.207	0.9873
hot,infected,time2	**- cold,uninfected,time3**	**-14.211**	**3.44**	**102**	**-4.126**	**0.0041**
hot,infected,time2	- hot,uninfected,time3	-7.251	3.66	102	-1.980	0.7056

cold,uninfected,time2 - hot,uninfected,time2	**11.476**	**3.37**	**102**	**3.400**	**0.0427**
cold,uninfected,time2 - cold,infected,time3	-2.145	3.60	102	-0.596	1.0000
cold,uninfected,time2 - hot,infected,time3	3.918	3.75	102	1.045	0.9962
cold,uninfected,time2 - cold,uninfected,time3	-5.694	3.37	102	-1.687	0.8699
cold,uninfected,time2 - hot,uninfected,time3	1.265	3.60	102	0.352	1.0000
hot,uninfected,time2 - cold,infected,time3	**-13.620**	**3.74**	**102**	**-3.643**	**0.0206**
hot,uninfected,time2 - hot,infected,time3	-7.558	3.88	102	-1.946	0.7278
hot,uninfected,time2 - cold,uninfected,time3	**-17.170**	**3.52**	**102**	**-4.871**	**0.0002**
hot,uninfected,time2 - hot,uninfected,time3	-10.210	3.74	102	-2.731	0.2267
cold,infected,time3 - hot,infected,time3	6.062	4.08	102	1.486	0.9411
cold,infected,time3 - cold,uninfected,time3	-3.550	3.74	102	-0.949	0.9984
cold,infected,time3 - hot,uninfected,time3	3.410	3.94	102	0.865	0.9993
hot,infected,time3 - cold,uninfected,time3	-9.612	3.88	102	-2.475	0.3678
hot,infected,time3 - hot,uninfected,time3	-2.652	4.08	102	-0.650	1.0000
cold,uninfected,time3 - hot,uninfected,time3	6.960	3.74	102	1.861	0.7795

B.1.3 Lysozyme activity

Table B.3 Results of pair-wise comparison test indicating interactions between temperature, *N. crassostreae* exposure and date on lysozyme activity of the cell-free haemolymph in *C. gigas*. Estimate, standard error (SE), degrees of freedom (df), t. ratio and probability value (p). Significant values ($p < 0.05$) are highlighted in bold

Contrast	estimate	SE	df	t.ratio	p.value
cold,infected,time1 - hot,infected,time1	0.000181	0.0389	106	0.005	1.0000
cold,infected,time1 - cold,uninfected,time1	-0.001545	0.0335	106	-0.046	1.0000
cold,infected,time1 - hot,uninfected,time1	0.052129	0.0344	106	1.515	0.9333
cold,infected,time1 - cold,infected,time2	**-0.159855**	**0.0320**	**106**	**-5.001**	**0.0001**
cold,infected,time1 - hot,infected,time2	**-0.159412**	**0.0327**	**106**	**-4.882**	**0.0002**
cold,infected,time1 - cold,uninfected,time2	**-0.201000**	**0.0335**	**106**	**-6.007**	**<.0001**
cold,infected,time1 - hot,uninfected,time2	**-0.162300**	**0.0320**	**106**	**-5.077**	**0.0001**
cold,infected,time1 - cold,infected,time3	**-0.362069**	**0.0344**	**106**	**-10.519**	**<.0001**
cold,infected,time1 - hot,infected,time3	**-0.243335**	**0.0356**	**106**	**-6.839**	**<.0001**
cold,infected,time1 - cold,uninfected,time3	**-0.277950**	**0.0335**	**106**	**-8.307**	**<.0001**
cold,infected,time1 - hot,uninfected,time3	**-0.201046**	**0.0335**	**106**	**-6.009**	**<.0001**
hot,infected,time1 - cold,uninfected,time1	-0.001726	0.0395	106	-0.044	1.0000
hot,infected,time1 - hot,uninfected,time1	0.051947	0.0404	106	1.287	0.9791
hot,infected,time1 - cold,infected,time2	**-0.160036**	**0.0383**	**106**	**-4.180**	**0.0033**
hot,infected,time1 - hot,infected,time2	**-0.159593**	**0.0389**	**106**	**-4.106**	**0.0043**
hot,infected,time1 - cold,uninfected,time2	**-0.201181**	**0.0395**	**106**	**-5.087**	**0.0001**
hot,infected,time1 - hot,uninfected,time2	**-0.162482**	**0.0383**	**106**	**-4.244**	**0.0027**
hot,infected,time1 - cold,infected,time3	**-0.362251**	**0.0404**	**106**	**-8.976**	**<.0001**
hot,infected,time1 - hot,infected,time3	**-0.243516**	**0.0414**	**106**	**-5.888**	**<.0001**
hot,infected,time1 - cold,uninfected,time3	**-0.278132**	**0.0395**	**106**	**-7.033**	**<.0001**
hot,infected,time1 - hot,uninfected,time3	**-0.201227**	**0.0395**	**106**	**-5.089**	**0.0001**
cold,uninfected,time1 - hot,uninfected,time1	0.053674	0.0352	106	1.525	0.9301
cold,uninfected,time1 - cold,infected,time2	**-0.158310**	**0.0328**	**106**	**-4.828**	**0.0003**
cold,uninfected,time1 - hot,infected,time2	**-0.157867**	**0.0335**	**106**	**-4.718**	**0.0004**
cold,uninfected,time1 - cold,uninfected,time2	**-0.199455**	**0.0342**	**106**	**-5.824**	**<.0001**
cold,uninfected,time1 - hot,uninfected,time2	**-0.160755**	**0.0328**	**106**	**-4.903**	**0.0002**
cold,uninfected,time1 - cold,infected,time3	**-0.360524**	**0.0352**	**106**	**-10.247**	**<.0001**
cold,uninfected,time1 - hot,infected,time3	**-0.241789**	**0.0363**	**106**	**-6.656**	**<.0001**
cold,uninfected,time1 - cold,uninfected,time3	**-0.276405**	**0.0342**	**106**	**-8.071**	**<.0001**
cold,uninfected,time1 - hot,uninfected,time3	**-0.199501**	**0.0342**	**106**	**-5.825**	**<.0001**
hot,uninfected,time1 - cold,infected,time2	**-0.211984**	**0.0338**	**106**	**-6.278**	**<.0001**
hot,uninfected,time1 - hot,infected,time2	**-0.211540**	**0.0344**	**106**	**-6.146**	**<.0001**
hot,uninfected,time1 - cold,uninfected,time2	**-0.253129**	**0.0352**	**106**	**-7.194**	**<.0001**
hot,uninfected,time1 - hot,uninfected,time2	**-0.214429**	**0.0338**	**106**	**-6.350**	**<.0001**
hot,uninfected,time1 - cold,infected,time3	**-0.414198**	**0.0361**	**106**	**-11.474**	**<.0001**
hot,uninfected,time1 - hot,infected,time3	**-0.295463**	**0.0372**	**106**	**-7.940**	**<.0001**
hot,uninfected,time1 - cold,uninfected,time3	**-0.330079**	**0.0352**	**106**	**-9.381**	**<.0001**
hot,uninfected,time1 - hot,uninfected,time3	**-0.253175**	**0.0352**	**106**	**-7.196**	**<.0001**
cold,infected,time2 - hot,infected,time2	0.000443	0.0320	106	0.014	1.0000

cold,infected,time2	- cold,uninfected,time2	-0.041145	0.0328	106	-1.255	0.9828
cold,infected,time2	- hot,uninfected,time2	-0.002446	0.0313	106	-0.078	1.0000
cold,infected,time2	**- cold,infected,time3**	**-0.202214**	**0.0338**	**106**	**-5.988**	**<.0001**
cold,infected,time2	- hot,infected,time3	-0.083480	0.0350	106	-2.388	0.4231
cold,infected,time2	**- cold,uninfected,time3**	**-0.118095**	**0.0328**	**106**	**-3.602**	**0.0232**
cold,infected,time2	- hot,uninfected,time3	-0.041191	0.0328	106	-1.256	0.9826
hot,infected,time2	- cold,uninfected,time2	-0.041588	0.0335	106	-1.243	0.9840
hot,infected,time2	- hot,uninfected,time2	-0.002889	0.0320	106	-0.090	1.0000
hot,infected,time2	**- cold,infected,time3**	**-0.202657**	**0.0344**	**106**	**-5.888**	**<.0001**
hot,infected,time2	- hot,infected,time3	-0.083923	0.0356	106	-2.359	0.4431
hot,infected,time2	**- cold,uninfected,time3**	**-0.118539**	**0.0335**	**106**	**-3.543**	**0.0278**
hot,infected,time2	- hot,uninfected,time3	-0.041634	0.0335	106	-1.244	0.9839
cold,uninfected,time2	- hot,uninfected,time2	0.038699	0.0328	106	1.180	0.9894
cold,uninfected,time2	**- cold,infected,time3**	**-0.161069**	**0.0352**	**106**	**-4.578**	**0.0008**
cold,uninfected,time2	- hot,infected,time3	-0.042335	0.0363	106	-1.165	0.9905
cold,uninfected,time2	- cold,uninfected,time3	-0.076950	0.0342	106	-2.247	0.5203
cold,uninfected,time2	- hot,uninfected,time3	-0.000046	0.0342	106	-0.001	1.0000
hot,uninfected,time2	**- cold,infected,time3**	**-0.199769**	**0.0338**	**106**	**-5.916**	**<.0001**
hot,uninfected,time2	- hot,infected,time3	-0.081034	0.0350	106	-2.318	0.4705
hot,uninfected,time2	**- cold,uninfected,time3**	**-0.115650**	**0.0328**	**106**	**-3.527**	**0.0291**
hot,uninfected,time2	- hot,uninfected,time3	-0.038745	0.0328	106	-1.182	0.9893
cold,infected,time3	**- hot,infected,time3**	**0.118735**	**0.0372**	**106**	**3.191**	**0.0758**
cold,infected,time3	- cold,uninfected,time3	0.084119	0.0352	106	2.391	0.4215
cold,infected,time3	**- hot,uninfected,time3**	**0.161023**	**0.0352**	**106**	**4.576**	**0.0008**
hot,infected,time3	- cold,uninfected,time3	-0.034616	0.0363	106	-0.953	0.9983
hot,infected,time3	- hot,uninfected,time3	0.042289	0.0363	106	1.164	0.9905
cold,uninfected,time3	- hot,uninfected,time3	0.076904	0.0342	106	2.246	0.5212

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References

Appendix